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Lipid mobilization of long-distance migrant birds *in vivo*: the high lipolytic rate of ruff sandpipers is not stimulated during shivering

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Summary

For long migrations, birds must rely on high flux capacities at all steps of lipid metabolism, from the mobilization of adipose reserves to fatty acid oxidation in flight muscle mitochondria. Substrate kinetics and indirect calorimetry were used to investigate key parameters of lipid metabolism in a highly aerobic shorebird: the ruff sandpiper *Philomachus pugnax*. In this study, we have quantified the effects of cold exposure because such measurements are presently impossible during flight. Lipolytic rate was monitored by continuous infusion of 2-[^{3}H]-glycerol and lipid oxidation by respirometry. Plasma lipid concentrations (non-esterified fatty acids, neutral lipids and phospholipids) and their fatty acid composition were also measured to assess whether cold exposure causes selective metabolism of specific lipids. Results show that shivering leads to a 47% increase in metabolic rate (44.4±3.8 ml O_2 kg^{-1} min^{-1} to 65.2±8.1 ml O_2 kg^{-1} min^{-1}), almost solely by stimulating lipid oxidation (33.3± 3.3 ml O_2 kg^{-1} min^{-1} to 48.2±6.8 ml O_2 kg^{-1} min^{-1}) because carbohydrate oxidation remains close to 11.5± 0.5 ml O_2 kg^{-1} min^{-1}. Sandpipers support an unusually high lipolytic rate of 55–60 μmol glycerol kg^{-1} min^{-1}. Its stimulation above thermoneutral rates is unnecessary during shivering when the birds are still able to re-esterify 50% of released fatty acids. No changes in plasma lipid composition were observed, suggesting that cold exposure does not lead to selective metabolism of particular fatty acids. This study provides the first measurements of lipolytic rate in migrant birds and shows that their capacity for lipid mobilization reaches the highest values measured to date in vertebrates. Extending the limits of conventional lipid metabolism has clearly been necessary to achieve long-distance migrations.

Key words: glycerol kinetics, lipolysis, lipid oxidation, fuel selection, shivering thermogenesis, cold exposure, indirect calorimetry, *Philomachus pugnax*.

Introduction

Long-distance migrant birds are renowned for their exceptional capacity to use lipids, but no *in vivo* measurements of lipid metabolism are available for these remarkable athletes. Indirect evidence shows that birds migrate at about twice the $\dot{V}_{O_2max}$ of same size mammals (Butler and Woakes, 1990), with most of the energy coming from lipids (McWilliams et al., 2004). Proteins represent the only other significant source of fuel and provide between 4% and 16% of the energy, depending on the size of fat reserves before the start of migration (Jenni and Jenni-Eiermann, 1998). The ruff sandpiper *Philomachus pugnax* is a long-distance migrant shorebird that flies from wintering areas in Africa to nesting grounds in northern Scandinavia. It must be able to extend the limits of lipid metabolism set by the best mammalian athletes to complete this annual round trip of up to 30 000 km (Cramp and Simmons, 1983). The metabolic rate of several bird species flying in wind tunnels has been reported (Lindstrom et al., 1999; Rothe et al., 1987; Ward et al., 2002; Ward et al., 2001), but specific measurements of lipid metabolism were not attempted on these animals. This is probably because quantifying the use of individual fuels is technically more challenging; it requires simultaneous monitoring of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) (indirect calorimetry), continuous infusion of tracers and blood sampling in catheterized animals (substrate kinetics), or a combination of these two approaches. In a first attempt to quantify lipid metabolism of migrant birds *in vivo*, we decided to use indirect calorimetry and substrate kinetics simultaneously. However, catheters could interfere with normal flying and, therefore, we opted to investigate active muscles during shivering rather than locomotion. This choice was influenced by recent results showing that >80% of the heat generated by cold-exposed sandpipers comes from lipid oxidation (Vaillancourt et al., 2005).

Successful migration depends on high flux capacity for all steps of lipid metabolism, from the mobilization of triacylglycerol reserves to fatty acid oxidation in flight muscle

mitochondria. Mobilization of fat stores, or lipolysis, has been measured as the rate of appearance of glycerol (R_a glycerol) in many species including humans (Beylot et al., 1987), other mammals (Himms-Hagen, 1968; Kalderon et al., 2000; McClelland et al., 2001; Shaw et al., 1975; Weber et al., 1993), rainbow trout (Bernard et al., 1999), and one bird: the king penguin (Bernard et al., 2002a; Bernard et al., 2003). R_a glycerol can only be measured by continuous infusion of labelled glycerol, and it is therefore not surprising that avian lipolytic rate is only known for large (>13 kg), easy-going penguins. Although extremely valuable, information on lipid metabolism of this non-flying bird cannot be extrapolated to long-distance migrants. Therefore, the goals of this study were to measure the effects of shivering on key parameters of lipid metabolism in a highly aerobic migrant shorebird: the ruff sandpiper. Rates of lipid mobilization (R_a glycerol) and lipid oxidation (measured by indirect calorimetry) were monitored during prolonged cold exposure. In addition, plasma lipid content (non-esterified fatty acids, neutral lipids and phospholipids), as well as the fatty acid composition of these three fractions, were quantified to determine whether shivering causes the selective mobilization/utilization of particular lipids. We anticipated that lipolysis and total lipid oxidation would be upregulated during cold exposure, in parallel with changes in metabolic rate. In migrant birds, it was also predicted that oleate (18:1) would be a preferred oxidative fuel over other fatty acids, as previously observed in other vertebrates (Blem, 1990; Leyton et al., 1987; Raclot and Groscolas, 1995; Weber et al., 2003).

