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THE EFFECTS OF DIETARY FATTY ACIDS ON AVIAN MIGRATORY PERFORMANCE

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ABSTRACT AND KEYWORDS:

Migratory birds use fat nearly exclusively to fuel their long distance movements. Little is known about how different types of fat affect their migratory performance, although dietary fats have been hypothesized to affect exercise through their roles as both oxidative substrates and membrane components. This thesis investigated the effects of fatty acids on avian flight performance and evaluated these two hypothesized mechanisms. I first investigated the selective mobilization of fatty acids from avian adipocytes. Unsaturated fatty acids and short chain fatty acids were most readily mobilized from avian adipocytes, indicating they may be important in increasing maximal metabolic rates. Further, this pattern did not change with migratory condition. I also investigated the effect of migratory condition and exercise on muscle phospholipid composition. Migratory disposition itself did not induce any endogenous changes to muscle phospholipid composition in preparation for migration, although exercise did have an effect on muscle phospholipids. Next, I demonstrated that birds fed a high ω6 fatty acid diet achieved higher peak metabolic rates than those fed a high ω3 diet. Further, I used a dietary manipulation to independently alter adipose triacylglycerol stores and muscle phospholipid composition. This experiment demonstrated that phospholipid composition does not drive exercise performance differences, but that triacylglycerol composition might be responsible for increased peak metabolic rate in birds fed the high ω6 diet. I also investigated the selectivity of the mitochondrial membrane fatty acid transporter carnitine palmitoyl transferase, and demonstrated that its activity was highest with shorter and polyunsaturated fatty acyl CoA substrates. Overall, maximal exercise

performance in birds may be affected by stored triacylglycerol composition due to selectivity of the fatty acid transport system, but does not appear to be affected by muscle phospholipid composition. Finally, I developed a theoretical framework for the study of avian fat composition that focuses on the tradeoffs between energy storage and transport of fatty acids.

KEYWORDS: adipose, birds, β-oxidation, carnitine palmitoyl transferase, exercise, fatty acid mobilization, membrane phospholipids, migration, muscle, omega 3, omega 6

CO-AUTHORSHIP STATEMENT:

A version of Chapter 2 was published with Anna Krokfors and Christopher Guglielmo as 2nd and 3rd authors, respectively. Ms. Krokfors was primarily responsible for development of methodology. Dr. Guglielmo aided methodological development, obtained most project funding and equipment, and provided editorial comments on the manuscript.

A version of Chapter 3 has been accepted for publication with Jay McFarlan and Christopher Guglielmo as 2nd and 3rd authors, respectively. Mr. McFarlan provided expertise and training in the techniques and analysis of real time PCR. Dr. Guglielmo obtained most project funding and equipment, provided methodological advice, and provided editorial comments on the manuscript.

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For my parents, Susan and Gary

And for my Aunt Becky and Great-Aunt Mabel who spurred my interest in birds

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CD36/FAT: fatty acid translocase

