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Immune challenge affects basal metabolic activity in wintering great tits

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The costs of exploiting an organism's immune function are expected to form the basis of many life-history trade-offs. However, there has been debate about whether such costs can be paid in energetic and nutritional terms. We addressed this question in a study of wintering, free-living, male great tits by injecting them with a novel, non-pathogenic antigen (sheep red blood cells) and measuring the changes in their basal metabolic rates and various condition indices subsequent to immune challenge. The experiment showed that activation of the immune system altered the metabolic activity and profile of immune cells in birds during the week subsequent to antigen injection: individuals mounting an immune response had nearly 9% higher basal metabolic rates, 8% lower plasma albumin levels and 37% higher heterophile-to-lymphocyte ratios (leucocytic stress indices) than sham-injected control birds. They also lost nearly 3% (0.5 g) of their body mass subsequent to the immune challenge. Individuals that mounted stronger antibody responses lost more mass during the immune challenge. These results suggest that energetic expenditures to immune response may have a non-trivial impact upon an individual's condition.

Keywords: immune challenge; basal metabolic rates; *Parus major*; heterophile-to-lymphocyte ratio; mass loss

1. INTRODUCTION

Over the past few decades, the role of parasites in the shaping of life-history patterns has been increasingly recognized by evolutionary animal ecologists (see Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Norris & Evans 2000). Several studies have suggested that immune function is directly involved in the mechanisms causing evolution and maintenance of ornamental traits (e.g. Hamilton & Zuk 1982; Westneat & Birkhead 1998), the generation of reproductive costs (e.g. Gustafsson *et al.* 1994; Møller 1997) and driving population cycles (e.g. Lochmiller 1996). In order to be involved in life-history trade-offs, the immune function has to be costly (e.g. Sheldon & Verhulst 1996; Owens & Wilson 1999). While the presence of such costs has been supported in studies on different organisms (see references above), a debate has emerged about the currencies in which the costs of using and maintaining an immune function are to be paid (Råberg *et al.* 1998; Westneat & Birkhead 1998). The traditional view of animal ecologists, namely that the costs involved in life-history trade-offs are basically energetic, has recently been challenged by evidence suggesting that the energetic demands required for maintenance of immune function (Klasing 1998; but see Lochmiller & Deerenberg 2000) and for mounting an immune response against specific foreign antigens (Svensson *et al.* 1998) are negligible. The latter study showed that, in captive blue tits (*Parus caeruleus*), cold-stressed individuals had lower immune responses to a diphtheria–tetanus vaccine than control birds, but that this could not be explained by energy limitation because the energetic cost of this immune response was considered to be very low. Hence, the assumption of energetic and nutritional costs of mounting an immune response has been questioned. However, the generality of such a conclusion awaits further research.

Consequently, the aim of this study was to assess the energetic and nutritional costs of mounting an immune challenge against a novel antigen in free-living, wintering great tits (*Parus major*). The great tit is a small (*ca.* 19 g), short-lived passerine that has been repeatedly used as a model organism in research into life-history evolution, not least in the context of ecological immunology (see e.g. Norris & Evans 2000). We studied the costs of mounting an immune response in wintering, male great tits by challenging them with sheep red blood cells (SRBCs) as a novel, multigenic antigen. Antibody response against SRBCs involves both T and B lymphocytes (e.g. Munns & Lamont 1991) and the magnitude of the response can be easily assessed by a haemagglutination assay. We expected the energetic cost of immune challenge to be revealed by increased basal metabolic rates (BMRs) as compared to control (saline-injected) individuals. The nutritional status of individuals was estimated on the basis of serum protein concentrations and profiles. A decrease in total serum protein concentration accompanies almost all diseases but is a particularly prominent symptom of malnutrition and infection, particularly when caused by a decline in albumin level (e.g. Kawai 1973; Coles 1997). A decrease in serum albumin content during inflammation is usually accompanied by a simultaneous increase in globulin concentration, mainly caused by the globulins belonging to the β- and γ-fractions. The γ-globulin fraction of serum includes most of the known antibodies involved in the immune response to protozoan, bacterial

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and viral infections, while the β-globulin fraction contains the acute-phase proteins, including fibrinogen (e.g. Coles 1997). In both acute disease and chronic infection or inflammation, diseased individuals reveal a higher total globulin concentration and lower albumin to globulin ratios than healthy individuals. In addition, we registered the changes in body mass, BMR and blood immune cell concentrations during the experiment and looked for correlations between these variables and the magnitude of the immune response.

