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Daily energy expenditure and cell-mediated immunity in pied flycatchers while feeding nestlings: interaction with moult

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Abstract Ecological immunology posits a trade-off between parental effort and immunocompetence underlying the cost of reproduction. The moult-breeding overlap observed in several bird species represents a conflict in resource allocation between two energy-demanding processes. Moult processes have been associated with enlargements of immune system organs. In the present study. we measured simultaneously daily energy expenditure (DEE) and the T-cell-dependent immune response of pied flycatchers, *Ficedula hypoleuca*, caring for grown nestlings. We used the doubly labelled water technique and the phytohaemagglutinin (PHA) injection assay on both males and females, while recording provisioning rates and moult scores. DEE and the PHA response were negatively correlated for females, but not for males. A significantly higher proportion of males than females initiated moult. Provisioning rates were strongly correlated with DEE for females but only for non-moulting males. The DEE of moulting males was marginally correlated with moult score. For moulting males, there was a marginally significant positive correlation between moult score and immune response. The trade-off between DEE and immunity for females could underlie the cost of reproduction. However, the moult-breeding overlap found in males may offset this trade-off, thereby reducing the implications of immunosuppression for parental survival.

Keywords Ecological immunology · Parental effort · Cost of reproduction · T-cell-dependent immunity · Moult-breeding overlap

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Introduction

Ecological immunology posits a trade-off between reproductive effort and immunocompetence (Gustafsson et al. 1994; Sheldon and Verhulst 1996) which would underlie the cost of reproduction (Roff 1992; Stearns 1992). Despite the widely held assumption that physiological mechanisms are the substrate of the most crucial life history trade-offs (Moreno 1993), progress in this field has been hampered by a methodological and conceptual separation between physiology/immunology and evolutionary ecology (Bernardo 1996). This gap is slowly being reduced through a confluence in approaches and cooperation across research fields (Sheldon and Verhulst 1996). Most ecological studies on this topic have concentrated on correlational studies of reproductive effort and parasite burden (Festa-Bianchet 1989; Møller 1997) or on experimental manipulations of brood demand and determination of health state (Ots and Horak 1996; Wiehn et al. 1999). These indirect estimations of the effort-immunity trade-off through its effects on health have lately given way to a more direct approach trying to determine directly the impact of exertion on immunocompetence (Sheldon and Verhulst 1996). Thus, recent experimental manipulations with birds have shown that different components of the immune system may be adversely affected by increased brood demand (Deerenberg et al. 1997; Nordling et al. 1998; Moreno et al. 1999a). The reciprocal experiment inducing an immune response to elicit effects on reproductive effort has met with contrasting results (Williams et al. 1999; Ilmonen et al. 2000). Activity levels of captive birds (Deerenberg et al. 1997) or provisioning rates in the wild (Moreno et al. 1999a) have shown negative correlations with antibody production or cell-mediated immunity. Thus, there is at present some empirical ground to sustain a claim for the ecological relevance of reproductive effort-immunity trade-offs.

An energetically costly activity which may coincide with breeding is avian moult, as many birds initiate the post-nuptial moult while raising young (Jenni and Winkler 1994). This moult-breeding overlap represents a conflict in resource allocation between two processes demanding energy and nutrients (Murphy 1996; Hemborg and Lundberg 1998). There is also a tendency for males of several species to initiate moult earlier than females, and thus increase the period of moult-breeding overlap (Dhondt 1981; Hemborg 1999; Sanz 1999). There are indications that birds moulting while raising young reduce their parental effort, presumably to cope with the energy demands of moult (Svensson and Nilsson 1997). This suggests that energy budgets of moulting and non-moulting individuals are different with respect to the activity component, moulting birds investing less in physical exercise. If immunosuppression is linked to increased activity, we should expect the trade-off between energy expenditure and immunity to be more marked for non-moulting individuals. In addition, moult processes have been associated with size increases in organs of the immune system like the thymus or spleen (Ward and D'Cruz 1968; Silverin et al. 1999; but see Alodan and Mashaly 1999 for poultry). Large components of the energy budget devoted to moult could therefore be associated with a higher, not lower immune response.

