Delayed effects of cold stress on immune response in laboratory mice

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This study was undertaken to examine the trade-off between the cost of thermoregulation and immune function in laboratory mice. Mice were maintained either at 23 °C or cold exposed at 5 °C for 10 days. Then, they were immunized with sheep red blood cells. Thus, the cold-exposed mice had either experienced or not experienced cold stress prior to immunization. Cold stress elicited a substantial increase in food intake, accompanied by a significant reduction in food digestibility. An increase in mass of metabolically active internal organs (small intestines, heart and kidney) was observed in cold-exposed mice. These findings reassured us that costs of increased thermoregulation caused by cold stress were substantial. The immune response of mice exposed to long-lasting cold stress was significantly lower, but immune response was not affected in short-exposed mice. Differences in immune response between experimental groups accompanied changes in mass of immunocompetent organs (thymus and spleen). Our findings indicate that studies of trade-offs should account for the fact that resource reallocation in response to an environmental challenge may not be immediate. In fact, resource reallocation may be postponed until the new environmental state becomes permanent or until an organism attains physiological adaptation to the current conditions.

Keywords: immunocompetence; thermal stress; trade-offs; resource allocation

1. INTRODUCTION

A common assumption in life-history theory is that a trade-off exists between the costs and benefits of various physiological functions. This assumption is generally supported by empirical studies (e.g. Stearns 1992).

Recently, the vertebrate immune system has become one of the most intensively studied components of life-history strategy, as it can compete for nutrient demands with various other functions including growth, reproduction, thermoregulation and work. Pathogens might be very harmful to their hosts by reducing the amount of acquired resources, which can lead to decreased survival. Thus, investments in defence mechanisms seem to be crucial for maximizing fitness. Although it is intuitively clear that immune functions should be costly since mounting an immune response requires resources that could otherwise be allocated to other physiological functions, little is known about the costs of immune defence (Lochmiller & Deerenberg 2000). There is evidence from several studies of birds that increased work and reproduction reduce the experimentally induced immune response (Deerenberg *et al.* 1997; Nordling 1998; Cichoń *et al.* 2001). Mounting the immune response against an injected non-pathogenic antigen in adult birds attending their nestlings was also found to negatively affect their feeding rate in the blue tit (*Parus caeruleus*; Råberg *et al.* 2000) and body mass and survival of nestlings in the pied flycatcher (*Ficedula hypoleuca*; Imonen *et al.* 2000). Increased energetic demands elicited

by an immune response were suggested in chickens immunized with sheep red blood cells (SRBC), as they consumed more food after immunization (Henken & Brandsma 1982). By contrast, Svensson *et al.* (1998) did not find any significant elevation in energy expenditure after an antigenic challenge in blue tits. However, the elevation of energy expenditure has been shown in mice with an ongoing immune response against keyhole limpet haemocyanin antigen (Demas *et al.* 1997).

In homeotherms, thermoregulation can constitute an important energy-demanding constraint to immune function. The energetic demands of thermoregulation cannot be met by simple reallocation of resources from other functions, but require a substantial increase in energy expenditure and rescaling of the whole metabolic machinery. In laboratory mice, for example, a decrease in temperature from 23 to 8 °C elicits an almost twofold increase in food intake, a one-and-a-half-fold increase in resting metabolic rate and a 30–70% increase in the mass of metabolically active internal organs (Konarzewski & Diamond 1994). Thus, thermal stress is a perfect means by which constraints to immune functions due to a trade-off over limited resources can be studied. The negative effect of cold stress on the immune response to two novel antigens has been found in blue tits (Svensson *et al.* 1998). However, such a reduction in immunocompetence was not found in a study on bobwhite birds (*Colinus virginianus*), in which individuals were exposed either to cold or heat (Dabbert *et al.* 1997). The contradiction between these two studies calls for further experimental work on the effects of increased costs of thermoregulation on immune function.

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Table 1. Metabolically active internal organ mass in mice immunized with SRBC.

(Mice were defined as H, H/L or L (see § 2). Least-squares means with standard errors are presented separately for each group. All variables were tested by means of analysis of covariance with body mass as a covariate. Differences between experimental groups are significant at $p = 0.01$ (after Tukey post-hoc test) if characters given in superscript are different.)

	experimental group			$F_{d.f.}$	p
	H	H/L	L		
small intestine	1.14 ± 0.051^a	1.46 ± 0.042^b	1.56 ± 0.043^b	23.88 _{4,29}	< 0.001
liver	1.39 ± 0.034^a	1.29 ± 0.027^b	1.42 ± 0.028^a	52.80 _{4,29}	< 0.001
kidney	0.38 ± 0.014^a	0.45 ± 0.011^b	0.49 ± 0.011^b	24.29 _{4,29}	< 0.001
heart	0.12 ± 0.004^a	0.15 ± 0.003^b	0.16 ± 0.003^b	29.66 _{4,29}	< 0.001

