# The role of disease risk and life history in the immune function of larks in different environments

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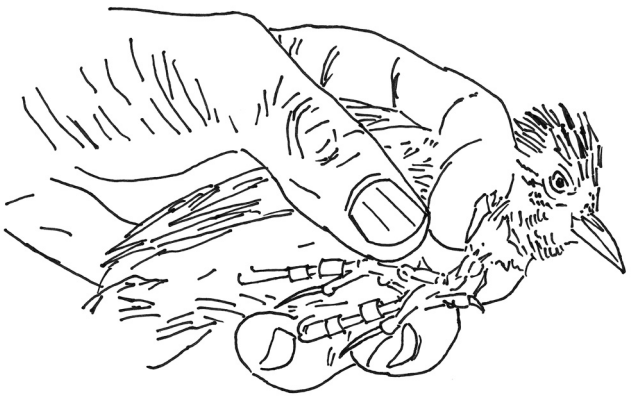

CHAPTER 2

# Pathogen pressure puts immune defence into perspective

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**Abstract**

The extent to which organisms can protect themselves from disease depends on both the immune defences they maintain and the pathogens they face. At the same time, immune systems are shaped by the antigens they encounter, both over ecological and evolutionary time. Ecological immunologists often recognise these interactions, yet ecological immunology currently lacks major efforts to characterise the environmental, host-independent, antigenic pressures to which all animals are exposed. Failure to quantify relevant diseases and pathogens in studies of ecological immunology leads to contradictory hypotheses. In contrast, including measures of environmental and host-derived commensals, pathogens and other immune-relevant organisms will strengthen the field of ecological immunology. In this paper we examine how pathogens and other organisms shape immune defences and highlight why such information is essential for a better understanding of the causes of variation in immune defences. We introduce the concept of 'operative protection' for understanding the role of immunologically-relevant organisms in shaping immune defence profiles, and demonstrate how the evolutionary implications of immune function are best understood in the context of the pressures that diseases and pathogens bring to bear on their hosts. We illustrate common mistakes in characterising these immune-selective pressures, and provide suggestions for the use of molecular and other methods for measuring immune-relevant organisms.

## Integrating immunology and ecology

The immune system bridges the divide between internal and external environments, integrating an organism's physiology and environment. In doing so, the immune system acts as a barrier to infection and disease, identifying threats and coordinating necessary responses. Despite its complexity, immunologists have elucidated many of the cellular processes and specific mechanisms that allow the immune system to function. Yet our knowledge of how evolutionary pressures shape immune systems is still incomplete.

Ecological immunology promotes the use of immunological measures to test ecological and evolutionary hypotheses. The field arose from a desire to explain the variation in immune function that is observed within and among individuals, populations and species, across environments and over time. Many factors influence, and can generate variation in, immune responses: these include sex, nutritional status, social dominance, exercise, and seasonality, as well as trade-offs in resource allocation between the immune system and other physiological systems such as reproduction (Sadd and Schmid-Hempel 2009; Schulenburg *et al.* 2009). Over both ecological and evolutionary timescales however, the most enduring selective pressures on the immune system are the myriad challenges posed by everything that immune systems encounter. Particularly important in terms of evolution are interactions between the immune system and organisms with the ability to live in, or on, a host and the potential to evolve in response to current immune defences. We refer to the specific suite of components that generate these evolutionary and ecological selective forces on the immune system as the 'immunobiome' and their ability to shape immune defences as 'immunobiotic pressure' (Fig. 2.1). Understanding the interactions of the immune system with immunobiomes is essential for helping to explain patterns of immunological variation.

In light of immunological costs, animals should match their immune defences* to the threats that they face (Sheldon and Verhulst 1996; Tschirren and Richner 2006). However, the nature of immunobiomes is poorly understood. For example, does their basic composition differ among environments? Which components most strongly shape immune defences in which hosts? Understanding these issues will be central to solving broader challenges in immunology such as the consequences of emergent infectious diseases (Jones *et al.* 2008) or the consequences for health of altering commensal microbial communities (Blaser and Falkow 2009). We propose that to advance ecological immunology, measures of the immunobiome that are independent of immune indices should be developed

* Any anatomical, chemical, physiological or behavioural barrier maintained by an animal that inhibits or controls the establishment and reproduction of any element of the immunobiome within or on the animal.