In the present study, we measured simultaneously daily energy expenditure (DEE) and T-cell-dependent immune response of parent pied flycatchers, *Ficedula hypoleuca*, caring for nestlings. For this purpose, we used the doubly labelled water (DLW) technique and the phytohaemagglutinin (PHA) injection assay on both males and females. The reason for measuring both variables simultaneously is that DEE may depend on weather factors that vary daily (Bryant 1989). Measuring the variables on different days might incorporate environmental noise in the DEE-immunity correlations. The PHA injection assay has earlier revealed a response of the cell-mediated component of the parental immune system to brood demand in the study population (Moreno et al. 1999a). Parental DEE has a direct effect on offspring fitness in this species (Merino et al. 1996; Moreno et al. 1997) and is consistent individually across years (Potti et al. 1999). However, it is difficult to manipulate experimentally (Merino et al. 1998; Moreno et al. 1999b), possibly due to compensatory adjustments among different components of the energy budget (Bautista et al. 1998; Deerenberg et al. 1998). We have not tried to experimentally alter parental DEE through brood manipulations, handicapping or food supplementation, because earlier experiments indicated this is not feasible in this species (Moreno et al. 1995, 1999b). We surmise that differences in metabolic activity due to physical exercise are reflected in the integrated measure of DEE, and therefore expect immunosuppression at higher levels of DEE. We also expect that an increased moult-breeding overlap will interact with the predicted DEE-immunity trade-off if moult is associated with increased immunocompetence.

Material and methods

Study area and species

The study was conducted in 1999 in a deciduous forest of pyrenean oak, *Quercus pyrenaica*, at 1,200 m above sea level in the vicinity of La Granja, Segovia Province, central Spain (40°48′ N, 4°01′ W). A population breeding in nestboxes in this area has been studied since 1991 (Sanz 1995). Nestboxes (125×117 mm bottom area) are cleaned every year after the breeding season. Each year, the nestboxes are checked for occupation by pied flycatchers, and the dates of clutch initiation, clutch sizes and number of fledged young are determined.

The pied flycatcher is a small (12–13 g) hole-nesting passerine of European woodlands (Lundberg and Alatalo 1992). It is a summer visitor, and adapts readily to breeding in artificial nestboxes. Egg laying in the population under study typically begins in late May, and clutch sizes range from 2 to 8 eggs with a mode of 6 eggs (mean 5.73). The female incubates alone and receives part of her food from her mate (Lundberg and Alatalo 1992). Young are brooded by the female only up to an age of 7 days (Sanz and Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days of hatching (Lundberg and Alatalo 1992). This occurs in the second half of June in our study area. There are no second clutches in the study population.

Doubly labelled water

When nestlings were 12 days old (hatching day=0), we captured 39 males and 24 females with nestbox traps, and used the DLW technique (Bryant 1989) to measure their DEE. Adults were weighed to the nearest 0.1 g, and then injected intraperitoneally with 0.1 ml of a mixture containing 10.21 g of 90.43 atom % (AP) $H_2{}^{18}O$ and 5.21 g of 99.9 AP D_2O. The birds were then placed in a small dark box for 1 h to allow equilibration of the isotopes in the body fluids. Several samples of blood were then taken from a brachial vein and kept in flame-sealed, heparinized capillary tubes until analysis. After bleeding stopped, the birds were released. Approximately 24±1 h later, birds were recaptured and more blood samples were taken from the brachial vein in the opposite wing. They were then reweighed and released. Blood samples were analysed for ^{18}O and D concentrations at the Centre for Isotope Research of the University of Groningen, The Netherlands. More than two capillaries for each isotope were analysed when isotopic concentration values differed by more than 2.5%, and the closest values were used to derive means. Six males and one female were not included in the analyses because values for one or both isotopes differed by more than 2.5% or due to leakage or badly sealed capillaries. Final sample sizes of birds used in the analysis were 33 males and 23 females.

Body water volume was derived from an estimated percentage of water of 67% obtained after drying ten fresh carcasses of adult birds recovered in nestboxes in the last 5 years (corresponding probably to recently arrived birds killed by con- or heterospecifics in competition for nestboxes). Daily CO_2 production was derived from fractional turnovers of the two isotopes using Eq. 35 in Lifson and McClintock (1966). CO_2 production in ml g^{-1} h^{-1} was converted to DEE (kJ/day) by assuming a respiratory quotient of 0.8 for insectivorous passerines (Williams 1988) and an energetic equivalent of 20 kJ l^{-1} of oxygen consumed (Schmidt-Nielsen 1990).

Provisioning rates

On the day before first capture, we filmed the entrance of the nestboxes with a video camera for 1 h in order to count the number of feeding trips performed by both mates. The video camera was placed 5–10 m away from the nestbox. However, all sessions in which one pair member we knew to be present in the study area did not turn up at the nest were excluded from analyses, because the absence could have been due to disturbance by the camera or other unknown factors. There was no effect on provisioning rates of the time of day when films were made, all films being obtained between 0930 and 1445 hours (correlations for males and females, $P>0.10$). We assume that provisioning rates during the day of DEE measurement were similar to those obtained on film the day before, as handling of female pied flycatchers for DLW measurements has little effect on their behaviour (Moreno et al. 1997). Not all males for which we had DEE information were filmed. In total, 64 nests were filmed.

