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ECOLOGICAL IMMUNOLOGY

Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology

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Summary

1. Ecoimmunologists aim to understand the costs, benefits, and net fitness consequences of different strategies for immune defense.

2. Measuring the fitness consequences of immune responses is difficult, partly because of complex relationships between host fitness and the within-host density of parasites and immunological cells or molecules. In particular, neither the strongest immune responses nor the lowest parasite densities necessarily maximize host fitness.

3. Here, we propose that ecoimmunologists should routinely endeavour to measure three intertwined parameters: host fitness, parasite density, and relevant immune responses. We further propose that analyses of relationships among these traits would benefit from the statistical machinery used for analyses of phenotypic plasticity and/or methods that are robust to the bi-directional causation inherent in host-parasite relationships. For example, analyses of how host fitness depends upon parasite density, which is an evolutionary ecological definition of tolerance, would benefit from these more robust methods.

4. Together, these steps promote rigorous quantification of the fitness consequences of immune responses. Such quantification is essential if ecoimmunologists are to decipher causes of immune polymorphism in nature and predict trajectories of natural selection on immune defense.

Key-words: bivariate statistics, *Daphnia*, evolutionary parasitology, immunocompetence, optimal immunity, random regression, resistance, tolerance

Introduction

Hosts vary greatly in the strength of their immune responses and their capacity to defend themselves against parasites. Ecoimmunologists shed light on this variation by characterizing optimal defense strategies in a world of life-history tradeoffs, unpredictable epidemics, polyparasitism, and genetic and environmental variation (Medley 2002; Rolff & Siva-Jothy 2003; Lazzaro & Little 2009; Sadd & Schmid-Hempel 2009). Accordingly, a basic requirement of empirical studies in ecoimmunology is to measure and interpret the fitness consequences of immune responses – in other words, to ascertain the impact of cellular or molecular responses to infection (hereafter, 'immune responses') upon the lifetime reproductive success (hereafter, 'fitness') of the responder. But this basic requirement poses serious challenges.

Ecoimmunologists increasingly appreciate that two 'shortcuts' to estimating the fitness consequences of immune responses must be avoided. The first is to count immunological cells or molecules and assume that hosts producing the most hemocytes or antibodies, for example, are the most fit (e.g. Nunn, Gittleman & Antonovics 2000 as critiqued by Read & Allen 2000). The second is to quantify parasite densities and assume that hosts bearing the most parasites are the least fit (e.g. see critique in Behnke, Barnard & Wakelin 1992). These shortcuts fail because the magnitude of an immune response does not always correlate positively with host fitness

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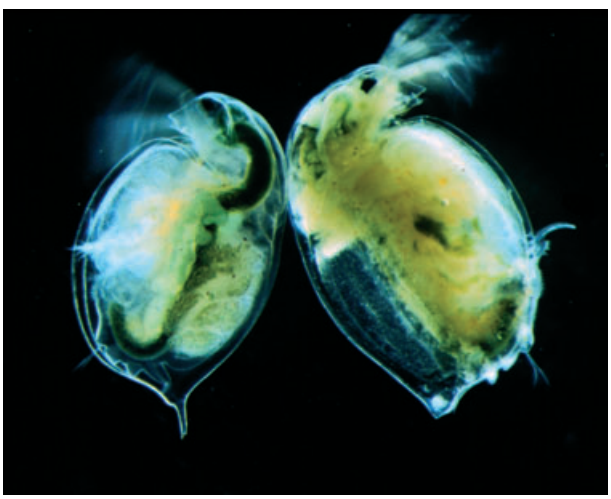
(Adamo 2004; Graham, Allen & Read 2005; Rolff & Siva-Jothy 2003, Sadd & Schmid-Hempel 2009, Viney, Riley & Buchanan 2005), and hosts that kill all of their parasites are not necessarily better off: host fitness may be maximal at some intermediate parasite density (Behnke, Barnard & Wakelin 1992; Viney, Riley & Buchanan 2005; Stjernman, Raberg & Nilsson 2008). As a result, the relationship between host fitness and parasite density – sometimes called tolerance by evolutionary ecologists – has received a lot of attention lately (Raberg, Sim & Read 2007; Ayres & Schneider 2008, 2009; Pagan, Alonso-Blanco & Garcia-Arenal 2009; Raberg, Graham & Read 2009); also see summary of controversy below.

Here, we aim to cement the view that ecoimmunologists should aim to quantify how host fitness is affected by both parasite density and immune response magnitude. Measuring this triad of traits offers the best opportunity to interpret ecological variation in immunity. We stress that each trait is likely to be the product of an interplay between host and parasite genes, which has important consequences for empirical practice and for inferring evolutionary outcomes. We propose that a combination of controlled experiments and statistical

Box 1. From evolutionary genetics to ecoimmunology in lab and field: *Daphnia magna–Pasteuria ramosa* as a 'model' system

Daphnia are small (~1–3 mm), ubiquitous freshwater crustaceans that have been the focus of a large and diverse literature, including toxicology, life-history, physiology, nutrition and parasitology. *Daphnia* were also the subject of pioneering work on invertebrate cellular immunology (Metchnikoff 1884), an area that has recently been revisited within the ecoimmunology framework (Auld, Scholefield & Little 2010) (Boxes 2 and 3). In the field, gathering epidemiological data is relatively straightforward because the clear carapace of *Daphnia* makes many infections easy to identify. In the photograph, the left *D. magna* is healthy (note embryos in the brood chamber), while the right *D. magna* is infected with the bacterium *Pasteuria ramosa,* which sterilizes hosts leading to an empty brood chamber (a clear indication of reduced host fitness). Epidemics are common and severe in this system, but highly variable in space and time (Stirnadel & Ebert 1997; Duncan, Mitchell & Little 2006; Lass & Ebert 2006; Duncan & Little 2007). With parasite density and indeed parasite fitness being further quantifiable because transmission spores are easily counted, the recommended triad of traits – host fitness, within-host parasite density, and immune response magnitude – are measurable.

