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Review

Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds?

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Activating the immune system has associated fitness costs, both immediate costs in the form of reduced current reproduction and long-term costs in the form of reduced life span and future reproduction. This indicates that immune system activation can be an important agent in life history trade-offs. In this review, we evaluate the importance of four currencies generally considered as potential mediators of the costs of immune responses in ecological studies: (1) energetic costs, (2) nutrient costs, (3) autoimmunity and (4) oxidative stress, which may be responsible for these trade-offs. A meta-analysis revealed significant elevation of energy consumption during an immune response; however, the magnitude of this energetic cost was only 5–15%. In a direct comparison using similar immune system activation in tits, energetic savings in terms of lowered feeding rate was seven times higher than energetic costs of mounting an immune response. These results do not support the hypothesis that energy is the key proximate currency mediating the costs of immunity. Nutrient savings from immunosuppression seem to be even less beneficial as this constitutes only a minor part of the daily nutrient turnover in the body. In our view, there are some indications that oxidative stress can be an important currency that could mediate both short-term and long-term costs of immune system activation, although direct evidence is so far limited. The importance of autoimmune responses is at this point hard to evaluate owing to limited empirical studies in wild animals.

The vertebrate immune system is an induced defence that has evolved to destroy and remove particles of foreign origin, such as viruses, bacteria and other parasites, from the body. The fact that the immune system is fully activated only when needed implies that an immune response is connected to costs. In line with this notion, experimental activation of the immune system has resulted in fitness costs. Immediate costs in the form of reduced current reproductive success have been demonstrated experimentally by immunizing parents during breeding, resulting in a prolonged incubation period, reduced feeding effort and/or reduced fledging success (Table 1). A substantial long-term cost associated with immune system activation has been found in terms of considerably reduced between-year return rate in eiders, *Somateria mollissima*. Incubating and hence fasting females that mounted antibody responses to two antigens simultaneously apparently suffered from impaired survival (Hanssen et al. 2004). Furthermore, induced immune responses commonly reduce growth rate in chicks (Fair et al. 1999; Soler et al. 2003; Brommer 2004; Grindstaff 2008), impair postfledging survival (Eraud et al. 2009) and reduce testosterone levels in adults (Boonekamp et al. 2008).

In addition, studies manipulating workload to measure effects on immune responses can indirectly provide data of importance for interpreting fitness costs associated with immune system activation. A meta-analysis of experiments increasing/decreasing clutch/brood size to alter parental effort found a significant negative relationship between parental effort and immune responses (Knowles et al. 2009). For other demanding tasks that can be experimentally manipulated, such as mating and incubation effort, flight cost and thermoregulation, negative associations with the strength of an immune response have also been found (Table 2). Thus, a trade-off between workload and immunocompetence seems to be common in free-living birds during physiologically demanding activities such as breeding.

One indirect method to study the potential fitness effects of immune system activation is to measure selection on artificially induced immune responses or natural parasite resistance. Survival until the breeding season of blue tits, *Cyanistes caeruleus*, injected with nonpathogenic antigens in winter revealed stabilizing selection on the diphtheria toxoid response (Råberg & Stjernman 2003). Blood parasite resistance among infected blue tits was also subjected to stabilizing selection (Stjernman et al. 2008), suggesting that individuals with the strongest and weakest immune responses disappeared before the breeding season. However, both these studies are correlational and causality is therefore unclear.

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Table 1
Effects of experimental immune system activation on fitness-related traits during breeding in studies of birds

Species	Immune challenge	Fitness-related trait	Effect on trait	Source
Blue tit, *Cyanistes caeruleus*	DT	Nestling feeding	Reduction	Råberg et al. 2000
Pied flycatcher, *Ficedula hypoleuca*	DT	Nestling mass	Reduction	Ilmonen et al. 2000
		Fledging success	Reduction	
Tawny owl, *Strix aluco*	Tetravac	Nestling size	Reduction	Gasparini et al. 2009
		Recruitment	Reduction	
Collared flycatcher, *Ficedula albicollis*	SRBC	Song rate*	Reduction	Garamszegi et al. 2004
		Testosterone level†	Reduction	
		Body mass	Reduction	
Blackbird, *Turdus merula*	SRBC	Bill coloration*	Reduction	Faivre et al. 2003
Great tit, *Parus major*	RRBC+DT	Plumage coloration*	Reduction	Fitze et al. 2007
House martin, *Delichon urbica*	NDV	Relaying interval	Prolonged	Marzal et al. 2007
		Fledging success	Reduction	
House sparrow, *Passer domesticus*	LPS	Nestling feeding	Reduction	Bonneaud et al. 2003
		Fledging success	Reduction	
		Relaying interval	No effect	
Blue-footed booby, *Sula nebouxii*	LPS	Foot coloration*	Reduction	Torres & Velando 2007
Zebra finch, *Taeniopygia guttata*	LPS	Bill coloration*	Reduction	Alonso-Alvarez et al. 2004a
Common eider, *Somateria mollissima*	SRBC+DT	Between-year return rate/survival	Reduction	Hanssen et al. 2004
Common eider, *Somateria mollissima*	SRBC	Length of incubation period	Prolonged	Hanssen 2006
Mallard, *Anas platyrhynchos*	SRBC	Bill UV-reflectance*	Reduction	Peters et al. 2004
		Testosterone level†	Reduction	
Zebra finch, *Taeniopygia guttata*	SRBC	Nestling growth rate	No effect	Verhulst et al. 2005
		Time to second clutch	No effect	
Starling, *Sturnus vulgaris*	SRBC	Relaying interval	No effect	Williams et al. 1999

Injections were with the following antigens: DT = diphtheria toxoid and tetanus toxoid vaccine, Tetravac = blend of diphtheria toxoid, tetanus toxoid, pertussis toxoid, filamentous haemagglutinin, D antigen of the poliovirus type 1, 2 and 3, SRBC = sheep red blood cells, NDV = Newcastle disease virus vaccine, LPS = lipopolysaccharide, RRBC = rabbit red blood cells.

