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Plasma metabolites reflect seasonally changing metabolic processes in a long-distance migrant shorebird *(Calidris canutus)*

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Summary

Migrant birds have tightly scheduled annual cycles consisting of several distinct life cycle (sub-)stages such as reproduction, migration, moult and overwintering, each of which have specific metabolic requirements (e.g., fattening during migration, protein build-up during moult). This study examines changes in fat and protein metabolism during the annual cycle of body mass and moult over 1.5 years in a captive flock of an arctic-breeding shorebird, the red knot *Calidris canutus islandica*. 2–5 h after food withdrawal, plasma uric acid levels were still decreasing and β-hydroxy-butyrate levels were low, indicating prolonged catabolism of dietary protein, probably linked with a conversion into lipids. Such a late-resorptive state is achieved much earlier in passerines, but only after several days in penguins and, thus, seems to depend on meal size or mass-specific metabolic rate. Substages of body mass gain and high body mass were characterized by increased plasma triglyceride levels reflecting increased turnover of lipids, and low levels of the ketone body β-hydroxybutyrate, indicating that the bird is not short of glucose. The high uric acid levels during these substages indicated an increased breakdown of nutritional protein. During moult, plasma triglyceride levels were low, suggesting that lipids were less available than at other times of the year. It is concluded that plasma metabolite levels indicate the metabolic processes related to migratory fuelling and moult and the influence of exogeneous factors.

Key words: annual cycle, fat metabolism, bird migration, moult, red knots

Introduction

Free-living migrant birds of temperate areas generally exhibit annual cycles which consist of reproduction, migration to the winter quarters and back to the breeding area, overwintering and moult (Murton and Westwood, 1977). In some passerine birds it has been established that this annual cycle is endogenous and is repeated in birds kept in captivity (Gwinner, 1986). In these captive passerines, the features of the annual cycle that are most easily observed are: (1) increases in body mass during migration periods, (2) moult, and (3) increased gonad size during the reproductive period. Several studies showed that such annual cycles also occur in captive shorebirds (Clark, 1983; Goede, 1993; Piersma et al., 1995; Melter and Bergmann, 1996) and a related study showed an annual cyclicity of thyroid hormones (Jenni-Eiermann et al., 2002). One study hints at endogenous control (Cadée et al., 1996).

Measurement of plasma metabolites offers a rapid and repeatable method to study the metabolism and physiological state of free-living birds (Jenni-Eiermann and Jenni, 1998). It reflects the momentaneous metabolic state (e.g., direct influence of food intake), but also different seasonal processes that have to do with moult and migratory fueling (Jenni-Eiermann and Jenni, 1996). Apart from the intrinsic value of knowledge about plasma metabolite levels in relation to metabolic processes, instantaneous determinations of specific metabolites might inform us about the longer-term physiological process (e.g., fuelling) in which the sampled individual is involved (Jenni-Eiermann and Jenni, 1994; Jenni and Schwilch, 2001).

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In contrast to several studies that examine metabolic correlates of body mass increases and moult in free-living migratory passerines (e.g., deGraw et al., 1979; Jenni-Eiermann and Jenni, 1996; Jenni and Schwilch, 2001), this study examines seasonal variations during the entire annual cycle in plasma metabolites in a standardized way. In this study, we examine a tundra-breeding shorebird, the red knot *Calidris canutus*, a member of another avian order containing many long-distance migrating species, the Charadriiformes (Piersma et al., 1996b). The species shows large changes in metabolic rate and body composition in the course of its annual cycle (Evans and Davidson, 1990; Piersma et al., 1995, 1996a, Piersma, 2002). The 1.5 year study period enabled us to describe the changes in fat and protein metabolism in relation to the seasonally differing physiological processes such as moult and migratory fattening. We measured plasma levels of triglycerides, β-hydroxy-butyrate and uric acid of birds kept in a small flock under the thermal and photoperiodic conditions of their temperate wintering area. The results were compared with similar changes in phylogenetically unrelated groups such as passerines and penguins.