CPT: carnitine palmitoyl transferase

CS: citrate synthase

FABPpm: plasma membrane fatty acid binding protein

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

H-FABP: cytosolic (heart type) fatty acid binding protein

HOAD: 3-hydroxyacyl-CoA dehydrogenase

HSL: hormone-sensitive lipase

Hω3: high ω3 diet group

Hω6: high ω6 diet group

Hω3RI: high ω3 diet group with restricted feeding and intermediate diet

Hω6RI: high ω6 diet group with restricted feeding and intermediate diet

KRBA: Krebs-Ringer buffer with 4% bovine serum albumin

LDH: lactate dehydrogenase

MIG: migratory treatment group

NEFA: non-esterified fatty acids

NL: neutral lipids

PMR: peak metabolic rate

PL: phospholipids

PPAR: peroxisome proliferator-activated receptor

PUFA: polyunsaturated fatty acids

VLDL: very low density lipoproteins

WIN: winter treatment group

WEX: winter with exercise treatment group

CHAPTER 1

AN INTRODUCTION TO DIETARY LIPIDS AND THEIR EFFECTS ON AVIAN MIGRATORY PERFORMANCE

Fat and migratory birds

Migratory animals across a wide variety of phylogenetic lineages tend to store fat rather than other macromolecules (carbohydrate and protein) as the energy source to fuel their long distance movements (Blem, 1980; Dingle, 1996). Although fat is more difficult to transport through aqueous media such as cytosol or plasma, it is much more energy-dense than carbohydrate or protein, making it useful for minimizing weight during long-distance transport (Alerstam, 1993; Blem, 1980; Klasing, 1998; McWilliams et al., 2004; Ramenofsky, 1990). Because of the power required to overcome gravity (Pennycuick, 1972) flying animals such as birds are under strong selection pressure to use stored fat to minimize weight during migration (Jenni and Jenni-Eiermann, 1998; Pennycuick, 1989). Additionally, the efficiency of conversion of dietary energy to fat is higher than that of protein, and the maintenance cost of adipose is lower than that of protein stores (Klasing, 1998). Indeed, birds primarily use fat to fuel their long-distance flights (Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004). However, very little is known about how different *types* of fat affect these flights. In this work I have sought to better understand how dietary fat composition can affect flight performance, and to understand the mechanisms underlying those effects. This chapter will largely consist of a literature review that will provide an introduction to the following 4 chapters that describe my experimental work.

Types of fatty acids and notation

Fatty acids consist of a carboxylic acid group with a long aliphatic carbon chain. Vertebrates generally utilize unbranched fatty acids of chain lengths ranging from 12 - 24 carbons (Raclot, 2003). Fatty acids may be saturated, in which all carbons are connected to each other by single bonds, or unsaturated, in which there exists at least one double bond between two carbon atoms. The location of the double bond can be indicated by the IUPAC system of nomenclature which names them from the carboxylic end of the molecule. Thus, the fatty acid commonly called linoleic acid is named cis,cis-Δ^9,Δ^{12}-octadecadienoic acid, indicating an 18 carbon fatty acid with double bonds located at the 9^{th} and 12^{th} carbons from the carboxyl end. This notation is unwieldy, however, because natural fatty acids nearly always have double bonds separated by exactly 3 carbons and, more importantly, enzymatic modification of fatty acids occurs near the carboxyl end, such that this notation obscures the relationship between fatty acids that are enzymatically related. A more common "ω" notation in physiology describes the position of just the first double bond from the methyl end of the molecule (Allport, 2006; Holman and Mohrhauer, 1963). Thus linoleic acid can be denoted 18:2ω6 (also written as 18:2n6 or 18:2n-6), indicating that it is 18 carbons long, contains 2 carbon-carbon double bonds, and the first of these double bonds occurs at the 6^{th} carbon from the methyl end. Further, it is readily apparent that 18:2ω6 is related to 20:4ω6 by two steps of enzymatic desaturation and 1 step of elongation (Figure 1.1).

Polyunsaturated fatty acids (PUFA) are those containing 2 or more carbon-carbon double bonds. They commonly occur as ω3 or ω6 fatty acids. Although they can be modified by desaturation and elongation, they cannot be synthesized *de novo*

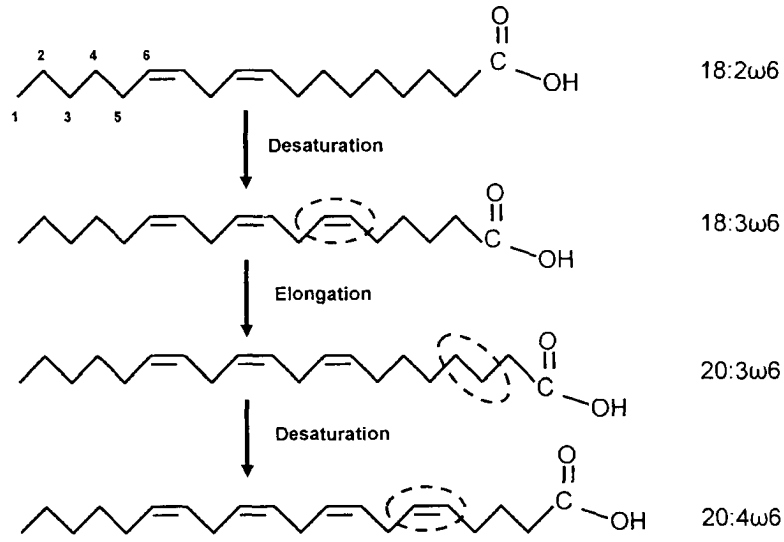

Figure 1.1. Enzymatic relationship between some common ω6 fatty acids. Dashed ovals indicate the location of enzymatic modification. Modification occurs close to the carboxyl end, such that the first double bond from the methyl end is always in the same position. All double bonds are *cis*-double bonds. Linoleic acid (18:2ω6) can be desaturated to add a double bond, producing γ-linolenic acid (18:3ω6). This fatty acid can be elongated by adding 2 carbons at the carboxyl end, producing 20:3ω6. Another step of desaturation creates arachidonic acid (20:4ω6).

by birds (Stevens, 1996). The ω6 fatty acids are also required for certain cell functions, making them essential nutrients (Stevens, 1996). The ω3 fatty acids are essential for mammals, and are suspected to be essential nutrients in birds (Watkins, 1991). Because of their essential nature, PUFA can have important effects on many aspects of physiology (Watkins, 1991). However, in wild birds, the focus to date has been on their effects on migration.

