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Published in:

Proceedings of the Royal Society of London. Series B, Biological Sciences

DOI:

[10.1098/rspb.2004.2952](https://doi.org/10.1098/rspb.2004.2952)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2005

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Landys, MM., Piersma, T., Guglielmo, CG., Jukema, J., Ramenofsky, M., Wingfield, JC., Guglielmo, C. G., & Wingfield, J. C. (2005). Metabolic profile of long-distance migratory flight and stopover in a shorebird. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 272(1560), 295-302. <https://doi.org/10.1098/rspb.2004.2952>

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Metabolic profile of long-distance migratory flight and stopover in a shorebird

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Migrating birds often complete long non-stop flights during which body energy stores exclusively support energetic demands. The metabolic correlates of such long-distance travel in free-living migrants are as yet poorly studied. Bar-tailed godwits, *Limosa lapponica taymyrensis*, undertake a 4500 km flight to their single spring stopover site and thus provide an excellent model in which to determine the energy fuels associated with endurance travel. To this end, we evaluated plasma concentrations of six key metabolites in arriving godwits caught immediately upon landing near their stopover site. Initial metabolite levels were compared with levels after 5 h of inactive rest to determine how flight *per se* affects energy metabolism. Birds refuelling on the stopover site were also examined. Arriving godwits displayed elevated plasma free fatty acids, glycerol and butyrate, confirming the importance of lipid fuel in the support of extended migratory activity. Furthermore, elevated plasma triglycerides in these birds suggest that fatty acid provisioning is facilitated through hepatic synthesis and release of neutral lipids, as previously hypothesized for small migrants with high mass-specific metabolic rates. Finally, elevations in plasma uric acid suggest that protein breakdown contributes to the support of long-distance movement, to possibly maintain citric acid cycle intermediates, gluconeogenesis and/or water balance.

Keywords: migration; plasma metabolites; energy fuel; flight; stopover

1. INTRODUCTION

Migrating birds often travel thousands of kilometres between summer breeding areas and winter feeding sites. Such migratory journeys can impose tremendous energetic demands. Bouts of uninterrupted flight may last several days (e.g. Battley *et al.* 2000), during which time migrants rely exclusively on body energy stores.

In support of migratory flight, birds primarily derive energy from lipids stored as triglycerides in adipose tissue (Berthold 1975; Ramenofsky 1990), because lipid fuel imposes a low degree of wing loading relative to protein or carbohydrate (Schmidt-Nielsen 1990). The extent to which energy is derived from lipid mobilization is exemplified by studies on passerine migrants, in which plasma free fatty acids and glycerol—breakdown products of triglycerides—are markedly elevated in association with active flight (Jenni-Eiermann & Jenni 1991). Migratory red knots, *Calidris canutus*, flying under controlled conditions in a wind tunnel also show significant elevations in fat breakdown metabolites (Jenni-Eiermann *et al.* 2002).

Although plasma free fatty acids increase in association with migratory flight, they may not suffice to support the

high energetic demands of extended travel: the rate of fatty acid transport in blood is limited by plasma binding proteins. Recent evidence suggests that migratory songbirds with high mass-specific metabolic rates may supplement plasma fatty acids with plasma triglycerides to provide additional energy to working muscle (Jenni-Eiermann & Jenni 1992). However, the importance of triglycerides during free flight in larger migrants remains unclear.

Although migrants rely primarily upon lipid stores, elevations in plasma uric acid in several short-flying passerines (Jenni-Eiermann & Jenni 1991) suggest that catabolism of body protein also occurs (Mori & George 1978; Robin *et al.* 1987). Furthermore, flight in a wind tunnel increases plasma uric acid in a migratory shorebird (Jenni-Eiermann *et al.* 2002). Thus, migrants do not seem to avoid protein use during flights: a strategy that would extend tolerable fasting time (Robin *et al.* 1987; Lindgård *et al.* 1992) and would provide a buffer against travel delays. However, the extent to which protein breakdown supports long-distance movement in a free-living migrant has not been determined, although large changes in the lean dry mass of some long-flying species suggest a heavy use of proteins (Biebach 1998; Battley *et al.* 2000; Bauchinger & Biebach 2001).

