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Functional Ecology, Volume 12, Issue 6 (Dec., 1998), 912-919.

Stable URL:
http://links.jstor.org/sici?sici=0269-8463%28199812%2912%3A6%3C912%3AESIATC%3E2.0.CO%3B2-7

Functional Ecology 1998
12, 912–919

Energetic stress, immunosuppression and the costs of an antibody response

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Summary

1. Recently, there has been much interest in physiological trade-offs between parasite resistance and fitness-related traits such as secondary sexual characters or reproductive effort. More specifically it has been suggested that (i) energetically costly activities may suppress the immune system and (ii) that this immunosuppression is caused by costly immune defences competing with other bodily demands for scarce resources, e.g. energy.

2. The possibility was investigated of an energetically based trade-off between humoral (antibody-based) immunocompetence and other costly activities, by immunizing Blue Tits, *Parus caeruleus*, with novel antigens (proteins) thereby inducing antibody responses, and performing two experiments. In experiment 1, one group of birds was subjected to cold stress, thereby increasing their daily energy expenditure and the effect on immune responsiveness was investigated. In experiment 2, the basal metabolic rate (BMR) of immunized birds was measured to investigate the energetic costs of mounting the antibody responses.

3. In experiment 1, birds subject to increased energy turnover had significantly lower antibody responses, consistent with the hypothesis that environmental stress could suppress immunocompetence. However, in experiment 2 the energetic costs of these antibody responses were found to be low and at most 8–13% of BMR, indicating that adaptive resource allocation of energy was an unlikely explanation for the lowered immune responsiveness in the cold stress treatment (experiment 1).

4. It is concluded that our data provide some support to the idea that there may be a trade-off between immunocompetence and energetically costly activities such as thermoregulation, reproduction or mate attraction, although this trade-off may not necessarily be based on energy or nutrient limitation (i.e. resource allocation models). Two non-energetic explanations are briefly discussed, one adaptive and one non-adaptive, that could explain the immunosuppression in our study as well as in other behavioural and ecological contexts.

Key-words: Cold stress, energy turnover, immunocompetence, *Parus caeruleus*, physiological trade-offs

Functional Ecology (1998) **12,** 912–919

Introduction

In recent years there has been a growing interest in links between individual immune system performance (immunocompetence) and fitness-related traits, e.g. reproductive effort (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996; Deerenberg *et al.* 1997) or secondary sexual characters (Folstad & Karter 1992; Wedekind & Folstad 1994; Saino, Bolzern & Møller 1997). It is usually assumed that costly immune responses are suppressed during stressful activities such as parental care, and that the released resources from this immunosuppression are then adaptively reallocated to cover other costly activities (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996). This hypothesis has been inspired by and is partly supported by medical and physiological studies which have shown that stressful activities such as strenuous exercise tend to suppress several aspects of immune function (e.g. Hoffman-Goetz & Pedersen 1994; Nierman & Nehlsen-Cannarella 1994). However, there is to our knowledge no study that has experimentally addressed the question about the existence of a resource-based trade-off, though several workers have more or less implicitly assumed this when

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interpreting observed cases of immunosuppression in experimental studies (Gustafsson *et al.* 1994; Saino *et al.* 1997; Deerenberg *et al.* 1997).

In this study we have experimentally investigated the importance of energetic stress and energy turnover on humoral (antibody-based) immunocompetence in Blue Tits (*Parus caeruleus*). By experimentally challenging the immune system with novel antigens and measuring the concomitant host antibody titres against these antigens, one gets a good estimate of host resistance to a variety of host pathogens, in contrast to more correlative approaches such as leucocyte counts (Luster *et al.* 1993). Accordingly, this kind of experimental approach has been advocated by several authors as the appropriate method to investigate the importance of immunocompetence as a cost mechanism in evolutionary ecology (Svensson & Skarstein 1997).

