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OXIDATIVE FUEL METABOLISM OF RUFF SANDPIPERS (*PHILOMACHUS PUGNAX*) DURING COLD EXPOSURE AND EXERCISE

Eric Vaillancourt

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Master of Science (2005)

(Biology)

Title: Oxidative fuel metabolism of ruff sandpipers (*Philomachus pugnax*) during cold exposure and exercise

Author: Eric Vaillancourt

Supervisor:

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OXIDATIVE FUEL METABOLISM OF RUFF SANDPIPERS (*PHILOMACHUS PUGNAX*) DURING COLD EXPOSURE AND EXERCISE

SUMMARY

Indirect calorimetry and nitrogen excretion measurements allow quantifying dynamic changes in the rates of carbohydrate, lipid and protein oxidation over time. Although measuring oxygen consumption and carbon dioxide production is relatively simple, only a few studies presented these two measurements in avian models, but none used the data to calculate rates of metabolic fuel oxidation. Therefore, the first goal of this study was to use indirect calorimetry and nitrogen excretion measurements to quantify the rates of lipid, carbohydrate and protein oxidation over time in ruff sandpiper during cold exposure and terrestrial locomotion (Chapter 2). My results show that during shivering and running the energy is provided almost exclusively by the oxidation of lipids, the most abundant metabolic fuel stored in migrant birds. During shivering, heat production was derived mainly from lipids (82% of $\dot{V}_{O_2}$), but carbohydrates (12% of $\dot{V}_{O_2}$) and proteins (6% of $\dot{V}_{O_2}$) played much minor roles. During running, lipids remained the preferred substrate (66% of $\dot{V}_{O_2}$), while carbohydrates (29% of $\dot{V}_{O_2}$) and proteins (5% of $\dot{V}_{O_2}$) were less important. This reliance on lipids is not consistent with the pattern of fuel selection seen in cold-exposed and exercising mammals. Because lipids had their greatest relative importance and contributed to a greater extent to heat production during cold exposure, I decided to study lipid metabolism in cold-exposed birds.

It is known that lipids are the main fuel used during flight, and that the metabolic rate of flying birds is much higher than the maximum metabolic rate of mammals. To

power flight (and other energy-demanding activities such as shivering thermogenesis), lipids probably have to be mobilized and oxidized at extremely high rates, thus my second goal was to determine if the rate of lipolysis is increased during cold exposure (Chapter 3). Because cold exposure nearly tripled both the metabolic rate and the rate of lipolysis in humans, I hypothesized that the rate of lipolysis in cold-exposed migrant birds would be increased in parallel with their metabolic rate.

In mammals, thermoregulation can be accomplished using shivering and non-shivering thermogenesis. Triacylglycerol:fatty acid (TAG:FA) cycling is one of the mechanisms that can be used for non-shivering thermogenesis, but it has only been studied in humans. Therefore, the third goal of my thesis was to determine the importance of TAG:FA cycling to total heat production in cold-exposed ruff sandpipers (Chapter 3). Because it has been shown that TAG:FA cycling is stimulated in cold-exposed humans, and that secondary cycling is largely responsible for this increase, I hypothesized that 1) in migrant birds, TAG:FA cycling increases during cold exposure, and 2) in cold-exposed migrant birds, the increase in total TAG:FA cycling is mostly caused by an increase in secondary cycling. To test my hypotheses for Chapter 3, the birds were subjected to a double catheterization allowing radiolabelled glycerol and palmitate to be continuously infused, and for blood samples to be taken at regular intervals without interrupting the infusion. Results show that the surgical procedure increases the plasma NEFA concentration, probably due to the heparin used as an anticoagulant. Although metabolic rate is increased, lipolysis is not affected at the temperature tested here. Therefore, I rejected my hypothesis that lipolysis increases proportionately to metabolic rate during cold exposure. However, the conclusion could

be different during cold exposure for a period longer than 3 h, or for lower exposure temperatures. Also, while lipid oxidation increases in response to cold exposure, NEFA turnover decreases in cold-exposed ruff sandpipers. Together, these observations suggest that the lipids oxidized during cold exposure may come from two stores. One possibility is that these birds use intramuscular lipid reserves to fuel shivering, while the other mechanism involves the oxidation of very low density lipoprotein-triacylglycerol (VLDL-TAG) released into the circulation by the liver. If VLDL-TAG is really used as an oxidative fuel, then the values I obtained for total cycling and secondary cycling are erroneous, because the equations used for my calculations require lipid oxidation to have NEFA as its sole source. In fact, calculations of the secondary cycling gave negative values, which also suggests that the values of TAG:FA cycling should be interpreted with caution. Therefore, I could not test the hypotheses related to TAG:FA cycling.

Even though the data obtained does not allow me to test these hypotheses, one finding is really interesting. The ruff sandpiper may use intramuscular lipid reserves to power muscle contractions, but these small reserves would probably be depleted within a short period of time, especially during flight. Therefore, this species may have the capacity to use a secondary mechanism to carry lipids from the adipocytes to the working muscles. Because plasma albumin is present in limited amounts, and because each albumin molecule can only bind a limited number of fatty acids, VLDL-TAG from the liver may be a good way to insure that the working muscles do not run out of fuel.

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TABLE OF CONTENTS

SUMMARY .... **iv**

REMERCIEMENTS .... **vii**

LIST OF FIGURES .... **xii**

LIST OF TABLES .... **xv**

LIST OF ABBREVIATIONS .... **xvi**

LIST OF APPENDICES .... **xviii**

CHAPTER 1. GENERAL INTRODUCTION **1**

Shivering thermogenesis *3*

Land locomotion *4*

Lipolysis *5*

Triacylglycerol:fatty acid cycling *7*

Aims of the study *9*

CHAPTER 2. ENERGETICS OF A LONG-DISTANCE MIGRANT SHOREBIRD (*PHILOMACHUS PUGNAX*) DURING COLD EXPOSURE AND RUNNING **12**

Introduction **13**

Methods **16**

Animals *16*

Indirect calorimetry *17*

Cold exposure experiments *18*

Exercise experiments *18*

Nitrogen excretion *19*

Calculations and statistical analyses *20*

Results **21**

Cold exposure experiments *21*

Exercise experiments *23*

Fuel selection during cold exposure and exercise *24*

Discussion **41**

Cold exposure *41*

Exercise *44*

Conclusions *46*

CHAPTER 3. GLYCEROL AND NEFA KINETICS OF A LONG-DISTANCE MIGRANT SHOREBIRD (*PHILOMACHUS PUGNAX*) **48**

Introduction **49**

Material and methods **53**

Animals 53
Indirect calorimetry 54
Cold exposure experiments 54
Surgical procedures 55
Infusate preparation 59
Preparation of blood samples 61
Glycerol analysis 62
Fatty acid analysis 63
Calculations and statistical analyses 65
Results **67**
Indirect calorimetry 67
Continuous infusion 68
Plasma lipids in NEFA, NL and PL fractions 70
Discussion **89**
Glycerol and NEFA kinetics 89
TAG:FA cycling 93
Composition of lipid fractions 95
Conclusions 97
CHAPTER 4 – GENERAL CONCLUSION **100**
REFERENCES **104**
APPENDICES **120**

LIST OF FIGURES

Figure 2.1. Changes in ambient temperature (A), oxygen consumption ($\dot{V}_{O_2}$) (B) and respiratory exchange ratio (RER) (C) of adult ruff sandpipers. Treatment temperatures are indicated as follows: 22°C control (▲), 15°C (◇), 10°C (●) and 5°C (▽). RER values were pooled (O) because they were not significantly different between treatments. Values are means ± SEM (N=6).26

Figure 2.2. Changes in rates of carbohydrate (CHO; open circles) and lipid oxidation (filled circles) over time for ruff sandpipers exposed to 22°C (control) (△,▲), 15°C (◇,◈), 10°C (O,●) and 5°C (▽,▼). Superscripts indicate significant differences from baseline at P<0.05 (*) and P<0.001 (‡). Values are means + SEM (N=6). 28

Figure 2.3. Rate of oxygen consumption ($\dot{V}_{O_2}$) (A) and respiratory exchange ratio (RER) (B) of ruff sandpipers at rest and during running at various speeds. The lines indicated were fitted by linear regression on the exercise values only. Superscripts indicate significant differences from rest at P<0.05 (*) and P<0.001 (‡). Values are means ± SEM and sample sizes are indicated in parentheses. 30

Figure 2.4. Rates of carbohydrate (O) and lipid oxidation (●) of ruff sandpipers at rest and during running at various speeds. The lines indicated were fitted by linear regression on the exercise values only. Values are means ± SEM (sample sizes as in Fig. 2.3). 32

Figure 2.5. Rate of energy expenditure of ruff sandpipers at rest (speed 0) and during running at various speeds. Line indicated was fitted by linear regression for exercise values only. The cost of transport of running ruffs (=slope of line) is 1.29 $J \cdot m^{-1}$. Superscript indicates a significant difference from rest at P<0.05 (*). Values are means ± SEM (sample sizes as in Fig. 2.3)......................... 34

Figure 2.6. Relative contributions of carbohydrates (CHO) (■), lipids (□) and proteins ( ) to total energy expenditure of ruff sandpipers during cold exposure (A) and running (B). Values different from controls (22°C for cold exposure and speed 0 for exercise) are indicated by * (P<0.05) and ‡ (P<0.001). Values are means + SEM (*N*=6 for cold exposure and as in Fig. 2.3 for exercise)......... 36

Figure 2.7. Relationships between rates of lipid oxidation or carbohydrate oxidation and metabolic rate ($\dot{V}_{O_2}$) of ruff sandpipers during cold exposure or running. 38

Figure 3.1. Changes in oxygen consumption ($\dot{V}_{O_2}$) (A), carbon dioxide production ($\dot{V}_{CO_2}$) (B) and respiratory exchange ratio (RER) (C) of adult ruff sandpipers before (22°C) and during cold exposure (5°C). Temperature was decreased using the same protocol as in Chapter 2 (See ▽ symbols in Fig. 2.1A). Superscripts indicate significant differences from baseline at P<0.05 (*). Values are means ± SEM (*N*=5). 72

Figure 3.2. Changes in rates of carbohydrate (CHO; ▽) and lipid oxidation (▼) over time for ruff sandpipers before (22°C) and during cold exposure (5°C). Superscripts indicate significant differences from baseline at P<0.05 (*) and P<0.001 (‡). Values are means ± SEM (*N*=5). 74

Figure 3.3. Relationships between rates of lipid oxidation (●) or carbohydrate oxidation (○) and metabolic rate ($\dot{V}_{O_2}$) of ruff sandpipers during cold exposure. The intercept (b), the slope (m) and the coefficient of determination (r^2) are also indicated for 5 individual birds at 7 different times (every 30 min between t=-15 min and t=165 min).. 76

Figure 3.4. Glycerol concentration (A), specific activity (B) and flux (C) in ruff sandpipers before (22°C) and during cold exposure (5°C). Superscript indicates a significant difference from steady-state values at P<0.05 (*). Values are means ± SEM and sample sizes are indicated in parentheses....... 78

Figure 3.5. Palmitate concentration (A), specific activity (B) and flux (C) in ruff sandpipers before (22°C) and during cold exposure (5°C). Superscripts indicate significant differences from steady-state values at P<0.05 (*). Values are means ± SEM (sample sizes as in Fig. 3.4). 80

Figure 3.6. Non-esterified fatty acid (NEFA) concentration (A) and flux (B) in ruff sandpipers before (22°C) and during cold exposure (5°C). Superscripts indicate significant differences from steady-state values at P<0.05 (*). Values are means ± SEM (sample size as in Fig. 3.4)... 82

LIST OF TABLES

Table 2.1. Oxygen consumption ($\dot{V}_{O_2}$), carbon dioxide production ($\dot{V}_{CO_2}$), respiratory exchange ratio (RER), carbohydrate (CHO_{ox}) and lipid oxidation (FAT_{ox}) in birds exposed to 22, 15, 10 or 5°C. 40

Table 3.1. Average plasma concentrations, specific activities and fluxes of glycerol and palmitate in adult European ruff sandpipers (*Philomachus pugnax*) exposed to 5°C. 84

Table 3.2. Fractional contribution of individual non-esterified fatty acids (NEFA), neutral lipids (NL), and phospholipids (PL) to total plasma NEFA concentration in adult European ruff sandpipers (*Philomachus pugnax*) that did not have surgery, or were used for a continuous infusion during cold exposure.......... 85

Table 3.3. Fractional contribution of individual non-esterified fatty acids (NEFA) to total plasma NEFA concentration in cold-exposed adult European ruff sandpipers (*Philomachus pugnax*) at various times during a continuous infusion. 86

Table 3.4. Fractional contribution of individual neutral lipids (NL) to total plasma non-esterified fatty acid concentration in cold-exposed adult European ruff sandpipers (*Philomachus pugnax*) at various times during a continuous infusion. 87

Table 3.5. Fractional contribution of individual phospholipids (PL) to total plasma non-esterified fatty acid concentration in cold-exposed adult European ruff sandpipers (*Philomachus pugnax*) at various times during a continuous infusion. 88

LIST OF ABBREVIATIONS

14:0	tetradecanoate, or myristate
16:0	hexadecanoate, or palmitate
16:1	hexadecenoate, or palmitoleate
17:0	heptadecanoate, or margarate
18:0	octadecanoate, or stearate
18:1	octadecenoate, or oleate
18:2	octadecadienoate, or linoleate
18:3	octadecatrienoate, or linolenate
20:0	eicosanoate, or arachidate
20:1	eicosenoate, or gadoleate
20:2	eicosadienoate
20:3	eicosatrienoate
20:4	eicosatetraenoate, or arachidonate
20:5	eicosapentaenoate (EPA), or timnodonate
22:0	docosanoate, or behenate
22:1	docosenoate, or erucate
22:3	docosatrienoate
22:5	docosapentaenoate (DPA), or clupanodonate
22:6	docosahexaenoate (DHA)
24:0	tetracosanoate, or lignocerate

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
CHO	Carbohydrates
CHO_{ox}	Carbohydrate oxidation
E_{metab}	Energy cost of locomotion per unit time
FAT_{ox}	Lipid oxidation
GC	Gas chromatography
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form of NAD)
NEFA	Non-esterified fatty acids
NL	Neutral lipids
PL	Phospholipids
RER	Respiratory exchange ratio
SA	Specific activity
SR	Sarcoplasmic reticulum
STPD	Standard temperature and pressure for dry gas
TAG	Triacylglycerol
TAG:FA	Triacylglycerol: fatty acid
TLC	Thin layer chromatography
$\dot{V}_{CO_2}$	Rate of carbon dioxide production
VLDL-TAG	Very low density lipoproteins-triacylglycerol
$\dot{V}_{O_2}$	Rate of oxygen consumption

LIST OF APPENDICES

Appendix 1. Catheters used for venous (A; right jugular vein) and arterial catheterization (B; left carotid artery). The venous catheter was made entirely using PE-50 tubing. The arterial catheter was made using PE-50 and a piece of PE-10. Note that one catheter loops over the longer length of PE-50, while the other catheter loops under; this facilitates the insertion into the vessels, and anchoring of the catheters.......... 120

Appendix 2. Diagram of an extended version of the TAG:FA cycling. This modified version combines the traditional mammalian TAG:FA cycling, and the VLDL-TAG pathway proposed by Jenni-Eiermann and Jenni (1992). Note that the shape, color, size and proportions of the various structures are not representative of *in vivo* structures. 121

CHAPTER 1. GENERAL INTRODUCTION

To stay alive, birds need to regulate their body temperature and to maintain it in a narrow range. Heat transfer between the animal and its environment can be controlled through diverse behavioral and physiological mechanisms. Behavioral mechanisms include moving to another environment where heat exchange conditions are more favorable (e.g. moving to sunlight or shade, social thermoregulation, or migration). Physiological mechanisms include changes in metabolic heat production (exercise and shivering), in the regulation of thermal exchange (vasoconstriction and vasodilatation), and in evaporation (Campbell, 1995).

Migration is one of the ways used by birds to escape unfavorable environments. This strategy encompasses alternating periods of flight and stopovers to refuel. Although flight itself is very costly in energy, stopovers also offer significant physiological challenges to migrant birds. Even though the rates of energy expenditure are lower during stopovers than during flights, stopovers can account for two thirds of all the energy spent during migration (Hedenström and Alerstam, 1997; Wikelski et al., 2003). The main activities responsible for the energy spent during stopovers are probably thermoregulation (cold exposure at night or at high altitude) and locomotion associated with feeding.

Ruff sandpipers (*Philomachus pugnax*) are long-distance migrant shorebirds that fly from wintering Afrotropical regions to nesting grounds in Northeastern Siberia. The distance traveled annually averages 12,000 km, but some individuals can cover up to 30,000 km during their roundtrip (Cramp and Simmons, 1983). To cover such large distances, these avian athletes must have physiological adaptations allowing them to store metabolic fuels in large amounts during stopovers and to oxidize these reserves at

extremely high rates during flight. Because measuring flying birds is extremely difficult to achieve and requires expensive, specialized equipment (e.g. wind tunnel), this study focuses on the main activities performed by birds during their migratory halts. During stopovers, ruff sandpipers may be exposed to cold temperatures and have to forage for extended periods of time to rebuild their lipid reserves rapidly. Therefore shivering thermogenesis and land locomotion are studied for this bird species.

Shivering thermogenesis

In cold environments, homeotherms thermoregulate by lowering heat loss and increasing heat production using chronic and acute mechanisms. In birds, acute changes in heat loss are performed *via* vasoconstriction/vasodilation, whereas chronic changes are made by altering the subcutaneous fat layer (Duchamp et al., 2002; Marjoniemi, 2000; Piersma et al., 1995) or feather quality (Campbell, 1995) to modify thermal conductance. The most important acute mechanism of heat production is shivering thermogenesis, whereby muscles use a mixture of carbohydrates, lipids and proteins as fuels. Because antagonist muscles are contracted simultaneously, no net movement is produced, and the chemical energy of these fuels is released as heat (Eckert et al., 1997). In migrant birds, pectoral muscles are the principal sites of shivering thermogenesis (Olson, 1994). Body composition analyses show that most of the energy obtained during stopovers is stored as lipids because this lighter fuel is preferred for flight. Because flight muscles are also used for shivering, it is reasonable to assume that lipids may also be the preferred fuel for heat production. However, some researchers have investigated changes in muscle glycogen content during shivering and have concluded that carbohydrates

could also play a very important thermogenic role (Parker and George, 1975). Therefore, the fuel selection pattern of shivering birds is unclear and the first goal of Chapter 2 was to measure the contributions of lipids, carbohydrates and proteins to total heat production in a long-distance migrant bird during cold exposure. Results show that lipids are responsible for more than 80% of total heat production in shivering ruff sandpipers.

Land locomotion

During stopovers, many migrating birds spend a lot of time and energy walking or running to feed rapidly and replenish their fat reserves before resuming flight. Even though lipids are stored in great amounts, two treadmill studies report that carbohydrates are the main fuel used in running chickens (Brackenbury and Vincent, 1988; Vincent and Brackenbury, 1988). However, the fuel selection pattern of running (highly aerobic) migrant birds is unknown, and could be very different from what is observed in sedentary (low aerobic) chickens. Therefore, the second goal of Chapter 2 is to characterize the pattern of fuel selection in ruff sandpipers during running. As for shivering, I found that lipid was the dominant substrate for land locomotion, contributing 58-72% of all the energy.

The cost of transport is defined as the amount of energy required to move a unit body mass over a unit distance. For running, it has been shown that this cost is lower for shorebirds than for other birds (Bruinzeel et al., 1999; Taylor et al., 1982). In long-distance migrants, energy reserves must be increased rapidly during stopovers, and it would make sense to minimize the energy cost of foraging to accelerate refueling. Therefore, ruff sandpipers would be expected to have a particularly low cost of transport

compared to non-migrant birds, and a secondary goal of Chapter 2 was to quantify this cost in this migrant species.

Lipolysis

In Chapter 2, it was shown that lipids are the main source of energy during shivering and land locomotion, and that lipid oxidation increases in parallel with metabolic rate. To allow this increase in lipid oxidation, lipolysis may be stimulated to mobilize fatty acids for contracting muscles. The only *in vivo* studies performed on birds show that lipolysis increases in penguins during glucagon infusion and after mercaptoacetate infusion (Bernard et al., 2002b; 2003). Similarly, *in vitro* studies have demonstrated that glucagon-stimulated lipolysis increases in adipocytes from cold-acclimated ducklings compared to thermoneutral ducklings (Bénistant et al., 1998), and in broiler chicken adipocytes pre-incubated with pancreatic polypeptide (Oscar, 1993). In mammals, lipolysis has been shown to increase in many species when they are exposed to a variety of stresses (Elia et al., 1987; Friedlander et al., 1999; Kalderon et al., 2000; McClelland et al., 2001; Mora-Rodriguez et al., 2001; Reidy and Weber, 2002; Weber et al., 1993; Wolfe et al., 1990), but humans are the only species tested during cold exposure (Vallerand et al., 1999), and their lipolytic rate increases proportionately to metabolic rate. Because lipolysis has never been measured *in vivo* in a long-distance migrant bird, even at rest, the goal of Chapter 3 was to quantify baseline lipolytic rate in ruff sandpipers and to determine the effects of cold exposure.

Shivering thermogenesis is the main thermogenic mechanism of birds because they lack brown adipose tissue (Bicudo et al., 2002; Cannon and Nedergaard, 2004;

Johnston, 1971; Saarela et al., 1991). However, other non-shivering mechanisms such as substrate cycles may be used by birds to increase heat production. Substrate cycles can occur when opposing reactions catalyzed by different enzymes are active simultaneously, and use adenosine triphosphate (ATP) without net conversion of substrate to product (Reidy and Weber, 2002). Calcium (Ca^{+2}) cycling is a similar mechanism that has been investigated in birds. Ca^{+2} cycling occurs when Ca^{+2} ions are released from, and then pumped back into the sarcoplasmic reticulum (SR) by Ca^{+2}-ATPase without triggering contraction. The Ca^{+2}-ATPase of the SR of skeletal muscle can interconvert different energy forms. During Ca^{+2} transport, the chemical energy derived from ATP hydrolysis is converted to osmotic energy. After Ca^{+2} has accumulated inside the SR, a Ca^{+2} gradient is formed across the membrane. This promotes the reversal of the catalytic cycle of the enzyme, and osmotic energy is converted back to chemical energy. During cycle reversal, Ca^{+2} leaves the SR through the Ca^{+2}-ATPase, and this is coupled with the synthesis of ATP. However, part of the Ca^{+2} leaks from the SR vesicles through the Ca^{+2}-ATPase without promoting synthesis of ATP, and this uncoupled Ca^{+2} leaking produces heat (De Meis, 1998). Although Ca^{+2} cycling has been characterized in ducklings as a potential means for heat production during cold exposure (Duchamp and Barré, 1993; Duchamp et al., 1993; Duchamp et al., 1992; Dumonteil et al., 1994), this mechanism may be absent in adult birds. For these reasons, the processes allowing heat production *via* brown adipose tissue or Ca^{+2} cycling have been ignored in this study. Instead, I started investigating another substrate cycle that has never been studied in birds, and that may play a significant role in this group of animals: the triacylglycerol:fatty acid (TAG:FA) cycle.

