Reviewing the effects of food provisioning on wildlife immunity

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Summary

While urban expansion increasingly encroaches on natural habitats, many wildlife species capitalise on anthropogenic food resources, which have the potential to both positively and negatively influence their responses to infection. Here we examine how food availability and key nutrients have been reported to shape innate and adaptive immunity in wildlife by drawing from field-based studies, as well as captive and food restriction studies with wildlife species. Examples of food provisioning and key nutrients enhancing immune function were seen across the three study type distinctions, as were cases of trace metals and pharmaceuticals impairing the immunity of wildlife species. More generally, food provisioning in field studies tended to increase innate and adaptive responses to certain immune challenges, whereas patterns were less clear in captive studies. Mild food restriction often enhanced, whereas severe food restriction frequently impaired immunity. However, to enable stronger conclusions we stress a need for further research, especially field studies, and highlight the importance of integrating nutritional manipulation, immune challenge, and functional outcomes. Despite current gaps in research on this topic, modern high throughput molecular approaches are increasingly feasible for wildlife studies and offer great opportunities to better understand human influences on wildlife health.

1. Introduction

With continued urban expansion and the loss of natural habitat, it is increasingly important to understand the effects of human activities on wildlife. While habitat destruction and the resulting deprivation of shelter and food resources are well-known consequences of human activities, the replacement or supplementation of natural food resources can have both beneficial and insidious effects on wildlife health. Provisioning of wildlife with food resources occurs through a range of deliberate means, such as backyard bird feeders and attracting game species for hunting and tourism, and as an unintended consequence of other activities, such as crop farming and waste disposal [1-3]. Food and nutrient availability are important limiting factors in nature [4, 5], and

many wildlife species capitalise, and in some cases rely, on these abundant and often predictable anthropogenic food sources. Their effects on the ecology of wildlife species are numerous: from the individual-level timing of maturation and reproduction, to altered population densities, community interactions and ecosystem functioning [1,6]. Key among these are the multifaceted effects of food provisioning on host-parasite dynamics.

In this review we refer to parasites broadly, including micro- and macro-parasites [7], and to food provisioning as any food source made available to wildlife by human activities. Becker et al. [8] proposed three primary mechanisms through which food provisioning can influence host-parasite dynamics; by altering 1) host contact and movement behaviours, 2) host demography, and 3) host immune defences. Immune defences are critical to a host's ability to resist and combat infection, but compete with other physiological traits for energy and nutrients [9]. This point is emphasised by the relationship between physiological condition - one of the most obvious external manifestations of access to food resources - and infection. Accumulating evidence indicates a "vicious circle" between poor condition and infection that is mediated by a hosts' immune capacity [10]. Under this scenario, individuals in the worst physical condition are least capable of resisting infections, which further worsen their condition and in turn increase infection loads. However, despite the obvious benefits conferred to wildlife by access to abundant and predictable food sources, their effects on host immunity may not be universally positive [2, 11]. Anthropogenic food may contain contaminants that impair immunity [12, 13], or lack important nutrients that are provided through a more balanced natural diet [14, 15]. These effects are especially likely when food provisioning is unintentional, and wildlife consequences have therefore often not been considered.

The countless forms of anthropogenic food, and their potential to both positively and negatively affect host immunity, make predicting the immunological implications of food provisioning difficult. Our aim here is to synthesise these studies and suggest hypotheses that may help resolve apparent discrepancies between them. Some of the concepts covered herein have been well studied using laboratory and biomedical model organisms. However, we argue that inherent differences between these species and the research settings necessitate a wildlife-specific line of investigation. For this reason, we give greatest attention to research carried out on wildlife species in their natural and semi-natural environments (such as large enclosures), which we complement with studies on wildlife that have been translocated to more controlled settings (such as laboratories). Due to their predominance in the literature, our focus is on terrestrial vertebrates, and we address both the abundance of food resources and the role of specific nutrients, as well as highlight the immune parameters that have been used to investigate this topic. Lastly, we draw attention to gaps in knowledge and the research required to progress understanding.

2. Wild immunology

(a) Optimising resource expenditure

Understanding how the immune systems of different species function in their natural habitat is a core aim of wild immunology. Maintaining immune defences and responding to immunological challenges is energetically and nutritionally costly [16, 17]. When food or nutrient availability is

limited, its allocation to immunity may mobilise resources that could otherwise be devoted to alternative processes, such as growth, reproduction or ornamentation [18, 19]. Conversely, investment in these other processes can constrain a hosts' ability to adequately respond to infection. Some of the strongest evidence for such trade-offs comes from experimental studies manipulating reproductive effort in avian species [20]. For example, nesting collared flycatchers (*Ficedula albicollis*) were immunized against Newcastle Disease Virus (NDV) and brood size was altered by removing, increasing or displacing eggs [21]. The researchers found that NVD-specific antibody responses decreased with reproductive effort, while in non-immunised birds, the intensity of *Haemoproteus* infections (a common haemosporidian in wild birds) increased.

An expanding body of research has also identified seasonal variation in the immune function of wildlife [9, 22, 23], which tends to covary with energetically demanding physiological processes [24]. As a consequence, seasonal rhythms in immune defences are suggested to have evolved to conserve energy reserves when food is scarce and/or infection risk is low [23, 24]. In line with this, Owen and Moore [25] identified reduced immune investment in three thrush species during migration. Interestingly, when one of the species was translocated to indoor aviaries and migratory restlessness induced by manipulating photoperiod, the birds displayed lower immune responses to phytohaemagglutinin challenge than controls with untampered photo period, despite both groups having access to *ad libitum* food [26]. This finding indicates that immune investment is at least partly a rigid trait, triggered by environmental cues, and raises questions regarding its flexibility in response to resource increases. However, it should be noted that short-term plasticity in wildlife immune responses have been documented for other host stressors, such as social interactions [27], suggesting the same may occur due to changes in food availability.

