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Effects of physiological state, mass change and diet on plasma metabolite profiles in the western sandpiper *Calidris mauri*

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Summary

We used a food restriction/refeeding protocol to put birds through a controlled cycle of mass loss and mass gain to investigate the effects of rate and phase of mass change on plasma metabolite levels in relation to diet. Despite marked differences in fat content of the two diets (18% *vs* 4%) mean rate of mass loss or mass gain was independent of diet. There was also no effect of diet on plasma levels of any of the four measured metabolite (triglyceride, glycerol, uric acid and β-OH-butyrate) during mass loss. However, during mass gain birds on the low fat diet had higher plasma levels of triglyceride and uric acid and lower β-OH-butyrate than birds gaining mass on the high-fat diet. Thus, diet composition can affect plasma metabolite profiles independently of differences in rates of mass change. Nevertheless, certain plasma metabolites were related to variation in rates of mass change across physiological states. Glycerol levels were negatively related to the rate of mass change (independent of diet), and butyrate was negatively related to the rate of mass change on both diets (though the slope of this relationship was diet dependent). Uric acid was positively related to the rate of mass change but only for birds on the low-fat diet. Our study therefore confirms that measurement of plasma metabolites can provide robust information on physiological state (gain, loss) and the rate of mass change (e.g. in free-living birds caught only once) although researchers should be cogniscent of potential confounding effects of diet composition for certain metabolites, both for field studies and for future experimental validations of this technique.

Key words: metabolites, mass change, migration physiology, fattening, captive shorebirds.

Introduction

Refueling or fattening rates are thought to be important determinates of the timing and success of long-distance migration in birds (e.g. Alerstam and Lindstrom, 1990), and recently decreases in refueling rates have been implicated in rapid population declines in some Arctic-breeding shorebirds (Brown et al., 2001; Baker et al., 2003). However, empirical data on rates of refueling, i.e. mass or fat gain, are difficult to obtain directly in free-living birds through measurement of body mass because of problems associated with recapturing individuals, especially in very large, asynchronously migrating populations, and potential effects of capture stress (Guglielmo et al., 2005; but see Dunn, 2000). Furthermore, single measurements of body mass or condition can provide unreliable information on dynamic rates of mass change (Williams et al., 1999).

Several recent studies have suggested that measurement of plasma metabolite concentrations might provide useful information on rates of mass change or physiological state (e.g. feeding *vs* fasting; Jenni-Eiermann and Jenni, 1994; Williams et al., 1999; Jenni and Schwilch, 2001), patterns of fuel utilization (Gannes, 2001; Jenni-Eiermann et al., 2002a) and inter-population or inter-site variation in fattening rates (Schaub and Jenni, 2001; Guglielmo et al., 2002). Direct validation of the use of plasma metabolite profiles to predict mass change in free-living birds will require independent assessment of fattening rates, which is difficult because of the problems associated with multiple recaptures of individuals, as outlined above (but see Guglielmo et al., 2005). Thus, the biological interpretation of changes in plasma metabolite concentrations in free-living individuals is currently based on relationships between metabolite concentrations and rate of mass change established in captive individuals (e.g. Jenni-Eiermann and Jenni, 1994; Williams et al., 1999). However, there have been relatively few thorough validations of this technique, and it is unclear how the relationship between metabolites and mass change is affected by captivity itself (see Lambrechts et al., 1999), or factors such as variation in diet quality, or the rate of mass change itself. For example, DeGraw et al. (1979) found that metabolite concentrations in captive white-crowned sparrows (*Zonotrichia leucophrys*) differed

from those in free-living individuals. Diet quality has been shown to influence fuel utilization and fattening rates in some passerines (e.g. Bairlein, 1998; Gannes, 2001), but, to our knowledge, the effect of diet composition on plasma metabolite concentrations has not yet been tested. This information is important since it can indicate how robust mass-change-dependent variation in metabolite levels will be in the context of even greater ecological variability experienced by free-living birds.

In this paper, we examine the effects of rate and trajectory of mass change and diet quality on plasma metabolite levels (triglyceride, glycerol, uric acid and β-OH-butyrate) in western sandpipers (*Calidris mauri*, Cabanis), a long-distance migrating, Arctic-nesting shorebird. The specific objectives of the study were (a) to experimentally manipulate rates of mass change and mass trajectory, using high- and low-fat diets, to investigate effects of diet quality on metabolite levels during different mass cycle phases or physiological states (i.e. mass loss, mass gain, and stable mass), and (b) to determine the effect of diet quality on the relationship between plasma metabolite levels and rate of mass change. Finally, since there is often marked individual variability in plasma metabolite levels, even for a given mass or physiological state (e.g. Guglielmo et al., 2002; Ydenberg et al., 2002) we also investigated whether measures of body condition or plasma metabolite levels at time of capture (i.e. in free-living individuals) predicted variation in these parameters in the same birds in captivity.

