THE ROYAL SOCIETY

Received 20 February 2003
Accepted 2 June 2003
Published online 12 August 2003

Costs of immune response in cold-stressed laboratory mice selected for high and low basal metabolism rates

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To study whether mounting an immune response is energetically costly, mice from two lines divergently selected for high (H-BMR) and low (L-BMR) basal metabolic rate (BMR) were immunized with sheep red blood cells. Their energy budgets were then additionally burdened by sudden transfer from an ambient temperature of 23 °C to 5 °C. We found that the immune response of H-BMR mice was lower than that of L-BMR mice. However, the interaction between line affiliation and ambient temperature was not significant and cold exposure did not result in immunosuppression in either line. At 23 °C the animals of both lines seemed to cover the costs of immune response by increasing food consumption and digestive efficiency. This was not observed at 5 °C, so these costs must have been covered at the expense of other components of the energy budget. Cold exposure itself elicited a considerable increase in food intake and the mass of internal organs, which were also heavier in H-BMR than in L-BMR mice. However, irrespective of the temperature or line affiliation, immunized mice had smaller intestines, while cold-exposed immunized mice had smaller hearts. Furthermore, the observed larger mass of the liver and kidneys in immunized mice of both lines kept at 23 °C was not observed at 5 °C. Hence, immunization compromised upregulation of the function of metabolically active internal organs, essential for meeting the energetic demands of cold. We conclude that the difficulties with a straightforward demonstration of the energetic costs of immune responses in these animals stem from the extreme flexibility of their energy budgets.

Keywords: basal rate of metabolism; artificial selection; laboratory mice; costs of immune response; organ masses; cold exposure

1. INTRODUCTION

Animals continuously face limitations of resources and the need to balance conflicting demands. One of the most intensively studied trade-offs has been recently the one involving immune function (Norris & Evans 2000). Life-history theory predicts that any significant increase of the investment in immunity is likely to compromise other vital processes contributing to the animal's fitness (Stearns 1992). The costs of immune function have most often been studied in terms of life-history components, such as reproductive output or behavioural traits (Norris & Evans 2000). The physiological processes underlying these costs, most notably energy expenditures generally, have been treated as a 'black box'. Surprisingly, studies aimed at demonstrating physiological trade-offs related to immune response have yielded conflicting results (Lochmiller & Deerenberg 2000). In particular, unequivocal demonstration of the energetic costs of mounting an immune response has proved difficult both in birds and mammals. For example, blue tits (*Parus caeruleus*) challenged with a non-pathogenic antigen did not increase their energy expenditures (Svensson *et al.* 1998). Such an increase in basal metabolic rate (BMR) following immune challenge was reported in wintering, free-living male great tits (*Parus major*) (Ots *et al.* 2001). Likewise, immune challenge elevated oxygen consumption in laboratory mice (Demas *et al.* 1997). However, contrary to expectations, lymphocyte-deficient mice showed higher BMR than mice with a fully functional immune system (Råberg *et al.* 2002).

One of the most likely reasons for the elusiveness of the energetic costs of immune response is the low repeatability of energy expenditures of individual animals in the wild (Berteaux *et al.* 1996; Potti *et al.* 1999). Hence, statistical analysis of variation in their energy budgets may lack sufficient power to detect such costs. This especially applies to the costs reflected in changes of BMR, which alone constitute only 25–30% of the daily energy expenditure in wild animals (Ricklefs *et al.* 1996). To circumvent the problems related to the non-repeatability of energy expenditures, we studied the immune responsiveness of two lines of laboratory mice divergently selected for high (H-BMR) and low BMR (L-BMR). The selected lines differ not only with respect to BMR but also with respect to maximum energy expenditure, which determines the metabolic limits of their energy budgets (Książek *et al.* 2003). They also differ in the relative sizes of metabolically active internal organs, such as the small intestines, kidneys and heart, which substantially contribute to repeatable, between-line variation in the BMR. Thus, we expected those genetically determined differences in both BMR and

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total energy expenditure between the selected lines to facilitate detection of the energetic costs of immune response.