2. METHODS

(a) *Study site and research protocol*

The study was performed between 8 February and 2 March 2000 at the Zvenigorod Biological Station of Moscow University (55°44′N, 36°51′E), which comprises two small villages surrounded by mixed and coniferous forest and is located 70 km west of Moscow. The daily ambient temperature during the study period ranged from −17.5 to 3.5 °C with the mean temperature being −3.0 °C. The weather was relatively mild as compared to the season's average (−7.2 °C for the previous five years). Great tits were captured with mist nets and spring traps and kept indoors in individual cages supplied with water, mealworms and sunflower seeds until night when their body masses and BMRs were recorded. In the morning following the first capture, blood samples were taken from the birds (for measuring leucocyte counts). The birds were then randomly assigned to immune challenge (injection with suspension of SRBCs) and control (injection with isotonic saline) treatments. Thereafter, they were released. Hence, the birds performed their normal activities in a natural environment during the experiment. Altogether, 42 male great tits were captured and assigned to control and antigen injections at first capture. Twenty-five of these birds (ten control and 15 experimentals) were subsequently recaptured after six to ten days (mean = 7.4 ± 1.5 days). All of these individuals could be used for estimating the effect of immune challenge on body mass change, while 24 individuals could be used for examining changes in their leucocyte profiles. The sample size was smaller (21 birds) for estimating changes in BMR because four birds did not sleep during the measurement of their BMRs during either the first or second capture and, hence, were excluded from the analyses. Control and experimental birds at first capture did not differ significantly in respect of their BMRs (3.9 ± 0.3 versus 4.0 ± 0.4 ml $O_2\,g^{-1}h^{-1}$ for controls and experimentals, respectively) ($t_{8,11} = 0.89$ and $p = 0.4$), body masses (18.6 ± 1.1 versus 18.8 ± 0.7 g for controls and experimentals, respectively) ($t_{9,14} = 0.52$ and $p = 0.6$) or heterophile-to-lymphocyte (H:L) ratios (1.4 ± 0.6 versus 1.5 ± 0.8 for controls and experimentals, respectively) ($z_{8,14} = 0.17$ and $p = 0.9$). Neither did the control and experimental birds differ in respect of date of initial capture (median 8 February versus 9 February for controls and experimentals, respectively) ($t_{14,9} = 1.05$ and $p = 0.3$) or the interval between captures (7.3 ± 1.7 versus 7.4 ± 1.4 days for controls and experimentals, respectively) ($t_{9,14} = 0.16$ and $p = 0.9$).

In order to account for interindividual differences in initial trait values, the effect of immune challenge on changes in trait values was examined in ANCOVAs including each trait's value at first capture as a covariate (see e.g. Merilä & Wiggins 1997). All models were tested for possible confounding effects of age, territorial status, date and between-capture interval, all of which (except the effect of date on BMR) turned out to have no significant effect on the variables examined ($p = 0.3$–0.7). Analyses were performed with the SAS GLM procedure (SAS Institute 1985) using type III sums of squares, thereby enabling us to account for all the effects of the independent variables simultaneously. Correspondence of residuals to a normal distribution was checked with the SAS UNIVARIATE procedure (Shapiro–Wilk's *W*-test) and in no case was the assumption of normality violated. Mann–Whitney *U*-tests were used when comparing group averages of non-normally distributed traits. All significance levels refer to two-tailed tests. Subscripts used in connection with test statistics refer to degrees of freedom. The values are means (± s.d.). The study was performed in accordance with the laws of the Russian Federation regarding the capturing and holding of wild animals and all individuals were released after the experiment.