Materials and methods

Animals

Twenty five adult ruff sandpipers *Philomachus pugnax* L. were obtained from a captive colony (Dr David Lank, Simon Fraser University, Burnaby, British Columbia, Canada). Average body mass for males and females was 166±4 g and 101±2 g, respectively. The birds were lean and not physiologically prepared for enhanced lipid oxidation associated with migration. They were kept at 22°C in a windowless room with artificial light (12 h:12 h L:D photoperiod) and enough space to fly (3.9 m×2.4 m×2.1 m). They had unlimited access to food (Zeigler trout pellets, Finfish Silver, Gardners, PA, USA; 42% protein, 10% lipid, 4% fibre) and running water (67 cm×42 cm×11 cm basin with ramp), and were acclimated to these conditions for at least 2 months before starting the measurements. Measurements were started 30–60 min after the animals had stopped having access to food. All experimental protocols were approved by the Animal Care Committee of the University of Ottawa.

Catheterizations

Two days before assessing metabolic parameters, catheters were placed in the right jugular vein and left carotid artery for *in vivo* measurement of glycerol kinetics. During surgery, the animals were placed on their left side, rather than on their back, to prevent air sacs from collapsing (as observed in preliminary experiments). After tracheal intubation, catheterization was performed under 1–2% halothane anaesthesia. Both catheters were prepared with 30 cm segments of polyethylene tubing (PE-50; Intramedic, Clay Adams, Becton Dickinson, Rutherford, NJ, USA) curved under steam into 270° loops, and sterilized with ethylene oxide. The carotid catheter was further extended with a 15 mm-piece of PE-10 tubing and assembled with Vetbond tissue adhesive 3M (St Paul, MN, USA). The catheters were fed 15 mm into their respective vessels, sutured in place, and exteriorized behind the neck. They were filled with saline containing heparin (20 U ml^{-1}) and Penicillin G (125 000 U ml^{-1}), and were flushed twice a day to maintain patency. The catheters were coiled and taped to the neck of the animal. Particular care was taken to avoid injecting heparinized saline in the circulation. Surgical success rate was low because these shorebirds are very sensitive to anaesthesia, and maintaining double catheter patency in animals of this size was particularly challenging. With the limited number (25) of these valuable birds available for our study, entire experiments including catheterization, rapid recovery from surgery (i.e. overnight return to normal feeding and activity levels), continuous tracer infusion and blood sampling were only completed successfully in five birds (109±16 g). Twenty individuals were used to practice surgery, failed to recover quickly from surgery, or lost catheter patency.

Indirect calorimetry and cold exposure

Food was withheld for 1 h before starting measurements. Rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were then measured using a calibrated Oxymax system (Columbus Instruments, Columbus, OH, USA) (for details, see Weber and O'Connor, 2000) connected to a modified respirometer (Vaillancourt et al., 2005) supplied with room air at 2–3 l min^{-1}. For each cold exposure experiment, animals were kept for 1 h at 22°C, before a 1 h cooling period down to 5°C, and a 2 h shivering period at 5°C. A control temperature of 22°C was selected because it is within the thermoneutral zone of most birds (particularly species commonly found in polar regions) (Scholander et al., 1950). We also observed that ruff sandpipers start panting at environmental temperatures of ~24°C (Eric Vaillancourt, unpublished observation).

Glycerol kinetics

During cold exposure experiments, the rate of appearance of glycerol (lipolytic rate) was measured by continuous infusion of radiolabelled glycerol (Bernard et al., 1999; Haman and Weber, 1996). Extensions reaching outside the respirometer were added to the catheters. The infusate was freshly prepared immediately before each infusion by mixing 80 μCi of 2-[^{3}H]-glycerol (Amersham, Oakville, Ontario, Canada; ~28 GBq $mmol^{-1}$) with 1 ml of plasma collected from ruff

sandpipers by venous puncture in the wing, more than 2 weeks before surgery. The infusate was adjusted to a final volume of 5 ml with sterile saline. Continuous infusion of 2-[^{3}H]-glycerol was performed through the venous catheter at 1 ml h^{-1} with a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA). Average infusion rate was 4,761±383×10^3 d.p.m. kg^{-1} min^{-1} (N=5). A priming dose of labelled glycerol equivalent to 30 min of infusion was administered before starting the actual infusion. This protocol ensured that isotopic steady state was reached in less than 40 min (Bernard et al., 2002a; Bernard et al., 2003). Blood samples (450 μl each) were drawn from the arterial catheter 50 and 60 min after starting the infusion to determine glycerol kinetics under thermoneutral conditions (22°C). Additional blood samples were taken every 45 min after starting the decrease in environmental temperature to measure the effects of cold exposure. Blood was centrifuged immediately after sampling to separate the plasma that was kept at –20°C until analyses.