A robust test of the significance of energetic trade-offs in upregulating the immune system can only be carried out under conditions that warrant exhaustion of the ability to increase energy acquisition. Otherwise, increased rates of energy acquisition rather than its reallocation from other essential components of energy budget (Van Noordwijk & de Jong 1986) may fuel the extra costs evoked by immune challenge. For this reason, we burdened the mice from the two selected lines not only with immune stress but also sudden low ambient temperature. This created a situation demanding a trade-off between immune response and cold acclimation. We predicted that the immune response of cold-exposed mice selected for H-BMRs should be particularly suppressed, because those mice are already burdened with the high energetic costs of maintenance of their enlarged internal organs. Alternatively, however, one might expect the cold-elicited energetic demands related to upregulation of metabolic machinery to be especially significant in mice characterized by L-BMR. If so, the high costs of rebuilding their metabolic machinery to meet high energetic demands of thermoregulation may cause reallocation of resources from the immune function, resulting in immunosuppression. Thus, to understand the physiological costs of the immune function it seems useful to follow the changes in the physiological parameters of mice subjected to experimental immune challenge and cold exposure. We measured daily food consumption, digestive efficiency and changes in the size of immunocompetent organs and internal organs involved in energy processing.

2. MATERIAL AND METHODS

(a) *Animals and their maintenance*

We selectively bred 15 generations of Swiss Webster mice to achieve two groups characterized by extremely high BMR or low BMR. This breeding regime was performed at the Institute of Biology, University of Bialystok, Poland. Briefly, the BMR of outbred 12-week-old Swiss Webster mice was measured for 3 h in an open-circuit respirometry system. Males and females characterized by the highest and lowest mass-specific BMR (ml $O_2 h^{-1}$) were chosen as progenitors of the H-BMR and the L-BMR line, respectively. A similar procedure was repeated in subsequent offspring generations, yielding a significant, genetically based differentiation of both lines with respect to BMR (Książek *et al.* 2003).

The experiment was conducted on 93 males (16–18 weeks old, 30–42 g body mass), following the BMR measurements, that formed a part of the divergent selection procedure. Subjects were randomly chosen from the pool of animals not qualified as progenitors. The body-mass-corrected BMRs of L-BMR and H-BMR mice were significantly different (51 ± 0.3 and 59 ± 0.4; ANCOVA, $F_{1,84} = 38.20$, $p < 0.0001$). A week before the experiment all animals were placed in individual plastic cages with an elevated bottom but no bedding material, on a 12 : 12 hour light schedule and ambient temperature of 23 °C. Mice had free access to food (murine laboratory chow, Łomna-Las, Poland) and water. All mice were weighed daily to the nearest 0.1 g.

(b) *Experimental procedures*

H-BMR and L-BMR mice were either immunized or assigned to non-immunized control groups. Mice from the immunized groups were injected intraperitoneally with 20 $\mu l\, g^{-1}$ body mass of a standard non-pathogenic antigen-sterile sheep red blood cells (SRBCs, Polish–American Institute of Pediatrics, Kraków, Poland) suspended in phosphate-buffered saline and adjusted to 6×10^6 cells mm^{-3}. Control groups were injected with an equal amount of sterile phosphate-buffered saline (PBS). Following injection the mice were assigned to two temperature treatments. They either were kept at 23 °C or were immediately exposed to ambient cold at 5 °C. Thus, we applied a $2 \times 2 \times 2$ factorial experimental design, with immunization, line affiliation and ambient temperature as the main factors.

(c) *Food intake, digestibility and body mass*

The development of a maximum immune response to SRBC in mice takes 6 days on average (Hudson & Hay 1989). This coincides with a time-course of reaching new steady-state values of body mass, food consumption and digestibility after a sudden change in ambient temperature (Toloza *et al.* 1991). We monitored these parameters for 6 days following SRBC injection and/or cold exposure. This allowed us to analyse the effect of immunization on food utilization.

Food remains (orts) and faeces dropping to the bottom of the cage were separated from each other, dried in an oven at 70 °C, and weighed to the nearest 0.001 g. Daily food intake was calculated individually for each mouse as the mass of food disappearing from the food dispenser that day minus orts (Konarzewski & Diamond 1994). Digestibility was calculated as the difference between food intake and faecal output, divided by food intake.

(d) *Haemagglutination*

Six days after injection the mice were killed by cervical dislocation. Blood was collected from the heart with a heparinized Pasteur pipette, and centrifuged (2500 r.p.m. for 15 min). The blood plasma was extracted and heat-inactivated (56 °C) for 30 min. Antibody production was assessed by a microhaemagglutination procedure, in which the number of titres showing positive haemagglutination represented antibody production (Hudson & Hay 1989). Titres refer to $\log_2$ antibody concentrations.