Immunization

The PHA injection test has been routinely used in poultry science (Goto et al. 1978). The main cellular response observed 6–12 h after injection consists of a prominent perivascular accumulation of T lymphocytes followed by macrophage infiltration (Goto et al. 1978). The PHA-stimulated hypercellularity normally disappears 48 h post-injection. The PHA skin test is considered a useful method to evaluate thymus-dependent function (Goto et al. 1978), and has been routinely used as an assay of T lymphocyte cell-mediated immune function in studies of poultry (Tsiagbe et al. 1987; Cheng and Lamont 1988; Lochmiller et al. 1993). It is being increasingly used also in field studies (Saino et al. 1997; Sorci et al. 1997; Moreno et al. 1998; Merino et al. 2000), given its benign character compared with other methods used to evaluate immunocompetence (Merino et al. 1999). Typically in birds, one wing web is challenged with PHA, while the opposite is injected with phosphate-buffered saline (PBS), and the difference between the swellings attained during 24 h is used as the response. We have used the simplified protocol proposed by Smits et al. (1999), which avoids the injection of PBS in the opposite wing web as a control. The advantages of eliminating the PBS injection are a decrease by half in the handling time of the birds (and thereby in stress), a reduction in the probability of errors when injecting into such a thin structure as the wing web of a passerine and a decrease in the coefficient of variation that is due to measurement inaccuracies (Smits et al. 1999). The latter authors have shown convincingly that this protocol reduces inaccuracies inherent in the technique.

After injection with DLW, birds were injected with 0.05 ml of a solution of PHA in PBS (5 mg of PHA in 10 ml of PBS) in the left wing web after measuring its thickness at the point of injection. Three measures were taken with a digital spessimeter with constant pressure (Mitutoyo 7/547, Tokyo, Japan) to the nearest 0.01 mm to calculate the repeatability of wing web measurements. On recapture and before blood sampling (see above), three new measurements of the thickness of the left wing web at the point of injection were taken. The three measurements were averaged, because wing web thickness has shown a high repeatability in earlier studies (Moreno et al. 1999a). The immune response was estimated as the difference between initial and final measurements.

Moult score

The stage of wing moult of the parents was determined on day 12 after injection with DLW. The moult score was calculated as a sum of the scores assigned to the individual primaries of both wings (0=old feather, 5=fully developed new feather; Ginn and Melville 1983), since the moult in the right and left wings was not always symmetrical. The moult status was defined either as moulting (moult score >0) or non-moulting (moult score=0).

Results

Males and females did not differ with respect to either DEE, cell-mediated immunity, provisioning rates or body mass (Table 1). Brood size showed no correlation with either DEE (males: $r_{32}=0.21$, $P=0.24$; females: $r_{22}=0.32$, $P=0.14$) or PHA response (males: $r_{38}=0.16$, $P=0.32$; females: $r_{23}=-0.03$, $P=0.88$) of males or females. DEE and the PHA response were negatively correlated for females, but not for males (Fig. 1). The absence of a trade-off for males could be due to a weakened association between physical exercise and DEE due to moult. In the study year, only 17.3% of 104 females were moulting while feeding 12-day-old nestlings, while 56.5% of 92 males were ($\chi^2=32.8$, $P<0.001$). Provisioning rates as a measure of activity were highly significantly correlated with DEE for females, but not for males (Fig. 2). However, controlling for moult state, male DEE was positively correlated with provisioning rates (ANCOVA, provisioning rate: $F_{1,28}=4.32$, $P=0.047$; moult state: $F_{1,28}=3.64$, $P=0.066$). The DEE of moulting

Table 1 Daily energy expenditure (DEE, kJ/day), cell-mediated immunity (PHA response, mm), provisioning rates (feedings/h) and body mass (g) of female and male pied flycatchers (sample size in *parentheses*)

	Females	Males	*t*	*P*
DEE	55.78±5.76 (23)	54.93±8.28 (33)	0.42	0.67
Cell-mediated immunity	0.170±0.098 (24)	0.162±0.096 (39)	0.30	0.77
Provisioning rate	20.58±8.96 (64)	19.38±9.44 (64)	0.74	0.46
Body mass	12.11±0.53 (24)	11.90±0.42 (39)	1.77	0.082