Material and methods

Animals

Eight *Islandica*-Knots, aged on the basis of plumage characteristics and all older than two years, were captured on 15 October 1988 in the Dutch Wadden Sea (53º29′ N, 6º12′ E). This subspecies breeds on high arctic tundra in northern Greenland and northeast Canada and spends the nonbreeding season (August through April) in western Europe (Davidson and Wilson, 1992). They were kept in an outdoor aviary in The Netherlands (see Piersma et al., 1995). Hence, during the nonbreeding season the birds encountered photoperiods and a temperature regime comparable to the wild.
Birds were fed *ad libitum* with trout food pellets (Trouvit) without any added hormones (Produits Trouw, Vervins, France, pers. comm.). During weekly cleaning, the birds were weighed and checked for moult. The intensity of the contour feather moult on the breast was scored as 0 = no moult, 1 = light moult (a few feathers growing), 2 = medium moult (many feathers growing), and 3 = heavy moult (about 30% or more of the feathers growing). Moult of the primary and secondary flight feathers was scored in the standard way (Ginn and Melville, 1983).

Blood sampling

Every 4–6 weeks blood samples were collected. Food was withdrawn from the cages in the morning (9:00–10:30 h) and birds were handled in the early afternoon (13:00–14:30 h). As quickly as possible after capture we bled the birds from the wing vein into a heparinized syringe, collecting a maximum of 2 ml of blood. It took 5–40 min between entering the aviaries and completing the bleeding of all individuals. Samples were held on ice water for 1–3 h until plasma was separated by centrifugation. Plasma samples were stored at –30 ºC until they were transported to Switzerland on dry ice for analysis.

Metabolite determinations and storage time

We determined the concentration of three metabolites. Triglycerides can be interpreted as an indicator of fat deposition (Jenni-Eiermann and Jenni, 1994; Jenni and Schwilch, 2001), uric acid as an indicator of protein catabolism (Cherel at al., 1988a,b) and β-hydroxy-butyrate as an indicator of fat catabolism and fasting (Elia et al., 1987; Cherel et al., 1988a; Jenni-Eiermann and Jenni, 1994).
All metabolites were determined in the plasma using standard test-combinations modified for small amounts of plasma (5–20 µl per determination): enzymatic UV-tests for β-hydroxy-butyrate (Sigma Diagnostics), enzymatic colorimetric tests for uric acid and triglycerides including free glycerol (Merckotest^{R}). As the amount of plasma available varied, not all metabolites could be determined in all individuals.
Blood samples were collected over a period of over 1.5 years and analysed ca. 3.5–5 years later (first blood sampling in November 1988, last blood sampling in September 1990, analyses made in October 1993). The long sampling period and variable interval until analysis might have influenced the comparability among samples and the absolute metabolite levels (e.g., Bustamante and Travaini, 1994). Tests using multiple regression analyses and analyses of variance (including date and time interval between capture and blood sampling as covariates) indicated that the variation in storage time between the first and the last sample of 1.5 years had no significant effect on plasma levels of the three metabolites examined. We therefore conclude that the comparability between samples within our study is ensured. Compared with the plasma metabolite levels in overnight fasted small passerines (Jenni-Eiermann and Jenni, 1991), the red knots showed only slightly lower values for triglycerides, uric acid and β-hydroxy-butyrate.

Data analysis

The only metabolite with a skewed distribution relative to normal was β-hydroxy-butyrate, but not if ln-transformed. Transformed values ln(β-hydroxy-butyrate +0.5) were used for all analyses of β-hydroxy-butyrate.

Table 1. Life cycle stages and substages as indicated in Fig. 1 and Fig. 2 together with the body mass and moult criteria on the basis of which they were assigned (see Jacobs and Wingfield, 2000; Piersma, 2002).

Life history stage	Substage	Body mass	Moult
Pre-basic moult	A	Stable low	Wing + body moult
Overwintering	B	Stable low	None
Pre-basic moult	C	Stable low	Wing + body moult
Pre-alternate moult	D	Stable low	Body moult
Migration	E	Strong increase	End of body moult
Migration	F	Stable high	None
Migration	G	Strong decrease	None
Oversummering	H	Stable low	None

The relationships between metabolites and life history stages were analysed in two ways.