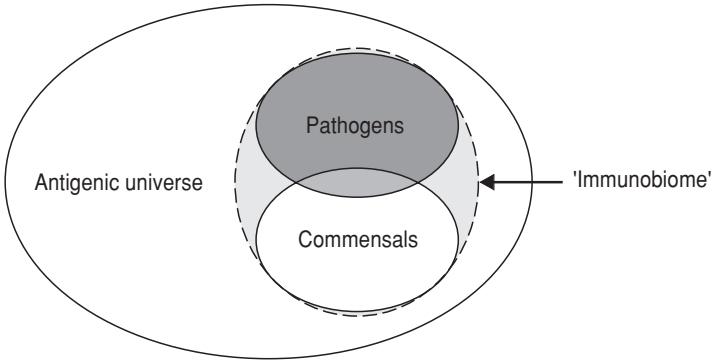


**Figure 2.1.** A representation of the antigenic universe, which consists of all the possible antigens that any immune system could ever encounter. This universe includes antigenic, immunogenic, inflammatory and toxic agents. Within the antigenic universe is the immunobiome. The immunobiome contains all the living organisms that can live in or on a host and with the potential to evolve in response to immune defences. The immunobiome does not include other immuno-reactive particles such as dust that cannot multiply. Two major components of the immunobiome, in terms of the evolution of the immune system, are commensals and pathogens. Since some commensals may be pathogenic under suitable conditions, these groups are not mutually exclusive. Immunobiome components that fall outside these two categories (light grey area) include environmental microbes such as 'pseudo-commensals' (Rook 2009), which although regularly encountered by hosts, do not gain any benefit from their temporary association with a host, yet may still shape regulatory circuits of the immune system. Scaling of the different subsets is arbitrary.

and incorporated into future studies. Much as data on availability of food are required for an understanding of diet selection (e.g. Belovsky 1981) and environmental temperature profiles are required for explaining heat balance (e.g. Tieleman and Williams 2002), knowledge of immunobiomes and immune stimuli is essential when testing hypotheses in ecological immunology. Rather than being relegated to anonymous, yet highly relevant sources of variation, the diverse constituents of the immunobiome and the evolutionary pressures they exert must be seen as central to ecoimmunological studies (Bordes and Morand 2009; Sadd and Schmid-Hempel 2009; Graham *et al.* 2011; Pedersen and Babayan 2011).

## Interactions with entire immunobiomes shape immune defences, but pathogens and commensals are particularly important

Animals live in diverse and variable environments and their immune systems must interact with and respond to equally diverse and variable immunobiomes. However, across immunobiomes, two categories of organisms that lie at all points

along a continuum from benign, or even beneficial, to harmful, are expected to be particularly important sources of immunobiotic pressure (Fig. 2.1). At the detrimental end of this continuum are pathogens. These microparasites (viruses, bacteria, fungi and protists) and macroparasites (e.g. helminths, ticks, lice) can potentially harm host tissues through their inherent ability to breach immune defences that normally restrict other organisms. Pathogens seek to circumvent host immune defences and may disrupt normal immune processes (Tortorella *et al.* 2000; Finlay and McFadden 2006). This might include immunosuppression (Babu *et al.* 2006; Jackson *et al.* 2009) or shifting of the immune system towards a specific mix of defences (Maizels and Yazdanbakhsh 2003).

Of equal importance to the evolution of the immune system, commensals normally sit at the benign end of the pathogenicity spectrum. Benefiting from intimate associations with their host, commensals can also modulate immune responses and play an essential role in development of the immune system (Rakoff-Nahoum *et al.* 2004; Mazmanian *et al.* 2005; Rook 2009; Round and Mazmanian 2009). Different commensal communities might offer distinct advantages or disadvantages in terms of immunomodulation (Jackson *et al.* 2009) and colonization by pathogens (Stecher and Hardt 2008; Stecher *et al.* 2010). Under some circumstances, organisms that normally behave commensally can become pathogenic (sensu 'amphibiosis'; Rosebury 1962). Commensals that escape immune controls by inappropriately breaching defensive barriers (e.g. the intestinal epithelium) may also become *de facto* pathogens (Blaser and Falkow 2009). Overall, interactions with the entire immunobiome, encompassing organisms associated with the full range of the pathogenicity spectrum, shape both immune defences and immunobiotic components themselves, and can have implications for health and survival (Round and Mazmanian 2009).

Hosts might encounter immunobiomes that vary temporally (e.g. at different times of the day, or across seasons) or spatially (e.g. small scale differences in use of habitat, or large-scale differences in biogeography). At ecological scales immunobiotic pressures mould individual responses and physiological condition. Immune function interacts with and is influenced by each antigen (pathogenic, commensal or otherwise) encountered over the lifetime of an individual, from *in utero* or *in ovo* (Boulinier and Staszewski 2008; Grindstaff 2008) to adulthood. Nonetheless, these relationships are ultimately governed by an immune system that has been shaped through evolution. At evolutionary scales, differences in composition and function of immunobiomes and levels of exposure to them are expected to lead to genetically-based changes in the organization of the immune system, with immunobiotic pressures shaping immuno-defensive architecture.