Triacylglycerol:fatty acid cycling

TAG:FA cycling is an alternative mechanism to shivering that could be used by birds to produce heat. In adipocytes, lipolysis occurs when triacylglycerol (TAG) is hydrolyzed to glycerol and non-esterified fatty acids (NEFA). NEFA can either be re-esterified without leaving the adipocyte (primary cycling), or can be incorporated back into the fat reserves after release into the circulation (secondary cycling). TAG:FA cycling (or total cycling) is the sum of primary cycling and secondary cycling (Wolfe et al., 1990). TAG hydrolysis and NEFA re-esterification require various enzymes, and form a substrate cycle that uses ATP and releases heat. In birds, a different mechanism has been proposed over a decade ago, and requires NEFA to be re-esterified to TAG in hepatocytes using circulating glycerol. The newly formed TAG could be sent to the circulation as VLDL-TAG (very low density lipoprotein-triacylglycerol), and transported to the shivering muscles to be oxidized, or go back to the adipocytes to be hydrolyzed to TAG by lipoprotein lipase (Jenni-Eiermann and Jenni, 1992). However, because there is little supporting evidence, and because no equations currently exist for this potential, alternative mechanism involving VLDL, I decided to study TAG:FA cycling for which equations have been validated in mammals. Total cycling, primary cycling, and secondary cycling rates have been quantified *in vivo* in mammals (Wolfe et al., 1990), and my goal was to use the same experimental approach to study TAG:FA cycling in ruff sandpipers (whereby, glycerol flux, NEFA flux and total lipid oxidation must be quantified). The only bird measurements of glycerol and NEFA fluxes have been performed in penguins (Bernard et al., 2002a; 2002b; 2003), and it was found that their lipolytic rate increases during glucagon infusion and after mercaptoacetate infusion (an

inhibitor of β-oxidation). Also, primary cycling was shown to decrease as fasting is prolonged, but secondary cycling and total cycling could not be calculated because fatty acid oxidation was unknown (oxygen consumption and carbon dioxide production were not measured).

In mammals, primary cycling and secondary cycling have been studied in a few species such as the rat (Kalderon et al., 2000; McClelland et al., 2001), the rabbit (Reidy and Weber, 2002) and the human (Vallerand et al., 1999; Wolfe et al., 1990). However, TAG:FA cycling during cold exposure was investigated only in humans (Vallerand et al., 1999). In that study, secondary cycling was increased more than 3-fold upon exposure to 5°C, but primary cycling remained unchanged. During exercise, humans were shown to increase primary cycling and secondary cycling (Wolfe et al., 1990), but rats exercised under normoxia and hypoxia showed no increase in those parameters (McClelland et al., 2001). McClelland et al. (2001) observed an increase in primary cycling in resting rats acclimated to hypoxia compared with resting rats under normoxia. Rats treated with thyroid hormone and Medica 16 (a thyromimetic) (Kalderon et al., 2000), and rabbits injected with leptin (Reidy and Weber, 2002) also demonstrated increased primary cycling. Because long-distance migrant birds may use substrate cycles as a way to increase heat production during migration stopovers in addition to shivering, and because no information could be found about glycerol and NEFA fluxes in cold-exposed birds, I decided to quantify glycerol and NEFA fluxes, and I tried to determine the importance of primary cycling and secondary cycling to heat production in ruff sandpipers (Chapter 3).

Aims of the study

The general aims of this thesis are:

i) to determine the relative contribution of the various metabolic fuels to metabolic rate in cold-exposed and exercising ruff sandpipers,

ii) to quantify the rate of lipolysis of ruff sandpipers at rest and during cold exposure, and

iii) to determine the thermogenic importance of TAG:FA cycling (as an alternative mechanism to shivering) in cold-exposed ruff sandpipers.

In Chapter 2, indirect calorimetry is used to quantify absolute rates of lipid, carbohydrate and protein oxidation in cold-exposed or running ruff sandpipers. The use of glycogen as a thermogenic fuel during extreme cold exposure has been investigated in the pectoral muscle of pigeons (Parker and George, 1975), and the authors concluded that carbohydrates could become a significant fuel for heat production during high-intensity shivering. Similarly, respiratory exchange ratio (RER) values suggest that carbohydrates are the main fuel used in running chickens (Brackenbury and Vincent, 1988; Vincent and Brackenbury, 1988; Ward et al., 2002). Based on mammalian fuel selection, my hypothesis for Chapter 2 is that carbohydrates are the main metabolic fuel oxidized by shivering or running migrant birds (hereafter referred to as Hypothesis I). Therefore, I predicted that, as the intensity of exercise increases during shivering and running, the

relative contribution of carbohydrates to total energy expenditures would increase, and the relative contribution of lipids to total energy expenditures would decrease.

In Chapter 3, I wanted to quantify lipolytic rates of resting and cold-exposed birds. Lipolysis can be quantified by measuring glycerol fluxes, and these measurements can be made using the double catheterization technique. This technique is commonly used in mammals to measure glycerol fluxes, but has been used in birds only in the king penguin. Thus, I needed to adapt this technique for small birds. The procedure requires the implantation of two catheters, one is used for the continuous infusion of radiolabelled compounds, and another is used for blood sampling. The labelled compounds used in this study are 2-[^{3}H]-glycerol and 1-[^{14}C]-palmitate. They were infused in cold-exposed birds to quantify glycerol and NEFA fluxes (glycerol flux = lipolysis), and to derive primary cycling, secondary cycling and total cycling. Because the rate of lipolysis of cold-exposed humans is nearly tripled during a proportional increase in metabolic rate, my primary hypothesis for Chapter 3 is that the rate of lipolysis in cold-exposed migrant birds increases in proportion to metabolic rate (Hypothesis II). Thus, I predicted that the glycerol flux of shivering ruff sandpipers would increase proportionally to the increase in oxygen consumption. Also, it has been shown that TAG:FA cycling is stimulated in cold-exposed humans, and that secondary cycling is largely responsible for this increase. Therefore, my secondary hypotheses for Chapter 3 are that 1) in migrant birds, TAG:FA cycling increases during cold exposure (Hypothesis III), and 2) in cold-exposed migrant birds, the increase in total TAG:FA cycling is mostly caused by an increase in secondary cycling (Hypothesis IV). Thus, my predictions were that 1) during cold exposure, TAG:FA cycling would increase in ruff sandpipers, and 2) in shivering ruff sandpipers,

the increase in secondary cycling would be the main cause of the increase in total cycling. The double catheterization technique requires implanting two catheters, but it is unknown whether the surgical procedures used to implant them have an effect on lipolysis or on the fatty acid profile. Therefore, my secondary goal was to determine if surgery affects metabolic rate, lipid oxidation or the fatty acid profile of plasma lipids.

The general conclusions of my thesis are reported in Chapter 4. Because the absolute rate of carbohydrate oxidation did not increase during shivering and running, and because the relative importance of carbohydrates did not increase in any of the treatments, I rejected Hypothesis I in Chapter 2. Similarly, the increased metabolic rate observed during cold exposure did not cause an increase in the rate of lipolysis, and Hypothesis II was rejected. Finally, I found that the equations previously used to calculate TAG:FA cycling in humans may not apply to migrant birds. The exact reasons for this are unknown, but Chapter 3 discusses possible differences in lipid metabolism that may explain this observation. Therefore, I could not determine the contribution of TAG:FA cycling to heat production in cold-exposed ruff sandpipers, and I could not test the two hypotheses (Hypotheses III and IV) related to TAG:FA cycling.

CHAPTER 2. ENERGETICS OF A LONG-DISTANCE MIGRANT SHOREBIRD (*PHILOMACHUS PUGNAX*) DURING COLD EXPOSURE AND RUNNING

Based on

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Introduction

The metabolic consequences of cold exposure and exercise have been well characterized in mammals (Himms-Hagen, 1996; Rowell and Shepherd, 1996), but little information is available for birds (Bicudo et al., 2001; Butler, 1991; Hohtola et al., 1998; St-Laurent and Larochelle, 1994), and the physiological changes caused by shivering and exercise are even less well understood for migrants (Jenni-Eiermann et al., 2002; Klaassen, 1996; Kvist et al., 2001; Piersma et al., 2003; Ramenofsky, 1990; Ward et al., 2002). Ruff sandpipers (*Philomachus pugnax*) are long-distance migrant shorebirds that fly from wintering areas in Africa, to nesting grounds in Northeastern Siberia: an annual roundtrip that can reach 30,000 km (Cramp and Simmons, 1983). These remarkable athletes have been able to extend the physiological limits of endurance capacity, but nothing is known on their pattern of metabolic fuel selection. Quantifying the relative contributions of the different fuels to total metabolism has proven very difficult, mainly because methods routinely used in mammalian research are not easily adaptable to birds.

Migration flights are energetically very demanding, but other activities than flying also offer significant physiological challenges. For instance, metabolic rate must be increased when low temperatures are encountered (during the night / at high altitude), or when the birds are rapidly building fat reserves and spend a lot of time running while feeding. In some species, leg muscles are even known to hypertrophy during stopovers (Piersma et al., 1999). Therefore, shivering thermogenesis and terrestrial locomotion are two ecologically relevant activities regularly performed by ruff sandpipers.

Acclimation to cold environments has been the subject of many bird studies (Ballantyne and George, 1978; Block, 1994). For acute cold exposure, however, most of the work has been carried out on juvenile birds, and it has been shown that they cannot thermoregulate (Østnes et al., 2001). As they age, shivering thermogenesis develops in leg and pectoral muscle, and the latter becomes a major site of heat production (Marjoniemi and Hohtola, 1999), although few studies have determined which oxidative fuels are being used. One study provides indirect information on metabolic fuels during cold exposure, and reports respiratory exchange ratios of 0.70 and 0.77 (RER= $\dot{V}_{CO_2}$ / $\dot{V}_{O_2}$) in fasted and fed Arctic terns (*Sterna paradisaea*), suggesting that lipid oxidation is dominant (Klaassen et al., 1989). However, the RER values given were not corrected for protein oxidation, and results from a single species exposed to two temperatures cannot be generalized. It is possible that the relative use of carbohydrates (CHO) for thermogenesis is higher in other species and at lower temperatures. For example, the use of glycogen as a thermogenic fuel during extreme cold exposure was investigated in the pectoral muscle of pigeons (Parker and George, 1975). The authors concluded that carbohydrates could become a significant fuel for heat production during high-intensity shivering.

Most of the information available on avian fuel metabolism during exercise is based on measurements of metabolite concentrations and total body composition in captive birds, or in wild animals caught at various stages of migration (Guglielmo et al., 2002a; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002). These studies have showed birds can use lipids at very high rates, but they have not provided much information on carbohydrate metabolism, on individual birds (i.e. only groups of animals

are compared), or on the time course of changes in fuel utilization (only start and end points have been measured). In a few cases, it has been possible to exercise birds in flying wheels [red junglefowl, (Chappell et al., 1996; Hammond et al., 2000); house sparrow, (Chappell et al., 1999)] or in wind-tunnels [pigeon, (Rothe et al., 1987; Rothe and Nachtigall, 1987); thrush nightingale, (Klaassen et al., 2000; Lindström et al., 1999); European starling, (Ward et al., 2001); barnacle and bar-headed geese, (Ward et al., 2002); red knot, (Jenni-Eiermann et al., 2002; Kvist et al., 2001)]. Changes in metabolite concentration reported in many of these studies are very useful, but they only provide an estimate of fuel utilization, and conclusions based on such measurements can be misleading (e.g. see Haman et al., 1997). In contrast, indirect calorimetry follows dynamic changes in substrate oxidation over time and this is the main reason it was selected for my experiments. Unfortunately, this method has rarely been applied to bird exercise studies in the past and the RER values reported have never been used to calculate fuel oxidation (Brackenbury and Vincent, 1988; Rothe et al., 1987; Ward et al., 2002; 2001). In this study, my goal was to quantify the rates of lipid, carbohydrate and protein oxidation in ruff sandpipers during cold exposure and terrestrial locomotion, using indirect calorimetry and nitrogen excretion measurements. Even though it is well established that long-distance flight is predominantly supported by lipid oxidation, the fuel selection patterns of running and shivering birds are not known. Based on mammalian fuel selection, I hypothesized that carbohydrates are the main metabolic fuel used by shivering and running migrant birds.

Methods

Animals

Adult European ruff sandpipers were obtained from a captive colony (Dr. David Lank, Simon Fraser University, Burnaby, British Columbia, Canada). The birds were kept indoors in a room allowing flight (2.1 × 3.9 × 2.4 m) with *ad libitum* access to food (Zeigler, Finfish Silver; 42% protein; 10% lipid; 4% fiber) and water (67 × 42 cm x 11 cm deep water basin with ramp). The room had no windows and was only supplied with artificial light. Photoperiod (12L:12D) and temperature (22°C) were kept constant. The animals were acclimated to these conditions for at least 2 months before starting experiments. Seven females and 3 males (110 ± 7g) were used for the experiments. The physiological parameters reported in this study showed no gender differences and, therefore, results for males and females were pooled. Average body mass for males and females (166 ± 6 g and 98 ± 2 g, respectively) were very similar to the values reported by Cramp and Simmons (1983) for wintering birds (December-February; 172 g for males and 99 g for females). Body mass is known to increase significantly before and during spring migration (March-April; 210 g for males and 132 g for females), and, therefore my experiments were performed in lean ruffs that were not physiologically prepared for enhanced lipid oxidation associated with migration. All experiments were started 30 min after the animals had stopped having access to food.

Indirect calorimetry

For cold exposure, rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were measured with an Oxymax system (Columbus Instruments, Columbus, Ohio, USA) supplied with room air at 2-3 $l \cdot min^{-1}$ as detailed previously (Weber and O'Connor, 2000). For exercise, gas exchange was measured with Applied Electrochemistry analyzers (models S-3A/II and CD-3A; Pittsburgh, Pennsylvania, USA), using an air flow rate of 2 $l \cdot min^{-1}$. Small fans ensured that the air was continuously mixed in the measuring chambers. Air flow rate was controlled by a mass flow regulator accurate to within 1% of full scale and calibrated with a reference volume meter (Porter Instruments, Pennsylvania, USA). Oxygen and CO_2 concentrations were measured in the inflow and outflow air after removing water vapor through calcium sulphate columns (Drierite, W.A. Hammond). New calcium sulfate was always exposed to air for 5 min before use to avoid CO_2 absorption during measurements. All analyzers were calibrated with known gas mixtures before and after each experiment. $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were corrected for dry gas under standard temperature and pressure conditions (STPD). Both experimental systems were accurate to within ±3 % after bleeding known amounts of CO_2 or N_2, or within ±2 % by burning 99 % ethanol in the respirometers. The method of indirect calorimetry was selected for these experiments because it is non-lethal, non-invasive, and it measures changes in the rates of metabolic fuel utilization in individual animals over time (i.e. rates of carbohydrate and lipid oxidation after correcting for protein oxidation, itself estimated by measuring the rate of nitrogen excretion). These advantages are not provided by alternative methods commonly used in

this field (i.e. changes in blood or tissue metabolite concentrations, or whole body composition analyses).

Cold exposure experiments

Shivering experiments were carried out in a closed respirometer (38 × 26 × 21 cm) connected to a cooling bath (PolyScience, Niles, Illinois, USA) containing winter (-40°C; methanol-based) windshield washer fluid. The refrigerated fluid was recirculated within the respirometer walls. All the cold exposure measurements were started between 9 and 11 am, and no food or water was available in the respirometer. While shivering, the birds stood quietly in place. No walking or jumping was observed. A piece of perforated Plexiglas covered the respirometer floor to protect the animal's feet. Before collecting data, each bird was placed in the respirometer for 5 h at 22°C on 2 separate occasions to familiarize it with the experimental set-up. After familiarization, each animal was measured at 4 temperatures in random order (22, 15, 10 and 5°C) with a minimum of 3 days between measurements. Each experiment included a 60 min baseline period at 22°C, a transition period of 30-60 min to reach the test temperature, and a 3 h period at the test temperature. Using an analog thermometer displaying 1 decimal, ambient respirometer temperature was recorded every 5 min.

Exercise experiments

The metabolism of running birds was quantified on a motorized treadmill enclosed in an acrylic respirometer (50 × 28 × 14 cm) at an incline of 8% (modified Simplex II, rat treadmill respirometer from Columbus Instruments, Columbus, Ohio,

USA). This incline was selected to reach as high a metabolic rate as possible without wing flapping. The animals were familiarized with the experimental set-up by running at different speeds for at least 3 practice sessions of 15-30 min. Baseline gas exchange values (speed 0) were obtained by leaving each animal quietly in the respirometer for 60 min in the dark. The last 10 min of this resting period were used to quantify pre-exercise $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. In each exercise session, 2 different running speeds were monitored until steady gas exchange values were reached (i.e. when the coefficient of variation remained <10% for at least 10 min). Each bird was measured at 15, 20, 25, 30, 35 and 40 $m \cdot min^{-1}$. The order of speeds tested was randomized and successive sessions for the same animal were separated by at least 24 h.

Nitrogen excretion

The rate of nitrogen excretion was measured by collecting excreta for 2 consecutive periods of 3 h in 8 fasting individuals kept at 22°C, and by quantifying the concentrations of uric acid (Marquardt, 1983) and urea (Sigma kit, St. Louis MO). A mean value of 0.534 ± 0.056 mg $N \cdot min^{-1} \cdot kg^{-1}$ (N=8) was measured and it was used for all birds in my calculations. Given that the rate of nitrogen excretion did not change between the 2 periods of fasting, I have assumed that the rate of protein oxidation was not affected significantly by cold exposure or running.

Calculations and statistical analyses

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 (1983) modified for uricotelic animals (Walsberg and Wolf, 1995), and for the units used in this study:

Rate of carbohydrate oxidation (CHO_{ox}) = $3.39\ \dot{V}_{CO_2} - 2.39\ \dot{V}_{O_2} - 0.65\ n$

Rate of lipid oxidation (FAT_{ox}) = $3.39\ \dot{V}_{O_2} - 3.39\ \dot{V}_{CO_2} - 5.28\ n$

where rates of carbohydrate and lipid oxidation are in ml $O_2 \cdot min^{-1} \cdot kg^{-1}$, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are in $ml \cdot min^{-1} \cdot kg^{-1}$, and n is the rate of nitrogen excretion in mg $N \cdot min^{-1} \cdot kg^{-1}$.

Percentages were transformed to the arcsine of their square root before analysis. For cold exposure, mean $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, respiratory exchange ratio (RER) and percentages were compared using one- or two-way, repeated measures analyses of variance (ANOVA). When the assumption of homoscedasticity or normal distribution was not met, the Friedman repeated measures ANOVA on ranks was used. For running data, one-way ANOVA was used because sample size differed between speeds. For each bird, the cost of transport was measured and compared to values calculated from allometric equations for shorebirds only (Bruinzeel et al., 1999) or for birds in general (Taylor et al., 1982). Mean observed and predicted values were compared using a one-way ANOVA. Comparisons between test and control means were performed using Bonferroni's adjustment. Decisional threshold was set at $P<0.05$ and all the values presented are means ± SEM.

Results

Cold exposure experiments

Changes in environmental temperature, metabolic rate ($\dot{V}_{O_2}$) and RER during cold exposure are presented in Fig. 2.1. After a progressive decrease from baseline temperature of 22°C, the animals were kept under constant cold conditions for 3 h at 15, 10 or 5°C (Fig. 2.1A). While standing quietly at 22°C, metabolic rate was 46.1 ± 1.0 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$. When temperature was decreased, metabolic rate increased proportionately with the intensity of cold exposure, and reached a maximum of 81.6 ± 9.1 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ after 80 min at the lowest test temperature of 5°C (Fig. 2.1B). RER was not significantly different between temperatures ($P>0.05$; Friedman repeated measures ANOVA on ranks), and, therefore, values for all treatments were pooled for this parameter (Fig. 2.1C). RER did not change from the mean baseline value of 0.756 ± 0.003 over time ($P>0.05$; Friedman repeated measures ANOVA on ranks). However, when the effect of time was tested separately for each temperature, RER was temporarily elevated from baseline between 25 and 30 min in the 5°C group ($P<0.05$; Friedman repeated measures ANOVA on ranks).

Changes in the rates of CHO and lipid oxidation (CHO_{ox} and FAT_{ox}) during cold exposure are presented in Fig. 2.2. At all times, FAT_{ox} was more than 3.2-fold higher than CHO_{ox} (the average ratio of FAT_{ox} / CHO_{ox} was 5.6). Overall, CHO_{ox} was higher at 5°C than at all other temperatures ($P<0.05$; one-way repeated measures ANOVA; Bonferroni t-test). Cold exposure had no effect on CHO_{ox} over time ($P>0.05$; one-way repeated measures ANOVA; Bonferroni t-test), except between 45 and 75 min at 5°C,

when it was higher than baseline ($P<0.05$; one-way repeated measures ANOVA; Bonferroni t-test). FAT_{ox} was increased in proportion with the intensity of cold exposure. All treatment temperatures were different from each other ($P<0.001$; one-way repeated measures ANOVA), and each treatment showed a significant increase in FAT_{ox} over time, except for the control group kept at 22°C ($P<0.001$ for 5, 10 and 15°C; one-way repeated measures ANOVA; Bonferroni t-test; $P>0.05$ for control; one-way repeated measures ANOVA). Figure 2.7 shows regression analyses between CHO_{ox} or FAT_{ox} and $\dot{V}_{O_2}$ for cold exposure and exercise experiments. The slope of the relationship between FAT_{ox} and $\dot{V}_{O_2}$ is highly different from 0 (Fig. 2.7A; slope=0.841; $r^2=0.920$; $P<0.001$; linear regression), while the relationship between CHO_{ox} and $\dot{V}_{O_2}$ is also significant, but the slope is much lower than for FAT_{ox} (Fig. 2.7C; slope=0.174; $r^2=0.338$; $P<0.01$; linear regression).

All the parameters measured in the cold exposure experiments reached steady-state values (see Fig. 2.1B) and they are summarized in Table 2.1. These values were calculated by averaging measurements over the last 25 min at each temperature, and they are presented for gas exchange ($\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and RER) and fuel utilization (CHO_{ox} and FAT_{ox}). Steady-state values were progressively higher for $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and FAT_{ox} as cold exposure intensified. However, RER and CHO_{ox} were not affected by temperature (Table 2.1).