Although generally behaving quite synchronously, there was temporal variation between individuals in the precise timing of the life cycle stages. In order to compare different stages of the annual cycle among individuals, we divided the individual annual cycles into a number of life cycle stages, based on body mass development (increasing, decreasing, stable) and presence/absence of body and wing moult (Jacobs and Wingfield, 2000; Piersma, 2002). Because certain moult periods did not occur in each individual (see below), body mass was used as the primary cue to determine stage and (alphanumeric) substage (Table 1). This procedure was considered best because no statistical procedure was found to take account of the many possible combinations of body mass development and events of moult (see also Jenni-Eiermann et al., 2002). Independently of SJ and LJ working from the raw data, TP arrived at the same (sub-)stages using a dataset based on a much larger series of observations on different captive flocks of red knots.

Individual blood sampling dates were assigned to these stages and the metabolite levels tested for differences among substages with a residual maximum-likelyhood analysis REML (Patterson and Thompson, 1971) in Genstat 5. This procedure is appropriate for the analysis of repeated measurements data in an unbalanced design. The full model included the metabolite as dependent variable, individuals as random effects, and time interval between capture and blood sampling and life cycle substage as fixed effects.

In a second step, metabolite levels were tested for dependence on various indices of body mass and moult, again with a REML analysis. The full model included triglycerides, uric acid and β-hydroxy-butyrate, respectively, as dependent variable, the individuals as random effects, and the following fixed effects: (1) sampling time, (2) relative body mass (deviation from the individual mean of those substages with low body mass, i.e. substages A1, B1, D1, H1, A2–D2, A3), (3) change in body mass (difference in body mass to the last body mass measurement/days; 5–7 days before), (4) presence or absence of body moult, (5) presence or absence of moult (wing and/or body moult), (6) the interaction term sampling time x change in body mass.

Results

Annual cycles of mass and moult

All eight red knots showed one distinct peak in body mass per year (example in Fig. 1A), a peak well synchronised between individuals. In May of both years, body mass increased by 43% over low body mass levels and decreased to pre-peak levels in June and early July. During the rest of the year, a small increase in body mass was observed in November and December of the first year (mean +14%), while this increase was less marked or absent in the second year (mean +3%). The annually recurring main body mass peak corresponds with large body mass changes during northward migration. The birds then find themselves oversummering on the non-breeding grounds, and hence do not need to fatten again for the return flight (Piersma et al. 1995, 1996a). A mid-winter peak is also observed in free-living birds (Piersma and Davidson, 1992).

All birds moulted their flight feathers (primaries and secondaries) and body feathers during two distinct phases (Fig. 1B); they were well synchronised among individuals. The main flight feather moult occurred in late summer and autumn and comprised most primaries (8 or 9 primaries out of 10 primaries, in 6 individuals during the second year) or all primaries (2 individuals) and part of the secondaries. Concurrent with the first part of this main phase of flight feather moult, soon after body mass had decreased to low levels, birds changed body feathers and attained the non-breeding plumage. Before and during the beginning of body mass increase, the breeding plumage was obtained. Concurrently, in individuals with suspended primary moult, the one or two remaining primaries and part of

