

Metabolic consequences of overlapping food restriction and cell-mediated immune response in a long-distance migratory shorebird, the little ringed plover *Charadrius dubius*

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Investment in immunity is commonly viewed as an energetically costly activity in birds. Although several studies have focused on the energy cost of mounting an immune response and its concomitant physiological trade-offs, nothing is known about the metabolic adjustments experienced by immunochallenged birds under resource limitation, or about the basal metabolism cost of mounting cell-mediated immune (CMI) responses in bird species other than non-migratory passerines. Here we measured the basal metabolic rate (BMR), inflammatory response, and body mass in *ad libitum* fed and food-restricted little ringed plovers *Charadrius dubius* challenged with phytohemagglutinin (PHA) in order to assess the energy cost, the strength, and the time course of the CMI response in a long-distance migratory bird in different nutritional states. We found that *ad libitum* birds injected with PHA significantly increased both mass-independent BMR and inflammatory response, whereas birds with an induced food restriction-immune response overlap experienced a mass-independent BMR downregulation and decreased inflammatory response relative to *ad libitum* birds. We suggest that both the BMR downregulation and the diminished inflammatory response observed in birds facing such an overlap could be energy-saving mechanisms to maintain the body mass above a critical level and maximize fitness.

Investment in immunity is commonly viewed as an energetically costly activity in birds (e.g., Ots et al. 2001, Bonneaud et al. 2003, Martin et al. 2003, Barbosa and Moreno 2004, Klasing 2004, Martin et al. 2006), and is often involved in trade-offs with other energy demanding processes such as growth, reproduction, thermoregulation, moult and self-maintenance (Sheldon and Verhulst 1996, Illmonen et al. 2000, Norris and Evans 2000, Zuk and Stoehr 2002, Hanssen et al. 2003, Ardia 2005, Martin 2005, Buehler et al. 2009). As a result of these physiological trade-offs, birds frequently display a substantial decline or even suppression of their immune function (Sheldon and Verhulst 1996, Nordling et al. 1998, Råberg et al. 1998, Svensson et al. 1998).

Food-restricted birds typically exhibit reduction of immunocompetence (e.g., Alonso-Alvarez and Tella 2001, Hangalapura et al. 2005, Brzęk and Konarzewski 2007). In the wild, immune response and food limitation are often closely linked energy challenges because organisms under nutrient limitation are more susceptible to parasite and pathogen infection (Rosen 1993, Nelson and Demas 1996, Apanius 1998, Barbosa and Merino 2004). Although several recent experimental studies have examined the costs of overlapping energy challenges in birds (e.g., Hemborg and Lundberg 1998, Svensson et al. 1998, Sanz et al. 2004,

Martin 2005, Hasselquist et al. 2007, Buehler et al. 2009, Vézina et al. 2010), metabolic responses to immune challenge and to food restriction have as yet only been studied separately. In birds dealing with food-restricted conditions, an additional immune challenge may present a much greater threat, and thus metabolic strategies of energy compensation (i.e., energy-saving mechanisms) could play a significant role in maximizing fitness. Studies of these and other overlapping energy challenges would help understand whether the metabolic response of birds facing different simultaneous energy limitations is similar to or different from what is observed when those limitations are imposed separately (Vézina et al. 2010).

There is growing evidence that energy-stressed birds can exhibit reductions in resting and basal metabolic rate (RMR and BMR) to reduce maintenance costs and reallocate resources among energy-demanding activities during both early development (Brzęk and Konarzewski 2001, Moe et al. 2004, Moe et al. 2005), and adult stages (Deerenberg et al. 1998, Bech et al. 2002, Piersma et al. 2004). Downregulation of the metabolic rate has been shown to be a common energy-savings strategy in birds coping with limited food resources (e.g., Shapiro and Weathers 1981, Laurila et al. 2005, Wiersma et al. 2005). However, food restriction does not always lead to decreased BMR when this parameter is

