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Immune response is energetically costly in white cabbage butterfly pupae

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Parasite-driven coevolution has led hosts to develop a complicated and potentially costly defence machinery, consisting mainly of the immune system. Despite the evidence for the trade-offs between immune function and life-history traits, it is still obscure how the costs of using and maintaining the immune function are paid. We tested whether immune challenge is energetically costly for white cabbage butterfly (*Pieris brassicae* L.) diapausing pupa. Individuals challenged with nylon implant raised their standard metabolic rate nearly 8% compared to the controls. Hence, costs of activation of immune system in insect pupa can be expressed in energetic currency.

Keywords: *Pieris brassicae*; immune challenge; metabolic rate; energetic costs

1. INTRODUCTION

Parasites pose a ubiquitous threat for all living organisms. Usually evolving faster than their hosts, they exert enormous selective pressures on host defence mechanisms. This coevolutionary process has forced hosts to develop an extremely complicated protection machinery consisting primarily of the immune system. The realization that such a sophisticated machinery is not cost-free has promoted an increasing interest of animal ecologists in the potential role of immune function in the shaping of life-history patterns (Lochmiller 1996; Sheldon & Verhulst 1996). By now, ample evidence from diverse taxa has been accumulated that suggests that immune function is indeed involved in basic life-history trade-offs (reviewed by Lochmiller & Deerenberg 2000; Zuk & Stoehr 2002; Schmid-Hempel 2003). However, the question about how the costs of using an immune function are paid appears to be under continuous debate (Råberg *et al.* 1998; Klasing & Leshchinsky 1999; Lochmiller & Deerenberg 2000). In the context of evolutionary ecology, it has been traditionally assumed that costs involved in immunity–life-history trade-offs are basically energetic. Additionally, the costs of an immune response may be manifested as immunopathological reactions or option costs (Zuk & Stoehr 2002).

Despite the long tradition of immunological research in human and veterinary medicine, attempts by animal ecologists to specify and quantify the costs of an immune response have remained scarce. Few successful experiments have demonstrated the energetic cost of mounting a humoral immune response (laboratory mice, Demas *et al.* 1997; free-living great tits (*Parus major*), Ots *et al.* 2001) and only a single study has indicated the direct energetic cost of a cell-mediated immune response (captive house sparrows (*Passer domesticus*), Martin *et al.* 2003). To our knowledge no such experiments have been performed in invertebrates.

Unlike vertebrates, invertebrates, such as insects, do not possess specific acquired immunity and rely on relatively simple and non-specific defence mechanisms resembling the innate immunity of vertebrates (Vilmos & Kurucz 1998). Hence, insect models enable one to estimate the costs of innate immune systems without the potentially confounding effects of acquired immunity. Indeed, over the last few years several studies have demonstrated immunity–life-history trade-offs using insects as model systems (see Zuk & Stoehr (2002) for a review). Unfortunately, the reviewed studies do not present any evidence in which currency the cost of immunity is being paid.

A particularly promising model system for studying the energetic costs of innate immunity is the insect pupa. A pupa is a semi-closed system, exchanging only gaseous substances with the environment. In the case of larvae and adults, the effect of immune challenge may remain unnoticed, since animals can compensate the loss of resources by consuming more food (Moret & Schmid-Hempel 2000).

The aim of the current study is to test whether immune challenge is energetically costly for insect pupae. To challenge the immune system, we used a nylon implant inserted into the haemocoel of an animal. A nylon implant induces both the humoral and cellular immune responses leading to melanization and/or encapsulation of foreign particles, similar to the reactions against parasitoid eggs or larvae, protozoans and nematodes (reviewed in Vilmos & Kurucz 1998). As a study object, we used diapausing pupae of the white cabbage butterfly (*Pieris brassicae* L.; Lepidoptera: Pieridae). We hypothesized that if the responses against a foreign implant are energetically costly, implanted individuals should raise their standard metabolic rate (SMR) compared to control animals.