Shorebirds complete migratory flights of especially long duration: they often cross large inhospitable barriers, such as the open ocean, and also have a limited number of

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suitable areas in which to stop to refuel. Thus, shorebirds provide an excellent model in which to examine the energy substrates involved in the support of long-distance movement. For example bar-tailed godwits, *Limosa lapponica*, undertake a two-day non-stop flight from west African wintering areas to their single stopover site in the Wadden Sea of Germany, The Netherlands and Denmark. The month-long stopover period that follows is indicative of the tremendous energetic demands encountered during travel. In fact, godwits display specialized adaptations to alleviate flight costs, for example they strategically adjust the size of internal organs before flight, possibly in an effort to minimize wing loading (Landys-Ciannelli *et al.* 2003).

To determine the extent to which specialized long-distance migrants use fat substrates and protein breakdown to support flight, godwits arriving at their stopover area were evaluated for plasma levels of six key metabolites: triglycerides, free fatty acids, glycerol, butyrate, uric acid and glucose. Triglycerides are the storage form of lipids. They are synthesized in the liver and are transported to peripheral tissues for deposition into fat bodies, but can also enter blood through dietary absorption (Robinson 1970). Free fatty acids and glycerol are released when triglycerides are hydrolysed, and indicate lipid mobilization from adipose tissue (Scow & Chernick 1970; Hurley *et al.* 1986; Elia *et al.* 1987). Butyrate (a ketone body) is synthesized from free fatty acids, and replaces some of the glucose requirements during energy shortage in tissues unable to catabolize fatty acids, such as the heart and brain (Robinson & Williamson 1980). Uric acid results from the breakdown of proteins that originate from body tissue or the diet (Mori & George 1978; Robin *et al.* 1987; Lumeij & Remple 1991; Lindgård *et al.* 1992).

We compared metabolite levels between just-landed godwits and birds subjected to 5 h of inactive rest to determine the degree to which flight *per se* affects energy metabolism. We also measured plasma metabolites in birds captured on the stopover site to determine how energy metabolism differs between periods of flight and refuelling.

2. METHODS

(a) *Study animals*

The godwit subspecies examined (*L. l. taymyrensis*; Engelmoer & Roselaar 1998) winters on the coastal mudflats of Mauritania and Guinea-Bissau and breeds just west and south of the Taymyr Peninsula, Russia (Boere & Smit 1981). The 9000 km distance between wintering sites and breeding areas is undertaken in only two bouts of flight, each of which takes *ca.* 2.6 days to complete (Piersma 1987; Landys-Ciannelli *et al.* 2002). Birds interrupt flight for a month-long refuelling stop in the Wadden Sea area (Piersma & Jukema 1990).

Northward-migrating godwits were captured at two sites: (i) in the dunes near Castricum, located on the western coast of The Netherlands and just 60 km short of the Wadden Sea (52°32' N, 04°37' E); and (ii) on their refuelling grounds in Texel: an island in the Dutch Wadden Sea (53°03' N, 04°48' E).

Godwits do not normally stop in Castricum, but can be lured to land during daylight hours with song playback and decoys, so are uniquely representative of migrants arriving after a long non-stop flight (Landys-Ciannelli *et al.* 2002). We henceforth refer to godwits captured in Castricum as 'arriving' birds. Arriving godwits were captured with clap nets on 29 April to 7 May 1998 and 30 April to 6 May 1999.

On the island of Texel, refuelling godwits were captured during daylight hours with a large wind-driven pull-net: the wilsternet (Jukema *et al.* 2001). We sampled refuelling birds on 13–22 May 1998 and 15–17 May 1999.

After birds were captured, blood samples were obtained by puncturing the alar wing vein with a sterile 23 gauge needle and then collecting pooling blood droplets (*ca.* 300 µl volume) into heparinized micro-haematocrit capillary tubes. Birds were subsequently weighed and measured for body morphometrics (as described in Piersma & Jukema 1990).

Differences in body mass between arriving and refuelling godwits were tested with a two-way ANOVA. Sex was included in the model as a factor. Body mass data were normalized through log-transformation.

Because godwits are sexually dimorphic, we corrected body mass for sex-related size differences by calculating body mass residuals for all birds according to wing length (see Landys-Ciannelli *et al.* 2002). Body mass residuals were used to categorize godwits captured in Texel as 'initial-refuelling' or 'advanced-refuelling' birds, according to negative or positive mass residual values, respectively. It should be noted that some birds show similar mass residual values even though they have been divided into different groups (i.e. when absolute residual values are close to zero), so group comparisons are conservative.

