The Journal of Experimental Biology 213, 3810-3818

doi:10.1242/jeb.045484

Relationships between metabolic status, corticosterone secretion and maintenance of innate and adaptive humoral immunities in fasted re-fed mallards

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Accepted 17 August 2010

SUMMARY

The prolonged exposure of birds to environmental stressors known to affect energy status and glucocorticoid secretion may have several physiological consequences including a decrease in immunocompetence, further compromising the survival of individuals. However, the relationships between these parameters remain poorly understood. To this end, changes in body energy content, plasma corticosterone, adaptive (total plasma immunoglobulin Y; IgY) and innate (natural antibodies; NAbs) immune systems were assessed in female mallards (*Anas plathyrhynchos*) throughout prolonged fasts of different intensities and subsequent re-feeding. Plasma IgY and NAb scores were decreased by 36% and 50%, respectively, during phase II of fasting (protein-sparing phase) and by up to 40% and 80%, respectively, during phase III (protein-wasting phase), indicating a selective regulation of immune function. These results are consistent with the hypothesis of a trade-off between immune function and other energy-demanding activities. However, despite full repletion of fuel reserves and NAbs, only 76% of initial IgY levels were recovered, further supporting a trade-off between innate and adaptive branches of immunity. Although fasting induced significant increases in corticosterone levels to up to 6 times higher than baseline levels during phase III, baseline levels were recovered within 1 day of re-feeding. Our data do not support the hypothesis of a direct regulation of immunocompetence by corticosterone, at least during periods of energy repletion. Finally, the mismatch between the kinetics of body fuels and the two arms of the immune system during fasting and re-feeding suggests that variations in immune system components do not strictly covary with body mass under fluctuating food conditions.

Key words: environmental variations, fuel reserves, plasma immunoglobulin Y, natural antibodies, trade-off.

INTRODUCTION

Over the course of their lifetime, organisms have to sustain seasonal fluctuations in energy availability as well as energy expenditure. Under such constraints the total energy budget must be optimally divided between different physiological functions, such as thermoregulation, locomotion or reproduction, which are critical to different degrees for immediate survival (Gwinner, 1990; Schmidt-Nielsen, 1990; Blem, 2000). In this context, the total energy balance or physiological condition might also determine the ability of individuals to mount an optimal immune response (French et al., 2007a; French et al., 2007b; Love et al., 2008). If so, the prolonged exposure to stressful factors such as strenuous exercise (Hoffman-Goetz and Pedersen, 1994; Nierman and Nehlsen-Cannarella, 1994) or reproduction (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000) may have several physiological consequences including a decrease in immunocompetence, which in turn may compromise survival. Reciprocally, the maintenance and deployment of the immune system are supposed to induce energetic or nutritional costs (Lochmiller and Deerenberg, 2000). For example, the experimental induction of the immune system in mice (Demas et al., 1997) and in house sparrows (*Passer domesticus*) (Martin et al., 2003) increased the energy expenditure of individuals, further negatively affecting their energy metabolism. Nevertheless, some studies failed to demonstrate the expected trade-off: the cost of mounting an immune response against an antigen was shown to be low in blue tits (*Parus caeruleus*) (Svensson et al., 1998) and negligible in zebra finches (*Taeniopygia guttata*) (Verhulst et al., 2005). However, in these experiments birds had access to *ad libitum* food enabling them to potentially compensate for any energetic cost through food intake (Nilsson, 2002; Amat et al., 2007).

Moreover, even when assuming that the immune system is being traded off against other resource-demanding activities in situations of energetic constraint, trade-offs might not apply equally to all the components of immune function (Norris and Evans, 2000; Klasing, 2004; Viney et al., 2005). The vertebrate immune system comprises both innate and adaptive immune pathways. The innate immune system provides a fast protection against pathogens while the adaptive immune system confers a delayed but more specific protection (Roitt et al., 2001). In birds, three classes of antibodies are expressed (Lundqvist et al., 2006): IgM and IgY, two circulating immunoglobulins, and IgA found in various body secretions (Higgins and Warr, 1993). Natural antibodies (NAbs), which mainly belong to the IgM class, are the immunoglobulins of the innate immune system (Casali and Notkins, 1989; Ochsenbein and Zinkernagel, 2000; Palacios et al., 2009) while IgY are the main systemic antibodies of the adaptive immune system (Lundqvist et al., 2006). By comparing the basal metabolic rate of knockout mice lacking adaptive immune responses with that of control mice, Råberg and colleagues (Råberg et al., 2002) concluded that maintenance is costlier for the innate immune system than for the adaptive immune system. Accordingly, Lee (Lee, 2006) predicted that both constitutive and induced innate immune systems should be down regulated to the benefit of acquired defences in slow-living species experiencing high energy demands. However, the maintenance of the constitutive innate immunity was shown to be less costly than deploying the adaptive immune system (induced immunological

response) in red knots (*Calidris canutus*) subjected to food shortages (Buehler et al., 2009). Likewise, Bourgeon and colleagues (Bourgeon et al., 2007a) showed in female common eiders (*Somateria mollissima*) that the induced innate immune response was maintained throughout the incubation fast while both cellular and humoral acquired components were negatively affected. Whether such trade-offs within the immune system apply to birds exposed to different nutritional stressors remains to be determined.