Plasma analyses

Heptadecanoate (17:0; 0.30 mg ml^{-1}), a lipid naturally absent from ruff sandpipers, was added to plasma as an internal standard for subsequent analysis of fatty acids by gas chromatography. All lipids were extracted twice with a mixture of chloroform:methanol (2:1 v/v) by the Folch method (Folch et al., 1957). The aqueous phase (containing the glycerol) and the organic phase (containing the lipids) were separated. Each phase was dried at 70°C under N_2 and resuspended in ethanol:water (1:1 v/v) for water-soluble compounds or hexane:isopropanol (3:2 v/v) for lipids. For the aqueous phase, measurements of glycerol concentration and glycerol activity were performed as described previously (Bernard et al., 1999). For the organic phase, neutral lipids (NL), non-esterified fatty acids (NEFA), and phospholipids (PL) were separated by filtration on Supelclean solid-phase extraction tubes (LC-NH_2, Sigma, St Louis, MO, USA). NL were eluted with chloroform:isopropanol (2:1 v/v), NEFA with isopropyl ether:acetic acid (98:2 v/v) and PL with methanol. After methylation (NEFA) or acid transesterification with acetyl chloride in methanol (NL and PL) (Abdul-Malak et al., 1989), the fatty acid composition of each fraction was analyzed by gas chromatography. Individual fatty acid methyl esters were separated on a Hewlett-Packard 5890 series II (with HP 7673 autosampler; Mississauga, ON, Canada) equipped with a flame-ionization detector and a 30 m fused silica column (Supelco 2330, Sigma). Helium was the carrier gas. The injector port was at 220°C and the detector at 240°C. Column temperature was kept at 185°C for 35 min, raised to 210°C at a rate of 5°C per min, and maintained at 210°C for 10 min. Exact retention times of individual fatty acids were determined with pure standards (Sigma).

Calculations and statistics

Rates of carbohydrate and lipid oxidation were calculated from $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and the rate of nitrogen excretion using the equations of Frayn (Frayn, 1983) modified for uricotelic animals (Walsberg and Wolf, 1995), and for the units used in our study:

$$\text{Rate of carbohydrate oxidation} = 3.39\dot{V}_{CO_2} - 2.39\dot{V}_{O_2} - 0.65n ,$$

$$\text{Rate of lipid oxidation} = 3.39\dot{V}_{O_2} - 3.39\dot{V}_{CO_2} - 5.28n ,$$

where rates of carbohydrate and lipid oxidation are in ml O_2 kg^{-1} min^{-1}, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are in ml kg^{-1} min^{-1}, and n is the rate of nitrogen excretion [0.534 mg nitrogen kg^{-1} min^{-1}; see previous study (Vaillancourt et al., 2005)]. The rate of appearance of glycerol was calculated with the steady-state equation (Steele, 1959). The rate of fatty acid re-esterification was calculated as follows: (3×R_a glycerol)–total fat oxidation (with both variables expressed in μmol FA kg^{-1} min^{-1}) (see Wolfe et al., 1990). Statistical comparisons were performed using one-way, repeated-measures analyses of variance (ANOVA) or Friedman repeated-measures ANOVA on ranks when the assumptions of normality or homoscedasticity were not met. When significant changes were detected, Bonferroni's adjustment was used to determine which means were different from thermoneutral values. Relationships between rates of fuel oxidation and $\dot{V}_{O_2}$, and between R_a glycerol and body mass were assessed by linear regression. All percentages were transformed to the arcsine of their square root before analysis. Significance threshold was set at P<0.05 and all the values presented are means ± standard error of the mean (s.e.m.).

Results

Gas exchange

The effects of shivering on the rates of oxygen consumption ($\dot{V}_{O_2}$), carbon dioxide production ($\dot{V}_{CO_2}$), and on the respiratory exchange ratio (RER) are presented in Fig. 1. At 22°C, the resting, thermoneutral metabolic rate of sandpipers was 44.4±3.8 ml O_2 kg^{-1} min^{-1}. As air temperature decreased, the animals started shivering and their metabolic rate increased above thermoneutral values after 45 min (P<0.001), to reach a maximum of 65.2±8.1 ml O_2 kg^{-1} min^{-1} after 105 min at 5°C (Fig. 1A). At 22°C, thermoneutral $\dot{V}_{CO_2}$ was 33.7±3.1 ml CO_2 kg^{-1} min^{-1}. This rate increased after 45 min at 5°C (P<0.001) and reached a maximum of 50.6±6.0 ml CO_2 kg^{-1} min^{-1} after 105 min (Fig. 1B). Thermoneutral RER was 0.758±0.013 and shivering had no effect on this ratio that averaged 0.767±0.019 throughout the experiments (P=0.162; Fig. 1C).