Triacylglycerol (or 'triglyceride') is the storage form of fatty acids, consisting of 3 fatty acids esterified to a glycerol backbone. Triacylglycerol is found mostly in adipose tissue and in muscles. Common phospholipids (PL) are similar in structure to triacylglycerol except that a phosphate-containing head group replaces one of the fatty acids. PL are major components of cellular and subcellular membranes. The term lipid is often used to refer to fats, but generally refers to any substance that can be extracted from cells by nonpolar organic solvents. Fat and oil usually refer to triacylglycerol, the difference between the two being only that fats are generally solid at room temperature, while oils are liquid.

From diet to oxidation: storage, incorporation, and use of dietary fat

Fatty acids are the only dietary macromolecules that can be directly incorporated into body tissues intact (Klasing, 1998). Further, dietary fats are known to affect the composition of a wide variety of bird tissues, ranging from muscle PL to preen wax (Klasing, 1998; Thil et al., 2003; Thomas et al., In prep.; Watkins, 1991; Xu et al., 1994), and thus they have the potential to affect a great number of physiological processes.

Dietary triacylglycerol is hydrolyzed to produce non-esterified fatty acids (NEFA) and monoacylglycerol in the intestine, followed by absorption across the epithelium of the intestine (Denbow, 2000; Leeson and Summers, 2001; Place, 1996; Ramenofsky, 1990). These components are reesterified to triacylglycerol and then transported in portomicrons via the venous portal system to the liver and other tissues (Bensadoun and Rothfield, 1972; Ramenofsky, 1990; Stevens, 1996). In the liver, fatty acids may be modified and then repackaged as triacylglycerol in very low density lipoproteins (VLDL) for circulatory transport to other tissues (Klasing, 1998; Stevens, 1996). Upon reaching tissues such as adipose or muscle, the triacylglycerol contained in portomicrons or VLDL is hydrolyzed by lipoprotein lipases to produce free fatty acids which are transported inside those tissues where they can be reesterified to reform triacylglycerol (Ramenofsky, 1990). Thus, dietary fatty acids may be incorporated into adipose and intramuscular triacylglycerol intact. Enzymatic elongation and desaturation may also modify dietary fatty acids prior to storage in muscle or adipose triacylglycerol. *De novo* hepatic synthesis of fatty acids from carbohydrate or protein substrates is also possible, primarily leading to the formation of 16:0, 16:1ω7, 18:0, and 18:1ω9. Composition of tissue triacylglycerols will thus reflect a combination of dietary fat composition, post-absorptive modification of dietary fatty acids, *de novo* synthesis, and potentially other processes such as preferential oxidation (Leyton et al., 1987).

Birds may oxidize intramuscular triacylglycerol for energy, but sustained flight requires the use of extramuscular lipid stores (Jenni and Jenni-Eiermann, 1998). Adipose triacylglycerol is hydrolyzed by hormone-sensitive lipase (HSL) and other lipases, and fatty acids are released to the circulation (Ramenofsky, 1990). There, fatty acids bind to

circulating albumin and are then transported to muscle. Translocation of fatty acids across the muscle membrane is a protein-mediated process (Luiken et al., 1999; McFarlan et al., 2009). Within myocytes, fatty acids are transported to the mitochondria bound to a cytosolic fatty acid binding protein H-FABP (Guglielmo et al., 1998). Fatty acids are converted to acyl-CoAs by acyl CoA synthetase, and then to acyl-carnitines by the enzyme carnitine palmitoyl transferase (CPT). The acyl-carnitine then crosses the mitochondrial membrane, acyl-CoA is reformed, and this acyl-CoA undergoes β-oxidation to eventually produce ATP. Fatty acids may also be partly catabolized in peroxisomes in a process similar to mitochondrial β-oxidation before transport to mitochondria for complete oxidation (Berg et al., 2002).