controlled for body mass. A recent experiment conducted on captive red knots *Calidris canutus* found that a 6-h limitation on access to food was not enough to cause a mass-independent BMR downregulation in this long-distance migratory shorebird (Vézina et al. 2009). However, the results of the second part of the experiment showed that the acute phase response caused by lipopolysaccharide (LPS) was suppressed in these birds (Buehler et al. 2009). LPS is an inert antigen that induces a systemic inflammatory response within few hours after injection (Janeway and Travers 1999). Although some authors have reported that the exposure to this antigen induce a remarkable drop in food intake and body mass as part of the acute phase response of birds (e.g., Cook et al. 1993, Parmentier et al. 1998, Bonneaud et al. 2003), food-restricted red knots challenged with LPS tended to increase feeding behaviour and reduce locomotor activity (Buehler et al. 2009). This support the results of earlier studies reporting that birds need to increase intake rate and/or decrease activity to cover the extra costs evoked by the immune challenge (e.g., Svensson et al. 1998, Barbosa and Moreno 2004, Lee et al. 2005).

Recent work suggests that producing cell-mediated immune (CMI) responses to phytohemagglutinin (PHA) stimulation entails significant energy costs in birds (reviewed in Ardia and Schat 2008), however, the small number of literature reports of this class of data have only focused on small non-migratory passerines (Martin et al. 2003, Lee et al. 2005, Martin et al. 2006, Nilsson et al. 2007). Indeed, the results reported in these studies seem to be contradictory, showing large differences in metabolic responses. The experiments conducted by Martin et al. (2003, 2006) on house sparrows *Passer domesticus* showed RMR increases of 29% and 32%, respectively. Conversely, PHA injection had little effect on the RMR of great tits *Parus major* (Nilsson et al. 2007), and even decreased the RMR of tree sparrows *P. montanus* (Lee et al. 2005). Although these differences in metabolic response might partly be due to methodological questions (see the discussions in Lee et al. 2005 and Nilsson et al. 2007), further work is needed to gain insight into these responses in immune challenged migratory (both passerine and non-passerine) birds in diverse physiological states.

In this context, we expected to find that severe food restriction coupled with a CMI challenge would induce a state of negative energy balance, so that the stressed birds might adopt metabolic strategies of energy compensation in order to save energy. Establishing a better understanding of the causes and consequences of the up- and down-regulation of BMR is essential to appropriately evaluate avian metabolic adaptations to the abiotic and biotic characteristics of their environments.

The little ringed plover *Charadrius dubius* (hereafter “plovers”) is a small-sized migratory shorebird that typically spends the non-breeding season in tropical Africa (Cramp and Simmons 1983). This bird is exposed to parasites (Figuerola 1999, Mendes et al. 2005), and, as do many other long-distance migratory shorebird species, it probably faces frequent limitations in food resources during the non-breeding season (Evans 1976, Goss-Custard 1980, Duffy et al. 1981, Zwarts and Wanink 1991). Furthermore, short-billed shorebirds such as plovers are more vulnerable to the detrimental effects of high temperatures since invertebrates living in soft sediments may move to greater depths to avoid

desiccation and stay beyond the reach of their beaks (Evans 1976).

The aim of this study was to test the effects of CMI challenge on the BMR, inflammatory response, and body mass of plovers in different nutritional states. We also estimated the energy costs of mounting a CMI response in terms of BMR to determine whether those costs are consistent with the above-mentioned experiments. Given the findings of previous studies (Alonso-Alvarez and Tella 2001, Hangalapura et al. 2005, Brzęk and Konarzewski 2007, Fair and Whitaker 2008), we expected there to be a weaker inflammatory response to PHA in individuals feeding under food-restricted conditions.

Methods

Animal capture and care

Twenty seven adult (non-moulting) little ringed plovers en route to the wintering grounds in Africa were caught with mist nets and clap nets in Extremadura, SW Spain (38° 58'N, 6° 29'W), in autumn 2009. The birds were housed under natural weather conditions (photoperiod of 10 h:14 h L:D and daily mean temperature range from 14° C to 19° C) in outdoor aviaries at the Univ. of Extremadura (Gutiérrez et al. 2011). They were provided with fly larvae *Protophormia terraenovae*, commercial food dry pellets (Dibaq-Diproteg SA, Segovia, Spain – consisting of 48% protein, 6% carbohydrate, 25% fat, and 11.5% ash), and fresh water ad libitum. They were allowed to adjust to captivity for two months, and prior to the experiment they were maintaining a stable body mass. They were then randomly assigned to either food ad libitum (n = 14) or food-restricted conditions (4 h food access; n = 13), and re-housed in eight cages (5 m × 2.5 m × 2 m, 3–4 birds per cage) each equipped with freshwater ad libitum for drinking and bathing in a large pool (2.5 m × 2 m, 2 cm deep). Diet restriction was imposed for a 3-d period before the immune challenge. Since the metabolic measurements were made overnight, and little ringed plovers mainly forage during daylight, we considered that the individuals assigned to the ad libitum treatment were fed without restriction. After the experiment, birds were fed until they maintained a stable body mass and were then released at the site of capture.