Trade-offs between the immune system and life-history traits were hypothesized to be mainly mediated by corticosterone playing a causal role in immunosuppression (Nelson and Demas, 1996). In non-mammalian tetrapods, glucocorticoids – which mediate adaptive physiological and behavioural responses to stressful events such as a large depletion of energy (Robin et al., 1998; Sapolsky et al., 2000) – could hence establish a close endocrine link between immunocompetence and stress (Apanius, 1998). However, humoral immunity did not significantly covary with natural corticosterone concentrations in breeding barn swallows (*Hirundo rustica*) (Saino et al., 2002). Moreover, while the administration of exogenous corticosterone did not affect T cell-mediated immunity (CMI) of captive common eiders (Bourgeon and Raclot, 2006) or constitutive innate immunity of wild barn owl nestlings (*Tyto alba*) (Stier et al., 2009), it did suppress humoral immunity of both species, further suggesting that corticosterone can selectively suppress different immune components. Therefore, it is still unclear whether naturally elevated corticosterone levels would induce immunosuppression, and the relationships between corticosterone and the different arms of the immune system need to be clarified. For example, although Eraud and colleagues (Eraud et al., 2008) showed that hatching asynchrony in collared doves (*Streptopelia decaocto*) resulted in junior chicks having lower CMI and higher corticosterone levels than senior chicks, the role of corticosterone as an immunosuppressor remains unclear.

Thus, to shed more light on the relationships between metabolic status, body energy content, corticosterone and the immune system we studied the impact of experimental modification of energy reserves (total fasts of different intensities followed or not by re-feeding) on these parameters. Long-term fast is first characterized by exhaustion of glycogen reserves (phase I) before a long period of protein sparing and preferential mobilization of fat stores (lipolysis; phase II), which is followed by a period of increased net protein catabolism (proteolysis; phase III) (Le Maho et al., 1981; Robin et al., 1988). Furthermore, phase III is associated with an increase in corticosteronaemia hypothesized to be implicated in the stimulation of proteolysis (Cherel et al., 1988; Robin et al., 1998).

In the present study, captive female mallards (*Anas plathyrhynchos*) of known age were subjected to fasts of various intensities, and subsequently some were re-fed until partial or full restoration of their initial body energy reserves. Total body energy content and adiposity, together with total plasma protein, plasma corticosterone, IgY and NAb levels were subsequently assessed in relation to metabolic status to determine the relationships between adaptive and innate immune components and body reserves. We predicted that food restrictions should have a negative impact on both branches of the immune function of female mallards but that these effects should be quickly reversed by re-feeding. However, the degree to which each immune component might be affected was more difficult to predict (see conflicting results of studies cited above) as it might be species and/or context specific. Finally, we proposed that natural increases in corticosterone levels might act as a direct mechanism for variations in immune parameters.

MATERIALS AND METHODS

Study subjects

The experiment was carried out between February and April 2004 on 49 captive female mallards from the wild species *Anas platyrhynchos* L. (9–10 months old) purchased from the Canarderie de la Ronde (registered breeding field station, Céré-la-Ronde, France). The experiment complied with the Principles of Animal Care publication no. 86-23, revised 1985 of the National Institutes of Health, and with current legislation (L87-848) on animal experimentation in France.

Once in the laboratory, ducks were placed in up to six communicating outdoor aviaries where they were subjected to ambient temperatures and natural photoperiods. From the day they arrived and until the start of the experiments, all females were fed *ad libitum* with a balanced commercial food (Standard duck food 7751, Sanders Corporation, Einville au Jard, Lorraine, France) and provided with running water in 1 m^2 basins. During this adaptation period, birds were weighed (±1 g) every day between 10:00 h and 12:00 h (local time) until their body mass stabilized (about 2 weeks after their arrival). Birds hosted in the same aviary were randomly assigned to different experimental groups.

Experimental groups

Prior to nutritional manipulation, birds were split into seven experimental groups (seven birds per group). Average initial body mass and size (tarsus length) did not differ significantly between groups (Table 1). The birds were then left to feed *ad libitum*, or put on the fasting, or fasting and then re-feeding regime for various durations so that at the time of killing the nutritional status in each group was as follows: fed *ad libitum* (Fed), early fasting (48 h), fasting up to the transition between the protein-sparing and the protein-wasting stage (phase II; PII) or up to the protein-wasting stage (phase III; PIII), fasting up to phase III and then re-feeding for 1 (R1) or 3 days (R3), or until full recovery of initial body mass (Rt; mean re-feeding duration: 15.6±2.1 days, N=7). In the last four groups, the fasting duration as well as the body mass reached at the end of the fast were not significantly different (Table 1). To avoid any time-related processes (infection, endocrine changes, etc.), the mean date on which the birds were killed did not significantly differ between the seven experimental groups (one-way ANOVA: $F_{6,42}$=0.99, P=0.44; Table 1). Birds were weighed every day and the metabolic stages of fasting (phase II and III) were determined using the changes in the specific daily body mass dm/mdt (i.e. the body mass loss per unit of time per unit of body mass). Briefly, dm/mdt values decrease during the early stage of fasting (phase I), are maintained at a low level during phase II and increase sharply in phase III (Le Maho et al., 1981; Cherel et al., 1988). The metabolic stage was subsequently confirmed by the changes in plasma concentrations of β-hydroxybutyrate (β-OH, a ketone body, by-product of fatty acid breakdown) and uric acid (the end-product of proteolysis in birds). Indeed, these indexes (dm/mdt, β-OH and uric acid) were shown to reflect the shifts in body fuel utilization during prolonged fasting (Le Maho et al., 1981; Cherel et al., 1988; Robin et al., 1988).