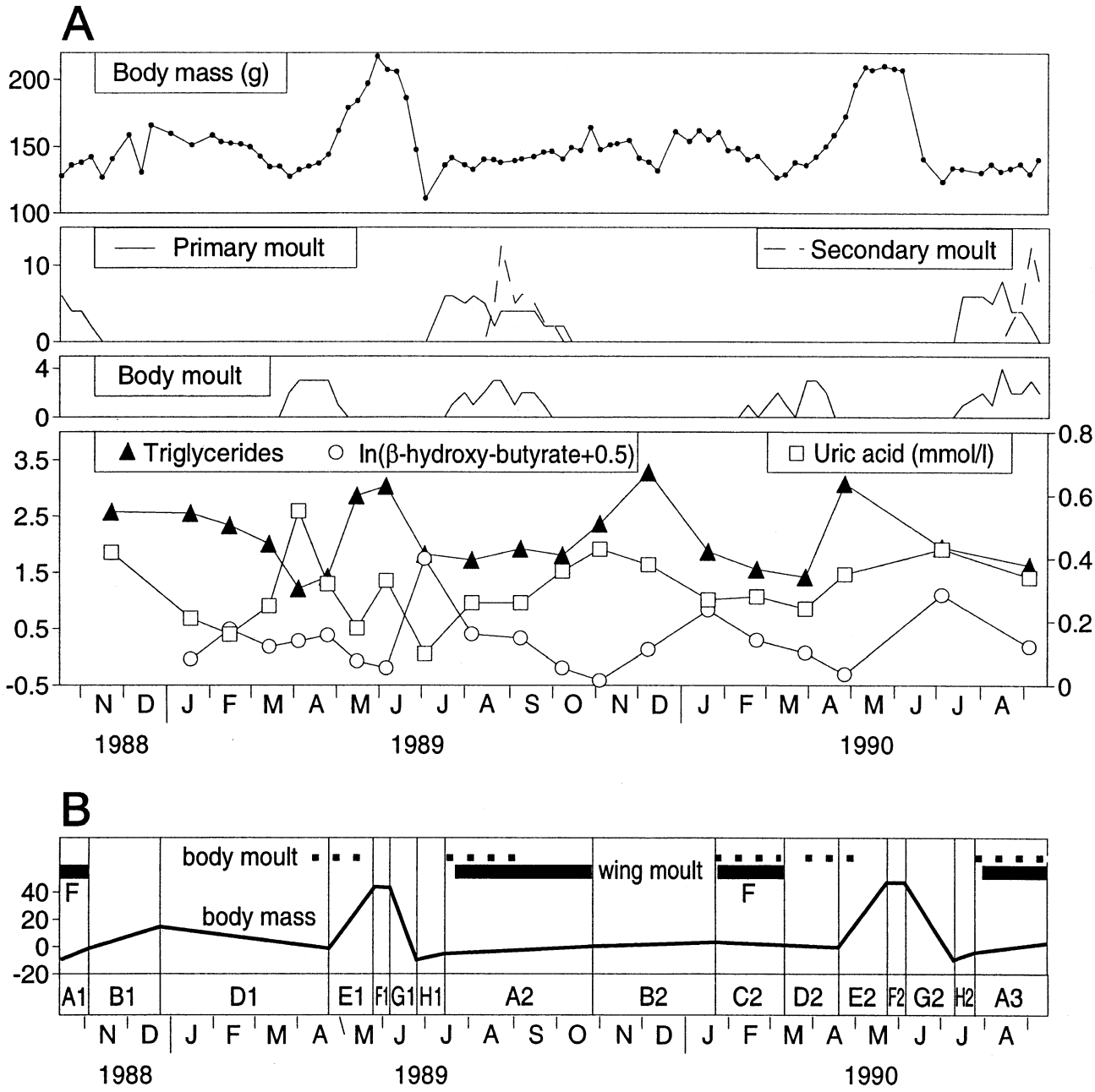

Fig. 1. **(A)** Body mass, moult and plasma metabolite levels of an individual knot (K409). The panels show (from top to bottom): body mass (g); number of growing primaries and secondaries; intensity of body moult (score ranging from 0 to 3); plasma levels of triglycerides and β-hydroxy-butyrate (left axis) and of uric acid (right axis) (mmol/l). **(B)** Mean body mass and moult substages in all eight red knots. The graph shows the mean duration of distinct annually recurring substages (A–H, see Table 1) determined from changes in body mass and moult of all individual birds. Letters from A–H denote the substages of one calender year (see Table 1). Numbers following letters denote annually recurrent substages. Body mass is expressed as percentage deviation from the individual mean body mass of substages with low body mass (see methods). The wing-moult period ocurring only in part of the individuals is marked with F.

the secondaries were moulted in March and April before the increase in body mass started. This resumed primary moult occurred in only two individuals in the first year, but in six individuals in the second year, the others having completed primary moult during the main period. The two moult periods corresponded well with those of free-living birds (Piersma and Davidson, 1992; Piersma et al., 1996a).

Annual cycle in plasma metabolite levels

The "average" annual cycle of the captive red knots and the division into eight different substages according to body mass and moult are shown in Figure 1B. The plasma levels of triglycerides and β-hydroxy-butyrate showed significant differences between the substages (Fig. 2). For both metabolites peaks of plasma levels were repeated at seasonally recurring stages. Triglycerides were high during all substages of body mass increase and of high body mass (substages B1, E1, F1, E2). In contrast, β-hydroxy-butyrate levels were high during substages of body mass decrease (G1, G2) and generally low during increase and stabilization of body mass (substages E1, F1). It appears that the annual cycles of metabolite levels were primarily correlated with changes in body mass and less so with the incidence of moult. These relationships will be examined in more detail in the following paragraph.