(b) Aims, challenges and opportunities

Two challenges need to be addressed in wild immunology: identifying and interpreting specific characteristics of each species' immune system (between-species genetic characteristics) that impact how they respond to specific parasites, and disentangling environmental sources of variation within species. Indeed, despite a high level of conservation, the immune system diverges in significant ways across taxa. While overall immune function is conserved [28], details can differ that may lead to diverse responses to identical pathogens. This has been particularly well-studied for mice and humans, where there are numerous divergences (e.g. the balance of leukocyte subsets, toll receptors, and B and T cell signalling pathway components) [29] that can be expected to apply similarly to wildlife. Such discrepancies make generalising mechanistic details of immune processes hazardous, and call for specific reagents and rigorous validation in the focal species.

As a consequence, disentangling the complex interplay between the immune system and environmental variation in exposure, seasonality, and resource availability in natural populations has been severely hampered by the lack of bespoke immunological assays. Furthermore, while ecologists and eco-immunologists have long focussed on natural variation as the underlying topic of their research agendas, immunologists have focussed on mechanism, which is best studied when all extraneous 'noise' is eliminated. There is therefore little understanding of the causes and consequences of variation in how immunity is expressed within populations. To properly account

for variation, wildlife studies require large sample numbers to detect significant effects. Wild immunology thus requires cross-fertilisation of the mechanistic studies within natural variability, of high throughput platforms that can help overcome current limitations in specific immunological reagents, and of the appropriate statistical and modelling approaches to link individual immune profiles with community and population dynamics [30].

(c) Practical considerations

To overcome the technical barrier of studying immunity in non-model systems, researchers typically rely on knowledge of, and reagents initially developed for, better-studied species. Several different assays have been used to measure the innate immune function of wild animals within the context of food provisioning (Figure 1). Microbial killing assays (MKA) are a common method to assess humoural innate immunity, especially in birds [31]. The major player in humoural innate immunity is the complement system, which upon activation forms an enzymatic cascade aimed at the destruction of target parasites [32]. Natural antibodies (nAbs) are another component of humoural innate immunity, which constitutively circulate in the host and are capable of binding antigens non-specifically. For MKAs, animal serum or plasma, consisting of complement proteins and nAbs, is incubated for a specified time with a parasite (the bacterium *Escherichia coli* is often used) *in vitro* and the resulting decrease in microbe viability assessed. Importantly for field studies, the blood sample can be stored frozen before the assay is performed (although, ideally at -80°C). A modified version of the MKA, the whole blood microbial killing assay (WBMKA), measures the activity of phagocytic cells (mainly macrophages and neutrophils) in addition to humoural innate immunity. Unlike MKAs, this method can only be conducted using freshly collected whole blood. While MKAs and WBMKAs don't specify the exact component responsible, they are an efficient and effective method for assessing complete innate immune function.

Cells of the immune system, primarily phagocytic cells and lymphocytes, are collectively referred to as white blood cells (WBCs) [32]. The number and proportions of phagocytic cells in blood can be used to measure innate immunity, with the ratio of neutrophils (mammals) or their equivalent heterophils (birds) to lymphocytes (N or H:L ratio) especially common [9]. For this assay, WBCs are counted using a fresh blood sample [31]. During an inflammatory response, neutrophil (and heterophil) increases are more pronounced than lymphocytes. Like the N:L ratio, total WBC counts can be assessed from a small amount of blood, but are again, difficult to interpret. As such, quantifying the magnitude of responses to immune challenge is more robust for assessing immune function than simply measuring constitutive levels of immune parameters, such as total or differential WBC counts. In general, assays for innate immunity are relatively straight forward. They can be conducted outside of sophisticated laboratories and require a single blood sample, rendering them suitable for many field-based studies [31, 33].

As compared to innate immunity, adaptive immunity is usually more difficult to measure under field conditions due to the need for invasive procedures (such as antigen administration) and repeated sampling. Similar to the innate system, adaptive immune responses involve humoural and cellular components that are mediated by T and B lymphocytes, respectively. Delayed type

hypersensitivity (DTH) assays are commonly used to assess cellular adaptive immunity towards an antigen *in vivo* [31,33]. Typically, this assay requires injection of an antigen to the skin, followed by assessment of swelling 2-3 days post-challenge. This delayed inflammatory reaction is mostly caused by the accumulation of T cells, which respond to antigen at the site of injection. For wildlife, a DTH assay based on phytohaemagglutinin (PHA) injection is widely employed, especially for birds [31, 34]. PHA is a T cell mitogen, and this test is commonly used to approximate T cell responsiveness in wildlife. However, as swelling results from multiple cell types becoming activated and entering the inflamed tissue, caution should be exercised when interpreting the outcome of this assay [34], and comparisons across species are often hindered by physiological differences, such as skin tightness. A more specifc way to assess T cell immunity is by measuring the proliferation and cytokine expression of T cells, isolated from blood or secondary lymphoid organs, in response to an antigen *in vitro.* The humoural arm of adaptive immunity consists of antigen-specific antibodies known as immunoglobulin (Ig) proteins, which are produced by B cells [32]. These are often measured as the magnitude of an antibody response towards a novel antigen (primary response) or a previously encountered antigen (secondary or memory humoural response). Sheep red blood cells (SRBCs) or keyhole limpet haemocyanin (KLH) are widely used for this purpose in mammals and birds [31, 33, 35].

