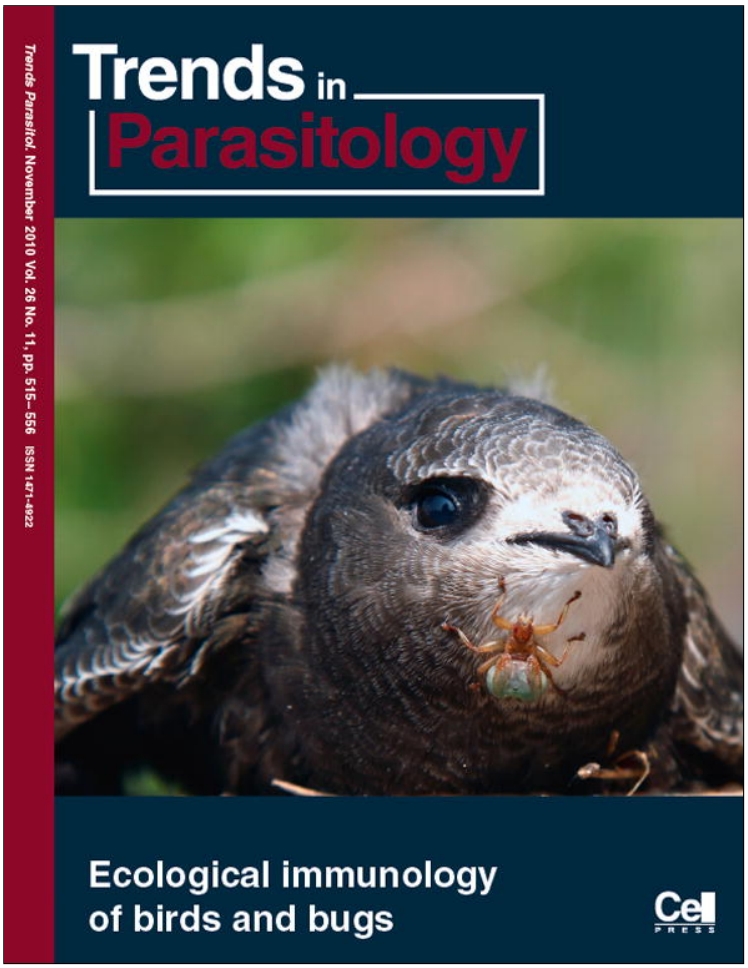

Review

Ecological immunology of bird-ectoparasite systems

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Ecological immunology is a rapidly expanding field of research that attempts to explain variation in immune function across individuals, populations and species. Birds and ectoparasitic arthropods have frequently been used in attempts to measure the cost of immune function in relation to adult condition, nestling growth and other life history challenges. Unfortunately, most studies in ecological immunology have relied on assays of general immunocompetence that are not connected to actual parasites. A summary of potential interactions between the avian immune system and ectoparasites is provided and methods that can be used to test ecological questions in the context of naturally occurring host–parasite interactions are proposed.

'The immune defense is a final line of protection and is therefore of great importance. Given this importance, variability in immune defense would seem counterintuitive, yet that is what is observed' [1].

Ecological immunology: an emerging field

Ecological immunology (EI) is a cross-disciplinary field of study focused on variation in immune function among individuals, populations and species. A major goal of EI is to understand the evolutionary significance of immunological variation, relative to different parasites, pathogens and environmental factors. A central prediction is that investment in immune function limits investment in other life-history traits, such as secondary sexual traits, or factors contributing to the growth and development of offspring (Figure 1) [1,2]. The assumption underlying this prediction is that individuals must allocate limited physiological resources among growth, reproduction and immunological defense. A concept called 'immunocompetence' has played a central role in EI [3,4]. Immunocompetence is the general ability of an organism to control infection with the immune system, so as to minimize fitness costs to the host [2]. In practice, immunocompetence is often assessed independently of specific parasites or pathogens. Current measures of immunocompetence are designed to assay the overall strength of the immune system using antigens that are novel to the animals tested (i.e. unrelated to actual parasite antigens) to circumvent differences in parasite exposure among the animals tested. Immunological responses to these novel challenges are expected to reflect the robustness of the immune system, assuming that the measures correlate with defense against natural parasite challenges. Though studies of immunocompetence have provided intriguing information about links between general immune function and other traits, we are still largely in the dark concerning the relationship of particular parasites and pathogens to host immunological trade offs. This gap in our understanding has slowed progress in the emerging field of EI [5]. Herein, a conceptual and methodological framework for EI is provided that hopefully will make real host–parasite relationships more accessible to EI. Although the focus is on avian–ectoparasite systems (Box 1), many of the points raised are broadly relevant to other systems.

The immune response to an ectoparasite results in the production of cells and proteins involved in defense against the ectoparasite and repair of tissue damage (Figure 1(a)) (Box 2). Immune defense is a physiologically costly process that can conceivably tax limited resources needed for other life-history traits. Alternatively, activation of physiological systems required for other life-history traits