(b) *Measurement of BMRs, immune responses and condition indices*

The metabolic rates of the great tits were determined as the rate of oxygen consumption by each resting bird at night (between 23.00 and 03.00) using a modified Kalabukhov closed system (Dolnik & Gavrilov 1979; Gavrilov 1979). Each bird was placed in a small cage and kept in the dark without food at 22–25 °C for at least 4 h. The cages and birds were then placed in 3-l glass metabolism chambers and kept in the dark in an incubator at 25.5–26 °C. One hour was allowed for adaptation and thermal stabilization. The chamber was then sealed and oxygen consumption was measured by measuring the flow of pure O_2. CO_2 in the chamber was removed using KOH. Oxygen consumption was measured over 10-min intervals for 60–70 min. The average of these 10-min intervals was used as an estimate of individual oxygen consumption. The temperature in the chamber was measured using a mercury thermometer. Birds were weighed after removal from a chamber. Oxygen consumption values were corrected to standard temperature and pressure (Depocas & Hart 1957). BMRs were expressed as the amount of oxygen consumed per gram of body mass per hour ($ml\,O_2\,g^{-1}h^{-1}$) (see also e.g. Røskaft *et al.* 1986; Schieltz & Murphy 1995). The use of five respirometers and two series of measurements enabled us to estimate the BMRs of up to ten birds per night. The first trial of BMR measurements started at 23.00 h (i.e. 6 h after sunset) and the second trial (for another five birds) started at 02.00.

In the morning after first capture, following the measurement of their BMRs, experimental birds were injected with 50 µl suspension of 40% SRBCs in isotonic saline into their pectoralis muscles, while the sham-operated control birds were injected with the same amount of saline. Prior to injection, SRBCs were double washed and resuspended in saline in order to achieve the desired concentration. Blood samples (*ca.* 150 µl) that were to be used for assessment of immune response and plasma proteins were obtained six to ten days after initial sampling. Serum was separated by centrifugation at 3000 r.p.m. for 10 min and preserved at −20 °C until analysis. Immune response (SRBC antibody titre) was measured using a haemagglutination assay (Wegmann & Smithies 1966; Lawler & Redig 1984) in 96-well microplates. A 12.5 µl aliquot of serum was added to 87.5 µl of saline in the first well of a plate and serially diluted using 50 µl of saline (0.5, 0.25, etc.). Then 50 µl of 1% suspension of SRBCs in saline was added to all samples. The microplates were incubated at 37 °C for 1 h. Titre was scored as the inverse of the dilution that contained sufficient antibodies to haemagglutinate SRBCs (hence, the higher the titre, the stronger the immune

Table 1. *Effect of immune challenge with SRBCs (treatment) on subsequent changes in BMR, body mass and the H:L ratio*
(For the direction of changes see figure 1. $R^2 = 0.75$ for BMR, $R^2 = 0.41$ for body mass and $R^2 = 0.77$ for the H:L ratio.)

	change in BMR		change in body mass		change in the H:L ratio	
effect	$F_{\text{d.f.}}$	p	$F_{\text{d.f.}}$	p	$F_{\text{d.f.}}$	p
treatment	$12.1_{1,17}$	0.003	$5.9_{1,22}$	0.024	$9.0_{1,21}$	0.007
initial value	$32.5_{1,17}$	< 0.001	$8.0_{1,22}$	0.010	$63.1_{1,21}$	< 0.001
date	$11.3_{1,17}$	0.004	—	—	—	—