Blood sampling

Since manipulation increases plasma corticosterone levels, only one bird per aviary was caught per day between 08:00 h and 09:00 h before any other disturbance occurred. Blood (5 ml) was sampled from the brachial vein within 135±11 s (N=49). It was immediately transferred into heparinized tubes and kept on crushed ice before being centrifuged for 10 min at 1400 ***g*** within 1 h. Plasma samples

Table 1. Profiles and fasting durations of fasted and fasted then re-fed female mallards

Experimental groups	Fed (*N*=7)	48 h (*N*=7)	PII (*N*=7)	PIII (*N*=7)	R1 (*N*=7)	R3 (*N*=7)	Rt (*N*=7)
Tarsus length (cm)	5.62±0.11[a]	5.44±0.08[a]	5.44±0.05[a]	5.61±0.06[a]	5.59±0.06[a]	5.57±0.12[a]	5.56±0.07[a]
Initial body mass (g)	922±50[a]	912±29[a]	920±32[a]	915±28[a]	923±39[a]	905±46[a]	905±38[a]
Body mass at the end of fasting (g)	–	810±32[a]	658±25[b]	567±11[c]	564±23[c]	548±12[c]	551±17[c]
Body mass at the end of re-feeding (g)	922±50[a]	–	–	–	658±33[b]	767±25[b]	942±39[a]
Fasting duration (days)	0.00±0.00[a]	2.00±0.00[a]	11.43±0.92[b]	12.00±1.94[b]	12.71±0.86[b]	12.57±1.02[b]	11.86±1.16[b]
Sampling day	65.57±4.90[a]	66.00±5.31[a]	63.57±3.82[a]	63.29±3.64[a]	66.29±3.99[a]	62.00±3.23[a]	74.86±4.51[a]
Total body protein mass (g)	161.25±8.14[a]	145.49±6.62[a,b]	128.59±5.79[b,c]	106.95±3.25[d]	112.35±6.93[c,d]	121.74±4.91[c,d]	159.34±6.61[a]
Total body lipid mass (g)	94.42±14.18[a,b]	87.31±10.97[a]	28.78±4.33[c,d]	10.05±0.77[c]	13.53±3.14[c]	36.69±4.60[d]	116.03±7.89[b]
Plasma β-hydroxybutyrate (mmol l^{-1})	0.16±0.06[a]	4.12±0.80[b]	2.68±0.87[c]	1.14±0.09[a]	0.12±0.03[a]	0.05±0.01[a]	0.18±0.03[a]
Plasma uric acid (mmol l^{-1})	0.52±0.06[a,b]	0.29±0.05[a]	0.76±0.20[b]	1.99±0.33[c]	0.41±0.09[a,b]	0.51±0.06[a,b]	0.35±0.08[a,b]

Fed: fed *ad libitum*; 48 h: fasted for 48 h; PII, fasted up to the end of phase II of fasting; PIII, fasted up to entry into phase III of fasting; R1, fasted up to entry into phase III and then re-fed for 1 day; R3, fasted up to entry into phase III and then re-fed for 3 days; Rt, fasted up to entry into phase III and then re-fed until recovery of initial body mass.

Values are means ± s.e. For each row, lower case letters indicate a significant difference between groups (Fisher's LSD *post-hoc* tests).

were stored at –20°C and subsequently used to assess levels of plasma metabolites, IgY and NAbs, plasma total proteins and corticosterone. All biochemical parameters were assessed in the same session.

Body composition assessment

Following blood sampling, the birds were weighed (±1 g), killed using a guillotine and plucked. The digestive tract was then cleared of its content and showed no evidence of intestinal parasites. The entire carcass was freeze-dried to constant mass and ground under liquid nitrogen to obtain a homogeneous powder for analysis. Nitrogen content was determined in triplicate using 150–200 mg aliquots following the Kjeldahl method. Protein content was calculated as nitrogen content ×6.25 (Campbell and Leatherland, 1980). Lipid content was determined in duplicate using 1 g aliquots following a gravimetric method derived from Folch et al. (Folch et al., 1957), i.e. lipids were extracted with a chloroform/methanol solution (2/1, v/v). Overall adiposity was calculated as the percentage of total fat mass contributing to body mass. The metabolizable energy content of each animal was calculated assuming an energetic equivalence of 39.3 and 18.0 kJ g^{-1} for lipid and protein, respectively (Schmid-Nielsen, 1990). The negligible amount of energy stored as carbohydrates was not taken into account (Cherel et al., 1992).

Assessment of plasma metabolites, total plasma proteins, plasma corticosterone, IgY and NAbs

Plasma uric acid and β-hydroxybutyrate levels were estimated using enzymatic methods on whole and deproteinized plasma, respectively (Scheibe et al., 1974; Williamson and Mellanby, 1974). Plasma total protein levels were determined using the Bradford method (Bradford, 1976).

Corticosterone concentration was determined by radio-immunoassay (RIA) using an ^{125}I RIA double antibody kit (ICN Biomedicals, Costa Mesa, CA, USA) designed for mouse and rat corticosterone analysis. Kits were validated for avian plasma using standard RIA analysis techniques described in common eiders (Bourgeon and Raclot, 2006) and American kestrels (*Falco sparverius*) (Love et al., 2003). The corticosterone RIA had an intra-assay variability of 7.1% (*N*=10 duplicates) and an inter-assay variability of 6.5% (*N*=15 duplicates). Corticosteronaemia is known to increase with the duration of handling but we checked that there were no significant differences in the mean time for blood sampling between the seven groups ($P>0.05$) and no significant relationship between the timing for blood sampling and corticosteronaemia within a group ($P>0.05$).