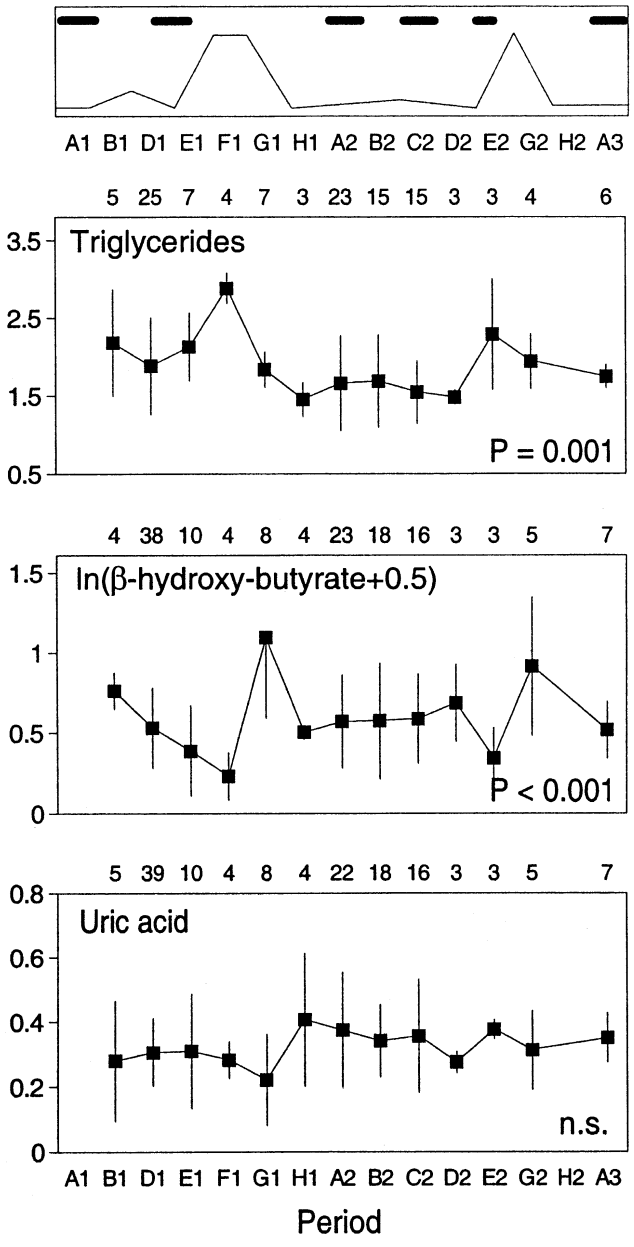

Fig. 2. Mean (± SD) plasma levels of metabolites (mmol/l) for the different stages (see Fig. 1B). Only averages based on >2 birds are shown. Numbers above the graphs indicate sample sizes. P-values indicate the significance of differences among substages (REML). For uric acid, sampling time had a significant effect on plasma levels ($P < 0.001$). For β-hydroxy-butyrate the individuals had a significant influence ($P < 0.001$) The top panel schematically represents body mass (line) and moult periods (bars), as determined in Figure 1B.

Relationships of metabolite levels with body mass, mass changes and moult

The REML analysis showed that relative body mass and presence/absence of moult had a significant effect on triglyceride levels, but this was not true for body mass change (Table 2). Analysed for substages with low body mass only, triglyceride levels were significantly lower in birds during moult of their body feathers than in birds not moulting body feathers (mean triglyceride level 1.58 ± 0.59 mmol/l (N = 43) versus 1.81 ± 0.48 mmol/l (N = 47) REML analysis: $P = 0.04$), while wing moult had no significant effect. Moreover, triglyceride levels decreased with increasing body moult intensity (Fig. 3).

Relative body mass and change in body mass had a significant effect on β-hydroxy-butyrate levels (Table 2). Even for substages of low body mass, no significant effect of moult on β-hydroxy-butyrate levels was found.