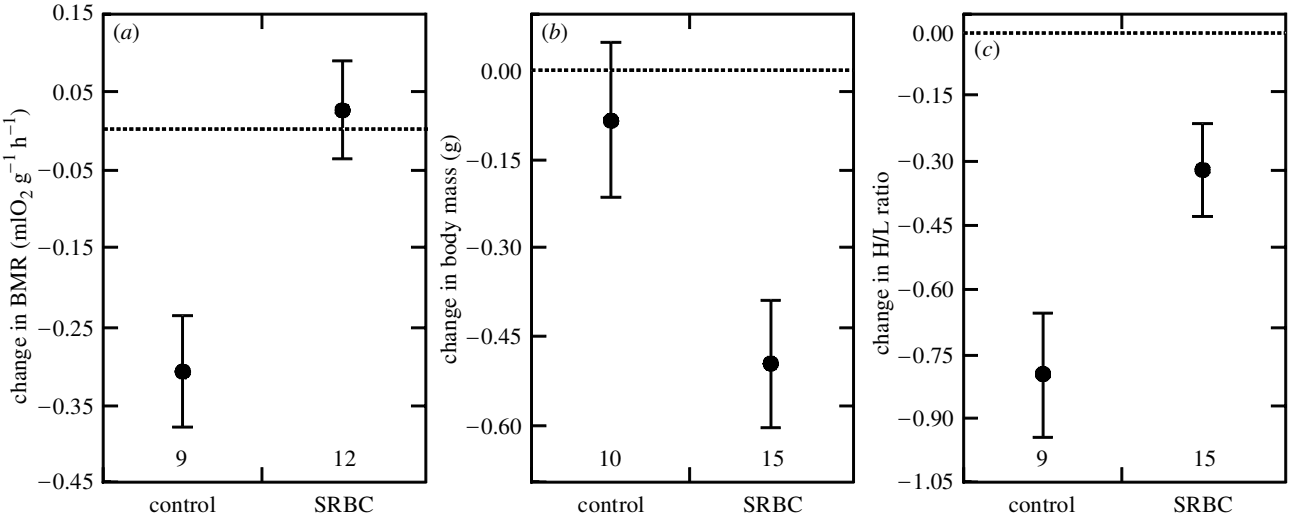

Figure 1. Comparison of changes in BMR, body mass and the H:L ratio between immune-challenged (SRBC-injected) and control (saline-injected) great tits. Least-square means and s.e.s derived from the models in table 1. The numbers under bars denote sample sizes.

response). The rest of the serum was used for determination of protein concentrations by standard agarose gel electrophoresis (see Ots *et al.* 1998 for details).

Leucocyte profiles and concentrations were estimated from thin blood smears that were prepared in the mornings following the measurement of BMRs. We concentrated on two leucocytic condition indices: the total leucocyte count (white blood cells, WBCs), the elevation of which is characteristic of inflammatory processes in response to microbial and macroparasite infections (e.g. Dein 1986) and the H:L ratio, which is widely used as a stress index in poultry (e.g. Gross & Siegel 1983; Maxwell 1993) but which is also used in studies of wild animals (e.g. Hõrak *et al.* 1998). In order to count leucocytes, a drop of blood was smeared on two individually marked microscope slides, air dried, fixed in absolute methanol and stained with azure-eosin. The proportions of different types of leucocyte were assessed on the basis of an examination of a total of 100 leucocytes under ×1000 magnification under oil immersion. Estimates of WBCs were obtained by counting the number of leucocytes per *ca.* 10 000 erythrocytes. The repeatabilities of leucocyte counts obtained using this method are high and significant, as shown by Ots *et al.* (1998).

3. RESULTS

The BMR decreased with calendar date over the course of the study period ($r = -0.41$, $p = 0.008$ and $n = 42$). In order to account for this effect, we included the date of capture as a covariate when analysing the effect of immune challenge upon the change in individual BMR values. The BMR decreased between the first and second capture among control birds, whereas there was no change among immune-challenged males (table 1 and figure 1*a*). As a result, immune-challenged birds had on average an 8.6% higher BMR at second capture than control birds (4.0 ± 0.2 versus 3.7 ± 0.3 ml $O_2\,g^{-1}h^{-1}$ for experimental and control birds, respectively) ($t_{11,8} = 2.80$ and $p = 0.011$) Birds injected with SRBCs on average lost 0.5 g (2.8%) of body mass between episodes of capture, while the mass of control birds did not change (figure 1*b* and table 1).

The H:L ratios of control birds decreased more than those of immune-challenged individuals between the first and second captures (figure 1*c* and table 1). This resulted in 37% higher H:L ratios in immune-challenged birds at second capture (1.11 ± 0.48) as compared to controls (0.70 ± 0.48). Antigen injection had no effect upon the change in total leucocyte count ($F_{1,21} = 0.37$ and $p = 0.5$). Immune-challenged birds had significantly lower total serum protein (21.9 ± 1.9 versus 24.1 ± 1.9 g l^{-1}) ($t_{13,9} = 2.63$ and $p = 0.015$) and albumin (17.8 ± 1.6 versus 19.4 ± 1.9 g l^{-1}) ($t_{13,9} = 2.30$ and $p = 0.031$) levels at second capture, while albumin to globulin ratios did not differ between experimental categories (4.5 ± 1.1 versus 4.3 ± 0.9) ($t_{13,9} = 0.45$ and $p = 0.7$). Hence, immune challenge resulted in a relative increase in metabolic rates and