Materials and methods

Animals and diets

Female juvenile western sandpipers were captured at two migratory stopover sites in the Fraser River Delta, Boundary Bay (49°04′, 122°58′) and Robert's Banks (49°03′, 123°08′), during the southward migration in August 2001 (*N*=10) and 2002 (*N*=36), using mist nets (Avinet, Dryden, New York, USA). When possible, blood samples were taken from individuals upon capture ('wild' phase) to obtain free-living plasma metabolite levels. Capture and banding was carried out under United States Fish and Wildlife Service, Washington Department of Fish and Wildlife, Environment Canada, and Simon Fraser University Animal Care permits. Birds were maintained in captivity at the Animal Care Facility at Simon Fraser University, in outdoor aviaries (3 m × 6 m × 2 m high) on a natural light cycle, with an infrared heat lamp and *ad libitum* access to water. Birds were provided with a diet of boiled egg (with shell) and mealworms for the first few days of captivity and were transitioned slowly to a diet of Clarke's Fry trout chow (see below). After birds were successfully transitioned to the standard diet, based on stabilization of body mass, all birds were blood sampled to obtain pre-treatment metabolite values with *ad libitum* food ('pre-treatment' phase) prior to further diet manipulation.

Two different diets were used in this experiment. Our standard diet was 1.5 mm (width) pelleted Clark's Fry trout chow (Moore-Clark, Vancouver, Canada); the same diet was used in our previous studies (Williams et al., 1999; Egeler et al., 2003) and is similar to that used in other studies (Jenni-Eiermann et al., 2002b). This high-fat, high-protein diet, referred to hereafter as the high-fat diet, is primarily fish meal-based and consists of 47% protein, 18% fat, 2% fiber, 9% ash and 16.5% nitrogen-free extract (NFE), with a total energy content of 18.2 kJ g^{-1}. The second diet used was Hikari Cichlid Baby 2.0 mm (diameter) pellet (Hikari USA, Hayward, California, USA), a low-fat, high-protein diet, referred to hereafter as the low-fat diet. This is primarily fish meal-based and consists of 35% protein, 4% fat, 5% fiber, 9% ash and 37% NFE, with a total energy content of 16.2 kJ g^{-1} (this second, low-fat diet more closely approximates to the fat content of natural prey items; see Egeler and Williams, 2000).

General experimental protocol

Daily food consumption (g $bird^{-1}$ day^{-1}) was measured as the combined amount for all birds in each experimental group throughout the experiment (see below for final sample sizes). All birds were weighed weekly (±0.1 g), and birds were weighed every other day during periods of mass loss and gain, to closely monitor mass change. Any individuals with body mass <22 g (lean mass in this species; Guglielmo and Williams, 2003) were separated out and excluded from the experiment. These individuals were held in a separate pen on the high-fat diet and if they recuperated to a mass >22 g they were used in subsequent experiments. Birds were caught a few at a time (4–5 birds per experimental group) and blood sampled indoors to allow the remaining individuals to return to normal foraging activity between disturbances; birds returned to foraging within 1 min of the researcher vacating the aviary. Total disturbance time, the number of minutes between the first disturbance of birds in a cage and the final bird being caught and sampled in that cage, averaged 23.7±14.9 min. Bleed time, between capture and blood sampling of each individual, averaged 7.6±3.7 min, and 90% of the bleed times were less than 12 min. All birds were blood sampled and mass recorded between 10.00 h and 12.00 h PST (Pacific standard time; so time of day, or time since last meal was not a confounding factor in our study, see Discussion). Birds were blood sampled *via* brachial venipuncture with a 26.5 gauge needle and blood (up to 300 μl) was collected with heparinized capillary tubes and centrifuged at 1800 ***g*** for 10 min. Plasma was drawn off using heparanized capillary tubes and was stored at –20°C until assayed.

Diet experiments

Experimental work was conducted over several months beginning in October 2002. *Ad libitum* food was defined as 12.5 g $bird^{-1}$ day^{-1}, based on preliminary experiments where average food consumption was 6.6±1.7 g $bird^{-1}$ day^{-1}. Birds were divided into two experimental groups (low-fat and high-fat diets), equally divided in terms of age (juveniles, *N*=36, caught in August 2002, and yearlings, *N*=4, caught in 2001) and capture site (Boundary Bay and Robert's Bank). Birds

were transitioned from the high- to low-fat diet over a 2 week period. In Trial 1, both the high-fat and low-fat groups were cycled through mass loss (food restriction) and mass gain (refeeding). Blood samples were taken in the middle of the 'Loss' phase (day –7 relative to the end of the experiment, see Fig. 1) and 2 days after refeeding during the 'Gain' phase (day –2, Fig. 1). Mass loss was achieved by food restriction, during which time birds received 85% of the average food consumption (g bird^{-1} day^{-1}) for that group. To ensure continued mass loss, food was further restricted to 80% of average consumption during the final 2 days of the Loss phase. At the end of the mass Gain phase, once body mass stabilized, birds were blood sampled again during *ad libitum* food consumption ('Adlib' phase), and the birds were left on that diet for at least 2 weeks after the last blood sampling before being switched to the alternate diet and used in Trial 2. The same cycle of mass loss/gain was then repeated on the alternate diet (Trial 2) so that each individual served as its own control. This random order design accounts for possible time of year effects, given that a minimum of 1 month separated the two cycles because of recovery and transition periods. This experimental design also controls for possible effects of experience (i.e. birds having previously been through a loss–gain cycle), because half of the birds experienced diet manipulation for the first time on the high-fat diet and the other half experienced the manipulation for the first time on the low-fat diet. Some birds (N=7) in the first group transitioned to the low-fat diet were unable to stabilize body mass on that diet and were isolated from the rest of the birds. As soon as their body mass did stabilize they were run through the mass cycle on the low-fat diet as a separate group. There was no significant difference between these two groups of birds in the rate of mass change during either the Adlib or Loss phases (P>0.7, both phases). The lighter birds that were separated achieved a marginally lower rate of mass gain than the original group ($F_{1,13}$=5.04, P=0.045); however, there was no significant difference in the percentage of mass gain or loss achieved between these two groups (P>0.1, both parameters). Therefore, the results of these two groups were pooled together and analyzed as a single set of birds on the low-fat diet in Trial 1. Data were pooled between trials and analyzed for each diet treatment (low- or high-fat). Final sample sizes were N=19 for birds cycled through a loss–gain cycle on the low-fat diet, N=28 for on the high-fat diet and N=15 for the mass cycle on both diets.