A sensitive ELISA (enzyme-linked immunosorbent assay) method was used to determine the amount of total IgY in mallard plasma (diluted to 1/16,000 in carbonate–bicarbonate buffer, 0.1 mol l^{-1}, pH 9.6). This method using commercial anti-chicken antibodies has so far been validated in seven wild avian species (Martinez et al., 2003) including Anseriforms [for the detailed protocol, see Bourgeon et al. (Bourgeon et al., 2006)]. The level of plasma IgY, the most important serum protein in the avian humoral immune response (Roitt et al., 2001), was subsequently used to assess the overall humoral adaptive immune function. However, the interpretation of this immune measure requires caution because it could also reflect blood parasite load (Morales et al., 2004). Furthermore, it could be influenced by the sex (Møller et al., 1998) and/or the age of organisms (Cichon et al., 2003) further leading to misinterpretation of physiological data and concealing significant effects of environmental factors (Lavoie, 2006; Martin et al., 2006a). Therefore, in an attempt to control for the main confounding factors responsible for inter-individual differences in immunoglobulin levels, we first worked on captive birds, which are, to a large extent, free of parasites that are shown to lower immune function (Ots and Hõrak, 1998; Fargallo and Merino, 2004). Additionally, only females were used, to avoid potential inter-sex differences in the allocation of resources to immune function (Møller et al., 1998; Moreno et al., 2001; Love et al., 2005; Love et al., 2008). Finally, all the birds used in our experiment belonged to the same age cohort.

Plasma NAbs are major humoral components of innate immunity (Palacios et al., 2009). The haemagglutination assay was carried out in 96-well plates using a small amount of plasma (50 µl) following Matson et al. (Matson et al., 2005). Plasma was introduced into column 1 and serially diluted (1:2) with phosphate-buffered saline (PBS; Sigma-Aldrich, Lyon, France) from column 2 through to column 11, with column 12 serving as a negative control (PBS only). A fixed amount of a sheep red blood cell (SRBC) suspension was added to each well and the plate was then incubated for 90 min at 37°C. The SRBCs were provided by the slaughterhouse (Haguenau, Alsace, France) under veterinarian authorization and

conserved in 50% Alsever's solution (Sigma-Aldrich). The SRBCs were washed 4 times as previously described (Matson et al., 2005) and resuspended in 0.1% PBS. As recommended by Matson et al. (Matson et al., 2005), the plate was tilted to an angle of 45 deg for 20 min at room temperature to enhance agglutination visualization. The agglutination titre (NAb score) for each individual was scored blindly by the same experimentator (S.G.) and reflects levels of NAbs only. Nevertheless, because plasma samples were serially diluted, NAb scores do not directly reflect the plasma concentrations of NAbs. The inter-assay variability was 5.1% (N=5 duplicates).

Statistical analyses

Statistical analyses were conducted using SPSS 17.0.2 (SPSS Inc., Chicago, IL, USA). Values are means ± standard error (s.e.). When data were not normally distributed (Kolmogorov–Smirnov test, $P<0.05$), values were either log-transformed (plasma corticosterone, β-hydroxybutyrate and uric acid levels) or square-root transformed (agglutination scores) to meet parametric assumptions (Kolmogorov–Smirnov test, $P>0.05$ in all cases). One-way ANOVAs were used to test for the effects of the nutritional treatment on body mass, body energy content (body lipid and protein masses), adiposity, and plasma concentrations of β-hydroxybutyrate, uric acid, total proteins, corticosterone, IgY and NAbs. Linear regression analyses were used to assess the relationships between these parameters. The criterion for significance was set at $P<0.05$.

RESULTS

Validation of the experimental design

After stabilization during phase II, dm/dmdt values were 2.5–3.5 times higher in phase III, during the last 2 days of the fast (one-way ANOVA: $F_{4,24}$=61.40, $P<0.001$). Plasma β-hydroxybutyrate and uric acid concentrations differed significantly between groups (one-way ANOVA: $F_{6,42}$=52.54, $P<0.0001$; $F_{6,42}$=9.58, $P<0.0001$, respectively). β-Hydroxybutyrate values were higher after a 48 h fast and at the end of phase II than in the other groups, whereas the highest uric acid values were observed during phase III (Table 1). The fluctuations of these three indexes (dm/dmdt, β-hydroxybutyrate and uric acid) between the different groups indicate distinct metabolic stages previously described in fasting birds (Le Maho et al., 1981; Cherel et al., 1988; Robin et al., 1988).

Effects of fasting and re-feeding on body mass, adiposity and total body energy content

Body mass was significantly affected by the nutritional treatments (one-way ANOVA: $F_{6,42}$=18.47, $P<0.0001$) (Fig. 1A). In comparison to fed birds, body mass was 29% and 40% lower at the end of phase II and during phase III, respectively. Initial body mass was restored to 26% and 61%, respectively, in females re-fed for 1 or 3 days after phase III. Initial body mass was recovered after 15.6±2.1 days (N=7) of re-feeding in Rt females.

Total body lipid mass was significantly lowered by 70% at the end of phase II and by 90% in phase III compared with that of fed females (one-way ANOVA: $F_{6,42}$=29.79, $P<0.0001$). In agreement with this, adiposity significantly decreased from phase II and reached its lowest value in PIII and R1 females (one-way ANOVA: $F_{6,42}$=40.26, $P<0.0001$) (Fig. 1B). Body protein mass was significantly decreased by 20% and 35% at the end of phase II and phase III, respectively (one-way ANOVA: $F_{6,42}$=12.49, $P<0.0001$). Accordingly, the total body energy content started to decrease significantly from phase II (one-way ANOVA: $F_{6,42}$=29.02, $P<0.0001$), with phase III females showing the lowest body energy content of all groups (66% lower than fed birds) (Fig. 1C). The body