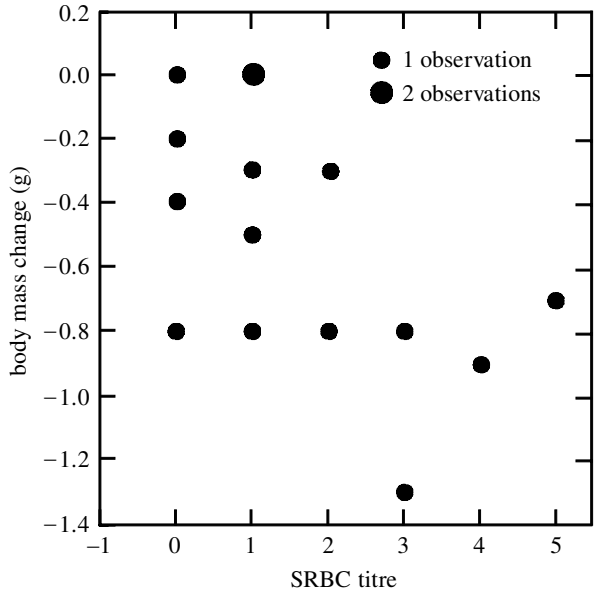

Figure 2. Relationship between SRBC titre and body mass change (mass in second capture minus mass in first capture) in male great tits ($r = -0.63$, $p = 0.027$ and $n = 15$).

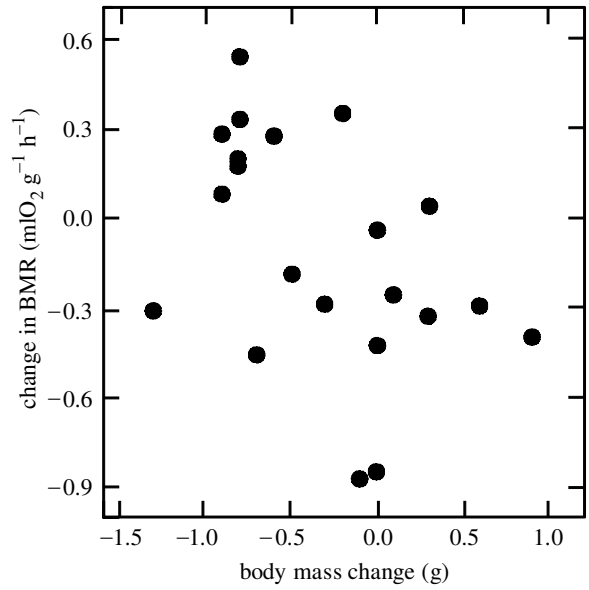

Figure 3. Relationship between body mass change (mass in second capture minus mass in first capture) and change in BMR (BMR in second capture minus BMR in first capture) in male great tits ($r_S = -0.45$, $p = 0.041$ and $n = 21$).

stress indices and loss of body mass and plasma nutrient content, while the plasma globulin content did not increase. The birds that mounted a stronger immune response against SRBCs lost more mass (figure 2), while the antibody titre did not correlate significantly with change in BMR ($r_S = -0.13$, $p = 0.7$ and $n = 12$). Body mass loss was accompanied by an increase in metabolic rate (figure 3) and the H:L ratio (figure 4).