Plasma metabolite assays

Free plasma glycerol and triglyceride were assayed using a sequential color endpoint assay (Trinder reagent A and B, respectively, Sigma-Aldrich Canada, Oakville, Ontario, Canada), using 5 µl of sample with 240 µl and 60 µl of reagents A and B respectively, with a reading taken at 540 nm after 10 min of incubation at 37°C after the addition of each reagent. Triglyceride concentration (mmol l^{-1}) was calculated by subtracting free glycerol from total glycerol. Uric acid was assayed via color endpoint assay (WAKO USA, Richmond, Virginia, USA), using 5 µl of sample with 300 µl of reagent, with a reading taken at 550 nm after 10 min of incubation at 37°C. Assays were run in 400 µl flat-bottom 96-well microplates (NUNC, Denmark) and read with a microplate spectrophotometer (Biotek 340EL). Each plate was run with a standard curve based on a serial dilution of 2.54 mmol glycerol (Sigma-Aldrich Canada, Oakville, Ontario, Canada) for the triglyceride-glycerol assay and 2.97 mmol uric acid (prepared in our laboratory) for the uric acid assays. Each plate also included a 19-day-old hen plasma pool used to calculate inter-assay coefficient of variation. Inter-assay coefficients of variation were 3.1% (N=11), 7.0% (N=11), 6.1% (N=4) and 9.4% (N=5), and intra-assay coefficients of variation were 3.2% (N=6), 3.9% (N=6) and 3.1% (N=17) for glycerol, triglyceride and uric acid respectively. β-OH-butyrate was analyzed by one of us (C.G.G.) *via* kinetic endpoint assay (Guglielmo et al., 2005).

Statistical analysis

All four metabolite levels were non-normally distributed, so we transformed the data using ln(metabolite) to approximate normality and transformed data were used in all analyses. Owing to the very short period of mass gain, we calculated ΔMass over the first two days of mass gain (between day –4 and –2). However, the period of mass loss was more prolonged. We therefore calculated ΔMass over the 3-day period prior to the Loss blood sample (i.e. between day –10 and day –7, Fig. 1). We considered the shorter intervals of mass gain and loss (2–3 days) as more biologically relevant since they approximate average flight and stop-over times for migrating western sandpipers (Warnock and Bishop, 1998).

The relationship between the rate of mass change (ΔMass; g day^{-1}) and residual metabolite values (controlling for covariates as necessary, see below) was tested for each metabolite independently on a pooled data set including individuals and in all three mass cycle phases, using a linear mixed model with repeated measures to account for repetition of individuals with diet as a main effect. Testing the mass change-metabolite relationship for all phases of the loss-gain cycle with a single model allowed for maximization of range of mass change. In each case if the ΔMass*diet interaction term was significant the relationship between ΔMass and metabolite concentration was tested separately by diet; otherwise the interaction term was dropped from the model. All statistical analyses were carried out using SAS (SAS Institute, 1990).

Results

Variation in rates of mass change in relation to diet manipulation

On both diets the trajectories of mass change were similar for Trials 1 and 2 (Fig. 1). On the low-fat diet, there was no difference in initial, lowest or final mass between trials (P>0.05 in all cases; Table 1). In contrast, on the high fat diet initial and lowest masses were lower in Trial 2 than Trial 1 (P<0.05), but there was no difference in final mass (Table 1). For rate of

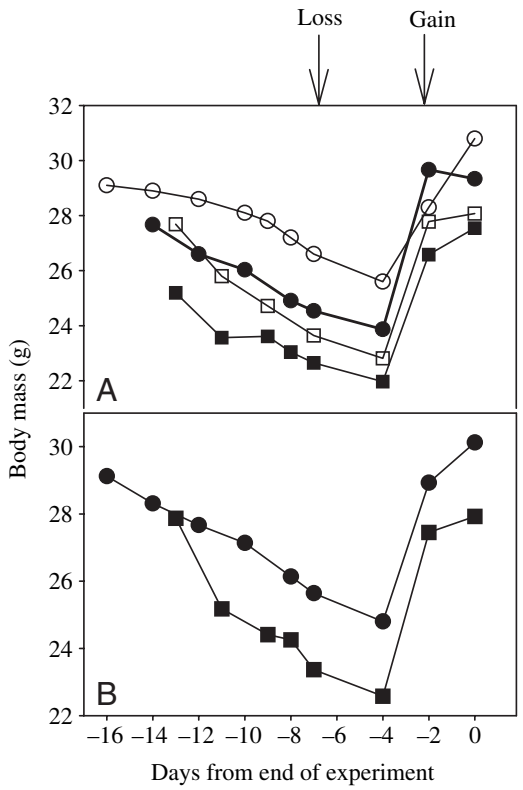

Fig. 1. Mean body mass of captive western sandpipers during mass loss and gain cycles (A) during Trial 1 (open symbols) and Trial 2 (closed symbols) on low-fat (squares) and high-fat (circles) diets, and (B) pooled data for the two trials on different diets. Days of experiment have been standardized from the end of the experiment. Arrows indicate time of blood sampling for the loss and gain phases.

mass change, there was no difference between trials for either mass gain or loss on the low fat diet (P>0.5). For the high-fat diet there was similarly no trial difference for mass loss (P>0.30), but rate of mass gain was higher in trial 2 compared with trial 1 (P<0.01). However, since we were primarily interested in the relationship between *variation* in mass change and plasma metabolites, and since there was a control for body mass, we pooled data from Trials 1 and 2 for subsequent analysis (see Fig. 1).