* Male secondary sexual ornament of importance for mating success.

† Hormone important for male mating success.

Thus, a negative relationship between demanding activities and the strength of immune responses is commonly observed in birds, implying that immune responses can induce significant ultimate costs. However, empirical evidence for the proximate nature of such costs, that is, the currencies mediating the conversion of immune response costs into fitness costs, is much scarcer. The most frequently suggested currencies in ecological studies are (1) energetic costs, (2) nutrient costs, (3) autoimmunity and (4) oxidative stress. The energetic costs hypothesis is based on 'the principle of allocation' and involves direct trade-offs for energy between the immune system and other demanding tasks (Sheldon & Verhulst 1996). This implies a cost of rather short duration, at least in physiologically nonexhausted individuals; that is, when the strenuous activities cease, immune responses should be restored within days or weeks (Verhulst et al. 2005). The same logic is plausible for nutrient costs related to synthesis and proliferation of immune cells (e.g. leucocytes, antibodies, cytokines), in particular for macronutrients (e.g. protein). In contrast, costs induced by oxidative stress or autoimmunity may be long lasting, acting over months or years (Råberg et al. 1998; von Schantz et al. 1999). We stress that this partitioning into short- and long-term costs is not clear-cut; still, we think it is a useful concept because it delineates different physiological mechanisms. Generally speaking, the short-term costs are more transient and can be (at least partly) compensated for after the end of the immune challenge, whereas the harmful effects of long-term costs accumulate and become more severe over time.

The main aim of the present review is to evaluate empirical evidence to elucidate the importance of different proximate mechanisms (currencies) proposed to mediate the cost of immune responses affecting fitness traits. Hence, we are mainly interested in natural systems under selection and because the majority of these studies have been conducted on birds, we focus our review on bird studies. Our choice of natural systems under selection puts the emphasis of this review on the ecological/evolutionary literature. The complementary, more mechanistic approach in studies of model organisms taken by immunologists is also important but will not be explicitly dealt with in this review. We first compile the

Table 2
Experimental manipulation of work rate, except manipulations of parental effort, and its effects on immunity in studies of birds

Species	Manipulated work rate	Immune measure	Effect on immunity	Source
Pied flycatcher, *Ficedula hypoleuca*	Mating effort	DT-H	Negative	Kilpimaa et al. 2004
Tree swallow, *Tachycineta bicolor*	Cost of flying	KLH-H	Negative	Hasselquist et al. 2001
Zebra finch, *Taeniopygia guttata*	General activity	PHA-C	Negative	Ewenson et al. 2003
Blue tit, *Cyanistes caeruleus*	Thermoregulation	DT-H	Negative	Svensson et al. 1998
Red knot, *Calidris canutus*	Flight endurance	PHA-C	No effect	Hasselquist et al. 2007
		DT-H	No effect	

Abbreviations of immune measures are: PHA-C = phytohaemagglutinin-induced (unspecific) cell-mediated immunity, DT-H = humoral immunity induced by injection with diphtheria toxoid and tetanus toxoid vaccine, KLH-H = humoral immunity induced by injection with keyhole limpet haemocyanin (KLH).

evidence for each of the short-term (energetic and nutrient) and long-term (oxidative stress and autoimmunity) costs and then evaluate their role as mediators of costs of immune responses in birds.

When sample size permitted we performed random-effect model meta-analyses (Lipsey & Wilson 2001). We treated each study as a separate sample, but combined data for different years, ages or sexes into one study-specific estimate used in further analyses. From the papers, we extracted means, standard errors/deviations and sample sizes to calculate standardized mean differences (d) between groups of immune-challenged and control birds (Table 3) and between carotenoid-fed and control birds (Tables 4, 5). If descriptive statistics were lacking, we used reported F or t values to calculate d.

IMMUNE RESPONSES

Eliciting an immune response involves increased cellular and molecular turnover rates, and it consists of two temporally separated parts. The acute phase response that accompanies many infections is characterized by a systemic cytokine-mediated inflammatory response involving, for example, recruitment of macrophages and fever (Klasing & Johnstone 1991; Klasing 1998). In the second phase, the acquired immune system (humoral and T-cell-mediated immunity) is activated involving T-cell and B-cell proliferation and antibody production. More details on vertebrate immune responses can be found in Murphy et al. (2008) and on inflammatory responses in wild animals in Sorci & Faivre (2009).

PROXIMATE COSTS OF IMMUNE RESPONSES

Energetic Costs

Energy is the most common currency predicted to underlie trade-offs between life history traits (Stearns 1992). This currency has also been assumed to be responsible for the trade-off between immune responses and other life history traits (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Wikelski & Ricklefs 2001). Accordingly, this mechanism, measured as changes in metabolic rate following an immune challenge, has attracted most attention among possible costs of mounting an immune response (Table 3). Energetic costs of immune responses can be induced by both the acute phase response and the ensuing increased proliferation rates of leucocytes and antibodies (Lochmiller & Deerenberg 2000; Klasing 2004).