Here, we studied the effect of cold exposure on laboratory mice and their immune response to a novel antigen, SRBC. In contrast to other studies (e.g. Dabbert *et al.* 1997), we directly measured the effect of cold on food consumption and mass of metabolically active internal organs. This ensured that the thermal stress applied had challenged the energy budget of the mice and elicited costs of adaptation to low temperature, which constituted a trade-off with immune function. As adaptation to cold requires time-consuming changes in physiology (Toloz *et al.* 1991), we predicted that cold exposure would have a negative effect on immune response and that this response would vary with the duration of exposure. Therefore, in contrast to the earlier-mentioned studies, we introduced an additional experimental group in which animals were exposed to cold for 10 days prior to immunization. This allowed us to test whether immunocompetence of cold-adapted mice differed from that of mice still undergoing adaptation to cold. We expected immune response to be suppressed in cold-adapted mice, as they had already tuned their allocation strategy to meet high thermoregulatory demands. Alternatively, one would expect mice undergoing the adaptation process to show low immunocompetence, as they had not had time to tune their physiology and were experiencing a negative energy balance.

2. METHODS

(a) *Animals and their maintenance*

The experiment was conducted on 34 male Swiss mice (10–12 weeks old, 22–27 g body mass) that were purchased from a licensed dealer (Breeding of Laboratory Animals, Warszawa, Poland). Mice were housed individually in plastic cages with an elevated bottom but no bedding material, in a room on a 12 L : 12 D cycle. Mice had free access to food (murine laboratory food, Łomna-Las, Poland) and water. All mice were weighed daily to the nearest 0.1 g.

Mice were randomly assigned to three experimental groups differing in the temperature and duration of thermal exposure and maintained in separate, but otherwise identical climatic chambers. After acclimatization to the laboratory conditions (21 days at 23 °C), one group of 11 mice (H mice) were maintained for the whole experimental period (16 days) at room temperature (23 °C). Another group of 12 mice (L mice) was kept in a climatic chamber at 5 °C for the whole experimental period. The third group of 11 mice was kept at 23 °C for 10 days, then transferred to the climatic chamber set at 5 °C (H/L mice). On the 10th day of the experiment, mice of all groups were immunized

with SRBC, as described below. A temperature of 5 °C is regarded as inducing of cold stress, as the most substantial increase in food consumption is associated with a decrease in the temperature from 23 to 8 °C in Swiss-Webster mice (Konarzewski & Diamond 1994). The development of a maximum immune response to SRBC usually takes 6 days (Hudson & Hay 1989) and this coincides with the mice reaching new steady-state values of body mass, gut mass and food digestibility after a change in ambient temperature (Toloz *et al.* 1991).

(b) *Food intake and digestibility*

Food remains (orts) and faeces that had dropped to the bottom of the cage were collected daily. They 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 mass. An apparent dry-matter digestibility (uncorrected for possible contributions of gut bacteria and shed protein to faecal mass) was calculated as the difference between daily food intake and a daily faecal output divided by food intake. As the retention time of digesta in the mouse's guts may exceed 24 h, to avoid pseudoreplication, both food intake and an apparent digestibility were analysed and presented for 2-day periods.

(c) *Immunization*

All animals were injected intraperitoneally with 20 µl g⁻¹ body mass of standard non-pathogenic antigen-sterile SRBC (Polish-American Institute of Pediatrics, Kraków, Poland) suspended in phosphate-buffered saline and adjusted to 6×10^6 cells mm⁻³. Six days after immunization, mice were killed by cervical dislocation. Blood was collected from the heart using a heparinized Pasteur pipette. Blood was centrifuged at 2500 rpm for 15 min, after which the plasma was extracted and heat-inactivated (at 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 log₂ antibody concentrations.

(d) *Morphometrics*

Following collection of the blood samples, the lymphatic organs (thymus, spleen) and metabolically active internal organs (small intestine, liver, kidney, heart) were excised after removing adherent fat. Freshly excised organs were weighed with accuracy to 0.001 g.

Before performing statistical analyses, assumptions of parametric tests were assured (Sokal & Rohlf 1995). Differences

between experimental groups were analysed with one-way ANCOVA with body mass as a covariate, because food consumption and mass of internal organs are related to body mass. Two persons dissected metabolically active internal organs and a significant effect of the person dissecting was revealed. Thus, we also accounted for the effect of dissector in the relevant ANCOVAs.