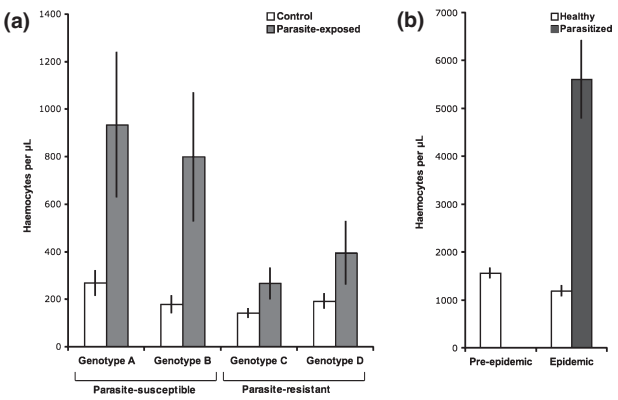


Adding power to these studies is the possibility to gain insight into genetic effects through controlled experimentation. Especially important for this experimentation is the fact that *Daphnia* are facultative parthenogens and can be cloned, which enables precise comparison of genetic backgrounds, or the study of different environments on replicates of the same genetic background. Experiments on susceptibility of *D. magna* to *P. ramosa* have revealed extensive genetic variation in both hosts and parasites (Ebert, Zschokke-Rohringer & Carius 1998; Little & Ebert 1999, 2000, 2001), including genetic specificity – that is, host genotype by parasite genotype interactions where the susceptibility of a host genotype is tightly dependent on the parasite strain to which it is exposed (Carius, Little & Ebert 2001). Similar 'context-dependence' has been revealed when hosts and parasites have been studied under different environmental conditions (genotype by environment interactions; Vale & Little 2009; Vale, Stjernman & Little 2008). Furthermore, the short generation time of *Daphnia* (~ 10 days) enables the study of real-time evolutionary responses to parasites (Little & Ebert 1999, Duncan & Little 2007, Zbinden, Haag & Ebert 2008). The *Daphnia* system is also unique for the accessibility of reconstruction of historical genetic changes via the resurrection of resting stages (Limburg & Weider 2002; Decaestecker *et al.* 2007).

Box 2. An immune measure for the dead

The study of a putative immune response in the crustacean *Daphnia* provides a simple yet striking example of the dangers of assuming that a stronger immune response represents greater host fitness. Immune responsiveness in *Daphnia* can be estimated by extracting a small amount of hemolymph and counting the abundant plasmatocytes (cells that appear to have phagocytic function). Different genotypes of *D. magna* show markedly different susceptibilities (Carius, Little & Ebert 2001) to the naturally coevolving bacterial pathogen *P. ramosa,* and recent work has revealed that immune responses are evident only in susceptible genotypes (see panel A below; Auld, Scholefield & Little 2010).

These data are from an experiment involving four long-term laboratory *Daphnia* lines for which resistance characteristics are well-established. Two lines are highly susceptible and two are highly resistant, and replicate hosts from each line were either exposed or not exposed (controls) to a spore suspension of *P. ramosa*. Compared to their controls, the susceptible genotypes showed a substantial increase in the number of circulating phagocytes in an 8-h period of exposure (data are a mean of six replicates studied from four time points: 2, 4, 6, and 8 h of exposure). An expanded data set on sixteen host genotypes largely confirmed this pattern (See Auld, Scholefield & Little 2010). Thus, *D. magna* may have a two-stage defence – a genetically determined barrier to parasite establishment, and a cellular response once establishment has begun. A strong immune response is a marker for susceptibility rather than resistance.



This result has since been borne out in field studies comparing hemocyte counts in naturally infected and uninfected hosts. Many *D. magna* populations experience summer epidemics of *P. ramosa,* and it can be shown that pre-epidemic hosts (which are of course not infected) have low hemocyte counts, comparable to healthy hosts during the epidemic period (panel B, above). The pre-epidemic samples represent a mean from three sampling dates in May, 2009, whilst the epidemic samples represent a mean of 13 sampling dates spread from June to October 2009 when *P. ramosa* was common in the population; S. K. J. R. Auld, A. L. Graham & T. J. Little, unpublished). *Pasteuria ramosa* sterilizes its host, and so hosts showing signs of infection (and thus high cell counts) will not directly contribute genes to the next generation. Thus, in the *D. magna–P. ramosa* interaction, a strong immune response is not associated with high fitness, but rather is tightly linked to being genetically dead.

methodologies borrowed from other branches of biology can disentangle relationships among the three traits. Our statistical advice is focused on rigorous exploration of relationships between host fitness and parasite density (i.e. evolutionary ecological tolerance).

WHEN MORE IS BLATANTLY NOT MORE: AN EXAMPLE

We begin by illustrating the benefits of three-trait data sets with an example, the crustacean *Daphnia magna* infected with the bacterium *Pasteuria ramosa* (Box 1). Several decades of both laboratory and field research have generated a deep understanding of the fitness consequences of parasitism in *D. magna* (Ebert 2005). Consequently, unlike ecoimmunological work in which hemocyte or white blood cell densities, for example, are quantified without knowledge of host fitness or relevant parasite biology, ecoimmunology of *D. magna* can be undertaken with extensive knowledge of potential evolutionary outcomes. Different host genotypes show markedly different susceptibilities to infection (Carius, Little & Ebert 2001), and yet after exposure, densities of responding hemocytes are highest in susceptible genotypes (Auld, Scholefield & Little 2010) (Box 2). Had hemocyte densities been measured in *D. magna* hosts without either prior knowledge of the system or knowledge of the infection status of individuals – that is, without the understanding that cellular responses are a marker for both genetic susceptibility and infection – we might have naively concluded that hosts with highest hemocyte densities would have the highest fitness. However, hosts with the most hemocytes actually tend to have the lowest fitness because they're infected with a sterilizing parasite! This example strikingly demonstrates that more is not necessarily more

Table 1. An array of ecoimmunological study designs which may be experimental or observational, performed in the field, the laboratory, or both

Design	Description	Possible measurements	Examples
1	Experimental: induce immune response to non-infectious agents in the field or lab	Host fitness *Immune response* *Density of natural parasites*	a, b, c
2	Experimental: infect with different doses of parasites, primarily in the lab	Host fitness *Immune response* *Parasite density*	d, e, f, g, h, i
3	Experimental: infect with different parasite genotypes, primarily in the lab	Host fitness *Immune response* *Parasite density*	j, k, l
4	Experimental: remove parasites in the field or lab	Host fitness *Immune response* Parasite density	m, n
5	Observational studies in the field	Host fitness Immune response Density of natural parasites	o, p