Basically three methods to trigger immune responses have been used to estimate the metabolic cost of immune system activation. (1) PHA (phytohaemagglutinin) is a plant lectin that is injected in the wing web and the strength of the immune response is measured as the size of the injection-induced swelling (Martin et al. 2003; Nilsson et al. 2007). Administration of PHA will trigger both the innate immune system and the (unspecific) cell-mediated arm of the adaptive immune system (Goto et al. 1978), the relative importance of these two systems for the reaction differing between species (Martin et al. 2006). (2) SRBC (sheep red blood cells) is a novel multigenic antigen triggering a response involving both innate and acquired (mainly humoral) immunity that is measured using a haemagglutination assay (Ots et al. 2001). (3) Diphtheria-tetanus is a vaccine containing two toxoid antigens mixed with adjuvant (e.g. aluminium phosphate) that will activate humoral immunity typically resulting in peak antibody titres after 10–14 days in birds (Hasselquist et al. 1999; Råberg et al. 2003; Bustnes et al. 2004). The antigen-specific antibody responses against diphtheria and tetanus are measured as antibody titres using ELISA (Svensson et al. 1998; Mendes et al. 2006; Reid et al. 2006).

Most studies have been performed on caged birds with ad libitum food, allowing for increased energy intake as a response to increased energy demands. A meta-analysis of these studies showed significant elevation of resting metabolic rate (RMR) in immune-challenged versus sham-injected individuals ($d + \mathrm{SE} = 0.94 + 0.35$, $Z = 2.68$, $P = 0.007$; 95% confidence limits: 0.25–1.62; Table 3). The

Table 3
Energetic costs caused by experimental activation of the immune system in studies of birds

Species	Immune challenge	Effect size (%)	*P*	Standardized mean difference	95% confidence limits	Source
House sparrow, *Passer domesticus*	PHA	28.8	<0.001	6.74	4.03–9.45	Martin et al. 2003
Collared dove, *Streptopelia decaocto*	SRBC	8.6	0.002	1.85	0.80–2.90	Eraud et al. 2005
Blue tit, *Cyanistes caeruleus*	DT					Svensson et al. 1998
Primary response		2.5	0.55			
Secondary response		7.2	0.14	1.20	0.06–2.34	
Red knot, *Calidris canutus*	DT					Mendes et al. 2006
Primary response			0.47			
Secondary response		13	0.038	1.03	0.08–1.99	
Great tit, *Parus major*	PHA	4.5	0.046	0.72	−0.20–1.65	Nilsson et al. 2007
Greenfinch, *Carduelis chloris**	SRBC	4.7	0.14	0.63	−0.12–1.38	Amat et al. 2007
Greenfinch, *Carduelis chloris*	SRBC		0.27	0.41	−0.29–1.11	Hõrak et al. 2003
Great tit, *Parus major*	SRBC	8.6	0.011	0.40	−0.47–1.27	Ots et al. 2001
Ruff, *Philomachus pugnax*	DT					Mendes et al. 2006
Primary response			0.49			
Secondary response		−15	0.11	−0.76	−1.67–0.15	
Pied flycatcher, *Ficedula hypoleuca**	PHA		0.031			Moreno et al. 2001
Zebra finch, *Taeniopygia guttata*	SRBC		>0.5			Verhulst et al. 2005

Significance levels are those reported in the original papers. Standardized mean difference and 95% confidence limits in metabolic rate between individuals with an activated immune system and control individuals are shown. SRBC = humoral immunity induced by injection with sheep red blood cells, PHA = phytohaemagglutinin-induced (unspecific) cell-mediated immunity and DT = humoral immunity induced by injection with diphtheria toxoid and tetanus toxoid vaccine. The standardized mean difference could not be calculated for pied flycatchers and zebra finches as these studies did not present data that could be included in the meta-analysis.

* Energy cost measured as variation in DEE, in all other cases as variation in RMR.

Table 4
Standardized mean difference and 95% confidence limits in the wing web swelling response induced by an injection of PHA (phytohaemagglutinin) between individuals being fed supplementary carotenoids and control individuals

Species	Standardized mean difference	95% confidence limits	Source
Moorhen, *Gallinula chloropus*	2.12	1.25–2.99	Fenoglio et al. 2002
Zebra finch, *Taeniopygia guttata*	1.25	0.30–2.21	Blount et al. 2003
Zebra finch, *Taeniopygia guttata*	0.99	0.19–1.79	McGraw & Ardia 2003, 2005
Mountain bluebird, *Sialia currucoides*	0.73	0.11–1.35	O'Brien & Dawson 2008
Grey partridge, *Perdix perdix*	0.63	0.38–0.89	Cucco et al. 2007
Grey partridge, *Perdix perdix*	0.34	0.10–0.58	Cucco et al. 2006
Society finch, *Lonchura domestica*	0.20	−0.68–1.08	McGraw et al. 2006
Greenfinch, *Carduelis chloris*	0.19	−0.37–0.75	Hõrak et al. 2006
Pheasant, *Phasianus colchicus*	0.17	−0.36–0.70	Smith et al. 2007
Greenfinch, *Carduelis chloris*	0	−0.58–0.58	Hõrak et al. 2007
Blue tit, *Cyanistes caeruleus*	0	−0.57–0.57	Biard et al. 2006
Great tit, *Parus major*	−0.09	−0.85–0.66	Biard et al. 2006
American goldfinch, *Carduelis tristis*	−0.18	−0.80–0.44	Navara & Hill 2003
Great tit, *Parus major*	−0.18	−0.61–0.25	Fitze et al. 2007

heterogeneity of the effect sizes was moderately significant ($Q_8 = 16.8$, $P = 0.033$) indicating that the variability across effect sizes exceeded the expected based on sampling error. Thus, variability between effect sizes depends partly on random differences across rather than within studies. Most of the effect sizes, expressed as change in RMR before and during the immune challenge, were in the range 5–15% increase in RMR.