(e) *Morphometrics*

Following collection of blood samples the lymphatic organs (inguinal nodes and spleen) and metabolically active internal organs (small intestine, liver, kidney and heart) were excised, cleared of blood, foodstuffs and adherent fat, and weighed to an accuracy of 0.001 g.

(f) *Statistics*

Differences between experimental groups were tested with ANCOVA, with line affiliation, temperature and treatment as the main effects and body mass as the covariate. Two persons dissected the metabolically active internal organs, and a significant effect of dissecting person was revealed, so the effect of dissector was also incorporated in the relevant ANCOVAs. In several analyses the effect of temperature was so overwhelming that it made between-factor interactions difficult to detect. In view of this, we additionally carried out separate analyses within each temperature. Body mass, daily food consumption and faecal output were analysed with repeated-measures ANOVA.

Table 1. Summary of repeated-measures ANOVA of the effect of immunization, ambient temperature, line affiliation and time-course of the experiment on daily changes of mouse body mass, food consumption and digestibility.

	body mass		food consumption		digestibility	
effect	$F_{\text{d.f.}}$	p	$F_{\text{d.f.}}$	p	$F_{\text{d.f.}}$	p
between subjects						
immunization	$1.55_{1,82}$	0.2	$11.0_{1,81}$	< 0.001	$23.7_{1,81}$	< 0.001
ambient temperature	$3.84_{1,82}$	0.05	$606.4_{1,81}$	< 0.0001	$38.7_{1,81}$	< 0.0001
line affiliation	$0.39_{1,82}$	0.5	$12.8_{1,81}$	< 0.001	$0.6_{1,81}$	0.4
immunization × temperature	$0.07_{1,82}$	0.8	$2.1_{1,81}$	0.15	$6.6_{1,81}$	0.01
immunization × line affiliation	$1.5_{1,82}$	0.2	$0.1_{1,81}$	0.7	$0.4_{1,81}$	0.5
temperature × line affiliation	$0.05_{1,82}$	0.8	$1.2_{1,81}$	0.3	$0.2_{1,81}$	0.7
within subjects						
time	$2.14_{5,78}$	0.07	$54.4_{5,77}$	< 0.0001	$1.37_{5,77}$	0.2
time × immunization	$5.65_{5,78}$	< 0.001	$2.95_{5,77}$	0.02	$0.20_{5,77}$	0.9
time × temperature	$0.65_{5,78}$	0.7	$19.58_{5,77}$	< 0.0001	$2.33_{5,77}$	0.04
time × line affiliation	$0.47_{5,78}$	0.8	$0.61_{5,77}$	0.7	$1.25_{5,77}$	0.3

Daily food consumption was first regressed on the body mass at a given day, to remove its effect, and the residuals obtained were then analysed.

Least-square means from ANCOVA analysis ± s.e. are reported throughout the paper. Before statistical analyses were run, the assumptions of parametric tests were assured (Sokal & Rohlf 1995). If not stated otherwise, significance was tested at $p = 0.05$.

3. RESULTS

(a) *Body mass, food consumption and digestibility*

Neither immunization nor line affiliation significantly affected changes in body mass, while the effect of temperature appeared significant (table 1). The interactions between the factors were not significant. However, there was a significant interaction between time-course and the effect of immunization, most probably resulting from a temporary decrease of body mass of immunized mice (table 1, figure 1*a*,*b*).

Food consumption was affected primarily by ambient temperature (table 1). In the course of the whole experiment, cold-exposed mice consumed considerably more food than mice kept at room temperature (58 ± 0.6 g over 6 days at 5 °C versus 40.7 ± 0.6 g over 6 days at 23 °C). It was also significantly affected by immunization (table 1). Separate comparisons of food consumption between immunized and non-immunized mice within each ambient temperature revealed that immunization elicited elevation of food consumption at 23 °C (figure 1*c*, $F_{1,41} = 17.5$, $p < 0.001$) but not at 5 °C (figure 1*b*, $F_{1,39} = 0.7$, $p > 0.4$). Food consumption increased significantly with time from the start of the experiment (table 1). This increase was affected by immunization and differed between temperatures, as indicated by the significant time × immunization and time × temperature interactions (table 1). Separate analyses within each temperature revealed that immunized H-BMR mice consumed significantly more food than immunized L-BMR mice at 23 °C ($F_{1,41} = 18.8$, $p < 0.001$) but not at 5 °C ($F_{1,39} = 0.7$, $p > 0.4$).