We have studied two aspects of immunocompetence as such a cost mechanism that should be of interest to ecologists. First, we investigated the consequences of an increased energy turnover on immune responsiveness by subjecting one group of birds to cold stress and comparing their immune responses with a non-stressed control group. This experiment should have some implications for avian winter ecology, since suppressed immune responses during cold winters could potentially affect disease risk and mortality rates and hence avian population dynamics (cf. Dabbert, Lochmiller & Teeter 1997), in addition to traditionally invoked factors such as predation and starvation (Lack 1966; McNamara & Houston 1987). Furthermore, suppressed immune responses during energetic stress are likely to be important in other ecological contexts as well (Gustafsson *et al.* 1994; Saino *et al.* 1997; Deerenberg *et al.* 1997).

We proceed by measuring the energetic costs of the antibody responses against the immunized antigens used in the first experiment. One interpretation of reduced immune responses during such a stressful activity could be that energy-stressed birds adaptively suppress their costly antibody responses and reallocate the saved energy towards other body functions. For such adaptive energy-saving immunosuppression to be important, there has to be a major energetic cost associated with the antibody response. Hence, we would expect that the basal metabolic rate (BMR) should increase substantially among immunized as opposed to non-immunized birds.

Methods

STUDY SPECIES AND GENERAL METHODS

Blue Tits are common, sedentary birds that spend the winter close to their breeding grounds in southern Sweden (see Nilsson & Svensson 1996). Blue Tits were captured by mist-netting and making nightly nestbox visits in a wide area east of Lund, southern Sweden, between December 1995 and March 1996. The population in which the birds were captured has previously been intensively studied with respect to reproductive strategies in the field, and hence the breeding and winter ecologies are well-known (see Nilsson & Svensson 1996; Svensson & Nilsson 1995, 1997 for examples of studies). Birds were transported to an indoor laboratory at the Department of Animal Ecology in Lund. They were kept in individual cages at 20 °C (except the birds subject to the cold stress treatment, see below) and an L:D cycle of 10:14 (an average of natural light conditions during the period). During the course of the study, the birds received daily replenished *ad libitum* food consisting of mealworms *Tenebrio molitor* and vitamin-enriched water. Captured birds were allowed to become accustomed to indoor conditions for 1–12 days before the experiments started.

EXPERIMENT 1: COLD STRESS AND IMMUNE RESPONSIVENESS

A total of 27 Blue Tits were subject to either of the following treatments: 11 birds served as controls and were kept at 20 °C from the day of capture and throughout the study. Sixteen birds were kept at 4 °C during the days and – 15 °C during the nights. To standardize housing conditions (other than temperature), the experiment was performed in three randomly ordered sequences: 9 in low temperature, 11 control birds and finally 7 in low temperature. All birds were kept in a climate room during the days (20 or 4 °C) and in a climate cabinet (Hereaus) next door during nights (20 or – 15 °C).

The magnitude of the cold experiment treatment aimed at increasing the 24-h energy expenditure of the birds by approximately 1 BMR up to a total level of about 3 BMR. This level of daily energy turnover level is similar to that experienced by Blue Tits during cold winter periods (cf. Nilsson & Svensson 1996). Furthermore, it is also similar to the level experienced by birds participating in parental care during the breeding season (Drent & Daan 1980; Masman *et al.* 1989). Literature data were used on the lower critical temperature of the thermo-neutral zone and thermal conductance below this temperature was used to calculate the increment in energy turnover rate due to thermoregulation (Gavrilov & Dolnik 1985). Since data for the Blue Tit were not available, data for the closely related Great Tit, *Parus major*, were used. The extra cost of thermoregulation is 33–63% of BMR at 4 °C and 91–151% of BMR at – 15 °C (cf. references in Gavrilov & Dolnik 1985).

Birds were immunized (day 1) intramuscularly in the pectoral muscle with 100 µl diphtheria–tetanus vaccine (Statens Seruminstitut, Copenhagen, Denmark). This vaccine contains two antigens novel to the birds and the antibody responses to each of them are highly correlated within birds (Pearson: $r = 0{\cdot}627$, $N = 26$,

$P=0{\cdot}001$, log-transformed data). This indicates that the immune responses against these antigens are not idiosyncratic, but give a general measure of humoral immunocompetence; that is, the ability to mount an antibody response against an arbitrary novel antigen (see also Luster *et al.* 1993). Birds were weighed in the morning and evening at the start and end of the experiment to get a measure of nightly body mass losses and their current energy balance. A blood sample (about 150 µl, taken from the jugular vein) was collected on day 1 (before immunization) and day 12 (after immunization) and centrifuged at 3000 r.p.m. in EDTA-containing tubes for 8 min. Plasma was extracted and stored at −50° until later ELISA (enzyme-linked immunosorbent assay) analysis.