We did not find any obvious difference in the energetic cost of an immune response dependent on which part of the immune system was challenged (Table 3), except for primary (humoral) responses to diphtheria-tetanus vaccine resulting in a lower energetic cost (mean $d = 0.49$) than antigens activating other parts of the immune system (mean $d = 1.79$). This may reflect that SRBC and PHA induce relatively strong activation of the innate immune system involving elevated production of inflammatory cytokines, which orchestrates a cascading acute phase response that is suggested to be especially energy consuming (Klasing & Leshchinsky 1999; Råberg et al. 2002; Lee & Klasing 2004).

Table 5
Effects of supplemental feeding of carotenoids on different experimentally induced immune responses

Species	Mode of immune challenge				Source
	SRBC	LPS	DT	BK	
Zebra finch, *Taeniopygia guttata*	+				McGraw & Ardia 2003, 2005
American goldfinch, *Carduelis tristis*	0				Navara & Hill 2003
Greenfinch, *Carduelis chloris*	0				Hõrak et al. 2006
Society finch, *Lonchura domestica*				+	McGraw et al. 2006
Red Junglefowl, *Gallus gallus*		0		+*	McGraw & Klasing 2006
Pheasant, *Phasianus colchicus*			0		Ohlsson et al. 2003

Immune responses were triggered by SRBC (sheep red blood cells), LPS (lipopolysaccharide), DT (diphtheria toxoid and tetanus toxoid vaccine) and BK which is a measure of the bacteria-killing capacity of blood. '+' denotes an enhanced immune response compared to control individuals and '0' denotes no significant difference between the two experimental groups.

* Only in males.

When measuring the energetic cost of an immune response as a net increase in metabolic rate, a potential problem might be the possible reallocations of energy to meet the increased demands, masking the true cost of the immune response. Such an underestimation of the cost is most serious when metabolic rate is measured over a 24 h period (daily energy expenditure; DEE), because reduced activity is a common behavioural response to an immune challenge (Hõrak et al. 2003; Lindström et al. 2003; Amat et al. 2007; Owen-Ashley et al. 2007). Thus, energy may be reallocated from activity to the immune response without affecting the overall DEE. However, the two studies based on DEE measurements (Table 3) have been conducted either in small cages to reduce activity also for control birds (Amat et al. 2007) or on parents feeding young (Moreno et al. 2001), which limits the possibilities for activity-induced differences in DEE between experimental categories. In the majority of studies (Table 3), metabolic measurements were conducted at night on inactive birds. Still, allocations among physiological systems within an individual can potentially underestimate the cost of an immune challenge (Derting & Compton 2003; Mendes et al. 2006). Because most studies have been performed on caged birds with ad libitum food, that is under conditions that do not call for the need to reallocate energy within the body, we find it most plausible that the costs in Table 3 are representative of the absolute energetic cost of an immune response.

Nutritional Costs

Macronutrients

An immune response is characterized by synthesis and proliferation of leucocytes and acute phase proteins, which potentially can induce an increased demand for micro- and macronutrients (Klasing 2004). A number of studies have manipulated the total amount of food or protein content in birds. Most of these studies are on young birds during the ontogenetic period and the general pattern is that food manipulation affects at least some aspect of immune function (e.g. Lochmiller et al. 1993; Saino et al. 1997; Hoi-Leitner et al. 2001; Fargallo et al. 2002; de Neve et al. 2007, Bize et al. 2010; but see Ohlsson et al. 2002). Based on poultry data, Klasing (1998) made some calculations to get a rough estimate of the nutritional resources needed for immune responses compared with normal growth. His calculations showed that leucocytes only make up 0.42% and total antibodies 0.1% of total body mass, that the normal turnover rate of immunoglobulins is less than 0.05% of body mass per day and that total immunoglobulin levels only increase by 25% even at hyperimmunization. Hence, the mass of the normal daily production of leucocytes and immunoglobulins (ca. 800 mg/kg body mass) is less than 1% of the daily increase in body mass of a growing chick (Klasing 1998), and even the cell production caused by an immune system activation would not increase this estimate to more than 2% of the daily body mass increase. Hence, Klasing (1998, page 1122) concluded that it is 'doubtful that the immune system would be a significant consumer of nutritional resources'. Further support for rather low nutritional demands of immune function comes from a meta-analysis of poultry. Lines selected for increased growth had compromised immune function, whereas in lines selected for increased immune function growth was unaffected (van der Most et al. 2011).