Surprisingly, immunization significantly affected food digestibility (table 1). The effect of immunization interacted with ambient temperature (table 1). Separate ANOVAs within each temperature revealed that the effect of immunization was significant at 23 °C ($F_{1,41} = 22.4$, $p < 0.001$, figure 1*e*) but not at 5 °C ($F_{1,39} = 3.5$, $p < 0.07$, figure 1*f*). Irrespective of the effect of immunization, cold exposure reduced digestive efficiency (table 1, compare figure 1*e*,*f*), while line affiliation did not affect it (table 1).

(b) *Immune response and mass of lymphatic organs*

The immune response to SRBC, measured as the number of titres in the haemagglutination test, was not affected by ambient temperature (ANOVA, $F_{1,42} = 0.34$, $p = 0.56$), but differed between lines. It was significantly higher in L-BMR than H-BMR mice and averaged 7.5 ± 0.4 and 6.2 ± 0.4, respectively (ANOVA, $F_{1,42} = 4.30$, $p = 0.04$; figure 2*a*). However, the expected interaction between temperature and BMR line appeared non-significant (ANOVA, $F_{1,42} = 0.09$, $p = 0.8$).

Spleen and inguinal nodes were significantly larger in immunized mice than in control animals (table 2). When the analyses were restricted to immunized mice only, L-BMR and H-BMR mice did not differ in spleen mass and inguinal nodes. Spleen mass was significantly higher in cold-exposed mice (ANCOVA, $F_{1,40} = 8.42$, $p = 0.006$, figure 2*b*), while inguinal nodes were not affected by temperature (ANCOVA, $F_{1,40} = 0.08$, $p = 0.7$, figure 2*c*). Interestingly, the difference in spleen mass between groups exposed to 5 °C or 23 °C was larger in H-BMR than in L-BMR mice, as indicated by the significant interaction between temperature and line affiliation (ANCOVA, $F_{1,40} = 4.61$, $p = 0.03$, figure 2*b*).

(c) *Metabolically active internal organs*

Immunization significantly affected the size of the small intestines, heart and liver (table 2). However, the masses of metabolically active internal organs changed in a temperature-dependent manner (figure 3). The livers and kidneys of immunized animals were larger at 23 °C but not at 5 °C (figure 3*a*,*b*). This was indicated both by the significant immunization × temperature interaction in four-way ANCOVA (table 2) and by three-way ANCOVAs