3. Reviewing the literature

In this section we discuss field-based, captive food provisioning, and captive food restriction studies separately to facilitate clarity. Within each of these, we group studies as they apply to different taxonomic assemblages and forms of food provisioning. Due to their ability to replicate the natural host environment, research conducted in large outdoor enclosures has been included under field studies, rather than captive studies, which we use to focus on highly controlled settings. Key aspects of the field studies are summarised in Table 1, while the captive food provisioning and food restriction studies are summarised in Table S1 in the supplementary material.

(a) Field studies

Few field studies have investigated the effects of food provisioning on immunity in wildlife. Those to have done so have often focussed on the abundance of food resources, rather than comparing the effects of specific nutrients. For example, in vampire bats, livestock biomass was positively associated with indices of innate immunity (increased neutrophils and MKA response) and decreased odds of *Bartonella* and hemoplasmas infection [36]. Interestingly, livestock biomass was a stronger predictor of the relationship between food resources and immunity than individual bat diet, indicating an inflammatory response associated with livestock density (increased N:L ratio), potentially caused by greater exposure to pathogens, lower quality resources, and/or changes in bat population structure.

A wide spectrum of immune outcomes were studied in cotton rats (*Sigmodon hispidus*) and field voles (*Microtus agrestis*) that were translocated to large outdoor enclosures. In cotton rats, mixed-ration and methionine-enhanced food supplementation elevated total WBC counts (specifically in response to methionine), but failed to influence the N:L ratio or T cell proliferation [37]. *Ad libitum*

supplementation of wild field voles with a protein-rich diet during the resource limited boreal winter enabled robust changes in differential WBC counts (increased N:L ratio) in response to helminth, but not *Eimeria* or *Bordetella bronchiseptica* infections [19, 38]. Food supplemented vole populations also displayed enhanced adaptive humoural immunity, as seen through higher circulating total IgG levels in response to helminth infections, as well as lower helminth infection prevalence [19, 39]. However, the opposite seemed to occur in *B. bronchiseptica* infected voles [38]. Little or no effects were seen on total IgG levels without pathogen challenge in field voles [19, 40], emphasising the importance of assessing functional parameters of innate immunity, such as MKAs.

Turning to avian species, increased humoural innate immunity (through MKAs) was shown following the supplementation of 11 different wild bird species with *ad libitum* birdseed [41]. Food supplementation also improved their general health and decreased the H:L ratio, suggesting reduced stress (less inflammation) [42]. A reduced H:L ratio together with enhanced cellular and humoural adaptive immunity, as detected by PHA-directed wing web swelling and antibody responses to SRBC, was also observed for serin (*Serinus serinus*) nestlings raised in a food resource-rich environment when compared to a food-scarce environment [43]. However, non-specific food provisioning may conceal potential negative effects on host immunity. Spanish Imperial Eagle (*Aquila adalberti)* nestlings were supplemented with wild and domestic rabbits with the goal of improving breeding productivity [44]. Unexpectedly, antibiotics in the domestic rabbits led to a reduction in eaglet complement activity (innate humoural response) and their overall health.

The effects of micronutrients on the immunity of non-captive wildlife species have rarely been investigated. In one study, carotenoid-fed female lesser black-backed gulls and the eggs they produced contained lower total immunoglobulin levels than non-supplemented birds [45]. This finding seemingly contradicts the suspected immune-boosting properties of carotenoids [46]. However, the authors suggest that it may in fact reflect a reduced need for immunoglobulins due to enhanced efficiency of the innate immune response at clearing infections during the period preceding sample collection (this may also explain the finding in voles discussed above [38]). Meanwhile, supplementing eggs with carotenoids boosted the T cell mediated immunity of nestling barn swallows, but did not affect their humoural immune response to Newcastle disease virus vaccine [47]. Other research indicates that the immunomodulating effects of carotenoids are dependent on a particular compound, β-carotene, as supplementation with β-carotene in combination with lutein and zeaxanthin, but not lutein and zeaxanthin alone, boosted DTH in nestling great tits [48].

We identified only a single study on reptiles. In this, MKAs were used to evaluate humoural innate immunity in response to elevated testosterone delivered via external patches and/or food provisioning with vitamin dusted mealworms and crickets in male Sagebrush lizards (*Sceloporus graciosus)* [49]. Food supplementation increased immune function in non-treated and testosterone-treated individuals. However, immune responses were greater in testosterone-treated than non-treated lizards, indicating an absence of testosterone-mediated immune suppressive effects.

(b) Captive food provisioning studies

Recapturing animals, administering treatments, and collecting and maintaining the integrity of samples tends to be more difficult in field than captive settings. As such, the majority of studies assessing the effects of food provisioning on wildlife immunity have been performed in captivity. Based on the distinction between captive and food restriction studies applied for this review, all captive studies have been conducted using bird species. The studies on non-captive birds discussed above found that supplementation with an energy-rich diet lowered the H:L ratio [41, 43], indicative of reduced stress and lower constitutive levels of innate immunity [50]. However, research on captive birds (curve-billed thrashers and hooded crows) [51, 52], comparing stable (predictable) to variable (non-predictable) feeding regimes, failed to detect any effect on the H:L ratio, despite reduced body mass for both species on the variable diets, and for the thrashers, also elevated levels of the stress hormone, corticosterone. Together these findings indicate that the stress associated with maintaining some species of wild birds in captivity may negate the positive effects of food supplementation on the H:L ratio.