For the pooled data, the lowest mass reached during Loss and the final mass reached during Gain were both lower for birds on the low-fat diet than for birds on the high-fat diet (lowest mass, day –4, t_{43}=–3.40, P<0.01; final mass, day 0, t_{41}=–2.26, P<0.05; Fig. 1); however, initial mass was independent of diet (t_{42}=–1.11, P>0.2; Fig. 1B). As we had anticipated, mass cycle phase (gain, loss, ad lib) had a highly significant effect on rate and trajectory of mass change (low-fat diet, $F_{2,16}$=75.2, P<0.0001; high-fat diet, $F_{2,21}$=32.3, P<0.0001; Table 2). For both diets, all pair-wise contrasts of mass change between mass cycle phases were highly significant (P<0.01 in all cases). However, there was no effect of diet on rate of mass change for either the Loss, Gain or Adlib phases of mass change (P>0.60 in all cases; Table 2). Mean

Table 1. *Initial, lowest and final body mass (g) of captive western sandpipers during mass loss and gain cycles by trial (Trials 1 and 2) and diet (high fat and low fat)*

Diet	Mass (g)	Trial 1	Trial 2	P
High fat (N=15,13)	Initial	29.1±2.4	27.7±5.3	0.020
	Lowest	25.6±1.9	23.9±2.5	0.037
	Final	30.8±3.2	29.3±5.0	0.572
Low fat (N=14,5)	Initial	27.7±4.4	25.2±1.6	0.247
	Lowest	23.2±1.4	22.0±0.9	0.081
	Final	28.5±3.6	27.5±3.0	0.709

Values are means ± S.D.; N values in parentheses are for Trials 1 and 2, respectively.

mass change was significantly different from zero for the Loss and Gain phases, but not when birds were on Adlib food (Table 2).

For each diet, size-corrected body mass (controlling for tarsus length) was positively correlated between all three mass cycle phases (low-fat: r>0.5, P<0.02; high-fat: r>0.4, P<0.04 all cases), i.e. the heaviest individual during Loss would also be the heaviest individual during Gain. In contrast, none of the four metabolite values were correlated within individuals between different phases of mass change on either diet (P>0.07, all cases).

Effects of bleed time, handling time and body mass on metabolite levels

Plasma glycerol levels were independent of plasma levels of the other three measured metabolites (P>0.1 for each comparison with the three other metabolites), so this metabolite was analyzed separately. There was a positive correlation between triglyceride and uric acid levels (r=0.47, P<0.0001), and both triglyceride and uric acid were negatively correlated with β-OH-butyrate (triglyceride, r=–0.47, P<0.0001; uric acid, r=–0.56, P<0.0001). Plasma triglyceride levels were positively related to bleed time (triglyceride: $F_{1,205}$=6.64, P<0.02, b=0.016), whereas glycerol, uric acid, and

Table 2. *Rates of mass change for captive western sandpipers during different phases of an experimentally induced mass loss and gain cycle on a low-fat and high-fat diet*

Phase	Diet	Mass change (g day^{-1})	Range
Loss	Low-fat	–0.57±0.12**	–1.14 to –0.17
	High-fat	–0.61±0.38*	–2.89 to 2.11
Gain	Low-fat	2.03±0.87**	0.75 to 4.43
	High-fat	2.16±1.31**	0.40 to 5.56
Adlib	Low-fat	–0.07±0.06	–1.75 to 0.98
	High-fat	0.00±0.95	–2.91 to 2.64

Values are means ± 95% CI, with ranges; N=18 (low-fat) and N=23 (high-fat) diet.

Asterisks indicate mass change is significantly different from zero, *P=0.01, **P<0.001.

β-OH-butyrate levels were independent of bleed time (P>0.1, in all cases). In addition, plasma triglyceride and uric acid were negatively related to total disturbance time (triglyceride, $F_{1,172}$=5.70, P<0.02, b=–0.007; uric acid: $F_{1,171}$=16.90, P<0.0001, b=–0.011), while β-OH-butyrate levels were positively related to total disturbance time ($F_{1,158}$=7.75, P<0.01, b=0.009). Plasma triglyceride and uric acid were positively related to body mass (triglyceride, $F_{1,205}$=56.68, P<0.0001, b=0.069; uric acid, $F_{1,203}$=3.26, P=0.07, m=0.018) whereas β-OH-butyrate was negatively related to body mass ($F_{1,178}$=12.97, P<0.001, b=–0.041). For subsequent analyses, of triglyceride, uric acid and β-OH-butyrate residual metabolite values were calculated from a regression analysis including mass, disturbance time or bleed time as factors where appropriate. Glycerol was independent of all three factors (body mass, bleed time, and total disturbance time, P>0.3, in all cases) and log transformed plasma glycerol concentrations will be referred to as glycerol levels hereafter.