EXPERIMENT 2: ENERGETIC COSTS OF ANTIBODY RESPONSES

In this experiment the birds' overnight BMR was measured (day 0–1). The measurements started at 18·00 and ended around 08·00 the following morning. BMR was calculated from oxygen consumption as measured in an open circuit respirometer (see Lindström & Kvist 1995 for methodological details). During the nightly measurements, the birds were kept in airtight boxes in total darkness in a climate chamber (Hereaus) at 25 °C (all the birds used in the respirometry experiment were kept in individual cages at 20 °C during daytime, as in experiment 1). Birds were measured each night in parallel channels with identical set-up. Oxygen consumption was calculated according to Hill (1972). BMR (kJ day^{-1}) was calculated by selecting the 10-min period with the lowest average oxygen consumption and assuming an energetic equivalence of 19·8 kJ l^{-1} O_2.

Birds that lost flight feathers were immediately excluded from further measurements to avoid effects on BMR from the confounding costs of feather synthesis (Lindström, Visser & Daan 1993). Out of 40 birds (20 immunized + 20 controls), 24 passed day 15 without losing any feathers, and 15 passed day 43. Out of these, one of the immunized birds did not have a detectable primary immune response (optical density value never exceeded the mean optical density of preimmunized birds plus three standard deviations, cf. Isogai *et al.* 1994 and below) and was therefore excluded from further analyses. The data set thus consists of 12 immunized and 11 control birds for days 0–15 and 7 immunized plus 7 control birds for days 28–43.

Body mass was measured before and after the metabolic measurements. On the day after the first respirometry night (day 1), blood samples were taken and plasma extracted in the same way as in experiment 1 (see above). After the first blood sampling occasion, some of the birds were randomly selected to be immunized (see below) and the others served as controls. The BMR measurements and blood sampling procedures were repeated on all individuals (both immunized and control birds) with weekly intervals, i.e. on days 7–8, 14–15, 21–22 and 28–29. On day 29, the immunized birds were subject to a second immunization in order to induce secondary immune responses. BMR measurements and blood sampling procedures were then repeated on days 35–36 and 42–43. The intervals between immunization and BMR measurements were chosen to include at least one data point for each experimental bird from the periods when peak antibody titres are expected; i.e. 10–14 days postimmunization for the primary and 6–9 days postimmunization for the secondary response (Feldmann 1996).

ELISA PROTOCOL

Analyses of antibody titres were performed as standard ELISAs. Polystyrene 96-well plates (Maxisorp, NUNC, Denmark) were coated overnight with either diphtheria toxoid, 3 µg ml^{-1}, or tetanus toxoid, 3 µg ml^{-1}, in 0·15 M carbonate buffer, pH 9·6. After three washings with buffer (29·2 g NaCl, 0·2 g KCl, 0·2 g KH_2PO_4, 1·15 g Na_2HPO_4, $2H_2O$, 10 ml Triton X-100, distilled water ad 1 l), dilutions of plasma from individual birds were applied to the wells (diluted in a buffer containing 10 g bovine albumin l^{-1}, PBS adjusted to 0·5 M and Tween 20 0·05%). A reference sample was prepared from a pool of plasma from immunized birds and was used as internal standard during all titrations. The titre of the standard sample was arbitrarily defined as 100 units ml^{-1}. This made it possible to recalculate optical density values to relative antibody titres that were comparable between plates. Serum samples that were tested were diluted 1:40 and the pooled standard sample was two-fold diluted (starting dilution 1:20). Plates were incubated over night and then washed three times with washing buffer. As a secondary antibody, a peroxydase-conjugated rabbit anti-chicken Ig (Cat. A9792, Sigma) was used. This secondary antibody was diluted 1:1000 and incubated for 2 h. After three additional washings, colour reactions were achieved in a citrate buffer (7·3 g citric acid, 11·86 Na_2HPO_4, $2H_2O$, ad 1 l). To 10 ml of the citrate buffer was added 4 µl H_2O_2 (Perhydrol, 30%) and 4 mg orthophenylene diamin (OPD, KemEnTec, Denmark). The colour was allowed to develop for 30 min, whereafter the reaction was stopped by the addition of 150 µl 1 M H_2SO_4. The reaction was read in an ELISA reader at 405 nm (ThermoMax, Molecular Devices). Titres were expressed as units in comparison with the defined standard, set as 100 units ml^{-1}, using a four-parameter plot. Intra-assay variation was < 5% and interassay variation < 12%.