Cellular immunity: PHA challenge

At the start of the experiment, all the birds were accustomed to the respirometry system for 3 d before the immune challenge. To evaluate the T-cell mediated immune response, we injected 60 µl of 1 mg/ml phytohemagglutinin-P (PHA-P; Sigma L-8754) in phosphate-buffered saline (PBS) intradermally into the left wing web of each treatment plover (seven individuals from the ad libitum group, and seven individuals from the food-restricted group). Control plovers (seven and six individuals from the ad libitum and food-restricted group, respectively) were injected with the same volume of PBS. The thickness of the wing web was measured in triplicate with a pressure-sensitive spessimeter (Mitutoyo) immediately before injection (0 h) and every night after the respirometry

measurements (24 h, 48 h, 72 h, 96 h, and 336 h post-injection). As the repeatability of the measurements was high ($r = 0.99$, $p < 0.001$), we used the mean for the subsequent statistical analyses (Smits et al. 1999). The CMI response was the difference in wing web swelling between control and treatment groups. The energy cost of mounting the CMI response was estimated according Martin et al. (2003). Thus, we calculated the difference in average BMR between PHA- and PBS-injected birds from the time of initial challenge (time 0 h) to the end of the experiment (time 96 h).

Metabolic measurements

We measured the BMR indirectly as oxygen consumption (VO_2) using an open-circuit respirometry system. All measurements were made overnight after the birds had been fasted for 4 h to create a post-absorptive state. Birds were placed in a darkened metabolic chamber (effective volume = 4.6 l) located in a temperature-controlled room at a constant temperature of 27°C, i.e., within the thermoneutral zone of little ringed plovers (Kendeigh et al. 1977). The metabolic chambers received a flow of atmospheric air at a rate of 1 l/min through calibrated mass flow controllers (MFS-5; Sable Systems, Las Vegas, USA). A multiplexer (TR-RM4; Sable Systems, Las Vegas, USA) allowed automatic switching between four channels. After taking a subsample at 150 ml min⁻¹ using a subsampler mass flow meter unit (SS-3; Sable System, Las Vegas, NV, USA), the air stream coursed through Drierite columns to remove the water vapour. Oxygen concentration was determined using a gas analyzer (FC-10 Oxygen Analyzer; Sable Systems, Las Vegas, USA) and automatically logged at a 1 Hz sampling rate onto a computer using ExpeData software (v. 1.1.25; Sable System), and a UI2 converter. Each sampling sequence started with logging ambient baseline air for 10 min, followed by sampling each chamber for 10 min with 2 min flushing the system between chambers to remove latent gases. This sequence was repeated six times, so that there were 6 records per bird per night. The birds were weighed immediately before and after the metabolic measurement, with the body mass reported for the BMR analysis being the mean of these two weights. Oxygen consumption was calculated according to the appropriate equations given by Hill (1972) on the basis of the lowest 5-minute average of O_2 consumption. Since respiratory quotient (RQ) was not measured and may have differed between ad libitum and food-restricted birds, we set RQ to 0.8 which would result in a maximal error of +2.6 – 4.7% in VO_2 when the true RQ is between 0.7 and 1 (Koteja 1996). Metabolic rate was calculated using an energy equivalent of 20 kJ/l O_2 , as has been used in other studies on shorebirds (e.g., Kersten and Piersma 1987, Kersten et al. 1998, Kvist and Lindtröm 2001).

