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ORIGINAL ARTICLE

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Two estimates of the metabolic costs of antibody production in migratory shorebirds: low costs, internal reallocation, or both?

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Abstract We measured the costs of mounting a humoral immune response using two novel antigens (tetanus and diphtheria) in two shorebird species (Scolopacidae): Red Knot (*Calidris canutus*, measured in autumn) and Ruff (*Philomachus pugnax*, measured in spring). Metabolic rate was measured during the preinjection phase, at the building phase of the primary immune response, and at peak secondary immune response by determining the oxygen consumption of the postabsorptive birds at rest. Confirming earlier studies, Red Knots and Ruffs responded with lower antibody titers to the diphtheria than to the tetanus antigen. Although Red Knots and Ruffs produced the same amounts of antibodies, Red Knots showed a significant 13% increase in basal metabolic rate (BMR) during the secondary antibody response, whereas Ruffs showed a 15%, but only marginally significant, reduction in BMR. The results from this study suggest that the energetic costs of an immune response may be small, but the "negative cost" in Ruffs hints at the possibility of resource reallocation and the concomitant difficulty of measuring such costs during "basal" metabolic rate measurements.

Keywords Energetics · Humoral response · Metabolic rate · Physiological trade-offs · Red Knots · Ruffs

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Introduction

An immune response is a plastic trait that is regulated to maximize pathogen control and at the same time minimize host damage (Segel and Bar-Or 1999). It is dependent on nutritional status and condition (Klasing 1988; Birkhead et al. 1999; Gonzalez et al. 1999). Like many other life-history traits, immune response is traded off against other fitness parameters (reviewed in Zuk and Stoehr 2002; Schmid-Hempel 2003), such as reproduction (Deerenberg et al. 1997; Råberg et al. 2000), thermoregulation (Svensson et al. 1998; Ots et al. 2001), and the growth and maintenance of secondary sexual characteristics (Folstad and Karter 1992; Wedekind and Folstad 1994; Saino et al. 1997). Despite increasing evidence for such trade-offs, the causes for the compromises remain unclear. During stressful situations the immune system may be downregulated to avoid excessive oxidative stress (von Schantz et al.1999) or autoimmune diseases (Råberg et al. 1998). If energy is limited, the immune system simply has to compete with other costly activities for energy resources (Nelson et al. 2002).

Reallocation of resources among physiological functions is probably common (Ricklefs 1991; Ricklefs and Wikelski 2002). For example, long-distance migrant shorebirds can reduce their digestive organs while increasing their flight muscle within the weeks prior to taking off on long flights (Piersma et al. 1999; Landys-Ciannelli et al. 2003). Reallocation of energy from the reproductive and digestive systems to the immune system has been described for small rodents (Derting and Compton 2003), but more commonly it is reported that

immune responses can be expressed with varying intensity (Schmid-Hempel 2003).

The vertebrate immune system has two main components, the innate and the acquired immune defense (Feldman 2000). Innate defense is the first line of defense against any pathogen and is maintained regardless of the presence of diseases in the environment. The acquired immune response needs prompting by an immunological challenge and then takes some time to build, but it is highly specific in fighting particular pathogens (Råberg 2002). Although immune responses always include both innate and acquired components, the relative participation of each varies.

We studied the energetic costs of mounting an acquired immune response in two related, similar-sized shorebird species (Scolopacidae) with contrasting ecologies (see Piersma 2003), the Red Knot (*Calidris canutus canutus*, Linnaeus 1758), which breeds in the High Arctic and winters in coastal areas, and the Ruff (*Philomachus pugnax*, Linnaeus 1758), which breeds much further south and winters in freshwater habitats. We challenged the birds with two nonpathogenic antigens and measured the amount of antibodies produced during primary and secondary immune response, as well as the direct energetic costs of an activated immune defense (Råberg et al. 2000, Mendes et al. 2006). We aimed to see whether the single species estimates of costs made so far for passerines (Svensson et al. 1998; Ots et al. 2001; Råberg et al. 2002) are relatively low in shorebirds as well. We also wanted to verify whether such estimates are actually robust in the light of the possibility that experimental treatment may induce reallocation of energy (Derting and Compton 2003; Piersma et al. 2004).