## Operative protection – balancing immune defences and immunobiotic pressure

How can ecological immunologists best understand what immune indices represent in terms of protection from infection and enhancement of fitness to an animal in the wild? We advocate that levels of immune defence be considered relative to the immunobiotic pressures that are encountered by an organism, focusing on effectiveness of protection rather than upon magnitude of response (see also e.g. Viney, Riley and Buchanan 2005; Graham *et al.* 2011; Pedersen and Babayan 2011). We refer to this immunobiome-specific assessment of immune defence as 'operative protection'. Operative protection encompasses the fitness-enhancing protection against immunobiotic pressure (and immunopathology) afforded by the immune system, relative to the immunobiotic pressure under which an organism is placed (Fig. 2.2). Put more simply, operative protection describes the goodness of fit between the immunobiotic pressure in a given environment and the immune defences of an animal in that environment. A mismatch between immunobiotic pressure and immune defences could result in three outcomes. Inappropriately low operative protection (i.e. immune defences inadequate to match immunobiotic pressure) could lead to increased infection and disease. Inappropriately high operative protection (i.e. immune defences exceeding the immunobiotic pressure of the current environment) could lead to immunopathology and unnecessary expenditure of energetic and nutritional resources on the immune system. This in turn might affect other physiological systems (e.g. reproduction) that must compete with the immune system for allocation of resources. In both instances a reduction in fitness is expected. In some specific instances, inappropriately high operative protection might indirectly correlate with increased fitness. For example, if an invasive species leaves behind the immunobiome with which it co-evolved (Torchin *et al.* 2003), then that species might encounter a less-threatening immunobiome that requires reduced investment in immune defences. The positive effects on fitness of this enemy release might partially outweigh any negative fitness consequences associated with superfluous immunological investment. On balance, this excess investment still has the potential to reduce fitness (i.e. the gross fitness benefits of enemy release might be even greater than the net, realized fitness benefits). Therefore any mismatch in operative protection may be transient on an evolutionary timescale. In general however, immune responses that are evolutionarily advantageous in one immunobiome may not be advantageous in a different immunobiome. Operative protection might change, for example, over an annual cycle or among environments, if immune defence or immunobiotic pressures also vary. Identifying and understanding such differences in operative protection would be an important advancement over simply identifying variation in immune indices. The magnitude of an immune response need not relate directly to fitness, while knowledge

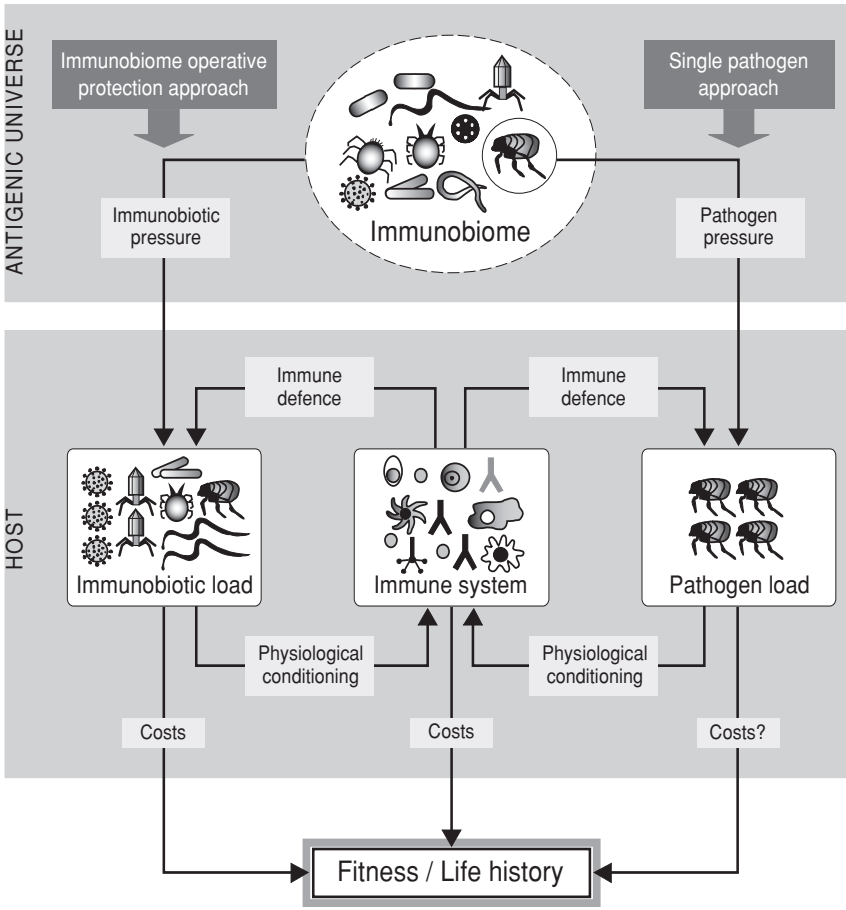


**Figure 2.2.** Operative protection requires the consideration of immune defences in terms of immunobiotic pressures. Immune systems and immunobiomes are complex, and these multivariate systems interact to shape immunobiotic load, fitness and life history. Immunobiotic load (or pathogen load in studies of single pathogens or parasites) relates to intensity of infection after the deployment of immune defences. The broadly integrative and multidimensional nature of the 'immunobiome operative protection approach' (left side of figure) offers several advantages over a 'single pathogen approach' (right side of figure). Specific components of the immunobiome may not be universal, making operative protection particularly relevant in comparative studies. Furthermore, simultaneous measurement of multiple components of the immunobiome allows for the fact that the effects of immunobiotic load (e.g. co-infections) on fitness may be interactive rather than simply additive. The costs involved in shaping fitness or life history trade-offs are also clearer when considering immunobiotic load than when considering the load of a single putative parasite or pathogen, which may impose negligible or even no fitness costs.

of changes in operative protection identifies when and where individuals, populations, or species are most at risk from disease and infection.