To investigate the reliability of the immunization procedures, optical density values were compared from the ELISA analyses (see above) of day 1 (background) and day 12 within all the birds used in

experiment 1. All birds (n = 27) had optical densities at day 12 that were substantially higher than their background optical density, indicating that all birds responded to immunization.

STATISTICAL ANALYSES

Antibody titres were log-transformed to achieve normally distributed residuals (Sokal & Rohlf 1995). Where data consist of repeated samplings of the same individuals, repeated measures ANOVA/ANCOVA have been used (von Ende 1993). Probability values of within-subject factors were Greenhouse–Geisser adjusted ε range: 0·74–0·93) to compensate for non-circularity in the variance–covariance matrices (von Ende 1993). All tests were performed with SYSTAT (SYSTAT 1992).

Results

COLD STRESS AND IMMUNE RESPONSIVENESS

The potential influence of confounding effects caused by sex or age differences among individual birds were first investigated. There were no significant effects of either of these variables either for the antitetanus response (sex: $F_{1,24}$=1·240, P=0·28; age: $F_{1,24}$=0·047, P=0·83) or for the antidiphtheria response (sex, $F_{1,24}$=0·003, P=0·96; age, $F_{1,24}$=0·151, P=0·70). Hence, differences in sex or age composition among our experimental and control groups are not confounding the results.

In experiment 1, cold-exposed birds lost significantly more body mass each night than controls (Table 1). That the birds remained in energy balance throughout the course of the experiment was illustrated by the fact the mass loss among the cold-exposed birds was compensated by significantly higher evening body mass than controls (repeated measures ANOVA of treatment effect: $F_{1,24}$ = 7·354, P = 0·012). In contrast, morning body masses did not differ between the experimental categories (repeated measures ANOVA of treatment effect: $F_{1,24}$ = 0·002, P = 0·96). This demonstrates that experiment 1 led to an increased energy expenditure (body mass loss) among cold-exposed birds, and that the birds apparently compensated for this by eating and storing more food as body reserves during the days in anticipation of the colder nights. Apparently, cold-exposed birds increased their subcutaneous fat reserves during the course of days (E. Svensson *et al.*, unpublished observations). Thus, although both experimental categories remained in energy balance due to *ad libitum* food regimes, cold-exposed birds had apparently a higher energy turnover rate, which was one of the aims with the experiment.

Cold-exposed birds had lower antibody responses to both the diphtheria antigen (Fig. 1a) and to the tetanus antigen (Fig. 1b). The effects of the experimental treatment were statistically significant for both antigens (diphtheria, $t_{1,25}$ = 5·93, P = 0·02; tetanus, $t_{1,25}$ = 9·92, P = 0·004).

Table 1. Repeated measures ANOVA with nightly body mass loss among Blue Tits between days 1–2 and 10–11 as dependent variables and treatment (−15 or + 20 °C) as independent variable

Type of response	df	MS	F	P
Between subjects				
Treatment	1	4·234	170·8	<0·001
Error	25	0·025		
Within subjects				
Night	1	0·061	4·695	0·04
Night × treatment	1	0·369	28·56	<0·001
Error	25	0·013		