Materials and methods

In July 2001, 19 adult Red Knots of the *canutus* subspecies were captured at night with mist nets in the western Wadden Sea (53°16′N, 5°08′E). These birds were captured postbreeding during southward migration, and to ease the comparison with Ruffs, we attempted to select females on the basis of their body dimensions and plumage characteristics (see Nebel et al. 2000). In April and May 2002, during northward migration, 20 female Ruff were captured with wilsternets in the meadows of the Dutch province of Fryslân (53°4′N, 5°30′E; see Jukema et al. 2001). We chose females to avoid the occurrence of aggressive behaviour typical of males in captivity (personal observation). In both species, sex was confirmed with a standard molecular PCR-DNA technique verified for Red Knots (Baker et al. 1999). Three of the 19 Red Knots (one in the experimental group and two in the control group) that were assigned as females on the basis of body dimensions and plumage were in fact males. However, these individuals had identical scores for all the measured values, including basal metabolic rate (BMR) and antibody titers, and therefore were included in the analysis. All experimental Ruffs were genetically confirmed to be females.

Birds were kept for 2 months in single flocks in the same large (7×7 m^2 and 2.5 m high) aviary at the Royal Netherlands Institute for Sea Research, Texel, The Netherlands, under a natural light:dark cycle at room temperature. Birds were allowed to acclimatize to captivity conditions and to stabilize their body mass for 2 weeks. Food and water were given ad libitum. Red Knots were fed trout pellets, and the Ruffs were fed mealworms (*Tenebrio* spec). Birds were randomly assigned to either a control or an experimental group (Table 1), but all birds were maintained together. All experiments complied with Dutch law.

To measure the cost of mounting a humoral immune response, we induced a primary and a secondary antibody response by twice injecting the experimental birds with a vaccine containing diphtheria and tetanus toxoid mixed with aluminium phosphate and suspended in phosphate-buffered saline (PBS; Statens Seruminstitut, Copenhagen, Denmark) in the pectoral muscles with a 0.5-ml sterile syringe. The primary response was obtained by giving the experimental birds 120 µl of the combined vaccine and the secondary immune response by injecting 100 µl of the same vaccine (see Hasselquist et al. 2001 for further details of procedures). Control birds were injected with the same amount of sterile phosphate saline solution. To assay antibody levels, blood samples were taken prior to the first injection and at the presumed peaks of the primary and secondary immune responses, day 14 after the first injection and day 7 after the second injection, respectively (Hasselquist et al. 1999; Råberg and Stjernman 2003). Blood was centrifuged for 12 min at 6,900 g and the plasma preserved at −30° C until analysis.

We used a modified quantitative enzyme-linked immunosorbent assay (ELISA) to measure blood concentrations of tetanus- and diphtheria-specific antibodies (Hasselquist et al. 2001). For ELISAs, we coated sets of polysterene 96-well plates (Costar) either with a diluted diphtheria toxoid or with a diluted tetanus toxoid (both diluted to 3 µg/ml with 0.15 M of carbonate buffer, at 9.6 pH). After an overnight incubation period at 4°C, plates were washed with washing buffer (0.01 M PBS with 0.05% Tween 20) three times to remove the unbound antigen and were then blocked for 2 h at room temperature with 3% milk powder diluted in the same buffer. After 2 h, we washed the plates two times with washing buffer. Then a 1:1,600 diluted plasma sample was added (plasma was diluted in a 1:2 serial dilution with 1% milk powder mixed in PBS/Tween20). Plates were allowed to incubate overnight and after being washed another three times with washing buffer, 1:1,000 diluted antiserum sample was added (rabbit anti-redwinged blackbird Ig antibody; Hasselquist et al. 1999) and allowed to incubate at 37°C. After 1 h, the plates were washed twice with washing buffer, and then a diluted peroxidase-labeled goat anti-rabbit antibody (Cat. A 6154, Sigma) was added and incubated for 30 min at

Table 1 Prevaccination values of body mass, body size (wing length), and basal metabolic rate (BMR) from control and experimental Red Knots and Ruffs

		Sex ratio (percentage of females)	Body mass (g)		Wing length (cm)		BMR (w)	
			Average ± SD	Range	Average ± SD	Range	Average ± SD	Range
Red Knot	Control ($n = 9$)	78	116 ± 6.7	105–125	170 ± 5.7	163–180	1.07 ± 0.11	0.94–1.21
	Experimental ($n = 10$)	90	119 ± 7.9	104–132	171 ± 4.5	168–181	1.00 ± 0.10	0.87–1.17
Ruff	Control ($n = 10$)	100	97.0 ± 5.3	88–106	159 ± 2.7	156–165	0.83 ± 0.10	0.67–1.04
	Experimental ($n = 10$)	100	96.6 ± 4.3	88–101	160 ± 2.2	155–162	0.89 ± 0.16	0.66–1.14