4. DISCUSSION

Our experiment clearly demonstrated the energetic and physiological impact of immune challenge in wintering, male great tits. Birds mounting antibody responses against the foreign antigen had nearly 9% higher metabolic rates, 37% higher H:L ratios and 8% lower plasma albumin levels than control birds and they also lost nearly 3% (0.5 g) of their body mass subsequent to the immune challenge. This means that non-pathogenic immune challenge (i.e. mere activation of the immune system) can affect basal metabolism and change immune cell profiles in adult birds. Furthermore, the cost of exploiting an immune system is suggested by the result that individuals that mounted stronger antibody responses against SRBCs lost more mass during the immune response (figure 2). To the authors' knowledge, this is the first study to show such an effect in a wild bird species. A study in red grouse (*Lagopus lagopus scoticus*) found a 16% increase in metabolic rate following a truly pathogenic challenge with nematodes (Delahay *et al.* 1995). Studies in chickens (Klasing *et al.* 1987; but see Henken & Brandsma 1982) and quails (Fair *et al.* 1999) have shown that SRBC-induced immune challenge can suppress growth and reduce plasma protein content. The reason for the latter is decreased protein synthesis in the muscle and an increase in the use of amino acids in the liver for the synthesis of acute-phase plasma globulins (Klasing & Austic 1984). Such a mechanism could explain the lower serum albumin and total protein concentrations of the experimental birds in our study. In addition, the low levels of plasma albumin in the immune-challenged great tits might have resulted from reduced food intake. Such SRBC-induced anorexia has been demonstrated in chickens (Klasing & Austic 1984); however, the functional explanations for this phenomenon are still obscure (see Kyriazakis *et al.* 1998).

The 37% higher H:L ratio of the experimental versus control birds in our study suggests that injection with SRBCs affected the concentrations of different immune cells in the peripheral blood of great tits. Elevated H:L ratios in studies of poultry have traditionally been regarded as general symptoms of almost any kind of stress (caused by injuries, infectious diseases, starvation or the administration of glucocorticoids) (e.g. Gross & Siegel 1983; Gray *et al.* 1989; Maxwell 1993; Dhabhar *et al.* 1995). H:L ratios have also been shown to increase in response to experimentally increased reproductive effort (Hõrak *et al.* 1998) and phytohaemagglutinin-induced immune challenge (Hõrak *et al.* 2000) in great tits. In the present study, the increase in the H:L ratio between the events of capture was associated with a decrease in body mass (figure 4), suggesting that stress levels increased in parallel with deterioration of body condition. The increase in the H:L ratio in response to antigen injection could be possibly ascribed to corticosterone-induced redistribution of lymphocytes to secondary lymphoid tissues (Dhabhar *et al.* 1995; Trout *et al.* 1996) and simultaneous migration of heterophils (which participate in the phagocytosis of antigen) into the peripheral blood in the course of an inflammatory response (Klasing & Austic 1984). Unlike Fair *et al.* (1999) in the study of growing quails, we failed to detect an increase in the total leucocyte count in response to SRBC injection.

Considering the lack of effect of antigen injection on peripheral immune cell proliferation and immunoglobulin production, the costs of mounting an immune

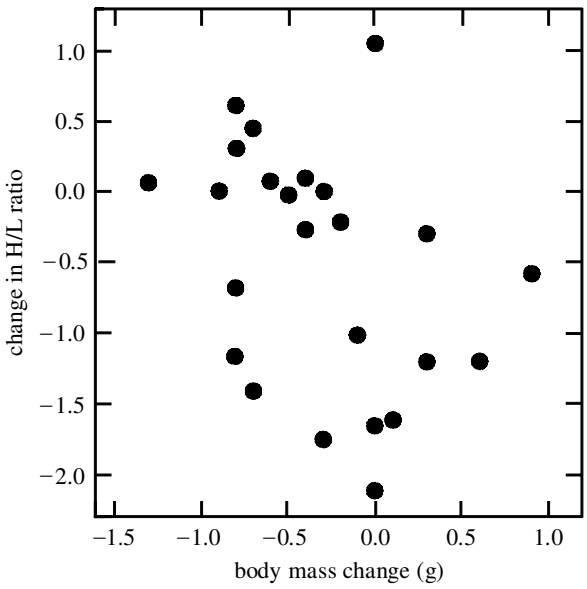

Figure 4. Relationship between body mass change (mass in second capture minus mass in first capture) and change in the H:L ratio (H:L ratio in second capture minus H:L ratio in first capture) in male great tits ($r_S = -0.46$, $p = 0.025$ and $n = 24$).