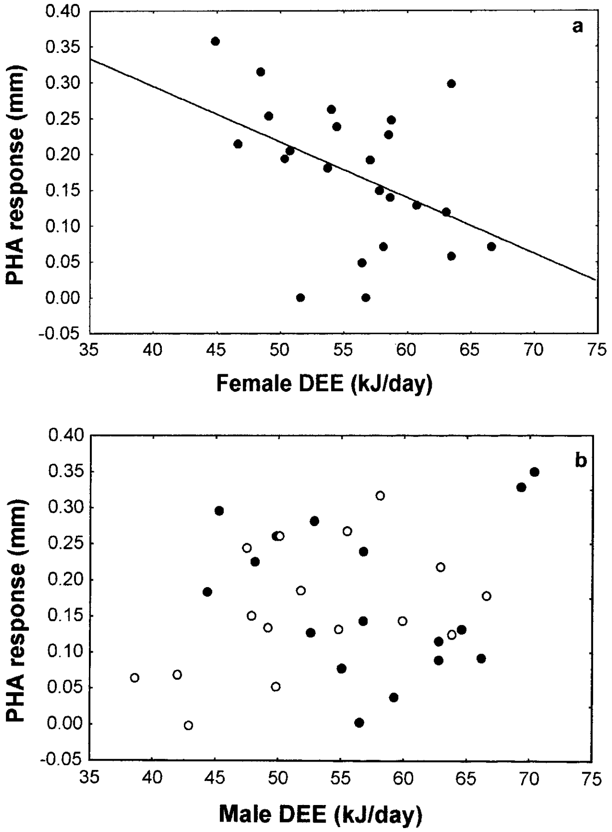

Fig. 1 **a** Regression of phytohaemagglutinin (PHA) response on daily energy expenditure (DEE) for females ($r=-0.45$, $F_{1,21}=5.36$, $P=0.031$). **b** Regression of PHA response on DEE for males ($r=0.20$, $F_{1,31}=1.33$, $P=0.258$) (*closed* and *open symbols* are non-moulting an moulting males, respectively)

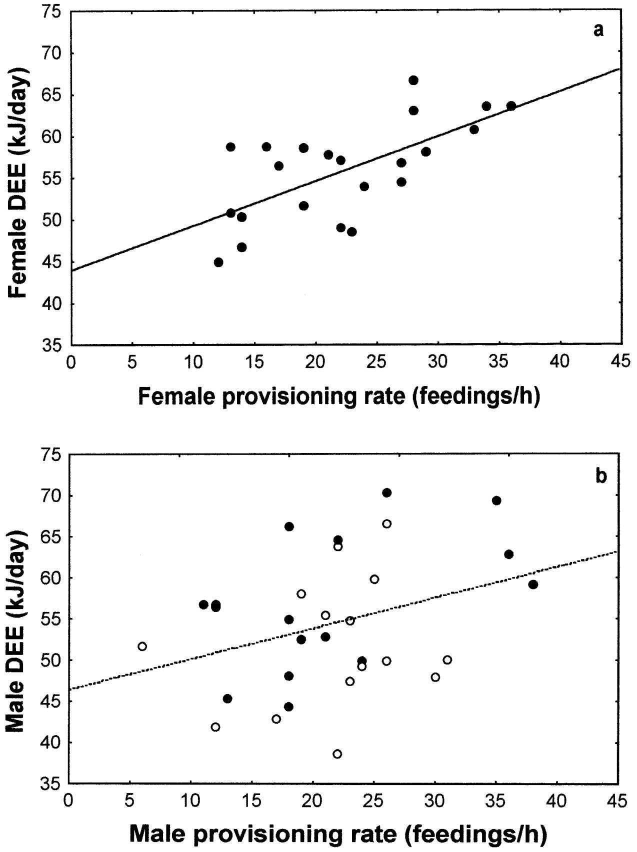

Fig. 2 **a** Regression of DEE on provisioning rate for females (r=0.66, $F_{1,20}$=15.33, P <0.001). **b** Regression of DEE on provisioning rate for males (r=0.33, $F_{1,29}$=3.72, P=0.063) (*Closed* and *open symbols* are non-moulting and moulting males, respectively)