An energy-rich diet positively influenced adaptive immunity in hooded crows (*Corvus corone*) given variable feeding regimes, with body mass loss negatively associated with T cell immunity, as assessed via PHA-directed wing web swelling [51]. Similarly, male Japanese quail (*Coturnix coturnix*) fed with standard poultry feed had stronger Ig responses to chucker partridge red blood cells and PHA-directed wing-web swelling, when compared to those fed with an energy-reduced corn-based diet [53]. The corn-based diet also had a negative effect on the secondary humoural responses of quail, but only in the presence of lead, a toxic compound common around shooting ranges. Lead was similarly found to decrease the peak of secondary (or memory) anti-KLH Ig responses in feral pigeons (*Columba livia*) [54]. However, these negative effects were mitigated by zinc supplementation, which also increased the primary Ig response and T-cell immunity as seen through a PHA-directed wing web swelling response.

In line with the field studies, high protein-content food provisioning was positively correlated with T cell immunity, as measured by PHA-directed wing web swelling in Northern bobwhite (quail) chicks (*Colinus virginianus*). It also increased spleen mass, but did not influence responses to SRBC or *ex vivo* lymphoproliferative reactions to a range of stimuli [55]. In contrast, pheasants (*Phasianus colchicus*) allocated to high-protein content food supplementation displayed higher adaptive humoural responses to diphtheria and tetanus antigens than those with low protein, while no effect was seen on PHA-induced, T cell-mediated wing web swelling [56]. A study of male house sparrows (*Passer domesticus*) found no effects of dietary protein content on antibody responses to diphtheria or tetanus antigens [57]. The researchers additionally replaced phenylalanine and tryptophan, precursors to melanin, with glutamic acid and demonstrated elevated antibody responses to the vaccines. However, in a secondary experiment without glutamic acid addition, no affect occurred following phenylalanine and tryptophan removal, indicating that glutamic acid may boost adaptive humoural immunity [57].

In addition to their suspected immune boosting activity, carotenoids are used for coloration in sexually-selected ornamentation traits [46]. Many evolutionary biologists have capitalised on this dual role to investigate trade-offs in their allocation. Carotenoids are distinguished between

xanthophylls, which contain oxygen, and carotenes, which do not. The studies discussed below are based on xanthophyll carotenoid supplementation unless otherwise stated. Carotenoids were found to boost WBMKA responses in male and female house finches (*Carpodacus mexicanus*) during the moult period (the same effect did not occur during the non-moult period) [58] and also in male society finches (*Lonchura domestica*) (females were not assessed) [59]. Society finches do not employ carotenoid-dependent coloration, supporting a direct immune modulating effect. WBMKA responses in male jungle fowl (*Gallus gallus*) were also enhanced by supplementation (no effect was seen in females) [60]. However somewhat surprisingly, carotenoid supplementation reduced macrophage phagocytosis in both sexes. Meanwhile, no effects were seen on the H:L ratio or oxidative burst of whole blood in Moorhen chicks (*Gallinula cholorpus*) [61] or male greenfinches (*Carduelis chloris*) [62]. Taken together, it seems that carotenoids have a specific, and also potentially antagonistic, effect on components of the innate immune system.

With regard to adaptive immunity, early work demonstrated that carotenoids supplemented into the drinking water of male zebra finches enhanced T-cell mediated immunity and humoural responses, as measured by PHA-stimulated wing swelling [63] and responses to SRBC, respectively [64]. Similar positive effects of carotenoid supplementation were seen on PHA-directed wing swelling in house finches during the moult period [58]. In male green finches, carotenoid supplementation enhanced antibody responses to *Brucella abortus* antigen in one study [65], but not in another [62]. The latter finding is consistent with studies of green finches, indicating no effect of carotenoids on adaptive humoural immunity, as measured by SRBC assays [65-67]. Similarly, carotenoid supplementation did not enhance PHA-stimulated wing swelling in male society finches [59] or male American goldfinches [68]. In addition to xanthophyll carotenoids discussed above, several studies have demonstrated beneficial effects of β-carotene on T cell immunity in chick and female, but not male, grey partridges (*Perdix perdix*) [69-71].

It has been argued that flavonoids may be a more important form of dietary antioxidants than carotenoids due to their higher bioavailability (from fruits) and more robust functions [72]. In support of this concept, flavonoids were shown to increase humoural responses to SRBCs in blackcaps (*Sylvia atricapilla*) [73]. However, a lack of additional research on this topic precludes general conclusions.

(c) Captive food restriction studies

Food restriction (FR) experiments are limited to highly controlled settings where food intake can be closely regulated. These studies, in which animals are given a proportion of their daily energy requirements, are often used to assess the level of food deprivation under which immunity can be maintained. The effects of FR on innate immunity in wildlife have received little research attention. Tuco-tuco's (*Ctenomys talarum*) were FR to levels that decreased body mass by 10-25%, which in turn increased their N:L ratio (indicating stress) but had no effect on MKAs or nAbs [74, 75]. A study using capybaras (*Hydrochoerus hydrochaeris*) similarly found that FR (40-50% reduced intake) increased nAbs and eosinophil levels [76]. In Siberian hamsters (*Phodopus sungorus*), MKAs were positively correlated with the number of fat deposits in FR animals, while no effect was seen on those receiving *ad libitum* food [77].