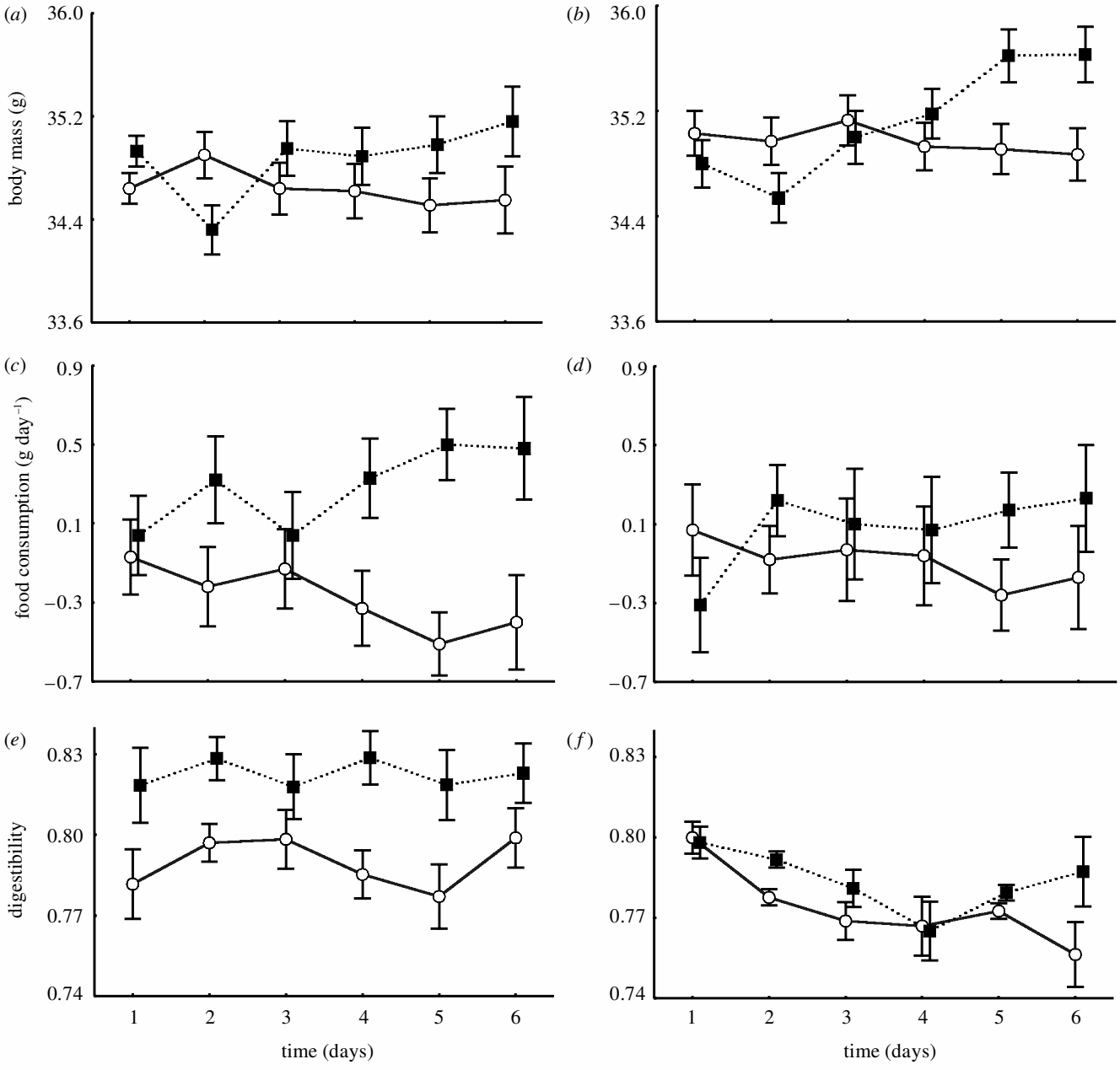

Figure 1. The effect of immunization on changes in body mass (*a*,*b*), food consumption (*c*,*d*) and food digestibility (*e*,*f*) under exposure to (*a*,*c*,*e*) 23 °C or (*b*,*d*,*f*) 5 °C. Food consumption is presented as least-square means from repeated-measures ANOVA and corrected for the effect of line affiliation and differences in body mass in consecutive days of the experiment. Circles, control; squares, SRBC. In this and subsequent graphs error bars denote ± s.e.

performed separately within each temperature (liver: $F_{1,47} = 23.8$, $p < 0.0001$ at 23 °C and $F_{1,44} = 0.03$, $p > 0.9$ at 5 °C; kidney: $F_{1,47} = 6.0$, $p = 0.02$ at 23 °C and $F_{1,44} = 1.1$, $p = 0.3$ at 5 °C). By contrast, the mass of the heart of immunized mice was lower in the cold (three-way ANCOVA; $F_{1,47} = 2.3$, $p = 0.1$ at 23 °C and $F_{1,44} = 5.65$, $p = 0.02$ at 5 °C, figure 3*d*). The small intestines of immunized and non-immunized mice were not affected by temperature.

All internal organs were significantly larger in cold-exposed mice, and independently these organs were significantly larger in H-BMR than in L-BMR mice (table 2). The interactions between line affiliation and ambient temperature or immunization were not significant.

4. DISCUSSION

We expected that mice divergently selected for L-BMR and H-BMR should differ in their response to SRBC, if mounting an immune response entails metabolic costs. We also expected that because mice maintained at room temperature might not be energetically limited, this difference should be more pronounced in the cold. Irrespective of the ambient temperature, H-BMR mice showed significantly lower antibody production, while the expected interaction between line affiliation and temperature treatment was not significant (figure 2*a*).