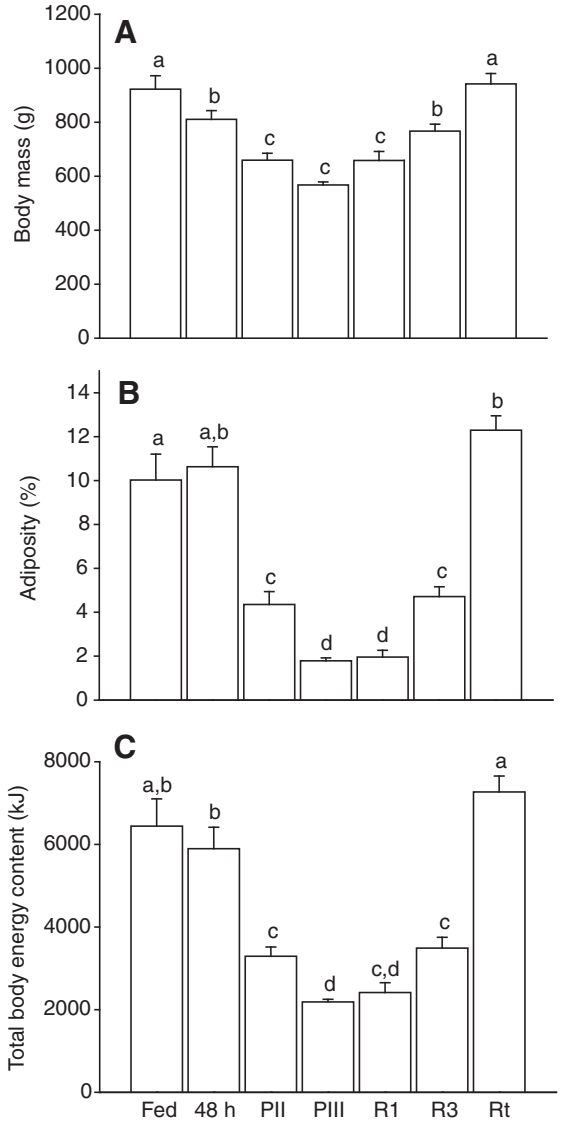

Fig. 1. Body mass (A), adiposity (B) and total body energy content (C) at various metabolic stages in fasted and fasted then re-fed female mallards. Fed: fed *ad libitum*; 48 h: fasted for 48 h; PII, fasted up to the end of phase II of fasting; PIII, fasted up to entry into phase III of fasting; R1, fasted up to entry into phase III and then re-fed for 1 day; R3, fasted up to entry into phase III and then re-fed for 3 days; Rt, fasted up to entry into phase III and then re-fed until recovery of initial body mass. Values are means ± s.e., N=7 for each group and parameter. Different letters indicate significant differences between groups (Fisher's LSD *post-hoc* tests).

energy content significantly increased after 3 days of re-feeding [PIII *versus* R3 birds; Fisher's least significant difference (LSD) *post-hoc* test, P=0.02] and, together with the lipid and protein masses, was fully recovered in Rt females (Fig. 1C).

Effects of fasting and re-feeding on plasma concentrations of total proteins, corticosterone, IgY and NAbs

Total plasma protein concentration remained unchanged up to the end of phase II but was significantly decreased by 40% during phase III (one-way ANOVA: $F_{6,42}$=15.09, $P<0.0001$) (Fig. 2A). As early as 3 days after re-feeding, total plasma proteins were 44% higher than levels reached during phase III (Fisher's LSD *post-hoc* test, P=0.01). Total plasma proteins in Rt females recovered to levels

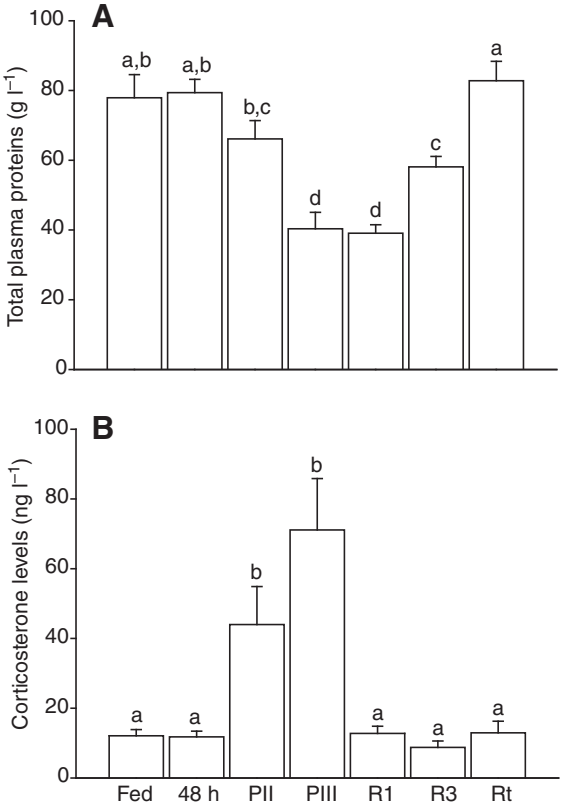

Fig. 2. Plasma total proteins (A) and plasma concentration of corticosterone (B) for various metabolic stages in fasted and fasted then re-fed female mallards. Refer to Fig. 1 for complete definition of the different experimental groups. Values are means ± s.e., *N*=7 for each group and parameter. Statistical tests were run using log-transformed plasma concentrations of corticosterone to meet parametric assumptions. Different letters indicate significant differences between groups (Fisher's LSD *post-hoc* tests).