We argue that nearly any design would benefit from inclusion of immune response and parasite density measurements, to accompany measurement of host fitness. Possible measurements in plain text are not optional; items in *italics* are optional but recommended (see text). [a]Bonneaud *et al*. (2003), [b]Moret & Schmid-Hempel (2000), [c]Raberg & Stjernman (2003), [d]Ben-Ami, Ebert & Regoes (2010), [e]Bleay *et al*. (2007), [f]Lundgren & Thorpe (1966a), [g]Lundgren, Thorpe & Haskell (1966b), [h]Nol, Olsen & Rhyan (2009), [i]Xiao *et al*. (2005), [j]Carius, Little & Ebert (2001), [k]Grech, Watt & Read (2006), [l]Raberg, Sim & Read (2007), [m]Hudson, Dobson & Newborn (1998), [n]Pedersen & Greives (2008), [o]Norris, Anwar & Read (1994), [p]Stjernman, Raberg & Nilsson (2008).

in immunology, that well-studied host-parasite systems may be poised to make major contributions to ecoimmunology, and that host fitness and parasite densities [or other readouts of the efficacy of defense (Adamo 2004; Viney, Riley & Buchanan 2005)] must be measured alongside immune responses.

Three key traits in the context of ecoimmunological study designs

Various study designs enable ecoimmunologists to quantify the fitness consequences of immune responses (Table 1). Here, we highlight the role that the three focal measurements (host fitness, immune response magnitude, and parasite density) can play in each, to emphasize that more measurements per study rather than radically new study designs will go a long way to improving empirical ecoimmunology. We illustrate with examples, but have not attempted to be exhaustive.

We make several qualifications from the outset. First, fitness in terms of lifetime reproductive success is not easy to measure, but it remains an aspiration. Proxies such as annual survival, annual fecundity, or health must have demonstrated relevance to true fitness for the system under study. Secondly, the appropriate immunological and parasitological measurement(s) will vary greatly from system to system. We discuss how to promote selection of relevant parameters below. Thirdly, when quantification of parasite density is impossible but longitudinal studies are feasible (for instance, studies undertaken on free-ranging animal populations in the wild), duration of infection (e.g. days parasite positive) might in principle serve as the parasitological readout, though we know of no such studies to date. Fourthly, we caution that multiple independently-derived stocks of the parasite or immunostimulant may be needed, depending on the level of generalization desired. For instance, if only one strain of *Plasmodium* was used in an experiment [or indeed in years of experiments, as frequently observed in laboratory infection models (Viney 2006)], it is difficult to generalize to the fitness consequences of malaria as these may differ dramatically across strains/species. Finally, field and laboratory research have different weaknesses. In particular, field studies may be confounded by unknown exposure histories of hosts, whereas lab studies often use both host and parasite strains of restricted genetic diversity (Viney 2006). We believe that the most powerful ecoimmunological studies will combine such data (e.g. Box 2) and would encourage development of more systems that span the field-lab divide while quantifying host fitness, parasite density, and immune response magnitude.

DESIGN 1: EXPERIMENTS IN THE ABSENCE OF INFECTION

A common ecoimmunological study design involves non-infectious experimental manipulations such as injection with agents that spark immune responses [e.g. lipopolysaccharide (LPS) or vaccines; Design 1 in Table 1]. For example, injection of LPS into house sparrows followed by fitness measurements demonstrated that reproductive costs of immune responses may be compensated for by greater investment in the next clutch, among other mechanisms (Bonneaud *et al*. 2003). Injection of LPS into bumblebees demonstrated that survival costs of immune responses might only be expressed when resources are limited (Moret & Schmid-Hempel 2000). A key advantage of using parasite mimics rather than true infections is avoidance of the confounding influence of the mechanisms the parasite uses to circumvent immune responses (Huxham, Lackie & Mccorkindale 1989; Barnes & Siva-Jothy 2000).

Studies of Design 1 can be enriched by measurement of cellular or molecular immune responses. A particularly good example is the study of blue tits injected with tetanus-diphtheria vaccine, in which survival was monitored and vaccine-specific antibodies measured; a major finding was stabilizing selection on primary antibody responses to diphtheria (Raberg & Stjernman 2003). In other words, birds with either very weak or very strong responses to that antigen were unlikely to survive the winter. The birds probably do not experience diphtheria. Instead, the titre of vaccine-induced antibodies might be considered an index of overall immune responsiveness: weak responders are presumably prone to infectious diseases in general, hence their high mortality rate, while the high mortality rate of strong responders might arise from general or vaccine-induced costs of immunity (Raberg & Stjernman 2003).

For any study of Design 1, a difficulty is that the relevance of the induced response to an animal's ability to fight a real infection is rarely known (Adamo 2004; Staszewski & Boulinier 2004; Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2006). For example, does the magnitude of response to LPS predict responsiveness to live bacteria? Similar questions arise for the assumed relationship between diphtheria-specific antibodies and resistance to real infections of the blue tits described above. In principle, studies of Design 1 can be broadened to include measurement of the within-host densities of relevant parasites. This enables researchers to address whether strong responses to immunostimulants are correlated with lower prevalence or intensity of real infections (e.g. Lee *et al*. 2006). Indeed, we support calls for studies of Design 1 to provide 'functional readouts' (Viney, Riley & Buchanan 2005) or 'host resistance tests' (Adamo 2004) that lend insight into the ability of hosts to fight real infections.

DESIGNS 2–4: EXPERIMENTS IN WHICH INFECTIONS ARE ADDED OR REMOVED

The fitness consequences of strong immune responses probably depend upon the number and genotype of parasites with which a host is infected. Ecoimmunological experiments in which infections are added to or removed from hosts (Designs 2–4) aim to test that hypothesis. Just as data on immune response magnitude and/or parasite density make Design 1 studies more informative, the same applies to these designs.