Metabolic fuel oxidation

The effects of cold exposure on the rates of lipid and carbohydrate oxidation are presented in Fig. 2. Before and during shivering, lipid oxidation was at least threefold higher than carbohydrate oxidation, and the ratio between lipid and carbohydrate oxidation averaged 4.0±0.4 throughout the experiments. Before cold exposure, oxidation rates were 33.3±3.3 ml O_2 kg^{-1} min^{-1} for lipids and 7.9±2.2 ml O_2 kg^{-1} min^{-1} for carbohydrates. Cold exposure had no significant effect on carbohydrate oxidation, except for a transient increase to 15.3±1.6 ml O_2 kg^{-1} min^{-1} at 105 min (P=0.006). In

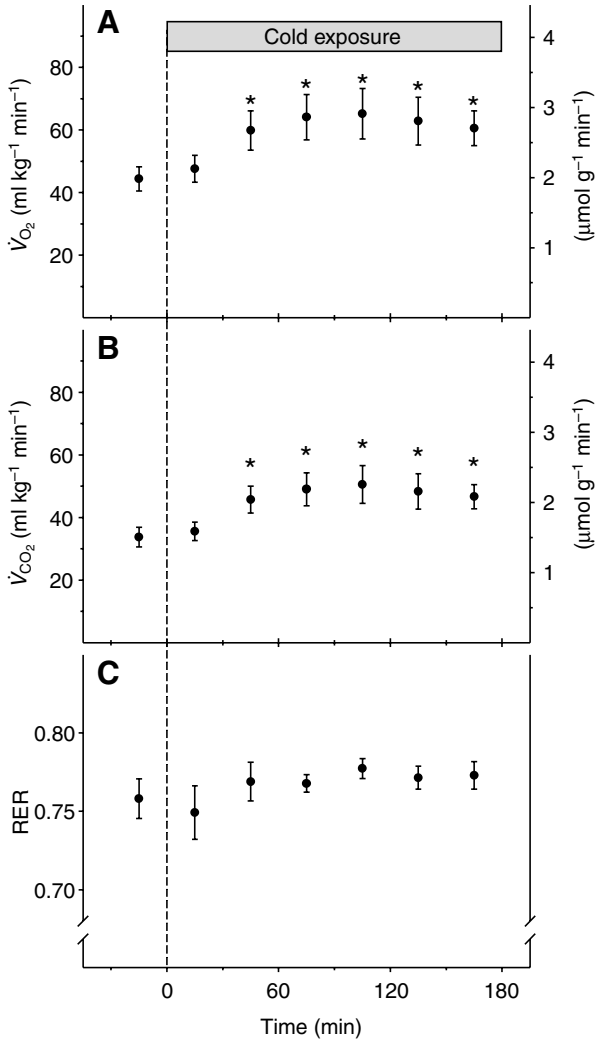

Fig. 1. Rates of (A) oxygen consumption ($\dot{V}_{O_2}$), (B) carbon dioxide production ($\dot{V}_{CO_2}$) and (C) respiratory exchange ratio (RER) of adult ruff sandpipers before (22°C) and during cold exposure (5°C). Values are means ± s.e.m. (*N*=5). *Means different from thermoneutral values (*P*<0.05).

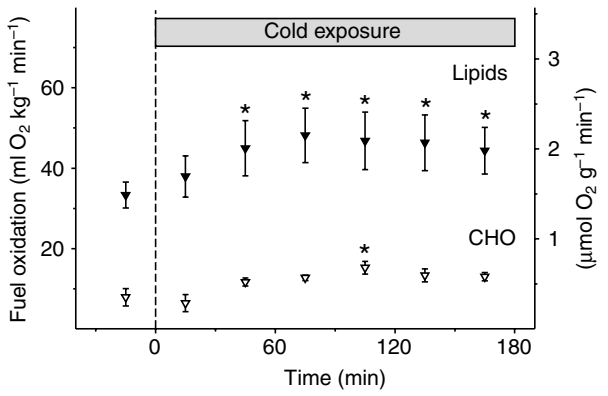

Fig. 2. Rates of lipid (filled triangles) and carbohydrate oxidation (CHO; open triangles) in adult ruff sandpipers exposed to cold (5°C). Values are means ± s.e.m. (*N*=5). *Different from thermoneutral values (*P*<0.05).

contrast, lipid oxidation increased above thermoneutral values after 45 min of cold exposure and stayed elevated until the end of the experiments (*P*<0.001). Lipid oxidation reached a maximum of 48.2±6.8 ml O_2 kg^{-1} min^{-1} after 75 min of cold exposure. Fuel selection of cold-exposed sandpipers is shown in Fig. 3 where the rates of carbohydrate and lipid oxidation are given in relation to metabolic rate ($\dot{V}_{O_2}$) for thermoneutral and shivering birds. For both metabolic fuels, the regression lines had a positive slope, significantly different from 0 (*P*=0.003 for carbohydrates and *P*<0.001 for lipids). However, most of the shivering-induced increase in $\dot{V}_{O_2}$ was accounted for by lipids (slope of regression line=0.858), with only a minor contribution from carbohydrates (slope=0.141). Surgical procedures and blood sampling had no measurable effects on metabolism because the changes in oxygen consumption and fuel selection reported here for catheterized birds were the same as previously observed in intact animals (Vaillancourt et al., 2005).