(b) *Initial metabolic profile*

Plasma levels of metabolites from collected blood samples were determined for arriving, initial-refuelling and advanced-refuelling birds. Jenni-Eiermann & Jenni (2001) have shown that levels of some metabolites change within 20 min of capture in night-migrating passerines. Thus, we tried to obtain blood samples from godwits as quickly as possible, i.e. within 4.2 ± 2.8 min (average \pm s.d.) of capture.

Differences in plasma metabolite levels among migratory stages were examined with one-way ANOVA tests. Tukey tests were used to conduct *post hoc* comparisons. To satisfy conditions of normality, all metabolite data were log-transformed.

(c) *Resting metabolic profile*

To determine the degree to which flight and stopover *per se* affect plasma metabolites, we compared initial metabolite levels of a subset of arriving and refuelling birds sampled immediately after capture with metabolite levels of the same birds kept inactive for 5 h. During captivity, birds were kept singly in a box without access to food. We assumed that 5 h would suffice to induce a resting energetic state because metabolite levels begin to return to baseline values within 30 min of flight in the pigeon (Schwilch *et al.* 1996). One-way repeated-measures ANOVAs were used to compare initial metabolite levels with levels resulting from 5 h of inactivity. Arriving and refuelling birds were analysed separately. Power analyses were conducted when significance fell just short of the $\alpha = 0.05$ level. Because of the small sample sizes obtained in this part of the study, we did not divide birds captured on the stopover site into different refuelling groups.

(d) *Assay techniques*

All blood samples were stored on ice and centrifuged within 10 h. Aspirated plasma was placed into micro-centrifuge tubes and subsequently stored at -80°C . Metabolite concentrations in blood plasma were determined on a powerwave 340 \times microplate spectrophotometer (BioTec Instruments). Assays were run in 400 µl flat-bottom, 96-well polystyrene microplates (NUNC) exactly as described in Landys *et al.* (2004a).

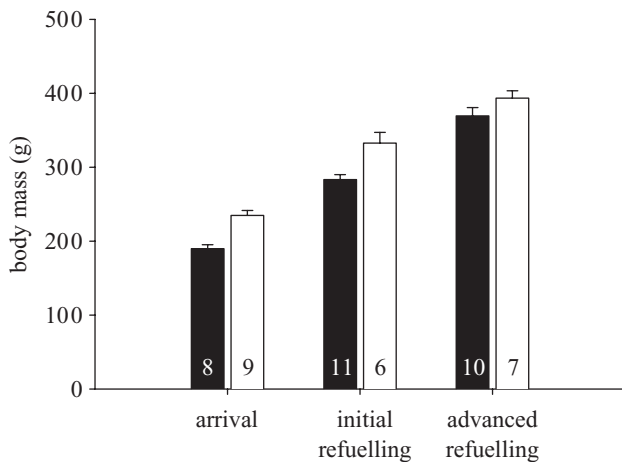


Figure 1. Body mass of godwits according to stage of migration. Even though statistical comparisons were made only between arriving and refuelling birds, all three stages are shown for purposes of evaluation. Males, filled bars; females, open bars. Error bars denote standard errors of the mean. Sample sizes are indicated.

3. RESULTS

(a) Arrival versus refuelling

As previously shown for this species (Landys-Ciannelli *et al.* 2002), body mass was significantly different between arriving and refuelling birds ($F_{1,47} = 151.533$, $p < 0.001$; figure 1). Female godwits were heavier than males ($F_{1,47} = 18.615$, $p < 0.001$).

Examined godwits showed considerable differences in five of the six examined metabolites (figure 2). Plasma free fatty acids ($F_{2,48} = 56.160$, $p < 0.001$), glycerol ($F_{2,47} = 7.810$, $p < 0.001$) and butyrate ($F_{2,48} = 27.170$, $p < 0.001$) were significantly different among migratory stages. Specifically, arriving birds had higher levels of free fatty acids, glycerol and butyrate than initial-refuelling or advanced-refuelling individuals ($p < 0.05$).

Migratory stages were also marked by significant differences in plasma triglycerides ($F_{2,47} = 36.212$, $p < 0.001$) and uric acid ($F_{2,48} = 9.533$, $p < 0.001$; figure 2). Triglyceride levels were lowest in arriving godwits and highest in advanced-refuelling birds ($p < 0.05$). Similarly, plasma uric acid was lower in arriving godwits than in initial-refuelling or advanced-refuelling birds ($p < 0.05$).