Design 2, in which hosts are challenged with varying doses of live parasites, is commonplace in biomedical research, with the dose at which 50% of hosts can no longer prevent infection (infectious dose, ID_{50}) or survive infection (lethal dose, LD_{50}) serving as indices of host susceptibility. Indeed, dose-response experiments can reveal whether completely resistant host genotypes exist and, more generally, quantify the distribution of host susceptibility in a population (Ben-Ami, Ebert & Regoes 2010). When accompanied by immunological measurements, such experiments can also demonstrate whether there is a threshold number of parasites above which immune elements are induced, qualitatively altered, or else overcome (Bleay *et al*. 2007). If hosts die above a particular inoculating dose despite controlling parasite numbers, then disease may be due to a cytokine storm (uncontrolled production of signalling molecules, particularly by the innate immune system) or other immunopathology (Graham, Allen & Read 2005). A virulence factor of methicillin-resistant *Staphylococcus aureus* (MRSA) exhibits such dose-dependence: at low doses it induces protective innate immune responses, while at high doses it induces septic shock (Yoong & Pier 2010). The severity of other infections may entail similar dose-dependent shifts to immunopathology (e.g. among microparasites of vertebrate hosts (Schmid-Hempel & Frank 2007)). Such patterns have even been observed in invertebrates. In *D. magna*, for example, very high spore doses of *P. ramosa* may lead to drastic reductions in host fitness, even though parasite density often decreases with increasing dose (Ebert, Zschokke-Rohringer & Carius 2000). The benefits and costs of strong immune responses can therefore be obscured in studies of Design 2 unless parasite density and/or immune response magnitude are also measured as experimental outcomes.

Design 3, in which the experimenter varies the parasite genotype or species to which hosts are exposed, is indispensable for identification of genetic specificity of attack and defense that underpins so much of co-evolutionary theory (e.g. Carius, Little & Ebert 2001; Grech, Watt & Read 2006). Again, parasite density and immunological measurements aid interpretation by providing some mechanistic detail of within-host events. For example, whether the sickest hosts bear high parasite densities, cytokine storms, or both, can be shaped by parasite genotype (Long *et al*. 2008) and lead to different evolutionary trajectories (Day, Graham & Read 2007).

Design 3, accompanied by parasite density measurements, was used in the first declared test for tolerance in animals (Raberg, Sim & Read 2007). The study demonstrated that host genetic background conditioned how fitness (i.e. health of laboratory mice, in this case anaemia and cachexia) changed with increasing malaria parasite density. Mouse strains that experienced the shallowest declines in fitness with increasing parasite density were considered the most tolerant (Raberg, Sim & Read 2007). However, interpretational problems arise when parasite diversity and density are confounded – more generally, when density is not experimentally controlled – or when tolerance mechanisms are unknown, as discussed in detail below.

For a variety of ethical and logistical reasons, both Designs 2 and 3 may be difficult to apply outside the laboratory. For example, one may (rightly) be forbidden to infect wild animals experimentally. A possible exception would be to add ecoimmunological analysis onto epidemiological susceptibility studies such as those used to assess the potential for wild hosts to sustain transmission of zoonotic infections such as rickettsia, brucellosis, or monkeypox (Lundgren & Thorpe 1966a; Lundgren, Thorpe & Haskell 1966b; Xiao *et al*. 2005; Nol, Olsen & Rhyan 2009).

Better yet, Design 4, in which parasites are experimentally removed from wild hosts, is likely to be informative and

applicable across a wide variety of systems. Such experiments have been used to quantify how parasites (particularly nematodes) regulate host population size (Hudson, Dobson & Newborn 1998; Pedersen & Greives 2008), but the experiments can also reveal costs of parasitism borne by individuals and, in principle, the costs and benefits of immune responses (Pedersen 2005, Pedersen & Greives 2008). For example, following clearance of nematodes, measurements of the density of other parasites and the magnitude of subsequent immune responses can disentangle mechanisms of within-host interaction, as has been advocated for observational studies (Bradley & Jackson 2008). Design 4 seems a rich vein for future experimentation in ecoimmunology.

DESIGN 5: ECOIMMUNOLOGICAL OBSERVATIONS

When fitness measurements are coupled with data on parasite densities and/or immune response magnitude, purely observational studies can also yield rich insights (Norris, Anwar & Read 1994; Stjernman, Raberg & Nilsson 2008). For example, blue tits with both very low and very high densities of Apicomplexan parasites exhibit reduced overwinter survival (Stjernman, Raberg & Nilsson 2008). The data suggest that strong immune responses themselves are associated with mortality risk, while weak immune responses increase risk of mortality due to infection. Such an inference would be supported by evidence that birds with the lowest parasite densities exhibit the strongest parasite-specific immune responses. To our knowledge, such a data set does not yet exist, though the data of Raberg & Stjernman (2003) on vaccine-specific antibody and survival of blue tits (discussed above) lend support. Another observational ecoimmunological study – of the Soay sheep of St. Kilda – gains tremendous power via longitudinal tracking of survival, fecundity, and lifelong parasite densities of individual sheep (Clutton-Brock & Pemberton 2004). Immunological measurements have now demonstrated an association between antibody titres and the ability of sheep to resist nematodes (Coltman *et al.* 2001) and to survive harsh winters (Graham *et al.* in press).

One problem with observational studies is that a wild host that bears few parasites might not necessarily be resistant to infection, but might instead have avoided exposure (Sheldon & Verhulst 1996). It is sometimes possible to pair observational data with experiments that distinguish these distinct causes of parasite density – for example, in the case of potential environmental influences on both exposure and susceptibility of amphibians to trematode infections (Rohr *et al.* 2008) or dose-response experiments on *D. magna* (Ben-Ami, Ebert & Regoes 2010). However, when controlled experiments are impossible, immune response measurements can also help to distinguish whether exposure or resistance best explains low parasite density (Bradley & Jackson 2008). For example, if helminth-free hosts bore high titres of IgE, then the inference of resistance to infection would be supported (Bradley & Jackson 2008).

WHICH PARASITES AND IMMUNE RESPONSES TO MEASURE?

The examples above highlight the value of measuring parasite density and/or immune response magnitude in the context of most ecoimmunology study designs, to 'open the black box' of mechanisms operating within hosts. For study systems that are not yet well characterized, exactly what to measure may not be obvious – for example, if the entire parasite fauna of the focal host species is unknown, or if the type of immune response required to kill a particular parasite is difficult to extract from the encyclopaedia of immunological possibilities. We suggest that opening the black box enough to permit evolutionary ecological inference does not require hugely specialized knowledge of parasitology and immunology. It does require dedication, however, and a willingness to think beyond LPS, phytohemagglutinin (PHA), sheep red blood cells (sRBC), and other tried and true but nonetheless limited workhorses of ecoimmunology (Adamo 2004; Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2006).