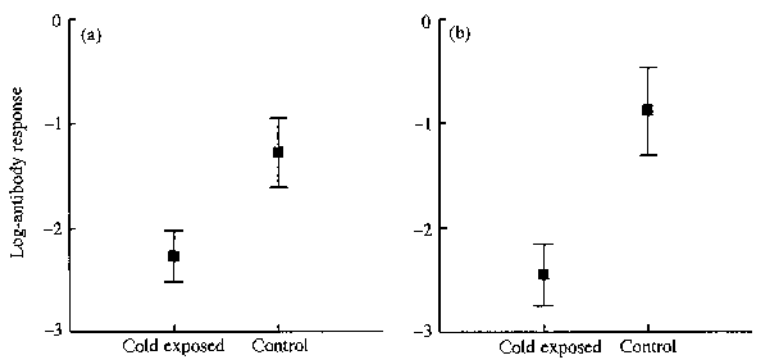

Fig. 1. Antibody responses (means ± SE) produced by the Blue Tits against (a) diphtheria antigen and (b) tetanus antigen. For statistical tests see text.

ENERGETIC COSTS OF THE ANTIBODY RESPONSES

Analysis of the serum samples obtained weekly in experiment 2 showed that antibody titres towards both diphtheria and tetanus followed a similar temporal pattern: the highest values in the primary immune response were 14 days postimmunization whereas the highest value in the secondary response was 7 days after reimmunization (Fig. 2). All individual birds followed the same temporal pattern although there was variation among birds in the strength of their antibody responses (Fig. 2). In accordance with previous knowledge from other vertebrate species (Feldmann 1996) the secondary response was much stronger than the primary response (Fig. 2).

Basal metabolic rates of immunized birds were higher than for control birds during days 7, 14, 28, 35 and 42 (Fig. 3). However, these BMR differences between the experimental categories were far from significant, both during the primary immune response (repeated measures ANOVA: $F_{1,21}$ = 0·37, P = 0·55), and during the secondary response (repeated measures ANOVA: $F_{1,12}$ = 2·53, P = 0·14). It is possible that the sample sizes in our experiment were too low to reject

the statistical null hypothesis of no costs of mounting an antibody response, i.e. a type II error. This is likely, since all physiological processes, including antibody responses, consume energy, the important question is only how much. Nevertheless the 95% confidence limits of the difference in BMR between the experimental categories can be used to estimate an upper limit of the magnitude of such a cost. Our calculations revealed these values to be 1·6 kJ day^{-1} at day 14–15 and 2·6 kJ day^{-1} at day 35–36 (the peaks of the primary and secondary immune responses, respectively). These values correspond to an 8% and 13% increase in BMR, respectively. It is emphasized that these percentages are not mean values, but are rather upper limits to the costs of the antibody responses and since the difference between the experimental and control group was far from significant, increased sample sizes would most probably narrow the confidence limits and hence lower these estimates. For the moment, it is concluded that, with 95% certainty, the energetic costs of the antibody responses against the antigens used in this experiment, are equal or lower than these percentages.

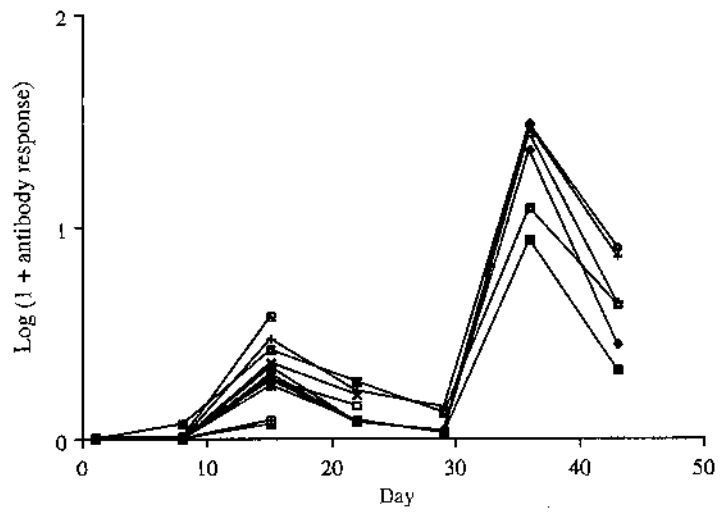

Fig. 2. Antibody titre (ab-titre, log-scale) for immunized Blue Tits against diphtheria antigen. The ab-titre against tetanus was very similar to the pattern for diphtheria and is hence not shown in the figure. Ab-titres estimated through ELISAs and calculated from optical density values to units ml^{-1}, using a standard (pooled serum from immunized birds) as common reference on all ELISA plates. Primary immune response: day 0–14, secondary immune response: day 28–42.