response in our study were unlikely to have resulted from the reallocation of limited energy, protein or other nutrients for the production of immune cells and molecules. However, while the quantitative need for nutrients for the proliferation of leucocytes and production of antibodies may be very small (Klasing 1998), there appear to be a number of indirect costs associated with systemic cytokine-mediated inflammatory and acute-phase responses, anorexia, cellular metabolic rates and cellular–molecular turnover (Klasing & Leshchinsky 1999; Lochmiller & Deerenberg 2000) which may lead to increased energy turnover and metabolism in the process of up-regulation of the immune system. Given that such metabolic costs could be readily detected in our study despite the modest sample size and relatively mild weather conditions, the contention of Lochmiller & Deerenberg (2000) that the costs of upregulation of the immune system may push an animal beyond the minimal levels of bodily reserves to survive should probably be given serious attention. Notably, increased metabolism was accompanied by a considerable loss of body mass in our great tits (figure 3), suggesting that energetic expenditures to an immune response may indeed have a non-trivial impact upon a host's condition.

Our results on the effect of antigen injection on BMR were of comparable magnitude to those found by Svensson *et al.* (1998) in a related species, i.e. the blue tit. Svensson *et al.* (1998) calculated that the upper limit of the energetic costs of an immune response may range up to 8 or 13% of BMR, but these differences (in a sample size comparable to that of the current study) turned out to be insignificant. However, in contrast to the great tits in our study that lost mass in response to antigen injection, their immune-challenged blue tits had significantly higher body masses than the control birds during the secondary immune response. We can see two (not mutually exclusive) explanations why the immune response in our study was accompanied by reduced body mass but not in blue tits. First, the antigen used for immunizing blue tits (a diphtheria–tetanus vaccine) might have induced a lower extent of inflammatory response than the SRBCs used in our study. Since the immune response against SRBCs involves both B and T lymphocytes and antigens that induce T-lymphocyte responses are thought to induce greater levels of inflammatory cytokines than those that induce immunoglobulin responses (Klasing & Leshchinsky 1999), the response to SRBCs may appear energetically more costly than the response to diphtheria–tetanus vaccine. Second, the blue tits in the experiment of Svensson *et al.* (1998) were kept in aviaries where they had access to food *ad libitum*, which evidently enabled them to retain an energy balance throughout the experiment. In contrast, the immune-challenged great tits in our study were released to natural conditions, which means that they were not deprived of the behaviourally mediated costs of finding and defending food sources, social interactions and anti-predator vigilance. Evidently, such behavioural costs might have been sufficient to boost the energetic costs of mounting an immune response, which could remain undetected in laboratory conditions. Hence, our study illustrates the necessity of measuring the costs of using the immune system under natural conditions.

Both the present study and that of Svensson *et al.* (1998) raise the question of the biological significance of elevation of the BMR in response to immune challenge. Although small passerines can work at a level of daily energy expenditure (DEE) that equals four times the BMR under extreme conditions (e.g. Stevenson & Bryant 2000), the optimal DEE:BMR ratio for passerine birds is considered to be equal to 2.5 (Dolnik 1982, 1996). This ratio is very close to that of non-territorial, wintering, male great tits (DEE = 2.7 BMR) in Zvenigorod (Nagy *et al.* 1999). At ambient temperatures similar to those of the present study, the DEE of the magnitude of 2.5 times the BMR was found to be very close to existence metabolism, which includes expenditures of *ca.* 1.8 times the BMR for basal metabolism and thermoregulation (Gavrilov *et al.* 1996). This means that wintering great tits normally spend energy at a magnitude of only 0.7–0.9 times the BMR for activities such as foraging and flocking behaviour, anti-predator vigilance, etc. This proportion of energy becomes still smaller with a decrease in ambient temperature. In this context, an extra increase in BMR of 8–13% due to activation of the immune system can be considered a serious challenge to the energy budget of an animal. The question of whether this challenge is serious enough to compromise investments in other vital functions of an organism remains to be solved in future studies measuring both the BMR and field metabolic rates of immune-challenged individuals.

In conclusion, our study shows that the traditional energetic and nutritional currencies in which the costs of life-history trade-offs are thought to emerge may also be relevant for research in ecological immunology. This of course does not mean that alternative costs of immune responses (such as immunopathology or free radical production) will be unimportant. Rather, our results suggest the need for careful consideration of all different expenditures related to activation of the immune system.

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