Of course, the final decision of what to measure hinges on both relevance and feasibility. Relevant parasites are likely to be the most prevalent/abundant in the environment or in hosts, though they might also be parasites that are rare but cause severe disease (Grenfell & Dobson 1995). The over 130 years of publications in parasitology and infection biology may provide excellent clues on what parasite(s) to measure, especially if related host species have received attention. Feasible parasites are those for whom samples can be obtained, ideally noninvasively, and for whom density (or at least prevalence) can be quantified. Blood and faeces are good places to begin looking for parasites of vertebrates (or invertebrates; e.g. Lazzaro, Sackton & Clark 2006), and for parasites such as helminths and protozoa, little more than vital stains and basic microscopes might be required. PCR-based techniques can make the detection of parasites feasible from almost any tissue.

The relevant immune response to measure often follows on from the relevant parasites, because the immune system to a large extent must tailor parasite killing mechanisms to the size, location (intracellular vs. extracellular, as well as gut vs. blood vs. other anatomical location), and route of entry of parasites (Schmid-Hempel 2005; Weaver & Murphy 2007). Thus, for example, if nematodes are prevalent and deadly, as among the Soay sheep, then it makes sense to target nematode-specific IgA for measurement (Clutton-Brock & Pemberton 2004). If instead blood-borne Apicomplexans are prevalent and deadly, as among Hawaiian birds, then it would be better to measure malaria-specific cytophilic IgY (Lee *et al.* 2006). Targeted measurement of cellular responses in *D. magna* (Box 2) and other invertebrates makes sense because many innate immune responses are based primarily on phagocytic cells (e.g. Elrod-Erickson, Mishra & Schneider (2000). These cells also generate non-specific reactive oxygen and nitrogen species or phenoloxidase that destroy pathogens and can

also be measured (Rolff & Siva-Jothy 2003; Rivero 2006). In vertebrates, it can be also be informative to measure non-specific molecules such as complement or natural antibody (Adamo 2004). Feasibility for immunological measurements is determined by the availability or development of appropriate tools for each host species (Bradley & Jackson 2008). We do not underestimate the difficulty of this enterprise (Matson *et al*. 2006), but we also feel that the benefits of working with real parasites and real immune responses (see also Martin, Weil & Nelson 2006) cannot be overstated.

Relationships among traits

Of course, choosing the right parasites and immune responses to measure is just one step. Next, the causal relationships among traits must be considered. This issue was highlighted at the beginning of this article with the *Daphnia* example, where a large immune response indicates susceptibility. The general point is that an immune response of a particular magnitude can either be a cause OR a consequence of a particular parasite density. For example, a high antigen-specific antibody titre can be indicative of resistance to infection by parasites bearing that antigen, but it can also indicate persistence of that antigen in the host.

Measuring both parasites and relevant immune responses is key to resolving directionality, because a negative correlation between them is predicted if immune responses cause resistance, whereas a positive correlation is predicted if immune responses merely reflect antigen load or present parasite density (see also Sheldon & Verhulst 1996; Lee *et al*. 2006, Whiteman *et al*. 2006; Bradley & Jackson 2008). If the magnitude of an appropriate effector immune response is uncorrelated with parasite density, then tolerance may be at work. That said, the magnitude and even the sign of these relationships can change over the course of infection. For example, early in infection, as immune responses ramp up, there may be a positive association between parasite densities and concentrations of immunological molecules. Later in infection, once most parasites have been cleared, the correlation may become negative. Controlled laboratory experiments will be critical to clarify these dynamics. Manipulative experiments in which immunological tools like monoclonal antibodies are used to alter levels of effector activity (e.g. Long *et al*. 2008) can reveal the extent to which particular immunological cells or molecules control parasite density in some systems. Longitudinal field studies – for example, of the dynamics of *Borrelia* exposure and *Borrelia*-specific antibodies in seabirds (Staszewski *et al*. 2007) – may also be informative. Indeed, theoretical groundwork for exploring relationships between parasite density and immune response magnitude has been laid, but data are lacking (Fenton & Perkins 2010).

Another key relationship in our triad of recommended traits is that between parasite density and host fitness. In the rest of this section, we outline analytical problems inherent in the study of this relationship and propose statistical solutions that should apply equally to relationships among all traits in the triad.

DEFINING TOLERANCE

Evolutionary ecologists have come to call the relationship between host fitness and parasite density tolerance (Raberg, Sim & Read 2007; Ayres & Schneider 2008, Ayres & Schneider 2009, Pagan, Alonso-Blanco & Garcia-Arenal 2009; Raberg, Graham & Read 2009). We note that this differs from the definition of tolerance in vertebrate immunology – that is, a lack of responsiveness to antigen that is actively maintained by cells of the immune system and essential to avoiding autoimmunity, for example (Abbas *et al*. 2004). However, we also note that cellular tolerance of parasite antigens can lead to organismal tolerance of parasites (Mills 2004), so the verbal definitions are not entirely at odds. The quantitative definition of tolerance poses greater challenges.

Tolerance according to the evolutionary ecological definition is the ability of hosts to limit the fitness costs of a given parasite density, but the quantitative definition has varied. In some theoretical (e.g. Roy & Kirchner (2000) and empirical (e.g. Ayres & Schneider 2008) studies, tolerance has been considered at a single parasite density, where two host genotypes bear the same number of parasites, but one genotype achieves higher fitness and is thus more tolerant of a given parasite density ['point tolerance' (Little *et al*. 2010)]. In other studies, tolerance has been considered a slope, quantifying how host fitness declines with increasing parasite density; more tolerant genotypes lose fitness less quickly as densities increase ['range tolerance' (Little *et al*. 2010)]. Genetic variation for range tolerance of rodent malaria was studied by Raberg, Sim & Read (2007), using an approach in line with studies of tolerance to herbivory (Tiffin & Rausher 1999; Simms 2000), though in plant studies the focus has been fitness (e.g. seed set) per unit of direct and measurable *damage* (e.g. leaf area lost due to herbivore chewing), while animal studies have thus far focused on fitness per parasite (see Baucom & de Roode in this issue). What is worrying is that alternative quantitative definitions – that is, point vs. range tolerance – can generate contradictory conclusions. For example, for two host genotypes that differ in range tolerance, their reaction norms will cross at some point in the range of parasite densities. If tolerance is estimated from relative fitness at a single parasite density, then the conclusion of which genotype is most tolerant depends upon where in the density range the underlying reaction norms cross, and the density at which point tolerance measurements are made (discussed in detail in Little *et al*. 2010).