The lack of more distinct immunosuppression of H-BMR mice in the cold is surprising. We suggest that this

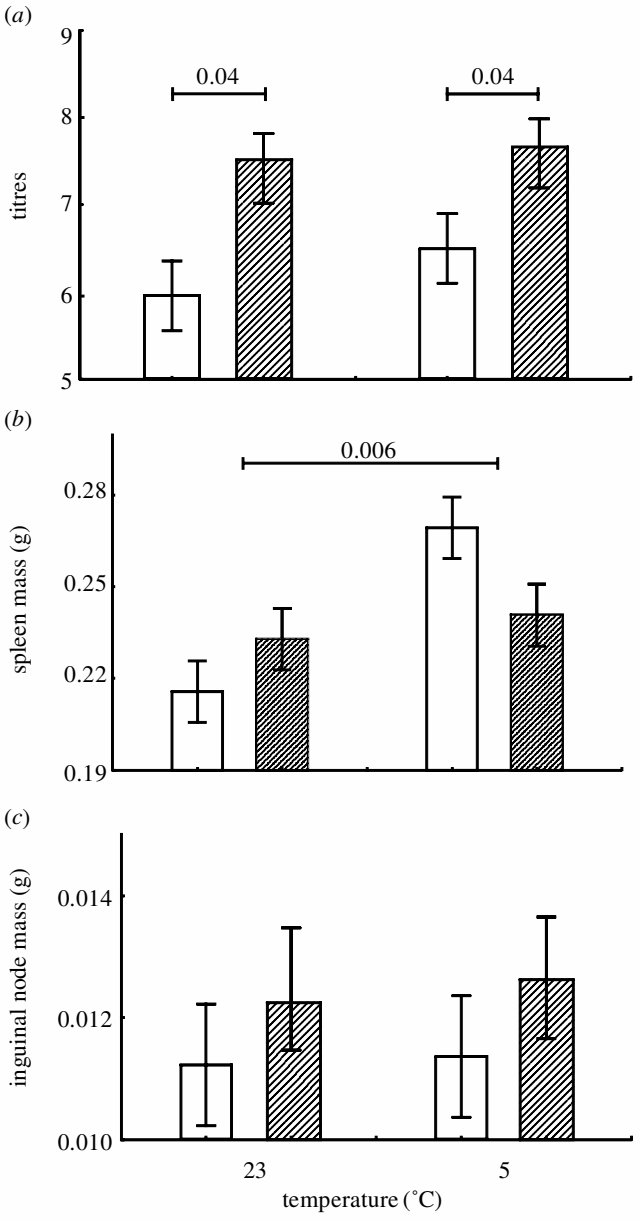

Figure 2. The effect of cold exposure and line affiliation on the immune response to (*a*) SRBC, (*b*) mass of spleen and (*c*) inguinal nodes. Values are least-square means from ANCOVA. Only data for immunized mice are presented. No shading, H-BMR; shading, L-BMR.

may stem from the animals' ability to cover the costs of combating the immune challenge from other components of the resource budget, rather than from the insignificance of those costs. At room temperature, mice of both lines covered them by increased food consumption and digestive efficiency (figure 1*c*,*e*), although the food consumption of L-BMR mice was lower than that of H-BMR animals. By contrast, there was no effect of immunization and line affiliation on food intake and digestibility at 5 °C. It is important to note here that cold exposure alone resulted in a substantial and sudden increase of food consumption and marked reduction of digestive efficiency (table 1, figure 1*e*,*f*). Cold-exposed immunized mice of both lines were therefore close to exhausting the digestive capacity of the gut. For this reason, unlike immunized mice maintained at 23 °C, cold-exposed animals were not

Table 2. The effect of immunization, temperature and line affiliation on lymphatic and metabolically active internal organs.
(Values are least-squares means (± s.e.) from ANCOVA corrected for the remaining two main effects and the effect of dissector and body mass.)

	experimental treatment											
	immunization				temperature				line affiliation			
	SRBC	control	$F_{d.f.}$	p	23 °C	5 °C	$F_{d.f.}$	p	H-BMR	L-BMR	$F_{d.f.}$	p
spleen	0.24 ± 0.006	0.16 ± 0.006	$83.31_{1,84}$	< 0.0001	0.19 ± 0.006	0.21 ± 0.006	$5.12_{1,84}$	0.02	0.20 ± 0.006	0.20 ± 0.006	$0.08_{1,84}$	0.7
inguinal nodes	0.012 ± 0.0005	0.007 ± 0.0005	$40.27_{1,84}$	< 0.0001	0.01 ± 0.0005	0.01 ± 0.0005	$0.03_{1,84}$	0.8	0.009 ± 0.0005	0.009 ± 0.0005	$0.95_{1,84}$	0.3
small intestine	1.18 ± 0.03	1.28 ± 0.03	$4.75_{1,81}$	0.03	1.1 ± 0.03	1.35 ± 0.03	$31.31_{1,81}$	< 0.0001	1.28 ± 0.03	1.17 ± 0.03	$5.92_{1,81}$	0.01
liver[a]	1.76 ± 0.02	1.66 ± 0.02	$11.44_{1,81}$	0.001	1.61 ± 0.02	1.81 ± 0.02	$40.34_{1,81}$	< 0.0001	1.75 ± 0.02	1.68 ± 0.02	$4.78_{1,81}$	0.03
heart	0.18 ± 0.002	0.19 ± 0.002	$7.28_{1,81}$	0.008	0.16 ± 0.002	0.2 ± 0.002	$127.66_{1,81}$	< 0.0001	0.19 ± 0.002	0.18 ± 0.002	$17.27_{1,81}$	< 0.0001
kidneys[b]	0.60 ± 0.007	0.59 ± 0.007	$0.78_{1,81}$	0.3	0.56 ± 0.007	0.64 ± 0.007	$58.59_{1,81}$	< 0.0001	0.61 ± 0.007	0.58 ± 0.007	$8.18_{1,81}$	0.005