3. RESULTS

(a) Body mass, food consumption and digestibility

Mice from different thermal treatments did not differ in body mass at the start of the experiment (ANOVA; $F_{2,31} = 1.52$, $p = 0.23$). However, after 10 days, L mice had lost significantly more mass than H and H/L mice (4.0, 0 and 0.4 g, respectively; ANOVA; $F_{2,31} = 30.8$, $p < 0.001$, followed by Tukey post-hoc comparisons). Between day 10 and the conclusion of the experiment, H/L mice lost 1.8 g, while H and L mice gained 0.7 and 0.5 g, respectively (ANOVA; $F_{2,31} = 17.0$, $p < 0.0001$, followed by Tukey post-hoc comparisons).

Food consumption measured during the first 2-day period after immunization differed significantly between all experimental groups (ANOVA; $F_{3,30} = 20.96$, $p < 0.0001$; figure 1a). A similar pattern was observed for the second, consecutive 2-day period (ANOVA; $F_{3,30} = 2.2$, $p < 0.0001$; figure 1a). However, during the final 2-day period, food consumption of H/L mice had increased to the level observed in L mice (figure 1a). It is important to note that differences in food consumption were inversely related to differences in apparent digestibility (figure 1b).

(b) Organ masses

Except for the liver, wet mass of the internal organs (small intestine, kidneys and heart) was significantly higher in cold-exposed mice (table 1). Experimental groups differed in the thymus mass (ANOVA; $F_{3,30} = 26.99$, $p < 0.001$) and spleen mass (ANOVA; $F_{3,30} = 16.22$, $p < 0.001$). L mice had smaller thymus and spleen than mice from the other experimental groups (figure 2a,b).

(c) Immune response

L mice showed inferior immune response against SRBC compared with H or H/L mice (ANOVA; $F_{2,31} = 5.7$, $p < 0.01$, figure 2c). The post-hoc comparisons revealed a significant effect between H and L mice (Tukey test: $p < 0.01$) and the difference between H/L and L approached significance ($p = 0.055$). The immune response was positively related to spleen mass (ANOVA with spleen mass as a covariate; $\beta = 0.53$, $F_{1,30} = 11.73$, $p < 0.002$, figure 3) and the relationship differed between experimental groups, being pronounced to the largest extent in L mice (test for parallelism in ANCOVA; $F_{2,28} = 5.03$, $p < 0.01$). Such a relationship for thymus mass was not significant ($\beta = 0.27$, $F_{1,30} = 2.35$, $p = 0.14$) and there were no differences in this relationship between experimental groups ($p = 0.64$).

4. DISCUSSION

The results showed that mice exposed to cold had an inferior ability to mount a humoral immune response to

Figure 1. The effect of experimental treatment on (a) food consumption and (b) digestibility, in mice immunized with SRBC. Mice were defined as H (circles, dotted lines), L (diamonds, solid lines) or H/L (squares, dashed lines) (see § 2). Least-squares means with standard errors are presented separately for three consecutive 2-day periods from the day of immunization (1, 2 and 3). Differences between experimental groups within each 2-day period are significant at $p = 0.01$ (after Tukey post-hoc test if characters given above bars are different). Note that the analyses were performed for differences between groups separately for each 2-day period, and not between periods.

a novel non-pathogenic antigen. Thus, mice forced to expend more energy on thermoregulation had possibly fewer resources available to their immune function. We can conclude, therefore, that thermoregulation constitutes another resource demanding physiological activity, which, like physical work and reproduction, compete for limited resources with immune functions (Deerenberg *et al.* 1997; Nordling 1998; Cichoń *et al.* 2001). Interestingly, we found that the suppression of the immune response was elicited only by long, 16-day exposure to cold and was absent following shorter, (6-day) cold stress. This is an important finding because it indicates that the apparent trade-off between immune function and thermoregulation may not be based on immediate resource partitioning but may occur only later when the new environmental state becomes permanent. Possibly, the immune challenge imposed simultaneously with cold stress (as in H/L mice) may still activate the immune system, as the animals have not yet adapted to the new environment. Moreover, a reduction in immune function should not be possible until ongoing infection is exterminated. Alternatively, the trade-off between the immune function and thermoregulation

Figure 2. The effect of experimental treatment on (a) thymus, (b) spleen and (c) level of antibodies in mice immunized with SRBC. Mice were defined as H, H/L or L (see § 2). Least-squares means with standard errors are presented separately for each experimental group. Differences between experimental groups are significant at $p = 0.01$ (after Tukey post-hoc test) if characters above bars are different.

may come into play only after the animals have depleted their current nutrient reserves.