Glycerol kinetics

Changes in plasma glycerol concentration and specific activity, as well as in the rate of appearance of glycerol over time are presented in Fig. 4. Neither glycerol concentration (Fig. 4A, *P*=0.173) nor R_a glycerol (Fig. 4C, *P*=0.075) were affected by shivering. They averaged 0.29±0.04 mmol l^{-1} (concentration) and 56.2±8.1 μmol kg^{-1} min^{-1} (flux) throughout the experiments. Mean values for glycerol concentration and R_a glycerol in control and shivering animals are presented in Table 1, together with calculated values for fatty acid re-esterification. No significant differences between control and shivering birds were detected for these parameters (*P*>0.05). The R_a glycerol of ruff sandpipers measured here is compared with published values for resting or exercising birds and mammals of different body sizes (Fig. 5). The comparison reveals that the lipolytic rate of ruff sandpipers reaches the highest values known in vertebrates (i.e. 60 μmol kg^{-1} min^{-1} in hypoxia-acclimated rats) (McClelland et al., 2001), even when exercising mammals are included.

Table 1. *Mean plasma concentration and rate of appearance of glycerol in adult ruff sandpipers at thermoneutrality (22°C) and during shivering (5°C)*

	Control (22°C)	Shivering (5°C)
Glycerol concentration (mmol l^{-1})	0.37±0.11	0.28±0.04
R_a glycerol (μmol kg^{-1} min^{-1})	60.3±14.1	55.2±5.9
Fatty acid re-esterification (μmol FA kg^{-1} min^{-1})	121.9±36.8	85.6±14.8
% Re-esterification of total FA made available by lipolysis	60.0±6.2	49.4±7.2

FA, fatty acid; R_a glycerol, rate of appearance of glycerol.

Absolute and relative rates of fatty acid re-esterification [(3R_a glycerol)–FA oxidation] are also indicated.

Values are means ± s.e.m. (*N*=5).

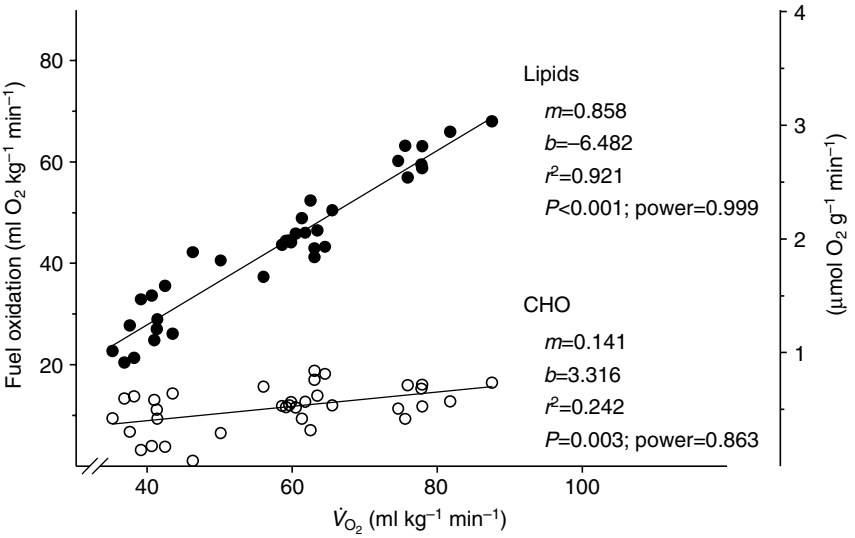

Fig. 3. Relationships between the rates of lipid oxidation (filled circles) or carbohydrate oxidation (CHO; open circles) and metabolic rate ($\dot{V}_{O_2}$) in adult ruff sandpipers exposed to cold (5°C). Lines were fitted by linear regression. Slope (*m*), intercept (*b*), coefficient of determination (r^2), probability that the slope is equal to 0 (*P*), and power of the analysis of variance are also indicated.