Pioneering work by Lochmiller et al. [78] found that moderate FR (80% *ad libitum*) increased, whereas severe FR (80% *ad libitum* followed by 40%) reduced adaptive immunity in cotton rats (*Sigmodon hispidus*), as measured by *ex vivo* splenocyte lymphoproliferative responses to lectins. Severe FR further dampened T cell immunity as revealed by DTH *in vivo* (the effect of moderate FR was not studied with this assay). Fasting in female Mongolian gerbils (*Meriones unguiculatus*) similarly lowered the DTH response [79]. Taken together with the study assessing innate immunity components in capybaras mentioned above [76], it seems that under stressful conditions, such as food shortage, immunity can at least transiently increase, whereas long-term or more severe FR can cause reduced immune function due to energetic shortages.

Other studies have similarly found that severe (75%) FR reduced DTH [80] and modest FR (25%) increase antibody responses [77] in Siberian hamsters, but only under short (not long) day lengths. This may reflect the ability of some species to boost immunity for winter, when infection risk is suggested to be high [81], although such generalisations regarding seasonal variation in disease risk are debatable [22]. Similar observations have been made for the calves of Iberian red deer (*Cervus elaphus hispanicus*), where 50% FR elevated total Ig levels [82]. FR has also been shown to downregulate T cell immunity in several bird species, including yellow-legged gulls, hand-reared sand martin nestlings and little ringed plovers [83-85]

It is clear that an adaptive immune response to a novel antigen is energetically costly due to increases in lymphocyte proliferation and protein production in the form of antibodies. However, the energetic demands of maintaining immunological memory (i.e. long-lived quiescent memory lymphocytes) to a specific antigen have not been well-addressed in wildlife. A modest and transient decrease in diet (70% *ad libitum*) prior to secondary KLH challenge in male deer mice (*Peromyscus maniculatus*) resulted in a reduced KLH-specific IgG response, which was accompanied by reduced numbers of splenic B220+ IgG producing B cells [86, 87]. It thus seems likely that the reduced secondary IgG response was due to diminished B cell numbers in response to food restriction. Unfortunately, the ability of transiently food restricted animals to respond to primary antigen challenge was not assessed in these studies.

4. Conclusions

Variation among studies in terms of the research settings, quantity and quality of food provisioning, sample numbers, and often immune parameters and host species, limits the appropriateness of direct comparisons. Importantly however, examples exist demonstrating the potential for food provisioning and specific nutrients to enhance wildlife immunity across the three study type distinctions applied for this review (field, captive food provisioning and captive food restriction), as do cases where contaminants associated with anthropogenic food sources impaired immune function (specifically, antibiotics and lead).

In the field studies, food provisioning positively influenced both innate and adaptive immune function in birds (Figure 2, Table 1), with several studies demonstrating elevated MKA and DTH responses to increased food availability, and especially to carotenoids. However, for mammals, the

same general conclusion is precluded by the lack of available data. In addition to the low number of studies being available, only some employed assays that measure functional immune responses (e.g. MKA, DTH). As the influence of a hosts' general health on constitutive levels of immune parameters are usually unknown, assessing immune function without immune stimulation is fraught with interpretational issues. The positive effect of methionine supplementation on WBC counts in cotton rats is supported by research on field voles provisioned with a protein-rich diet. These highlight, the importance of qualitative components, specifically proteins, in energy-rich diets for immune cell proliferation.

In contrast to wild birds, food provisioning did not decrease the H:L ratio in captive birds. In addition to being a measure of cellular innate immunity, elevated H:L ratios are likely to reflect host stress [50]. Thus, it appears that if food resources are readily available, wild birds are less stressed than their captive counterparts. Meanwhile, and consistent with the field studies, carotenoids boosted innate immunity and protein-rich diets elevated cellular and humoural adaptive immunity in captive birds. However, the effect of carotenoids on adaptive immunity are less clear for captive than for wild birds. On one hand, this could be because captivity-related stress has less influence on innate than adaptive immunity. On the other hand, carotenoid-driven immunity might be more sensitive to stress than immune function based on the quantity of energy input. Importantly, captive studies revealed that not only carotenoids, but also other micronutrients (zinc, glutamic acid, flavonoids), can have positive effects on the immunity of birds.

Although the amount and duration of food availability often varied, clear trends presented in the food restriction studies; specifically, immune function was often elevated by moderate food restriction and suppressed by severe food restriction (Figure 2, Table S1). This conclusion is further supported by research with laboratory mice [88]. It is thus likely that stress related to moderate FR increases immunity as a prophylactic measure towards fighting future infections, whereas extensive FR decreases immunity due to energy shortages.

5. Future prospects

The strongest evidence for the effects of anthropogenic food provisioning on wildlife immunity will come from studies carried out in natural settings, which indeed form the basis of wild immunology. While this review has highlighted important field contributions, further research is clearly required to enable robust conclusions. These will ideally integrate nutritional manipulation (with appropriate controls), immune challenge and measures of functional immunity, and should also aim to assess the indirect effects of food provisioning on wildlife immunity. For example, abundant resources usually support higher population densities and can cause wildlife to move into less favourable environments; both of which can amplify host stressors that impair wildlife immunity, such as intraspecific competition and predation [27, 89, 90]. This line of research calls for multi-disciplinary research agendas, and astute study designs to elucidate mechanisms leading to variation in immune function.

As the reports above demonstrate, while antibody levels, visible signs of inflammation, and infection burdens have been widely utilised for wildlife and non-model species more generally,

these techniques can only provide limited insight into how observed changes in immune responses are effected: are immune responses controlled, for example, by the central nervous or hormonal system as part of an evolutionary adaptive strategy, or are they are starved of necessary nutritional elements, which prevents it from functioning normally; do discrete arms of the response, such as anti-bacterial vs. anti-helminthic, respond differently to changes in food quality and quantity; does the immune system prioritise responses to certain classes of pathogens over others, and if so, how does that change with age, climate, or parasite communities? Answering such questions, be they about genetic adaptation, phenotypic plasticity, or resource competition, would all benefit from a more detailed characterisation of underlying immune processes, and using immune stimuli that more closely mimic natural antigens than KLH, PHA, or SRBC, which remain very crude. In addition to increasing our understanding of the immune system at a fundamental level, the implications for translational activities, including drug intervention, vaccine design and coverage, and disease control, are profound.