males was marginally positively correlated with moult score when controlling for provisioning rate and body mass in a multiple regression (β=0.64, t_{11}=2.17, P=0.053). For non-moulting males, there was a nearly significant positive correlation of DEE with provisioning rate (r_{14}=0.48, P=0.057). This suggests that the DEE of males is dependent on activity when non-moulting, but is more dependent on the moult process once moult has been initiated. For moulting males, there was a marginally significant positive correlation between PHA response and DEE (r_{14}=0.47, P=0.068), while no such trend was detected for non-moulting males (r_{15}=0.04, P=0.87). Moulting and non-moulting males did not differ with respect to either DEE, PHA response or provisioning rate (P>0.10 in all cases). Moult score was almost significantly correlated with PHA response in moulting males when correcting for provisioning rates in a multiple regression analysis (β=0.48, t_{14}=2.06, P=0.058). In the same analysis, provisioning rates were not correlated with PHA response (β=0.25, t_{14}=1.08, P=0.30). This may indicate that the cell-mediated immune response in moulting birds is elevated due to growth of immune defence organs.

Discussion

One potential criticism of the simultaneous measurement of DEE and reaction to PHA injection in the wing web is that the pain induced by the PHA injection and the subsequent swelling could impair flight behaviour and the propensity to engage in flight. This could explain the negative trade-off found in females if a higher response was associated with a reduction in flight activity and therefore in DEE. This possibility seems unlikely because the PHA treatment has been shown to be unstressful for birds injected in the wing web (Merino et al. 1998). In the same population, females caring for enlarged broods had higher provisioning rates and lower PHA responses than control females (Moreno et al. 1999a), suggesting that the causal relationship is from effort to immune response, and not the other way round as would be predicted by the pain-flight effort link. Furthermore, the sexual difference could not be explained by this potential methodological bias. Finally, one could also argue that flight alleviates pain through air circulation around a structure not directly associated with flight effort, predicting a positive trend of DEE with PHA response. We detected evidence for a sex-dependent trade-off between effort expended in provisioning young and cell-mediated immunity in our study population. For females, there is a clear negative association between parental effort and response to PHA as predicted by ecological immunology theory (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000). DEE is closely associated (r^2>40%) with a measure of physical exercise like provisioning rate, which is presumably associated with offspring benefits. Activity presumably explains the negative association between DEE as a measure of the total energy budget and the cell-mediated immune response. This trade-off may imply a cost for parents involved in intense physical exercise while caring for young and could represent a cost of reproduction as expected from life history theory (Roff 1992; Stearns 1992).

However, the picture becomes less clear for males. More than half of the males initiated moult before the young fledge, while only a small proportion of females did. This agrees with other studies of the same (Ojanen and Orell 1982; Hemborg 1999) and other (Dhondt 1981; Sanz 1999) species. Males may thus be experiencing a compromise between devoting energy to provisioning young and to moult (Svensson and Nilsson 1997; Hemborg and Lundberg 1998). Provisioning activity does not explain variation in DEE of moulting males, while it shows a marginally significant association with DEE for non-moulting males (probabilities slightly above 0.05 turn up repeatedly in this study, presumably due to the small sample sizes imposed by the costly DLW technique). The DEE of moulting males is better explained by moult score, indicating a link between total energy expended and advancement of the moult process (Murphy 1996). A preliminary conclusion from the pattern shown by males is that physiological processes linked to moult constitute an important component of the field metabolism of males at the end of the nestling period.

There is some evidence that moulting males with a higher moult score also had a higher PHA response and a higher DEE. As a higher moult score of these males could be linked to a more advanced moult process, our results hint at increases in immunocompetence associated with moult. Increases in spleen and thymus size have been detected in relation to moult in passerines (Ward and D'Cruz 1968; Silverin et al. 1999). This hypertrophy may result from a need for increased lymphocyte production associated with an expansion of the circulation (Ward and D'Cruz 1968) or to the need to avoid infection of wounds resulting from the breakage of growing feather follicles (Silverin et al. 1999). This increase in immunocompetence in moulting males would completely offset any decrease resulting from parental effort, removing the negative correlation observed for females. If general, the increase in immunocompetence associated with post-nuptial moult would make it difficult to detect trade-offs between reproductive effort and immunity in species with a moult-breeding overlap. It could also offset any lasting damage from immunosuppression during reproductive activities, thus reducing its life history implications.

To conclude, we found evidence for a trade-off between parental effort and immune response in females caring for young. This trade-off could potentially underlie the cost of reproduction as envisaged by ecological immunology (Sheldon and Verhulst 1996). However, the moult-breeding overlap found in males offsets this trade-off due to energy investment in moult processes which do not detract from resources needed by the immune system but appear to actually increase the cell-mediated immune response. Moult processes may reduce or negate the implications of reproductive immunosuppression for parental survival.

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