2. MATERIAL AND METHODS

Female butterflies were collected from a field in southeast Estonia in July 2001. Females were held in individual cages where they were fed with honey solution (10%) *ad libitum* and allowed to lay eggs on cabbage plants (*Brassica oleracea* L.). Larvae were fed on cabbage leaves in laboratory conditions. To control for possible origin-related effects, larvae of different females were raised separately. Diapausing pupae were kept at 10 °C until the start of the experiment. One day before the experiment pupae were transferred to 20 °C. The time lag between pupation and experiment (hereafter referred to as pupal age) varied from 28 to 65 days. Only male pupae were used in the experiment. Before the experiment pupae were weighed to the nearest milligram with a Mettler-Toledo AB-S electronic balance.

For challenging the immune system, 2 mm long nylon filament implants (diameter of 0.1 mm) were used. Implants were inserted into the sixth abdominal segment. Control animals were punctured on the same location with a sterile 0.1 mm needle and the opening was closed with bee wax. As indicated by the blackening of the implants, all experimental animals produced melanizing encapsulation responses.

The experiment lasted 3 days during which the SMR of individual animals was recorded microcalorimetrically. On day 1, animals were placed in the calorimeter. The next day, an implant was inserted (experimental group) or puncturation was made (control group). One day after manipulation (2 days from the start of the experiment) the

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Table 1. The effects of treatment (implanted versus control) and pupal age (period from pupation until experiment in days) on the SMR over the 3 day period in repeated measures ANOVA.
(A significant time × treatment term means that the change in SMR differed between treatments. For the direction of the effect, see figure 1. MS, mean square.)

type of response	MS	$F_{\mathrm{d.f.}}$	p
between subjects			
treatment	8.1×10^{-4}	$0.75_{1,23}$	0.395
pupal age	26×10^{-3}	$24.4_{1,23}$	< 0.001
within subjects			
time	2.4×10^{-4}	$9.24_{2,46}$	< 0.001
time × treatment	1.5×10^{-4}	$5.95_{2,46}$	0.005
time × pupal age	7.5×10^{-5}	$2.88_{2,46}$	0.066

implant was removed and the hole was closed with wax. With control animals the hole was opened and closed again with wax. Thereafter, the SMR was recorded for another day.

For measuring the SMR, a differential microcalorimeter was used. The calorimeter consists of two thermally isolated copper cylinders. Temperature changes in the animals' chamber were measured against an empty reference chamber. The signal from the thermocouples (connected to the chambers) was amplified and recorded for further analysis with the computer-based data acquisition system at a 0.016 Hz sampling rate (one reading per minute). All experiments were made at 20 °C, *ca*. 60% relative humidity in complete darkness. SMR is expressed as average heat production (μW) during the measuring period. To avoid handling effects on the SMR, a 3 hour period after every handling was excluded from calculations. Also periods of CO_2 bursts were excluded from the SMR calculations (see Kuusik *et al.* (1994) for details).

The effect of treatment on changes in the SMR was tested with a repeated measures ANOVA. Only the factors having a significant effect on SMR were included in the final model. In other analyses, Spearman rank correlations (r_s) and Kruskal–Wallis ANOVAs were used.

3. RESULTS

We found no effect of pupal mass on the SMR ($r_s = 0.074$, $p = 0.72$, $n = 26$). Therefore, in all analyses the absolute SMR values were used. As we also found no significant brood (or origin of pupae) effect on the initial SMR ($H_{10,26} = 15.2$, $p = 0.12$, Kruskal–Wallis ANOVA), individual SMR values were used in the analyses. The treatment groups did not differ with respect to the initial SMR (table 1 and figure 1) or pupal age ($Z_{14,12} = 0.334$, $p = 0.74$).

After treatment, implanted individuals raised their SMR by nearly 8% compared to the controls (table 1 and figure 1). Pupal SMR also increased with pupal age (table 1).