Whenever possible (e.g. via dose-response experiments) range tolerance seems preferable to point tolerance to provide more comprehensive information about the fitness consequences of different defense strategies. However, range tolerance also raises complex analytical issues familiar to evolutionary biologists who study traits shaped by phenotypic plasticity or co-evolution.

HOW FITNESS DEPENDS ON PARASITE DENSITY: TOLERANCE AS PLASTICITY

If fitness is measured across a range of parasite densities, then range tolerance is directly analogous to the concept of plasticity under a linear reaction norm model (Scheiner 1993). It therefore seems likely that recent methodological advances in modelling phenotypic plasticity might usefully be applied to studies of tolerance. For example, fitness (W) of host genotype i at parasite density D might be modelled as:

$$W_{iD} = \mu + d.D + g_i + e \quad \text{Model 1}$$

where μ is the overall mean fitness, d is the average regression of fitness on parasite density (i.e. the mean range tolerance), g_i is the effect (relative to the overall mean) of having genotype i, and e is a residual error. In practical terms, this model could be parameterized as a linear mixed effect model with g_i fitted as a random effect. This would allow estimation of the variance in g_i, which is properly interpreted as an estimate of the genetic variance for host fitness (under a parasite challenge) in the population from which tested host genotypes were drawn. However, Model 1 is only appropriate if the host genotypes differ in their average fitness (i.e. there is among-genotype variance in g_i) and not in the slopes of their regressions on parasite density. When this holds, estimates of point tolerance will yield the same fitness ranking of host genotypes regardless of the value of D at which they are tested [i.e. the reaction norms do not cross (Little *et al.* 2010)].

Alternatively, g_i may itself depend on D if range tolerances differ between genotypes. Assuming that a linear model of this dependence of g_i on D is appropriate we should then expand our model such that:

$$W_{iD} = \mu + d.D + g_{\text{int}.i} + g_{\text{slope}.i}.\ D + e \quad \text{Model 2}$$

where $g_{\text{int}.i}$ is a genotype-specific effect on mean host fitness (relative to μ) while $g_{\text{slope}.i}$ is a genotype-specific effect on the regression of host fitness on parasite density. This model could be parameterized by adding a genotype by parasite density term to the random effect structure of the mixed model in a random regression (so-called because the regression is contained with the random effect structure of the model). This approach is increasingly being used to model reaction norms across environmental gradients (Nussey, Wilson & Brommer 2007). On a practical note, it is often useful to zero-centre the D axis such that the estimate of variance in g_{int} can be interpreted as the genetic variance for fitness under an average parasite density (i.e. when $D = 0$).

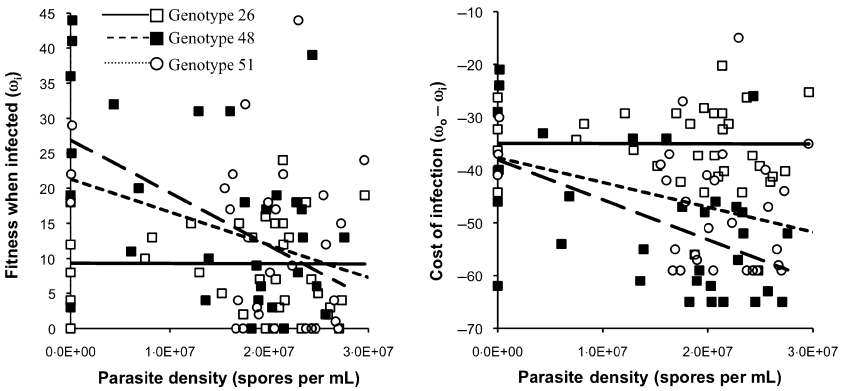
However, the key point to take from Model 2 is that, as outlined verbally by Little *et al.* (2010), if genotypes differ in their reaction norm slopes (i.e. there is variance in g_{slope}) then we expect the relative fitness ranking of different genotypes to change with D (though not necessarily within the range of parasite densities tested, nor within a biologically relevant range). A second point to note is that by formulating Model 2 as a mixed effect model a researcher can – and should – explicitly account for the covariance between reaction norm slopes and intercepts. Failure to account for this covariance can generate biologically misleading results because the information needed for evolutionary inference will often be influenced by the way in which tolerance relates to fitness in the absence of infection (the intercept). Host genotypes will almost certainly show fitness differences in the absence of infection – that is, genetically determined life-history variation is common (Stearns 1992). These differences may be linked to variation in the traits that contribute to defense via pleiotropy, as follows. One scenario is where defense against parasites is traded-off against vigor – that is, where a host possessing an allele that confers more potent defense is less fit than other genotypes when parasites are not around. But even in the absence of trade-offs, measurement of fitness of both infected and uninfected hosts is key, and *a priori* omission of intercepts from analyses of range tolerance (e.g. Raberg, Sim & Read 2007) may greatly limit inference about evolutionary outcomes. In Box 3, we illustrate this using data from the *D. magna–P. ramosa* system. Measuring the intercept of the reaction norm should be routine in laboratory studies of tolerance in which it is feasible to include control animals that are unexposed to the focal infection.

HOW FITNESS DEPENDS ON PARASITE DENSITY: CAUSATION AND CO-EVOLUTION

Another concern about the study of range tolerance in animals centres on the issue of causation. This is because parasite density, host fitness, and even immune responses are likely to be under the joint control of the host and the parasite. For example, leaving aside environmental effects on exposure, parasite density within a host is the result of the parasite's intrinsic replication rate and the host's ability to kill parasites. Immune response magnitude is the result of the host's intrinsic responsiveness and the immunogenicity of, or immunosuppression by, the parasite. Finally, host fitness when infected depends on all of the above, plus parasite virulence, plus host tolerance (Little *et al.* 2010)! Parasite growth within hosts is therefore difficult to experimentally control [even when controlling for genotype-by-genotype or genotype-by-environment interactions (e.g. Box 1)]. This problem may not apply to macroparasites such as helminths that do not replicate within the host (Bleay *et al.* 2007) or that have resting stages (Stopper *et al.* 2002), and thus their densities can be largely controlled via inoculating dose, but the problem certainly pervades the study of microparasites. Consequently, microparasite density at time t can be considered an uncontrolled outcome of the experiment, as opposed to an explanatory variable in the sense of regression or analysis of covariance (Sokal & Rohlf 1995). Here, it is not possible to disentangle whether parasite density determines host health (and by extension, host fitness), or if host health determines parasite density: they fundamentally confound each other.