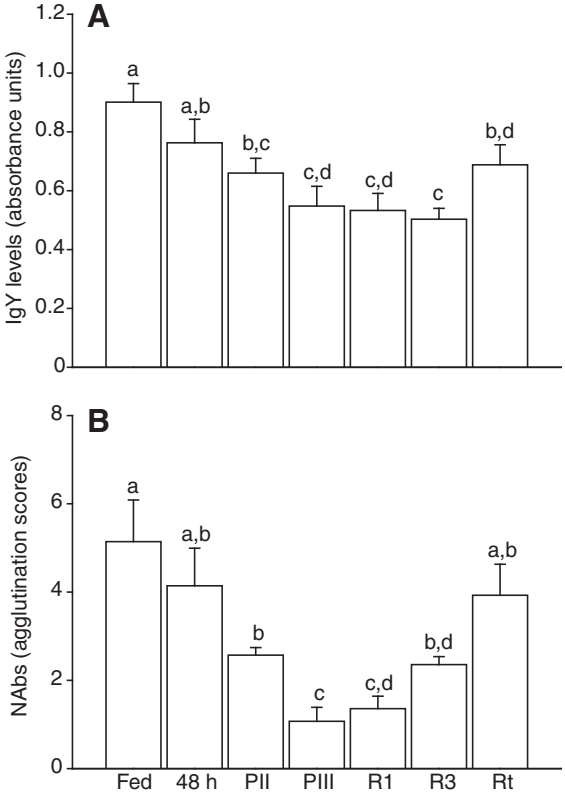

Fig. 3. Plasma concentration of immunoglobulin Y (IgY; A) and natural antibodies (NABs; B) for various metabolic stages in fasted and fasted then re-fed female mallards. Refer to Fig. 1 for complete definition of the different experimental groups. Values are means ± s.e., *N*=7 for each group and parameter. Statistical tests were run using square-root transformed plasma NAbs to meet parametric assumptions. Different letters indicate significant differences between groups (Fisher's LSD *post-hoc* tests).

similar to those observed in fed birds (Fisher's LSD *post-hoc* test, P=0.47) (Fig. 2A). Finally, there was a significant positive relationship between total body protein mass and total plasma proteins (linear regression: R^2=0.48, N=49, P<0.0001), further suggesting that proteins in body tissues and fluids follow the same pattern during fasting and re-feeding.

Plasma corticosterone levels recorded in fed birds were maintained at baseline levels after 48 h of fasting while they were significantly (4 and 6 times) higher during phase II and phase III, respectively (one-way ANOVA: $F_{6,42}$=17.36, P<0.0001) (Fig. 2B). Baseline levels (observed in fed birds) were restored after 1 day of re-feeding (Fig. 2B). While corticosterone levels were not significantly related to body mass (R^2=0.01, N=49, P=0.51), they were negatively correlated to body energy content (R^2=0.16, N=49, P=0.005).

A significant decrease in IgY levels was observed during phase II and reached 40% in phase III birds (one-way ANOVA: $F_{6,42}$=5.40, P=0.0003) (Fig. 3A). Even after 3 days of re-feeding, IgY levels were not significantly higher than the lowest levels recorded during phase III (Fisher's LSD *post-hoc* test, P=0.60) (Fig. 3A). Moreover, whereas total body energy content and total plasma proteins were fully restored in Rt birds (Fig. 1C and Fig. 2A, respectively) only 76% of the IgY levels observed in fed birds were recovered (fed *versus* Rt birds; Fisher's LSD *post-hoc* test, P=0.02) (Fig. 3A). Despite IgY and plasma proteins showing different kinetics during re-feeding, a weak but significant positive relationship was observed between the two parameters (R^2=0.21, N=49, P=0.001). Likewise, IgY levels were positively correlated to body mass (R^2=0.10, N=49, P=0.01), body energy content (R^2=0.19, N=49, P=0.002), total body protein and lipid masses (R^2=0.15, N=49, P=0.005; R^2=0.19, N=49, P=0.002, respectively), and adiposity (R^2=0.21, N=49, P=0.001). However, plasma IgY and corticosterone were not significantly related (R^2=0.03, N=49, P=0.21). The estimates for each of these relationships are shown in Table 2.

Agglutination scores, strictly reflecting the level of NAbs, decreased significantly from 5.1±0.9 in the fed state to 2.6±0.2 in phase II and up to 1.1±0.3 during phase III (one-way ANOVA: $F_{6,42}$=8.01, P<0.0001) (Fig. 3B). However, 3 days of re-feeding were enough to significantly increase agglutination titres above the lowest scores reached during phase III (Fisher's LSD *post-hoc* test, P=0.01) (Fig. 3B). Moreover, Rt birds showed scores not significantly different from those observed in fed birds (Fisher's LSD *post-hoc* test, P=0.30) (Fig. 3B). Agglutination scores were significantly and positively related to total plasma proteins (R^2=0.51, N=49, P<0.0001) (Fig. 4), body mass (R^2=0.21, N=49, P=0.001), body energy content (R^2=0.25, N=49, P<0.0001), total body protein and lipid masses (R^2=0.20, N=49, P=0.001; R^2=0.24, N=49, P<0.0001, respectively), and adiposity (R^2=0.31,

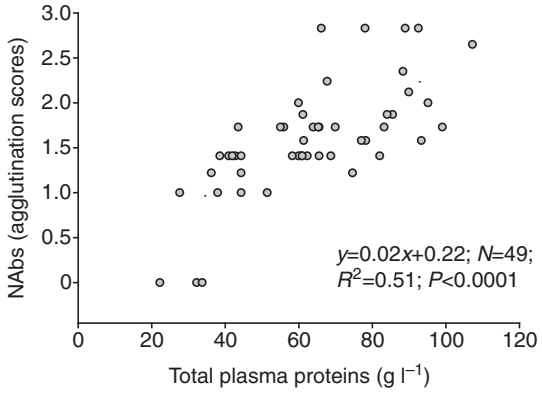

Fig. 4. Relationship between total plasma proteins and plasma NAbs (square-root transformed) in female mallards that were fasted up to phase III then re-fed until complete restoration of initial body mass after they reached phase III. Each point represents an individual value.

N=49, P<0.0001). By contrast, there was a negative relationship between agglutination scores and plasma corticosterone (R^2=0.09, N=49, P=0.02). The estimates for each of these relationships are shown in Table 2.