Exercise experiments

Oxygen consumption and RER of birds running at various speeds are shown in Fig. 2.3. Baseline values for animals resting on the treadmill at 22°C were 51.7 ± 4.0 ml·min^{-1}·kg^{-1} for $\dot{V}_{O_2}$ and 0.761 ± 0.008 for RER (*N*=11). These resting rates were not different from baseline values measured in the cold exposure experiments (Fig. 2.1; P>0.05; Mann-Whitney rank sum test). $\dot{V}_{O_2}$ increased progressively with running speed (Fig. 2.3A; P<0.05; t-test), and the highest exercise $\dot{V}_{O_2}$ of 78.9 ± 3.5 ml O_2·min^{-1}·kg^{-1} was measured at a speed of 35 m·min^{-1}. RER was elevated from baseline at low and intermediate speeds (P<0.05 at 15, 30 m·min^{-1}; t-test; P<0.001 at 25 m·min^{-1}; t-test), but it was not different from baseline at the 2 highest speeds (Fig. 2.3B; P>0.05; t-test for speed of 35 m·min^{-1}; Mann-Whitney rank sum test for speed of 40 m·min^{-1}). The slope of the regression line for RER *vs* speed was not different from zero (P>0.05; linear regression).

Figure 2.4 shows CHO_{ox} and FAT_{ox} as a function of speed. At all times, FAT_{ox} was more than 1.6-fold higher than CHO_{ox} (the average ratio of FAT_{ox} / CHO_{ox} was 2.5). Baseline CHO_{ox} in animals resting on the treadmill was 9.07 ± 1.28 ml O_2·min^{-1}·kg^{-1}. The slope of the regression line for the CHO_{ox} *vs* running speed was not different from zero (P>0.05; linear regression). In contrast, FAT_{ox} increased progressively as speed increased (slope of regression line higher than zero; P<0.01; linear regression).

Figure 2.5 shows the relationship between the energy cost of locomotion per unit time (E_{metab}) and running speed. Mean resting rate of energy expenditure was 2.21 ± 0.23 J·s^{-1} (speed =0), and the slope of the linear regression between E_{metab} and speed was different from 0 (P<0.01; linear regression). The slope of this relationship, or cost of transport (energy cost per unit distance) for running ruff sandpipers was 1.29 J·m^{-1}. This

value is significantly lower than predicted from the allometric equation for birds in general [2.49 $J \cdot m^{-1}$; P<0.01; Mann-Whitney rank sum test; (Taylor et al., 1982)]. However, it is not different from the value predicted from the allometric equation specifically derived for shorebirds [1.68 $J \cdot m^{-1}$; P>0.05; Mann-Whitney rank sum test; (Bruinzeel et al., 1999)].

Fuel selection during cold exposure and exercise

The relative contributions of carbohydrate, lipid and protein oxidation to $\dot{V}_{O_2}$ during cold exposure and running are summarized in Fig. 2.6. In the cold exposure experiments (Fig. 2.6A), lipid oxidation accounted for more than 80% of $\dot{V}_{O_2}$, the same value observed in the control animals held at 22°C. In contrast, protein oxidation (4-7%) and carbohydrate oxidation (10-14%) had much lower relative contributions. The importance of carbohydrates and lipids did not vary between temperatures (P>0.05; one-way repeated measures ANOVA), but the percent contribution of proteins decreased with temperature (P<0.001; Friedman repeated measures ANOVA on ranks).

In the exercise experiments (Fig. 2.6B), lipid oxidation contributed 75% of $\dot{V}_{O_2}$ at speed 0. This contribution was lower at 15, 25 and 30 $m \cdot min^{-1}$ (58-62%; P<0.05; one-way repeated measures ANOVA) but did not differ from the resting value at the other speeds measured (65-72%; P>0.05; one-way repeated measures ANOVA). Exercise caused an increase in relative carbohydrate oxidation above resting levels (P<0.001; one-way repeated measures ANOVA), but only at the lower running speeds (15 to 30 $m \cdot min^{-}$

[1]). The relative contribution of protein oxidation to $\dot{V}_{O_2}$ was decreased during exercise compared to rest (P<0.001; one-way repeated measures ANOVA).

Figure 2.1. Changes in ambient temperature (A), oxygen consumption ($\dot{V}_{O_2}$) (B) and respiratory exchange ratio (RER) (C) of adult ruff sandpipers. Treatment temperatures are indicated as follows: 22°C control (▲), 15°C (◇), 10°C (●) and 5°C (▽). RER values were pooled (○) because they were not significantly different between treatments. Values are means ± SEM (*N*=6).

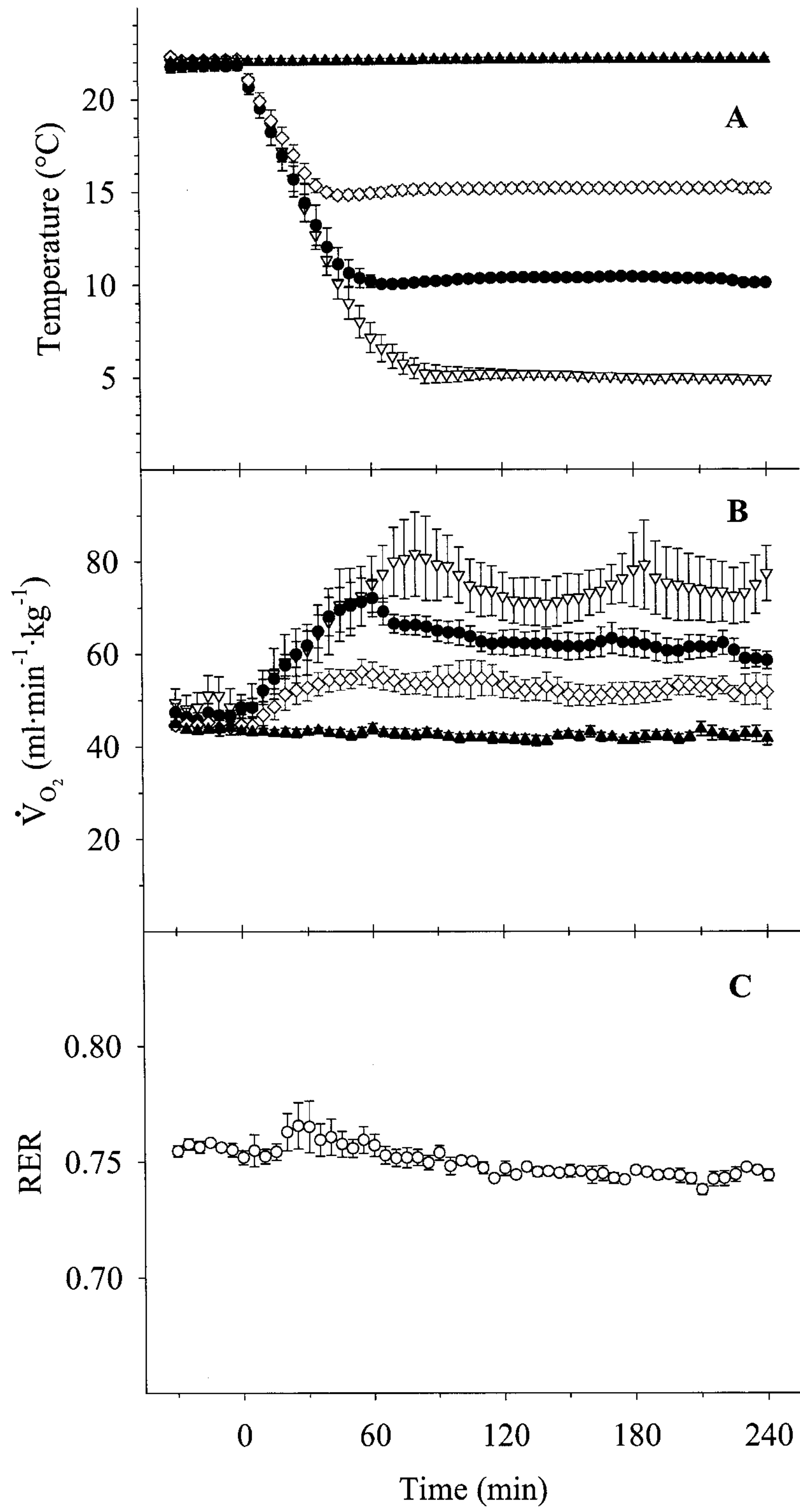
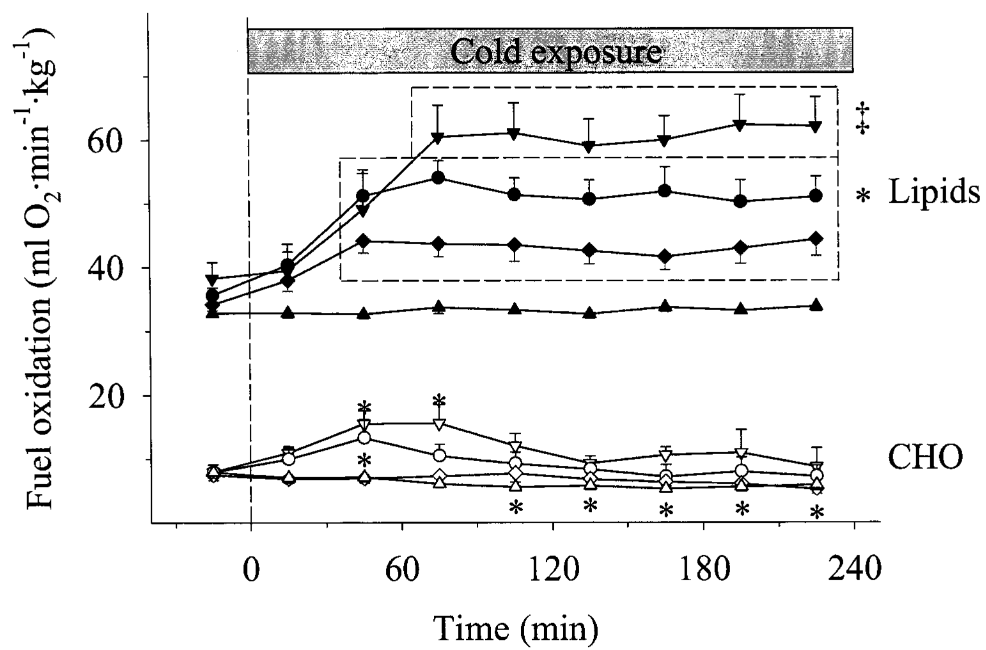
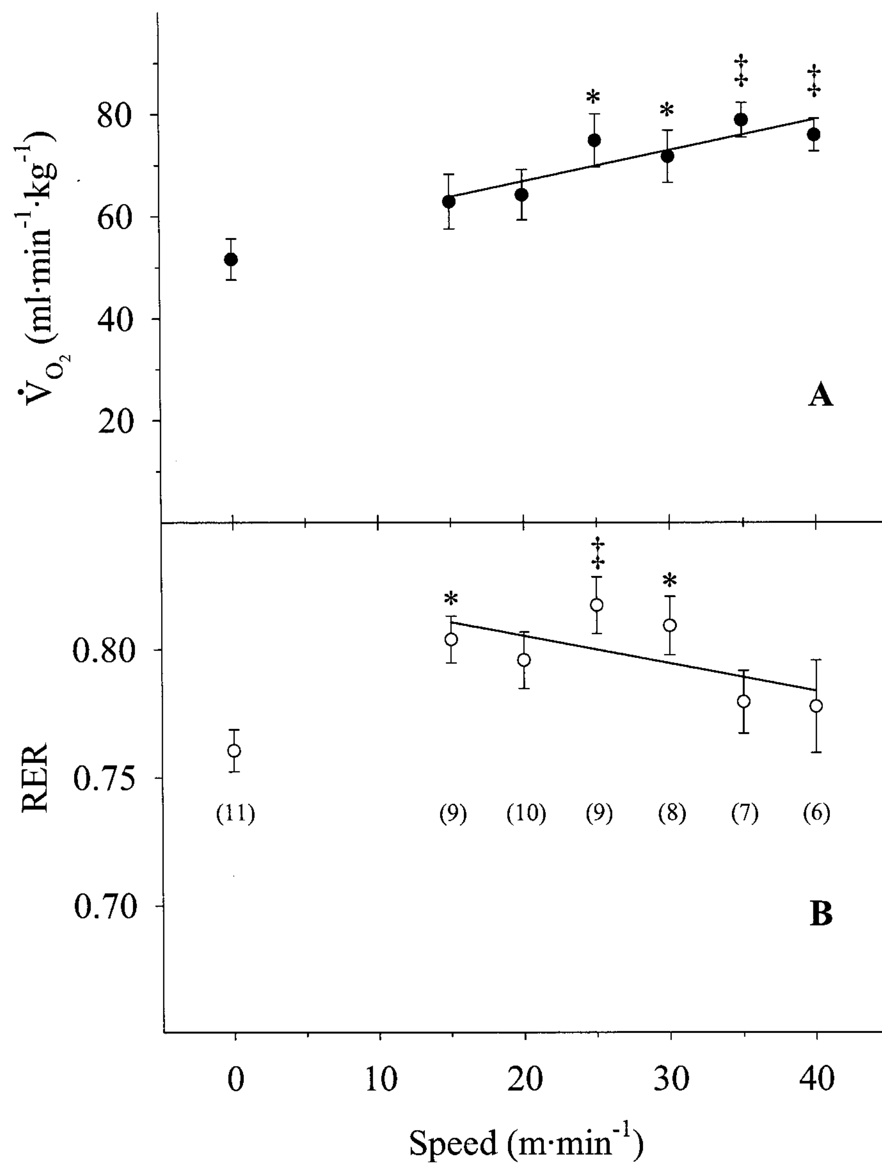

A
B
C
Temperature (°C)
20
15
10
5
$\dot{V}_{O_2}$ (ml·min^{-1}·kg^{-1})
80
60
40
20
RER
0.80
0.75
0.70
0
60
120
180
240
Time (min)

Figure 2.2. Changes in rates of carbohydrate (CHO; open circles) and lipid oxidation (filled circles) over time for ruff sandpipers exposed to 22°C (control) (△,▲), 15°C (◇,◆), 10°C (○,●) and 5°C (▽,▼). Superscripts indicate significant differences from baseline at $P<0.05$ (*) and $P<0.001$ (‡). Values are means + SEM (N=6).



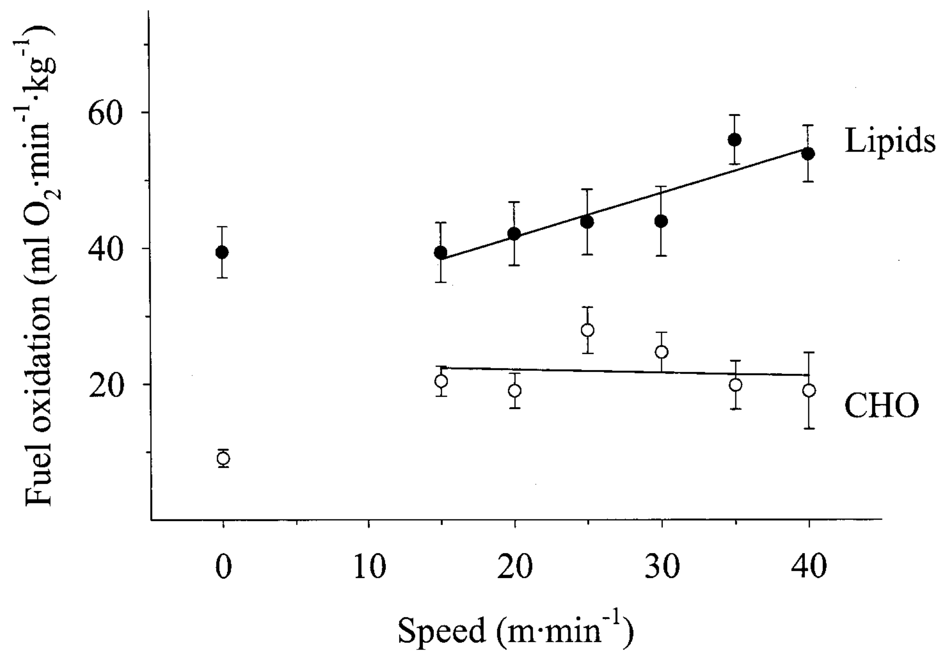
Cold exposure
Fuel oxidation (ml $O_2 \cdot min^{-1} \cdot kg^{-1}$)
Time (min)
‡
* Lipids
CHO
60
40
20
0
60
120
180
240

Figure 2.3. Rate of oxygen consumption ($\dot{V}_{O_2}$) (A) and respiratory exchange ratio (RER) (B) of ruff sandpipers at rest and during running at various speeds. The lines indicated were fitted by linear regression on the exercise values only. Superscripts indicate significant differences from rest at $P<0.05$ (*) and $P<0.001$ (‡). Values are means ± SEM and sample sizes are indicated in parentheses.



80
60
40
20
A
0.80
0.75
0.70
RER
(11)
(9)
(10)
(9)
(8)
(7)
(6)
B
0
10
20
30
40
Speed (m·min-1)

Figure 2.4. Rates of carbohydrate (○) and lipid oxidation (●) of ruff sandpipers at rest and during running at various speeds. The lines indicated were fitted by linear regression on the exercise values only. Values are means ± SEM (sample sizes as in Fig. 2.3).



Fuel oxidation (ml O2·min-1·kg-1)
60
40
20
Lipids
CHO
0
10
20
30
40
Speed (m·min-1)

Figure 2.5. Rate of energy expenditure of ruff sandpipers at rest (speed 0) and during running at various speeds. Line indicated was fitted by linear regression for exercise values only. The cost of transport of running ruffs (=slope of line) is 1.29 $J \cdot m^{-1}$. Superscript indicates a significant difference from rest at $P<0.05$ (*). Values are means ± SEM (sample sizes as in Fig. 2.3)..

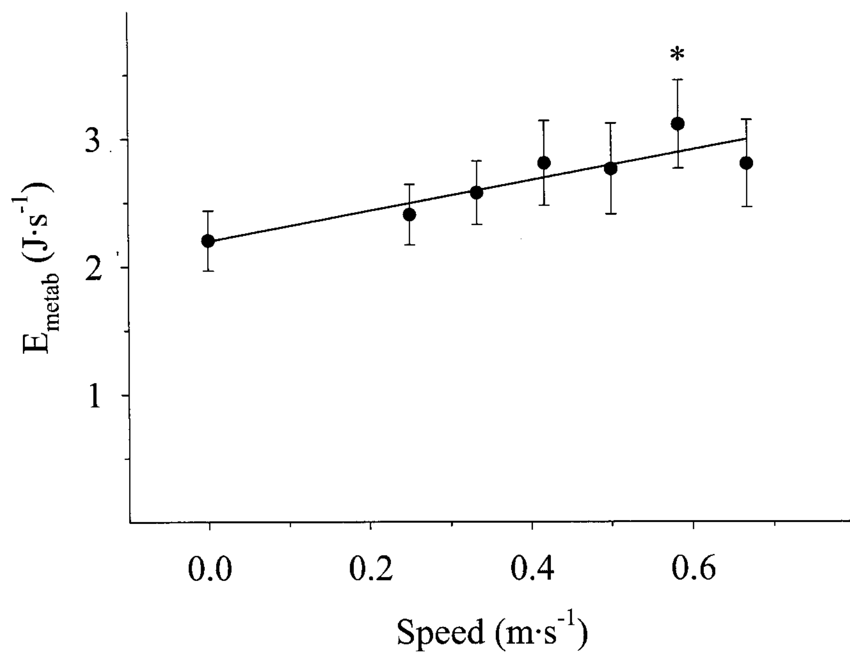
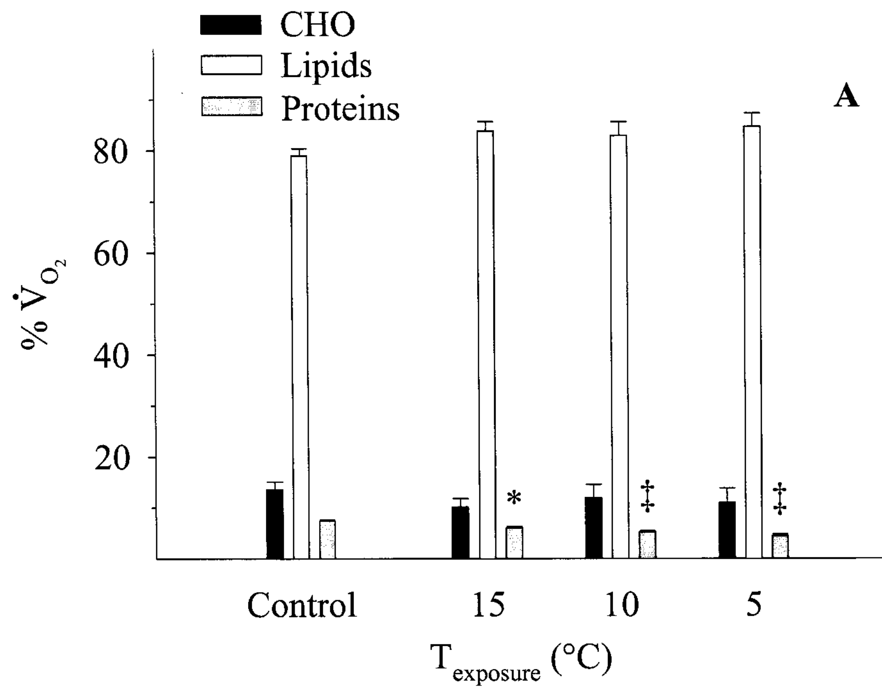

*
3
2
1
E_{metab} (J·s^{-1})
0.0
0.2
0.4
0.6
Speed (m·s^{-1})

Figure 2.6. Relative contributions of carbohydrates (CHO) (■), lipids (□) and proteins ( ) to total energy expenditure of ruff sandpipers during cold exposure (A) and running (B). Values different from controls (22°C for cold exposure and speed 0 for exercise) are indicated by * (P<0.05) and ‡ (P<0.001). Values are means + SEM (*N*=6 for cold exposure and as in Fig. 2.3 for exercise).


CHO
Lipids
Proteins
A
80
60
40
20
% $\dot{V}_{O_2}$
Control
15
10
5
$T_{exposure}$ (°C)

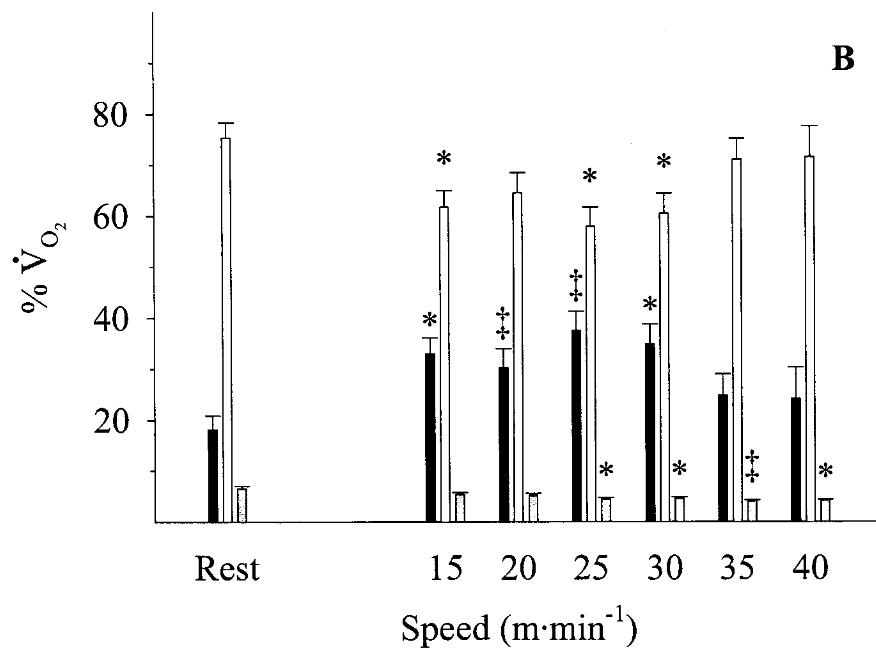

B
80
60
40
20
% $\dot{V}_{O_2}$
Rest
15
20
25
30
35
40
Speed (m·min^{-1})

Figure 2.7. Relationships between rates of lipid oxidation or carbohydrate oxidation and metabolic rate ($\dot{V}_{O_2}$) of ruff sandpipers during cold exposure or running.