Data analysis

The CMI response, BMR, and body mass data were analyzed by linear mixed modelling (GLMM) using SAS PROC MIXED (SAS Institute 1999), with treatment and time course as fixed factors, 'individual' as random effect, and outdoor cage temperature and body mass as covariates. By

including body mass as covariate, we assessed mass-independent BMR. The BMR (whole-organism and mass-independent) and body mass of the two PBS control groups did not differ from each other at any time (GLMM: $p > 0.08$ in all cases), so both PBS control groups were combined into a single control group. The fixed factor 'treatment', therefore, included three levels: birds from the ad libitum group injected with PHA, birds from the food-restricted group injected with PHA, and birds from both groups injected with PBS. A general linear model was performed to assess initial plover condition (before injection) in BMR and body mass. The means are given \pm SE.

Results

Basal metabolic rate

BMR just before injection was similar between treatments ($F_{2, 24} = 1.18$, $p = 0.32$). The trend in BMR over time differed significantly between treatments (Table 1). The induced food restriction-immune response overlap resulted in a significant reduction in the PHA_food-restricted birds' BMR compared to the PHA_ad libitum and control birds (Table 1; Fig. 1). The BMR decreased in PHA_food-restricted birds 48 h after immune activation, with the decrease peaking 72 h and 96 h after the immune challenge (Fig. 1).

In contrast, the PHA_ad libitum birds significantly increased their BMR above control values (time \times treatment: $F_{4, 72} = 4.01$, $p < 0.01$). This increase peaked 48 h after PHA-injection, when the BMR increment was 19.1% relative to the PBS-injected birds. We estimated the energy cost of mounting a CMI response to be 5.83 kJ per day (i.e., +15.6% BMR relative to controls). Return to BMR baseline values was reached within 10 d after termination of the experiment (Fig. 1).

Body mass

Body mass did not differ between treatments at the time of group formation (PHA_ad libitum birds: 42.31 ± 1.42 g, PHA_food-restricted birds: 41.45 ± 1.84 g, PBS birds: 38.16 ± 1.39 g; $F_{2, 24} = 2.16$, $p = 0.14$). The food-restricted birds' body mass just before PHA-injection (0 h) was on average 11.0% and 0.3% lower than that of the PHA_ad libitum and PBS birds, but these difference were not significant ($F_{2, 24} = 2.30$, $p = 0.12$). Body mass trend over the course of the experiment differed significantly between treatments (Table 1). PHA_ad libitum and control birds showed no significant body mass changes throughout the experiment (GLM: $p > 0.24$ in all cases). Food restriction per se did not cause a significant body mass loss throughout the experiment ($F_{4, 20} = 1.03$, $p > 0.42$), while the food restriction-immune response overlap led to a significant 11.6% body mass loss after PHA-injection ($F_{4, 24} = 45.90$, $p < 0.001$) (Fig. 2). The body masses in the three groups returned to the pre-experiment values within 10 d after the termination of the experiment.

Table 1. Mixed general linear model analysis testing for effects of experimental conditions on basal metabolic rate (BMR), cellular-mediated immune response (CMI), and body mass.

| | Covariates | | Fixed factor | | Random factor | |
|-----------|----------------------|----------------------|-----------------------|------------------------|-------------------------|-----------------------|
| | Body mass | Temperature | Treatment | Time | Treatment × time | Individual |
| BMR | F = 29.9 p < 0.01 | F = 0.23 p = 0.63 | F = 0.40 p = 0.67 | F = 1.54 p = 0.19 | F = 2.25 p < 0.01 | Z = 3.06 p < 0.05 |
| CMI | F = 0.38 p = 0.44 | F = 0.59 p = 0.44 | F = 0.67 p < 0.001 | F = 96.17 p < 0.001 | F = 29.52 p < 0.0001 | Z = 3.14 p < 0.001 |
| Body mass | — | F = 1.07 p = 0.3 | F = 3.56 p < 0.03 | F = 21.98 p < 0.001 | F = 4.38 p < 0.001 | Z = 3.42 p < 0.01 |

Wing web swelling

The wing web swelling increased significantly in PHA-injected plovers (in both *ad libitum* and food-restricted groups), peaking in both groups 24 h after PHA-injection (Fig. 3). Food-restricted birds always had significantly lower values of wing web swelling than *ad libitum* birds, and the time course of the immune response differed between groups (Fig. 3, Table 1).