Glossary of terms

Adaptive immunity: elements of the immune system that: (i) are up-regulated by T- and B- lymphocytes in response to a parasite, (ii) specifically adjust to molecular properties of a parasite, (iii) have memory, and (iv) differentiate self and non-self.
Androgen: a male sex hormone (e.g. testosterone)
Antibody: proteins produced by B-lymphocytes that bind to foreign molecules.
Cell-mediated immunity: elements of the immune system involving cellular interactions, such as production of chemical signals (cytokines), phagocytosis, and antibody secretion.
EI: ecological immunology
Granulocyte: white blood cells that contain and release molecules that influence inflammation and damage parasite tissues (e.g. mast cells, eosinophils, basophils, heterophils).
Humoral imunity: elements of the immune system involving soluble molecules in extracellular fluid (e.g. lymph or serum), such as secreted antibodies and complement proteins.
Ig: immunoglobulin (same as antibody)
Inflammation: a response in tissue involving coordinated interaction between all components of the immune system that results in physical changes to the tissue (e.g. swelling and immigration of immune cells).
Innate immunity: elements of the immune system that are available prior to infection and are not specifically adjusted to a parasite.
KLH: Keyhole limpet hemocyanin (novel protein that stimulates an antibody response)
Lymphocyte: white blood cells that are responsible for coordinating an adaptive immune response (T_H-cells) and producing antibodies (B-cells).
MHC: major histocompatibility complex (key to adaptive immune response)
NAbs: natural antibodies (constitutive antibodies involved with innate immunity)
PHA: phytohaemagglutinin (cell mitogen)
PMSS: parasite-mediated sexual selection
SRBC: sheep red blood cell (used as novel antigen)
WBC: white blood cell; immunological cell (lymphocyte, granulocyte, phagocytic cell)

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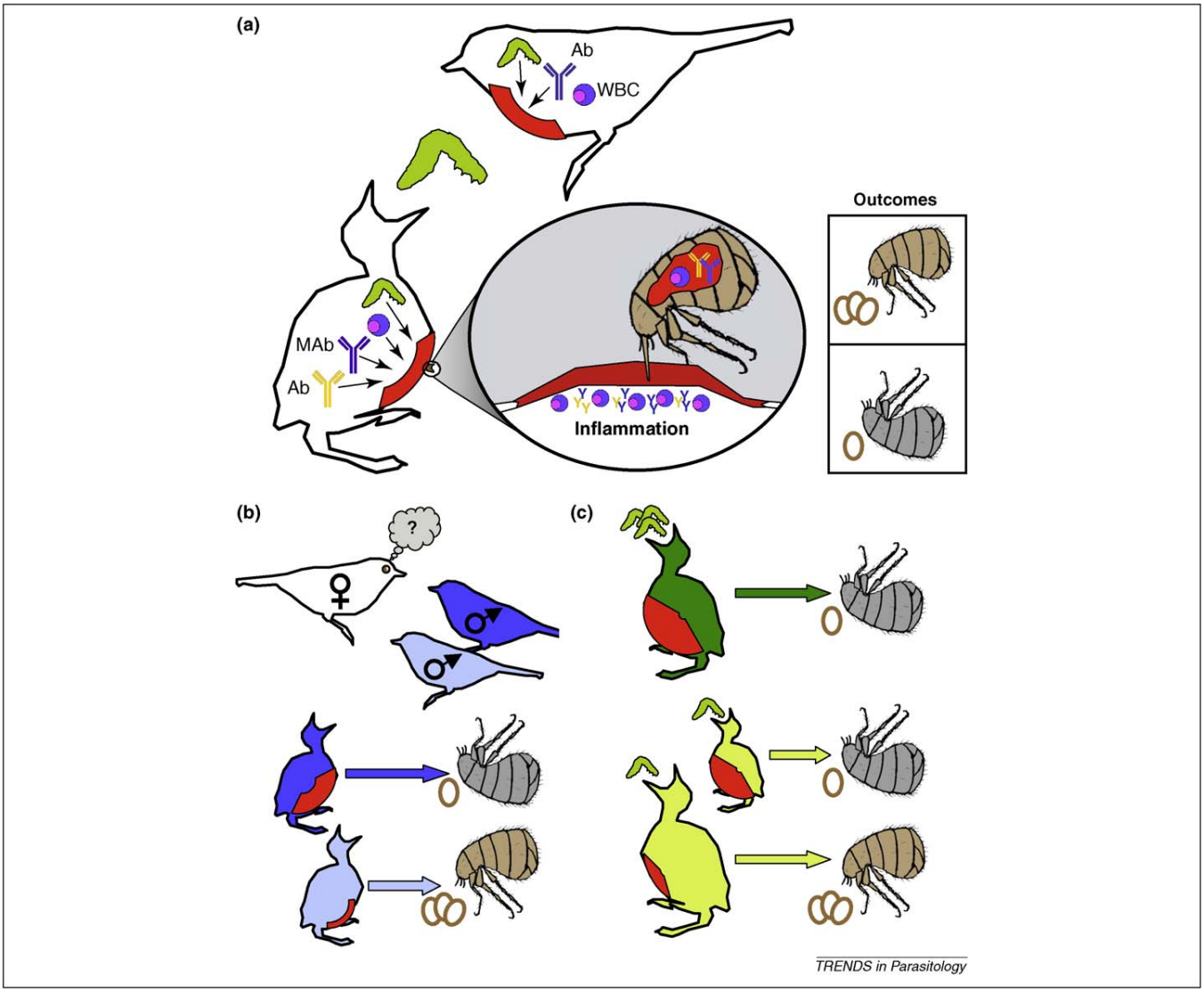