4. DISCUSSION

Subsequent to the immune challenge, white cabbage butterfly pupae increased their SMR by nearly 8% compared to the controls (table 1 and figure 1). To our knowledge, this is the first direct evidence indicating that activation of the immune system is energetically costly in insects. Seemingly in contrast to our results, the metabolic rates of tobacco hornworm (*Manduca sexta*) larvae infected by the braconid wasp *Cotesia congregata*, decreased 1 day after parasitization (Alleyne *et al.* 1997). However, polydnaviruses (or venom) accompanying wasp eggs are known to temporally suppress the host's cellular immunity (Strand & Pech 1995; Shelby *et al.* 2000). Hence, the lower metabolic rate of parasitized hornworms probably results from their reduced investment in immune

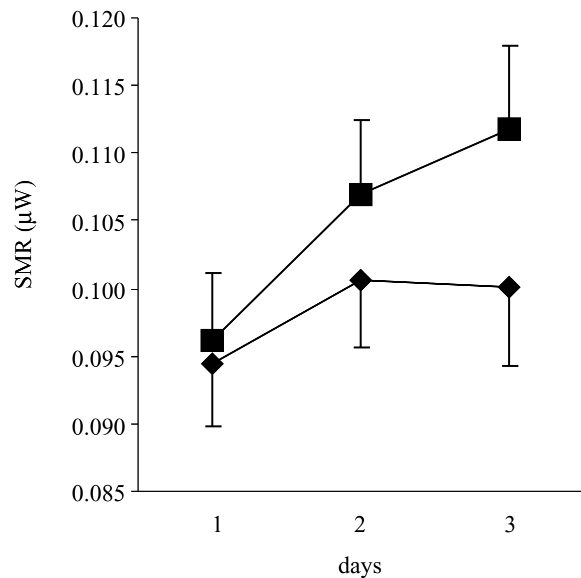

Figure 1. Change in SMR (least squares means ± s.e. from the model described in table 1) in implanted (squares) and control (diamonds) white cabbage butterfly pupae over 3 days. 1, 1 day before treatment; 2, 1 day after treatment; 3, 2 days after treatment. For the statistical tests see table 1.

function. In vertebrates, a single study has shown that a non-specific cell-mediated immune response is energetically costly. House sparrows challenged with phytohaemagglutinin significantly elevated their resting metabolic rate relative to controls (Martin *et al.* 2003).

In our study both implanted experimental pupae and puncturated control pupae tended to increase their SMR during the day following manipulation (figure 1). The first response after wounding is the rapid induction of several immune peptides providing effective defence against various micro-organisms (see Bulet *et al.* 1999; Lowenberger 2001). Induced early proteins might not be directly involved in immune responses, but might instead contribute to haemolymph coagulation and/or wound healing (Han *et al.* 1999). The production of immune peptides may explain initial SMR elevations in both treatment groups in our experiment.

Despite the fact that implants were removed 1 day before the end of experiment, the SMR in immune challenged animals continuously rose until the end of the experiment (figure 1). Such a pattern may be explained by the increased production of haemocytes after the

activation of a immune reaction (Eslin & Prévost 1998; Russo *et al.* 2001), which may concur expenditure of extra energy. An alternative, but not mutually exclusive explanation for the increase of SMR after removing the implant is the effect of oxidative stress accompanying melanotic encapsulation. After pathogen recognition the prophenol oxidase cascade is activated, which leads to the melanization of a foreign object (Chapman 1998; Barillas-Mury *et al.* 2000). The quinones generated by the activated phenoloxydase are thought to be toxic to the invading organism owing to their ability to generate reactive free radicals (Nappi *et al.* 1995; Barillas-Mury *et al.* 2000). Free radicals are toxic not only to the pathogens, but also to the host tissues (see von Schantz *et al.* 1999; Barillas-Mury *et al.* 2000). It is thus possible that the increase of SMR subsequent to implant removal could be at least partly ascribed to energy-demanding repair functions and clearance of necrotic tissue caused by oxidative damages.

The result that pupal SMR increased significantly with pupal age (table 1) can be explained by the typically U-shaped pattern of metabolic rate in diapausing insects. That is, the metabolic rate of insect pupae normally decreases during the first half of diapause and starts to increase afterwards (Kuusik *et al.* 1994; Chapman 1998). Evidently, our study period coincided with the increase phase of SMR.

In conclusion, we have presented clear evidence that insect responses against foreign implants are energetically costly. However, the question about which part of different physiological and biochemical reactions following immune challenge is mainly responsible for this cost requires further investigation.

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