Finally, there was a significant positive relationship between plasma IgY and agglutination scores (R^2=0.28, N=49, P<0.0001; Table 2), with birds showing high IgY levels also showing high levels of NAbs.

DISCUSSION

In the current study, we found that fasting had a negative effect on both plasma IgY levels (adaptive immunity) and agglutination scores (reflecting NAbs; innate immunity) which decreased significantly during phase II of fasting and by up to 40% and 80% at the end of phase III, respectively. Similarly, fasting captive male king penguins (*Aptenodytes patagonicus*) showed a 40% decrease in their IgY levels when reaching phase III of fasting (Bourgeon et al., 2007b). Overall, our results provide evidence for a trade-off between resources available to body maintenance and both arms of the immune system in female mallards, further supporting the hypothesis of an energetic cost associated with the maintenance of the immune system (Viney et al., 2005). During energy deficit episodes, body reserves might be hierarchically allocated to other energy costly activities more critical to survival than immunity, such as, among others, thermoregulation and/or fat store maintenance (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Buehler et al., 2008). Nevertheless, even during the most protein-demanding fasting phase (phase III), levels of IgY and NAbs, which are both proteins, never reached null values, suggesting that a basal immunity is maintained even when body energy reserves reach their lowest levels. Indeed, fully compromised immune function would be likely to lead to a greater susceptibility to infectious diseases, while a partially effective immune response can ensure a greater fitness at a lower cost (Medley, 2002; McNamara and Buchanan, 2005; Viney et al., 2005).

In the current study, innate immunity decreased much more than adaptive immunity during phase III of fasting. This result is in line with the hypothesis suggesting that innate immunity is more energy consuming than adaptive immunity (Lochmiller and Deerenberg, 2000; Råberg et al., 2002). However, the extent of the decrease of each component might not necessarily lead to a proportional cut in its respective cost. Although NAbs suffered a greater reduction than IgY levels during fasting, innate immunity was paradoxically restored more rapidly than adaptive immunity during re-feeding. Overall, the mismatch between the kinetics of depletion and restoration of the two immune parameters supports the hypothesis of an immune redistribution, i.e. a trade-off within different branches of the immune system (Martin et al., 2006b) (but see Gasparini et al., 2009). Nevertheless, this antagonistic interaction between innate and adaptive immunity cannot solely be explained by an energy-based trade-off and the underlying mechanisms remain unclear. First, independently of its cost, innate immunity could be restored as a priority because of the higher immunological benefit it might confer. For example, since the gut mucosa is an important route for infection, re-feeding leads to modifications of the gut flora which in turn stimulate the secretion of NAbs (Manz et al., 2005). Additionally, innate immunity might be more closely linked to variations in body reserves than adaptive immunity. Although IgY and NAbs are both proteins (immunoglobulins), we showed that plasma variations of NAbs are more strongly related to concentrations of total plasma proteins than IgY, independent of the treatment. Innate immunity might therefore vary more in relation to protein catabolism during

Table 2. Matrix of parameter estimates (intercept, slope, R^2, F- and P-values) for linear regressions relating dependent and independent variables of fasted and fasted then re-fed female mallards

		Independent variables								
Dependent variables		Total plasma proteins	Body mass	Body energy content	Total body protein mass	Total body lipid mass	Adiposity	Plasma cortico-sterone	NAbs	IgY
IgY	Intercept	0.359	0.307	0.471	0.241	0.548	0.518	0.779	0.376	*
	Slope	0.005	0.000	4.25×10^{-5}	0.003	0.002	0.021	–0.101	0.176	*
	R^2	0.21	0.10	0.19	0.15	0.19	0.21	0.03	0.28	*
	F	13.88	6.58	10.99	8.62	10.82	12.78	1.64	19.88	*
	P	0.001	0.01	0.002	0.005	0.002	0.001	0.21	<0.0001	*
NAbs	Intercept	0.221	0.125	0.919	0.076	1.201	1.065	2.285	*	0.486
	Slope	0.022	0.002	0.000	0.011	0.007	0.081	–0.567	*	1.692
	R^2	0.51	0.21	0.25	0.20	0.24	0.31	0.09	*	0.28
	F	48.22	13.76	16.71	12.86	16.41	22.59	5.86	*	19.88
	P	<0.0001	0.001	<0.0001	0.001	<0.0001	<0.0001	0.02	*	<0.0001

Variables are plasma immunoglobulin levels (IgY; absorbance units), natural antibodies (NAbs; agglutination scores), total plasma proteins (g l^{-1}), body mass at the time of killing (g), total body energy content (kJ), total body protein and lipid mass (g), adiposity (%) and plasma corticosterone (ng l^{-1}).
To meet parametric assumptions, plasma corticosterone was log-transformed while plasma NAbs were square-root transformed.
N=49 mallards in all cases.

fasting, and to protein synthesis during re-feeding, and thus be more specific to the changes in body reserves than the acquired immunity. This further raises the question of the relationships between immune parameters and nutritional status.

Although components of both arms of the immune system decreased during fasting, they did not strictly vary in synergy with the depletion of body reserves. Namely, while IgY and NAbs showed a significant decrease in their plasma levels at the end of phase II, the diminution of body and plasma proteins was slow during that phase, as suggested in previous studies (Le Maho et al., 1981; Robin et al., 1988). Likewise, during re-feeding, body fuels and innate and adaptive immunity followed different restoration patterns. Indeed, when plasma proteins along with body energy reserves and body mass were fully recovered at the end of the re-feeding period, only NAbs were restored to their pre-fasting values whereas plasma IgY levels remained significantly (25%) lower than the initial values observed in fed birds. The difference in depletion and restoration rates of the two immunoglobulin classes can be explained by the turn-over of their secretive cells. Indeed, in mammals, NAbs (IgM) are secreted by activated B1 lymphocytes, short-lived cells with a constant turn-over, whereas specific antibody levels such as IgY are thought to be maintained over time by both short- and long-lived cells from the B2 lineage in order to protect the organism against re-infection (Manz et al., 2005). Nevertheless, whether these mechanisms apply to birds is unknown. Furthermore, the extent to which such a decrease might affect the efficiency of the adaptive component in the case of exposure to pathogens requires further investigation, such as functional assays of the humoral immune response.