Effect of mass cycle phase and diet on plasma metabolite levels

On the low-fat diet, there was an overall phase effect for all four metabolite values, i.e. metabolite levels varied significantly in relation to phase of mass change (repeated measures ANOVA, P<0.001 in all cases; Fig. 2). In contrast, on the high-fat diet, there was a significant overall phase effect for glycerol and uric acid (P<0.002 in both cases), but triglyceride and β-OH-butyrate levels were independent of phase of mass change (P>0.1; Fig. 2). Birds losing mass had higher glycerol levels than birds in both Gain and Ad Lib phases of mass change, on both diets (P<0.05), with no difference in glycerol levels between Gain and Adlib phases (Fig. 2B). Similarly, on the low fat diet, birds losing mass had lower levels of uric acid and higher levels of β-OH-butyrate than birds in both Gain and Adlib phases (P<0.005; Fig. 2C,D). On the high fat diet, birds losing mass also had lower uric acid levels than in the Adlib phase (P<0.001), but uric acid levels were only marginally different between Loss and Gain phases (P=0.08; Fig. 2C). There were few significant differences in metabolite levels between Gain and Adlib phases of mass change, except that triglyceride levels were higher during the Adlib phase on the high-fat diet (P<0.05; Fig. 2A).

Comparing metabolite levels between diets, within each phase of mass change there were no significant differences in metabolite levels between the low- and high-fat diets for the Loss phase (P>0.1; all four metabolites). For both the Gain and Adlib phases of mass change, birds on the low-fat diet had higher triglyceride and uric acid levels and lower β-OH-butyrate levels than those on the high-fat diet (P<0.001 in all cases), but glycerol levels were independent of diet (P>0.4 in both cases; Fig. 2).

Relationship between metabolites and rate of mass change

For triglyceride and glycerol levels, the ΔMass*diet interaction was not significant (P>0.09), so it was dropped from the model. Triglyceride levels were independent of the rate of mass change ($F_{1,41}$=2.02, P>0.1; Fig. 3A). However, there was a significant effect of diet for this relationship with birds on the low-fat diet having higher triglyceride levels than birds on the high-fat diet ($F_{1,27}$=83.63, P<0.001), consistent with the analysis of mean metabolite levels for each phase. Glycerol levels were negatively related to the rate of mass change ($F_{1,40}$=14.59, P<0.005, b=–0.10) and this relationship was independent of diet ($F_{1,24}$=0.19, P>0.6; Fig. 3B). For uric acid and β-OH-butyrate, there was a significant ΔMass*diet interaction term (uric acid: $F_{1,32}$=6.02, P<0.02; β-OH-butyrate:

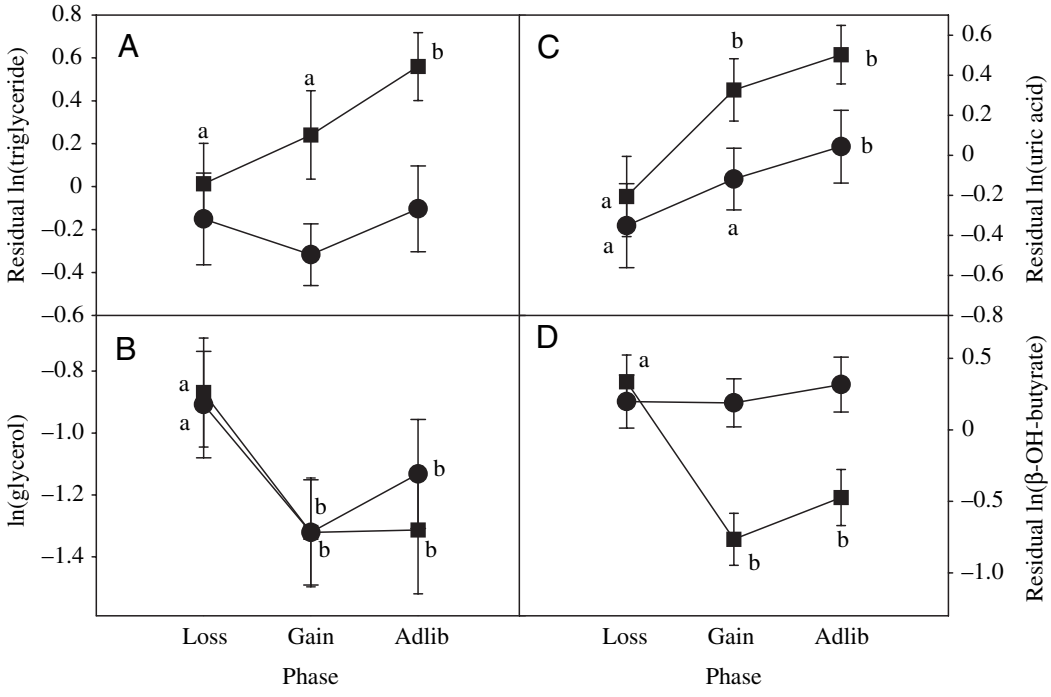

Fig. 2. Variation in plasma metabolite levels in relation to phase of mass change for (A) triglyceride, (B) glycerol, (C) uric acid and (D) β-OH-butyrate levels in captive western sandpipers on low-fat (squares) and high-fat (circles) diets. For each metabolite, and within each diet treatment, points with the same letter are not significantly different (P>0.05).