PL are synthesized from NEFA and glycerol 3-phosphate, initially sharing a common anabolic pathway with that of triacylglycerols. This synthesis from free fatty acids allows the composition of PL to be affected by dietary fat. While certain classes of PL are relatively invariant with regard to fatty acid composition (i.e. phosphatidyl inositol), most PL classes can vary greatly with respect to the composition of the fatty acids that make up 'tails' of the molecule (Berg et al., 2002). Nonetheless, the refractivity of certain tissues to dietary fat changes (Thil et al., 2003) and imperfect reflection of dietary lipids in PL composition (Nagahuedi et al., 2009) indicate that substantial regulation of fatty acid composition of PL can occur.

Evidence for an effect of dietary lipid composition on migratory flight performance

It is now well established that birds, like other vertebrates, require a minimal level of dietary intake of PUFA to achieve normal growth and maintenance (National Research

Council Board on Agriculture, 1994; Stevens, 1996; Watkins, 1991). Beyond this basic level of nourishment, however, the effects of dietary composition are poorly understood in vertebrates (McKenzie, 2001). In recent years, the effects of dietary fatty acids have been of particular interest to ornithologists because of their potential to affect migratory performance (Pierce and McWilliams, 2005; Weber, 2009). Below, I discuss several lines of evidence that dietary lipid composition can affect migratory performance.

Seasonal changes in stored lipids

Early studies of migratory birds described the composition of stored fats and how that composition changed seasonally (reviewed by Blem, 1976; Blem, 1980; Blem, 1990; Pierce and McWilliams, 2005). This work generally focused on the proportion of unsaturates carried by migrants, due to the suggestion that unsaturated fatty acids should be more readily mobilized (Blem, 1976; Conway et al., 1994; Johnson and West, 1973; Johnston, 1973). In his review of lipid unsaturation in birds, Blem (1980) concluded that there is no consistent difference between migrants and non-migrants. Pierce and McWilliams (2005) noted that only six bird species have been studied during both migratory and non-migratory seasons, and only half of these have shown increases in unsaturation during the migratory period. Even with a recent addition to this list showing an increase in unsaturation in adipose of migrating white-throated sparrows (*Zonotrichia albicollis*) (Klaiman et al., In press), these limited data do not represent strong support for migration-related changes in composition.

Proximately, adipose composition is determined largely by dietary lipid composition (Pierce and McWilliams, 2005), although other processes such as *de novo*

synthesis and endogenous modification cannot be ignored (Egeler et al., 2003; Klasing, 1998; Napolitano and Ackman, 1990; Pierce and McWilliams, 2005; West and Meng, 1968). This is significant, as it may provide some explanation for seasonal fat composition patterns (or the lack thereof, if certain desirable fatty acids are in limited supply). Birds are known to sometimes switch diets during the migratory period (Bairlein and Gwinner, 1994), and this could result in some of the observed seasonal patterns. Further, if the composition of stored lipids affects exercise, it presents a mechanism by which birds could enhance their flight performance simply by adjusting their diets. It is unclear, however, whether these diet switches are performed for the purpose of altering fat depot composition as suggested by some authors (Bairlein, 1996; Conway et al., 1994; Heitmeyer and Fredrickson, 1990) or are simply a result of birds adjusting to seasonal changes in food item abundance in order to maximize weight gain. In controlled trials with red-eyed vireos, Pierce and McWilliams (2005) found no seasonal changes in preference for dietary fatty acids. Thus, diet switches may improve overall migratory performance by increasing refueling rate and survival at stopover, but there is little evidence that these diet switches have the purpose of improving exercise performance during flight.

Previous authors have also commented on seasonal changes in the ratio of monounsaturates (16:1 + 18:1) to 18:2ω6 in stored fat (Blem, 1976; Pierce and McWilliams, 2005). Migrants have been thought to carry larger proportions of monounsaturates relative to 18:2ω6, although the data are limited and equivocal, with only 4 of 7 species exhibiting the 'pattern' across seasons in the wild (Klaiman et al., In press; Pierce and McWilliams, 2005). A possible mechanism for such a seasonal pattern

lies in the essential nature of 18:2ω6 combined with deposition of 18:1. The fatty acid 18:1 can be deposited directly from dietary sources or formed *de novo* from non-fat substrates, such that migratory hyperphagia combined with increased conversion of carbohydrates or protein to fat could drive this pattern (Egeler and Williams, 2000; Napolitano and Ackman, 1990). Nonetheless, in a captive study of red-eyed vireos under natural light conditions and controlled diet, Pierce and McWilliams (2005) found no evidence for a pattern of increased ratio of (16:1 +18:1)/18:2ω6 in adipose during the migratory season. Further, Blem's original assertion (1976) that the 18:1/18:2ω6 ratio is higher in migrants may have been skewed by a phylogenetic or diet bias: his non-migrant sample included a large proportion of gulls and galliformes, many of which have diets that are quite different from his mostly passerine migrant sample.