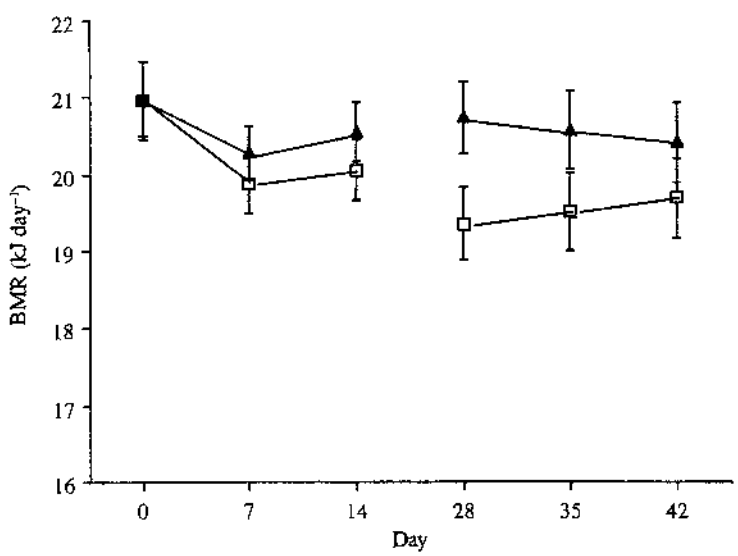

Fig. 3. Basal metabolic rates (BMRs, means ± SE) of (▲) immunized and (□) control Blue Tits. Primary immune response during day 0–14 and secondary immune response during day 28–42. For statistical tests see text and Table 2.

Immunized birds had somewhat higher average daily body masses than control birds (Fig. 4), although this difference was significant during the secondary immune response only (repeated measures ANOVAs of treatment effects: primary response, $F_{1,21} = 1{\cdot}19$, $P = 0{\cdot}29$; secondary response, $F_{1,12} = 8{\cdot}26$, $P = 0{\cdot}01$).

Among individuals, there was a strong positive relationships between BMR and body mass (linear regression of BMR on body mass days 0–1, 7–8, 14–15, 28–29, 35–36 and 42–42; $r^2 = 0{\cdot}40$–$0{\cdot}66$, P always $< 0{\cdot}01$). Since both inter- and intra-individual variation in body mass apparently influences BMR (this study; Daan *et al.* 1989; Scott, Mitchell & Evans 1996), a statistical comparison between the experimental categories was performed using their body mass-corrected BMRs (i.e. the residuals of BMR regressed on body mass). Using this statistical procedure, no significant effects of the immunization experiment on BMR was found (Table 2). Finally, there were no significant effects of neither sex nor age on BMRs during any of the days (ANCOVAs with mass and treatments as covariates; all $P > 0{\cdot}10$ for days 0, 7, 15, 21, 29, 36 and 45).

Discussion

The first experiment has clearly demonstrated that stress in the form of cold exposure has a negative impact on humoral immunocompetence. These results point to the possibility that suppressed immune responses may be a potential mortality factor in avian winter ecology; e.g. during hard cold spells immune responses may be suppressed leading to increased risks of infectious diseases (Dabbert *et al.* 1997; see also Nelson *et al.* 1996). Our results differ from those obtained by Dabbert *et al.* (1997), who found no evidence for suppressed humoral immunocompetence following experimentally induced cold stress in the Northern Bobwhite, *Colinus virginianus*. This difference could possibly be due to species-specific differences in insulation capacity and hence thermoregulatory costs.