Box 3. Inferring evolution from linear relationships between parasite density and host fitness

Many empirical studies have considered the linear relationship between parasite density (within hosts) and a measure of host fitness. Although a linear relationship may not always be representative, it can be adequate over some ranges of parasite density. But even in these cases, there are nuances to consider, in particular regarding the role played by host fitness in the absence of infection, that is, the *y*-intercept. Perhaps the majority of studies on the relationship between parasite density and host fitness have sought to gain insight into parasite evolution (evolution of virulence studies; e.g. De Roode *et al*. 2005), and thus the measurement of host traits in the absence of infection has been understandably ignored. Similarly, tolerance studies might not consider fitness in the absence of infection (which we call ω_0) because tolerance, by definition, does not include ω_0. And yet, it is difficult to make inference about selection on tolerance when ω_0 is not measured. First, the fitness of a particular genotype will be determined by both ω_0 and its fitness across parasite densities. These two components of fitness may not be independent due to pleiotropic effects, but even when they are, jointly considering how they covary sheds light on what the rank fitnesses of different genotypes might be. Secondly, it may not be realistic to estimate ω_0 from a *y*-intercept of a parasite density-host fitness relationship in a study that has not directly measured ω_0.



To highlight these points, we present the results of an experiment that exposed the crustacean *D. magna* to the bacteria *P. ramosa* (see Box 1 and Appendix S1, Supporting information). Fifteen replicates of each of twelve host genotypes were exposed to the parasite, and the number of offspring produced by infected hosts was counted. Later, infected hosts were killed and the density of parasite transmission spores (per mL of host tissue) was estimated. Thus, we gained the data necessary to plot parasite density (within-hosts) against host fitness (in this case measures of fecundity). For convenience, we use ω_i to represent the 'fitness of infected hosts'. We also measured the reproductive output of control hosts, that is, the fitness of those hosts not exposed to the parasite, ω_0. Full experimental details are presented in Supporting information.

We studied the relationship between parasite density and host fitness in two ways. First, we studied only 'fitness of infected hosts', ω_i. Secondly, we incorporated host fitness in the absence of infection (ω_0), by studying simply $\omega_0 - \omega_i$. As ω_0 represents what hosts can achieve in the absence of infection, $\omega_0 - \omega_i$. is the cost of infection. The two graphs above compare fitness when infected (ω_i) and the cost of infection ($\omega_0 - \omega_i$) across parasite densities.

For clarity, results for only three of the 12 genotypes are depicted, and we multiplied the cost of infection by (−)1 so that higher values represent greater fitness, making the two graphs visually comparable. Of particular note here is how inference regarding which is the most fit genotype changes depending on the fitness measure used. When examining only ω_i (fitness when infected), left graph, the genotype (26) that is the most tolerant in terms of range tolerance (i.e. shows the flattest slope) is less fit than the less range tolerant genotypes, except at the very highest parasite densities. However, in looking at the cost of infection, that is, once the response variable incorporates information about fitness in the absence of infection ($\omega_0 - \omega_i$, right graph), the most tolerant genotype is also potentially the most fit. The other two genotypes also switch their rank order of fitness over most, but not all parasite densities. The reason for these differences is that ω_0 is not accurately estimated by the relationship between parasite density and ω_i. Indeed, including all 12 host genotypes, a correlation between the *y*-axis intercept, as estimated from linear functions such as those in the left graph, shows no relationship with the actually measured fitness in the absence of infection ω_0 (spearman $\rho = -0{\cdot}2028$, $P = 0{\cdot}51$). How the cost of infection will ultimately determine the winner of a competition between genotypes will be determined by the local frequency of epidemics.

Thus, host genetic variation for range tolerance represents how genotypes differ in the strength of a relationship (typically studied as a regression) between parasite density and health/fitness, but it is difficult to say why. This becomes pertinent when considering the process of natural selection: without understanding the cause of differences in the

strength of relationships, it is not clear what trait is being selected upon and what evolutionary response to selection we should expect to see. For instance, it is possible that molecular mechanisms of tolerance control the relationship. If, for example, an immunological mechanism [e.g. anti-toxin or anti-inflammatory molecules (Raberg, Graham & Read 2009)] can be shown to alleviate disease severity as parasite numbers increase, it becomes more straightforward to interpret how natural selection will act on variation in range tolerance. This is because the immunological mechanism might then be understood to be the trait subject to natural selection. In the absence of such a mechanism, however, it is equally possible that different genotypes are just more or less sensitive to the laboratory environment, leading to differences in health and then parasite load. Here, we run the risk of confounding tolerance of the environment with tolerance of the infection. Interpreting the relationship between parasite density and fitness requires considerable caution because it is explicitly the product of two interdependent measures.

Similar issues have been discussed in other fields, and seem dangerous to ignore. For example, Ridley (1988), in his treatment of the benefits of multiple mating in insects, contrasted 'experimental comparisons' (with controlled explanatory variables), with 'non-experimental comparisons' (the uncontrolled, descriptive approach). In the latter kind of study, the risk is that experimental individuals in a sense self-select which treatments groups (once mated, twice mated, etc.) they are in, perhaps due to their condition. This self-selection may seem justified if randomly allocating individuals to treatments beforehand (the correct approach) entails significant loss of experimental subjects if some proportion of replicates fail to complete the required number of matings. However, it has become clear that different conclusions have been drawn about insect mating behaviour depending on the method used (Ridley 1988); see also Torres-Vila, Rodriguez-Molina & Jennions (2004). The similarities to experimental infection studies are obvious, as hosts (and parasites) may 'self-select' how a given dose turns into a given parasite density. Although this imposes a constraint on experimental design and inference, it cannot be ignored.