Our data show that increased heat loss resulted in a substantial decrease in body mass, increase in food consumption and upregulation of the function of internal organs involved in its processing (figure 1; table 1; see also Konarzewski & Diamond 1994; McDevitt & Speakman 1994; Koteja 1996). It usually takes several days to attain new steady-state values of gut and body mass after transferring mice to cold (Tolozza *et al.* 1991). At the very beginning of cold exposure, mice rely on fat stores and unutilized digestive capacity of the gut (the so-called digestive safety margin) to balance heat loss and accommodate increased food intake (Tolozza *et al.* 1991). Exhaustion of fat reserves and the digestive safety margin triggers hypertrophy of the gut, in anticipation of prolonged energy stress. Thus, an

Figure 3. The relationship between spleen mass and immune response to SRBC in mice experiencing different temperatures. Mice were defined as H (circles, dashed line), H/L (squares, dotted line) or L (diamonds, solid line) (see § 2). The correlation coefficients calculated separately for each group are as follows; L: $r = 0.77$, $n = 11$, $p = 0.005$, H/L: $r = 0.41$, $n = 12$, $p = 0.18$, H: $r = 0.32$, $n = 11$, $p = 0.34$. Lines on the graph are least-square regressions shown to visualize the relationships.

immediate resource reallocation resulting in a trade-off may not take place while mice rely on this safety margin. Therefore, only L mice may have traded the immune response for the costs of upregulation and maintenance of increased metabolic machinery, as indicated by low immune responsiveness of L mice (figure 2).

The effect of thermal stress on immune function has already been studied. For example, Svensson *et al.* (1998) showed that the immune response of blue tits exposed to cold was suppressed. However, in a study on bobwhites, Dabbert *et al.* (1997) failed to detect any effect of cold or heat exposure on immune response. In neither of these studies has the effect of the duration of cold stress been considered. Furthermore, mere application of cold exposure does not guarantee that thermal stress is strong enough to induce a trade-off between immune function and physiological adaptations to increased heat loss. For example, cold-exposed mice can partly buffer the effect of low temperatures by behavioural adaptations: they stay motionless for most of the time, with their tails curled beneath the body and with their eyes closed (Konarzewski & Diamond 1994). Clearly, before reaching any conclusion relating to the effect of the applied stressor on immune function, it is therefore essential to show that the stressor significantly challenged the animal's physiology. Our experiment meets this condition. We demonstrated that food consumption of cold-stressed mice almost doubled, while their apparent digestibility had been reduced (figure 1). This, along with a reduction of body mass in L mice, indicates that their metabolic capacity to maintain a positive energy balance was close to its limit, despite a substantial increase in the mass of the internal organs involved in energy processing (table 1). Thus, it is unlikely that the resources necessary to mount an immune response were solely obtained by an increase of food consumption, but rather that mice were subject to a trade-off with thermoregulation. Furthermore, a reduced ability to mount an immune response that was observed in L, but

not in H/L mice, indicates that under prolonged cold exposure thermoregulatory mechanisms are given priority.

The exposure to cold significantly affected lymphatic organs. The antibodies are produced by B-cells, which proliferate in the spleen and lymph nodes (Roitt *et al.* 1998). Thus, the size of those organs should constrain the immune response. Indeed, the spleen was smaller in L mice that also showed a low immune responsiveness. Interestingly, spleen mass was positively correlated with the level of immune response and this relationship was pronounced to the largest extent in L mice. This clearly suggests that L mice were indeed constrained and therefore it is only in this group the trade-off between thermoregulation and immune response came into play. Antibody response to most antigens, among others SRBC, requires antigen-specific T-cell help. These antigens are unable to induce antibody response in animals in which the thymus fails to generate specific T cells (Roitt *et al.* 1998). Antigen recognition occurs shortly after antigen contraction. Thus, H/L mice were presumably still able to recognize and mount a strong immune response because they were transferred to cold while immunized and their thymus was not yet affected by cold stress (one should bear in mind that the lymphatic organs were extracted only 6 days after immunization).

It is important to point out that, like many other authors (e.g. Dabbert *et al.* 1997; Svensson *et al.* 1998), we studied only one specific part of the immune function. We lack information on whether the function of the whole immune system was affected by cold stress or whether it affected only the humoral response. As the antigens that already entered an organism should not have been left unattended, it is possible that under stressful conditions non-specific immune functions predominate and compensate for suppression of humoral immunity and that during stressful conditions there could be a trade-off between investment in acquired and innate immunity. However, in any case, the observed reduction in humoral response after cold exposure indicates that mounting an immune response is costly and that the specific response tends to be compromised when resources are limited. Our data show that only long-lasting resource limitation generates reallocation of resources resulting in a trade-off. This may have very important implications for studies of life-history strategies and trade-offs.

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