37°C. Finally, after a two-times final wash with washing buffer, substrate solution [200 µl of 0.2 mM ABTS (Cat. A 1888, Sigma), 80 µl of 30% H_2O_2 diluted in 1:40 in distilled H_2O in 20 ml of citrate buffer (pH 4.0)] was added to achieve a color reaction. The kinetics of color reactions were read every 30 s for 14 min at 405 nm with a Vmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Antibody titers were calculated based on the slope of the substrate conversion, in millioptical density/min (mOD/min).

A duplicate of each sample was run (the within-individual intersample variability accounted for less than 3% of the total variability), and to access interplate variability, a dilution series (from 1:600 to 1:7,700) of the same reference sample from a previous challenged Red Knot was also analyzed (interplate variability accounted for less than 16% of the total variability). In the subsequent analyses, we used the average of the duplicate sample values. We corrected for interplate variability by reducing all data to the same reference plate, using the Red Knot reference sample. Note that, for any antigen, there will be a background binding of antibodies because antibodies are not absolutely antigen-specific, and many different "clones" of B-cells produce antibodies at the same time, of which some may show at least moderate affinity to an antigen despite not being its main target.

In all birds we measured BMR (that is, the metabolic rate needed to maintain all vital body functions) of postabsorptive animals in thermoneutrality and at rest. The measurements were conducted over a period of 6 h on birds kept in darkened metabolic chambers at night in thermal neutrality (25°C; Piersma et al. 1995). Birds were withheld food for 4 h prior to measurements to ensure their postabsorptive state.

BMR was measured three times in each bird. The first time, we measured BMR in the prevaccination situation, the second time during the building phase of the primary immune response (approximately at day 7 after the first immune challenge), and a third time at day 7 after the second immune challenge (corresponding to the peak antibody production of the secondary immune challenge). We measured two sets of two birds per night. The first set was measured from 1700 to 2400 and the second set from 2400 to 0800. To make successive BMR measurements fully comparable, individuals were always measured in the same sequence and at the same time of day.

Oxygen consumption was measured using an open circuit respirometer, a Servomex paramagnetic oxygen analyzer, model 4100. The respirometry system was calibrated as described by Piersma et al. (2004). BMR was calculated according to Hill (1972) based on the 60-min running average with the lowest average oxygen consumption and assuming an energetic equivalence of 19.8 kJ l^{-1} O_2 (Piersma et al. 2004).

To normalize residuals, antibody titers were transformed logarithmically (Sokal and Rohlf 1995). We used ANOVAs with post hoc Scheffe tests to examine differences between the successive vaccination stages, and *t*-tests to examine differences between species. We treated body mass and BMR separately. To fully account for variation in body mass and BMR, we calculated the ratios between individual values for body mass and BMR before immune challenge with the individual values after the primary and secondary immune challenges, respectively. These ratio values were normally distributed; thus we could use two sample *t*-tests to test for differences in BMR values between control and experimental groups. All tests were performed in SYSTAT.

Results

Antibody levels

The antibody level against the diphtheria antigen changed significantly throughout the experimental period in both Red Knots (ANOVA: $F_{2,27} = 9.720$, $P = 0.001$) and Ruffs (ANOVA: $F_{2,27} = 14.707$, $P < 0.001$). Primary immune response to diphtheria vaccine did not differ from prevaccination levels in experimental birds of either species (Red Knots: post hoc Scheffe test, $P = 0.958$; Ruffs: post hoc Scheffe test, $P = 0.888$; Fig. 1). However, during the secondary immune response, experimental birds had higher antibody titers than the prevaccination samples (Red Knots: post hoc Scheffe test, $P = 0.002$; Ruffs: post hoc Scheffe test, $P < 0.001$; Fig. 1). Although both Red Knots (ANOVA: $F_{2,27} = 16.597$, $P < 0.001$) and Ruffs (ANOVA: $F_{2,27} = 17.210$, $P < 0.001$) also had significant increases of the prevaccination levels against