Plasma levels of glucose did not change with stage of migration ($F_{2,46} = 1.270$, $p = 0.290$).

(b) Inactive fasting

Energy metabolism was significantly affected during the 5 h inactivity trial (table 1; figure 3). In arriving godwits, plasma free fatty acids, glycerol and triglycerides decreased with inactivity. Plasma triglycerides also decreased in refuelling godwits, whereas plasma free fatty acids and glycerol did not change. However, refuelling godwits subjected to inactive fasting showed a trend to increase plasma butyrate (power of performed test with $\alpha = 0.05$ was 0.328).

Forced inactivity resulted in alterations to protein metabolism. In response to 5 h of confinement, plasma uric acid significantly decreased both in arriving and in refuelling birds.

Finally, plasma glucose markedly increased during confined inactivity in arriving godwits and showed a strong

trend to increase in refuelling birds (power of performed test with $\alpha = 0.05$ was 0.466).

4. DISCUSSION

In this study, we compared the metabolic profile of bar-tailed godwits during arrival onto their stopover site after a 4500 km bout of flight with that of birds refuelling during the subsequent stopover period. To our knowledge, this is the first study to address the metabolic correlates of long-distance flight in a free-living migrant.

(a) Lipid metabolism

Our results confirm the importance of lipid stores in the support of long-distance migration: plasma free fatty acids and glycerol were markedly elevated in arriving godwits as compared with refuelling birds. Moreover, arriving godwits subjected to 5 h of inactivity showed a dramatic decrease in plasma free fatty acids and glycerol, suggesting a high degree of lipolysis specifically in association with migratory travel.

A significant increase in plasma free fatty acids and glycerol also occurs with endurance flight in a non-migratory species: the pigeon, *Columba livia* (Bordel & Haase 1993; Schwilch *et al.* 1996). Although this suggests that lipids may play a general role in the support of extended movement, migrants may nevertheless show specific adaptations for rapid lipid mobilization and transport (Guglielmo *et al.* 2002b): plasma fatty acids in arriving godwits (3.2 ± 0.2 mmol l⁻¹; average \pm s.e.m.) are almost twice as high as in pigeons flown for up to 22 h (*ca.* 1.9 ± 0.1 mmol l⁻¹; Bordel & Haase 1993). Fatty acids in arriving godwits are also higher than in migratory red knots flying for 10 h in a wind tunnel (1.1 ± 0.1 mmol l⁻¹; Jenni-Eiermann *et al.* 2002). This threefold difference suggests that wind tunnel flight may produce relatively lower energetic demands, for example flight altitude need not be adjusted. Furthermore, captive birds cannot anticipate the timing of departure and may not fully upregulate lipid transport mechanisms before flight.

Triglyceride levels in arriving godwits decreased with forced inactivity, suggesting a distinct elevation in association with flight. Thus, the triglyceride pathway may play a role in fatty acid delivery not only in small birds with high mass-specific metabolic rates (as hypothesized by Jenni-Eiermann & Jenni (1992)), but also in larger migrants. In fact, flying godwits circulate as much lipid energy in triglycerides as in free fatty acids (1 mmol of triglycerides = 3 mmol of fatty acids; see figure 2). Even in pigeons, triglyceride levels surpass resting values with increased flight time (Schwilch *et al.* 1996). Energy provisioning through the triglyceride pathway may function to maintain flight velocity: speed in marathon runners is limited mainly by rate of fatty acid transport (Guppy 1988).

Although plasma triglycerides decreased with forced inactivity both in arriving and refuelling godwits, we argue against the possibility that captivity stress may be a plausible alternative cause of observed changes in triglycerides. Psychological stress in humans (Stoney *et al.* 2002) and restraint stress in mice (Kurihara *et al.* 2002) disrupts the clearance rate of triglycerides and, thus, elevates rather than lowers plasma triglyceride levels.