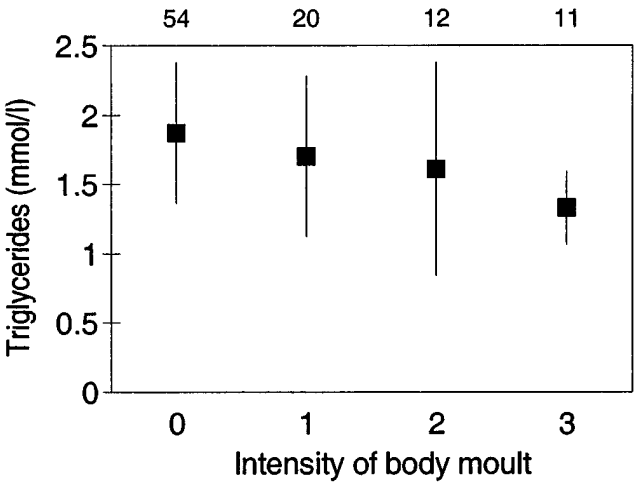

Fig. 3. Mean (± SD) plasma triglyceride levels during substages of low body mass at different body moult intensities. Sampling time and individuals were n.s. (0 = no moult, 3 = heavy body moult (REML analysis: $P = 0.01$).

Table 2. REML analysis of plasma metabolite levels. The full model is as described in Material and Methods. The individuals were not significant for triglycerides and uric acid, significant for β-hydroxy-butyrate ($P < 0.002$). The fixed effects were evaluated sequentially from top to bottom as shown in the table. Non-significant effects were removed and the most parsimonious model is shown. The slopes (standard error) and Wald statistics are given with their significance level. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Dependent variable	Fixed effects	Slopes (s.e.)	Wald statistic	N
Triglycerides	Relative body mass	0.014 (0.003)	27.6***	120
	Moult, no moult	0.000		
	in moult	–0.206 (0.097)	4.5*	
	Constant	1.926 (0.070)		
ß-hydroxy-butyrate	Relative body mass	–0.005 (0.002)	14.6***	143
	Body mass change	–0.105 (0.025)	18.0***	
	Constant	0.197 (0.082)		
Uric acid	Sampling time	–0.006 (0.001)	29.8***	144
	Body mass change	–0.022 (0.008)	7.0**	
	Constant	0.456		

Uric acid levels were positively correlated with change in body mass and sampling time. No significant relationship with moult, relative body mass or the interaction term change in body mass x sampling time could be detected.

Discussion

Metabolic responses to body mass changes and body mass

The deposition of body stores takes place during periods when energy intake exceeds energy expenditure. During short fasting periods in the phase of storage, birds have been shown to rely more on hepatic and muscular lipids than outside the storage phase (Jenni-Eiermann and Jenni, 1996). Hence, metabolic correlates with migratory fattening have to be considered at various time scales relative to food intake, i.e. relative to the last food intake (sampling time), relative to daily body mass increase, and relative to the life cycle (sub) stage.

In this study, birds were bled 2–5 h after food withdrawal. Plasma uric acid concentrations were still decreasing after this period (sampling time significant in Table 2), which indicates a prolonged catabolism of dietary protein, probably linked with a conversion into lipids. The decreasing uric acid levels and the low plasma β-hydroxy-butyrate level reflect the typical pattern of a late resorptive state. This contrasts with small passerines that show significantly increased plasma β-hydroxy-butyrate levels after already 90 min of fasting (Jenni-Eiermann and Jenni, 1991). The fact that it takes large sized king penguins *Aptenodytes patagonica*, during phase I and II of the breeding-fast, five days to show an increase in β-hydroxy-butyrate (Cherel et al., 1988b), suggests that the change into the fasting state is size-dependent, and correlates with mass-specific metabolic rate.

An important metabolic feature of the migratory period is the large increase in bodily fuel stores prior to long-distance flights. The red knot is a long-distance migrant and shows the typical changes in body mass, corresponding to the migratory periods, and these are also apparent in the captive birds (Fig. 1A, B). Body stores for migratory flights consist mainly of lipids, but also of protein (Lindström and Piersma, 1993; Piersma et al., 1999). In red knots that feed on a protein-rich diet of molluscs in the wild and trout pellets in captivity, the build-up of lipid stores is expected to be partly due to the transformation of proteins into lipids with a corresponding loss of nitrogen via uric acid, the end product of protein catabolism in birds.