## Current hypotheses about pathogen pressure, a component of immunobiotic pressure, require additional data and more testing

Understanding operative protection requires insight both into immune defences and immunobiotic pressure (Fig. 2.2). In terms of pressure, researchers have generally focused their thinking on only pathogens and parasites, and not on other components of an immunobiome such as commensals. We refer to this subset of immunobiotic pressure as 'pathogen pressure'. Several hypotheses link differences in pathogen pressure (e.g. abundance, diversity) to ecological or environmental variation (Table 2.1). These hypotheses have generally been tested using measures of either host-associated pathogens (i.e. pathogen load) or immune defence (Table 2.1), but neither approach provides a complete and independent picture, or direct measures, of environmental pathogen pressure (Fig. 2.3).

### *i) Using pathogen load to test hypotheses about pathogen pressure*

In one approach, pathogen load is used as an indicator of exposure to pathogens and hence as a proxy for pathogen pressure. Pathogen load is measured as the prevalence or intensity of infection by a single pathogen, or as parameters of host-associated pathogen guilds such as species richness (Table 2.1A). A greater pathogen load is taken to indicate a stronger pathogen pressure. Yet pathogen load provides information about intensity of infection only after immune defences and other behavioural counterstrategies have been deployed (Moyer, Drown and Clayton 2002). Intrinsic to these studies are assumptions that immune defence only involves eliminating or reducing pathogen load (i.e. resistance); processes that limit the damage caused by a given pathogen load without necessarily reducing it (i.e. tolerance), are neglected (Råberg, Graham and Read 2009). Resistance, tolerance, and pathogen pressure together dictate pathogen load. High pathogen loads could indicate high pathogen pressure, but also low (or compromised) immune defences, or tolerance to the measured entities (Fig. 2.3). Pathogen load need not always equate to pathogen pressure.

### *ii) Using measures of immune function to test ideas about hypothesized pathogen pressure*

In another approach, differences in immunity among groups or locations are attributed to *a priori* assumptions regarding differences in pathogen pressure, but pathogen pressure is usually not directly measured (Table 2.1B). This approach arises from theoretical predictions that relate the inherent costs of immune defences to the evolution of immune systems that optimally match pathogen

pressure (Lochmiller and Deerenberg 2000; Bonneaud *et al.* 2003; Tschirren and Richner 2006). That is, when pathogen pressure is low, immune defences are expected to be low, and vice versa. It is unclear over short timescales however, to what extent assays of immune function reflect previous exposure to pathogens, current state of health or disease, or the degree of evolved protection (Matson 2006; Bradley and Jackson 2008). Even immune assays that are seen as measuring fundamental attributes of individuals (i.e. are significantly repeatable; Buehler *et al.* 2008) exhibit large amounts of unexplained variation. Further complications can arise when immune measures are not clearly linked to known aspects of the immunobiome. Even when identified, any such links are expected to be highly specific and might not extend beyond the circumstances of a given study system. Since later studies often continue to cite initial reports and poorly substantiated hypotheses as a basis for further predictions and new conclusions, the need for direct measures of immunobiotic pressure is real. Combined measures

**Table 2.1.** Examples of studies testing predictions about pathogen pressure.

| **A) Studies that use pathogen load only to test hypotheses about pathogen pressure** | |
|---|---|
| Variable examined | Reference |
| Cooperative breeding | Poiani 1992 |
| Sociality and group size | Snaith *et al.* 2008 |
| Migration | Figuerola and Green 2000 |
| Saline vs. freshwater environments | Piersma 1997; Figuerola 1999; Mendes *et al.* 2005 |
| Latitude | Rohde and Heap 1998; Guernier, Hochberg and Guegan 2004; Nunn *et al.* 2005; Salkeld, Trivedi and Schwarzkopf 2008 |
| **B) Studies that use immune measures only to test hypotheses about pathogen pressure** | |
| Variable examined | Reference |
| Diet | Blount *et al.* 2003 |
| Sexual promiscuity | Nunn 2002; Nunn, Gittleman and Antonovics 2003 |
| Cooperative breeding | Spottiswoode 2008 |
| Population size, group size or sociality | Nunn 2002; Semple, Cowlishaw and Bennett 2002; Nunn, Gittleman and Antonovics 2003; Wilson *et al.* 2003; Stow *et al.* 2007 |
| Migration | Møller and Erritzoe 1998 |
| Life history strategy | Nunn 2002 |
| Substrate use | Nunn 2002; Nunn, Gittleman and Antonovics 2003 |
| Risk of injury | Semple, Cowlishaw and Bennett 2002 |
| Tropical vs. temperate environments | Møller 1998 |
| Continental vs. insular environments | Matson 2006 |
| Saline vs. freshwater environments | Mendes *et al.* 2006 |

of pathogen load and immune defences may be more instructive about broad patterns of potential pathogen pressure than either measure alone. Nonetheless, conclusions regarding pathogen pressure drawn from just these combined data must still be regarded as incomplete (Fig. 2.3).

