Fuel use and metabolic response to endurance exercise: a wind tunnel study of a long-distance migrant shorebird

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Summary

This study examines fuel use and metabolism in a group of long-distance migrating birds, red knots *Calidris canutus* (Scolopacidae), flying under controlled conditions in a wind tunnel for up to 10 h. Data are compared with values for resting birds fasting for the same time. Plasma levels of free fatty acids, glycerol and uric acid were elevated during flight, irrespective of flight duration (1–10 h). Triglyceride levels, the estimated concentration of very-low-density lipoproteins (VLDLs) and β-hydroxybutyrate levels were lower during flight, while glucose levels did not change. In flying birds, plasma levels of uric acid and lipid catabolites were positively correlated with the residual variation in body mass loss, and lipid catabolites with energy expenditure (as measured using the doubly labelled water method), after removing the effect of initial body mass. The plasma metabolite levels indicate: (i) that the rates of catabolism of lipids from adipose tissue and of protein are higher during flight; (ii) that low ketone body concentrations probably facilitate fatty acid release from adipose tissue; (iii) that low triglyceride and VLDL levels do not indicate the use of an additional pathway of fatty acid delivery, as found in small birds; and (iv) that the relationships between energy expenditure, body mass loss and metabolic pattern suggest that a higher individual energy expenditure entails a higher rate of catabolism of both lipids and protein and not a shift in fuel substrate.

Key words: bird, flight, red knot, *Calidris canutus*, plasma metabolite, energy expenditure, lipid catabolism, protein catabolism, migration.

Introduction

Many species of migrating bird are known to fly non-stop for thousands of kilometres. This is particularly true for shorebirds, which often cross oceans on their migration. These non-stop flights are exceptional among vertebrates for three reasons: they may last for more than 100 h (Piersma and Baker, 2000; Battley et al., 2000); they are performed without food or water intake, thus relying exclusively on body energy stores and on body and metabolic water; and the birds have to fly at a very high metabolic rate (well above the maximum sustainable rate of exercising small mammals; Butler and Woakes, 1990).

The metabolism of birds during endurance flight is still poorly understood because of the difficulties of studying birds in flight (Butler and Bishop, 2000). Lipids stored in adipose tissue are the main fuel during long flights, but several studies have also shown that there is a certain amount of protein catabolism during flight (for a review, see Jenni and Jenni-Eiermann, 1998). The proportion of protein contributing to energy expenditure is as low as in long-term fasting resting birds, slightly lower than in long-term fasting resting mammals

and considerably lower than in mammals during endurance locomotion (Jenni and Jenni-Eiermann, 1998). Therefore, it can be assumed that the delivery of lipids from adipose tissue to the working muscles is facilitated in various ways. Fatty acids are insoluble in the aqueous medium of the blood and cells and need to be bound to a carrier to be transported. The re-esterification of fatty acids in the liver and delivery to the flight muscles as very-low-density lipoproteins may be a means of circumventing constraints in the bloodstream of fatty acid supply to the muscles in small passerines (Jenni-Eiermann and Jenni, 1992). This results in high concentrations of triglycerides in the plasma. The transport of fatty acids within muscle cells is likely to be optimized by the highest concentration of fatty-acid-binding protein found in any vertebrate (Guglielmo et al., 1998).

The studies available so far on the metabolism and fuel utilization of free-flying birds involved either birds caught during nocturnal migratory flight whose duration was unknown (Jenni-Eiermann and Jenni, 1991, 1992) or homing pigeons

Columba livia (Bordel and Haase, 1993; George et al., 1989; George and John, 1993; John et al., 1988; Schwilch et al., 1996; Viswanathan et al., 1987). Homing pigeons, although usually trained to a certain extent, are not migratory birds, their flights are not voluntary and long flights of several hours are not a routine part of their life. Migratory birds can now be flown in a wind tunnel for up to 16 h (Pennycuick et al., 1996; Lindström et al., 1999, 2000; Klaassen et al., 2000; Kvist et al., 2001), which allows their metabolism to be studied under controlled conditions.

The first aim of this study was to investigate the metabolic responses to endurance flight of known duration in a shorebird, the red knot *Calidris canutus*, which routinely performs long non-stop flights during migration (Piersma and Davidson, 1992). We measured the plasma concentration of seven key metabolites. We compared knots flying in a wind tunnel for up to 10 h with knots fasting and resting for the same time. In particular, we investigated whether protein catabolism was detectable in birds performing very long non-stop flights, i.e. when the bird could be expected to maximize lipid utilization over protein utilization. Furthermore, we examined whether knots use the pathway of fatty acid delivery *via* very-low-density lipoproteins proposed for small passerines (Jenni-Eiermann and Jenni, 1992).