Figure 1. Bird-ectoparasite interactions relevant to ecological immunology. Hypothetical immune responses by a songbird to an ectoparasitic flea **(a)**, including the potential influence of mate choice **(b)**, and differential resource provisioning **(c)** on effectiveness of the immune response. The flea (panel **(a)**, center) obtains blood from both the mother bird (top) and her chick (bottom left). Antigens (e.g. flea saliva), as well as direct damage to skin, can both trigger inflammation (red regions), which is coordinated by antibodies (Ab) and white blood cells (WBC). Maternal antibodies (MAb), transferred to the chick before it hatches, might also aid the chick's inflammatory response. Nutrients (green larvae) aid inflammation by providing energy and resources to the immune system. Inflammation can block the flea's access to blood vessels, and damage the flea's tissues through the action of ingested host antibodies and WBCs (e.g. granulocytes). The effect of inflammation can vary from weak, in which case flea survival and fecundity are unaffected (top 'Outcomes' box), to strong, in which case flea survival and/or fecundity are reduced (bottom box). **(b)** Females choosing males with 'good' immune defense genes (dark blue) have chicks with immune defenses that reduce flea survival and/or fecundity, while females choosing males with poor immune defenses (pale blue) have chicks with immune defenses that are relatively ineffective against fleas. **(c)** Chicks receiving plentiful resources (dark green) can grow quickly and have effective immune responses. Chicks receiving fewer resources are forced to trade growth for an effective immune response (small yellow chick), or trade an effective immune response for growth (large yellow chick). The overall figure is based on the Great Tit *(Parus parus)* - hen flea (*Ceratophyllus gallinae*) system [26,36,37,38,39]; however, not all of the interactions shown have been tested in this particular system.

might interrupt immune responsiveness. The hypothesized tradeoff between demands of the immune system and other physiological traits is an active area of research in EI. Two major life history components for which immunological tradeoffs have been tested are: (i) adult condition/appearance (Figure 1(b)) and (ii) offspring growth/development (Figure 1(c)).

Tradeoffs between immune function and adult condition/appearance

Hormones have been central to the hypothesis that variation in immunological responsiveness is maintained by tradeoffs, or even antagonistic interactions, with other life-history traits. This is because hormones mediate adult bird condition and have direct or indirect effects on the immune system. The sex-hormones testosterone and estrogen establish male- and female-specific behaviors and morphology [6]. Testosterone has been linked to impaired immune function and increased parasite susceptibility in a number of vertebrate groups, whereas estrogen is often associated with increased resistance against infection [7]. For example, in domestic chickens, roosters had significantly higher northern fowl mite populations than hens [8], and roosters given varying dosages of estrogen had lower

Box 1. Bird–ectoparasite systems in EI

Studies involving birds and ectoparasitic arthropods form one cornerstone of ecological immunology for two reasons. First, bird–ectoparasite systems are experimentally tractable. The ecology of many bird species is well characterized and researchers can manipulate aspects of their life-histories, such as clutch size and parenting (cross-fostering). The macroscopic nature of ectoparasites, coupled with their on-host, or nest-dwelling, habit allows their populations to be quantified and manipulated more easily than most endoparasites, or microbes. Second, birds have life-histories with components that are central to EI, such as sexual selection and parental investment.

Birds are exploited by a diverse community of ectoparasitic arthropods, ranging from insects, such as lice (Phthiraptera), fleas (Siphonaptera), true bugs (Hemiptera), and flies (Diptera), to ticks and other mites (Acari) [40,41]. Many studies have demonstrated that ectoparasites reduce avian fitness [20]. They can reduce several components of host reproductive success, including mating, hatching, nestling survival, and fledging. They can also reduce adult condition and survival. Ectoparasites use resources that hosts might otherwise devote to life history demands such as survival, growth and reproduction, and they further tax the host in requiring it to engage in defenses that might be costly. Furthermore, ectoparasites can serve as vectors for a variety of pathogens and other parasites, including viruses, bacteria, protozoa and filarial worms [41].

Ecological immunology studies of birds and ectoparasites are rapidly accumulating, and many have made important contributions to our understanding of immune function in an ecological context. However, most of the studies have failed to establish effective links between host immune responses and defense against particular ectoparasites. These problems stem, in part, from overly simplified assays of immune function, and a failure to examine the biology of both the host and the parasite [42].

Birds also have a variety of non-immunological defenses against ectoparasites, ranging from behaviors such as preening, to chemical composition of the feathers. For a recent comprehensive review of non-immunological defenses see Ref. [43].

Box 2. Inflammation and ectoparasites

Inflammation, which is mediated by the immune system, is one of the most important defenses that birds have against blood feeding ectoparasites. Nonetheless, most of the details we know of the inflammation response are from work on mammals [40]. The basic events orchestrating the inflammatory response in the skin are as follows: (i) initial tissue damage and the introduction of foreign molecules (antigens) cause host tissue to release chemical signals (cytokines) that stimulate phagocytic cells (e.g. macrophages and dendritic cells) of the innate immune response to migrate out of circulation into the tissue at the site of damage; (ii) the responding phagocytic cells engulf antigens, degrade them, and subsequently present fragments of the antigens on the surface of the cells in conjunction with the class II major histocompatibility complex (MHC); (iii) helper T lymphocytes (T_H) of the adaptive immune response interface with these antigen-MHC complexes, activating the T_H cells; (iv) activated T_H cells trigger the production and secretion of antigen-specific antibodies (Ab) by B lymphocytes; (v) T and B lymphocytes form long-lived memory cells that are quickly activated upon re-exposure to the antigens; (vi) re-exposure to the antigens (subsequent parasitism) is met with specific antibodies, which bind, neutralize and tag the antigens; activated memory T_H cells recruit phagocytic cells and granulocytic cells (containing proteolytic compounds); and (vii) the combined actions of these immune effectors cause normal skin cells (box with arrow, Figure I(a)) to increase in size or number (box with arrow, Figure I(b)), the skin to swell with immunological cells and fluid (circle, Figure I(b)), and foreign molecules to be degraded or removed (for additional details see Ref. [44]).