Manipulations of food availability in adult birds (when organs including immune system components are fully developed) are less common but might be more relevant for analysing costs of immune responses, because they are separated from the nestling period, which is characterized by high energetic and nutritional demands. Results from such studies show that the amount of food can affect both cell-mediated immunity (measured as skin swelling after PHA injection; Birkhead et al. 1999; Gonzalez et al. 1999a; Bourgeon et al. 2006; but see Hõrak et al. 2000; Smith et al. 2007) and humoral immunity (Kidd 2004; Bourgeon et al. 2006; Smith et al. 2007; but see Poston et al. 2005). The interpretation of these studies is hampered, however, by the positive effect that the food manipulation has on general condition. For example, the PHA-induced skin swelling test is known to be highly sensitive to changes in body mass, condition, blood parasite infection and stress (Moreno et al. 1999; Gonzalez et al. 1999b; Alonso-Alvarez & Tella 2001; Lifjeld et al. 2002; Navarro et al. 2003; Ewenson et al. 2003). Thus, the effects of food supplementations/restrictions on immune function may ultimately depend on a general effect on stress and activity levels, and hence on condition, instead of a direct enhancing effect on the immune system.

The acute phase may potentially be the most nutrient-consuming part of an immune response (although it also involves liberating nutrients through catabolism of skeletal muscle tissue), in particular the demand for amino acids to support gluconeogenesis and synthesis of acute phase proteins, but also the need for proteins and carbohydrates to meet the increased energy demand of keeping the higher body temperature characteristic of fever (Klasing 2004). However, there are no quantitative measures of how much the demand for different nutrients increases in connection with an acute phase response. An acute phase response can be induced by the injection of lipopolysaccharide (LPS; Bonneaud et al. 2003; Bertrand et al. 2006; Owen-Ashley & Wingfield 2006) or Freund's complete adjuvant (Harlow & Lane 1988). In most studies of LPS immunization, injected individuals lose mass. This may be an effect of reduced energy intake, however, as anorexia has been suggested to be an adaptive reaction to an infection (Kyriazakis et al. 1998). In line with this, LPS-challenged Japanese quail, *Coturnix japonica*, and poultry chicks experienced a 14–15% reduction in growth rate (Klasing & Leshchinsky 1999; Klasing 2004); however, this effect was mainly due to anorexia and the calculated protein cost of the immune response was only 6.7% (measured as turnover of lysine, an amino acid mainly used as substrate for protein synthesis; Klasing 2004). Still, we feel that more studies of the acute phase response and its demand for resources are needed to rule out the possibility that this immune response has dramatically higher demands for nutrients, and therefore could underlie trade-offs relevant for life history strategies.

Micronutrients: direct effects

Micronutrients may also have the potential to limit immune responses. Several micronutrients, for example carotenoids, selenium and vitamin E, may directly enhance immune responses as the requirements for these compounds are generally higher when mounting an immune response than during basic growth and maintenance (Klasing 1998; Surai 2002; Hartley & Kennedy 2004). Thus, if these micronutrients are in short supply and/or involved in other important physiological functions, they may restrict the strength of immune responses. We call these effects 'direct' to separate them from the antioxidant capacity of many of these micronutrients, which could exert an 'indirect' effect as they may facilitate enhanced immune responses owing to their reducing effect on oxidative stress (see below).

To our knowledge, there has so far not been any direct attempt to calculate the amount of micronutrients needed for different immune responses. Instead, tests of the potential for these compounds to restrict the strength of immune responses come from experimental additions of micronutrients to the diet. Domesticated birds generally respond with a stronger immune response when fed extra micronutrients (reviewed in Surai 2002). In a meta-analysis, we also found a significant effect on the strength of the immune response to PHA when carotenoids are supplemented in the diet ($d + \mathrm{SE} = 0.31 + 0.12$, $Z = 2.55$, $P = 0.011$; 95% confidence limits: 0.07–0.55; Table 4). The heterogeneity in this data set was nonsignificant ($Q_{13} = 16.6$, $P = 0.2$) indicating that the variability among effect sizes did not exceed that expected from sampling error. Among other immune challenges the number of studies are small and the results ambiguous (Table 5), although it can be noted that both studies measuring the bacteria-killing activity in the blood reported enhanced innate responses in the carotenoid-supplemented group.

Autoimmunity Costs

One drawback of the vertebrate immune system is the risk of autoimmune reactions, that is when the organism's own immune system mistakenly identifies self-antigens as foreign and elicits responses against the organism's own cells (Theofilopoulos 1995). The severe consequences of autoimmune reactions and diseases are well known in humans, mice, *Mus musculus*, and chickens, *Gallus gallus domesticus* (Goldsby et al. 2003; Erf 2008). Based on these observations, autoimmune responses have been proposed as a potential mediator of the trade-off between immune responses and heavy work also in wild animals (Råberg et al. 1998). The logic behind this idea is that physiological stress in the form of strenuous work (e.g. a hard-working parent bird during the nestling-feeding period) or other stressors can cause cell and muscle damage (including T-cell depletion) increasing the self-antigen repertoire (Munck et al. 1984; Munck & Náray-Fejes-Tóth 1995; King et al. 2004), with a concomitant increased risk of hyperstimulation of the immune defence (Råberg et al. 1998). Strenuous work may also activate secretion of stress hormones (glucocorticoid steroids), which eventually suppress the immune system (Munck et al. 1984; Besedovsky & del Rey 1996; Owen-Ashley et al. 2004). Thus, to decrease the risk of autoimmune responses in situations requiring strenuous work (i.e. elevated stress levels) the immune system may be adaptively down-regulated (e.g. through secretion of corticosteroids), or reciprocally, in cases when the immune system needs to be activated it could be adaptive to reduce the workload (Råberg et al. 1998).