The few available studies suggest that take-off and short flights are fuelled by small muscular and hepatic carbohydrate stores (George and Berger, 1966; Rothe et al., 1987). In homing pigeons, plasma metabolite concentrations and respiratory quotient show that a gradual switch to a lipid-based energy delivery occurs during the first 1–2 h of flight (Bordel and Haase, 1993; George et al., 1989; John et al., 1988; Rothe et al., 1987; Schwilch et al., 1996; Viswanathan et al., 1987). However, there are no detailed studies on true migrants with known flight durations.

The second aim of this study was, therefore, to investigate how quickly the metabolism of a true migrant bird switches to maximal fat utilization. We followed the metabolic changes, as observed by metabolite concentrations in the plasma, during the first few hours of flight.

The types of fuel used during endurance flight (i.e. the proportion of lipids and proteins) may vary among and within individuals depending on body mass, flight style and ambient conditions. Protein from wet tissue is 8–10 times less energy-dense than lipids in adipose tissue (Jenni and Jenni-Eiermann, 1999). Therefore, if water balance is maintained, variation in the types of fuels used will affect body mass loss during flight.

The third aim of this study was to examine whether the types of fuels used (as observed from concentrations of key metabolites in the plasma) varied with energy expenditure (as measured by the doubly labelled water technique) and the rate of body mass loss.

Materials and methods
Birds and maintenance conditions
Red knots *Calidris canutus* of both the *canutus* and the *islandica* subspecies, all adults, were trapped in the Dutch Wadden Sea between April and September 1998. The birds were kept in the experimental shorebird facilities at NIOZ, Texel, The Netherlands, for periods of 1 week to 7 months before they were brought in groups to Lund, Sweden.

In Lund, the birds were kept together in an aviary (3 m×1.5 m×2 m) in the wind tunnel building. Fresh water was supplied in drinking bowls and in a 1 m×1.5 m basin. The birds were fed commercial trout pellets and mealworms *ad libitum* (but see below for experiments). Trout pellets mimic the chemical composition of the knots' natural diet of molluscs, and red knots successfully go through the annual routines of seasonal fattening and moult when fed these pellets (Piersma et al., 1996). Temperature in the wind tunnel building dropped from around +20 °C at the start of the experiments in early September to +7 °C in December when the study finished. The seasonal decrease in air temperature followed that experienced by birds in the wild. Temperature during flights (7–14 °C) was always within the range in which the birds were in water balance (A. Kvist, unpublished data). The birds were kept on a 12 h:12 h L:D cycle, with lights on at 09:00 h local time. Apart from short hovering flights in the aviary, the birds flew only in the wind tunnel. The time in captivity has no discernible effect on flight performance (for further information on bird husbandry, see Lindström et al., 2000).

Test protocol
Blood was sampled from individuals in two different physiological states: flight in the wind tunnel and resting while fasting. For each of these physiological states, samples were taken 0, 1, 2, 4 and 10 h after lights on. In this study, we analysed samples from seven birds during flight and compared them with the same seven individuals and five additional birds during fasting at rest. Each individual was used repeatedly for alternating flight and resting experiments. The interval between experiments for an individual was usually 1–7 days, but occasionally up to 30 days. The aim was to sample each individual at least once in each of the nine different combinations of state (flight or resting) and time of day (=resting or flight duration). Because not all the birds would fly for long enough periods in the wind tunnel, it was not possible to follow this procedure strictly, and each individual was used for 1–9 flight experiments and 1–8 fasting experiments.

The evening before the day of flight, food was removed between 18:00 h and 22:00 h. This procedure ensured that the birds were flying with an empty digestive tract, so that mass loss could be used as an additional estimate of fuel consumption during flight (Kvist et al., 1998).

Each flight in the wind tunnel started at 09:00 h, irrespective of flight duration. Before the 1, 2 and 4 h flights, the birds had access to water until the flight started. Before the 10 h flights, the birds had no access to water during the last hour before flight, because the 10 h flights were part of a study measuring energy expenditure using doubly labelled water (DLW) (Kvist et al., 2001). During the last hour before flight, the injected

DLW (0.4 g) was diluted in the body, and the birds were kept in darkness, where they were inactive. We have no reason to assume that the initial water balance was markedly different before the 10 h flights.

In the wind tunnel, the birds flew continuously at $15 \, m \, s^{-1}$. The 1 h flights were non-stop. During the 2, 4 and 10 h flights, the birds were briefly taken out of the wind tunnel for 40–80 s and weighed after 1, 2, 4, 6 and 8 h, where applicable. For each flight experiment, only one blood sample was taken at the end of flight.