The inflammatory response to ectoparasites (Figure I(b)) negatively impacts the parasite by physically preventing access to blood due to tissue swelling (edema) at the feeding site, forcing the ectoparasite's mouthparts away from capillaries, neutralizing salivary compounds that normally prevent host haemostatasis or immune responses, and directly damaging parasite tissue (e.g. release of proteolytic compounds by granulocytes) [40,45]). These events affect the ectoparasite by reducing blood meal size and quality, prolonging feeding, reducing the ability to digest the blood meal, and/or promoting host grooming behavior via itching. These effects, in turn, can reduce ectoparasite fecundity, inhibit molting, or even cause death of the parasite [40,45,46].

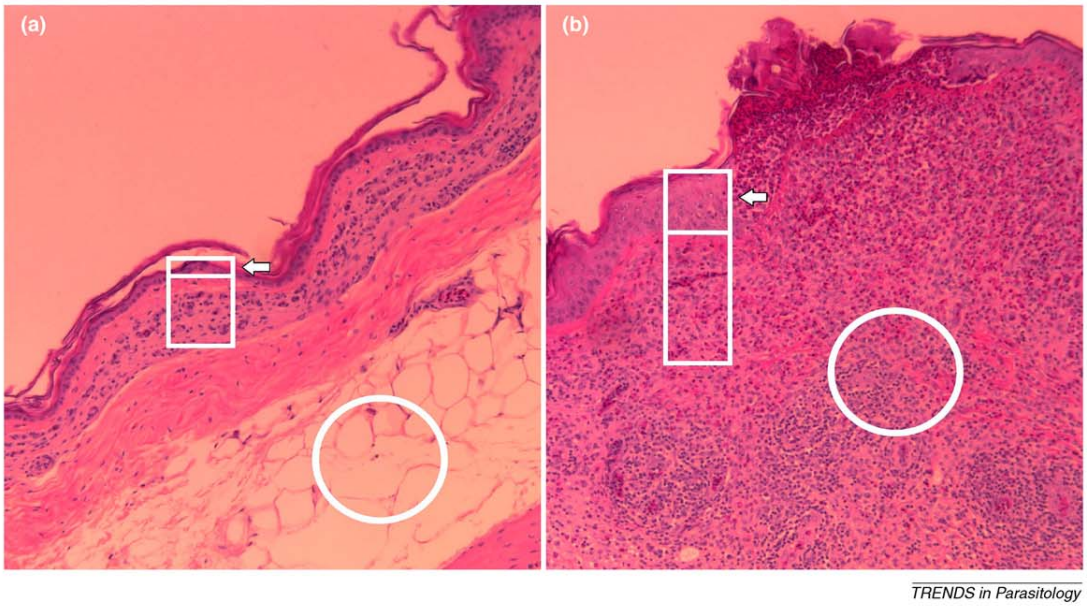

Figure I. Skin changes from inflammatory response to ectoparasite feeding. Micrographs show a cross-section of normal chicken skin **(a)** and chicken skin after several weeks of blood-feeding by northern fowl mites **(b)** [45]. In each panel, the upper boxes surround the top, epidermal layer of skin cells, the lower boxes surround cells in the dermis, and the circles surround tissue in the subcutis layer.

Box 3. Parasite-mediated sexual selection

Hamilton and Zuk [47] hypothesized that hosts can increase the frequency of 'good' genes for parasite resistance in their offspring through parasite-mediated sexual selection (PMSS), in which individuals choose mates on the basis of honest (cheat proof) signals given during courtship (Figure 1(b)). For example, plumage coloration in house finches (*Carpodacus mexicanus*) appears to be an honest indicator of susceptibility to infection with *Mycoplasma* bacteria [48]. Ectoparasite load could also be revealed by traits such as bird song [49]. Assuming that parasites adapt to the resistance genes over time, individuals will be expected to choose mates with rare, advantageous alleles. The resulting co-evolutionary cycles maintain heritable variation in host immune defenses over time.

Several groups have addressed the Hamilton–Zuk hypothesis by measuring immune response to novel antigens in relation to sexually selected traits [50]. However, to our knowledge, none of the studies assessed actual parasite load in response to immune defense and, given the uncertain relationship between response to artificial antigens and parasitism, their conclusions must be considered tentative. One of the most thorough explorations of PMSS is work by Roulin and colleagues. In a series of elegant studies of barn owls (*Tyto alba*), they showed that female spottiness is attractive to males, negatively correlated with parasitism, and positively correlated with chick antibody response to novel antigens (SRBC and human serum albumin) [51].

Another way in which females might be able to increase the parasite resistance of their offspring is by mating with genetically dissimilar males. This hypothesis differs from the Hamilton–Zuk model in that heterozygosity is a non-additive genetic trait. Inbreeding avoidance is thought to be under strong selection due to the negative fitness consequences of deleterious recessive alleles, which include increased susceptibility to ectoparasites [52]. Genetic diversity in the galapagos hawk (*Buteo galapagoensis*), a species particularly vulnerable to inbreeding, was found to be negatively correlated with louse load (*Colpocephalum turbinatum* and *Degeeriella regalis*), and louse load was positively correlated with natural antibody (NAb) agglutination titre, with the effects being stronger in smaller, more inbred populations [53]. Other studies have tested the importance of heterozygosity in defense against parasites by comparing inbreeding coefficients to immune responses to novel antigens [54]. Whether such immune responses are indicative of actual parasite resistance still needs to be determined.

northern fowl mite levels in the first two weeks of infestation than control roosters [9]. In wild birds, EI researchers have focused heavily on testosterone because of its links to the expression of secondary sexual traits and parasite-mediated mate choice (Figure 1(b)) (Box 3). However, a recent literature review found that testosterone is not a general immunosuppressant in free-living birds [10], nor does it generally enhance trait expression [11]. Thus, for wild birds it is unclear what effects androgens really have on variation in immune function and, ultimately, resistance to ectoparasites.