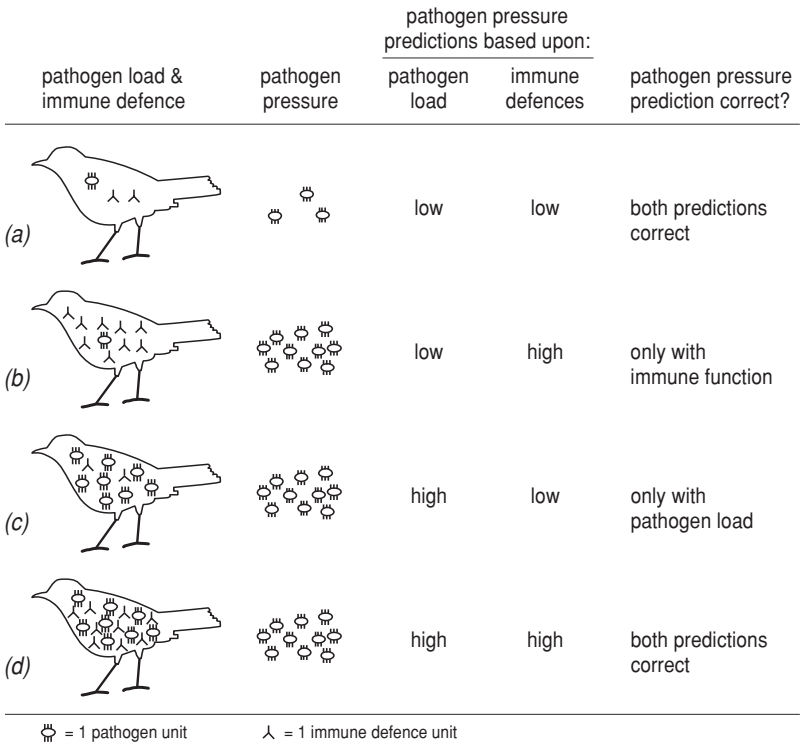


**Figure 2.3.** Many studies investigating immunobiotic pressure actually focus just on pathogen pressure. However, pathogen pressure is rarely measured directly. Instead researchers rely on pathogen load or immune function (Table 2.1 in the main text), without actually calibrating these indices with host-independent measures of pathogens. On their own, neither type of index consistently predicts pathogen pressure correctly. In the leftmost column, the birds (which could equally symbolize non-avian taxa) represent four host individuals, populations or species. In each case, pathogen loads (indicated by the number of pathogens in each bird) and immune defences (indicated by the number of antibodies in each bird) have been measured independently of each other. More antibodies equate to stronger or more fitness-enhancing immune defences; more pathogens equate to higher pathogen loads. Pathogen pressure is usually unknown in ecological immunology studies, but to illustrate our point we provide hypothetical values of pathogen pressure in the second column. More pathogens equate to greater environmental pathogen pressures. In the third and fourth columns we list predictions about pathogen pressure based on typical assumptions: higher pathogen loads indicate a greater pathogen pressure and stronger immune defences also indicate a greater pathogen pressure. In the rightmost column these predictions are evaluated in light of the hypothetical pathogen pressures.

### *An example: Immunobiotic and immune-defence perspectives lead to divergent predictions*

The study of immune defence as a life history correlate is an active area of research in ecological immunology (Tella, Scheuerlein and Ricklefs 2002; Tieleman *et al.* 2005; Martin II, Hasselquist and Wikelski 2006). For the purpose of these studies, related organisms from distinct environments and with different life history strategies are compared (Martin II *et al.* 2004). However, life history strategies tend to co-vary with environmental conditions (e.g. Tieleman, Williams and Visser 2004) and immunobiotic pressure might also co-vary with these conditions. As a result, all three factors (life history strategy, immunobiotic pressure, and abiotic environment) are potentially correlated and are difficult to disentangle. Neglecting to explicitly measure immunobiotic pressure ignores the possibility that immunological variation among different environments may relate directly to differences in immunobiotic pressure (Buehler, Piersma and Tieleman 2008) and only indirectly to disparate life history strategies. Experimental studies of individual animals could involve similarly confounded factors; manipulations aimed at directly altering immune defence could indirectly change either composition of the immunobiome or levels of exposure (Moyer, Drown and Clayton 2002). For example, experimental inflammation which makes animals more sedentary (Adelman *et al.* 2010) might increase exposure to vector-borne diseases through reduced anti-vector behaviours (e.g. less grooming). Similarly, experimental removal of immunobiotic components (e.g. specific pathogens) can alter immune phenotypes and might affect infections by other pathogens or members of immunobiomes (Ezenwa *et al.* 2010).

Using birds as an example, we illustrate one instance of confounded factors in a comparative study. Specifically, we show how simplistic predictions about immune parameters differ depending on whether they are derived from the perspective of life history strategy or immunobiotic pressure. Long-lived species generally have long development periods, which allow for diverse repertoires of antibody-producing B-lymphocytes (Lee *et al.* 2008). Over a lifetime long-lived species potentially encounter the same immunobiotic components repeatedly, so antibody-mediated acquired immunity and immunological memory may be especially valuable (Boots and Bowers 2004). Long-lived birds include those inhabiting the tropics (Wiersma *et al.* 2007), open oceans (Ricklefs 1990), and deserts (Tieleman, Williams and Visser 2004). However, these three environments are predicted to differ in terms of immunobiomes. Marine (Piersma 1997; Mendes *et al.* 2005) and xeric (Moyer, Drown and Clayton 2002; Valera *et al.* 2003) environments are hypothesized to be relatively pathogen-free, while wet tropical environments might harbour abundant and diverse pathogens (Møller 1998; Guernier, Hochberg and Guegan 2004). From a life history perspective, these 'slow-living' birds are all predicted to invest similarly in immunity and self maintenance (Lee 2006). Yet from the view point of immunobiotic (and more specifi-

cally, pathogen) pressure, tropical land birds are predicted to invest differently in immune defence than do oceanic or desert birds. In fact, the extent to which environments truly differ in either pathogen pressure or broader immunobiotic pressure is not at all clear.