Birds during fasting experiments were treated the same as flying birds during the day before the experiment. Instead of flight, however, the bird was transferred to a cage (1 m×0.7 m×0.7 m) and remained there without food, but with access to water. For each fasting experiment, only one blood sample was taken at the end of the pre-determined fasting period of 0, 1, 2, 4 or 10 h. Because food was withdrawn the evening before the experiment, the birds were actually fasting for an additional 11–15 h. During fasting and resting, the birds typically remained motionless or walked around in the cage. Hence, their energy expenditure was several times lower than during flight.

Blood sampling and plasma analysis

Blood samples were taken within 1–5 min after the flight had ended. Samples from resting birds were taken ±20 min around the intended end time.

Between 50 and 300 μl of blood was collected into heparinised 50 μl capillary tubes after puncturing the alar vein. The capillaries were immediately centrifuged for 15 min at 10 000 revs min⁻¹ The plasma was transferred into 2 ml test tubes and stored at –82 °C within 15 min. The samples were sent frozen to Switzerland in March 1999 for analysis of metabolite levels.

Quantitative enzymatic tests were used to measure β-hydroxybutyrate (Sigma, Procedure No. 310), triglyceride and glycerol (Sigma, Procedure No. 337), glucose (HK, Sigma, Procedure No. 16-UV), free fatty acid (Boehringer Mannheim, Catalogue No. 1383175) and uric acid (Sigma, Procedure No. 685) concentrations. The assays were adapted to a total volume of 500–600 μl, and 5–10 μl of plasma was used for each metabolite.

Lipoprotein levels were determined with the standard agarose gel electrophoresis system Paragon (Beckman), used according to the instructions given by the manufacturer. The lipoproteins were visualised with Sudan Black B and quantified by densitometric scanning (Appraise Junior densitometer, Beckman). The peaks had been characterized previously by ultracentrifugation (Jenni-Eiermann and Jenni, 1992). The fraction (percentage) of very-low-density lipoproteins (VLDLs) was used for this study. To estimate the plasma concentration of VLDLs from the VLDL fraction, we assumed that all circulating triglycerides are bound either in VLDLs or in low-density lipoproteins (LDLs) and that VLDLs contained 60 % triglycerides and LDLs contained 10 % triglycerides (by analogy with humans, see Jungermann and

Möhler, 1980). Hence, a rough estimate of the plasma concentration of VLDLs is:

(fraction of VLDLs×0.6)/[(fraction of VLDLs×0.6) +
(fraction of LDLs×0.1)] × (concentration of triglycerides) .

Because a few samples contained very little plasma, we could not determine the β-hydroxybutyrate concentration of one sample, free fatty acid levels in four samples and VLDL levels in nine samples.

Measurements of energy expenditure during 10 h flights

In birds flying for 10 h, energy expenditure was determined using the doubly labelled water method as described in detail by Kvist et al. (2001). Briefly, prior to flight, the birds were injected intraperitoneally with a dose of approximately 0.4 ml of a DLW mixture. After an equilibration period of 1 h, during which the bird was fasting, approximately five glass microcapillaries were each filled with 15 μl of blood after puncturing the brachial vein. The capillaries were immediately flame-sealed, and the bird was placed in the wind tunnel. Approximately 10 h later, the sampling procedure was repeated, and the bird was reinjected quantitatively with the same DLW mixture. Another blood sample was taken 1 h later. This reinjection procedure was undertaken to estimate the size of the bird's body water pool at the end of the flight period. We also took blood samples from four uninjected birds to determine the natural enrichments of ^2H and ^{18}O in the birds' body water pools prior to the measurements.

Samples were always stored at 4 °C until analysis at the Centre for Isotope Research. We followed the analytical procedure outlined by Visser et al. (2000), in which the sample was first microdistilled in a vacuum line. ^{18}O enrichments were determined using CO_2 equilibration, and subsequent measurement with an SIRA 10 isotope ratio mass spectrometer, and ^2H enrichments were determined after reduction over a uranium oven and subsequent measurement with the SIRA 10. In all cases, samples were analysed in quadruplicate, with added internal gas and water standards to monitor the linearity of the analytical procedure for each batch of samples.

Rates of CO_2 production were calculated using equations 3 and 5 of Visser et al. (2000) taking an average of the bird's body water pool at the start and end of the flight and assuming that 85 % of the water efflux was through evaporative pathways (see also Kvist et al., 2001). Finally, these rates of CO_2 production were converted to levels of energy expenditure assuming an energetic equivalent of $27.33 \, kJ \, l^{-1} \, CO_2$ following Gessaman and Nagy (1988).