Discussion

We did not find significant differences in neither whole-organism nor mass-independent BMR over time between PBS-injected plovers from the *ad libitum* and food-restricted group, so the food-restricted conditions *per se* did not cause a significant variation in BMR. However, we have shown that overlapping food restriction and CMI response in little ringed plovers triggered a significant BMR downregulation and diminished the CMI response. This suggests that when long-distance migratory birds such as little ringed plover are simultaneously faced with the two energy challenges, resources can be allocated between immunity and body mass maintenance. It is obvious that under natural conditions birds indeed face such simultaneous energy challenges, so that one should not look at energy challenges in isolation. This may also explain why single immune challenges can trigger

physiological and behavioural responses that are different from when individuals are subjected to extra constraints such as limitation in food availability (see Buehler et al. 2009).

A range of physiological processes such as moult (e.g., Dietz et al. 1992, Lindström et al. 1993, Klaassen 1995, Portugal et al. 2007, Vézina et al. 2009), egg production (e.g., King 1973, Nilsson and Råberg 2001, Vézina and Williams 2002, reviewed in Williams 2005), thermoregulation (e.g., King and Farner 1961, Grossman and West 1977, Cooper and Swanson 1994, Williams and Tieleman 2000, Arens and Cooper 2005), and mounting immune responses (e.g., Ots et al. 2001, Martin et al. 2003, Eraud et al. 2005, Nilsson et al. 2007, but see Svensson et al. 1998, Mendes et al. 2006) are reported as increasing the metabolic rate in both wild and captive birds. In contrast, other studies have found that, under certain circumstances or in physiological states such as result from limitation in food resources (e.g., Shapiro and Weathers 1981, Laurila et al. 2005, Wiersma et al. 2005, but see Vézina et al. 2009), or workloads (e.g., Bautista et al. 1998, Deerenberg et al. 1998, but see Nilsson 2002), reductions in avian metabolic rates can be expected. Since interactions among these multiple situations often occur in nature, there is a need for explanations of the possible metabolic strategies adopted by birds coping with overlapping constraints and their possible consequences for fitness. In an immunological context, such interactions

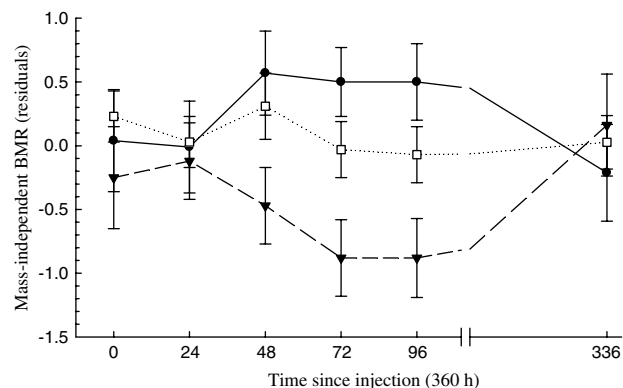


Figure 1. Mass-independent BMR (residuals from GLMM model, with body mass and temperature as covariates) of PHA_ *ad libitum* birds (●, n = 7), PHA_ *food-restricted* birds (▼, n = 7), and PBS_ *combined* birds (□, n = 13). Injection took place at 0 h. Values that reached 10 d after the finalization of the experiment are shown for reference (note break and scale change in x-axis). For statistical tests see Table 1.

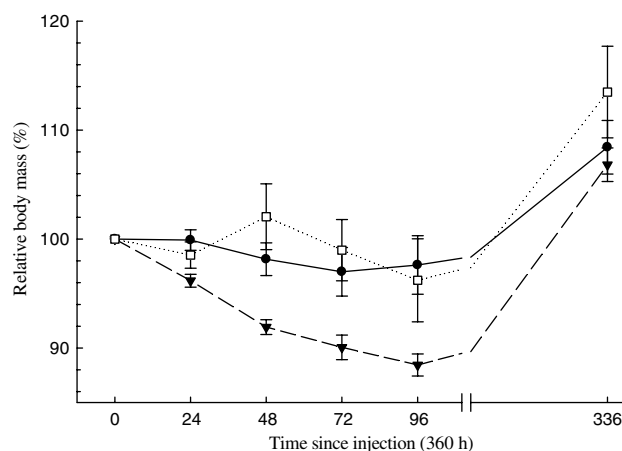


Figure 2. Relative body mass expressed in percent of body mass measured on d 0 of PHA_ *ad libitum* birds (●, n = 7), PHA_ *food-restricted* birds (▼, n = 7), and PBS_ *combined* birds (□, n = 13). Injection took place at 0 h. Values that reached 10 days after the finalization of the experiment are shown for reference (note break and scale change in x-axis). For statistical tests see Table 1.