## Measuring immunobiotic pressure: an immunobiome-wide approach

Veterinarians, parasitologists, ecologists and immunologists all think differently about the role of disease. Often, single pathogens or diseases are identified or analyzed outside of the contexts of ecology and evolution. In some field studies, one or more key pathogens may have been identified, and the effects of these infections on fitness might be clear. For the majority of wild hosts however, the fitness-reducing effects of most individual pathogens or parasites (or the fitness-enhancing effects of most commensals) are poorly understood. Studying the costs of infection of single pathogens is informative and should not be abandoned, but to understand immunobiotic pressure and operative protection we advocate a broader strategy. Increasingly, more attention is being devoted to understanding multiple infections, better reflecting the ecological context of individual animals, populations or species and their diseases (e.g. Jolles *et al.* 2008; Behnke *et al.* 2009; Ezenwa *et al.* 2010). Earlier authors have touched upon the importance of incorporating ecological measures (i.e. immunobiomes) into ecological studies of immunology (e.g. Sadd and Schmid-Hempel 2009; Schulenburg *et al.* 2009; Pedersen and Babayan 2011), but these ideas require further development. The assessment of operative protection necessitates descriptions of immune defence profiles that are protective against all (or at least a diverse range of) encountered pathogens and other immunobiotic components. Immunobiomes are central players, and an 'immunobiome approach' must treat them as such. We envisage researchers examining the diverse immunobiotic selective pressures that animals encounter, by exploring the immunobiomes relevant to their study systems (Alcaide *et al.* 2010). Although a daunting task, smart use of relevant technologies will make it feasible. Such an integrative effort is comparable to the Human Microbiome Project (HMP; http://nihroadmap.nih.gov/hmp), which focuses on identifying all human-associated microbes and analyzing the role of these microbes in health and disease. Similar to the HMP, we expect that the microbial component of the immunobiome will provide a particularly rich and diverse landscape to describe, since for wild animals it remains unexplored.

To start this daunting task, we suggest focusing first on microbial pathogens and microbial pathogen pressure, before expanding to the rest of the microbial immunobiome (Appendix 2.1). A range of methods, including microbiological and metagenomic techniques will be required for initial surveys and description.

Subsequently, the most relevant pathogens can be identified, and connections to specific components of immunity can be established, allowing the identification of protective immune phenotypes (Pedersen and Babayan 2011). Eventually, other subsets of the immunobiome can also be evaluated (e.g. commensals, multicellular pathogens). Then, a more complete picture can be drawn of how the immunobiome shapes immune defences, and conclusions regarding operative protection can be made (Fig. 2.2).

### *Description is innovation*

From some perspectives (e.g. that of a parasitologist), attempting to measure all the organisms associated with a host might not appear particularly novel. From the viewpoint of ecological immunology, however, assembling detailed descriptions of host-associated and habitat-associated immunobiomes is highly novel (Appendix 2.1). The first step in analyzing any community is to identify who is present and in what numbers, and without this essential foundation progress in ecological immunology will be stunted. Furthermore, it is essential for understanding the selective pressures that immunobiomes exert on the immune system. While it may not be possible to measure all relevant factors, simultaneous measurement of multiple components of the immunobiome will always be desirable because the effects of immunobiotic load (e.g. co-infections) on fitness may be interactive rather than simply additive (Jolles *et al.* 2008; Behnke *et al.* 2009; Ezenwa *et al.* 2010). As such, description represents the first level of innovation in the immunobiome approach that we promote, and will be a major advancement. By freeing researchers from the constraints of microbial culture techniques, molecular-genetic approaches dramatically increase the number of components of the immunobiome that can be surveyed. At the same time, the battery of indices available in comparative immunology continues to diversify. Integrating these indices of immunity with the newfound understanding of immunobiotic pressure will lead to a second level of innovation. Linking broadly defined immunobiotic pressures to evolution of the immune system and to operative protection remains an ultimate goal.

### *Collaboration is key*

A generalist approach to quantifying immunobiotic pressure is not without challenges, and requires careful consideration of sampling schemes and laboratory techniques. For example, samples should be collected with the interactions between host and immunobiome in mind. Outside surfaces of hosts and locations where external environment meets internal physiology, such as mucous membranes, should be targeted. Environmental substrates that hosts commonly contact, such as water sources and sleeping areas should be evaluated. The most probable routes of infection, such as ingestion with food, must also be considered. Ideally, potential pathogens found in the wider environment, including micropar-

asites and macroparasites, will be linked to hosts through ecology and habitat.