In the present study body mass was recorded once a week. Plasma uric acid levels were positively and plasma β-hydroxy-butyrate levels negatively correlated with body mass change. Uric acid is an indicator of protein catabolism and can be elevated either during resorption (reflecting the breakdown of nutritional protein; Jenni-Eiermann and Jenni 1991, 1994), or during long-term fasting (reflecting the breakdown of body protein; Cherel et al., 1988a, b). In contrast, β-hydroxy-butyrate has been shown to be an indicator of fat catabolism and to be negatively correlated with body mass gain (Jenni-Eiermann and Jenni, 1994; Jenni and Schwilch, 2001). As low β-hydroxy-butyrate levels indicate a resorptive or early postresorptive state, the high uric acid levels of red knots gaining body mass can be interpreted as an increased intake and breakdown of proteinaceous nutrients. Congruent with the findings in two passerine migrants showing that triglycerides indi-

cate fat deposition rate during the last hours before blood sampling only, not for the previous 24 hrs (Jenni-Eiermann and Jenni, 1994; Jenni and Schwilch, 2001), triglyceride levels were not correlated with body mass change measured over a period of 5–7 days. However, an intense recent study suggests a correlation with body mass change rates after all (M.W. Dietz, S. Jenni-Eiermann, T. Piersma unpubl. obs.).

Triglyceride levels correlated positively with relative body mass and were high during substages of high body mass and body mass increase. This is also in accordance with studies on passerines showing that during periods of fat deposition more triglycerides are available after food intake (Jenni-Eiermann and Jenni, 1996). It also reflects increased lipid storage during the day associated with migratory fattening as shown for Canada geese *Branta canadensis*, dunlin *Calidris alpina*, white-crowned sparrows *Zonotrichia leucophrys*, blackcaps *Sylvia atricapilla*, garden warblers *S. borin* and European robins *Erithacus rubecula* (Mori and George, 1978; deGraw et al., 1979; Jenni-Eiermann and Jenni, 1996; Mattig, 1998).

In summary, the high uric acid levels indicate an increased breakdown of nutritional protein, probably reflecting the conversion of proteins into lipids, which still takes place well after the last food intake and correlated with body mass increase over the last week. Triglyceride levels did not reflect the fat deposition rates averaged over one week, but reflect the higher availability of triglycerides during substages of body mass gain and of high body mass. This accords with the especially low β-hydroxy-butyrate levels during these life-history substages.

Metabolic correlates of moult

During moult a high proportion of body proteins are replaced (Newton, 1966, 1969; Murphy, 1996). Hence, moult may affect the metabolism by increased energy requirements and protein synthesis (Lindström et al., 1993). In the red knots plasma triglyceride levels decreased with body moult intensity. During moult, when an increased protein metabolism takes place, fat stores tend to be low. Small moulting passerines show a more moderate increase in triglyceride levels over the day than during migration (Jenni and Jenni-Eiermann, 1996), in accordance with lower daily body mass and fat store gains (Jenni-Eiermann and Jenni, 1996). Reduced plasma lipids during feather formation were also found in white-crowned sparrows (deGraw et al., 1979). The finding that in red knots triglyceride levels were dependent on the intensity of moult among substages with low body mass levels, suggests that lipids are less available during intense moult. Increased plasma levels of uric acid (indicating a higher net protein breakdown) known from moulting penguins (Groscolas, 1982), were not observed, however. This can be explained by the fact that penguins, but not red knots, are fasting during moult and have to catabolize bodily protein for the build-up of feathers.

Conclusion

This study showed that plasma metabolite levels change with the different requirements of the annual cycle. The results confirm earlier studies of small passerines and penguins in the field and show that plasma metabolites offer the possibility, also in charadriiforms, to rapidly assay the main concurrent metabolic profile. Plasma metabolites may therefore enable studies on the influence of exogenuous factors (such as weather or habitat characteristics) on physiological processes (e.g., fuelling status, moult intensity) by single sampling of individuals. We are presently following up this line of enquiry.

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