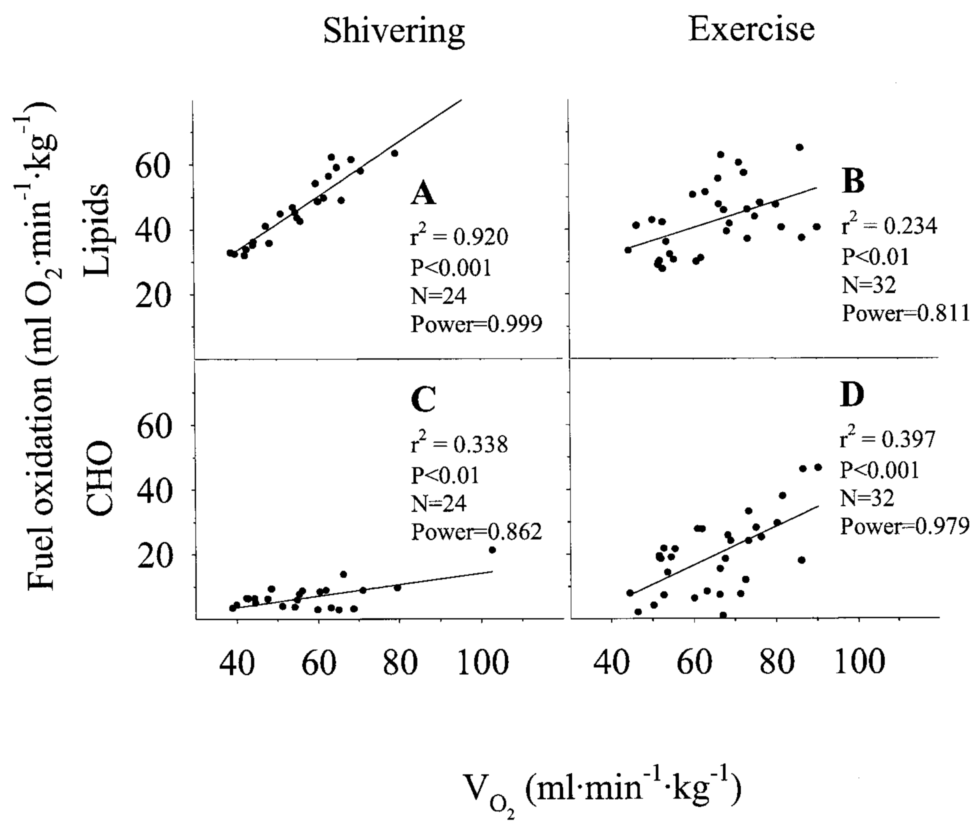

Shivering
Exercise
Fuel oxidation (ml O2·min-1·kg-1)
Lipids
CHO
60
40
20
A
r2 = 0.920
P<0.001
N=24
Power=0.999
B
r2 = 0.234
P<0.01
N=32
Power=0.811
C
r2 = 0.338
P<0.01
N=24
Power=0.862
D
r2 = 0.397
P<0.001
N=32
Power=0.979
40 60 80 100
VO2 (ml·min-1·kg-1)

Table 2.1. Oxygen consumption ($\dot{V}_{O_2}$), carbon dioxide production ($\dot{V}_{CO_2}$), respiratory exchange ratio (RER), carbohydrate (CHO_{ox}) and lipid oxidation (FAT_{ox}) in birds exposed to 22, 15, 10 or 5°C.

	Control (22°C)	15°C	10°C	5°C
$\dot{V}_{O_2}$ (ml·min^{-1}·kg^{-1})	42.9 ± 1.3^{a}	52.7 ± 2.1^{ab}	61.4 ± 2.4^{bc}	74.0 ± 7.0^{c}
$\dot{V}_{CO_2}$ (ml·min^{-1}·kg^{-1})	32.1 ± 1.2^{a}	38.8 ± 1.4^{ab}	45.6 ± 1.7^{bc}	54.9 ± 5.7^{c}
RER	0.747 ± 0.005^{a}	0.737 ± 0.005^{a}	0.742 ± 0.008^{a}	0.739 ± 0.008^{a}
CHO_{ox} (ml O_2·min^{-1}·kg^{-1})	5.9 ± 0.8^{a}	5.2 ± 0.8^{a}	7.2 ± 1.7^{a}	8.7 ± 3.0^{a}
FAT_{ox} (ml O_2·min^{-1}·kg^{-1})	33.8 ± 0.7^{a}	44.3 ± 2.6^{ab}	51.1 ± 3.1^{bc}	62.1 ± 4.6^{c}

Values are means ± SEM (*N*=6). Values not sharing a common exponent are significantly different ($P<0.05$).

Discussion

My goal was to quantify the pattern of oxidative fuel utilization of a long-distance migrant shorebird during cold exposure and running. Results show that shivering thermogenesis and land locomotion of ruff sandpipers are predominantly supported by lipid oxidation. The large lipid reserves of this endurance athlete provide most of the energy for heat generation and running, even at the lowest temperature and at the highest exercise intensity tested in this experiment.

Cold exposure

The metabolic rate of ruff sandpipers is stimulated in proportion with the intensity of cold exposure (Fig. 2.1B) and lipids are responsible for fueling shivering thermogenesis (except for a minor, transient contribution from carbohydrates; see Figs. 2.2, 2.6A, and Table 2.1). This conclusion is further supported by the observation that the slope of the regression line between FAT_{ox} and $\dot{V}_{O_2}$ is much higher than for CHO_{ox} *vs* $\dot{V}_{O_2}$ (0.841 and 0.174, respectively). This study is the first to quantify the use of oxidative fuels for heat production in an avian species exposed to cold. It shows that the relative use of lipids and carbohydrates is not affected by shivering, and, therefore, is independent of environmental temperature (Fig. 2.6A). More than 80% of $\dot{V}_{O_2}$ is accounted for by lipid oxidation at all temperatures, even during intense shivering at 5°C. During shivering, the relative contribution of carbohydrates to total energy expenditures decreases as the intensity of the exercise increases; therefore, I must reject Hypothesis I (see Chapter 1). At the highest FAT_{ox} measured here (~62 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$), and assuming that the

same pattern of fuel utilization is maintained, I can calculate that a 110 g ruff sandpiper with lipid reserves of 20% body mass [or half the maximal value observed just before migration (Van Rhijn, 1991)] could shiver continuously for 4.5 days.

How does this fuel selection pattern compare with what has been observed in other cold-exposed endotherms? In the absence of information on birds, I looked for shivering studies on mammals of similar size (and surface to volume ratio) as my sandpipers, but without much success. Using arterio-venous differences in substrate concentrations, the only study I was able to find reports that lipid oxidation accounts for more than 90% of $\dot{V}_{O_2}$ in cold-exposed rats (Adán et al., 1995). However, comparing these results with my results is not appropriate because the rats were acclimated to 22°C and kept in individual cages. Under such housing conditions, rats are known to produce significant amounts of brown adipose tissue, because they are well below their thermoneutral zone [29-31°C for Wistar rats (Romanovski et al., 2002)] and cannot use social thermoregulation. Therefore, heat production from brown adipose tissue may explain the high FAT_{ox} reported for rats by Adán et al. (1995), whereas the presence of this specialized thermogenic tissue has never been demonstrated in birds (Saarela et al., 1991). In addition, I have recently carried out experiments on group-housed rats acclimated to 28°C, to eliminate the thermogenic contribution of brown adipose tissue. Under these conditions, lipids were responsible for 52% $\dot{V}_{O_2}$ and carbohydrates for 37% $\dot{V}_{O_2}$ during prolonged exposure to 5°C (E. Vaillancourt, F. Haman and J.-M. Weber, unpublished).

Surprisingly, the only other mammal whose fuel metabolism has been well characterized during shivering is the adult human. In this experimental model, it was established that lipids play a much less important role than observed in ruff sandpipers. For cold exposure conditions

eliciting a 2-fold increase in metabolic rate, lipid oxidation is responsible for 50% of $\dot{V}_{O_2}$ in humans (Haman et al., 2002). The exact reasons for these discrepancies are unknown, but may be due to differences in the fiber composition of shivering muscles. In humans, it has been shown recently that CHO_{ox} for thermogenesis depends on the specific recruitment of type II, fast glycolytic fibers, responsible for "burst shivering" (Haman et al., 2004). In long-distance migrant birds, large pectoral muscles produce most of the heat, and the metabolic machinery of their fibers is specifically geared for lipid oxidation. Therefore, the high metabolic capacity of flight muscles for fat catabolism could explain why, on their own, lipids account for over 80% of $\dot{V}_{O_2}$ in cold-exposed ruffs. This high capacity for lipid oxidation and the large lipid reserves available in migrant birds mean that carbohydrates and proteins only play minimal roles during shivering, each accounting for less than 14% of $\dot{V}_{O_2}$. Taken together, these results show that lipids probably play a much more prominent role for shivering thermogenesis in migrant birds than in mammals.

At the lowest temperature tested in this study, metabolic rate appeared to fluctuate with a period of ~90 min (Fig. 2.1B) and this pattern may be related to a well known heat-saving strategy previously observed in birds. Similar cyclic variation in $\dot{V}_{O_2}$ and leg temperature has been observed in cold-exposed pigeons; these changes are caused by vasoconstriction/vasodilation cycles of the leg geared to decrease heat loss from this uninsulated region of the body. Interestingly, pigeons exposed to a lower temperature than tested here (-10°C) showed a shorter period of only 20 min (Østnes and Bech, 1998). Further research is

needed to establish whether this energy-saving strategy is found in all birds or only in species regularly exposed to cold conditions.

Exercise

As experimental models, long-distance migrant birds provide a unique vantage point to study the extreme performance of metabolic systems during exercise. Unfortunately, making physiological measurements during migration or simulating prolonged flight in the laboratory are extremely difficult and rarely attempted. As a compromise, and because little *in vivo* information is presently available on avian exercise, I reasoned that investigating land locomotion would be an important first step towards a better understanding of fuel metabolism in exercising migrants. However, it is clear that running does not simulate migration because leg and flight muscles are different, and maximal metabolic rates achievable during running are low compared to flight.

Indirect calorimetry measurements in running or flying birds have only been performed in a few studies that report $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (or RER), but these parameters have never been used to determine oxidation rates of metabolic fuels (Brackenbury and Vincent, 1988; Rothe et al., 1987; Suarez et al., 1990; Tucker, 1968; Vincent and Brackenbury, 1988; Ward et al., 2002; Ward et al., 2001). Here, I have quantified $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and nitrogen excretion to calculate absolute rates of fat, carbohydrate and protein oxidation as well as the relative contribution of each fuel to $\dot{V}_{O_2}$ during running (Figs. 2.3, 2.4 and 2.6B). Results show that, as for cold exposure, lipids play a dominant role in energy metabolism during running (Fig. 2.6B). When

exercise intensity is increased, FAT_{ox} is augmented whereas CHO_{ox} remains independent of running speed (Fig. 2.4). The overall fuel selection pattern of exercising ruff sandpipers stays relatively constant across speeds, with lipids providing more energy than all other fuels combined (58-72% $\dot{V}_{O_2}$) and carbohydrates being responsible for about half the contribution of lipids (24-38% $\dot{V}_{O_2}$) (Fig. 2.6B). The increased reliance on carbohydrates associated with higher running speeds commonly observed in mammals does not occur in birds, showing that the fuel selection patterns of exercising birds and mammals are different. Even though lipids are well known to provide most of the energy for sustained flying in long-distance migrant birds (Guglielmo et al., 2002a; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002), this study is the first to investigate running, and to provide detailed information about the use of all oxidative fuels under controlled exercise conditions of different intensities.

For comparison, I have calculated FAT_{ox} and CHO_{ox} from published bird exercise studies reporting $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, but without correcting for protein oxidation (because rates of nitrogen excretion are not available). The only two treadmill studies I could find show that CHO oxidation accounts for >80% of $\dot{V}_{O_2}$ in running chickens (Brackenbury and Vincent, 1988; Vincent and Brackenbury, 1988). The divergent fuel selection patterns observed in highly aerobic ruff sandpipers and sedentary, domesticated chickens can probably be explained by differences in the fiber composition of leg muscles (and associated enzymatic machinery), a parameter known to vary with species, age and gender (Guglielmo et al., 2002a; Olson, 2001). From studies on flying birds, I calculated that lipid oxidation accounts for >65% of $\dot{V}_{O_2}$ [budgerigar, (Tucker, 1968); pigeon, (Rothe et al., 1987); european starling, (Ward et al., 2001);

barnacle and bar-headed goose, (Ward et al., 2002)], except for hovering hummingbirds that can temporarily rely entirely on carbohydrates while feeding on nectar (Suarez et al., 1990). Migrant birds must clearly rely predominantly on lipids during non-stop, long-distance flights because alternative fuels are only stored in very small quantities, and would therefore be rapidly depleted.

Land locomotion is particularly important for long-distance migrant shorebirds because it allows them to replenish energy reserves rapidly during short stopovers. Strong selection pressure for decreasing the cost of walking and running may therefore be responsible for the very low cost of transport (energy cost per unit distance) observed here in ruff sandpipers. This cost is 48% lower than predicted from the allometric equation established for birds in general (Taylor et al., 1982), but it is not different from the value predicted from the allometric equation for shorebirds only (Bruinzeel et al., 1999). This observation suggests that economical running is not an exclusive attribute of migrants, but that it is a common feature of all shorebirds, possibly related to their particular leg morphology.

Conclusions

The energy necessary to support shivering and running in ruff sandpipers is provided almost exclusively by the oxidation of lipid reserves. Their pattern of oxidative fuel selection does not depend on shivering or running intensity. During shivering, total ATP production is unequally shared between lipids (82%), CHO (12%) and proteins (6%). During land locomotion, lipids remain the dominant substrate (66%), with CHO (29%) and proteins (5%) playing more minor roles. The prevailing use of lipids during intense shivering and high-speed running is not consistent with the fuel selection pattern observed in exercising and cold-exposed mammals.

Long-distance flying is well known to be supported primarily through lipid oxidation and this study shows that the same source of fuel is also dominant during other activities like intense running and shivering. The exact mechanisms allowing birds to use lipids at extremely high rates are still largely unexplored, and quantifying the exact importance of proteins and carbohydrates during long-distance flight remains a major challenge for future research.

CHAPTER 3. GLYCEROL AND NEFA KINETICS OF A LONG-DISTANCE MIGRANT SHOREBIRD (*PHILOMACHUS PUGNAX*) DURING COLD EXPOSURE

Introduction

In chapter 2, I used indirect calorimetry to determine the pattern of oxidative fuel selection in ruff sandpipers during cold exposure and treadmill running. Results show that lipids are the dominant fuel source used to regenerate ATP for both, shivering (>80% of $\dot{V}_{O_2}$) and running (58-72% of $\dot{V}_{O_2}$). Moreover, the absolute rate of lipid oxidation increases with metabolic rate, showing that lipid reserves must be mobilized to become available for shivering and exercising muscles. The high reliance on lipids observed during intense shivering and running in ruff sandpipers is inconsistent with the pattern of fuel selection seen in cold-exposed and exercising mammals of the same size, and indicates that these birds may be able to use lipids at higher rates than mammals. Birds must be able to mobilize their lipid reserves rapidly to allow their muscles to access sufficient amounts of energy when performing high-intensity exercise.

The mobilization of TAG reserves is called lipolysis. It releases glycerol and NEFA into the circulation. The circulating fatty acids can then be taken up and oxidized by contracting muscles. Our knowledge of avian lipolysis is almost non-existent because very few bird studies have investigated this physiological process, either at the organismal level or at the tissue or cellular level. *In vitro* studies of avian lipolysis have demonstrated that glucagon-stimulated lipid mobilization is higher in adipocytes from cold-acclimated ducklings than in their counterparts kept under thermoneutral conditions (Bénistant et al., 1998). Our knowledge about *in vivo* lipolysis of birds is very limited, because it was only measured in king penguins (Bernard et al., 2002b; 2003). These studies show that the lipolytic rate of this non-flying species

increases during glucagon infusion and after mercaptoacetate infusion (an inhibitor of β-oxidation). The information provided in these studies is very valuable, but data from a single, non-migrant bird species of relatively large size cannot be extrapolated to other birds such as small, long-distance migrants. The serious difficulties associated with complex surgical procedures and reduced blood volumes available for sampling in small birds explain why lipolysis has only been measured *in vivo* in >13kg, tamed penguins (Bernard et al., 2002a; 2002b; 2003). In contrast, mammalian lipolysis has been well studied. In mammals, lipolytic rate has been shown to increase in many species and under a variety of stresses such as fasting [humans (Elia et al., 1987)], thyroid hormone and Medica 16 injections [rats (Kalderon et al., 2000)], leptin injection [rabbits (Reidy and Weber, 2002)], and exercise [rats (McClelland et al., 2001); goats (Weber et al., 1993); humans (Friedlander et al., 1999; Mora-Rodriguez et al., 2001; Wolfe et al., 1990)]. Surprisingly, the effects of cold exposure on lipolysis have only been measured in humans who increase lipid mobilization in parallel with metabolic rate (Vallerand et al., 1999). In this chapter, my goal was to quantify lipolysis of ruff sandpipers *in vivo* as a first attempt to characterize this important aspect of lipid metabolism in a small, highly aerobic bird species. In view of the known mammalian response to cold exposure, I hypothesized that lipolysis increases proportionately to metabolic rate in cold-exposed migrant birds (see goal "*ii*" in Chapter 1).

In addition to shivering thermogenesis, alternative mechanisms of heat production may be used in avian thermoregulation. For example, mammals possess brown adipose tissue (BAT), a specialized, highly vascularized, thermogenic tissue that can oxidize lipids to generate heat through proton leak (Himms-Hagen, 1996). However, it has been demonstrated that BAT is

absent in birds (Bicudo et al., 2002; Brigham and Trayhurn, 1994; Cannon and Nedergaard, 2004; Johnston, 1971; Saarela et al., 1991). Other mechanisms such as substrate cycles may be used by birds to increase heat production (by converting chemical energy into heat) without requiring muscle contractions. One such potential cycle, calcium (Ca^{+2}) cycling, has been studied in birds. Ca^{+2} cycling occurs when Ca^{+2} ions are released from, and then pumped back into the sarcoplasmic reticulum by Ca^{+2}-ATPase without triggering muscle contraction. Even though Ca^{+2} cycling has been found to produce significant amounts of heat in ducklings (Duchamp and Barré, 1993; Duchamp et al., 1993; Duchamp et al., 1992; Dumonteil et al., 1994), this non-shivering mechanism may be absent in adult birds. For the above reasons, thermogenesis from brown adipose tissue and Ca^{+2} cycling have been ignored in this study.

Triacylglycerol:fatty acid (TAG:FA) cycling is a substrate cycle that could be used by birds to produce heat. In adipocytes, lipolysis results in the hydrolysis of triacylglycerol (TAG) to glycerol and non-esterified fatty acids (NEFA). NEFA can either be re-esterified without leaving the adipocyte (primary cycling), or can be incorporated back into the fat reserves after release into the circulation (secondary cycling). TAG:FA cycling (or total cycling) is the sum of primary cycling and secondary cycling. Total cycling, primary cycling, and secondary cycling rates have been quantified *in vivo* in mammals (Wolfe et al., 1990), and my goal was to use the same experimental approach to study TAG:FA cycling in ruff sandpipers (whereby, glycerol flux, NEFA flux and total lipid oxidation must be quantified). Glycerol and NEFA fluxes have only been measured in one bird species [penguins; (Bernard et al., 2002a; 2002b; 2003)]. The glycerol flux of penguins has been shown to be unaffected by fasting, while their NEFA flux is increased as fasting is prolonged (Bernard et al., 2002a; 2003). In the same studies, intracellular

cycling was also shown to decrease as fasting is prolonged, but extracellular cycling and total cycling could not be calculated because fatty acid oxidation was unknown (oxygen consumption and carbon dioxide production were not measured).

In mammals, primary cycling and secondary cycling have been studied in a few species such as the rat (Kalderon et al., 2000; McClelland et al., 2001), the rabbit (Reidy and Weber, 2002) and the human (Vallerand et al., 1999; Wolfe et al., 1990). During exercise, humans were shown to increase primary cycling and secondary cycling (Wolfe et al., 1990), but rats exercised under normoxia and hypoxia showed no increase in those parameters (McClelland et al., 2001). However, an increase was observed in primary cycling in resting rats acclimated to hypoxia compared with resting rats under normoxia (McClelland et al., 2001). Similarly, rats treated with thyroid hormone and Medica 16 (a thyromimetic) (Kalderon et al., 2000), and rabbits injected with leptin (Reidy and Weber, 2002) showed increased primary cycling. In humans, cold exposure (5°C) caused a 3-fold increase in secondary cycling, while primary cycling remained unchanged (Vallerand et al., 1999). Therefore, this chapter attempts to test the following hypotheses: 1) as an additional means of generating heat besides shivering, migrant birds increase TAG:FA cycling during cold exposure, and 2) the increase in total TAG:FA cycling is mostly caused by an increase in secondary cycling.

Finally, I estimated whether the catheterization procedure used in this chapter or cold exposure had an effect on the composition of plasma lipids. The concentrations and fatty acid compositions of non-esterified fatty acid (NEFA), neutral lipids (NL) and phospholipids (PL) will be measured in catheterized and non-catheterized birds, and potential changes elicited by cold exposure will be monitored.