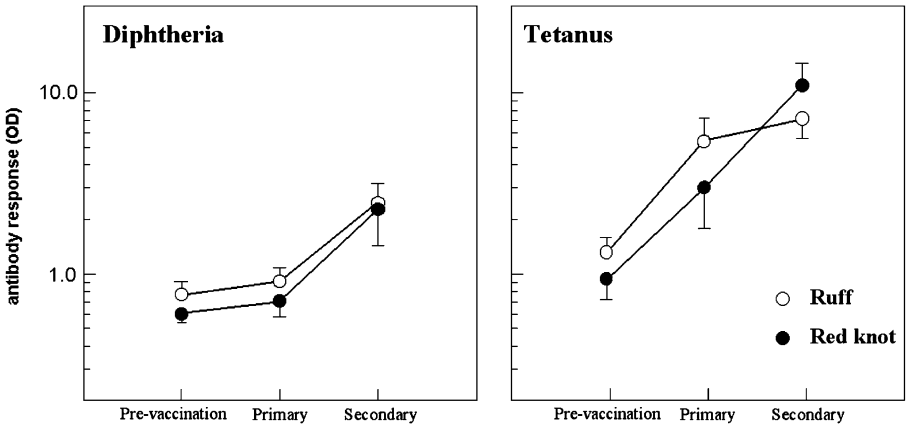

Fig. 1 Antibody levels (means ± SE) produced by Red Knots and Ruffs against diphtheria antigen and tetanus antigen during primary and secondary immune responses

the tetanus antigens, a different pattern was found for the immune response against this pathogen. During the primary immune response, both Red Knots (post hoc Scheffe test, $P = 0.019$) and Ruffs (post hoc Scheffe test, $P < 0.001$) had higher antibody titers compared with their prevaccination levels (Fig. 1). In contrast, during the secondary immune response, Red Knots continued to increase their primary response antibody level against tetanus (post hoc Scheffe test, $P = 0.037$), but Ruffs maintained primary response levels (post hoc Scheffe test, $P = 0.714$; Fig. 1).

The difference between Red Knots and Ruffs in pretreatment antibody levels (diphtheria, Student's t-test: $t = -1.434$, $df = 18$, $P = 0.169$; tetanus, Student's t-test: $t = -1.278$, $df = 18$, $P = 0.217$) was not significant at the 5% level. Furthermore, there was no difference in the diphtheria antibody titers between species, neither in the primary immune response (Student's t-test: $t = -1.073$, $df = 18$, $P = 0.298$) nor in the secondary immune response (Student's t-test: $t = -0.273$, $df = 18$, $P = 0.788$). The same was true for the responses against the tetanus antigen, where both species responded with equal intensity during the primary (Student's t-test: $t = -0.894$, $df = 18$, $P = 0.383$) as well as the secondary immune response (Student's t-test: $t = -1.213$, $df = 18$, $P = 0.241$).

Body mass

There was no difference between experimental and control groups in the initial body mass, either in Red Knots (Student's t-test: $t = -0.741$, $df = 17$, $P = 0.469$; Table 1) or in Ruffs (Student's t-test: $t = -0.046$, $df = 18$, $P = 0.963$; Table 1). There were also no differences in body mass ratio between the experimental and control Red Knots during the primary (Student's t-test: $t = 0.470$, $df = 17$, $P = 0.644$; Fig. 2a) or the secondary immune response (Student's t-test: $t = -0.268$, $df = 17$, $P = 0.792$; Fig. 2b). Similarly, in Ruffs there was no difference in body mass between groups during the primary (Student's t-test: $t = -0.014$, $df = 18$, $P = 0.989$; Fig. 2c) or the secondary response (Student's t-test: $t = -0.159$, $df = 17$, $P = 0.875$; Fig. 2d).

Basal metabolic rate

There were no differences in the initial BMR values between control and experimental Red Knots (Student's t-test: $t = 1.393$, $df = 17$, $P = 0.182$; Table 1) and Ruffs (Student's t-test: $t = -0.989$, $df = 18$, $P = 0.336$; Table 1). During the primary immune response, experimental and control birds showed similar BMR ratios in both Red Knots (Student's t-test: $t = -0.743$, $df = 17$, $P = 0.468$; Fig. 2e) and Ruffs (Student's t-test: $t = 0.705$, $df = 18$, $P = 0.490$; Fig. 2g).