In refuelling godwits, elevated plasma triglycerides clearly suggest pronounced lipid deposition. Results support the changes in body fat content previously described in this

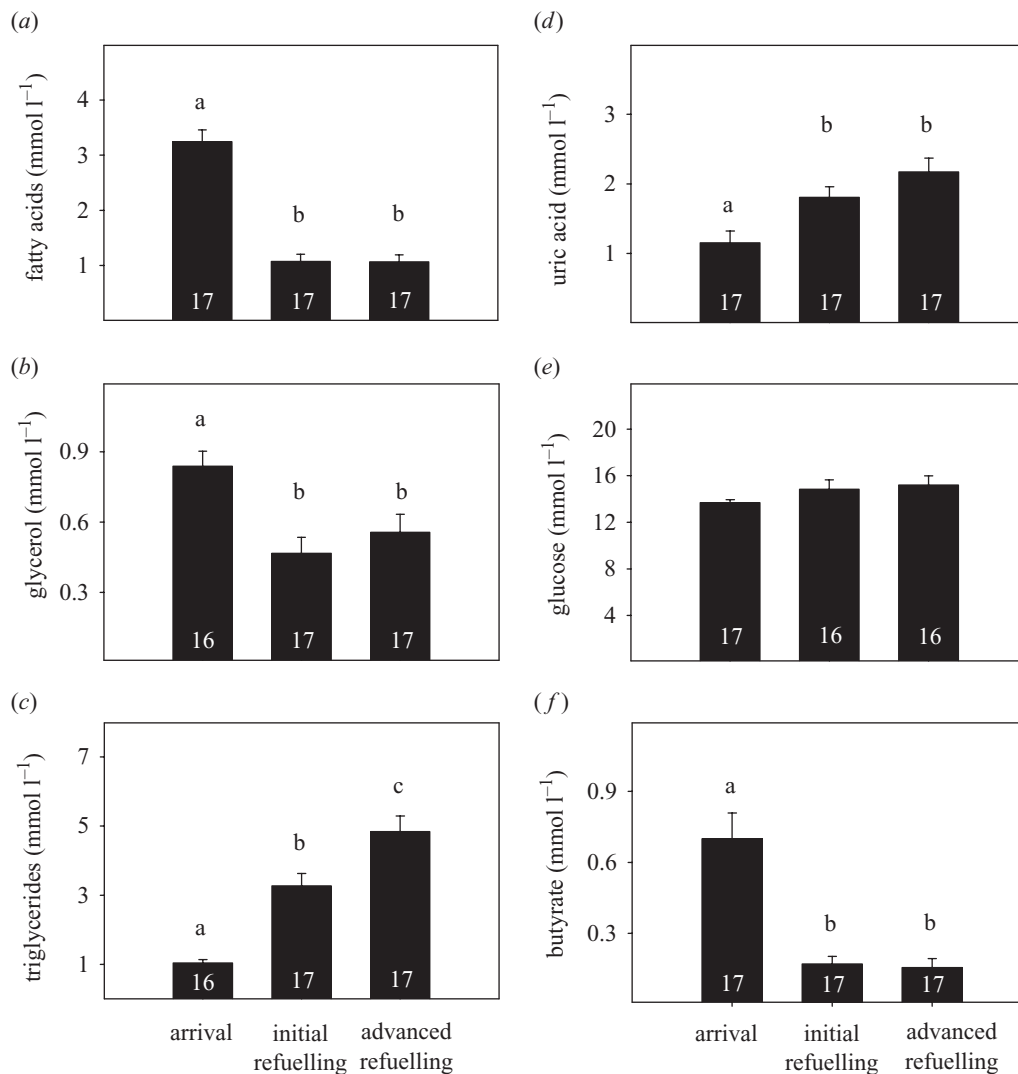


Figure 2. Plasma levels of six key metabolites in godwits according to stage of migration: (a) fatty acids; (b) glycerol; (c) triglycerides; (d) uric acid; (e) glucose; and (f) butyrate. Different letters above bars indicate significant differences in metabolite levels among migratory stages. Error bars denote standard errors of the mean. Sample sizes are indicated.

species (Landys-Ciannelli *et al.* 2003) and are commensurate with the observed pattern of increased plasma triglycerides during migratory fattening in other birds (see, for example, Mori & George 1978; deGraw *et al.* 1979; Jenni-Eiermann & Jenni 1996; Guglielmo *et al.* 2002a). In fact, because plasma triglycerides positively correlate with body mass gain in many migrants (Jenni-Eiermann & Jenni 1994; Williams *et al.* 1999; Jenni & Schwilch 2001), the higher triglyceride levels in advanced-refuelling godwits as compared with those in initial-refuelling birds (see figure 2) suggest that mass gains may be especially pronounced during the later stages of stopover. Mass deposition in initial-refuelling birds may be constrained because godwits recently arrived at the stopover site show reduced digestive machinery (Landys-Ciannelli *et al.* 2003) and may express limitations in the assimilation of ingested food matter.