Data analysis

The data were analysed with a residual maximum-likelihood analysis (REML) (Patterson and Thompson, 1971) in Genstat 5, release 3.22. This procedure is appropriate for the analysis of repeated-measurements data in an unbalanced design. REML yields the same results as conventional analysis of variance (ANOVA) in balanced designs, but avoids the bias

introduced by ANOVA in unbalanced designs (Robinson, 1987).

Our main model included one of the metabolite levels as the dependent variable, the individual bird as a random effect and the following fixed effects: physiological state (flying or resting), duration of flight or resting since 0900 h (in min) (categorical) and the interaction term state × duration. The concentrations of uric acid, glycerol and β-hydroxybutyrate were $\log_e$-transformed since their distribution was skewed.

From this model, residual metabolite levels were calculated and correlated with residual body mass loss and residual energy expenditure. This indicates whether an above- or below-average metabolite level was correlated with a mass loss or an above- or below-average energy expenditure given the individual, its starting mass and flight duration. Residual body mass loss was calculated from an REML analysis, which revealed that body mass loss was dependent on individual ($P=0.048$) and positively related to flight duration ($P<0.001$), to initial body mass ($P<0.01$) and to the interaction between flight duration and initial body mass ($P<0.05$), showing that initial body mass was positively correlated with body mass loss for long flight durations. In birds flying for 10 h, with DLW measurements of flight energy expenditure, residual energy expenditure was calculated from an REML analysis, which showed that energy expenditure was positively related to initial body mass ($P<0.001$). Furthermore, body mass loss was positively related to energy expenditure ($P<0.001$) and initial body mass ($P<0.001$), with a significant effect of individual ($P<0.001$) (A. Kvist, Å. Lindström, M. Green, T. Piersma and G. H. Visser, unpublished data).

Results

Comparison between flying and resting knots

Flying birds differed significantly from resting birds in levels of six metabolites (Tables 1, 2). Concentrations of free fatty acids and glycerol, both products of lipid mobilization from the adipose tissue, were significantly higher in plasma in flying than in resting birds. Similarly, the concentration of uric acid, the end product of protein catabolism in birds, was significantly higher in plasma in flying than in resting birds. The concentration of triglycerides and the estimated concentration of VLDLs, the form in which lipids are transported from the intestine or the liver to the adipose tissue, however, were significantly lower in flying than in resting birds. The same held for β-hydroxybutyrate, a ketone body produced from lipid catabolism which partly replaces glucose in the brain during fasting. No difference between resting and flying birds was found in the fraction of VLDLs and in glucose levels.

Plasma levels of free fatty acids, uric acid, triglycerides and β-hydroxybutyrate showed no significant change with flight duration or with resting duration (Table 1; Fig. 1). Glycerol levels showed a small increase between 1 and 2 h when resting and flying birds were combined, but there was no significant difference among flight durations ($P=0.09$). The estimated

Table 1. *Comparison of plasma metabolite levels between flying and resting fasting birds*

Dependent variable	State	Duration	State × duration	N
Free fatty acid concentration	13.7***	NS	NS	89
$\log_e$[glycerol]	83.1***	12.3*	NS	93
Triglyceride concentration	38.0***	NS	NS	93
VLDL fraction	NS	NS	NS	84
VLDL concentration	11.7***	3.5	15.8**	84
$\log_e$[β-hydroxybutyrate]	11.2***	NS	NS	92
$\log_e$[uric acid]	146.5***	NS	NS	93
Glucose concentration	NS	NS	NS	92

VLDL, very-low-density lipoprotein.

The Wald statistics of the effects of two fixed variables on metabolite concentrations in an REML analysis is shown (see Table 2 for their effects). In a separate REML analysis for each metabolite, the full model included the metabolite level as the dependent variable, individual as a random effect (not shown) and physiological state (flying or resting), duration of flight or resting (categorical) and its interactions with physiological state as fixed effects.

The effect of individual was significant ($P<0.05$) in all analyses, except in that for glucose.

The fixed effects were evaluated sequentially from left to right as given in the Table (as in a type I analysis of variance).

Non-significant effects were removed (NS), and the most parsimonious model is shown.

The significance of the Wald statistics is indicated: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 2. *Plasma metabolite levels of flying and resting fasting birds derived from the analysis given in Table 1*

Dependent variable	Flight	Rest
[Free fatty acid] (mmol l^{-1})	1.09±0.111 (34)	0.75±0.094 (55)
$\log_e$[glycerol]	−0.96±0.135 (35)	−2.21±0.107 (58)
[Triglyceride] (mmol l^{-1})	1.16±0.117 (35)	1.60±0.108 (58)
VLDL fraction (%)	11.9±5.57 (84)	
VLDL concentration (mmol l^{-1})	0.94±0.108 (33)	1.16±0.099 (51)
$\log_e$[β-hydroxybutyrate]	0.14±0.118 (35)	0.52±0.097 (57)
$\log_e$[uric acid]	−0.56±0.089 (35)	−1.67±0.071 (58)
[Glucose] (mmol l^{-1})	18.5±2.09 (92)	

Values are means ± S.E.M. (N).