Thankfully, technological progress is beginning to help overcome the lack of reagents for quantifying specific immune indices in non-model species. The recent development of polymerase chain reaction (PCR)-based sequencing assays has the potential to revolutionise ecoimmunological research [91, 92]. For instance, cytokine expression profiling assays, which conventionally employ species-specific recombinant antibodies and have therefore been restricted to model species, can now be used for non-model organisms, such as wildlife, via genome-wide RNA sequencing. By using this method, it is possible to quantify messenger RNA expression levels across the genome (e.g. to evaluate cytokine expression) with relatively small volumes of cellular samples, such as whole blood. Sequencing the genome or assembling a transcriptome *de novo* are the quickest ways to generate the required immunological information. We encourage researchers to explore progressive methods, such as this, in future studies.

Competing interests

We have no competing interests.

Authors' contributions

All authors contributed to the structure design, and prepared different sections for the review. All authors then edited and approved the final document.

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Figure and table captions

Figure 1. Assays used in ecoimmunology. The immune system can be broadly divided into innate and adaptive arms. Complement proteins, natural antibodies (nAbs) and phagocytic cells (mainly neutrophils but also monocytes and macrophages) are the main components of innate immunity, whereas T and B lymphocytes, the latter responsible for immunoglobulin (Ig) production, mediate adaptive immunity. Microbial killing assays (MKA) assess the functionality of complement proteins and nAbs in blood, while whole blood microbial killing assays (WBMKA) additionally include phagocytic cells of the innate immune arm. The N:L ratio measures the relative number of

neutrophils, as compared to T and B lymphocytes, in blood, and total white blood cell (WBC) counts measure the total number of immune cells (phagocytes and lymphocytes). Delayed-type hypersensitivity (DTH) assays quantify Th1 lymphocyte responses, while assays measuring total or specific Ig levels approximate adaptive B lymphocyte responses.

Figure 2. Key findings from different study types. We distinguished between field-based, captive food provisioning, and captive food restriction studies. Due to their ability to replicate the natural host environment, studies conducted in large outdoor enclosures were included under field studied rather than captive studies, which focussed on more controlled settings such as laboratories.

Table 1. Key aspects of field studies assessing the effects of food provisioning on immunity in wildlife species.

Table 1.

Host species	Type of food provisioning	Immune assay(s)	Type of immunity	Key results	Reference
Male cotton rats (*Sigmodon hispidus*)	1) *ad libitum* mixed ration + methionine 2) *ad libitum* mixed ration 3) no supplementation	PHA-directed skin swelling, total complement activity, total and differential WBC counts	T cell immunity, humoral innate immunity, cellular immunity	Elevated WBC levels with methionine, no other effects.	[37]
Field voles (*Microtus agrestis*)	1) *ad libitum* high protein 2) no supplementation	Total WBC counts, N:L ratio	Cellular immunity	Elevated WBC levels, no other effects.	[38]
Field voles (*Microtus agrestis*)	1) *ad libitum* 30% protein 2) *ad libitum* 1% protein 3) no supplementation	Total IgG	Constitutive humoral immunity	No overall effect. Elevated in females at end time point.	[39]
Field voles (*Microtus agrestis*)	1) *ad libitum* high protein 2) no supplementation	Total IgG, N:L ratio	Constitutive humoral immunity, cellular immunity	Increased in helminth infected voles. No effects in eimeria infected voles.	[19]
Vampire bats (*Desmodus rotundus*)	Variations in livestock biomass (non-manipulated)	Total WBC, differential WBC counts, total IgG, MKA	Humoral innate immunity, cellular immunity constitutive humoral immunity	WBC and MKA levels positively associated with livestock biomass, Ig and lymphocytes decreased	[36]
Eleven different avian species	1) *ad libitum* birdseed 2) no supplementation	H:L ratio, MKA	Humoral innate immunity and cellular immunity	Decreased H:L ratio, increased MKA.	[41]
Serin nestlings (*Serinus serinus*)	Variation in food availability around nest site (non-manipulated)	PHA-directed wing web swelling, SRBC assay, H:L ratio	Cellular immunity, adaptive humoral and T cell immunity	Wing web swelling and SRBC responses positively associated with food availability, H:L ratio decreased.	[43]

Spanish Imperial Eagle nestlings (*Aquila adalberti)*	1) wild or domestic rabbits from markets (for human consumption) 2) wild or domestic rabbits from farms (high risk for veterinary drugs) 3) no supplementation	Complement activity	Humoral innate immunity	Decreased due to pharmaceuticals in domestic rabbits.	[44]
Lesser blackbacked gulls *(Larus fuscus)*	1) 2mg carotenoids + 20g vegetable fat daily 2) 20g vegetable fat daily	Total Ig	Constitutive humoral immunity	Decreased	[45]
Barn swallows (*Hirundo rustica*)	1) Carotenoid (lutein) supplementation of eggs + corn oil 2) Corn oil alone 3) Egg displacement (no supplementation)	PHA-directed wing web swelling, antibody response to Newcastle disease virus vaccine	Adaptive humoral and T cell immunity	T cell immunity increased, no effect on humoral response	[47]
Great tit nestlings (*Parus major*)	1) carotenoids (lutein and zeaxanthin) every other day 2) carotenoids (lutein, zeaxanthin, β-carotene) every other day 3) no supplementation	PHA-directed wing web swelling after diphtheria toxin + SRBC immunisation	T cell immunity	β-carotene had positive effect but only in immunised nestlings, no other effects	[48]
Male Sagebrush lizards (*Sceloporus graciosus)*	1) vitamin dusted mealworms and/or crickets 2) no supplementation	MKA	Humoral innate immunity	Increased (especially is lizards given testosterone patches)	[49]

Supplementary material

Table S1. Key aspects of captive and food restriction studies assessing the effects of food provisioning on immunity in wildlife species. References are listed in the main document.