Material and methods

Animals

Adult European ruff sandpipers were obtained from a captive colony (Dr. David Lank, Simon Fraser University (SFU), Burnaby, British Columbia, Canada). The birds were kept indoors in a room allowing flight (2.1 m × 3.9 m × 2.4 m) with *ad libitum* access to food (Zeigler, Finfish Silver; 42% protein; 10% lipid; 4% fiber) and running tap water (67 cm × 42 cm x 11 cm deep water basin with ramp). The room had no windows and was only supplied with artificial light. Photoperiod (12L:12D) and temperature (22°C) were kept constant. The animals were acclimated to these conditions for at least 2 months before starting experiments. Out of the total 25 birds received from SFU, 5 were used to adapt the double catheterization surgery procedure to ruff sandpipers, and for practise purposes, leaving 20 animals for experimentation. Ten of them died at various stages through the surgery (they are very sensitive to blood loss while under anaesthesia, and to anaesthetic overdose), while 10 of them recovered. One bird died after 135 min of cold exposure, thus all samples from this bird were used only to practise blood metabolite analysis. Blood coagulated in the arterial catheter of one bird, and blood withdrawal would have been impossible, thus the animal was sacrificed. Another bird was found dead in its cage less than an hour before the scheduled time for the infusion. Out of the 7 birds which completed the experimental protocol, 2 had glycerol fluxes that were extremely high (an ANOVA performed to detect the effect of individual birds declared that these 2 specific birds were statistical outliers; SYSTAT 10), so all data for these birds were discarded, leaving data from 3 females and 2 males (109 ± 16 g). For one of these 5 birds, one plasma sample was lost,

which explains the smaller sample size (*N*=4) in Figs 3.4, 3.5 and 3.6, ten minutes before the onset of cold exposure (t= -10 min). Once all data were corrected for body mass, the physiological parameters reported in this study showed no gender differences and, therefore, results for males and females were pooled. Average body mass for males and females (148 ± 6 g and 83 ± 3 g, respectively) were lower than the values reported by Cramp and Simmons (1983) for wintering birds (December-February; 172 g for males and 99 g for females). Although males had similar body masses than males used in chapter 2 ($P>0.05$; t-test), females in this chapter had lower body masses than the females exposed to cold in the previous chapter ($P<0.05$; t-test). Body mass is known to increase significantly before and during spring migration (March-April; 210 g for males and 132 g for females), and, therefore these experiments were performed in lean ruffs that were not physiologically prepared for enhanced lipid oxidation associated with migration. All experiments were started 30-60 min after the animals ceased to have access to food.

Indirect calorimetry

All indirect calorimetry measurements were done with the same instruments that were used for cold exposure experiments as described in Chapter 2.

Cold exposure experiments

Besides the following modifications, the cold exposure experiments were performed as described in Chapter 2. The first adjustment was made to minimize the risk of injury by having their neck or wings entangled in the catheters during the experiments; the birds needed to be

restrained. A piece of Styrofoam was fixed in the respirometer to compartmentalize it into two sections, and was perforated to allow air to be well mixed between the two compartments and give good gas measurements. To prevent the bird from being injured by the pointy tip of the analog thermometer, the thermometer and the animal were kept in different compartments. Thus the temperature experienced by the bird may have been higher than the temperature measured by the thermometer. The animal was restrained by a respirometer wall on one side and the Styrofoam panel on the other side. The bird could still move back and forth inside the respirometer, but it could not open its wings nor perform a 180° rotation. The second adjustment was done after familiarization, as each animal was measured only at a single cold exposure temperature (5°C). The experiment included a 60 min baseline period at 22°C, a 75 min transition period to reach 5°C, and a cold exposure period at 5°C. The sum of the transition and cold exposure periods was equal to 180 min, compared to 240 min for experiments performed in Chapter 2 (Fig. 2.1A).

Surgical procedures

The catheterization was performed 2 days before the experiments. Two hours before the surgery, the animals were set aside in a dog carrier cage and had access only to water. This was done to help minimize the amount of food present in the stomach, thus minimizing the risk of food reflux that could occur when birds are anaesthetized and lay on their side. A plastic cone was covered with a stretched piece of latex cut from a latex glove, and maintained in place by rubber bands. A Y-shape incision was then made to allow the beak of the bird to fit in the cone. The cone was linked to a halothane vaporizer using coiled tubing. The birds had their beak

inserted in the plastic cone in such a way that even their nostrils were inside the cone, thus preventing the bird from breathing room air. The halothane mixture was set to 2% to induce anaesthesia. Anti-bacterial gel was put in the eyes of the birds to prevent eye dryness after the surgery. The cone was then removed and the bird had an endotracheal intubation, thus maximizing the space available around the neck of the bird to manipulate the surgical instruments. The plastic shield of an intravenous catheter needle (14G x 51mm for males; 16G x 45 mm for females) was lubricated with a water-base gel (KY) and carefully inserted into the trachea of the bird. To prevent the air sacks from collapsing, the bird was laid down on its left side, never on its back. The tube was then fixed to the upper beak of the bird using surgical tape. The ventral-right side of the neck was then defeathered, cleaned with 4% Hibitane, and then cleaned with iodine. The halothane mixture was then reduced to 1-1.5%, as required to maintain the surgical anaesthetic plane, but to prevent the loss of these anaesthesia sensitive, prone to overdose birds. A ventral-right longitudinal incision (2 cm) was made to allow access to both the right jugular vein and the left carotid artery. The right jugular vein was isolated over a length of 1.5 cm using *Mosquito* forceps and *Moria* forceps, and the anterior part of the vein was tied with 3 half-hitch knots (double reef knot; see (Waynforth, 1980) using 3-0 nylon surgical silk to stop blood flow. Another length of 15 cm silk was passed around the posterior part of the vein, a half-hitch was made, but the knot was not tied up. Instead, the loop was reduced to a 1-cm diameter and the 2 ends of the silk length were seized with *Straight* forceps. By gently pulling the forceps posteriorly, a slight pressure was exerted to compress the vein walls and prevent blood reflux. Surgery lamps tend to emit heat and to dry body tissue. Thus, to prevent the vessels from drying and cracking, sterile saline was applied on the surgery site at regular

intervals. The excess saline was removed, forceps were used to take hold of the vein, and 2-mm incision was made in the vein wall using an iris scissor. A piece of polyethylene tubing (PE-50) had previously been cut to a length of 30 cm, curved under steam into a 270° loop (see Appendix 1A), the shorter end was blunted, and the tubing was sterilized under ethylene oxide. A blunt 23G sterile needle was connected to a sterile1-ml syringe filled with sterile 0.9% saline, the needle was inserted tightly in the PE-50 and all air bubbles were removed by bleeding the apparatus. The short end of the PE-50 was slowly fed into the vein and the tension exerted on the half-hitch knot was diminished to allow the PE-50 to be fed posteriorly 1.5 cm into the jugular vein. The tubing was then secured inside the vein using the half-hitch already in place to complete a double reef knot, and a second knot was made at the posterior end of the tubing. The silk that had been utilized to tie the anterior end of the jugular was then used to secure the tubing to the vein. A fourth double reef knot was made at the junction where the PE-50 loops 270° to prevent the tubing from being pulled and kinked during the exteriorization procedure. The catheter was tested for patency, filled with heparinized saline containing Penicillin G (20 units heparin ml^{-1} saline, 125 000 UI PenG ml^{-1} saline), (but precaution was taken to minimize the amount of heparinized saline injected in the vessel) to maintain patency, a multiple layer piece of gauze was wrapped around the free end of the tubing to prevent damage, and forceps were used to compress the tubing and prevent blood from filling it. A filed, rounded stitching needle was inserted firmly in the tubing to seal it, and the forceps and gauze were removed. Forceps were tunnelled under the skin and a slight pressure was exerted dorsally until the tip of the forceps could be seen through the thin skin. A 2-3 mm incision was made using a scalpel, and the tubing was fed through that opening to exteriorize the tubing dorsally at the neck level, halfway

between the shoulders and the head. The same technique that was applied to insert, secure, maintain patency, and exteriorize the right jugular vein catheter was used for the left carotid artery, but the catheter had a small modification. Instead of using only PE-50 tubing, a 15 mm length of PE-10 tubing was cut and fed 5 mm into the larger PE-50 tubing (see Appendix 1B). A small amount of contact glue was put on the PE-10/PE-50 junction and left to dry, then was tested for patency, and sent for ethylene oxide sterilization. The smaller PE-10 could probably have been used alone without PE-50, because it has a greater elasticity, and would probably have been crushed by the tight knots necessary to hold it firmly in place. Once both catheters were exteriorized, the larger ventral incision was sutured using 4-0 Prolene monofilament. The smaller dorsal incision was also sutured, and was used as an anchoring site for the exteriorized catheters. The ends of the monofilament were wrapped 4-6 times (one clockwise, the other counter clockwise) around the catheters in a knot called the *Chinese purse string*, and secured with a double reef knot. Upon completion of the surgery, the intubation tube was removed, and the bird was allowed to recover for an hour in a dog carrier sitting on a water-perfused rubber mattress set to 35-40°C. After this 60 min recovery period, bowls of food and fresh water were inserted in the dog carrier. The rubber mattress was perfused with warm water for 24 h after the end of the surgery. Until the day of the experiment, food and water were replaced twice daily, at the same time the catheters were flushed with heparin/PenG/saline solution mentioned above to maintain patency. Tribissen was injected intramuscularly soon after the catheters had been flushed. Torbugesic (intramuscular injection; morning) and Metacam (subcutaneous injection; afternoon) were administered only once daily. All medication was administered according to the dosage sheets supplied by the manufacturers. All experimental protocols (including cold

exposure and surgical procedures) were approved by the Animal Care Committee of the University of Ottawa in accordance with guidelines provided by the Canadian Council for Animal Care.

Infusate preparation

Radioactive compounds were prepared as described previously (Bernard et al., 1999; Haman et al., 1997). The infusate was prepared daily with 2-[^{3}H]-glycerol and 1-[^{14}C]-palmitate as tracers. Ruff plasma had been collected more than 2 weeks before surgery, and lipid composition was analyzed (see Table 3.1). The plasma that had been left over was used as a source of fatty acid-binding proteins. The 1-[^{14}C]-palmitate was supplied commercially in toluene, and an 80 μCi aliquot was pipetted in a sterile vial, dried under nitrogen and immediately resuspended and well mixed in ethanol. NaOH (2 mM in ethanol) was added to excess and the mixture was evaporated to dryness. The water-soluble 1-[^{14}C]-palmitate sodium salt was dissolved in heated sterile saline (60-70°C), and the solution was cooled to 30°C before adding 1 ml ruff plasma. Eighty μCi 2-[^{3}H]-glycerol were added and the infusate volume was adjusted to 5.0 ml by adding sterile saline. The vial was capped and vortexed after the addition of each substance. The infusate was transferred from the vial to a sterile 5 ml syringe. A small amount of infusate was left at the bottom of the vial and 2-3 aliquots of 50 μl were counted in 10 ml Safety Solve to evaluate the activity of the infusate prior to the infusion. Infusion of the infusate was performed using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA) at 1 ml h^{-1}. Infusion rates averaged 4,761±383 x10^{3} DPM kg^{-1} min^{-1} and 1,025±173 x10^{3} DPM kg^{-1} min^{-1} for 2-[^{3}H]-glycerol and 1-[^{14}C]-palmitate, respectively.

Infusion protocol and blood sampling

When the infusate was ready, the animal had PE-50 extensions (60-70 cm) connected to the existing catheters. A 23G needle was cut to obtain a 10 mm piece of stainless steel tubing. The extremities of this tubing were filed to remove sharp edges, and the whole piece was submerged in 70% ethanol for disinfection. Another 23G needle was blunted, rinsed in 70% ethanol, connected to a 1-ml syringe filled with heparinized saline (10 U ml^{-1}), and a PE-50 extension was inserted over the blunted needle. The stainless steel tubing was fed 5 mm inside the free extremity of the PE-50 tubing, and water was pushed in the extension to remove any air bubble. A piece of gauze was wrapped around one of the catheters and compressed using forceps, the blunted stitching needle used to plug the catheter was removed, the remaining 5 mm of the 23G stainless tubing was inserted in the catheter, and was then secured in place with a drop of contact glue. Blood was withdrawn (from the arterial catheter) until it was visible through the exteriorized segment of the catheter, and was reintroduced into the animal's bloodstream by pushing heparinized saline (10 U ml^{-1}) in the catheter to prevent loss of patency. Care was taken to minimize the amount of heparin injected in the bloodstream. The same procedure was applied to install the venous catheter extension, but this extension was connected to the 5 ml syringe containing the infusate paired to the calibrated syringe pump. The animal was then introduced in the respirometer, ready to start the infusion. In order to reach isotopic steady-state within 60 min of infusion, a priming dose equivalent to a 30 min infusion was infused during the first 3 min of the infusion (10 $ml \cdot h^{-1}$ for 3 min). After this priming dose, and for the remainder of the experiment, the infusion pump rate was set to 1 $ml \cdot h^{-1}$. Because I needed 200 μl plasma samples for analysis, and because I assumed the hematocrit would be near

0.5, blood samples of 450 μl were taken. Because of the small size of the birds and its proportionately small blood volume, I determined that I could not take more than 6 samples over the time course of the experiment. To determine if isotopic steady-state had been reached upon the onset of cold exposure, I needed at least 2 samples before the beginning of the cooling process, leaving 4 samples to be taken during cold exposure. Therefore, I chose to take samples ten min before the onset and at the onset on cold exposure, and every 45 min for the remainder of the experiment (t= -10 min, t= 0 min, t= 45 min, t= 90 min, t= 135 min and t= 180 min, respectively). Immediately after sampling, the blood was centrifuged and the plasma was separated and frozen at -20°C until further analysis. Heparinized saline was used to fill the arterial catheter after each blood sampling, and thus prevent a blood clot. Again, care was taken to minimize the amount of heparin injected into the bloodstream.

Preparation of blood samples

For the non-radioactive samples analyzed from birds that had no surgery, the procedure was the same as for the radioactive samples, but non-radioactive samples were not counted in the Beckman beta-counter. Once thawed, 200 μl plasma were mixed in 6 ml of chloroform:methanol 2:1 (Folch 2:1) (Folch et al., 1957) in 12 ml glass centrifuge tubes. Twenty μl 17:0 (30 mg/100 ml hexane) were added as an internal standard for NEFA GC analysis. The solution was vortexed and left to sit for 10 min. The solution was centrifuged for 10 min at 3000 RPM (Beckman centrifuge). Pieces of Kimwipe tissues were inserted in Pasteur pipets to obtain homemade filters, the Folch solution was pipetted on top of the filters, and 2 ml Folch 2:1 was added to the pellet. The solution containing the pellet was vortexed, left to sit, filtered, and

added to the first 6 ml. Two hundred μl of Folch mixture (out of total 8 ml) were taken and counted in 10 ml scintillation fluid (Safety Solve) to obtain total lipid activity before washing with water. To get rid of water soluble compounds, 0.25 volume water (2.0 ml) was added, tube was capped, shaken, and placed in a water bath (70°C) to separate the two phases. The top (aqueous) and bottom (organic) phases were separated in 12 ml centrifuge tubes and re-extracted with 3 ml chloroform and 2 ml methanol:water (4:3), respectively. Tubes were placed in the water bath for a second 10 min period, and respective aqueous and organic phases were merged. Each pooled phase was then dried under nitrogen using a heating block (70°C). The aqueous phase was resuspended in 2 ml ethanol:water (1:1) and the organic phase in 2 ml hexane:isopropanol (3:2).

Glycerol analysis

Out of the total 2.0 ml ethanol:water mixture, two aliquots of 50 μl were counted in 10 ml scintillation fluid to obtain total tritium (that was found mainly in glycerol and glucose, although other unidentified compounds accounted for a minor percentage of the activity), 300 μl were transferred to a 5 ml disposable tube to perform TLC, and the remaining 1.6 ml (equivalent of 160 μl plasma) was dried under nitrogen and resuspended in hydrazine base buffer. Twenty μl of this solution were added to 200 μl hydrazine/ATP/NAD/glycerophosphate dehydrogenase buffer, and absorbance of NADH was read at 340 nm. Ten μl glycerokinase were added and the preparation was left to incubate at room temperature for 30 min before absorbance was read again at 340 nm. Percentage activity in glycerol was obtained by separating the glycerol, the glucose and the unidentified compounds (which were negligible) by thin-layer chromatography

(TLC). The 300 μl aliquot (equivalent of 30 μl plasma) of the aqueous phase was evaporated and resuspended in 12 μl ethanol:water (1:1) before spotting the entire volume on a 10 cm x 5 cm silica gel plate (60 F_{254}, Merck, Germany) using a hair dryer. The plate was revealed using chloroform:methanol (8:3), and the glucose and glycerol fractions were scraped into separate scintillation vials. They were resuspended in 2 ml ethanol:water (1:1) and counted in 8 ml Safety Solve scintillation fluid. Percentage glycerol activity was calculated from the sum of the glycerol and glucose activities, and a correction was applied to total tritium activity measured earlier.

Fatty acid analysis

The neutral lipids, non-esterified fatty acids (NEFA) and phospholipids contained in the organic phase were separated by filtration on Supelclean solid-phase extraction tubes (LC-NH2; 1-ml capacity; Supelco). The neutral lipids were eluted using 1.8 ml chloroform and isopropanol (2:1), the NEFA with 1.6 ml isopropyl ether: acetic acid (49:1), and the phospholipids with 6.0 ml methanol. One hundred μl heptadecanoate (17:0; 30 mg/100 ml hexane) was added to NL and PL fractions (as a GC internal standard), but not to NEFA fraction, as the 17:0 added earlier was eluted with the NEFA. An aliquot of each fraction (500 μl for NL and PL, 400 μl for NEFA) was counted to determine the distribution of ^{3}H and ^{14}C activity in plasma lipids. Because no ^{14}C is incorporated into fatty acids other than palmitate, the activity found in the NEFA fraction was equal to palmitate activity. The remainders of the 3 fractions were used to determine the relative distribution of individual fatty acids using gas chromatography (GC) analysis as follows.

NEFA fraction was evaporated under nitrogen and left to cool down to room temperature. Methanol (100 μl), dimethoxypropane (1 ml) and concentrated HCl (40 μl) were added, and the tube content was vortexed after each addition. After the addition of HCl, the temperature of the centrifuge tube was felt (a decreased temperature indicates the exothermic methylation reaction is taking place), and the mixture was left at room temperature for 20-30 min, and then dried under nitrogen. After the sample was dry and the tube had cooled down, it was redissolved in 60 μl isooctane, and was ready for GC analysis.

NL and PL fractions were analyzed the same way using acid transesterification. Samples were evaporated under nitrogen, and 2 ml acetyl chloride in methanol (1 M; density of 1.104; = 7.9 g / 100 methanol = 7.2 ml acetyl chloride in 100 ml methanol) were added. Acetyl chloride was always prepared fresh and kept in the freezer for no longer than a week. Centrifuge tubes were capped tightly, place on the heating block (90°C) for 2 h, then left to cool to room temperature. Samples were evaporated under nitrogen, redissolved in 2.0 ml methanol and evaporated under nitrogen to get rid of residual HCl and water. After the samples were dry and the tubes had cooled down, they were dissolved in 60 μl isooctane, and were ready for GC analysis

The GC (5890 series II with HP 7673 autosampler) had a flame-ionization detector and a 30 m fused silica column. Helium was used as the carrier gas, while hydrogen and air were the detector gases. Injector port and detector temperatures were set to 220 and 240°C, respectively. Column temperature was maintained at 185°C for the first 35 min following injection, raised to 210°C at a rate of 5°C min^{-1}, and maintained at 210°C for an additional 10 min to cumulate a total analysis time of 50 min. Fatty acid retention times were determined using individual fatty

acid methyl esters as well as various commercially available fatty acid methyl ester mixes such as AOCS N° 6 (Sigma), 189-19 (Sigma), and RM-3 mix (Supelco).

Calculations and statistical analyses

Glycerol flux (rate of lipolysis) and palmitate flux were calculated using the steady-state equation of Steele (1959). NEFA flux was determined by dividing palmitate flux by the fractional contribution of palmitate to total NEFA. Triacylglycerol:fatty acid cycling (TAG:FA cycling; total cycling) is the sum of primary and secondary cycling (see Introduction). Total, primary and secondary cycling were calculated according to Wolfe et al. (1990) as follows:

Total cycling = (3 x glycerol flux) – fatty acid oxidation

Primary cycling = (3 x glycerol flux) – NEFA flux

Secondary cycling = NEFA flux – fatty acid oxidation = total cycling – primary cycling

However, these equations have only been validated for mammals and results suggest that they may not be applicable to birds (see Discussion).

Rates of carbohydrate and lipid oxidation (CHO_{ox} and FAT_{ox}) were calculated as in Chapter 2.

Percentages were transformed to the arcsine of their square root before analysis. For cold exposure, mean $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, respiratory exchange ratio (RER), CHO_{ox}, FAT_{ox} and percentages were compared using one-way, repeated measures analyses of variance (ANOVA), while relationships between $\dot{V}_{O_2}$ and CHO_{ox} or FAT_{ox} were analyzed by linear regressions. Glycerol

and palmitate concentrations, specific activities and fluxes, as well as NEFA concentration and flux were compared using one-way, repeated measures ANOVA, Friedman repeated measures ANOVA on ranks (when samples were heteroscedastic) and linear regressions to find trends. When significant changes were detected with ANOVA, Bonferroni's adjustement was used to determine which means were different from basal values (t= -15 min for Figs 3.1 and 3.2; t= 0 min for Figs 3.4, 3.5 and 3.6). Decisional threshold was set at $P<0.05$ and all the values presented are means ± SEM.

Results

Indirect calorimetry

Changes in metabolic rate ($\dot{V}_{O_2}$), carbon dioxide production ($\dot{V}_{CO_2}$) and respiratory exchange ratio (RER) during cold exposure are presented in Fig. 3.1. While standing quietly at 22°C, metabolic rate was 44.4±3.8 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$. When temperature was decreased, metabolic rate increased gradually above baseline value after 45 min ($P<0.05$; one-way repeated measures ANOVA) and reached a maximum of 65.2±8.1 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ after 105 min at 5°C (Fig. 3.1A). After 165 min of exposure to 5°C, $\dot{V}_{O_2}$ was almost identical to the value measured after 165 min at 10°C in chapter 2 (60.6±5.6 and 62.4±2.9 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$, respectively; $P>0.05$; t-test). The baseline value for $\dot{V}_{CO_2}$ was 33.7±3.1 $ml \cdot min^{-1} \cdot kg^{-1}$, it increased progressively above baseline level after 45 min at 5°C, and reached its maximum value of 50.6±6.0 $ml \cdot min^{-1} \cdot kg^{-1}$ after 105 min in the cold (Fig. 3.1B). At the beginning of the experiment, RER value was 0.758±0.013, and this value is practically the same that was observed in chapter 2 (Fig. 3.1C; compare with Fig. 2.1C; $P>0.05$; Mann-Whitney rank sum test). As for the observation made in the previous chapter, RER remained unchanged throughout the experiment ($P>0.05$; one-way repeated measures ANOVA) and averaged 0.767±0.019.