Glucocorticoids are another class of hormones that might create trade-offs between immune function and other life-history traits. Glucocorticoids play a central role in the stress response, and are elevated with the activation of the hypothalamic–pituitary–adrenal axis; they are also known to be immunosuppressive [7]. Several studies have shown negative relationships between glucocorticoid level and general immunocompetence in birds. For example, dark-eyed juncos (*Junco hyemalis*) with high glucocorticoid levels were found to have less inflammation in response to phytohaemagglutinin (PHA) injections [12]. However, only one study to date has examined glucocorticoid level in relation to a specific parasite; Bortolotti *et al.* [13] reported a positive correlation between glucocorticoid level and nematode abundance in red grouse (*Lagopus logopus scoticus*). These data are consistent with an immunosupressive effect of glucocorticoids that leads to increased levels of parasitism. Therefore, they are more likely than testosterone to mediate tradeoffs between parasite resistance and other life history challenges.

Pigments such as melanin and carotenoids are integral to both the immune system and adult appearance and/or condition. As such, they are potentially limiting resources that can mediate tradeoffs between parasite resistance and attractiveness to mates. Carotenoids are immunologically important because they can destroy free radicals produced during an immune response; these free radicals pose a risk to the integrity of host tissues. Carotenoids are also required for the expression of brightly colored traits, which are often signals of condition and competitive ability [14]. Several studies with birds have tested the hypothesis that carotenoid pigmentation of sexually selected traits is costly to immune function [14,15]. Experimental activation of the immune system has been shown to reduce the intensity of carotenoid dependent display traits, indicating that carotenoids are shared between the immune system and overall appearance [15]. However, only one study has demonstrated a direct connection between plumage carotenoids and parasite resistance. Parasitized male blackbirds (*Turdus merula*) supplemented with carotenoids developed more brightly colored bills than non-supplemented males, and had enhanced resistance against the protozoan parasite *Isospora* [16].

Many EI studies focusing on carotenoids tend to treat these pigments as a single entity. Nonetheless, carotenoids are a class of molecules, and accounting for the relative roles of different carotenoids in immunity and trait expression is important for testing the trade-off hypothesis. For example, a study of great tits involving experimental supplementation of carotenoids normally deposited in brightly-colored feathers (lutein and zeazanthin) resulted in no differential immune response to a novel antigen (PHA) [17]. However, supplements that included β-carotene, which is not deposited in the feathers, led to enhanced immune responses to PHA. Nestlings fed all three carotenoids showed enhanced responsiveness to PHA, but reduced plumage coloration. Taken together, these findings suggest that the carotenoids used in the immune response are not the same as those used in plumage coloration. They do indicate, however, that activation of the immune system impairs the delivery of plumage carotenoids to the feathers. This study highlights the importance of identifying the roles of individual carotenoid molecules in thorough investigations of ecological immunology.

Melanin-based coloration in birds is controlled by genes with pleiotropic effects on immune function [18]. Thus, melanin might represent another shared resource with indirect interactions between bird coloration and immune

function. In tawny owls (*Strix aluco*), melanin determines the degree of plumage reddishness. Dark red females (high melanin) produced a stronger antibody response to a cocktail of human vaccines, but also had greater loss of body mass during immune challenge than light red females [19]. Whereas this study suggests that melanin could be mediating tradeoffs between trait expression and immune function, experimental studies are needed to test this hypothesis directly.

Tradeoffs between immune function and offspring growth/development

Nest-dwelling ectoparasites are known to have negative effects on the growth and development of chicks [20]. These effects could be mitigated by differences in provisioning (Figure 1(c)), whereby more (or better) nutrition provides additional resources to be divided between growth and immune defense, resulting in increased resistance against ectoparasites without compromised growth. Several studies of nutrition have demonstrated links between nutrition and growth, immune function, and/or ectoparasite resistance. Early work by Matthysse *et al.* [8] showed that reduced levels of lysine in the diet of chickens decreased the intensity of the inflammatory response, and lowered resistance of birds to northern fowl mites (*Ornithonyssus sylviarum*). This work indicated that limited nutritional resources can impair immune function and ectoparasite resistance. In a cross-fostering study, during a period of low food availability, kittiwake (*Rissa tridactyla*) nestlings were shown to have weak resistance to *Ixodes uriae* ticks and had reduced growth as a result of parasitism [21]. In contrast, during a period of high food availability, growth was not impaired, and nestlings were able to limit blood feeding by ticks. Together, these observations suggest that nutritional resources can govern both growth and ectoparasite resistance.

More recently, the effects of the dietary supplement methionine were tested on the T-cell activity and growth of great tit (*Parus major*) nestlings experimentally infested with nest-dwelling hen fleas (*Ceratophyllus gallinae*) [22]. Methionine supplements increased T-cell activity and resulted in faster nestling growth rates during parasitism. This study shows that better nutrition can increase immune function and growth under parasite pressure. Nevertheless, studies that simultaneously explore the relationship between nutrition, growth, immune function and ectoparasite resistance are still needed. Although nutrition clearly affects both immune function and growth, no study yet has demonstrated a clear trade off between the immune system and growth, with nutrition as the limiting factor.