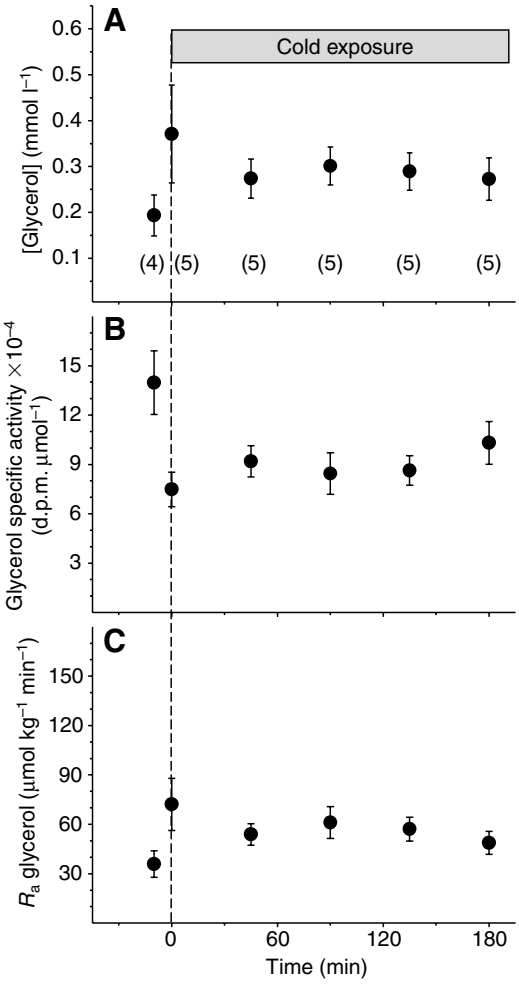

Fig. 4. (A) Plasma glycerol concentration, (B) glycerol specific activity, and (C) rate of appearance of glycerol (R_a glycerol) in adult ruff sandpipers before (22°C) and during cold exposure (5°C). Units for specific activity are disintegrations per min (d.p.m.) per micromole (d.p.m. μmol^{-1}). Sample sizes for all panels are indicated in parentheses in A. *Differences from thermoneutral values (*P*<0.05).

Fatty acid composition of plasma lipids

The plasma lipid composition of ruff sandpipers is presented in Table 2. For each animal, plasma samples taken throughout the cold-exposure experiments were analyzed separately. Shivering only had trivial effects on lipid composition, and, therefore, average values are reported in Table 2. Detailed plasma fatty acid composition (% contribution of individual fatty acids to total fatty acids within each fraction) is given for three separate lipid fractions (NEFA, NL and PL). Overall, shivering had no effect on the fatty acid composition of plasma lipids (*P*>0.05). Oleate (18:1), stearate (18:0), and palmitate (16:0) were the most abundant fatty acids in NEFA, whereas docosenoate (22:1), 18:1 and 18:0 were the main constituents of NL. The predominant fatty acids found in phospholipids were 18:0, 16:0, arachidonate (20:4) and docosahexaenoate (22:6 or DHA). The bottom line of Table 2 shows total concentration of each fraction in nmol of fatty acids per ml plasma. More than 75% of total plasma fatty acids were within the PL fraction, whereas NEFA only accounted for <15%.

Discussion

This study provides the first *in vivo* measurements of lipolysis in long-distance migrant birds. It shows that resting ruff sandpipers match the highest capacity to mobilize lipid reserves measured to date in vertebrates (60 μmol kg^{-1} min^{-1} in hypoxia-acclimated rats), higher even than for exercising mammals (Fig. 5). This high lipolytic rate not only supplies enough fatty acids for oxidation under thermoneutral conditions, but is also adequate during shivering because it is not stimulated in the cold (Fig. 4C; Table 1). The results show that lipids are primarily responsible for shivering thermogenesis (Fig. 3). At all temperatures, lipolysis exceeds the needs of energy metabolism and also provides enough extra fatty acids to support high rates of reesterification (see Table 1).

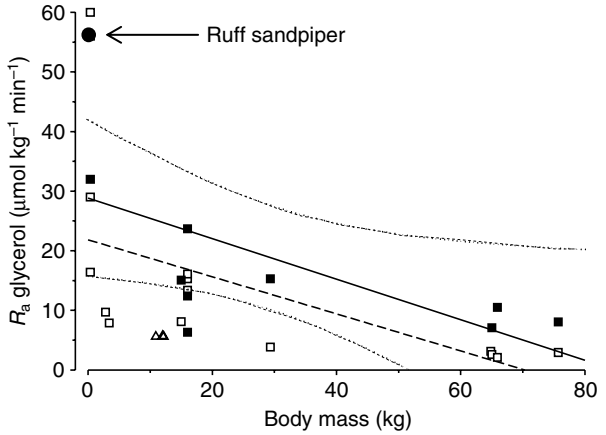

Fig. 5. Relationship between the rate of appearance of glycerol (R_a glycerol=lipolytic rate) and body mass for resting mammals (open squares), exercising mammals (filled squares), resting penguins (triangles) and ruff sandpipers (filled circle). The mean value plotted here for ruff sandpipers is for shivering birds (note that the mean value for non-shivering birds was even higher, although not significantly so; see Table 1). Each point is the mean value from a different study [rat (Kalderon et al., 2000; McClelland et al., 2001), rabbit (Himms-Hagen, 1968; Reidy and Weber, 2002), dog (Issekutz et al., 1975; Shaw et al., 1975), pigmy goat (Weber et al., 1993), sheep (Bergman, 1968), human (Bahr et al., 1990; Mora-Rodriguez et al., 2001; Wolfe et al., 1990) and king penguin (Bernard et al., 2002a; Bernard et al., 2002b; Bernard et al., 2003)]. Lines were fitted by linear regression for resting mammals (broken line; y=–0.31x+21.8; r^2=0.333) and exercising mammals (solid line; y=–0.34x+28.8; r^2=0.390). Curved dotted lines indicate 95% confidence limits around the values for exercising mammals.