Captive studies					
Host species	Type of food provisioning	Immune assay(s)	Type of immunity	Key results	Reference
Hooded Crow ***(Corvus corone)***	Variations in food quantity and predictability	H:L ratio, PHA-directed wing web swelling	T cell immunity and cellular immunity	No effect of H:L ratio despite body mass changes due to low food quantity and predictability, T cell immunity decreased with low predictability	[51]
Male curve-billed thrashers (*Toxostoma curvirostre)*	1) Variable food amounts (30 to 200% of daily food intake) 2) Constant (predictable) food amounts	H:L ratio	Cellular immunity	No significant effect despite decrease in body mass by variable feeding	[52]

Male Japanese quail *(Coturnix coturnix)*	1) standard poultry feed 2) ground corn (± lead or corticosterone for both groups)	Primary and secondary antibody response to chukar partridge red blood cells, PHA-directed wing web swelling, differential WBC counts	Adaptive humoral and T cell immunity, cellular immunity	Elevated H:L ratios in corn + lead, corn-diet decreased primary and together with lead also secondary IgG responses, lower T cell immunity with corn	[53]
Feral pigeons (*Columba livia*)	1) Lead + zinc supplementation in drinking water 2) Lead supplementation alone 3) Zinc supplementation alone 4) No supplementation	Primary and secondary IgY response to KLH, PHA-directed wing web swelling	Adaptive humoral and T cell immunity	Zinc increased primary and buffered the negative effect of lead on the secondary humoral response, Zinc also increased T cell immunity	[54]
Northern Bobwhite chicks (*Colinus virginianus*)	1) *Ad libitum* 8% dietary-protein feed 2) *Ad libitum* 15% dietary-protein feed 3) *Ad libitum* 33% dietary-protein	Total WBC counts, PHA-directed wing web swelling, SRBC assay, lymphocyte proliferation in response to conA, PWM and STM	Adaptive cellular and humoral immunity, Cellular immunity	No effect on total WBCs, T cell responsiveness to PHA positively associated with dietary protein, no effect on primary humoral response or	[55]

		(Salmonella typhimurium antigen), spleen mass		lymphoproliferation, spleen mass increased	
Ring-necked pheasant (*Phasianus colchicus*)	1) High protein food (24%) + canthaxanthin carotenoid in drinking water 2) High protein food alone 3) Low protein food (8%) + canthaxanthin carotenoid in drinking water 4) Low protein food alone	PHA-directed wing web swelling, primary and secondary response to diphtheria and tetanus toxoids	Adaptive humoral and T cell immunity	High protein content increased humoral responses but did not affect cellular immunity, carotenoids did not affect either immune parameter	[56]
Male house sparrows (*Passer domesticus*)	1) High protein (10%) 2) Low protein (6%) 3) Median protein (8%) with 50% reduced phenylalanine/tryptophan content with/without compensating increase of glutamic acid	Secondary response to diphtheria and tetanus (DT) vaccine	Adaptive humoral immunity	Glutamic acid elevated secondary antibody response to DT, no effect with other treatments	[57]

House finches (*Carpodacus mexicanus*)	1) Carotenoid supplementation in drinking water 2) No carotenoid supplementation	PHA-directed wing web swelling, WBMKA	T cell immunity and functional innate immunity	Increased during molt period, no effect during non-molt period	[58]
Male society finches (*Lonchura domestica*)	1) White millet + carotenoid beadlets (lutein and zeaxanthin) 2) White millet alone	PHA-directed wing web swelling, WBMKA	T cell immunity and functional innate immunity	Innate immunity increased, no effect on T cell immunity	[59]
Red junglefowl (*gallus gallus*)	1) Commercial chicken feed + carotenoids (lutein and zeaxanthin) 2) Commercial chicken feed alone	WBMKA, macrophage phagocytosis assay	Functional innate immunity	WBMKA increased in males, phagocytosis decreased in males and females	[60]
Male greenfinches (*Carduelis chloris*)	1) Carotenoid supplementation (lutein zeaxanthin) in drinking water 2) No carotenoid supplementation	Whole-blood oxidative burst response to lipopolysaccharide, antibody response to *Brucella abortus* antigen	Adaptive humoral immunity, innate cellular immunity	No effect	[61]

Moorhen chicks (*Gallinula cholorpus*)	1) Basic diet (cereal and earthworms) + daily canthaxantin carotenoid supplementation 2) Basic diet alone	PHA-directed wing web swelling, differential WBC	T cell immunity and cellular immunity	T cell immunity increased, no effect on WBCs	[62]
Male zebra finches (*Taeniopygia guttata*)	1) Carotenoids (lutein and Zeaxanthin) *ad libitum* in drinking water 2) No carotenoids in drinking water	PHA-directed wing web swelling	T cell immunity	Increased	[63]
Male zebra finches (*Taeniopygia guttata*)	1) Carotenoids (lutein and zeaxanthin) in drinking water 2) No carotenoids in drinking water	PHA-directed wing web swelling, SRBC assay	Adaptive humoral and T cell immunity	Increased	[64]