Another way in which parental provisioning could influence tradeoffs between immune function and nestling growth and development is through the maternal transfer of antibodies. One possibility is that the mother can transfer antibodies to the chick *in ovo,* thus protecting the offspring against parasites after they hatch. Maternally transferred antibodies might also influence the development of the chick's own immune system [23]. Recent experiments confirm that the antibodies deposited in eggs are, in fact, the same as those present in the mother, suggesting that maternal antibodies produced in response to ectoparasites can provide some defense for the chick (reviewed in Ref. [24]). Evidence was reported for this phenomenon in a cross-fostering study using great tits and the nest-dwelling hen flea [25]. Fleas that fed on chicks from parasitized parents survived less time than fleas fed on chicks from parents that were not parasitized; however, a correlational follow-up study found no significant relationship between total maternal antibody transfer and chick development, or resistance to hen fleas [26]. Recent experimental work with house sparrows (*Passer domesticus*) determined that specific maternal antibodies are transferred in very low amounts and persist in offspring for too little time to provide real protection against ectoparasites [27]. It is also unclear whether maternal antibodies prime the offspring's developing immune system [28] or, alternatively, impair immune system development by interfering with the ability of the offspring to detect foreign antigen and initiate an antigen-specific immune response [27]. In short, the importance of maternal antibody transfer to variation in immune function and immunological interaction with ectoparasites remains unclear.

Yet another form of provisioning by mother birds is to invest carotenoids in the yolks of their eggs. Support for this hypothesis comes from a recent study of New Zealand stitchbirds, also known as hihis (*Notiomystis cincta*), and the tropical fowl mite (*Ornithonyssus bursa*), which reduces nestling survival and growth [29]. Females supplemented experimentally with carotenoids (lutein and zeaxanthin) laid eggs with more carotenoids than non-supplemented females. Parasitized nestlings hatched from the eggs of supplemented females did not differ in size from nestlings in unparasitized nests. Thus, this study suggests that carotenoids might be an important mechanism underlying maternal effects on the parasite resistance of nestlings. This, in turn, might explain why some studies have observed maternal effects on parasite resistance yet failed to find maternally transmitted antibodies.

Evidence continues to accumulate that immune function is partly mediated by tradeoffs between the costs of immune defense and other life history traits. Such tradeoffs could influence the observed variation in immune function across individuals, populations and species. However, demonstrations of tradeoffs governing ectoparasite resistance in birds are still few and far between. A more concerted effort to incorporate parasites into studies of immunological tradeoffs will provide a vital missing piece of the EI puzzle: the impact of immunological variation on actual host–parasite interactions.

Methodological issues

Many EI studies focus on four discrete facets of the immune system, which are cellular versus humoral immunity and innate versus adaptive immunity. Cell-mediated immunity, involving actions of cells (e.g. phagocytosis), is distinguished from humoral immunity that involves actions mediated by soluble molecules (e.g. antibodies and complement proteins). Innate immunity, involving cells and proteins with fixed recognition of antigens, is distinguished from adaptive immunity that involves cells and proteins

Table 1. Relationships between avian immunological parameters and ectoparasites

Immunological Parameter[a]	Host	Ectoparasite	General method[b]	Relationship[c]	Refs.
Antibody Response (S)	chicken	northern fowl mite	Obs.	+	[8,68]
		Argas tick	Obs.	+	[69]
		red chicken mite	Obs.	+	[70]
	pigeon	*Argas* tick (adult)	Obs.	n	[46]
		Argas tick (larvae)	Obs.	+	[46]
		Argas tick (larvae)	Obs.	+/−	[71]
	medium ground-finch	*Philornis* nest fly	Obs.	+	[57]
Skin inflammation (S)	chicken	northern fowl mite	Mnp.[d]	−	[8]
	pigeon	*Argas* tick	Obs.	+/−	[72]
	chicken	northern fowl mite	Obs.	+/−	[45]
Antibody response (N)	barn owl	*Carnus* fly	Obs.	−	[73]
		Ixodid tick	Obs.	n	[73]
	barn swallow	lice	Mnp.[e]	+	[74]
		louse fly	Obs.	n	[74]
		tropical fowl mite	Obs.	n	[74]
		louse fly	Obs.	n	[75]
		lice	Obs.	n	[75]
		tropical fowl mite	Obs.	n	[75]
	galapagos hawk	blood-feeding louse	Obs.	−	[53]
		feather-feeding louse	Obs.	n	[53]
	small ground-finch	feather mites	Obs.	+	[76]
	florida scrub jay	sticktight flea	Obs.	n	[77]
	house martin	martin bug	Mnp.[f]	+	[65]
			Obs.	+	[78]
	sand martin	*Ixodes* lividustick	Mnp.[g]	+	[66]
	great tit	hen flea	Obs.	+	[38]
Skin inflammation (N)	great tit	*I. ricinus* tick	Mnp.[h]	n	[73]
	black-legged kittiwake	*I. uriae* tick	Mnp.[i]	n	[21]
	small ground- finch	feather mites	Obs.	−	[76]
	European roller	*Carnus* fly	Obs.	+	[79]
	barn swallow	louse fly	Mnp.[j]	n	[80]
	house martin	martin bug	Obs.	n	[81]
			Obs.	−	[78]
			Obs.	−	[82]
	sand martin	*I. lividus* tick	Mnp.[g]	n	[66]
	great tit	hen flea	Obs.	n	[36]
			Obs.	n	[39]
			Obs.	-	[83]
	mountain bluebird	blowfly	Obs.	n	[84]
	house sparrow	*Pellonyssus* mite	Mnp.[k]	n	[85]
	alpine swift	louse fly	Mnp.[l]	+/−	[86]