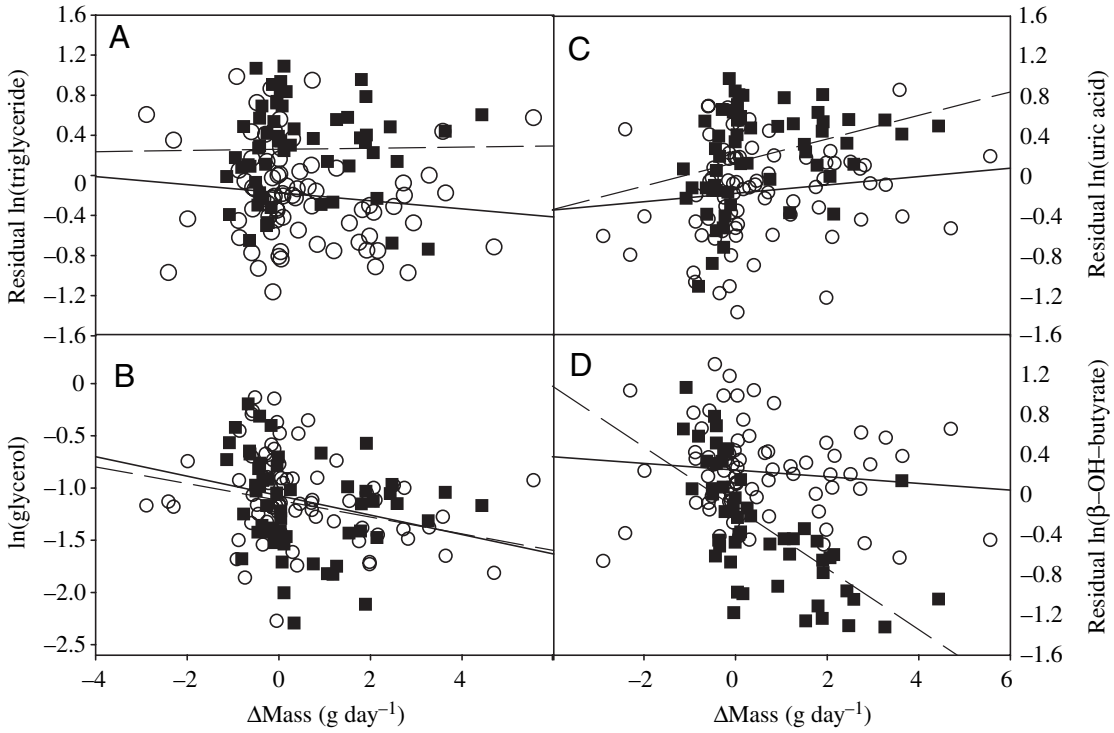

Fig. 3. Relationship between the rate of mass change (ΔMass) and (A) triglyceride, (B) glycerol, (C) uric acid and (D) β-OH-butyrate levels for captive western sandpipers on a low-fat diet (closed squares, broken line) and a high-fat diet (open circles, solid line).

$F_{1,28}$=25.64, P<0.001); therefore we analyzed each diet separately for these metabolites. Uric acid was positively related to the rate of mass change for birds on the low-fat diet ($F_{1,23}$=10.02, P<0.005, b=0.12); however, uric acid levels were independent of the rate of mass change on the high-fat diet ($F_{1,32}$=0.83, P>0.4; Fig. 3C). β-OH-butyrate was negatively related to the rate of mass change on both diets (low-fat: $F_{1,15}$=73.81, P<0.001, b=–0.30; high-fat: $F_{1,27}$=5.13, P=0.03, b=–0.06; Fig. 3D).

Comparison of mass and metabolite levels in free-living and captive birds

There was no correlation between size-corrected body mass and any metabolite values between birds at the time of original capture and the Pre-treatment phase (P>0.2, all parameters). Similarly, there was no correlation for these parameters between time of capture and birds in the Wild and Gain phases on either diet (P>0.1, all cases). Birds after transition into captivity, given an *ad libitum* high-fat diet, prior to diet manipulation (Pre-treatment) had lower uric acid levels and higher glycerol and β-OH-butyrate levels than they did at the time of original capture (glycerol: t_{35}=2.54, P<0.02; uric acid: t_{31}=–4.04, P<0.0005; β-OH-butyrate: t_{17}=4.48, P<0.0005). However, triglyceride levels did not differ between pre-treatment and time of capture (t_{33}=–1.17, P>0.2). Birds actively gaining mass (Gain) on the same high-fat diet had higher values of β-OH-butyrate and lower values of the other three metabolites than at the time of capture (triglyceride: t_{25}=–5.83, P<0.0001; glycerol: t_{26}=–2.82, P<0.01; uric acid: t_{23}=–5.11, P<0.0001; β-OH-butyrate: t_{15}=5.19, P<0.0001). In contrast, birds during the Gain phase on the low-fat diet did not differ from the original time of capture in any of the metabolite levels (glycerol: P>0.05; all others: P>0.4), i.e. the plasma metabolite profiles on the low-fat diet were more similar to those of free-living, migratory birds.