Previous studies on captive birds have shown that T-cell mediated acquired immunity is directly and positively linked to food intake and body mass in Northern bobwhite chicks (*Colinus virginianus*) (Lochmiller et al., 1993), zebra finches (Birkhead et al., 1999; Love et al., 2008) and yellow-legged gulls (*Larus cachinnans*) (Alonso-Alvarez and Tella, 2001). Likewise, it was shown that trade-offs between reproduction and immune capacity were only found when food resources were experimentally reduced in female zebra finches (Love et al., 2008) and female tree lizards (*Urosaurus ornatus*) (French et al., 2007a). Our results tend to support the so-called energy-based (or resource-driven) trade-off between immunity and life-history stages. Nevertheless, they also point out that the effect of various life-history traits on immunity might be confounded by metabolic status. Namely, while fed (control) and Rt females did not differ in body mass, total body energy content, plasma proteins or corticosterone levels, Rt females showed significantly lower acquired immunity (IgY levels) but similar innate immunity (levels of NAbs) than control females. These results emphasize that variations in immune function do not seem to follow a simple energy allocation model in which the highest body masses would be associated with the highest immune responses (Mauck et al., 2005). Therefore, various branches of the immune system observed at one stage in an individual's life cycle may not necessarily reflect inherent quality differences between individuals when environmental conditions such as food availability vary. This emphasizes the need for controlling for body condition when carrying out experiments to assess the effects of life-history traits on immunocompetence of birds, as previously suggested (Alonso-Alvarez and Tella, 2001; Brzek and Konarzewski, 2007). In natural conditions, sub-optimal feeding conditions (i.e. when food intake is less than that required for energy balance) and poor environmental factors experienced during winter can negatively affect body condition, further compromising survival. A feeding experiment showed that captive mallards fed food of sub-optimal nutritional value (soybeans) decreased their body mass by up to 35–40% (Loesch and Kaminski, 1989). This loss of body mass is similar to that experienced by the PIII birds in the current experiment. This implies that immune suppression resulting from body mass loss could be a physiological mechanism responsible for the higher mortality in wintering birds suffering high body mass loss. A lowered immunity could indeed make the birds more vulnerable to viruses (Flint and Franson, 2009).

Whatever hypothesis best supports the variations in immune response during periods of fluctuating energy availability, the physiological mechanisms linking body reserves and the immune system remain largely unknown. Glucocorticoid hormones, primarily involved in mediating adaptive behavioural and physiological responses to stress (the adrenocortical stress response) (Wingfield et al., 1998) have also been hypothesized to regulate immune function (McEwen et al., 1997; Apanius, 1998). However, their role as an endocrine link between immunocompetence and stress remains controversial (for a review, see Martin, 2009). In this context, plasma corticosterone levels were examined in relation to immune components to identify possible indirect immunomodulating effects of variable energy reserves *via* a stress-induced endocrine pathway. During prolonged fasting, increased plasma corticosterone levels are physiological markers of metabolic phase transitions (Cherel et al., 1988; Robin et al., 1998). In all our experimental groups, corticosterone levels were negatively related to body energy content but not to body mass. In agreement with the time lag observed between the decrease in IgY levels and the increase in corticosterone levels during fasting, we observed no significant relationship between these two parameters. Nevertheless, a negative, weak but significant, relationship was observed between plasma corticosterone and NAbs. However, barn owl chicks (*Tyto alba*) implanted with exogenous corticosterone maintained plasma NAbs (constitutive innate immunity) at levels similar to those observed in sham-implanted chicks (Stier et al., 2009). Overall, in the current study, the variations in the two branches of the immune system do not seem to be causally driven by corticosterone secretions. However, our correlational results cannot rule out this hypothesis. How food intake and energy metabolism are influencing and mediating the immune system are still under debate (Demas and Sakaria, 2005). Adipose tissue is perceived as an active participant in the regulation of essential and prominent body processes such as immune homeostasis (Matarese and La Cava, 2004). How adipose-derived factors such as leptin link the immune system to lipid stores remains to be investigated.

In conclusion, we have shown, through an experimental manipulation of food availability in captive female mallards, that a reduction in energy resources negatively affected both innate immunity (plasma NAbs) and adaptive immunity (plasma IgY), further suggesting an energy-based trade-off between body maintenance and immunity. Nevertheless, while innate immunity suffered a greater decrease than adaptive immunity during fasting, innate immunity was restored earlier than the adaptive immunity after re-feeding. Moreover, we found that corticosterone does not seem to directly mediate the observed immunosuppression. Furthermore, the trade-offs observed between the two arms of the immune system and body mass suggest that the variations in immune responses do not strictly match the variations in body mass in relation to fluctuating resources. More work is therefore required to investigate other avian endocrine or nutritional factors that could favour the reallocation of energy reserves to functions not directly related to immediate survival, such as immune defences.

ACKNOWLEDGEMENTS

We wish to thank Sandrine Zahn and Fabrice Bertile for their help in collecting tissue samples. We are indebted to René Groscolas for his helpful comments on the manuscript and to François Criscuolo for his advice on the NAb protocol. This research was funded by grants from CNRS. During the tenure of this study, S.B. was supported by a fellowship from the French MENRT.

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