[a]The parameter of the immune system measured. The letter indicates if the parameter was explicitly linked to a particular ectoparasite (specific, 'S') or not (non-specific, 'N').
[b]Method used if the experiment was purely observational (Obs.) or involved an experimental manipulation of the parasite and/or immune response (Mnp.).
[c]Relationship between immune measure and ectoparasite load characterized as '+' (higher value associated with more parasites), '−' (higher value associated with fewer parasites), '+/−'; (value varied relative to time, or treatment), or 'n' (no significant relationship/pattern).
[d]Antigen injection using parasite proteins to test for a parasite-specific inflammatory response, coupled with diets with specific nutrient treatments.
[e]Implantation of testosterone.
[f]The parasite loads in nests were controlled with fumigation and inoculation.
[g]Pyrethrin used to experimentally lower tick loads.
[h]PHA injection used to characterize inflammatory responsiveness of chicks of different ages, and parasite loads were separately measured on chicks of different ages.
[i]Chicks cross-fostered to test tick fitness on sympatric versus allopatric hosts.
[j]Comparisons made in PHA responses among nestlings from nests with normal versus artificially increased parasite loads.
[k]PHA responses among cross-fostered chicks correlated with natural parasite loads.
[l]Parasite feeding success and PHA responses measured on chicks with manipulated diet and methionine supplementation.

adjusted to specific antigens [30]. These subsets of immunity are useful categories for discussing the immune system, but they are frequently and erroneously treated as rigid divisions within immune responses, leading to an oversimplified concept of immunity. The immune response to a parasite involves all four components of the immune system in a series of coordinated cellular and molecular interactions (Box 2). These coordinated interactions are vital steps in the immune response and continually involve multiple elements of the immune system. As discussed below, the network of interactions in the immune response invokes a range of genes, which can drive tradeoffs with other physiological demands. Moreover, a functional immune response results in parasite-specific cellular and humoral defenses. Some current and popular methods used to assess immune function in EI are problematic because they bypass many elements of the immune response.

For example, a very common assay of immune function in EI involves PHA (phytohaemagglutinin). PHA is a

plant-derived molecule that triggers division of T-lymphocytes, resulting in a local inflammatory response where the material is injected [30]. The intensity of the swelling response is interpreted as an indicator of cellular immunity strength. KLH (keyhole limpet hemocyanin) is a large protein obtained from a gastropod that strongly promotes an antibody response. The antibody response is measured and interpreted as an indicator of the strength of humoral immunity. These assays share two beneficial features: (i) they are completely novel to the immune systems of birds under natural conditions, and (ii) they stimulate strong, measurable immune responses. Immunological novelty is important because investigators cannot control for the past exposure of wild birds to parasites. Adaptive immune responses measured against actual parasite antigens might reflect a primary (new) immune response, or a secondary immune response via memory lymphocytes retained from past exposure to the parasite (Box 2). The use of novel antigens that are unassociated with actual parasites circumvents this problem.

Unfortunately, the use of materials like PHA and KLH creates other problems. There are no consistent data demonstrating that bird responses to these materials are correlated with immune responses to parasites, or resistance to parasites (Table 1) [3]. Thus, it is unclear how to interpret immune responses to these novel antigens in the context of parasite-specific immune responses, or host defense [3]. Another problem is the unusual type of immune response stimulated by PHA, in particular. This protein directly and indiscriminately stimulates T-cell proliferation and activity. In contrast, a natural cell-mediated immune response follows a cascade of cellular interactions and chemical signals (Box 2), including antigen processing and presentation, which selectively activate lymphocytes. These vital steps in the immune response to parasites are bypassed in the response to PHA. Thus, it is

Box 4. Adding parasites to EI

To add parasites to EI studies, researchers need access to particular components of host–parasite interaction: specificity and dynamics. Adaptive immune responses to parasites are inherently specific at the molecular level (e.g. antigen presentation and antibody binding [30]) and the tissue level (location of the immune response [55]). Immune responses fluctuate through time as a function of host condition, parasite pressure and regulatory processes of the immune system itself [30,45]. Immune effectors are linked through complex genetic, molecular and cellular interactions that are relevant to the ultimate outcome of an immune response [30,31]. Salvante [56] reviewed techniques that are often applied to avian ecological immunology. Below, immunological assays are outlined that are parasite-specific, and methodological approaches to incorporate parasite-specific immune responses into ecological studies are described.

Specificity

To link host immune responses to parasites, it is important to demonstrate that immune effectors change relative to parasite exposure, or parasite-specific molecules. The first step in testing specificity is to use parasite-produced molecules as test substrates for antibody activity and cell-mediated responses (Table I). Parasite antigens can be extracted from whole organisms [57], or from specific tissues (e.g. salivary glands) [58,59].

Dynamics

Researchers can incorporate various methodological approaches in EI research to capture the dynamic nature of the immune system, including: (i) assessing immune function at multiple points in time, (ii) controlling factors that may affect immune responses, or (iii) tracking multiple parameters of the immune system (Table II).

Table I. Methods for assessing immune function relative to parasites

Assay	Information	Ref.
Enzyme-linked immunosorbent assay (ELISA)	Parasite-specific antibodies. Determine if a specific parasite stimulates the adaptive immune response (humoral).	[57]
Hypersensitivity	Inflammatory response (Box 2) to parasite antigens. Determine if a specific parasite stimulates the adaptive immune response (humoral and/or cellular).	[59]
Monoclonal antibody interference	Determine if antibody binding to parasite-produced molecules impairs parasite fitness. Test if antibody is protective for the host.	[60]
RNA interference	Determine if parasite-produced molecules introduced to the host are important to parasite fitness.	[61]
Histology	Cellular and molecular components of tissue inflammatory responses to parasites.	[45,62]
Cytology/Flow Cytometry	Cellular responses (in circulation) to parasites.	[63]
In vitro activity	Cell activity relative to parasite exposure.	[64]

Table II. Experimental approaches to using parasites in EI

Method	Information	Ref.
Recapture, repeated sampling	Changes in immune effectors relative to changes in parasite pressure.	[21,57]
Manipulative field studies	Changes in immune effectors relative to levels or types of parasite pressure (field conditions).	[65,66]
Captivity studies	Changes in immune effectors relative to levels or types of parasite pressure (environment controlled).	[67]
Lab models	Changes in immune effectors relative to levels or types of parasite pressure (environment and host genetics controlled).	[45]
Multiple effectors; multivariate analysis	Interactions among different parts of the immune system.	[31,56]

also unclear what responses to PHA reflect in the mechanistic sense of immune function.