Male greenfinches (*Carduelis chloris*)	1) Carotenoid supplementation (5.5 µg/ml of xanthophylls into water) (88% zeaxanthin, 5% zeaxanthin) 2) Xanthophylls (1.1µg/l) 3) Xanthophylls (0.55µg/ml) 4) No carotenoid supplementation	SRBC assay, antibody response to BA	Adaptive humoral immunity	No effect in SRBC assay, increased in response to BA	[65]
Greenfinches (*Carduelis chloris*)	1) Carotenoid supplementation (12mg/l; lutein, zeaxanthin 95:5) and vitamin E (0-500 mg/l) 2) No carotenoid supplementation	PHA-directed wing web swelling, SRBC assay	Adaptive humoral and T cell immunity	No effect	[66,67]
Male American goldfinches (*Carduelis tristis*)	1) 0.01g/l Carotenoid supplementation (lutein and zeaxanthin) 2) 0.1g/l Carotenoid supplementation	PHA-directed wing web swelling, SRBC assay	Adaptive humoral and T cell immunity	No effect	[68]

	3) 1.0g/l Carotenoid supplementation				
Adult grey partridges, *Perdix perdix*	1) Standard partridge diet + 2.7 mg/kg β-carotene 2) Standard partridge diet + 27 mg/kg β-carotene	PHA-directed wing web swelling	T cell immunity	Increased in females, no effect in males	[69]
Chick grey partridges, *Perdix perdix*	β-carotene (0.22% to 2.2% in pellets)	PHA-directed wing web swelling	T cell immunity	Increased	[70,71]
Blackcaps (*Sylvia atricapilla*)	1) Standard diet + 2·8 mg flavonoids daily 2) Standard diet	SRBC assay	Adaptive humoral immunity	Increased	[73]
Food restriction studies					

Tuco-tucos (*Ctenomys talarum*)	1) FR (resulting in 10% weight loss) 2) No FR 3) No FR + intramuscular methionine injections	PHA-dependent skin swelling, MKA, total WBC, differential WBC, SRBC agglutination (nAbs)	T cell immunity, humoral innate immunity, cellular immunity	FR decreased T cell immunity and increased N:L ratio, no effect on other parameters, methionine supplementation did not affect any parameters	[74]
Tuco-tucos (*Ctenomys talarum*)	1) Slight FR (85% initial body weight) 2) Severe FR (75% initial body weight)	SRBC antigen assay, MKA, total WBC, differential WBC	Adaptive humoral immunity, Innate humoral immunity, cellular immunity	N:L ratio increased with severe FR, no differences between slight and severe FR for other parameters	[75]
Capybaras (*Hydrochoerus hydrochaeris)*	1) 40-50% reduced normal food intake 2) *Ad libitum* food	Spleen mass, Differential WBC counts, Total Igs, Nabs	Cellular immunity, humoral innate immunity, constitutive humoral immunity	Eosinophils and Nabs increased with FR, spleen mass borderline reduced, no effect on other WBCs or total Igs	[76]

Female Siberian hamsters (*Phodopus sungorus*)	1) FR (70% *ad libitum*) 2) No FR	IgG response to KLH, MKA	Innate and adaptive humoral immunity	Adaptive humoral immunity increased (only in short-day conditions), innate immunity positively correlate with body fat in FR but not *ad libitum*-fed animals	[77]
Cotton rats *(sigmodon hispidus)*	1) Severe FR (80% then 40% *ad libitum*) 2) Moderate FR (80% *ad libitum*) 3) no FR	lymphocyte proliferation in response to conA and PWM, DTH response to oxazolone	Adaptive cellular immunity	Moderate FR increased and severe FR reduced lymphoproliferative responses, DTH reduced in severe FR	[78]
Female Mongolian gerbils (*Meriones unguiculatus*)	1) Fasting (3 days) 2) No fasting	Footpad thickness in response to PHA, total WBC	Cellular immunity and T cell immunity	Reduced with fasting	[79]
Siberian hamsters (*Phodopus sungorus*)	1) FR (75% *ad libitum*) 2) No FR	DTH response to 2,4-dinitro-1-fluorobenzene	T cell immunity	Reduced with FR during short-day conditions, no effect during long-day conditions	[80]

Iberian red deer hinds and calves (*Cervus elaphus hispanicus*)	1)FR (50-60% of energy requirements) 2) No FR	Total Ig	Constitutive humoral immunity	Increased in calves, no effect on hinds	[82]
Yellow-legged gulls (*Larus cachinnans*)	1) FR (33% daily intake) 2) No FR 3) Fasting (no food) (treatments for 8-18 days)	PHA-directed wing web swelling	T cell immunity	Reduced with FR and fasting	[83]
Little ringed plovers (*Charadrius dubius*)	1) FR (4 hours food access) 2) No FR	PHA-directed wing web swelling	T cell immunity	Reduced in FR	[84]
Hand-reared sand martins nestlings (*Riparia riparia*)	1) Severe FR (40% *ad libitum*) 2) Intermediate FR (70% *ad libitum*) 3) No FR	PHA-directed wing web swelling	T cell immunity	Reduced with severe and intermediate FR. Lower with severe than intermediate FR.	[85]

Deer mice *(Peromyscus maniculatus)*	1) FR (70 % *ad libitum*) 2) No FR	Secondary Ab response to KLH, number of IgG-expressing splenic B cells	Adaptive humoral immunity	Reduced in FR	[86,87]