In contrast, during the secondary immune response, experimental Red Knots had a 13% higher BMR ratio than controls (Student's t-test: $t = -2.251$, $df = 17$, $P = 0.038$; Fig. 2f). This result, incidentally, was even stronger when we removed the three males from the analysis (Student's t-test: $t = -2.580$, $df = 14$, $P = 0.022$). Experimental Ruffs showed a tendency for a lower BMR ratio (a 15% decrease) compared with controls (Student's t-test: $t = 1.695$, $df = 17$, $P = 0.108$; Fig. 2h). If the one outlier was excluded, however, the latter difference became significant (Student's t-test: $t = 3.578$, $df = 16$, $P = 0.003$).

Discussion

Experimental Red Knots and Ruffs responded with lower antibody titers, implying a weaker and possibly slower humoral response, to the diphtheria than to the tetanus antigen. This result supports previous findings in other wild bird species where the intensity of antibody production was lower against diphtheria compared with tetanus, highlighting that humoral immune responses can be highly dependent on pathogen type (Westneat et al. 2003; Owen-Ashley et al. 2004). One possible explanation for this difference may be that clostridial diseases are more common among livestock and wildlife

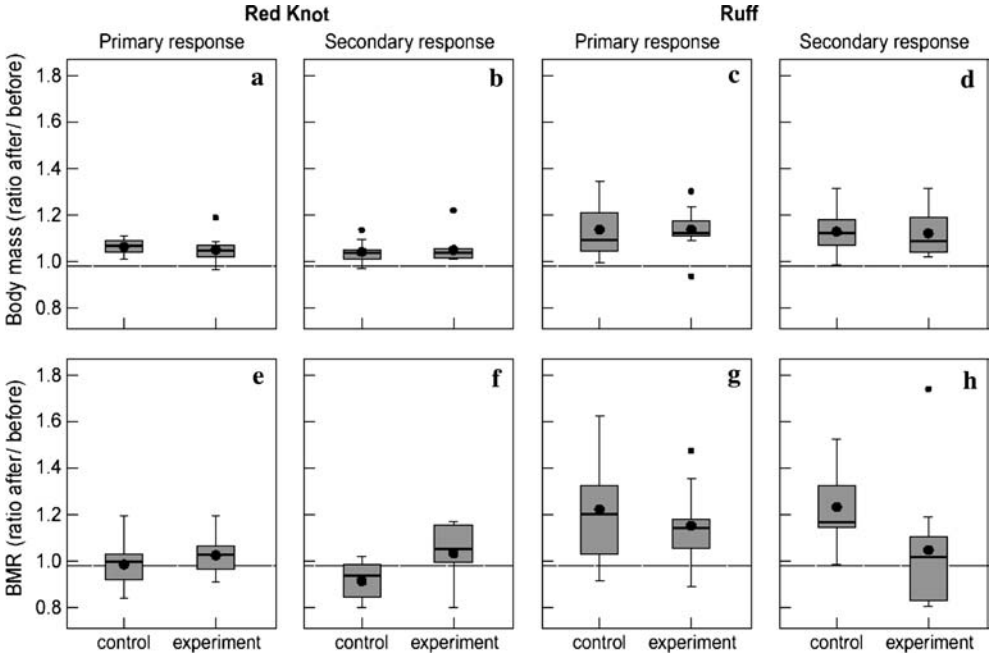

Fig. 2 Changes in the ratio between realized and pretreatment values of body mass (*top panels*) and basal metabolic rate (*bottom panels*) during the primary and the secondary immune response in Red Knots and Ruffs. Box plots are presented in which the boxes contain 50% of the data points (with the midline indicating the median value), and the remaining data points are within the vertical line (with outliers indicated by *separate dots*); the *closed circle* indicates the average. The *dotted line*, as ratio = 1, shows no change compared with pretreatment values.

(Mackintosh et al. 2002) than the diseases provoked by the bacteria of the genus *Corynebacterium*, to which diphtheria belongs. Tetanus infections have been detected in deer, whereas clostridial diseases (to which tetanus belongs) is a major source of mortality among wild birds. In particular, the closely related botulism bacterium (*Clostridium botulinum*) is a major source of mortality among wild populations (Friend and Franson 2001).

An alternative explanation for the differences in humoral immune responses against these two antigens is that the immune system activation against diphtheria may incur higher costs in terms of survival than immune responses induced by tetanus, as has been found in female Common Eiders (*Somateria mollisima*; Hanssen et al. 2004). However, we were unable to assess the individual costs of each antigen because we gave the two toxoids together.