To resolve these issues with novel antigens, researchers have suggested that a panel of assays can be used to assess immunocompetence across many parts of the immune system [31]. However, although more diverse assays promise to characterize the immune system more completely, they too lack any definitive connection to actual parasites. Although the concept of immunocompetence is ultimately meaningful only in the context of parasite resistance [2], many, if not most, EI studies do not even consider parasites [5]. The field of EI will benefit greatly by incorporating parasites, such that causes of immunological variation can be interpreted directly in the context of actual host defense [1].

Concluding remarks and future directions

Bringing parasites into EI requires two modifications to most experimental approaches currently in use. First, in each study system, investigators need to determine how novel antigen assays, such as PHA or KLH, are actually related to host resistance or susceptibility to parasites; such assays cannot serve as an umbrella approach to all host–parasite systems. On the other hand, if consistent relationships can be determined for a particular host–parasite system, then a novel antigen assay might be useful for assessing immune function.

Second, investigators need to develop parasite-specific assays, i.e. assays that use parasite-derived proteins, or intact parasites (Box 4). Such assays can assist in determining how immune function varies relative to parasite antigens, which will help elucidate sources of variation in parasite resistance. As discussed earlier, parasite-specific assays have been avoided, in part, because of concerns about how to discriminate current versus past exposure. Though labor intensive, these issues can be resolved through a combination of experimental approaches that include repeated measures of birds (recaptures), manipulative experiments, studies in captivity and use of laboratory-based, model host species (Box 4). These approaches will enable researchers to interpret the subtle changes in parasite-specific immune responses over time and under variable conditions. As a result, it should be possible to address three outstanding questions in EI: (i) what is the immunological response to a given parasitic infection?, (ii) what are the host fitness consequences of immunological variation?, and (iii) what are the parasite fitness consequences of immunological variation?

Another important future direction for EI is to identify the mechanisms that underlie functional immune responses by incorporating genetics and epigenetics. From a genetic perspective, widespread variation in immunity is particularly interesting because immunological traits, which are critical for survival and reproduction, should be purged of variation [1]. Yet, as we have discussed, studies in EI and immunogenetics show that variation is the norm. A fruitful area of investigation will be to identify the roles of genetic and non-genetic factors in maintaining variation in immune responsiveness.

Identifying the genetic basis of the immune response is an essential step in understanding variability in immunity. One critical, but often untested, hypothesis is that variability in host immunological loci provides a defense mechanism against individual, co-evolving parasite species. Alternatively, because hosts are exposed to co-infections by multiple parasite taxa, host immunogenetic variation might be partly the result of shifting selective landscapes, because of changes in parasite communities. For both hypotheses, alleles that confer resistance to one parasite can confer susceptibility to another parasite, resulting in the observed variation in immune function and parasite resistance. Moreover, immunological genes are known to have pleiotropic effects on other immunological factors and traits outside the immune system. This has been demonstrated in bird–ectoparasite systems for the major histocompatibility complex (MHC), a set of immunological genes with extreme allelic variation that influences immune function and parasite resistance [32]. Thus, pleiotropy might also explain the apparent linkage between immune function and other life-history traits. An essential future direction for EI will be to test for these possible pleiotropic relationships.

It is probable that many undiscovered genes contribute to immune function and parasite resistance. The use of quantitative trait loci (QTL) mapping, expression microarrays, and functional genomics will help identify these genes and determine their contribution to immune variation. A recent study found that 21 QTLs (including ten candidate genes) in domestic chickens contribute to resistance to coccidiosis [33]. The results of this study suggest that heritable variation in parasite resistance is caused by multiple genes and, possibly, their epistatic interactions. Susceptibility to parasites might also be caused by variation in gene expression and regulation. For example, a study using expression microarrays found that house finches (*Carpodacus mexicanus*) experimentally infected with *Mycoplasma* differentially regulated 34 genes with known homologues, as well as many novel transcripts [34]. Though many of these homologues are known to be immunological loci, some are not, suggesting that regulation of gene expression might link the immune system to other traits, further explaining immunological variation. By identifying the genetic bases of susceptibility to parasitism, these studies of bird-endoparasite systems provide a starting point from which to better understand population-wide variation in immune function and susceptibility to ectoparasites.

Finally, another important line of inquiry concerns the possible contribution of epigenetic effects to variation in immunity. Transgenerational epigenetic inheritance might contribute to such variation if hosts are able to 'prime' their offspring against parasites by reprogramming DNA methylation profiles of specific genes involved in immune defense [35]. Furthermore, the parasites themselves might also be able to induce phenotypic variation in host offspring via epigenetic changes. Although transgenerational epigenetic effects are still largely unstudied, they might yield new insights into factors contributing to the extensive variation in parasite resistance that is so prevalent in nature.

Ecological immunology is an exciting and very active area of research at the interface of ecology, immunology

and parasitology. Incorporating parasitology more fully into ecological immunology will shed considerable light on the paradox of rampant variation in the immune responses of host individuals, populations, and species. Bird-ectoparasite systems have played a prominent role in the history of ecological immunology, and they can contribute significantly to its future.

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