If our results can be generalized into other energetically stressful ecological situations such as during production or maintenance of secondary sexual characters (Wedekind & Folstad 1994; Saino *et al.* 1997) or reproductive effort (Gustafsson *et al.* 1994; Deerenberg *et al.* 1997), these data do thus provide some support to the idea that suppressed immune

responses could mediate reproductive costs or costs of sexual ornaments. Additional support for this interpretation is provided by the fact that the suppressed antibody responses among the cold-exposed birds are similar in magnitude (albeit slightly lower) to those observed among free-living Blue Tits participating in parental care during the breeding season (Svensson 1997).

Since the immune responses discussed above seem to be sensitive to stress, it seems reasonable to ask why they are suppressed. The most straightforward interpretation of the results in experiment 1 is that birds that spend more energy on thermoregulation have less energy available for their immune responses, that is, energy limitation. This is the basic assumption of the adaptive resource allocation model of immunocompetence as a cost mediator in evolutionary ecology (Gustafsson *et al.* 1994; Wedekind & Folstad 1994; Sheldon & Verhulst 1996; Deerenberg *et al.* 1997). However, for two reasons we do not think that energy limitation is the correct explanation for the lowered humoral immunocompetence observed in experiment 1. First, both control and experimental birds had access to *ad libitum* food and apparently remained in energy balance throughout the experiment as indicated by their mass dynamics. Second, experiment 2 showed that the energetic costs of mounting antibody responses against the two antigens used in the study were very small, and only in the order of a few percentage of BMR. Although very small costs of immune responses may be important in various situations of energy limitation, the energy savings from the suppression of these antibody responses should be very small compared with the overall costs of thermoregulation in experiment 1. Furthermore these energetic savings are also likely to be small compared with the overall costs of parental care during the breeding season. We will therefore briefly discuss some other explanations to why immune responses were suppressed in our first experiment, which may also be applicable to other ecological situations.

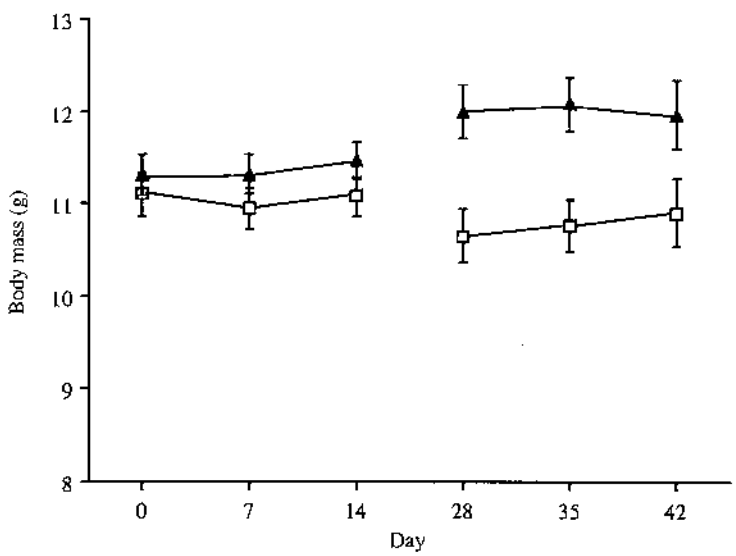

Fig. 4. Body masses (means ± SE) of (▲) immunized and (□) control Blue Tits. Primary immune response during day 0–14 and secondary immune response during day 28–42. For statistical tests see text.

Table 2. Repeated measures ANOVAs with residuals of BMR regressed on mass for the primary immune response (days 0–1, 7–8, 14–15) and secondary immune response (days 28–29, 35–36, 42–43) as dependent variables and treatment (immunized or control) as independent variable

Type of response	Variable	df	MS	*F*	*P*
Primary response					
Between subjects	Treatment	1	0·22	0·17	0·68
	Error	21	1·30		
Within subjects	Day	2	0·58	1·46	0·24
	Day × treatment	2	0·16	0·40	0·65
	Error	42	0·40		
Secondary response					
Between subjects	Treatment	1	0·51	0·28	0·68
	Error	12	1·83		
Within subjects	Day	2	0·12	0·44	0·63
	Day × treatment	2	0·14	0·52	0·58
Error	24	0·27			