VLDL, very-low-density lipoprotein.

For glycerol concentration and VLDL fraction, values varied significantly with the duration of flight, and the overall means are given.

concentration of VLDLs decreased significantly with flight duration (Fig. 1).

Flying knots

Residual metabolite levels (from the analysis given in Tables 1 and 2 and Fig. 1) indicate whether metabolite levels are above or below average, given the effect of flight duration and
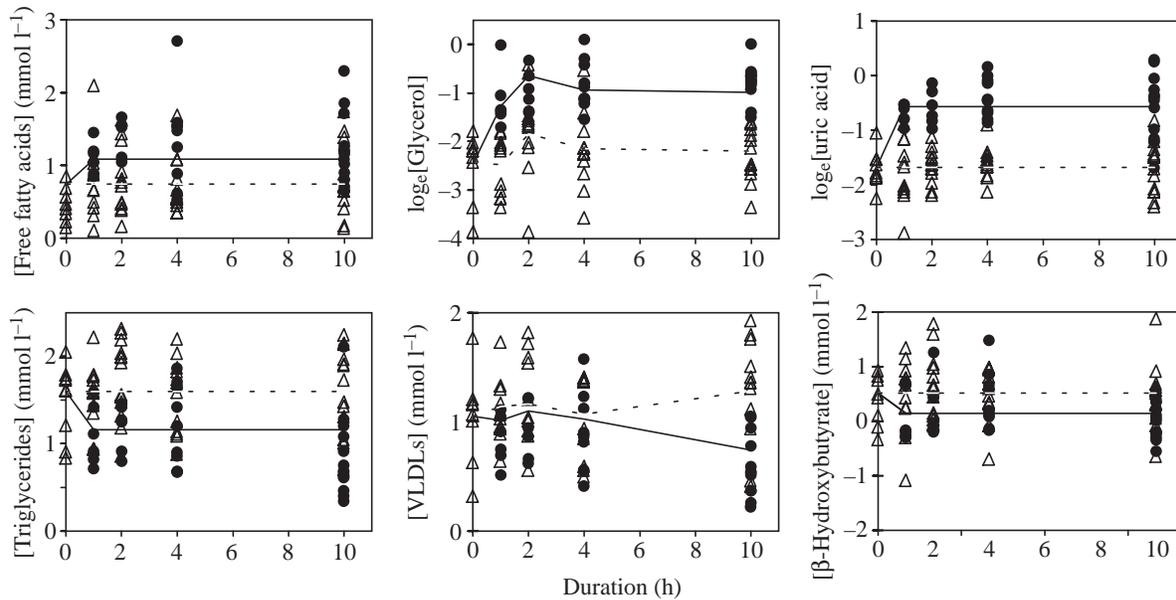
Fig. 1. Relationship between levels (mmol l^{-1}) of six metabolites and the duration of resting (triangles) or flight (circles). The lines for resting (broken lines) and flying birds (continuous lines) are derived from the analysis given in Tables 1 and 2. VLDLs, very-low-density lipoproteins.

individual. We examined whether these residual metabolite levels were correlated (i) with the residual body mass loss, indicating whether the bird lost an above- or below-average amount of body mass, given the initial body mass and flight time of that individual, and (ii) with residual energy expenditure in birds flying for 10 h, indicating whether the bird expended an above- or below-average amount of energy, given the individual and its initial body mass.

Residuals of free fatty acid, uric acid and β-hydroxybutyrate levels showed a significant positive correlation with residual mass loss (Fig. 2), but residuals of glycerol (r=0.220, N=35), triglyceride (r=0.022, N=35), very-low-density lipoprotein (fraction r=0.241; estimated concentration r=0.062, N=33) and glucose (r=0.017, N=35) concentrations did not. Thus, in flights with an above-average body mass loss, levels of uric acid, free fatty acids and β-hydroxybutyrate were higher than in flights with a below-average body mass loss.

In the 13 cases of knots flying for 10 h, residuals of free fatty

acid, β-hydroxybutyrate levels (Fig. 3) and glycerol showed a significant positive correlation with residual energy expenditure (corrected for initial body mass; see Materials and methods), but the relationship for glycerol level (not shown in Fig. 3) was significant only because of a single high value. Residual uric acid levels showed the same trend, although not significantly so (Fig. 3). The remaining residual metabolite levels showed no significant correlation with residual energy expenditure. Birds flying for 10 h with an above-average energy expenditure therefore had higher free fatty acid and β-hydroxybutyrate levels than birds with a below-average energy expenditure.