Table 2. *Relative contributions of individual fatty acids to non-esterified fatty acids, neutral lipids and phospholipids in plasma of European ruff sandpipers*

	NEFA	NL	PL
16:0	13.2±3.9	8.9±2.4	18.9±1.5
16:1	2.2±0.8	1.8±0.7	ta
18:0	18.7±2.3	10.9±1.8	25.5±1.3
18:1	34.2±3.1	21.9±1.3	11.0±0.4
18:2	9.5±0.5	7.8±0.9	7.6±0.4
18:3	1.0±0.2	ta	ta
20:0	1.5±0.4	1.0±0.3	ta
20:1	2.8±0.4	1.8±0.2	ta
20:4	2.2±0.3	2.6±0.2	14.4±1.5
20:5	1.4±0.3	3.2±0.4	2.8±0.3
22:0	ta	1.4±0.2	ta
22:1	nd	27.3±6.4	nd
22:5	2.9±0.6	1.7±0.1	2.8±0.5
22:6	8.4±1.9	5.5±0.2	14.0±1.4
24:0	ta	2.1±0.2	ta
Others	2.0±0.5	2.1±0.1	3.0±0.3
Total concentration	1604±218	1041±286	8068±621

NEFA, non-esterified fatty acids; NL, neutral lipids; PL, phospholipids. Nd, not detected; ta, trace amounts.

Only fatty acids contributing >1% of total fatty acid concentration in at least one lipid fraction are tabulated. For each fraction, total concentration (in nmol FA ml^{-1} plasma) is also indicated (bottom line).

Values are means ± s.e.m. (N=5).

Unusually high lipolytic rates in migrant birds

The rate of appearance of glycerol is a reliable measure of lipolysis at the whole-organism level (Wolfe, 1992; Wolfe et al., 1990), and it has been quantified in a few species of mammals, birds and fish under resting or exercise conditions [(Bernard et al., 1999); see also references in Fig. 5]. This relative paucity of information is probably due to the technical difficulties associated with double catheterization and continuous tracer infusion. Nevertheless, only direct measurements of glycerol flux provide useful information in this context because indirect evidence from changes in concentration can be grossly misleading (see Haman et al., 1997).

Fig. 5 compares lipolytic rates among endothermic vertebrates and shows that, except for hypoxia-acclimated rats, sandpipers have the highest capacity to mobilize lipid reserves, even when body size differences are taken into account. Lipolysis proceeds ~2–3 times more quickly in sandpipers than in resting or exercising rats, even though both species are of similar size. When compared to values for larger mammals, the lipolytic rate of ruff sandpipers is higher by sixfold (rabbit and dog), 15-fold (goat), 18-fold (sheep) and 30-fold (human). Overall, body size effects on metabolic rate can only explain <50% of these large differences [calculated using the classic allometric equation for resting mammals (Schmidt-Nielsen, 1990)]. Lipolytic rate is also one order of magnitude higher in sandpipers than in king penguins (Bernard et al., 2002a; Bernard et al., 2002b; Bernard et al., 2003), the only other avian species measured to date. In addition, it is clear that the values of R_a glycerol reported here lie at the lower end of the range of lipolytic rates achievable by ruff sandpipers for several reasons: (1) migration flights would simply not be possible without activating lipolysis well beyond the rates measured in this study (see next paragraph); (2) the animals used here were not physiologically prepared for migration (Vaillancourt et al., 2005) and they were not acclimated to hypoxia (a treatment known to stimulate lipolytic rate) (McClelland et al., 2001); (3) ruff sandpipers were measured here only 1–5 h after the cessation of feeding whereas all the mammalian species mentioned in Fig. 5 for comparison were fasted for much longer durations (18–24 h); and (4) it has been suggested that incomplete hydrolysis of triacylglycerol may take place in bird adipose tissue (Goodridge and Ball, 1965), and significant production of mono- and diacylglycerol in bird adipocytes would make R_a glycerol underestimate true lipolytic rate (a situation that does not exist in mammals) (Brooks et al., 1982). It should also be noted that the highest $\dot{V}_{O_2}$ value reached during shivering only represents <30% of the estimated $\dot{V}_{O_2max}$ of ruff sandpipers, a metabolic rate at which high rates of lipid mobilization and oxidation would be expected.

Using the allometric equation for exercising birds [see Schmidt-Nielsen (Schmidt-Nielsen, 1984), p. 156], we can calculate that a flying sandpiper of 110 g has a metabolic rate of 271 ml O_2 kg^{-1} min^{-1}. Assuming that 84% of the necessary ATP is derived from lipid oxidation (i.e. that the contribution of proteins is maximal at 16%) (Jenni and Jenni-Eiermann, 1998) and that re-esterification is nil, this migrant bird would require a R_a glycerol of 133 μmol kg^{-1} min^{-1} to release enough fatty acids just to support locomotion. This minimal lipolytic rate for flight is already 2.4 times higher than measured here at rest, but this calculated value is still a conservative estimate because lipolysis usually exceeds the needs for oxidation. For example, mammals re-esterify 29–62% of all the fatty acids released during exercise and 57–85% of those released at rest (Kalderon et al., 2000; McClelland et al., 2001; Reidy and Weber, 2002; Vallerand et al., 1999; Weber et al., 1993; Wolfe et al., 1990). Taken together, these results clearly show that migrant birds can reach record rates of lipolysis, well beyond the maximum capacity of the best endurance athletes among mammals.