[a] Significant interaction between immunization and ambient temperature ($F_{1,81} = 9.53$, $p = 0.003$).
[b] Significant interaction between immunization and ambient temperature ($F_{1,81} = 6.92$, $p = 0.01$).

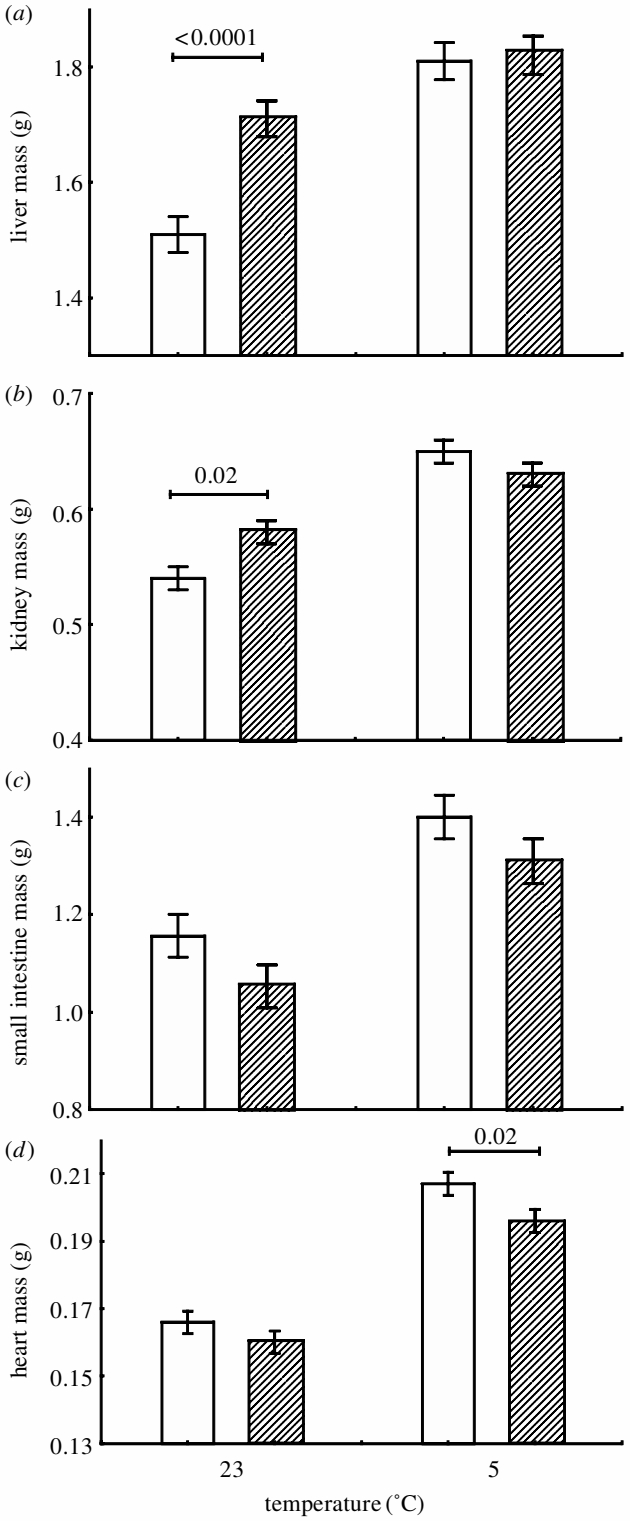

Figure 3. The effect of immunization on the mass of metabolically active internal organs: (*a*) liver, (*b*) kidneys, (*c*) small intestines and (*d*) heart. Values are least-squares means from ANCOVA within each temperature, corrected for the effect of line affiliation, body mass and dissector. No shading, control; shading, SRBC.

able to cover the costs of immune response by increasing food consumption over that required to cover heat loss. This suggests that the immune response was fuelled at the expense of other components of energy budget. To our knowledge, the present study is the first to demonstrate such effects of immune challenge on energy allocation.