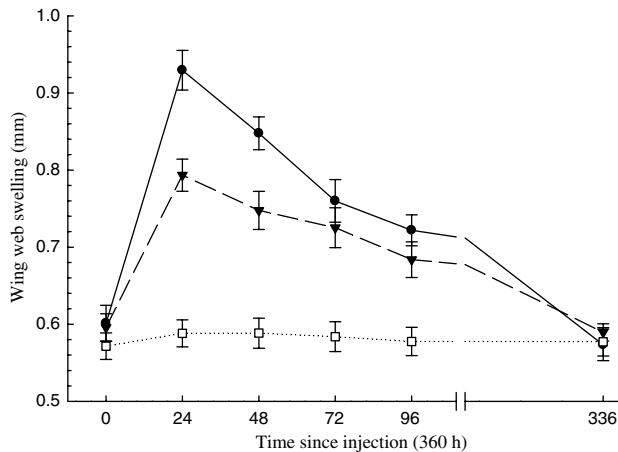


Figure 3. Time course of the CMI response to PHA in PHA_ad libitum birds (●, n = 7), PHA_food-restricted birds (▼, n = 7) and PBS_combined birds (□, n = 13). Injection took place at 0 h. Values reached 10 d after the finalization of the experiment are shown for reference (note break and scale change in x-axis). For statistical tests see Table 1.

could play a significant role in the prevalence of infections, and consequently negatively affect different aspects of fitness (e.g., reproduction, growth, or survival). It seems reasonable to expect that the degree of intensity of the physiological demands imposed by such interactions (i.e., potential trade-offs) would shape the optimal investment in immunity, with the more stressful interactions having more immunosuppressive effects. For example, trade-offs between investment in immunocompetence and investment in reproduction (considered to be an especially costly event in birds; Lindén and Møller 1989, Gustafsson et al. 1994, Nilsson and Svensson 1996) generally have patent immunosuppressive effects (Gustafsson et al. 1994, Deerenberg et al. 1997, Nordling et al. 1998, Hanssen et al. 2003, reviewed in Sheldon and Verhulst 1996, Martin et al. 2008). However, other less stressful trade-offs may not cause this expected immunosuppression (e.g., Ros et al. 1997, Hasselquist et al. 1999, Hasselquist et al. 2007, McGraw and Ardia 2007).

Birds under stressful conditions reduce self-maintenance by reducing mass-specific BMR, body mass, and the size of organs (e.g. Rønning et al. 2008). Specifically, non-breeding shorebirds such as red knot, when faced with food stress, may resort to energy saving mechanisms that involve reductions in BMR (Piersma et al. 2004). In our case, the BMR downregulation experienced by immunochallenged food-restricted plovers, therefore, could be an energy-saving strategy. We suggest that in the wild, where birds frequently encounter periods of low food availability, the relationship between metabolic rate and immune response could be confusing, and not reflect the real state of immunocompetence. The relationship between the immune response and metabolic rates in wild-captured birds needs to be interpreted with caution.

Little ringed plover challenged with PHA increased BMR by 15.6%. The large differences in metabolic adjustments to PHA-challenge found in non-migratory passerines species, ranging in magnitude from -25% to 32% for metabolic rate (see Lee et al. 2005 and Martin et al. 2006),

make difficult a comparison with our species. However, such an energy cost was of comparable magnitude to the 13% increase in BMR found by Mendes et al. (2006) in red knots mounting a humoral immune response against tetanus and diphtheria. In a recent review of avian eco-immunology, Ardia and Schat (2008) has pointed out that CMI response to PHA seems to entails greater energy costs than humoral immune responses. This observation highlights the need for more data on immune-related BMR adjustments in species other than non-migratory passerine species.

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