Although autoimmunity is a potentially severe cost induced by immune system activation and does occur in domestic chickens (Erf 2008), it is so far unknown to what extent it occurs in wild birds. The paucity of such studies results from a lack of methods to measure autoimmune reactions in wild organisms. An indirect way to learn more about potential risks of autoimmune reactions is to analyse studies that have measured stress indicators (glucocorticoids or heat shock proteins) in relation to heavy workload and immune system activation, because elevated levels of these stress indicators could reflect an increased risk of autoimmune reactions. In wild birds, an increased level of stress indicators has been found in relation to both strenuous work (nestling feeding, nestling growth; Moreno et al. 2002; Merino et al. 2006; but see Saino et al. 2002) and disease (Merino et al. 1998, 2002; Tomás et al. 2005; Morales et al. 2006; but see Merino et al. 2001; Morales et al. 2004). Note also that glucocorticoids (Fowles et al. 1993; Evans et al. 2000; Owen-Ashley et al. 2004; Roberts et al. 2004) and heat shock proteins (Merino et al. 2001; Morales et al. 2006; but see Garamszegi et al. 2006) have been found to correlate negatively with immune responses in wild birds. This could be interpreted as a prudent reaction to avoid a hyperactivated immune system;

however, an alternative interpretation is that high-quality individuals can produce higher immune responses and are less exposed to stress (Morales et al. 2006). More studies on autoimmune responses in wild animals are required before we can evaluate its potential as mediator of the cost of immune responses.

Oxidative Stress Costs

An immune response not only entails costs related to the activation of the response, but it also carries potential costs related to the actions and 'rest products' of these responses. Such costs may be caused by oxidative stress, the damaging action of reactive oxygen species (ROS) on nucleic acids, proteins and lipids. ROS are produced as a by-product of oxidative phosphorylation during metabolism (von Schantz et al. 1999; Barja 2004). In birds, costly investment in reproduction and growth has been associated with increased blood levels of ROS (Costantini et al. 2006) or reduced levels of antioxidant defence (Wiersma et al. 2004; Alonso-Alvarez et al. 2004a, 2007). In addition, however, activated immune cells release ROS as part of the defence against intruding organisms (Klasing 1998; Halliwell & Gutteridge 1999; von Schantz et al. 1999; Surai 2002; Bertrand et al. 2006). In particular, the parts of an immune response involving macrophage activity, inflammation and fever generate high levels of ROS (Klasing 2004). Accordingly, experimental infection (Kurtz et al. 2006) or immune system activation (Costantini & Dell'Omo 2006; Hõrak et al. 2007; Stier et al. 2009) can increase oxidative stress levels in wild animals. The latter experiments are especially informative as the increase in oxidative stress can be directly ascribed to the immune system activation without any effects of parasites. Hence, these studies imply that immune system activation can potentially have negative consequences in terms of oxidative stress. Furthermore, in light of an ROS-dependent decrease in survival and fecundity (Beckman & Ames 1998; Vleck et al. 2007; Bize et al. 2008), organisms may be reluctant to engage in two ROS-producing activities simultaneously, such as high workload and immune system activation (von Schantz et al. 1999). Instead, one of the ROS-producing activities may be adaptively down-regulated, potentially explaining the trade-off between the strength of an immune response and parental effort (Tables 1, 2).

To alleviate the level of oxidative stress, the ROS that are produced have to be immediately scavenged by endogenous and exogenous antioxidants. The quality of the endogenous antioxidant system may differ between genotypes (von Schantz et al. 1999) and is probably enhanced by good physical condition, whereas the exogenous antioxidants have to be ingested through the diet (Surai 2002). Consequently, the detrimental effects on important biomolecules induced by ROS will be most severe when antioxidants are in short supply (Vleck et al. 2007).

Micronutrients identified as exogenous antioxidants, mostly from studies on chickens (Surai 2002), include carotenoids, vitamin C and vitamin E. Most studies of wild vertebrates have focused on carotenoids because in many species these are also important as labels of condition/quality in the context of mate choice (Lozano 1994; von Schantz et al. 1999). The overall significantly enhanced immune response resulting from supplemental feeding of carotenoids (Table 4) that was reported above as a 'direct' effect could just as well be an 'indirect' effect resulting from the antioxidant capacity of carotenoids. Furthermore, the fact that the bactericidal capacity of the blood seems to be positively related to carotenoid supplementation (Table 5) could also be interpreted as support for the indirect effects hypothesis, because the innate immune system generates ROS during the bactericidal process and a high level of carotenoids could effectively scavenge ROS allowing stronger immune responses (McGraw & Klasing 2006). However, the antioxidant capacity of carotenoids has recently been questioned (Hartley & Kennedy 2004; Costantini & Møller 2008). Empirical results are conflicting as maternally derived carotenoids seem to be important in protecting embryos and hatchlings from oxidative damage (Blount et al. 2002a; McGraw et al. 2005), whereas the relationship between circulating carotenoids and the antioxidant defence in the blood of adult birds is unclear (Alonso-Alvarez et al. 2004b; Costantini et al. 2006, 2007; Hõrak et al. 2006; Tummeleht et al. 2006; Cohen et al. 2007; but see Blount et al. 2002b; Hõrak et al. 2007). A potential problem with all these studies is that they have measured circulating carotenoids, while the antioxidant activity may be highest when carotenoids are part of cell membranes. At the moment, we do not know to what extent carotenoid levels in plasma reflect carotenoids bound in membranes.