Discussion

This is the first study to examine fuel use and metabolic changes by measuring levels of blood metabolites in migratory birds flying under controlled conditions in a wind tunnel. This approach offers several advantages compared with studies on
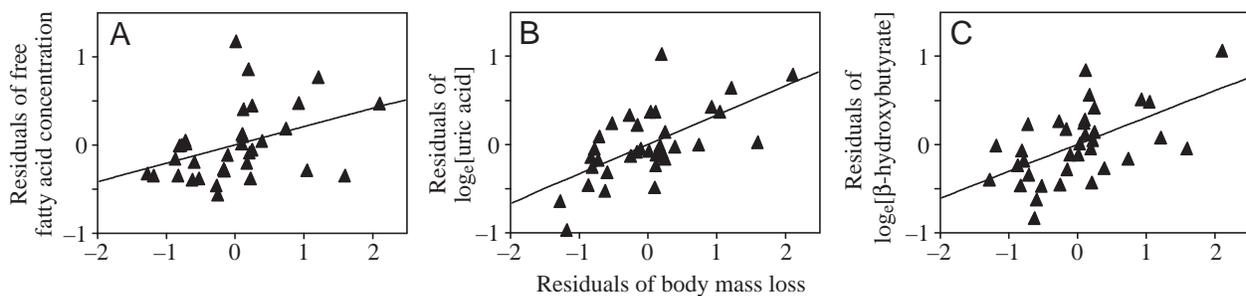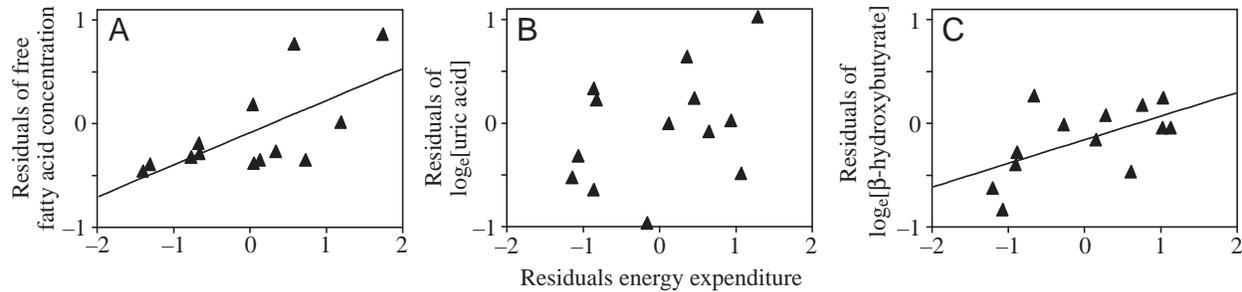

Fig. 2. Residuals of free fatty acid (A), uric acid (B) and β-hydroxybutyrate (C) levels from the analysis given in Table 1 *versus* the residuals of body mass loss after removing the effects of initial body mass and the duration of flight. This indicates whether an above- or below-average metabolite level was correlated with an above- or below-average body mass loss given the flight duration and initial body mass. Correlations were: (A) r=0.392, P=0.022, N=34; (B) r=0.633, P<0.001, N=35; (C) r=0.565, P<0.001, N=35.

Fig. 3. Residuals of free fatty acid (A), uric acid (B) and β-hydroxybutyrate (C) levels from the analysis given in Table 1 *versus* the residuals of energy expenditure after removing the effects of initial body mass for birds flying for 10 h. This indicates whether an above- or below-average metabolite level was correlated with an above- or below-average energy expenditure given the initial body mass. Correlations were: (A) $r=0.663$, $P=0.013$, $N=13$; (B) $r=0.399$, $P=0.177$, $N=13$; (C) $r=0.582$, $P=0.037$, $N=13$.

flight in free-living birds. The flight duration and the immediate 'history' of the bird before it started its flight are known, and metabolic responses can be measured during and directly after flight. Previous studies of metabolite levels from blood samples were performed in free-living small passerines (Jenni-Eiermann and Jenni, 1991) and in homing pigeons (see Bordel and Haase, 1993; George and John, 1993; John et al., 1988; Schwilch et al., 1996). In the study of small passerines, the flight duration of the night migrants was based on the assumption that they started their migratory flight in the evening. However, there are indications that some may have started later during the night (Bolshakov and Bulyuk, 1999; Åkesson and Hedenström, 2000). In homing pigeons, the time between starting a flight and arriving in the loft is known, but the flight route is unknown and it is difficult to know whether the birds took any rest, especially during longer flights. Also, different types of food or different wind and weather situations might influence the metabolic responses of free-flying birds to an unknown extent.