In conjunction with elevations in other lipid breakdown metabolites, plasma butyrate, which is synthesized from free fatty acids and supplements energy requirements in glucose-dependent tissues, was markedly higher during arrival than during refuelling. Plasma butyrate in arriving birds remained high during inactivity, suggesting that

butyrate supports general energetic requirements (such as those associated with fasting), rather than the specific energetic demands of flight. This elevation contradicts the hypothesis that butyrate is maintained at a low level to facilitate lipid mobilization during extended travel (Jenni-Eiermann & Jenni 1991; Jenni-Eiermann *et al.* 2002). In fact, butyrate levels steadily increase with flight duration in the pigeon (Schwilch *et al.* 1996). Thus, migrants may be able to minimize ketogenesis only in association with short flights that minimize fasting time.

In godwits captured on the stopover site, plasma butyrate showed a strong trend to increase during inactivity. Thus, short-term fasting may necessitate glucose-supplementation by fat substrates even during a phase of fuel deposition. Results are in agreement with past studies that indicate a marked increase in plasma butyrate within several hours of food removal in captive passerine migrants (Jenni-Eiermann & Jenni 1997; Landys *et al.* 2004a).

(b) Protein metabolism

Uric acid levels decreased with forced inactivity in godwits arriving at the stopover site, suggesting the occurrence of a higher rate of protein catabolism in flying godwits than in

Table 1. Results of the one-way repeated-measures ANOVAs used to examine effects of inactive fasting on plasma metabolites (mmol l^{-1}) in arriving and refuelling godwits. Data were evaluated separately for the stages of arrival and refuelling, represented by 15 and 6 birds, respectively.

independent variable	arriving godwits		refuelling godwits	
	<i>t</i> -statistic	<i>p</i> -value	<i>t</i> -statistic	<i>p</i> -value
free-fatty acids	2.689	0.018	0.943	0.389
glycerol	3.332	0.005	1.683	0.153
triglycerides	3.239	0.006	6.906	< 0.001
uric acid	7.184	< 0.001	9.543	< 0.001
glucose	-6.044	< 0.001	-2.536	0.052
butyrate	0.148	0.884	-2.135	0.086

inactive fasting birds. This interpretation is commensurate with observations of a low total protein content in arriving godwits (Landys-Ciannelli *et al.* 2003). Although uric acid levels also decreased in refuelling birds that were kept inactive, we argue that changes in uric acid levels were not an effect of captivity stress: the administration of glucocorticoids (hormones typically associated with unpredictable perturbations) increases rather than decreases plasma levels of uric acid in birds (see, for example, De La Cruz *et al.* 1981; Simon 1984), as does restraint stress in rats (Al-Qirim *et al.* 2002).

An interspecific comparison indicates that uric acid levels in arriving godwits ($1.2 \pm 0.2 \text{ mmol l}^{-1}$; average \pm s.e.m.) were as high as those found in migratory songbirds sampled during short nocturnal travel (pied flycatcher: *ca.* $1.2 \pm 0.1 \text{ mmol l}^{-1}$; garden warbler: *ca.* $0.9 \pm 0.1 \text{ mmol l}^{-1}$; robin: *ca.* $1.1 \pm 0.1 \text{ mmol l}^{-1}$ (Jenni-Eiermann & Jenni 1991)). This suggests that long-bout migrants do not spare protein to a greater degree than migrants travelling in short bouts (as previously suggested by Jenni-Eiermann & Jenni (1991)), even though lower protein use would extend tolerable fasting time (e.g. Robin *et al.* 1987; Lindgård *et al.* 1992) to provide a buffer against unforeseen travel delays. However, further reductions in the relative contribution of protein towards energy provisioning may not be possible: compared with mammals, birds already show an amazing ability to spare protein, i.e. the proportion of protein that contributes to total energy expenditure is kept relatively constant even with increasing exercise intensity (Jenni & Jenni-Eiermann 1998). However, compared with non-migratory birds, migrants may show some metabolic adaptations in this regard: uric acid levels of arriving godwits are considerably lower than those of pigeons having flown for up to 22 h (*ca.* $1.8 \pm 0.1 \text{ mmol l}^{-1}$; Bordel & Haase 1993).