In terms of methodology, adapting procedures already used for routine monitoring of microbial communities in other applications, such as in wastewater management, air-monitoring and soil science, will be fruitful (Appendix 2.1). Likewise, reagents or methodologies already established in model and commercially-exploited animals may be suitable for studies of wild and non-model animals (Abolins *et al.* 2011). Early pioneers of ecological immunology realized that if broad comparisons were to be made, then the complexity of immune systems dictates that multiple immune components must be measured (Norris and Evans 2000). Similarly, multiple axes (e.g. diversity and abundance of archaea, bacteria, fungi and viruses) will be required to encapsulate the complexity of microbial immunobiotic pressure. Available molecular techniques allow this to be done (Appendix 2.1, Table A2.1). Individual pathogenic strains can be identified and known markers or genes of pathogens can be targeted. Of course, a sequencing approach alone does not guarantee that all pathogens and virulence-factors will be recognized. Moreover, sequence data has a limited capacity to predict the impact that a given immunobiotic element has on a particular host. Pathogenicity may vary depending on hosts' characteristics at the individual, population and species levels. Collaboration among parasitologists, microbiologists, veterinarians and other wildlife-disease experts will be essential for overcoming these issues relating to methodology and interpretation.

A next step will then be to classify components of newly described immunobiomes according to their mode of action. This will more easily allow individual components of immunobiotic pressure to be related to the particular classes of immune responses that they elicit. At some levels this will be obvious (e.g. extracellular parasites should elicit extracellular immune responses), but identifying which specific components of the immunobiome elicit which specific responses may be less clear. On one hand, pairing immune challenges and immune responses in this way is a nod to traditional immunology. On the other hand, the comparative nature and ecosystem-wide scope of this 'immunobiome approach' firmly plants this research outside the bounds of immunology and anchors it within ecology. Joining these opposing factors will allow for the development of more targeted immune assays. Eventually, specific knowledge of immunobiotic pressures will direct choice of immune assay. A clearer understanding of which parameters of immunity are most important for hosts when exposed to immunobiomes with different compositions (e.g. more or less diversity) will ultimately emerge.

## Future directions and broader impacts

From the standpoint of ecological immunology, the necessity for measuring elements of the immunobiome is to gain a better handle on the selective pressures

that shape the immune systems and define the immune-strategies of animals living in the wild. In a wider context however, these novel evolutionary and ecological perspectives on immune systems also have much to offer, both to ecologists interested in the immune system and to immunologists who wish to place immunology in the context of a more 'real' world. For example, studying the evolution of immunological defences may provide insight into the rise of autoimmune diseases (i.e. inappropriately high operative protection) and allergies in industrialized societies (Bach 2002), which in turn might point towards therapeutic treatments (Fallon and Alcami 2006). Comparing related organisms from environments with different immunobiomes (Alcaide *et al.* 2010) may shed light on the effects of artificially reduced antigenic diversity and increased sanitation in contemporary human environments (Blaser and Falkow 2009). Global transport and climate change can have potentially major repercussions in terms of additional anthropogenic alterations to the immunobiomes of humans and other organisms, e.g. birds (Van Riper III *et al.* 1986) and corals (Rosenberg and Ben-Haim 2002). The emergence of new diseases and the spread of current ones can affect societies globally. Wild animals can serve powerfully both as reservoirs and sentinels of these diseases (Daszak, Cunningham and Hyatt 2000), and these functions further highlight the value of comparative studies of immunology, ecology, and disease.


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## Appendix 2.1. Methods for measuring pathogens.

*Direct molecular techniques*

Molecular techniques have transformed microbial ecology, and many of these tools lend themselves to the development of indices of microbial immunobiotic pressure. For example, 'finger-printing' methods (sequence-based metagenomics) allow the richness of microbial assemblages to be assessed. These metagenomic techniques use genetic material recovered directly from environmental samples containing mixed populations, to study naturally occurring microbial assemblages. Function-based metagenomic approaches can provide additional information, such as the presence of markers of pathogenicity. Table A2.1 in this appendix lists molecular techniques for assessing microbial immunobiotic pressure (with a focus on pathogens), along with the specific types of questions for which different techniques are most appropriate.

Many techniques have been developed for assaying pathogens in wastewater, sewage, soil or clinical samples (Gilbride, Lee and Beaudette 2006; Malik *et al.* 2008). These methods can be easily applied both to environmentally-derived samples and those collected directly from study animals, such as faeces or surface swabs (e.g. skin, mucosa). Both ambient and exhaled air contains diverse microbial communities that can also be sampled and analyzed (West *et al.* 2008; Muscatello, Gilkerson and Browning 2009). Microbial concentrations in ambient air have previously been linked to levels of immunological responses (Huttunen *et al.* 2010).

*Other techniques*

Indirect assessments of pathogen pressure can be a helpful first step for revealing broad patterns potentially related to immunobiotic pressure. For example, disease-transmitting vectors might be a useful proxy for some subsets of pathogen pressure in some systems. However, indirect measures that rely on this type of relationship require detailed knowledge of study systems to appreciate their limitations (Keesing *et al.* 2009) and pitfalls (Fig. 2.3) and ideally always should be combined with direct approaches to validate and calibrate actual immunobiotic pressure.