Nutrient limitation, such as the availability of proteins (Lochmiller, Vestey & Boren 1993), carotenoids (Lozano 1994) or certain scarce amino acids (Klasing & Austic 1994a,b), are known to have a negative impact on the immune system and the development of immune responses. Such nutrient limitation is, however, an unlikely explanation for the suppressed immune responses in our first experiment, since both categories of birds received the same quality and type of food. Furthermore, in experiment 2, immunized birds had significantly higher body mass than non-immunized controls, further contradicting such an explanation. Since neither energy nor nutrient limitation is likely to explain the suppressed antibody responses in our study, we offer two not mutually exclusive explanations for the observed immunosuppression: one adaptive (cf. Wedekind & Folstad 1994) and one non-adaptive (see below).

Several authors have suggested that the major costs of immune responses are tissue damage and/or risks of autoimmune reactions associated with failure of the immune system to distinguish 'self' from 'non-self' (e.g. Nesse & Williams 1994; Jurd 1995; Adams 1996; Nelson & Demas 1996). The physiological response to stress is largely mediated by glucorticoids ('stress hormones'; Munch & Náray-Fejes-Tóth 1995). It has been proposed that the main function of these hormones is to protect the organism from stress-activated immune defence mechanisms that may cause tissue damage (Munch & Náray-Fejes-Tóth 1995). Immunosuppression to avoid such damage costs may thus be adaptive even in the absence of resource limitation (Råberg *et al.* 1998).

An alternative, non-adaptive explanation for the suppressed antibody responses among the cold-exposed birds could be that a high metabolic rate may

have a negative effect on the immune system even if the animal remains in energy balance. More specifically, a high level of energy expenditure and concomitant oxygen consumption will increase the production of free oxygen radicals, that arise as a by-product of aerobic metabolism (Rose 1991; Kirkwood & Rose 1991). These highly reactive substances are known to have several non-specific damaging effects on body tissues, including the immune system (Huang *et al.* 1992; Jenkins 1993; Hollan 1995). According to this scenario, energy turnover, rather than a negative energy balance, is the critical factor causing immunosuppression (cf. Barja *et al.* 1994). Daan, Deerenberg & Dijkstra (1996) suggested that this could account for the delayed reproductive costs following brood size manipulations in the Eurasian Kestrel, *Falco tinnunculus*: parent birds had higher mortality rates several months after a breeding season with experimentally increased workload, even though they remained in energy balance during the whole course of the manipulation (Masman *et al.* 1989; Daan *et al.* 1996).

Conclusion

Our study has clearly demonstrated that humoral immunocompetence in the Blue Tits is sensitive to stress, but the ultimate causes and proximate mechanisms behind the observed immunosuppression remain unclear. Our results give no clear support for adaptive resource allocation of energy as the important factor behind the immunosuppression against these antigens. As a cautionary note, we suggest that workers should seriously consider non-energetic explanations for the observed suppression of antibody responses in experimental studies of sexual selection (Saino *et al.* 1997) or reproductive effort (Deerenberg *et al.* 1997). We have studied the energetic costs of immune responses to non-living antigens, but it remains to be investigated if other types of immune responses, preferably to relevant living pathogens, are energetically more costly than the one studied here (cf. Baracos, Whitmore & Gale 1987). We note, however, that even the simple and energetically inexpensive immune responses in this study seem to be sensitive to stress, supporting the need for non-energetic explanations of these patterns.

Acknowledgements

We are grateful to Bettina Eide-Holm for performing the ELISA analyses. We also wish to thank Marcel Klaassen, Anders Kvist and Åke Lindström for assistance with the respirometry equipment. Constructive comments on previous drafts of this manuscript were provided by Victor Apanius, Ivar Folstad, Jan-Åke Nilsson, Frode Skarstein and Claus Wedekind. Financial grants were provided by The Royal Swedish Academy of Sciences ('Hierta Retzius Stipendie-fond'), Uddenberg-Nordingska Stiftelsen, Helge Axson Johnssons Stiftelse and Lunds Djurskyddsfond to ES.

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Received 1 September 1997; revised 5 January 1998; accepted 21 January 1998