Although the functional relevance of protein breakdown during migratory flight is as yet unclear, several hypotheses have been proposed (reviewed in Bauchinger & Biebach (2001)). For example, protein breakdown may maintain citric acid cycle intermediates that are necessary for fatty acid oxidation and may also provide energy to working muscles through gluconeogenesis (Veiga *et al.* 1978; Dohm 1986). Protein breakdown has also been postulated to liberate metabolic water for the maintenance of water balance (Bintz & Strand 1983; Klaassen 1996), and might explain the lack of dehydration in godwits arriving after their two-day flight (Landys *et al.* 2000).

In contrast to arriving godwits, refuelling birds showed exceptionally high levels of plasma uric acid, clearly

suggesting the breakdown of dietary protein for use as an energy substrate or for transformation into lipid (Cherel & Le Maho 1988; Lumeij & Remple 1991). Indeed, godwits rely on protein-rich Tipulidae larvae during refuelling on the Wadden Sea.

(c) Carbohydrate metabolism

Similar glucose levels between arriving and refuelling godwits are in agreement with the idea that glucose in blood plasma is regulated within narrow limits. Stable glucose levels in various other migrants sampled during flight support our results (Jenni-Eiermann & Jenni 1991; Jenni-Eiermann *et al.* 2002). Furthermore, endurance flight in the pigeon does not elevate plasma glucose (Bordel & Haase 1993), although a transient increase (Schwilch *et al.* 1996) may reflect an initial dependence on glycogen stores: sudden bursts of activity, such as take-off or rapid acceleration, are primarily performed by glucose-dependent 'fast-twitch' muscle fibres (Parker & George 1975). However, because the contribution of glycogen to overall energy reserves in migrants is negligible (Farner *et al.* 1961), glucose levels in blood plasma are probably maintained through gluconeogenesis and the substitution of glucose by butyrate.

Although plasma levels of glucose were similar between arriving and refuelling godwits, glucose levels in both groups increased after 5 h of confinement. This glucogenic response may be a consequence of a handling-induced increase in the glucocorticoid hormone—corticosterone (Landys-Ciannelli *et al.* 2002)—and may assist animals in overcoming immediate energetic demands associated with unpredictable perturbations. Alternatively, increased glucose levels after recent endurance exercise may occur as a consequence of reduced glucose uptake by muscles (Schwilch *et al.* 1996), although this does not explain the strong trend for plasma glucose to increase also in refuelling godwits.

5. CONCLUSIONS

Through the examination of plasma metabolites in godwits arriving onto their stopover site and in subsequently refuelling birds, we have verified that long-distance migratory flight is primarily fuelled by lipids. Corticosterone (as well as other lipolytic agents) may contribute to the mobilization of lipid stores: corticosterone is elevated specifically in association with migratory flight in many species (Landys-Ciannelli *et al.* 2002; Landys *et al.* 2004b,c) and has been shown to promote fatty acid release in a passerine migrant (Landys *et al.* 2004a). Furthermore, our results indicate that in association with extended travel, fatty acid transport