The observed changes in the sizes of metabolically active internal organs provide another hint of the nature of the costs underlying the immune response. Internal organs were generally heavier in H-BMR mice (table 2). However, irrespective of line affiliation, immunized mice had significantly larger liver and kidneys at 23 °C. As these organs are involved primarily in processing acquired resources and managing waste products, their enlargement most probably reflected an increased rate of protein metabolism (Hammond & Janes 1998) associated with the costs of mounting an immune response. We did not detect a similar enlargement of liver and kidneys in immunized mice exposed to cold. Therefore the observed increase of the mass of these organs can be attributed solely to cold exposure (table 2, see also Toloza *et al.* 1991; Cichoń *et al.* 2002). The lack of significant immunosuppression in cold-exposed mice suggests that the liver and kidney functions associated with the immune response remained unaffected and were most probably covered at the expense of their functions related to heat production. Likewise, irrespective of ambient temperature, immune challenge resulted in a significant reduction of the mass of the intestines (table 2), one of the most energetically expensive internal organs (Konarzewski & Diamond 1994). Heart mass was not affected by immune challenge at 23 °C but it was significantly lower in SRBC-treated mice at 5 °C than in non-immune-challenged cold-exposed animals (figure 3*d*). This means that the load of immune stress slowed the rate of increase of heart mass to the level elicited by cold stress itself. These results suggest that the need for enlargement of internal organs to fuel thermoregulatory demands may interfere with the immune response.

Mice would not be able to cope with the metabolic stress elicited by immunization and sudden transfer to the cold without recourse to the excessive, usually unused physiological capacities built into their metabolically active internal organs. Such excess capacities form so-called safety margins (defined as the ratio of maximum capacity to average load), that typically range from 2 for the kidneys and intestines to 10 for the pancreas (Diamond 1993). They serve to tide over sudden and unpredictable metabolic challenges such as those simulated in our experiment. Maintenance of such safety margins may be crucial for survival, as physiological adaptation to a new environmental condition is not immediate and requires expensive upregulation of the metabolic machinery (Secor & Diamond 1998). Thus, if a metabolic stress such as immune challenge can be absorbed within the safety margins, the organism can still maintain a positive energy balance.

As we showed in an earlier paper, only long-term exposure to cold preceding immune challenge results in immunosuppression (Cichoń *et al.* 2002). This suggests that resource reallocation from the immune function occurs only when the environmental stress is perceived by an animal to be permanent. Otherwise the immune response is prioritized over the costs of adaptation to the cold, and covered primarily by utilization of the safety margins. Adaptation to cold is then postponed until the antigen is recognized and the immune response starts to develop. This seems likely in the light of our results: the observed increase in the sizes of metabolically active

organs in immunized, cold-exposed mice was lower than that in cold-exposed but not immunized animals.

In conclusion, we showed that H-BMR mice had lower immune response than L-BMR mice. However, cold exposure was not immunosuppressive. Moreover, the immune response of H-BMR and L-BMR did not differ much when the mice were simultaneously exposed to an additional metabolic threat—low ambient temperature. Our data on the changes in physiological parameters after immunization suggest that the difficulty in demonstrating the energetic costs of mounting an immune response may not lie in their non-significance. Rather, it stems from the animals' apparent ability to prioritize the most threatening metabolic challenge, and to combat it at the expense of other components of the energy budget. We suggest that this fascinating metabolic flexibility of organisms is what has made the energetic costs of an immune response elusive in many previous studies on the costs of the immune function.

P. Koteja, J. Kozłowski and J. Taylor provided helpful comments and discussion. M. Jacobs helped edit the manuscript. All procedures described in this paper were conducted under a permit from the Ethical Committee (permit no. 2001/2). Financial support was provided by the Committee for Scientific Research of the Republic of Poland to A.K. (grant no. 3 PO4F 014 22).

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.