Vitamins C and E have immunostimulating effects (reviewed in Surai 2002) and as they are considered to be more effective antioxidants than carotenoids (Hartley & Kennedy 2004) the effects on the immune system may therefore be a consequence of enhanced free radical scavenging and reduced oxidative stress. However, experimental tests of the antioxidant capacity of these vitamins in nondomesticated birds are few and inconclusive. Dietary addition of vitamin E did not reduce oxidative damage in greenfinches, *Carduelis chloris* (Hõrak et al. 2007), but it enhanced growth in nestling barn swallows, *Hirundo rustica* (de Ayala et al. 2006) indicating that the defence against ROS was enhanced permitting a higher energy turnover rate.

DISCUSSION

Although many studies indirectly support the notion that eliciting immune responses can entail fitness costs in wild birds (Table 1) there are actually rather few direct studies of this aspect (Graham et al. 2011), in particular in the same system as that in which the potential currencies of such costs have been investigated (for exceptions, see Svensson et al. 1998; Verhulst et al. 2005). Such studies are warranted to understand further the connection between investment in immune function and fitness.

Still, a rather large number of studies demonstrate that birds adjust their immune response in relation to workload as well as their workload to the level of immune system activation (Deerenberg et al. 1997; Ilmonen et al. 2000; Råberg et al. 2000; Tables 1, 2). This trade-off is often inferred as underlying life history strategies, for example investment in current as opposed to future reproduction, because investment in immune responses can be seen as investment in self-maintenance and investment in strenuous activities often equals reproductive effort (Gustafsson et al. 1994; Sheldon & Verhulst 1996). However, the proximate reasons for this trade-off and the mechanisms (currency) that mediate it are more enigmatic. This lack of knowledge is unfortunate as it reduces our ability to predict life history strategies of organisms in different environments, as well as the constraints and patterns of selection on immune system and life history factors.

Short-term Costs of Immune Responses

In terms of energetic costs of immune responses no relationship has been found between the magnitude of the energetic cost (cost of RMR) and the strength of the immune response (Ots et al. 2001; Eraud et al. 2005; Nilsson et al. 2007).

Still, our meta-analysis of bird studies implies that mounting an immune response entails a significant energetic cost, although of a rather low magnitude (Table 3). The 5–15% increase in metabolic rate is, for example, comparable to the cost of thermoregulation at a temperature 2–3 °C below the thermoneutral zone (Eraud et al. 2005). Such an increase in metabolic rate may of

course prove to be significant at the individual level if that individual is living on tight margins. Nevertheless, from a life history perspective, the energetic cost of an immune response may seem to be too low in comparison to the increase in metabolic rate of three to four times RMR (300–400% increase) during breeding (Drent & Daan 1980; Nilsson 2002; Hasselquist & Bensch 2008), to be of key importance in trade-offs between mounting an immune response and work rate. However, this comparison may be misleading because it is the energy saved by decreasing workload that is the relevant entity to compare with the energetic cost of an immune response.

We therefore tried to obtain a more relevant comparison of energetic cost of immune responses versus energetic gain by lowering workload, by using data from two closely related, same-sized (ca. 11 g) tit species on immune system activation (diphtheria-tetanus vaccination; blue tit) and the energetic cost of nestling feeding (marsh tit, *Poecile palustris*). Based on data from Råberg et al. (2000; their Figure 1c), we estimated that blue tits during a primary immune response decrease their feeding rate by 4 feeding trips/h (13.3%) 7–10 days after antigen injection. Females feed their nestlings at similar rates between 0500 and 1900 hours (Råberg et al. 2000), so vaccinated females decreased their daily workload by 4 feedings/h $\times$ 14 h = 56 feedings. Next we calculated the energetic cost of feeding nestlings (based on data from marsh tit females in Figure 3 in Nilsson 2002): the energetic cost per feeding trip is 0.047 kJ per day. We then calculated how much energy females are saving 7–10 days after a diphtheria-tetanus vaccination by decreasing feeding rate by 13.3%; this amounts to 56 feedings $\times$ 0.047 kJ per day = 2.63 kJ per day.

From Figure 3 in Svensson et al. (1998), we estimated the energetic cost of mounting a primary antibody response against diphtheria-tetanus toxoids in blue tits 7 days after vaccination to be 0.368 kJ per day. Thus, the relative level of energy savings gained by lowering feeding effort versus the energetic cost of mounting a primary immune response at 7–10 days after vaccination is 2.63 kJ per day divided by 0.368 kJ per day which equals 7.15. In other words, the decrease in work rate of vaccinated females lowers the daily energy consumption ca. seven times more than the energetic cost induced by mounting immune responses against the same vaccine. Given that lowering feeding by 13% can potentially induce substantial fitness costs in terms of quality and survival of offspring (e.g. Ilmonen et al. 2000 and data in Table 1), and that the ratio between energetic gain by decreasing feeding and energetic cost of mounting an immune response is 7:1, we interpret these data as not supporting the hypothesis that energy is the key currency mediating the trade-off between immune responses and strenuous work. However, there are still few studies that have directly compared the energetic trade-off between immune response costs and workload reduction using the same immune challenge in similar species. Such studies, using different immune challenges, are needed before we can draw any firm conclusions regarding the importance of energetic costs in constraining immune responses. We also acknowledge that energy savings of 5–15% can be of importance in birds exposed to physiologically pressing conditions, and could be problematic in situations in which recovering condition (including immune function) after an immune response is hampered by restricted food availability.