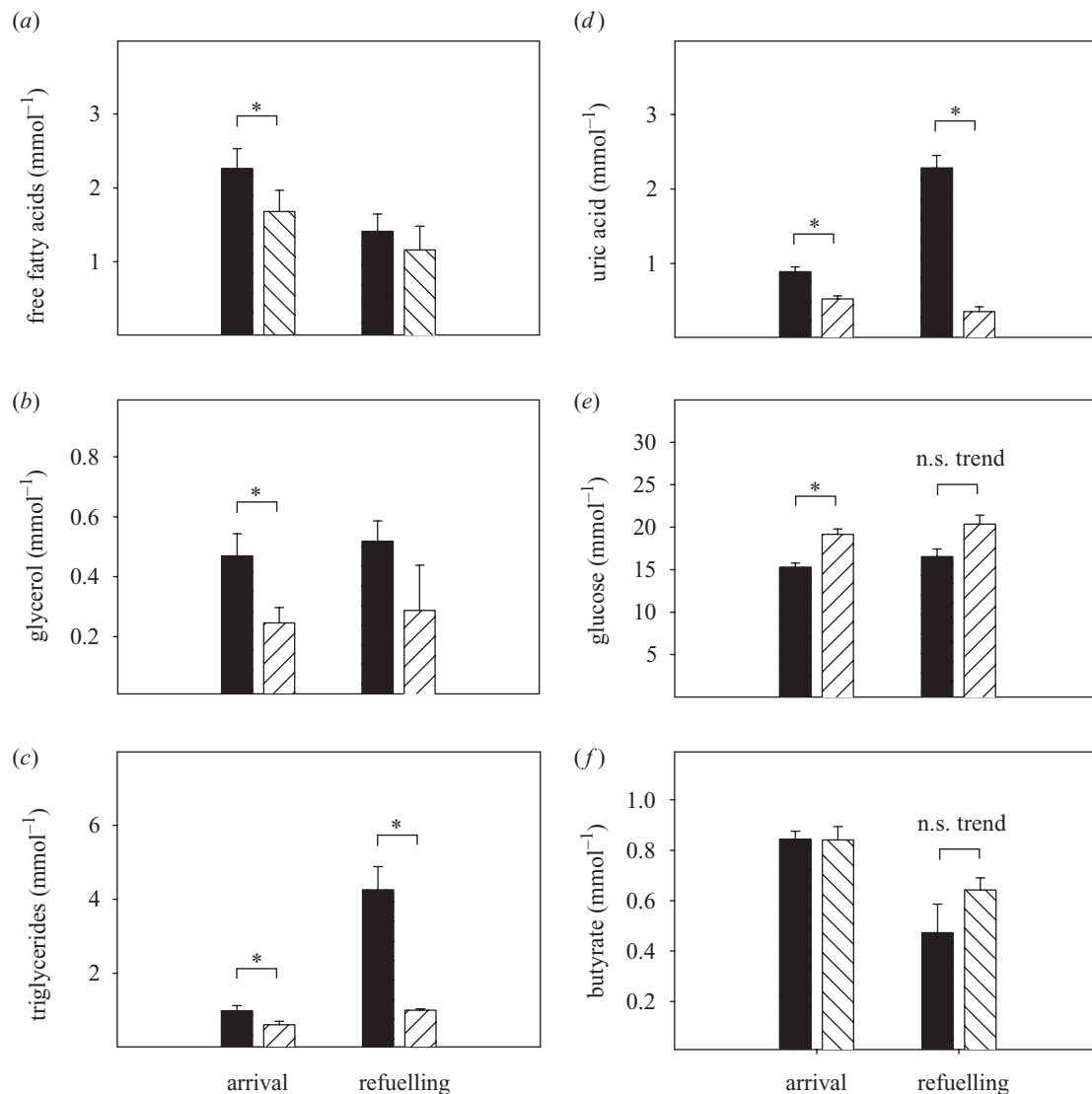


Figure 3. Effects of inactive rest on plasma metabolites in arriving and refuelling godwits: (a) fatty acids; (b) glycerol; (c) triglycerides; (d) uric acid; (e) glucose; and (f) butyrate. Filled bars represent initial metabolite levels and hatched bars represent metabolite levels after 5 h of rest. The asterisks indicate significant differences between initial and resting metabolite levels within a migratory stage. Non-significant trends are indicated. Error bars denote standard errors of the mean. Sample sizes include 15 arriving birds and 6 refuelling birds.

is facilitated through the circulation of plasma triglycerides: a pathway thought to apply only to small migrants with high mass-specific metabolic rates. Finally, results suggest that protein breakdown contributes to the support of long-distance flight even though protein sparing would be beneficial in extending tolerable fasting time. Future studies will hopefully confirm the importance of the triglyceride pathway in the support of long-distance flight in larger migrants and will elucidate the manner in which physiological mechanisms and hormones such as corticosterone support the many specialized metabolic pathways used during long-distance travel.

We gratefully acknowledge members of the Castricum Ringing Group for capture of arriving godwits. We thank the Provinciale Waterleiding Noord-Holland for providing lodging to M.M.L. and for permission to sample birds in the Castricum dune reserve. We are indebted to Lukas Jenni and two anonymous reviewers for comments on earlier drafts. Project work was supported by the Seattle ARCS Foundation, a PIONIER grant awarded to T.P. from the Netherlands

Organization for Scientific Research (NWO), and a National Science Foundation (NSF) grant (OPP-991133) awarded to J.C.W.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.