Effects of shivering on lipid metabolism

Upon exposure to 5°C, ruff sandpipers had to increase their metabolic rate by 47% to support shivering (Fig. 1). This increase was almost entirely due to the upregulation of lipid oxidation because carbohydrate oxidation only showed a marginal change (Figs 2 and 3). The birds were able to accommodate this increase in lipid oxidation without stimulating the high lipolytic rate already observed under thermoneutral conditions (Table 1, Fig. 4C). Even though the absolute re-esterification rate did not change significantly in the cold, a trend towards a possible reduction was apparent (Table 1). In theory, increasing oxidation without stimulating lipolysis could occur *via* a reduction in re-esterification. In this study, however, variability in re-esterification rate among individuals was too high to demonstrate whether this strategy is used by sandpipers coping with cold exposure. In humans, shivering causes parallel increases in metabolic rate, lipid oxidation and re-esterification (~2.8-fold) (Vallerand et al., 1999). Therefore, relative rates of re-esterification remain constant between thermoneutral and shivering humans (56% of total fatty acids released are re-esterified) at a value similar to that observed here in birds (50–60%, Table 1). Counterintuitively, the shivering-induced increase in metabolic rate was much lower in ruff sandpipers (1.4-fold) than observed in humans (2.8-fold) for identical cold exposure protocols (3 h in 5°C-air). The superior thermal insulation provided by ruffled feathers appears to overcompensate for the higher surface to volume ratio of these small birds. Other studies reporting the lipolytic response of animals deal with exercise regimes causing much larger increases in metabolic rate (5–8 times) that cannot be fairly compared with our data on shivering birds (Bahr et al., 1990; Issekutz et al., 1975; Klein et al., 1996; Weber et al., 1993; Wolfe et al., 1990). It could be argued that the metabolic changes measured here do not only support shivering, but also contribute to non-shivering thermogenesis. However, this is not the case because birds do not have brown adipose tissue (Cannon and Nedergaard, 2004) and other forms of non-shivering thermogenesis such as Ca^{+2} cycling have only been demonstrated in juvenile animals (Duchamp and Barré, 1993; Dumonteil et al., 1994).

Plasma lipid composition

Contrary to expectation, cold exposure had no effect on the concentration of the different plasma lipids (NEFA, NL and PL) or their fatty acid composition. Therefore, we were not able to uncover any evidence supporting selective mobilization or utilization of particular fatty acids during shivering. Two scenarios can explain our findings: (1) all available fatty acids are metabolized equally in shivering sandpipers, or (2) preference for particular fatty acids occurs *via* selective upregulation of flux without detectable effects on concentration.

In ruff sandpipers, circulating fatty acids are mainly transported as lipoproteins (85% of total plasma fatty acids), whereas NEFA only play a minor role (15%) (see Table 2). This has been observed in several other species of migrant birds [western sandpiper (Guglielmo et al., 2002); European robin, garden warbler and pied flycatcher (Jenni-Eiermann and Jenni, 1992), red knot (Jenni-Eiermann et al., 2002)] and in migrant fish [sockeye salmon (Magnoni et al., 2006)], suggesting that long-distance migrants use lipoproteins (instead of NEFA) to shuttle energy between adipose reserves and locomotory muscles. Future studies should investigate this strategy that differs drastically from the classic use of albumin-bound NEFA seen in mammals. From Table 2, we can calculate a PL/NL ratio of 7.75, indicating that high density lipoprotein (HDL) is the most abundant class of lipoproteins in the plasma of ruff sandpipers (Babin and Vernier, 1989). Our analysis shows that lipoproteins are made of a NL core containing mainly monounsaturated fatty acids (22:1 and 18:1) surrounded by phospholipids with high levels of saturated fatty acids (18:0 and 16:0).

Conclusions

High flux capacities at all steps of lipid metabolism are theoretically essential for endurance flight, but difficult to measure. As the first step in the mobilization of fat reserves, lipolysis plays a strategic role in supplying enough fatty acids to locomotory muscle mitochondria for β-oxidation. This study provides the first *in vivo* values for lipolytic rate in a long-distance migrant bird, and it shows that resting sandpipers match the highest capacity for mobilizing lipid reserves measured to date in vertebrates. With their impressive thermoneutral fluxes of 60 μmol glycerol kg^{-1} min^{-1}, these migrant birds do not need to stimulate lipolysis for supporting the increase in lipid oxidation caused by shivering. Furthermore, they are still able to re-esterify 50% of all the fatty acids released, even during prolonged cold exposure. During migration, calculations reveal that birds must reach lipolytic rates at least 2–3 times higher than observed here at rest. A better understanding of fuel metabolism in long-distance

migrants will ultimately depend on measuring metabolite fluxes in flying birds: a major technical challenge for the future.

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