Discussion

Food restriction/refeeding allowed us to successfully put birds through a controlled cycle of mass loss and mass gain, to rigorously investigate the effects of phase of mass change and diet, on plasma metabolite levels. Surprisingly, even though we used experimental diets of very different fat content (18% and 4%) there was no difference in the mean rate of mass loss or mass gain between diets. Consistent with this lack of difference in rate of mass change, during mass loss there was no effect of diet on plasma levels of any of the four measured metabolites (triglyceride, glycerol, uric acid and β-OH-butyrate). However, during mass gain birds on the low-fat diet had higher plasma levels of triglyceride and uric acid and lower β-OH-butyrate than birds gaining mass on the high-fat diet, despite the fact that rate of mass gain was not different. This diet effect on plasma metabolites was also apparent in birds on *ad libitum* food when body mass was more or less stable, confirming that diet composition can affect plasma metabolite profiles independently of differences in rates of mass change. Nevertheless, we confirmed that certain plasma metabolites can provide information on variation in rates of mass change

across physiological states. In particular, in this study, glycerol levels were negatively related to the rate of mass change (independent of diet), and β-OH-butyrate was negatively related to the rate of mass change on both diets (though the slope of this relationship was diet dependent). Uric acid was positively related to the rate of mass change but only for birds on the low-fat diet. Finally, neither mass or metabolite levels of free-living birds at capture predicted their mass or metabolite responses during captivity when on the high-fat diet. It is important to note that the low fat diet (4%) we used more closely approximates the average lipid content of marine invertebrate prey targeted by western sandpipers on mudflats (2%; Egeler and Williams, 2000). Consistent with this, the plasma metabolite profiles of captive birds gaining mass on the low-fat diet were not significantly different from those of free-living birds at the time of capture (which we assume were fattening at the stop-over site where they were caught).

There are several factors that could have complicated our experimental design, e.g. time of year and the effects of molt. Jenni-Eiermann et al. (2002b) showed that in a congeneric shorebird, the red knot, there is a naturally occurring cycle in fattening captivity, which is reflected in changes in plasma metabolite concentrations. However, in our experiment birds went through the mass change cycle on the two different diets in random order. In addition, our entire experiment took place between October and January, which falls within a stable mass phase in the natural cycle reported in the study of Jenni-Eiermann et al. (2002b). We are, therefore, confident that time of year effects do not confound our results. Plasma triglyceride levels have been shown to vary with timing of molt (Jenni-Eiermann and Jenni, 1996; Totzke and Bairlein, 1998; Jenni-Eiermann et al., 2002b). However, our study (October to January) occurred almost exclusively outside of the periods of body molt for the western sandpiper. No individuals in our study experienced wing molt. Body molt was observed in a few of the individuals, however, only toward the end of the second trial (January), so we are confident that molt does not confound our results.

Plasma metabolite concentrations and physiological state

Numerous studies in captive passerines have shown that plasma metabolite concentrations reflect the physiological state of a bird in relation to fattening or fasting (DeGraw et al., 1979; Jenni-Eiermann and Jenni, 1994; Totzke and Bairlein, 1998). Jenni-Eiermann et al. (2002b) showed that certain plasma metabolites (triglyceride and β-OH-butyrate) reflect seasonally changing metabolic processes among different life cycle stages (molt, migration, oversumering) in captive red knot (with birds on a similar diet to our high-fat diet). Our study confirms some of the results of Jenni-Eiermann et al. (2002b; β-OH-butyrate), and extends their results for other metabolites (glycerol), but there are also differences between their study and ours for uric acid and triglyceride. In both the red knot and the western sandpiper periods of mass gain are characterized by low β-OH-butyrate levels, relative to phases of mass loss. However, this relationship between mass gain and β-OH-butyrate was only evident on the low-fat diet in our study: on the high-fat diet β-OH-butyrate did not differ for birds losing or gaining mass, even though absolute rate of mass gain was the same as for the low-fat diet. In western sandpipers we found higher uric acid levels associated with mass gain, but again this was only significant on the low fat diet. Jenni-Eiermann et al. (2002b) found no significant change in uric acid levels across different life stages, despite birds showing clear periods of body mass increase.

In their red knot study, Jenni-Eiermann et al. (2002b) reported triglyceride including free glycerol (rather than reporting free glycerol separately; see Williams et al., 1999) so their triglyceride results are not directly comparable to our study. However, Jenni-Eiermann et al. (2002b) concluded that life-stages with body mass gain were also associated with increased plasma triglyceride levels, reflecting increased turnover of lipids. In contrast, correcting for free glycerol, we found no difference in plasma triglyceride levels between phases of mass gain and loss on the low-fat diets, and again on the high-fat diet triglyceride levels did not vary with any phase of mass change. Free glycerol was higher during mass loss than during mass gain, consistent with results from some passerine studies (Jenni-Eiermann and Jenni, 1994) and this was the only metabolite in our study for which this relationship was independent of diet. Glycerol has traditionally been assumed to increase during mass loss (i.e. when birds are in negative energy balance) because it is released into the plasma during lipolysis of triglycerides in adipose tissue (Ramenofsky, 1990). Recently Guglielmo et al. (2005) suggested that glycerol might demonstrate a U-shaped relationship with body mass, such that it was also high in birds with very high rates of mass gain, but we found no evidence to support this in our study.

The effect of diet composition on plasma metabolite concentrations of avian migrants has not, to our knowledge, been tested before, although Bairlein (1998) tested for effects of diet lipid and protein content on rates of migratory fattening in the passerine garden warbler *Sylvia borin*. Bairlein (1998) measured fattening on a more exhaustive suite of 13 different diets and the results offer insight into our study. In our study, the diets varied dramatically in lipid content (18% *vs* 4%) but protein content was only slightly higher for the high-fat diet (47% *vs* 35%). Bairlein (1998) demonstrated that the fattening rate achieved by the warblers depended on the percentage protein and the relative proportion of protein to fat, as well as the percentage lipid in the diet. The warblers achieved the highest rate of fattening on a diet consisting of 5% protein and 10% fat (5:10), which is lower in protein but similar in fat to their natural diet (15:10). Furthermore, the warblers achieved a higher rate of fattening on the 5:10 diet than on a diet of identical protein content but higher fat content (5:20). Similarly, in our study, the birds on the diet lower in both fat and protein (~4:35 *vs* ~18:47) had plasma metabolite concentrations indicative of higher fattening (although we cannot rule out an effect of the different NFE content of our two diets).