Changes in the rates of lipid and carbohydrate oxidation (FAT_{ox} and CHO_{ox}) during exposure to 5°C are shown in Fig. 3.2. FAT_{ox} was always at least 3.7-fold higher than CHO_{ox}, while the average ratio of FAT_{ox}/CHO_{ox} was 4.0, a value comparable to the value observed in chapter 2. In this experiment, baseline level for CHO_{ox} was 7.9±2.2 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$, and was

practically identical to the corresponding value of 7.8±0.6 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ measured in chapter 2 (see Fig. 2.2) ($P>0.05$; Mann-Whitney rank sum test). Besides a transient twofold increase of CHO_{ox} above baseline levels after 105 min of cold exposure (15.3±1.6 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$; $P<0.05$, one-way repeated measures ANOVA), the treatment had no other effect on CHO_{ox}. FAT_{ox} increased above baseline levels after 45 min in the cold, and attained its maximal value of 48.2 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ after 75 min of cold exposure. FAT_{ox} baseline level of 33.3±3.3 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ was similar to the corresponding value of 35.2±0.7 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ observed in the previous chapter (see Fig. 2.2) ($P>0.05$; Mann-Whitney rank sum test).

Fig. 3.3 shows regression analyses of FAT_{ox} and CHO_{ox} with $\dot{V}_{O_2}$ as the independent factor. Although the minor slope of the relationship between CHO_{ox} and $\dot{V}_{O_2}$ is significantly different from 0 (slope=0.141; $r^2=0.242$; $P<0.01$), a much more pronounced slope is observed for the relationship between FAT_{ox} and $\dot{V}_{O_2}$ (slope=0.858; $r^2=0.921$; $P<0.001$). These correlations are similar to the relationships observed for the cold exposure experiments in chapter 2 (Figs 2.7A and C).

Continuous infusion

Glycerol concentration, specific activity and flux in cold-exposed ruff sandpipers are presented in Fig. 3.4. Glycerol concentration (Fig. 3.4A) and flux (Fig. 3.4C) were not affected by cold exposure ($P=0.173$ and $P=0.075$, respectively; Friedman repeated measures ANOVA on ranks), and averaged 0.288±0.044 mM and 56.2±8.1 $\mu mol \cdot min^{-1} \cdot kg^{-1}$, respectively. Glycerol specific activity measured after 50 min of infusion (or 10 min before the beginning of cold

exposure; t= -10 min; 1.40±0.19 x10^5 DPM·μmol^{-1}) was almost twice its baseline value of 7.48±1.05 x10^4 DPM·μmol^{-1} (t= 0 min; P<0.05; one-way repeated measures ANOVA), which indicates there may have been a potential problem with the isotopic steady-state at the start of the infusion, or may result from the smaller sample size (*N*=4) analyzed for this particular point in time (Fig. 3.4B). Assuming that the isotopic steady-state was reached after 60 min of infusion, glycerol specific activity did not change after the onset of cold exposure.

Fig. 3.5 shows palmitate concentration, specific activity and flux in ruff sandpipers during cold exposure. Palmitate concentration was not affected by a decrease in environmental temperature, and averaged 0.199±0.059 mM throughout cold exposure (Fig. 3.5A). Palmitate specific activity (Fig. 3.5B) and flux (Fig. 3.5C) baseline levels were 1.03±0.66 x10^6 DPM·μmol^{-1} and 1.36±0.32 μmol·min^{-1}·kg^{-1}, respectively. Palmitate specific activity showed a transient 2.6-fold increase (2.72±0.80 x10^6 DPM·μmol^{-1}; P<0.05; one-way repeated measures ANOVA; Bonferroni t-test) 135 min after the onset of cold exposure. Palmitate flux decreased significantly after 90 min at 5°C, reaching its minimum value of 0.63±0.16 μmol·min^{-1}·kg^{-1} (P<0.05; one-way repeated measures ANOVA; Bonferroni t-test) by the end of the experiment.

NEFA concentration and flux in cold-exposed ruff sandpipers are presented in Fig. 3.6. NEFA concentration showed no response to cold exposure, and averaged 1.57±0.21 mM for the whole duration of the experiment (Fig. 3.6A; P>0.05; one-way repeated measures ANOVA). However, NEFA flux showed significant decreases from the baseline value (9.01±1.18 μmol·min^{-1}·kg^{-1}) after 90 and 180 min of cold exposure, and reached its lowest value of 5.52±1.26 μmol·min^{-1}·kg^{-1} after 180 min at 5°C (Fig. 3.6B). Also, a regression analysis detected

that the slope of NEFA flux, with time as the main factor, was significantly different from zero (slope= -0.0196; r^2=0.190; P=0.018; linear regression).

Because most parameters presented in figures 3.4, 3.5 and 3.6 did not show much change, their average values over time are given in Table 3.1. In addition, average fatty acid oxidation, primary cycling, secondary cycling and total cycling are presented in the same table. The most intriguing result is probably the negative value obtained for secondary cycling.

Plasma lipids in NEFA, NL and PL fractions

Total fatty acid concentration and fractional contribution of various individual fatty acids to total fatty acid concentration in ruff sandpipers that had surgery (this chapter) or no surgery (chapter 2) are presented in Table 3.2. Because the changes detected in the relative contribution of the various fatty acids over time were statistically significant, but did not seem physiologically significant (Tables 3.3, 3.4 and 3.5), values for all sampling times were pooled, averaged, and presented in Table 3.2. Only fatty acids which contributed to at least 1% of total fatty acid concentration in at least one lipid fraction and in at least one series of animals (with or without surgery) were tabulated. The fractional contribution of these fatty acids was determined in the non-esterified fatty acid (NEFA), neutral lipid (NL, mainly triacylglycerol) and phospholipid (PL) fractions. Although total NL and PL concentrations were not significantly different between the treatments, NEFA concentration increased 1.7-fold (from 956±75 to 1,604±218 nmol NEFA·ml^{-1} plasma) in the 5 birds that were subjected to surgery and used for continuous infusions compared to birds that had no surgery (P<0.01; t-test). Palmitate (16:0), stearate (18:0) and oleate (18:1) were generally the most abundant fatty acids in the NEFA, NL and PL

fractions, but linoleate (18:2), arachidonate (20:4) and docosahexaenoate (22:6) were also important constituents of those same fractions. In the NEFA fraction, the abundant 18:1 increased its contribution to total plasma NEFA by 1.5-fold, while 18:0, 20:4 and 22:6 showed similar increases in the PL fraction. Interestingly, in the NL fraction, 22:1 was the fatty acid that had its relative contribution to total plasma NEFA increase the most (6.2-fold). Noticeable decreases in the contribution of 16:0 and 18:1 occurred in the PL fraction, while the NL fraction had a decline in the relative importance of 18:0 in birds that had surgery. The only fatty acid that showed a consistent trend is 20:5, which diminished its relative contribution to total plasma NEFA in all three fractions for birds that had a surgery. It is also worth noting that the relative importance of all lipid fractions was unequal, PL always represented >75% of all plasma lipids while NEFA contributed to no more than 15% of total circulating lipids.

Figure 3.1. Changes in oxygen consumption ($\dot{V}_{O_2}$) (A), carbon dioxide production ($\dot{V}_{CO_2}$) (B) and respiratory exchange ratio (RER) (C) of adult ruff sandpipers before (22°C) and during cold exposure (5°C). Temperature was decreased using the same protocol as in Chapter 2 (See ▽ symbols in Fig. 2.1A). Superscripts indicate significant differences from baseline at P<0.05 (*). Values are means ± SEM (*N*=5).

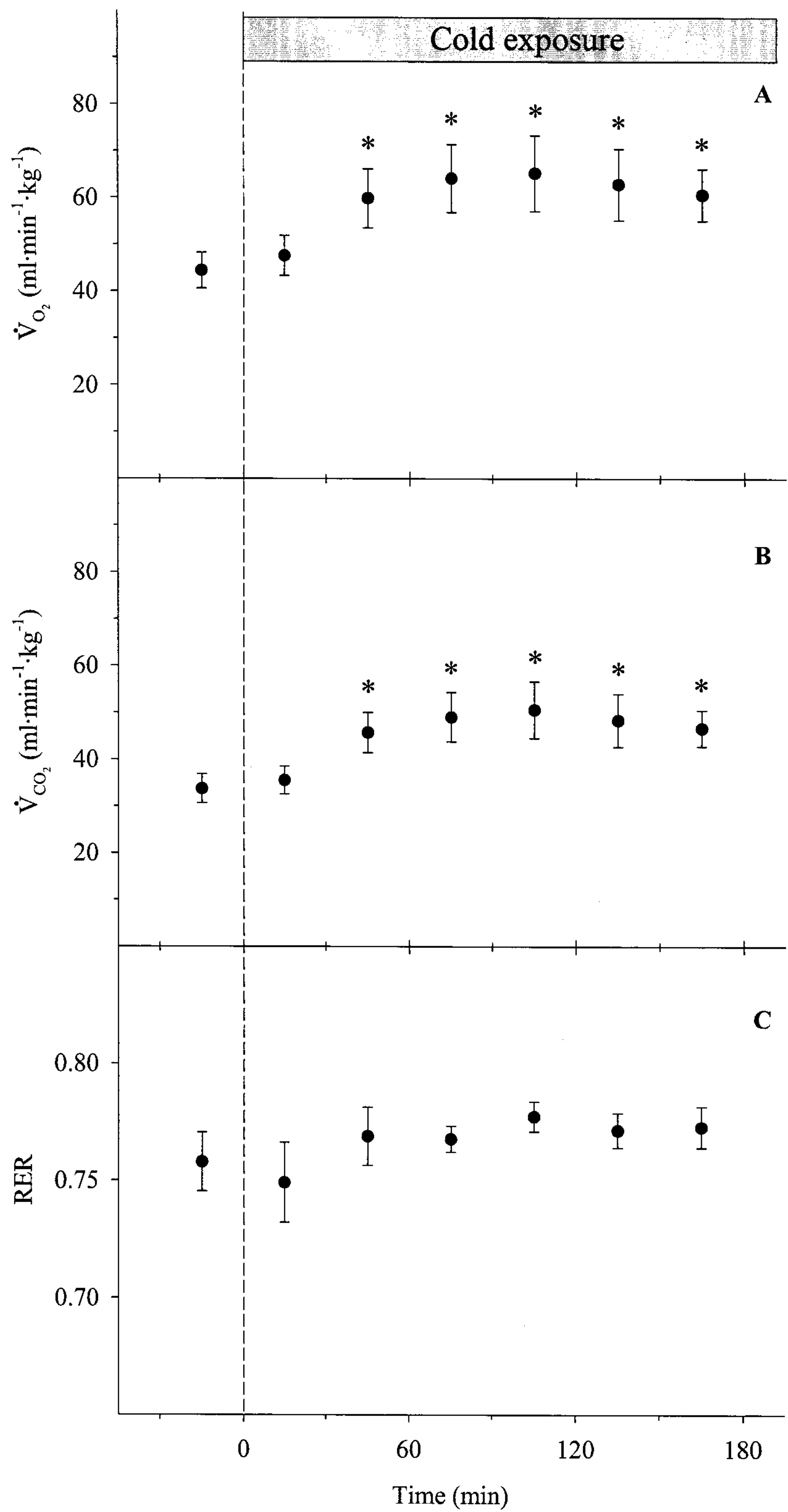

Cold exposure
A
B
C
80
60
40
20
0.80
0.75
0.70
0
60
120
180
Time (min)
RER

Figure 3.2. Changes in rates of carbohydrate (CHO; ▽) and lipid oxidation (▼) over time for ruff sandpipers before (22°C) and during cold exposure (5°C). Superscripts indicate significant differences from baseline at $P<0.05$ (*). Values are means ± SEM (N=5).

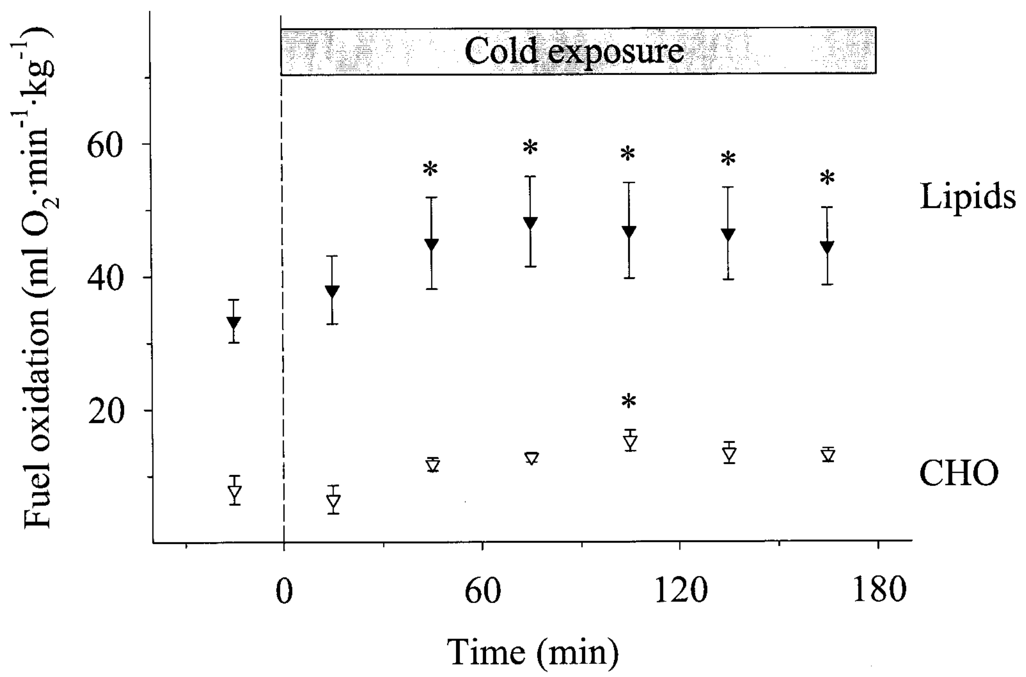
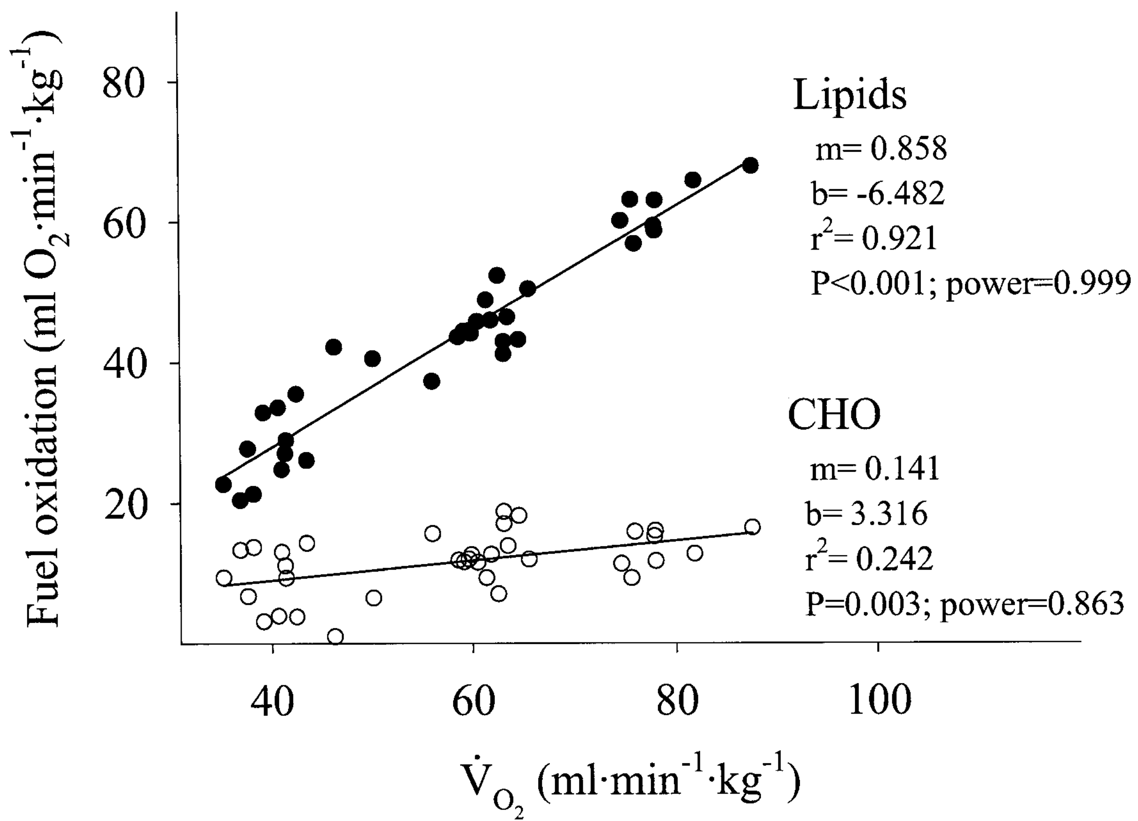

Cold exposure
Fuel oxidation (ml O2·min-1·kg-1)
60
40
20
*
Lipids
CHO
0
60
120
180
Time (min)

Figure 3.3. Relationships between rates of lipid oxidation (●) or carbohydrate oxidation (○) and metabolic rate ($\dot{V}_{O_2}$) of ruff sandpipers during cold exposure. The intercept (b), the slope (m) and the coefficient of determination (r^2) are also indicated for 5 individual birds at 7 different times (every 30 min between t=-15 min and t=165 min).



Fuel oxidation (ml O2·min-1·kg-1)
80
60
40
20
Lipids
m= 0.858
b= -6.482
r2= 0.921
P<0.001; power=0.999
CHO
m= 0.141
b= 3.316
r2= 0.242
P=0.003; power=0.863
40
60
80
100
VO2 (ml·min-1·kg-1)

Figure 3.4. Glycerol concentration (A), specific activity (B) and flux (C) in ruff sandpipers before (22°C) and during cold exposure (5°C). Superscript indicates a significant difference from steady-state values at $P<0.05$ (*). Values are means ± SEM and sample sizes are indicated in parentheses.

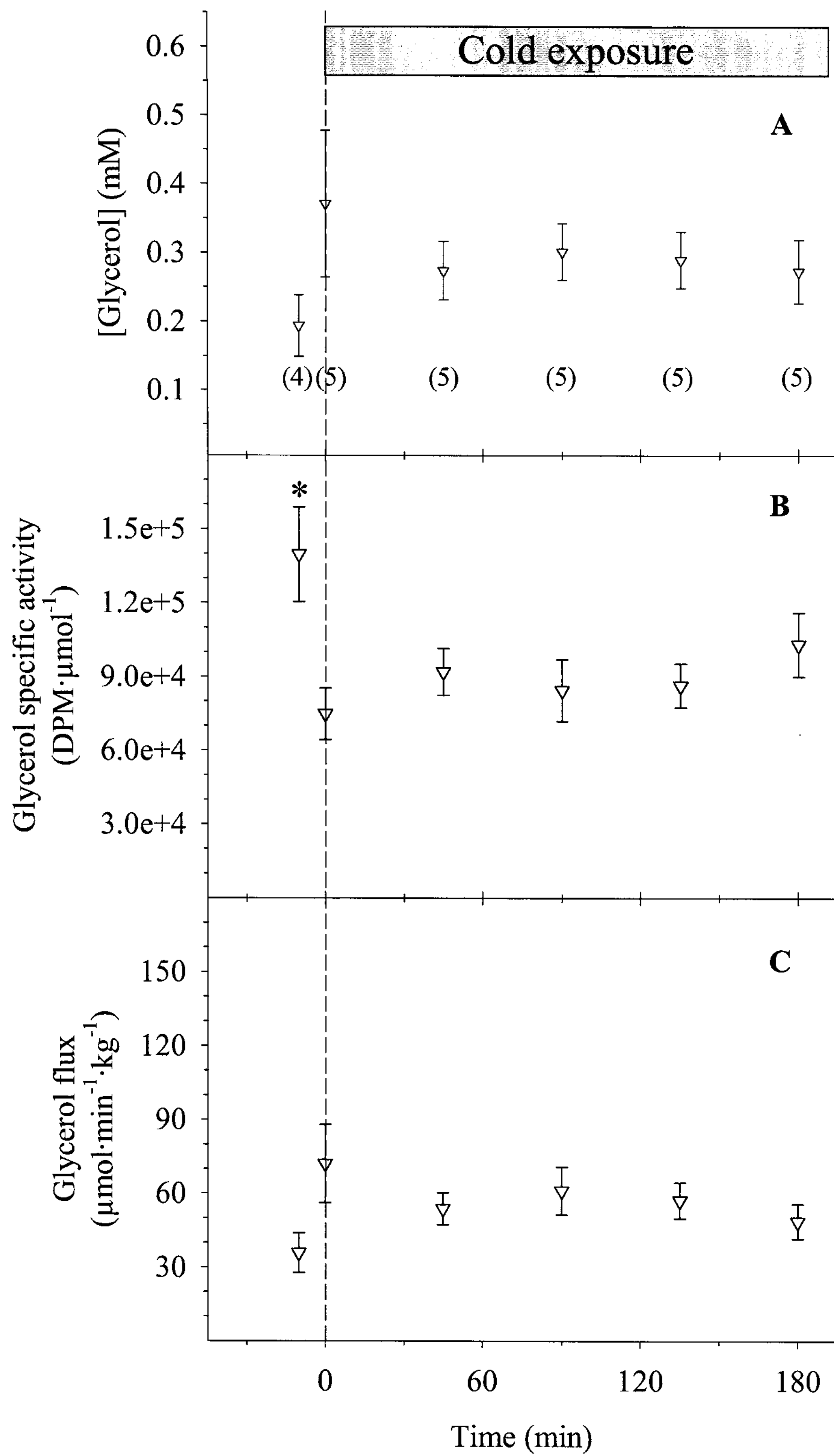
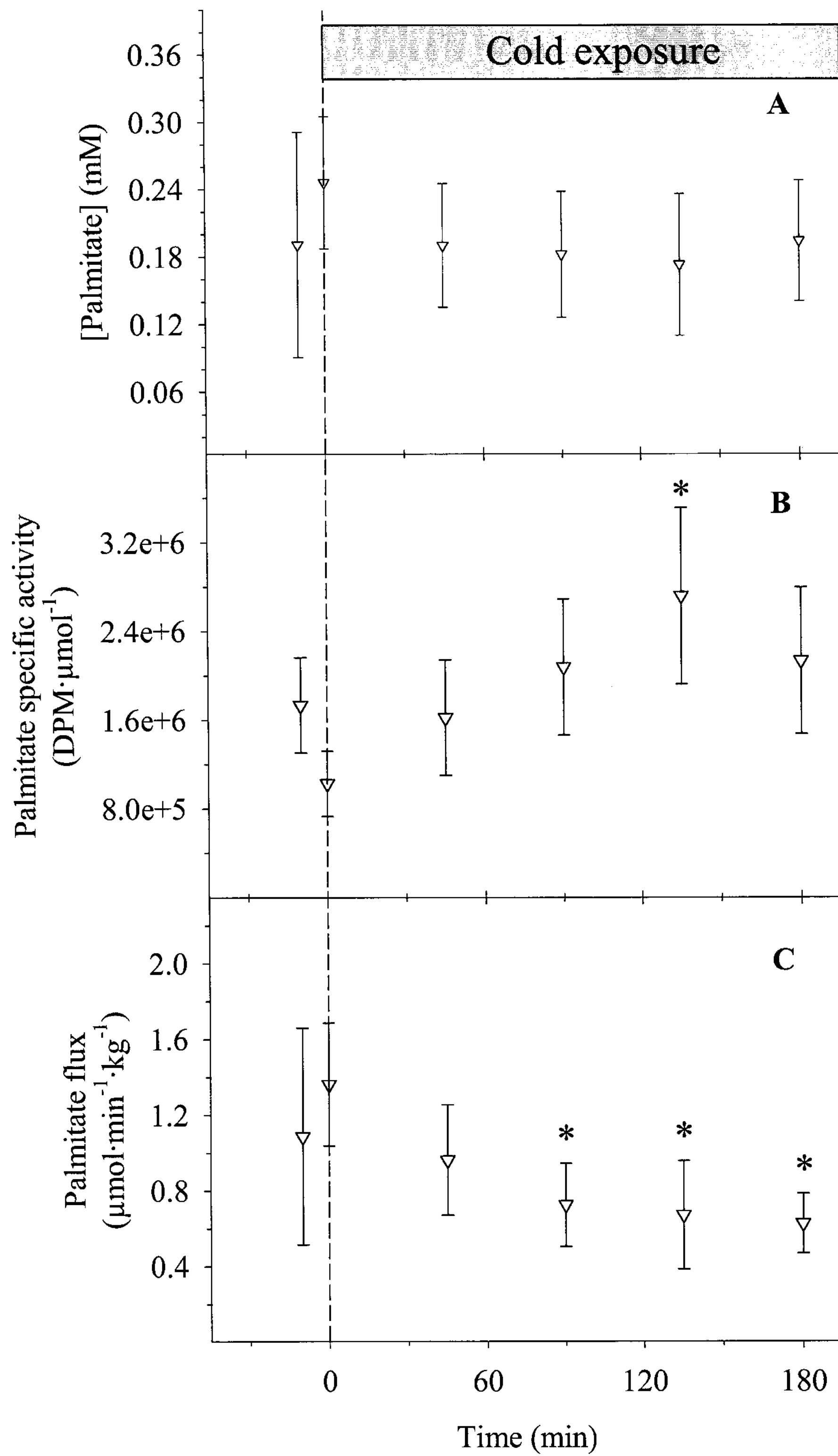

Cold exposure
A
B
C
[Glycerol] (mM)
0.6
0.5
0.4
0.3
0.2
0.1
(4) (5) (5) (5) (5) (5)
*
Glycerol specific activity (DPM·µmol-1)
1.5e+5
1.2e+5
9.0e+4
6.0e+4
3.0e+4
Glycerol flux (µmol·min-1·kg-1)
150
120
90
60
30
0
60
120
180
Time (min)

Figure 3.5. Palmitate concentration (A), specific activity (B) and flux (C) in ruff sandpipers before (22°C) and during cold exposure (5°C). Superscripts indicate significant differences from steady-state values at $P<0.05$ (*). Values are means ± SEM (sample sizes as in Fig. 3.4).