For birds flying in a wind tunnel, flight speed and duration are controlled. The knots were well accustomed to the tunnel, they were more-or-less hand-tame and they flew voluntarily for up to 10 h, which accords with their well-known long non-stop flights from one stopover site to the next (Piersma and Davidson, 1992).

We have good reasons for assuming that plasma concentrations of free fatty acids and glycerol indicate the rate of lipid catabolism and that the plasma concentration of uric acid indicates the rate of protein catabolism (Jenni-Eiermann and Jenni, 1991, 1996, 1998; Jenni et al., 2000).

Metabolic responses to flight

Most of the energy needed for prolonged exercise in migrating birds is provided by the oxidation of lipids, which are stored as triglycerides mainly in adipose tissue. In the plasma of flying knots, we measured increased levels of free fatty acids and glycerol in comparison with resting birds fasting for the same period, indicating increased hydrolysis of triglycerides from adipose tissue. This finding is in agreement with all studies of exercising birds, e.g. domestic fowl running on a treadmill (Brackenbury and El-Sayed, 1984), flying

pigeons (Bordel and Haase, 1993; Schwilch et al., 1996; Vallyathan and George, 1969; John et al., 1988; George et al., 1989) and small passerines (Jenni-Eiermann and Jenni, 1991) as well as studies on exercising mammals and humans (e.g. Paul, 1975; Keul, 1975).

We observed lower plasma triglyceride and VLDL concentrations in flying than in resting knots. This is in accord with most studies on endurance exercise of mammals and larger birds (Keul, 1975; Liesen et al., 1975; Paul, 1975; Brackenbury and El-Sayed, 1984) in which decreased plasma triglyceride levels were recorded during heavy endurance exercise. In homing pigeons, triglyceride levels and the fraction of VLDLs were lower (short flights) or only slightly higher (longer flights) in flying than in resting birds (Bordel and Haase, 1993; Schwilch et al., 1996). However, small migrating passerines have been found to have significantly elevated triglyceride and VLDL levels (Jenni-Eiermann and Jenni, 1991, 1992). It was suggested that the high plasma triglyceride and VLDL levels in small passerines indicate an additional pathway for delivering free fatty acids to the working muscles. This would circumvent constraints in free fatty acid supply imposed by limitations in the cardiovascular system and blood viscosity, particularly in small birds with very high mass-specific metabolic rates (Jenni-Eiermann and Jenni, 1992). However, the mean mass-specific rate of energy utilization of flying red knots ($0.105\,\mathrm{W\,g^{-1}}$; Kvist et al., 2001) is similar to that of a small passerine (thrush nightingale *Luscinia luscinia*) measured in flight in the same wind tunnel ($0.073\,\mathrm{W\,g^{-1}}$; Klaassen et al., 2000) and to that of pigeons (0.067, 0.069, 0.104 and $0.106\,\mathrm{W\,g^{-1}}$ in four different studies; Norberg, 1996). The difference in triglyceride and VLDL levels during flight among several species of small passerine, on the one hand, and knots and pigeons, on the other, might be because the latter two studies were for birds fasted for prolonged periods before flight or to real differences in metabolism between passerines and non-passerines.

β-Hydroxybutyrate is synthesized during fasting from free fatty acids and replaces some of the glucose requirements of tissues unable to catabolize fatty acids, e.g. the brain (Robinson and Williamson, 1980). Elevated levels of β-hydroxybutyrate reduce glucose utilization and play an important role in the sparing of carbohydrate and protein (Robinson and

Williamson, 1980). Consequently, the plasma β-hydroxybutyrate levels of flying knots are increased compared with feeding knots (flying, $1.33\pm0.79\,\text{mmol}\,l^{-1}$, $N=35$; feeding, $0.78\pm0.43\,\text{mmol}\,l^{-1}$, means ± S.D., $N=55$; t-test on $\log_e$-transformed values: $P<0.001$). However, they are lower than those of resting knots without food (Table 2; Fig. 1). Similar observations were made in small passerines (Jenni-Eiermann and Jenni, 1991), but not in flying homing pigeons, which have higher plasma β-hydroxybutyrate levels than fasting resting pigeons, or in fasting exercising humans, who show a return to stable pre-exercise levels after an initial decrease (Féry and Balasse, 1986; Hurley et al., 1986). Elevated levels of β-hydroxybutyrate are known to reduce free fatty acid release from adipose tissue (Robinson and Williamson, 1980). Hence, it seems that true migrant birds (small passerines and red knot), whose success in migration critically depends on a very high proportional contribution of fat to total energy expenditure, may have low β-hydroxybutyrate levels during flight in order not to impair fatty acid release from adipose tissue.