Relationship between metabolite levels and rate of mass change

The relationship between the *rate* of mass change and plasma concentrations of three of the four metabolites tested (triglyceride, β-OH-butyrate and uric acid) was also dependent on diet composition in our study. For β-OH-butyrate we found a negative relationship between metabolite level and rate of mass change on both diets, even though the slope of this relationship differed among diets. The same relationship has been reported previously for several passerines (Jenni-Eiermann and Jenni, 1994; Jenni and Schwilch, 2001) and the red knot (Jenni-Eiermann et al., 2002b). Fewer studies have reported on the relationship between mass change and uric acid, but Jenni-Eiermann and Jenni (1994) also found a positive relationship, as we found on our low-fat diet. However, this result contrasts with that of Jenni-Eiermann et al. (2002b) in which the uric acid levels of red knots were negatively related to the rate of mass change over a period of 5–7 days. The reasons for this difference are not clear; although in our study and that of Jenni-Eiermann et al. (2002b) birds were fed trout chow, it is possible these differed in protein content, which is known to affect uric acid levels (Jenni and Jenni-Eiermann, 1998). Furthermore, elevated uric acid levels can be associated with both protein catabolism and high protein turnover during hyperphagia and fattening (Jenni-Eiermann and Jenni, 1994; Jenni-Eiermann and Jenni, 1998). Therefore, differential representation of range of mass change (i.e. loss or gain) in the two studies is a possible explanation for the positive versus negative relationship between uric acid and rate of mass change detected by our study and that of Jenni-Eiermann et al. (2002b).

We also detected a significant negative relationship between the rate of mass change and plasma glycerol in captive western sandpipers, which is consistent with the relationship previously demonstrated in this species by Williams et al. (1999). As with data on phases of mass change, this relationship was independent of diet. We did not detect any relationship between plasma triglyceride levels and mass change in the present study, which contrasts with the similar study of the same species by Williams et al. (1999). Nevertheless, Williams et al. (1999) failed to detect a relationship between triglyceride and mass change over 7 days in western sandpipers, whereas a significant positive relationship between plasma metabolite concentrations and the rate of mass change was detected over 1–2 days, i.e. the relationship between mass change and triglyceride was less robust than that for glycerol and mass change. Williams et al. (1999) also generated mass loss over 1–2 days *via* food removal, not food restriction, so metabolic responses might have reflected short-term starvation, with much higher rates of mass loss than in the present study. Conversely, the sampling periods of 7 days that were tested by Williams et al. (1999) were under natural mass change conditions and reflected less extreme mean rates of mass change (± S.E.M.; gain, 0.13±0.08 g day^{-1}; loss, –0.36±0.08 g day^{-1}, cf. rates in Table 2) than those experienced by the birds on the experimentally induced mass cycle in the present study (see Table 1). Nevertheless, Jenni-Eiermann et al. (2002b) also found no relationship between triglycerides and body mass change in their study of captive red knot. These inconsistent results for triglyceride in captive sandpipers contrast with generally consistent positive relationships between triglycerides and mass change in passerines (e.g. Jenni-Eiermann and Jenni, 1994; Jenni and Schwilch, 2001). This difference might be due, in part, to the fact that some studies report total triglyceride including free glycerol whereas others calculate triglyceride as total glycerol − free glycerol; free glycerol can represent a substantial, and highly variable, proportion of total glycerol. It is interesting that the relationship between plasma triglyceride and mass change is the most inconsistent of all the metabolites in experimental studies of captive birds, whereas plasma triglyceride appears to be the most robust indicator of mass change in studies of free-living birds (e.g. Schaub and Jenni, 2001; Guglielmo et al., 2002, 2005; D. S., unpublished data). Our study suggests that one reason for these inconsistencies might be effects of different quality diets used in experimental studies: high-fat, trout chow diets typically used in shorebird studies generally appear to result in very different plasma metabolite profiles, and different relationships between metabolites and physiological state or mass change, than do low-fat diets (which more closely approximate natural shorebird diets). Plasma metabolite profiles of western sandpipers gaining mass on the low-fat diet were more consistent with data from field studies and, in our study, were more similar to those of free-living birds at the time of capture.

In conclusion, at least on the low-fat diet (4%), which more closely represents the average lipid content of marine invertebrate prey targeted by western sandpipers, our study confirms that measurement of plasma metabolites can provide robust information on physiological state (gain, loss) and the rate of mass change in free-living shorebirds caught only once, as has been demonstrated for passerines (e.g. Jenni and Schwilch, 2001; although we should stress that these results are only valid for birds that have been feeding for several hours prior to sampling). Nevertheless, in migratory species where the lipid composition of the natural diet can be high, or highly variable (e.g. passerines feeding on fruit, or sandpipers feeding on crab or fish spawn), researchers should be cogniscent of effects of diet composition for certain metabolites. Future studies should consider possible confounding effects of experimental diets in validating, or comparing, plasma metabolite profiles in relation to mass change for free-living versus captive birds, particularly for non-passerines.

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