With respect to the study of tolerance, we gain some traction on the problem by applying a range of parasite doses, although (as outlined above) dose will often show complex relationships with microparasite density – for instance it may be highly nonlinear [e.g. *Pasteuria* in *Daphnia* (Ebert, Zschokke-Rohringer & Carius 2000)], or dose may influence the timing but not the magnitude of peak parasite density [e.g. *Plasmodium* in *Mus* (Timms *et al*. 2001)]. Alternatively, it may be feasible to inoculate with a single parasite dose and then apply a range of subcurative doses of an anti-parasite drug, although we know of no examples of this approach in which tolerance was quantified and we would caution that various potential confounding effects, especially if the drug has a direct impact on host health or if initial dose is all that matters, require careful thought. Injection of LPS or heat-killed bacteria might be informative for quantifying tolerance of septic shock. Lastly, there is the potential to use a range of parasite genotypes that differ in the density they tend to reach (Raberg, Sim & Read 2007), although this tendency would have to be independent of host genotype – that is, host genotype by parasite genotype interactions (*sensu*; Carius, Little & Ebert 2001) would confound this approach. Overall, statistical approaches that can account for the interdependency of measures in ecoimmunological data sets seem warranted.

BEYOND REGRESSION-BASED APPROACHES

In our discussion of phenotypic plasticity, we highlighted ways in which statistical methods such as random regression might benefit ecoimmunology. However, our advocacy of such methods should not distract from the fact that important, but largely unrecognized, statistical issues arise when neither experimental control of parasite density nor investigation into mechanism are feasible. First, if parasite density is not experimentally controlled it will necessarily be measured with error that is typically unaccounted for in regression based analyses of tolerance. Under simple (type I) linear regression, measurement error in the explanatory variable will lead to underestimation of the magnitude of the slope (i.e. overestimate tolerance) (Sokal & Rohlf 1995). This problem could be avoided by use of type II or major axis regression. However, a second issue is that any regression model specifies and assumes a uni-directional cause-effect relationship between parasite density (the independent variable) and host fitness (the response). As outlined above, however, there are good biological reasons to expect that the relationship to be bi-directional. Statistical models must always make simplifying assumptions and we do not suggest that regression be abandoned, only that violated assumptions be more widely recognized and that alternative, complementary types of analyses warrant consideration. For instance, while correlation can never prove causation, path analysis and structural equation modelling might allow different models of causal relationships between the measured host and parasite processes to be considered (and in some cases statistically compared) (Mitchell 1992; Shipley 1997).

Alternatively, there is considerable logic in choosing to treat both parasite density and host fitness as response variables in a bivariate analysis. For instance, using a bivariate mixed model (Lynch & Walsh 1998), the observed covariance between parasite density (D) and host fitness (W) can be modelled and decomposed into components attributable to factors of biological interest (e.g. host genotype or source population) and experimental design (e.g. block). For example, by fitting host genotype as a random effect (and assuming that repeated observations on each genotype are available) the total variance (V) in a trait (x) can be decomposed into a portion attributable to host genotype and a residual component (attributable to unmodelled environmental effects and measurement error). In a bivariate model the total variance–covariance matrix for two traits can be similarly partitioned such that:

$$\mathbf{P} = \mathbf{G} + \mathbf{R}$$

where **P** is the phenotypic variance–covariance matrix between n (in this case 2) traits, **R** is the matrix of residuals (usually interpreted as environmental effects), and **G** is the genetic covariance matrix

$$\mathbf{G} = \begin{bmatrix} V_{G(W)} & \mathrm{COV}_{G(WD)} \\ \mathrm{COV}_{G(WD)} & V_{G(D)} \end{bmatrix}$$

where $V_{G(W)}$ and $V_{G(D)}$ are the among-host genotype (i.e. genetic) variances for fitness and parasite density, respectively, while $\mathrm{COV}_{G(WD)}$ is the genetic covariance term. If so desired these parameters could be rescaled to yield the heritabilities of W and D (seen as traits of the host) as well as the genetic correlation, although it should be noted that these will typically be broad-sense (as opposed to additive) genetic parameters if clonal replicates are used. Moreover, these models are not limited to the study of genetic correlations, and they are not limited to bivariate. Researchers could include all response variables in a single model, and can then extract almost any pairwise linear relationships, including regressions, that are of interest.

This approach also provides an unexploited link to quantitative genetic models of trait evolution, since the genetic covariance between a trait and (relative) fitness actually provides an unbiased prediction of the expected selection response (Robertson 1966; Morrissey, Kruuk and Wilson, in press). A simple corollary of this is that even if there is an association between host fitness and parasite density, evolution of the host mechanisms for controlling the parasite density is not expected if $\mathrm{COV}_{G(WB)} = 0$ and all covariance arises from environmental sources of covariance (portioned into **R**). Given suitable data, further partitioning of **P** is readily achieved by addition of further random effects. While additional random effects may certainly be used to test specific hypothesized sources of environmental covariance between D and W (e.g. maternal effects, host cage effects), a second genetic covariance structure may be estimated in the event that multiple parasite genotypes were used (with replicate observations for each). Thus, it is possible to model W and D as traits that vary, and covary, as a consequence of interacting host and parasite genotypes, and to estimate the relative contributions of each to observed (co)variance. In this way genetic control of W and D need not be assumed to lie with either the host or the parasite, but rather can be influenced by both. We encourage ecoimmunologists to explore these approaches in more detail across a range of organisms.

Optimal studies of optimal immunity

With this article, we suggest three primary improvements to the empirical framework for ecoimmunology. In brief, we urge researchers to make more measurements, to choose them wisely, and to analyse them using some of the statistical techniques that have permeated other fields and are recommended above. The additional measurements (immune response magnitude and parasite density, to complement host fitness in the context of various study designs; Table 1) help to dissect important details of within-host dynamics – for example, are hosts more likely to die of high parasite densities or of immunopathology (Graham, Allen & Read 2005)? Wise choice of which immune elements and parasites to measure ensures relevance to fitness but requires basic knowledge of the infection biology of the target hosts or of related, well-investigated model systems (Bradley & Jackson 2008). Finally, statistical methods used in other branches of evolutionary biology appear more appropriate than current methods for dealing with inherent issues in ecoimmunological data sets (e.g. bi-directional causal relationships). We provide preliminary statistical advice for studying tolerance, but the suggested methods should apply to any data on host fitness, parasite density and/or immune responses. Together, our suggestions promote robust quantification and interpretation of fitness consequences of immune responses. We hope to prompt researchers to tailor suggestions according to what is most reasonable and appropriate for their systems and research goals. Most studies are imperfect (including those of the authors), but with steps such as those explored here, studies of ecoimmunology and optimal immunity (Viney, Riley & Buchanan 2005) can better approximate perfection.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1. Details of methods and statistical analyses for data in Box 3.

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