Cold exposure
A
B
C
[Palmitate] (mM)
0.36
0.30
0.24
0.18
0.12
0.06
Palmitate specific activity
(DPM·µmol-1)
3.2e+6
2.4e+6
1.6e+6
8.0e+5
Palmitate flux
(µmol·min-1·kg-1)
2.0
1.6
1.2
0.8
0.4
0
60
120
180
Time (min)

Figure 3.6. Non-esterified fatty acid (NEFA) concentration (A) and flux (B) in ruff sandpipers before (22°C) and during cold exposure (5°C). Superscripts indicate significant differences from steady-state values at $P<0.05$ (*). Values are means ± SEM (sample sizes as in Fig. 3.4).

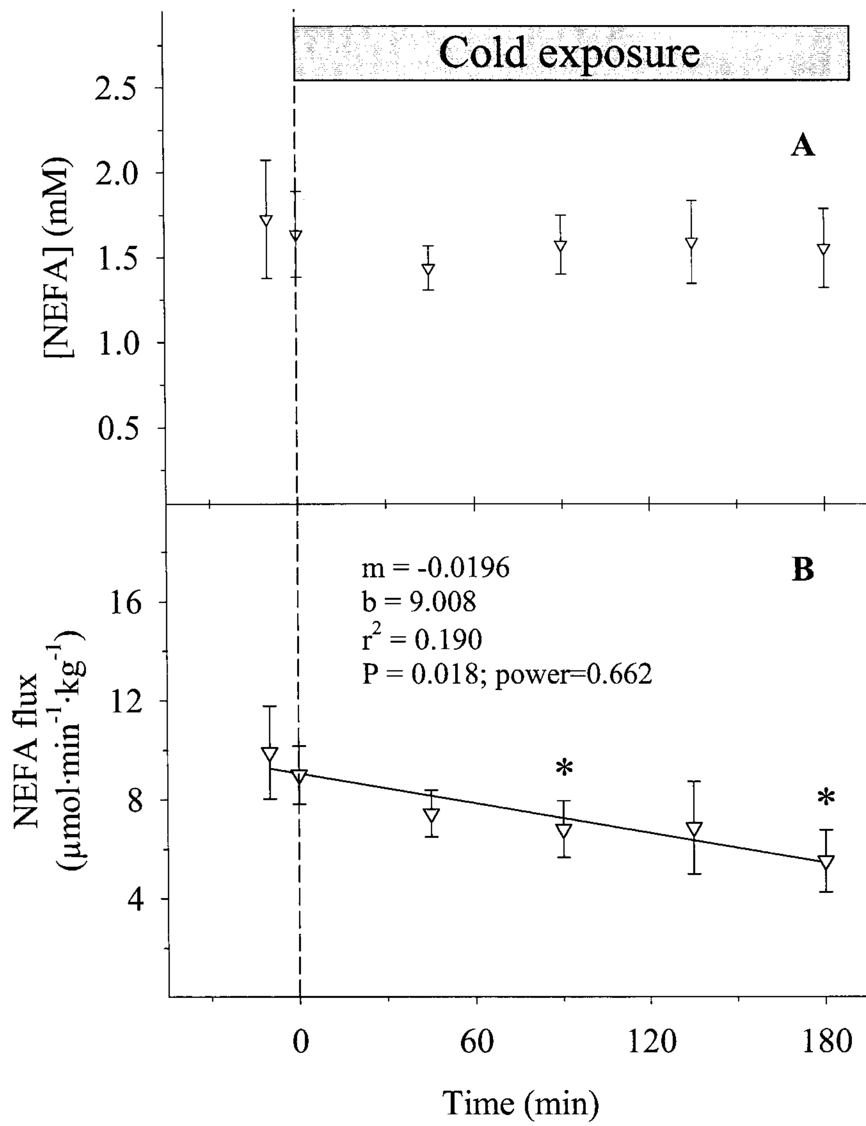

Cold exposure
A
B
[NEFA] (mM)
2.5
2.0
1.5
1.0
0.5
m = -0.0196
b = 9.008
r² = 0.190
P = 0.018; power=0.662
NEFA flux
(µmol·min⁻¹·kg⁻¹)
16
12
8
4
*
*
0
60
120
180
Time (min)

Table 3.1. Average plasma concentrations, specific activities and fluxes of glycerol and palmitate in adult European ruff sandpipers (*Philomachus pugnax*) exposed to 5°C.

[Glycerol] (mM)	0.288±0.044
Glycerol SA (DPM·μmol^{-1})	94,531±8,757
Glycerol flux (μmol·kg^{-1}·min^{-1})	56.2±8.1
[Palmitate] (mM)	0.199±0.059
Palmitate SA (DPM·μmol^{-1})	1,847,661±495,834
Palmitate flux (μmol·kg^{-1}·min^{-1})	0.91±0.27
% palmitate in NEFA	13.4±3.9
NEFA flux (μmol·kg^{-1}·min^{-1})	7.4±1.3
Fatty acid oxidation (μmol·kg^{-1}·min^{-1})	75.4±9.9
Total cycling (μmol·kg^{-1}·min^{-1})	93.3±18.3
Primary cycling (μmol·kg^{-1}·min^{-1})	161.2±25.1
Secondary cycling (μmol·kg^{-1}·min^{-1})	-67.9±10.2

Average fractional contribution of palmitate to non-esterified fatty acid (NEFA) fraction, NEFA flux, fatty acid oxidation, total cycling, primary cycling and secondary cycling are also given. Values are means ± S.E.M.

Table 3.2. Fractional contribution of individual non-esterified fatty acids (NEFA), neutral lipids (NL), and phospholipids (PL) to total plasma NEFA concentration in adult European ruff sandpipers (*Philomachus pugnax*) that did not have surgery, or were used for a continuous infusion during cold exposure.

	Without surgery (11)			With surgery and infusion (5)		
	NEFA	NL	PL	NEFA	NL	PL
16:0	14.5 ± 3.0	14.4 ± 0.8	24.6 ± 0.7	13.2 ± 3.9	8.9 ± 2.4	18.9 ± 1.5†
16:1	2.2 ± 0.6	2.4 ± 0.2	ta	2.2 ± 0.8	1.8 ± 0.7	ta
18:0	18.5 ± 1.6	16.6 ± 0.8	19.7 ± 0.4	18.7 ± 2.3	10.9 ± 1.8†	25.5 ± 1.3‡
18:1	23.5 ± 1.5	26.8 ± 1.4	14.4 ± 0.5	34.2 ± 3.1†	21.9 ± 1.3	11.0 ± 0.4†
18:2	11.8 ± 0.8	8.2 ± 0.4	7.3 ± 0.3	9.5 ± 0.5	7.8 ± 0.9	7.6 ± 0.4
18:3	1.0 ± 0.3	ta	ta	1.0 ± 0.2	ta	ta
20:0	0.7 ± 0.1	0.8 ± 0.1	ta	1.5 ± 0.4	1.0 ± 0.3	ta
20:1	0.8 ± 0.2	0.7 ± 0.0	ta	2.8 ± 0.4‡	1.8 ± 0.2†	ta
20:4	3.3 ± 0.4	2.7 ± 0.1	10.5 ± 0.5	2.2 ± 0.3	2.6 ± 0.2	14.4 ± 1.5†
20:5	6.2 ± 0.8	8.3 ± 0.8	7.7 ± 0.5	1.4 ± 0.3‡	3.2 ± 0.4‡	2.8 ± 0.3‡
22:0	ta	1.7 ± 0.1	ta	ta	1.4 ± 0.2	ta
22:1	nd	4.4 ± 0.5	nd	nd	27.3 ± 6.4‡	nd
22:5	2.2 ± 0.3	1.4 ± 0.1	2.4 ± 0.1	2.9 ± 0.6	1.7 ± 0.1*	2.8 ± 0.5
22:6	11.6 ± 1.6	6.6 ± 0.3	10.1 ± 0.4	8.4 ± 1.9	5.5 ± 0.2	14.0 ± 1.4†
24:0	ta	2.7 ± 0.1	ta	ta	2.1 ± 0.2*	ta
Others	3.7 ± 0.3	2.3 ± 0.1	3.3 ± 0.1	2.0 ± 0.5*	2.1 ± 0.1	3.0 ± 0.3
Total [ ]	956 ± 75	727 ± 62	11,435 ± 1,089	1,604 ± 218†	1,041 ± 286	8,068 ± 621

* $P<0.05$; † $P<0.01$; ‡ $P<0.001$; nd = not detected; ta = trace amounts. Values are means ± S.E.M; sample sizes in parentheses.

For NEFA, "Others" includes trace amounts of 14:0, 20:2, 20:3, 22:0, 22:3, and 24:0. For NL, "Others" includes trace amounts of 18:3, 20:2, 20:3, and 22:3. For PL, Others" includes trace amounts of 14:0, 16:1, 18:3, 20:0, 20:1, 20:2, 22:0, 22:3, and 24:0. All values are expressed as a percent of total NEFA, except for "Total [ ]" which is expressed as nmol NEFA·ml^{-1} plasma.

Table 3.3. Fractional contribution of individual non-esterified fatty acids (NEFA) to total plasma NEFA concentration in cold-exposed adult European ruff sandpipers (*Philomachus pugnax*) at various times during a continuous infusion.

	22°C		5°C			
NEFA	t = -10	t = 0 (control)	t = 45	t = 90	t = 135	t = 180
16:0	10.4 ± 4.3	15.2 ± 3.7	13.4 ± 4.0	12.5 ± 4.3	11.7 ± 4.2	13.1 ± 3.3
16:1	1.7 ± 0.8	2.6 ± 0.8	2.1 ± 0.8	2.0 ± 0.8	2.0 ± 0.8	2.0 ± 0.8
18:0	20.7 ± 2.5	18.0 ± 2.4	19.3 ± 2.9	19.0 ± 2.6	19.1 ± 2.4	17.3 ± 1.7
18:1	35.5 ± 3.6	35.0 ± 3.1	34.4 ± 3.3	34.2 ± 3.5	33.3 ± 3.7	34.6 ± 2.4
18:2	8.8 ± 0.5	10.1 ± 0.6	9.7 ± 0.5	9.4 ± 0.6	9.1 ± 0.8	9.8 ± 0.5
18:3[§]	0.5 ± 0.2	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.2	1.1 ± 0.2	1.3 ± 0.4
20:0	1.9 ± 0.6	1.1 ± 0.8	1.0 ± 0.3	1.6 ± 0.4	2.1 ± 1.1	1.3 ± 0.4
20:1	2.9 ± 0.6	2.6 ± 0.5	2.5 ± 0.4	2.9 ± 0.6	2.9 ± 0.5	3.2 ± 0.5
20:4	2.4 ± 0.2	1.9 ± 0.1	2.1 ± 0.3	2.1 ± 0.3	2.3 ± 0.4	2.5 ± 0.4
20:5	1.4 ± 0.5	1.3 ± 0.2	1.5 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.2
22:5	2.5 ± 0.8	2.6 ± 0.7	3.0 ± 0.7	3.3 ± 0.8	3.3 ± 0.8	2.8 ± 0.5
22:6	9.0 ± 2.4	7.1 ± 1.3	8.3 ± 2.0	8.6 ± 2.3	9.4 ± 2.5	8.4 ± 2.0
Others	2.3 ± 0.7	1.7 ± 0.5	1.8 ± 0.4	2.2 ± 0.6	2.3 ± 0.6	2.4 ± 0.5
Total [ ]	1,764 ± 350	1,667 ± 255	1,466 ± 132	1,614 ± 179	1,631 ± 249	1,596 ± 243

[§] Indicates a significant increase in the relative contribution of 18:3 to total plasma NEFA concentration, determined using a linear regression (P=0.045). Times (t) are expressed in minutes. Values are means ± S.E.M; N=5 at every time, except at t= -10 min where N=4). "Others" includes trace amounts of 14:0, 20:2, 20:3, 22:0, 22:3, and 24:0. All values are expressed as a percent of total NEFA, except for "Total [ ]" which is expressed as nmol NEFA·ml^{-1} plasma.

Table 3.4. Fractional contribution of individual neutral lipids (NL) to total plasma non-esterified fatty acid concentration in cold-exposed adult European ruff sandpipers (*Philomachus pugnax*) at various times during a continuous infusion.

NL	22°C		5°C			
	t = -10	t = 0 (control)	t = 45	t = 90	t = 135	t = 180
16:0	7.6 ± 2.4	9.6 ± 2.6	10.1 ± 2.5	8.1 ± 2.9	6.8 ± 1.9*	9.5 ± 2.5
16:1	1.5 ± 0.6	1.6 ± 0.5	1.7 ± 0.4	3.0 ± 1.9	1.3 ± 0.4	1.4 ± 0.4
18:0	10.0 ± 2.2	11.1 ± 2.1	9.5 ± 1.2	11.1 ± 2.3	11.9 ± 2.3	10.5 ± 1.4
18:1	21.7 ± 0.3	22.5 ± 1.1	23.7 ± 2.0	18.2 ± 2.1	20.5 ± 1.2	24.6 ± 2.8
18:2	7.3 ± 0.4	8.0 ± 0.6	9.1 ± 1.5	6.2 ± 0.7	6.8 ± 0.8	8.8 ± 1.6
20:0	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	2.4 ± 1.7*	0.9 ± 0.1	0.6 ± 0.1
20:1	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	2.2 ± 0.3	1.9 ± 0.1	1.7 ± 0.4
20:4	3.1 ± 0.6	3.0 ± 0.4	2.9 ± 0.2	2.2 ± 0.3	2.4 ± 0.1	2.3 ± 0.6
20:5	3.5 ± 0.5	3.4 ± 0.4	3.6 ± 0.5	2.7 ± 0.4	3.0 ± 0.4	3.2 ± 0.5
22:0	1.6 ± 0.2	1.1 ± 0.1	1.1 ± 0.3	1.2 ± 0.2	1.8 ± 0.2	1.7 ± 0.7
22:1	30.8 ± 4.5	26.4 ± 5.5	25.1 ± 7.2	29.7 ± 6.8	31.8 ± 6.0	24.3 ± 8.7
22:5§	1.2 ± 0.1	1.4 ± 0.1	1.6 ± 0.2	2.2 ± 0.5	1.6 ± 0.1	2.0 ± 0.3
22:6	5.3 ± 0.2	5.8 ± 0.1	5.9 ± 0.5	5.0 ± 0.4	4.9 ± 0.1	6.1 ± 0.8
24:0	2.1 ± 0.3	2.1 ± 0.3	1.7 ± 0.1	2.7 ± 0.7	2.4 ± 0.3	1.4 ± 0.4
Others	1.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.0	2.9 ± 0.7	2.0 ± 0.2	1.9 ± 0.2
Total [ ]	1,003 ± 279	904 ± 240	1,072 ± 243	1,098 ± 453	1,040 ± 336	1,220 ± 272

* $P<0.05$. § Indicates a significant increase in the relative contribution of 22:5 to total plasma NEFA concentration, determined using a linear regression ($P=0.019$). Times (t) are expressed in minutes. Values are means ± S.E.M; N=5 at every time, except at t= -10 min where N=4). "Others" includes trace amounts of 18:3, 20:2, 20:3, and 22:3. All values are expressed as a percent of total NEFA, except for "Total [ ]" which is expressed as nmol NEFA·ml^{-1} plasma.

Table 3.5. Fractional contribution of individual phospholipids (PL) to total plasma non-esterified fatty acid concentration in cold-exposed adult European ruff sandpipers (*Philomachus pugnax*) at various times during a continuous infusion.

	22°C		5°C			
PL	t = -10	t = 0 (control)	t = 45	t = 90	t = 135	t = 180
16:0	20.1 ± 1.4	19.7 ± 1.8	17.9 ± 1.5	19.6 ± 1.9	17.8 ± 1.8	18.8 ± 1.5
18:0	26.0 ± 1.7	26.0 ± 1.9	24.5 ± 1.0	25.7 ± 1.5	25.7 ± 1.3	25.4 ± 1.3
18:1§	10.6 ± 0.5	10.4 ± 0.5	11.0 ± 0.2	11.0 ± 0.5*	11.3 ± 0.4†	11.5 ± 0.4‡
18:2	7.2 ± 0.4	7.2 ± 0.5	7.6 ± 0.2	7.6 ± 0.4	7.8 ± 0.3*	7.9 ± 0.4†
20:4	14.9 ± 1.8	14.1 ± 1.5	15.0 ± 1.6	14.1 ± 1.6	14.5 ± 1.5	14.1 ± 1.4
20:5	2.6 ± 0.5	2.8 ± 0.4	2.9 ± 0.3	2.8 ± 0.3	2.8 ± 0.3	2.7 ± 0.3
22:5	2.5 ± 0.6	2.9 ± 0.5	3.0 ± 0.5	2.7 ± 0.4	2.9 ± 0.5	2.8 ± 0.4
22:6	13.3 ± 1.5	13.9 ± 1.9	15.1 ± 1.4	13.6 ± 1.4	14.2 ± 1.3	13.6 ± 1.5
Others	2.8 ± 0.3	3.0 ± 0.3	3.0 ± 0.2	2.9 ± 0.2	3.0 ± 0.3	3.2 ± 0.3
Total [ ]	8,905 ± 874	8,142 ± 664	8,030 ± 508	7,617 ± 604	7,991 ± 706	8,093 ± 728

* $P<0.05$; † $P<0.01$; ‡ $P<0.001$. § Indicates a significant increase in the relative contribution of 18:1 to total plasma NEFA concentration, determined using a linear regression ($P=0.048$). Times (t) are expressed in minutes. Values are means ± S.E.M; $N=5$ at every time, except at t= -10 min where $N=4$). Others" includes trace amounts of 14:0, 16:1, 18:3, 20:0, 20:1, 20:2, 22:0, 22:3, and 24:0. All values are expressed as a percent of total NEFA, except for "Total [ ]" which is expressed as nmol $NEFA \cdot ml^{-1}$ plasma.

Discussion

The aim of this chapter was to quantify the rate of lipolysis in resting and cold-exposed long-distance migrant shorebirds. In addition, I tried to determine the relative contribution of the TAG:FA cycle to total heat production, but this attempt was unsuccessful. One possible reason for this may be that the equations developed for mammals do not apply to migrant birds. These equations assume that lipid oxidation is accounted for by NEFA oxidation; however, it is possible that birds may transport their lipids from storage sites to working muscles as lipoproteins instead of NEFA (Jenni-Eiermann and Jenni, 1992). If this is the case, the mammalian equations can no longer be used for calculating TAG:FA cycling, and revised (bird) equations have not yet been developed. The effect of surgery on the fatty acid profile of plasma lipid fractions was also investigated. Results show that the rate of lipolysis of resting ruff sandpipers is higher than in all other avian and mammalian species measured to date, and that shivering has no effect on the rate of lipolysis. Results also suggest that ruff sandpipers may be sensitive to the small amounts of heparin used to keep catheters patent, and that the use of a different anticoagulant (e.g. citrate) may be required in future studies of lipid metabolism in birds.

Glycerol and NEFA kinetics

In Chapter 2, it was determined that lipids are the main metabolic fuel used for thermogenesis. To quantify lipolysis, continuous infusions of 2-[^{3}H]-glycerol were

performed in cold-exposed ruff sandpipers. 1-[^{14}C]-palmitate was also infused to try measuring TAG:FA cycling. The negative values obtained for secondary cycling are somewhat surprising, and indicate that the mammalian equations used to calculate cycling do not allow testing Hypotheses III and IV. It is important to keep in mind that to obtain lipid kinetics data in 5 birds, the difficult, time-consuming surgical technique necessary for catheterization had to be performed on 20 animals. The surgeries are difficult because these birds are very sensitive to anaesthetic overdose and to blood loss while under anaesthesia. In addition, keeping catheters patent is a major challenge because blood clots are difficult to avoid and the blood vessels of these birds tend to collapse easily. Although many animals were used for surgery, all experimental protocols (including cold exposure and surgical procedures) were approved by the Animal Care Committee of the University of Ottawa in accordance with guidelines provided by the Canadian Council for Animal Care. Also, a veterinarian assisted to the surgeries and insured that all approved protocols were followed.