We can more firmly conclude that macronutrient limitations seem unlikely as the currency mediating the cost of immunity. First, immune system cells make up a very small part of the total body mass (<1%). Thus, even though there is a high increase in turnover rate when mounting an acquired immune response, in terms of immune cell proliferation and synthesis, the nutritional resource demand would still not exceed 0.1% of total body mass per day (recalculated from Klasing 1998). Hence, in a bird with a body mass of 20 g, this is comparable to 0.02 g per day whereas the daily resource need (measured as protein; data on enthalpy of combustion from Alexander 1999) when resting (1$\times$ BMR, data from Lasiewski & Dawson 1967) is 1.3 g per day. We conclude that there are few nutrients to be saved and used for other activities by suppressing acquired immune responses. Second, we argue that if nutrients were the limiting resource when mounting an immune response, then the typical behavioural reaction of anorexia (Owen-Ashley & Wingfield 2007) in conjunction with an activated immune system would be maladaptive. If macronutrients were the most important factor limiting the immune response, we would instead have predicted increased foraging effort during immune system activation.

Judging from supplemental feeding experiments, carotenoids seem able to restrict the strength of an immune response (Table 4). The problem with carotenoids and other micronutrients lies in the interpretation of their actions. Carotenoids, vitamins and selenium have been suggested to enhance immune responses directly (Koutsos et al. 2003; Hartley & Kennedy 2004), but also to act as important ROS scavengers (Surai 2002). Although we found some studies indicating that the immune responses generating the highest levels of ROS (i.e. innate immunity) were also most affected by access to carotenoids (Table 5), the number of studies is too few for any firm conclusion.

Long-term Costs of Immune Responses

In general, autoimmune responses could induce long-term costs of immunity with potentially severe consequences for survival and fitness. Unfortunately there are so far no empirical studies that have directly investigated this in wild birds. One way of investigating the importance of autoimmune responses would be to use captive birds to identify MHC genotypes that are more prone to cause autoimmune (hyperactive) responses, and then sample natural or seminatural populations to search for a paucity of these MHC genotypes when exposed to natural selection under outdoor conditions. Presently, with limited knowledge of the occurrence of autoimmune reactions in wild birds, it is hard to evaluate whether autoimmunity can be a decisive mediator of the cost of immune responses. However, we envision that the potential costs for individuals exposed to autoimmune reactions are high enough for prudence to be favoured by selection.

Oxidative stress is another potential cost of immunity with the decisive negative effects acting over a prolonged time frame (Monaghan et al. 2009). Running the two ROS-generating processes, viz. strenuous work and immune system activation, simultaneously may induce cumulative damages eventually leading to the syndrome of oxidative stress with potentially severe consequences such as lower physical performance, senescence and death (von Schantz et al. 1999; Vleck et al. 2007). In our view, oxidative stress has potential to explain several of the observed patterns of the cost of immune responses found in studies of birds, in particular long-term costs. This is because the cost of immune responses has been reported to have adverse long-term effects on important fitness factors such as physical condition and survival (Beckman & Ames 1998; Bize et al. 2008). It is also worth noting that the degree of fluctuating asymmetries (FA), the failure of an individual to produce bilaterally symmetrical traits (Parsons 1990), might increase during oxidative stress. For example, FA is higher in organisms exposed to toxic compounds (Parsons 1990), probably because the biotransformation systems needed to degrade the toxic compounds are generating ROS at a high rate (von Schantz et al. 1999). In all studies in which relevant data were collected, there was a significantly increased degree of FA in immune-challenged individuals (Fair et al. 1999; Fair & Myers 2002; Fair & Ricklefs 2002; Whitaker & Fair 2002; Amat et al. 2007).

In conclusion, nutrition seems unlikely to be a key (proximate) factor mediating the cost of immune responses. For energy limitations the pattern is less clear. Although an immune response is connected to significant energetic costs (5–15%), the magnitude of these costs in relation to the level of workload sacrificed in response to an activated immune system does not support the hypothesis that energy is the key proximate currency for the cost of immune responses. In our view the damaging effects that ROS-generating immune system activation can have on both short-term (e.g. current reproduction) and long-term (survival) success make oxidative stress an interesting candidate for being a key factor mediating costs of immune responses. This is also supported by the fact that micronutrients important for immune system activity (carotenoids, vitamins) also are important antioxidants. However, we stress that our belief in oxidative stress as a proximate currency for the observed ultimate life history trade-offs is based more on data on the relatively small energy and nutrient savings gained by suppressing immunity as compared with strenuous work than on firm evidence for the importance of oxidative stress. Furthermore, it is worth noting that the importance of oxidative stress as a key factor in ageing has recently been questioned (Speakman & Selman 2011). Thus, we urge more studies to be done that focus on investigating the currencies that may mediate the cost of immune responses in life history trade-offs preferably in the same species; this is particularly true for studies of oxidative stress and immunopathology costs of immune responses, but also for detailed studies of energetic trade-offs of immune responses and workload using the same immune challenge.

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