Red Knots and Ruffs eventually produced similar levels of antibodies against the two antigens (Fig. 1), but they showed quite different relative changes in BMR. Red Knots showed a significant 13% increase in BMR during the secondary antibody response, but Ruffs had a nonsignificant decrease (15%). The increase in BMR in Red Knots is comparable to values for two passerine birds exposed to antigen challenge during winter conditions. Blue Tits (*Parus caeruleus*) showed a nonsignificant increase in BMR of 8–13% when challenged with the same antigens (Svensson et al. 1998), and a significant increase in BMR of about 9% was found in Great Tits (*Parus major*) challenged with sheep red blood cells (SRBC; Ots et al. 2001).

The finding that in Ruffs the challenge with diphtheria and tetanus vaccine did not result in an increase, but perhaps a decrease, in BMR is consistent with rather low costs, but additionally suggests the possibility of reallocation. Ruffs may have decreased their investment in other costly functions (and reallocated energy to the immune system) to such an extent that BMR actually became lower. Male wild-footed mice (*Peromyscus leucopus*) challenged with SRBC showed reduced testicle and intestine mass (Derting and Compton 2003). In this case, resources for the reproductive and digestive systems were reallocated to the immune system. In contrast, in a study on incubating female Common Eiders injected with the diphtheria-tetanus vaccine as well as with SRBC, the females that responded with antibody production did not lose more weight than control females, arguing against energy reallocation as a means of compensating for the energetic costs of immune system activation (Hanssen et al. 2004). Instead, female Eiders incurred long-term fitness costs in terms of reduced survival.

Whether the differences between Red Knots and Ruffs reflect fundamental differences between their ecophysiological responses related to differences in habitat selection, migration strategies, and/or disease risk, or whether the interspecific differences found here just reflect differences in the season of measurement, remains to be shown (but see Mendes et al. 2006).

Conclusions

Antibody titers appeared more dependent on antigen type (i.e., mimicking different parasite types) than on host species. Therefore, when drawing conclusions about costs of an immune defense, one must keep in mind that different pathogens incur different risks and provoke different defense strategies in the host.

In addition, our results suggest that the costs of an immune response, or the ways in which these costs are managed, may differ between hosts. In our view, a detectable increase in the BMR ratio can be regarded only as a minimum estimate of the energetic cost of mounting an immune response, since part of the necessary energy may be reallocated from other physiological systems. This means that future studies should incorporate assessments of the potential trade-offs between the immune system and other costly physiological functions, such as the maintenance of reproductive and digestive systems (see Derting and Compton 2003).

Zusammenfassung

Zwei Abschätzungen der Kosten der metabolischen Kosten für Antikörperproduktion bei ziehenden Limikolen: geringe Kosten, interne Umverteilung oder beides?

Wir haben die Kosten für den Aufbau des humoralen Immunsystems mit Hilfe zweier neuer Antigene (Tetanus und Diphterie) in zwei Limikolenarten (Scolopacidae) gemessen, beim Knutt (*Calidris canutus*; Messungen während des Herbstzugs) und beim Kampfläufer (*Philomachus pugnax*; Messungen während des Frühjahrszug). Sowohl in der Phase vor der Injektion der Antigene, während der Aufbauphase der primären Immunantwort, wie auch zur Zeit der stärksten sekundären Immunreaktion wurde der Stoffwechselumsatz ermittelt, indem der Sauerstoffverbrauch von ruhenden Tieren im postabsorptiven Zustand gemessen wurde.

Im Vergleich mit der Reaktion auf die Tetanus-Antigene reagierten beide Arten auf die Diphterie-Antigene mit einem niedrigeren Antikörper-Titer. Dieses Ergebnis bestätigt frühere Studien. Obwohl Knutts und Kampfläufer die gleichen Anzahlen Antikörper bildeten, zeigten Knutts während der Phase der sekundären immunologischen Reaktion einen signifikanten Anstieg der Stoffwechselrate um 13%. Kampfläufer dagegen zeigten einen 15%igen, obgleich nur geringfügig signifikanten, Rückgang des Stoffwechselumsatzes. Die Ergebnisse dieser Studie legen die Vermutung nahe, dass die eigentlichen Kosten einer humoralen Immunantwort eher gering sind. Die bei den Kampfläufern gemessenen ''negativen Kosten'' weisen jedoch möglicherweise darauf hin, dass Ressourcen intern umverteilt werden. Allerdings deuten sie gleichzeitig auch an, dass es schwierig sein könnte, solche Kosten durch Messen der ''basalen'' Stoffwechselrate zu ermitteln.

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