In addition to indices of immune function in adults, defences by eggs and maternally transferred antibodies are promising avenues of research for ecoimmunologists interested in pathogen pressures. Avian eggs harbour microbes on their shells and under certain conditions some of these microbes can penetrate the eggshell, infect the egg's contents, and reduce viability (Cook *et al.* 2003). Chemical barriers protecting the embryo include three quantifiable antibacterial proteins (avidin, lysozyme and ovotransferrin) in the albumen (Shawkey *et al.* 2008). Combined with indices of microbes on the surface of the shell, comparisons between matched species or populations of hosts may reveal the relative pathogenicity associated with different environments or nesting strategies (Godard *et al.* 2007). This approach could also be applicable to eggs from non-

avian taxa. For example, lysozyme is present in invertebrate (Matsuura *et al.* 2007) and fish (Yousif, Albright and Evelyn 1994) eggs, and avidin occurs in amphibian eggs (Korpela *et al.* 1981).

Through pre-natal and post-natal transfer of antibodies, mothers transmit their experience of the environment to offspring (Boulinier and Staszewski 2008).

**Table A2.1.** Molecular approaches to measuring pathogens.

| Level / Question being asked | Possible methods for measuring pathogens | Pathogens applicable to | Comments |
|---|---|---|---|
| *Host-pathogen interaction:*<br>• How does the presence of a single type of pathogen correlate with immune function? | • Prevalence/abundance counts<br>• Microscopy (e.g. blood of smears, coproscopy)<br>• Culture-based techniques<br>• qPCR with pathogen-specific primers (Whyte *et al.* 2002) | • Ecto- and endoparasites<br>• <1% microbial species<br>• Any microbial species | Does not reflect the complexity of pathogen assemblages. Ignores possibility of interactions among co-infecting pathogens.<br>Often unclear which immune parameter(s) is/are most appropriate to test. Any observed correlation may be between an unmeasured pathogen and immune function.<br>Unlikely that single pathogen measures are representative of overall pathogen pressure.<br>The link between host fitness and infection with individual pathogens may be very weak. |
| *Interaction of host with the pathogen assemblage (descriptive):*<br>• How does the diversity of the pathogen assemblage correlate with immune function?<br>• How does the abundance of components of the pathogen assemblage correlate with immune function? | • Prevalence/abundance counts<br>• Microscopy<br>• Culture-based techniques<br>• Sequence-based metagenomics (community 'fingerprinting'):<br>- DGGE (Klomp *et al.* 2008<br>- RISA (Ruiz-Rodríguez *et al.* 2009)<br>- TRFLP (Hackl *et al.* 2004)<br>- Gene sequence libraries (Corby-Harris *et al.* 2007)<br>- Bar-coded pyrosequencing (Cox-Foster *et al.* 2007)<br>- Taxonomic Microarrays (Gentry *et al.* 2006) | • All microbes, either one taxonomic group at a time, or simultaneously (i.e. microarray; containing DNA sequences from candidate community members of many taxa e.g. bacteria, archaea, fungi etc.) | Prevalence/abundance counts are laborious and sample size affects the pathogen diversity recorded (Walther *et al.* 1995). Alongside visual counts, diagnostic assays dapted from the livestock/pet industries could be a used to measure large pathogens (e.g. endoparasites) (Traversa and Otranto 2009).<br>Fingerprinting methods are uninformative about the identity of members of the assemblages described; with some techniques (e.g. DGGE), follow-up sequencing can achieve this.<br>Unrepresentative of true diversity; only the most abundant members are represented. The exception is bar-coded pyrosequencing, which allows deeper coverage of microbial diversity.<br>Provides some information about which features of the assemblage might correlate with immune function. Unclear as to cause and effect; is diversity of the pathogen assemblage driven by immune defences, or vice versa? (Bordes and Morand 2009). |

Levels of antibodies in eggs are proportional to levels circulating in the mother (Grindstaff 2008), and recent or repeated exposure to pathogens probably increases the level of antibodies in the circulation. Specificity and amounts of maternally transmitted antibodies may therefore be informative about those pathogens found in the local environment that young first encounter.

| Level / Question being asked | Possible methods for measuring pathogens | Pathogens applicable to | Comments |
|---|---|---|---|
| *Interaction of host with the pathogen assemblage (functional):*<br><br>• Which attributes or components of the pathogen assemblage correlate with immune function?<br><br>• Do microbial assemblages with higher 'pathogenic function' (e.g. greater no. of pathogenic markers) correlate with higher indices of host immune defence? | • Function-based metagenomics:<br>- Large insert libraries (Treusch *et al.* 2004)<br>- Meta-transcriptomes - sequencing expressed genetic information e.g. mRNA, protein (Bailly *et al.* 2007)<br>- Functional microarrays - containing DNA sequences for genes of known function e.g. virulence factors (Gentry *et al.* 2006) | • All microbes, but more difficult with eukaryotic microorganisms due to amount of genetic information present. | Markers of pathogenicity include pathogenicity islands, antibiotic resistance, virulence factors, genes associated with secretion systems, sequence homology with other known pathogenic species (Finlay and Falkow 1997).<br>Even though the identity of members of the pathogen assemblage may be initially unknown, it is still possible to examine functionality.<br>Provides more information about which features of the pathogen assemblage might correlate with immune function e.g. diversity, abundance or presence of certain members of the assemblage.<br>Currently more expensive and technically advanced than other techniques |

Abbreviations: qPCR - quantitative PCR; DGGE - denaturing gel gradient electrophoresis; RISA - ribosomal intergenic spacer analysis; TRFLP - terminal restriction fragment length polymorphism