In many avian studies, changes in the concentration of glycerol and NEFA have been reported under various conditions, but conclusions about fluxes derived from concentration changes are misleading (Haman et al., 1997). Therefore, it is better (although more difficult) to measure metabolite fluxes (or turnover rates) rather than metabolite concentrations. The rate of lipolysis (glycerol flux) has previously been measured only in penguins. Glycerol fluxes in resting ruff sandpipers are greater than the glycerol fluxes measured on three occasions in resting penguins (Bernard et al., 2002a; 2002b; 2003). Because no other values were available in birds, mammalian data were used here for comparison. The rat is the only mammal which has a size similar to my

birds, and for which the rates of lipolysis were measured. The glycerol fluxes in the ruff sandpipers used here are 1.9- to 3.5-fold those of resting rats (Kalderon et al., 2000; McClelland et al., 2001). Similarly, the resting rates of lipolysis in ruff sandpipers are higher than for rabbits [5.6-fold; (Reidy and Weber, 2002)], pigmy goats [15-fold; (Weber et al., 1993)], and humans [22- to 32-fold; (Elia et al., 1987; Friedlander et al., 1999; Mora-Rodriguez et al., 2001; Vallerand et al., 1999; Wolfe et al., 1990)]. From these observations it is obvious that migrant birds have the capacity to mobilize fatty acids at a greater rate than mammals. However, resting lipolytic rates are sufficient to provide enough lipid fuel for shivering, and cold exposure has no effect on glycerol flux; thus Hypothesis II (lipolysis increases in parallel to metabolic rate in cold-exposed migrant birds) must be rejected.

Contrary to expectations, cold exposure decreases the NEFA turnover rate of ruff sandpipers (Fig. 3.6B). The slope of the regression line between NEFA flux and time was significantly lower than zero (Fig. 3.6B). As for glycerol turnover rates, avian NEFA fluxes have been measured only in fasting penguins. It was observed that during prolonged fasting, glucagon infusion and mercaptoacetate infusion, NEFA turnover rates increase in king penguins (Bernard et al., 2002a; 2002b; 2003). Only one study has looked at NEFA turnover rates in cold-exposed mammals, and it reveals that NEFA fluxes are stimulated under these conditions (Vallerand et al., 1999). Although exercise had no effect on NEFA fluxes in goats (Weber et al., 1996) and in one rat study (McClelland et al., 2001), NEFA turnover rate was increased in exercising dogs (Weber et al., 1996) and humans (Friedlander et al., 1999; Wolfe et al., 1990). At least two other physiological stresses are known to stimulate NEFA turnover: a second rat study shows

that NEFA fluxes increase during thyroid hormone-induced calorigenesis (Kalderon et al., 2000) and in leptin-injected rabbits (Reidy and Weber, 2002). To my knowledge, the decreased NEFA turnover observed in this chapter in cold-exposed ruff sandpipers is the only decrease ever observed for any endotherm under any stressful situation. Together with the increased fatty acid oxidation (Fig. 3.2), this reduced NEFA turnover suggests that the amount of fatty acid mobilized decreases during cold exposure, although fatty acid oxidation is shown to increase. This situation could be explained by changes in TAG:FA cycling or if birds rely predominantly on lipoproteins to shuttle energy between TAG stores and shivering muscles.

The first mechanism requires that TAG undergo lipolysis to release glycerol and NEFA. Once into the circulation, NEFA can be carried by albumin to the shivering muscle where they are oxidized. If a decrease in NEFA turnover occurs, then shivering can compensate for this decrease by increasing the rate of intramuscular (or endogenous) lipid oxidation. In the muscle, if the TAG is not completely oxidized, glycerol may be released into the bloodstream, which would give the impression that lipolysis is maintained together with glycerol mobilization. Even though this is a possibility, the mechanism would probably be sustained for a short period during low intensity shivering, after which intramuscular lipid reserves would be depleted quickly. Therefore, a second mechanism, more probable and more sustainable, may be used by ruff sandpipers. Instead of being transported to the shivering muscles, NEFA-albumin can be sent to the liver and re-esterified to TAG using circulating glycerol. The newly formed TAG can be sent to the circulation as VLDL-TAG (very low density lipoprotein-triacylglycerol), and transported to the shivering muscles to be oxidized. Because the number of albumin

molecules is limited and that each molecule has a limited number of binding sites, it is possible that the use of the first mechanism alone may not be sufficient to deliver fatty acids to the highly active muscles of a bird at flight. Therefore, VLDL-TAG may be used alone or as a complement allowing for a sufficient fatty acid delivery to the muscles of these endurance athletes. This mechanism has been proposed over a decade ago, but there was little supporting evidence (Jenni-Eiermann and Jenni, 1992). In addition to being oxidized, VLDL-TAG can go back to the adipocytes to be hydrolyzed to TAG by lipoprotein lipase; converting TAG to NEFA and glycerol, then to VLDL-TAG, then back to TAG could also simply be an extended, more complicated (and possibly more energetically costly) version of the well-known secondary cycling that can contribute to non-shivering thermogenesis through TAG:FA cycling (see Appendix 2). To test this possibility, new equations need to be developed, which would incorporate new parameters (e.g. VLDL-TAG flux, VLDL-TAG oxidation, VLDL-TAG uptake by adipocytes, and VLDL-TAG conversion to TAG by lipoprotein lipase) to the existing equations. However, the tools required to accurately quantify such parameters are not yet available.

TAG:FA cycling

In this chapter, glycerol flux, NEFA flux and fatty acid oxidation were used to determine the importance of TAG:FA to total heat production in cold-exposed ruff sandpipers. The values obtained for total, primary and secondary cycling did not change over time, and were averaged in Table 3.1., but cannot be used with confidence because 1) the calculated value for primary cycling is much greater than the value for total cycling

and 2) secondary cycling yields a negative value (Table 3.1). Therefore, I cannot calculate the proportion of heat production that can be accounted for by TAG:FA cycling in cold-exposed ruff sandpipers, and I cannot test Hypotheses III and IV (Hypothesis III: TAG:FA cycling increases during cold exposure in migrant birds; Hypothesis IV: the increase in total TAG:FA cycling is mostly caused by an increase in secondary cycling in cold-exposed migrant birds). The reason why secondary cycling value is negative could be either that NEFA flux was underestimated, or that fatty acid oxidation was overestimated. Because the calculations of TAG:FA cycling assume that all the lipids oxidized are non-esterified fatty acids, then if a different source of lipids is oxidized (for example, VLDL-TAG), the fatty acid oxidation value calculated from lipid oxidation will be overestimated, because it will encompass this other lipid source. This overestimation of the fatty acid oxidation rate may explain why secondary cycling values were negative, and would affect the values for total cycling as their calculation also relies on the fatty acid oxidation rate.

In this study, total cycling did not change, but what are the observations made in studies were total cycling does change under a physiological stress? The only studies that looked at TAG:FA cycling in birds show results only for primary cycling; secondary cycling and total cycling could not be quantified because indirect calorimetry was not used to determine fatty acid oxidation rate (Bernard et al., 2002a; 2003). One study in humans showed that secondary cycling is responsible for the increased total cycling observed during cold exposure. In contrast, many studies in mammalian models show differences or trends that indicate that the increase in total cycling is mainly due to an increase in primary cycling. These studies were performed in high-altitude acclimated

rats (McClelland et al., 2001), in thyroid hormone-treated rats (Kalderon et al., 2000), in leptin-injected rabbits (Reidy and Weber, 2002), and in exercising humans (Wolfe et al., 1990). Because of the lack of confidence I have towards my TAG:FA cycling data, it is impossible to test the hypotheses that TAG:FA cycling increases in cold-exposed migrant birds, or that the increase in total TAG:FA cycling is mostly caused by an increase in secondary cycling.

Composition of lipid fractions

The surgical procedure performed to implant the catheters seems to affect at least one of the plasma lipid fractions. While total plasma NL and PL concentrations are not different between birds that had surgery and birds that did not undergo surgery, total plasma NEFA concentration increased 1.7-fold in birds used for the continuous infusions of labelled glycerol and palmitate (Table 3.2), and I must reject the hypothesis that surgery (and probably heparin) does not affect the fatty acid concentration of the plasma lipid fractions in migrant birds. This increase in NEFA concentration may be due to stress, but is more likely an effect of the lipolysis-stimulating heparin used to keep the catheter patent. While flushing the catheters, all efforts were made to minimize the amount of heparinized saline injected into the animal's bloodstream. However, even though it was possible to withdraw and discard all the heparinized saline from the carotid artery catheter, it was often very difficult to remove even a small fraction of the saline contained in the jugular vein catheter. To keep both lines in working condition, the heparinized saline was changed in the carotid catheter, but more saline sometimes had to be forced inside the jugular catheter to remove an obtrusive blood clot and maintain the

catheter patency. The amount of heparin that was injected in the bird's bloodstream, as minimal as it may have been, could have caused an increase in the rate of lipolysis, thus increasing the plasmatic concentrations of NEFA and glycerol. This possible effect of heparin may explain why glycerol concentration at the onset of cold exposure (t=0 min) tended to be higher than for t= -10 min. During the infusion, a more diluted solution of heparinized saline (10U heparin ml^{-1} saline, or half the concentration used to maintain catheter patency after the surgery) was used to keep the catheters patent until the next blood sampling, and it seems that the delay of ten min between the blood samples taken at t= -10 min and t=0 min was not sufficient the allow the rate of lipolysis to return to its baseline level. However, this trend is less evident for each subsequent blood sampling (t≥45 min), where 45 min could allow the rate of lipolysis to go back to baseline levels (Fig. 3.4A).

In general, the most abundant fatty acids in the NEFA, NL and PL fractions were 16:0, 18:0 and 18:1, although 18:2, 20:4 and 22:6 were also important in all those fractions (Table 3.2). To obtain the same basic fatty acid profile observed in western sandpipers by Guglielmo et al. (2002b), only 18:2 needs to be substituted by 20:5. The most extreme variation of the relative importance of a specific fatty acid to a lipid fraction is observed for 22:1. Therefore, it can be concluded that surgery (and possibly heparin) affects the fatty acid profile of plasma lipid fractions in migrant birds. Dietary factors can probably not explain this enormous 6.2-fold increase in the relative importance of 22:1 to total plasma NEFA in the NL fraction, because all the ruffs used in this thesis were fed the same diet. However, 22:1 is part of the omega-9 (n-9) metabolic pathway generating long-chain, monounsaturated fatty acids, and the n-9 pathway is diet-

independent. Although it is improbable because of the quality control measures in place in most companies, it is possible that the 22:1 was obtained directly *via* the diet. Although the percentage of lipids contained in the food should be similar between food lots, it is possible that the fatty acid profile of the food may have been different for the food lots given to the birds that did not undergo surgery and the food lots given to the birds used during the continuous infusion. There was a long period of time separating both experiments, and different ingredients may have been used to prepare the various food lots. If one food lot was prepared using sardine [sum of 20:1 and 22:1 = 7% of total fatty acid in the flesh (Gurr et al., 2002)], and another with cod, mackerel or herring [sum of 20:1 and 22:1 = 25%, 26% and 39% of total fatty acid in the flesh, respectively (Gurr et al., 2002)], then ruffs fed with the latter diet would most likely accumulate a similar proportion of 22:1 (and 20:1) in their lipid reserves. Interestingly, even though 20:1 was found only in small amounts in all lipid fractions of the birds that did not undergo surgery, its relative importance to total NEFA was higher in the plasma NEFA and NL fractions of the birds used during continuous infusions, although its relative importance remained below 3% (Table 3.2).

Conclusions

Lipids are the main metabolic fuel contributing to shivering thermogenesis in cold-exposed ruff sandpipers. Although lipid oxidation increases during cold exposure, lipolysis does not need to be increased to allow sufficient fatty acid mobilization. Together with the increased lipid oxidation observed during cold exposure, the decrease in NEFA turnover rate suggests migrant birds may not simply oxidize NEFA, but may

prefer to oxidize VLDL-TAG, or to use both mechanisms to allow their muscles to use lipids at extremely high rates during flight. Although I could no test my hypotheses, a more productive path may be to establish whether VLDL-TAG can be used as a fuel by long-distance migrant birds.

CHAPTER 4 – GENERAL CONCLUSION

The main goals of my thesis were 1) to determine the relative contribution of the various metabolic fuels to metabolic rate in cold-exposed and exercising ruff sandpipers, 2) to quantify the rate of lipolysis of ruff sandpipers during cold exposure, and 3) to attempt to determine the importance of TAG:FA cycling to support thermogenesis in cold-exposed ruff sandpipers. The first assignment was to determine the relative contribution of lipids, carbohydrates and proteins to total energy expenditures during cold exposure and running. Oxygen consumption, carbon dioxide production and nitrogen excretion were measured in cold-exposed or exercising ruffs (Chapter 2). The second task was to quantify lipolysis in resting and cold-exposed ruff sandpipers. In addition, I attempted to determine the relative importance of TAG:FA cycling for heat production in cold-exposed ruff sandpipers, and to determine whether surgery has an effect on the fatty acid profile of the plasma lipid fractions. To do so, the birds were subjected to a double catheterization allowing radiolabelled glycerol and palmitate to be continuously infused, blood samples to be taken at regular time intervals, and oxygen consumption and carbon dioxide production were measured (Chapter 3). The following conclusions can be drawn from my thesis:

1. Pattern of fuel selection during shivering and running:

At all times, temperatures and running speeds, cold exposure and exercise were mainly fueled by lipids, the most abundant substrate available. During shivering, heat production was derived mainly from lipids (82% of $\dot{V}_{O_2}$), whereas carbohydrates (12% of $\dot{V}_{O_2}$) and proteins (6% of $\dot{V}_{O_2}$) played much minor roles. During running, lipids were

also the preferred substrate (66% of $\dot{V}_{O_2}$), while carbohydrates (29% of $\dot{V}_{O_2}$) and proteins (5% of $\dot{V}_{O_2}$) were less important. This high reliance on lipids is not consistent with the pattern of fuel selection seen in cold-exposed and exercising mammals. This work allows concluding that the pattern of fuel selection of this long-distance migrant shorebird is different from the well known pattern of selection reported for mammals.

2. Lipolysis:

Although lipid oxidation increases during cold exposure, glycerol flux is unaffected by shivering at the temperature tested in this chapter. These observations suggest that ruff sandpipers have resting lipolytic rates that are sufficient to provide lipid fuel for prolonged shivering at environmental temperatures as low as 5°C.

3. Triacylglycerol:fatty acid cycling:

This cycle could not be quantified here, but results suggest that birds may use an interesting strategy to shuttle energy to their muscles. Even though the data obtained does not allow me to test my hypotheses, one finding is really interesting. The ruff sandpiper may be able to use endogenous (intramuscular) lipid reserves to power muscle contractions, but these small reserves are probably rapidly depleted within a short period of time, especially during long flights. Therefore, this species may also have the capacity to use a secondary mechanism that allows carrying lipids from adipocytes to working muscles. Because plasma albumin is present in finite amounts, and because each albumin molecule can only bind a limited number of fatty acids, hepatic VLDL-TAG may be a good mechanism insuring that the working muscles do not run out of fuel. Although the

surgeries are difficult to perform on this fragile species, future research on the energetic metabolism of these birds should focus on the VLDL-TAG as a source of energy during flight.

4. Effect of surgery:

Even though cold exposure does not affect lipolysis, surgery (and possibly the small amounts of heparin used during and after surgery) seems to increase NEFA concentration compared to birds that do not undergo surgery. Surgery also affects the fatty acid profile of plasma lipid fractions. In general, the main fatty acids are the same without and with surgery, but a large increase in 22:1 was detected in the NL fraction. Ruff sandpipers seem to be very sensitive to anaesthetic overdose and to blood losses when they are anaesthetized, and many animals were lost under anaesthesia and during recovery. It is imperative to minimize the amount of anaesthetic used to maintain the surgical plane, to minimize blood loss, and to flush the endotracheal intubation apparatus at regular intervals to prevent mucus buildup at the tip of the intubation apparatus. During surgery, it may be observed that some birds have fragile blood vessels; it would be a good idea to discard these birds, because their vessels could be punctured by the catheters (even if the catheter tip is blunt), and the bird could die of a hemorrhage. The female ruff sandpipers (80-100 g) are a little too small, and consequently have a small blood volume allowing only a small number of small blood samples. The male ruff sandpipers (150-180 g) have the ideal size and blood volume, but are not anaesthesia-tolerant, and they are difficult to obtain is large numbers as they are often a minority within a mostly female population. Given that blood volume is the limiting factor in

these experiments, it would be good to either develop analytical techniques that require smaller blood volumes, or to find a long-distance migrant species that is slightly bigger and, thus, has a larger blood volume.

In this thesis, I showed for the first time that the pattern of fuel selection of ruff sandpipers is unaffected by exposure to temperatures between 5-22°C or by running at speeds up to 40 $m \cdot min^{-1}$. I also performed the first measurements of lipolysis of a long-distance migrant bird, and results show that these birds have lipolytic rates that are higher than those previously measured in penguins and mammals. Finally, the observations seem to support the idea that migrant birds may use lipoproteins (instead of NEFA) to mobilize their lipid reserves.

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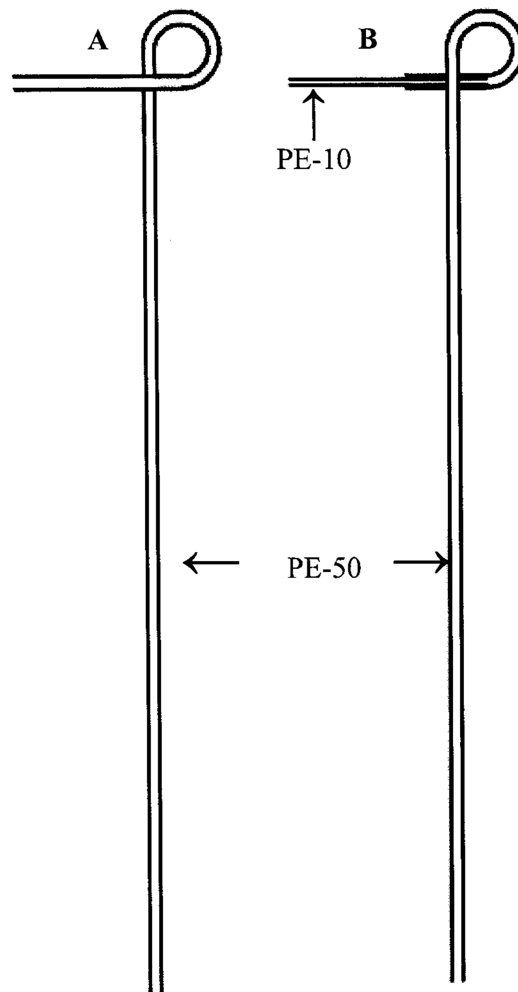
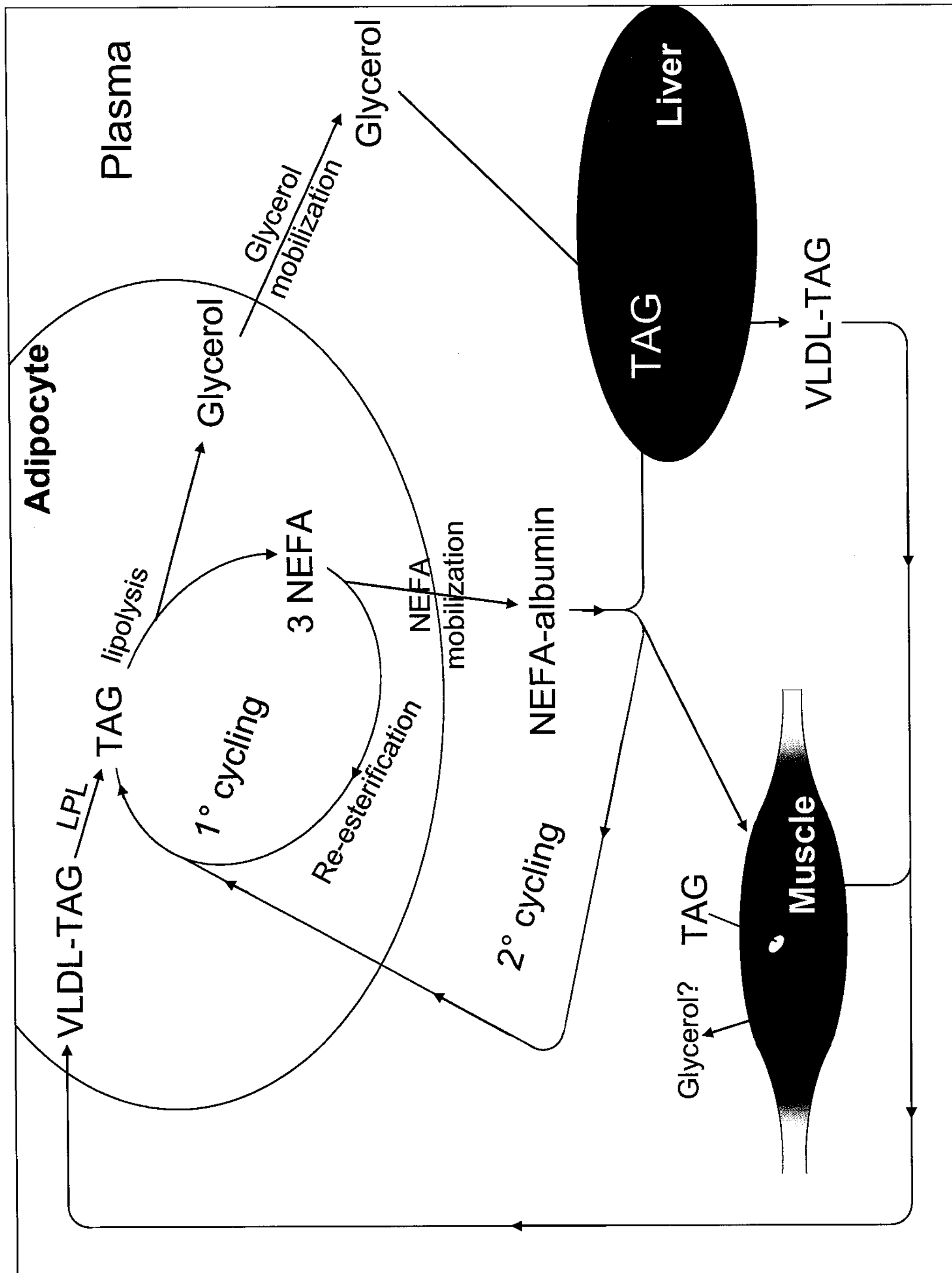
APPENDICES

Appendix 1. Catheters used for venous (A; right jugular vein) and arterial catheterization (B; left carotid artery). The venous catheter was made entirely using PE-50 tubing. The arterial catheter was made using PE-50 and a piece of PE-10. Note that one catheter loops over the longer length of PE-50, while the other catheter loops under; this facilitates the insertion into the vessels, and anchoring of the catheters.



Appendix 2. Diagram of an extended version of the TAG:FA cycling. This modified version combines the traditional mammalian TAG:FA cycling, and the VLDL-TAG pathway proposed by Jenni-Eiermann and Jenni (1992). Note that the shape, color, size and proportions of the various structures are not representative of *in vivo* structures.



Adapted from Jenni-Eiermann and Jenni (1992)