Plasma glucose levels did not differ between resting and flying knots. This accords with the idea that blood glucose levels are kept stable. Gluconeogenesis from glycerol and glucoplastic amino acids and the replacement of glucose by β-hydroxybutyrate help to maintain plasma glucose levels and spare glucose. In two out of three species of flying small passerine, no changes in plasma glucose levels were found in comparison with fasted individuals (Jenni-Eiermann and Jenni, 1991). In flying pigeons (Bordel and Haase, 1993; Schwilch et al., 1996) and in exercising domestic fowl (Brackenbury and El-Sayed, 1984), unchanged or slightly lower glucose levels were found compared with resting birds.

The level of uric acid, the end product of nitrogen metabolism and an indicator of protein catabolism, was substantially increased in flying knots. Elevated uric acid levels were also observed just after flight in pigeons (Bordel and Haase, 1993; Schwilch et al., 1996) and small passerines (Jenni-Eiermann and Jenni, 1991; Jenni et al., 2000). A small amount of protein catabolism during fasting is metabolically inevitable, and the most likely function is to replace glucose *via* glucoplastic amino acids and to replace intermediates of the citric acid cycle (anaplerotic flux) so that fatty acids can continue to be oxidized (see Jenni and Jenni-Eiermann, 1998). The catabolism of protein from the flight muscles and other organs has the concomitant advantage that flight muscle mass can be continuously adapted to the decreasing body mass (Pennycuick, 1998) and the body mass to be carried is reduced (Piersma and Lindström, 1997). Pectoral muscle thickness decreases during long flights in the same knots as used in this study (Lindström et al., 2000).

In conclusion, the metabolic pattern obtained from knots flying in a wind tunnel, in agreement with data from other flying birds, showed an increased catabolism of lipids from adipose tissue, as indicated by the high plasma levels of free fatty acids and glycerol. Although protein provides only a low proportion of the energetic needs (probably approximately 4–7 %; Jenni and Jenni-Eiermann, 1998), compared with resting birds its catabolism is increased, as shown by higher uric acid levels. Plasma levels of β-hydroxybutyrate are lower than in resting fasting birds, probably to facilitate free fatty acid release from adipose tissue. Low triglyceride and VLDL levels indicate that flying knots do not use the pathway of fatty acid resynthesis in the liver and delivery as VLDLs.

Metabolic switch at the beginning of flight

For take-off and during short flights, flight is powered by small muscular and hepatic carbohydrate stores (George and Berger, 1966; Rothe et al., 1987), while endurance flight is fuelled by lipids, contributing approximately 93–96 % to total energy expenditure (Jenni and Jenni-Eiermann, 1998). Hence, the organism has to switch from a carbohydrate-based to a lipid-based energy delivery.

In the knots flying in the wind tunnel, we found that all metabolites had reached their flight level after 1 h of flight (Fig. 1). This is earlier than in homing pigeons, which achieved a mainly lipid-based energy delivery after 1–2 h of flight (Rothe et al., 1987; Schwilch et al., 1996). The metabolic switch may have been quicker than in pigeons because knots are adapted to endurance flight. However, it might also have been facilitated by the fasting time of 11–15 h prior to flight. In pigeons, previous fasting elicited a quicker metabolic shift than that occurring in unfasted pigeons (Rothe et al., 1987).

Fuel types, body mass loss and energy expenditure

To the best of our knowledge, this is the first study to investigate the relationships between the fuel types used (indicated by plasma metabolite levels) and energy expenditure (measured using the doubly labelled water method) and body mass loss (direct measurements) in a migrant bird during endurance flight. The body mass loss of knots flying in the wind tunnel varied among individuals (A. Kvist, Å. Lindström, M. Green, T. Piersma and G. H. Visser, unpublished data). Is this variability in body mass loss caused by a change in the proportion of fuel types (protein or lipids) used?

We found that a higher residual body mass loss occurs in parallel with higher plasma levels of both protein catabolites and lipid catabolites (Fig. 2), indicating that rates of both protein and lipid metabolism increased and that there is no major change in the proportion of fuel types with increasing residual body mass loss. Furthermore, during 10 h flights, there were similar correlations between residual energy expenditure and both protein catabolite (although not significantly) and lipid catabolite (Fig. 3) levels, and body mass loss was positively correlated with energy expenditure (A. Kvist, Å. Lindström, M. Green, T. Piersma and G. H. Visser, unpublished data). The knots had no problem maintaining water balance at the ambient conditions prevalent during the experiments (A. Kvist, Å. Lindström, T. Piersma and G. H. Visser, unpublished data). Hence, the higher residual body mass loss was not due to a negative water balance.

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