Egeler et al. (2000) demonstrated that activity of Δ^9-desaturase, the liver enzyme that converts 18:0 to 18:1, increases during the migratory period in western sandpipers (*Calidris mauri*). This may, in part, be responsible for the increase in adipose fatty acid unsaturation during the migratory period (Egeler and Williams, 2000), although it may also be simply associated with the overall increase in *de novo* fatty acid production during migration (Egeler et al., 2000). In either case, this finding demonstrates that some degree of unsaturation appears to be necessary in the deposition of adipose triacylglycerol, regardless of whether there is an increase in unsaturation during the migratory period.

In summary, there does not seem to be strong evidence yet that migrants increase the overall level of unsaturation of their adipose stores during migration. Nonetheless, a certain amount of unsaturation may be necessary and/or beneficial in the storage of fat or

the fueling of long-distance flight. Seasonal changes in the fatty acid composition of adipose stores is largely determined by diet, but is influenced by *de novo* synthesis and selective deposition, use, and modification of dietary fatty acids.

Diet choice experiments

Studies of dietary preference in controlled cafeteria-style choice experiments have revealed preference with respect to dietary fatty acid composition. Bairlein (1991), using purified triacylglycerols and natural oils, showed that garden warblers prefer to consume 18:1 to 18:0 and also prefer 18:1 (but less so) to 18:2ω6. McWilliams et al. (2002) similarly showed that yellow-rumped warblers (*Dendroica coronata*) prefer to consume unsaturated free fatty acids over saturated ones, and that this preference is stronger when 18:1 was the unsaturate used than when 18:2ω6 was used. Pierce et al. (2004) also found preferences for 18:1 over 18:0, but found no difference in preference between 18:1 and 18:2ω6 in red-eyed vireos. This work was extended to show that vireos preferred 18:1 to either saturated fatty acid (16:0 and 18:0) independently of season (Pierce and McWilliams, 2005). The reason for this preference has generally been attributed to differences in assimilation efficiency, as 18:1 is easier to digest than its saturated counterpart 18:0 (McWilliams et al., 2002; Pierce et al., 2004; Place, 1996; Renner and Hill, 1961). However, the preferences exhibited by migratory birds also open the possibility that birds choose dietary fats that benefit migratory flight performance (Bairlein, 1996). Further, these preference trials have focused on major components of bird adipose tissue (16:0, 18:0, 18:1, 18:2ω6), but minor components (e.g., 15:0, 18:3ω3, 20:0, 20:4ω6, 20:5ω3, 22:6ω3,) have been hypothesized to play important roles in

affecting whole-organism and cellular performance (Guderley et al., 2008; Guglielmo et al., 2002b; Hulbert and Else, 1999; Infante et al., 2001; Weber, 2009). Maillet and Weber (2006) suggested that semipalmated sandpipers (*Calidris pusilla*) choose ω3-enriched food items at stopover, but did not conduct a controlled experiment to investigate this potential preference. It remains to be tested whether birds are able to distinguish these usually minor dietary fats and actively choose to consume them.

Direct effects of diet on exercise performance

Experiments in non-avian taxa have shown that dietary fatty acids can affect whole-animal exercise performance. Focusing on different ratios of ω6 to ω3 fatty acids, Ayre and Hulbert (1997) demonstrated that altering the composition of fats in the diet can affect endurance running in rats. McKenzie et al. (1998) found that maximum swimming speed in salmon was affected by the blend of oils provided in their experimental diets. In birds, Pierce et al. (2005) showed that lipid composition of the diet could affect the peak metabolic rate achieved during exercise-wheel (burst performance) trials. Similarly, McWilliams and Pierce (2006) found that dietary fat composition can affect energy efficiency during long-distance flights in a wind tunnel.

Other work has demonstrated that dietary ω3 fatty acids are correlated with slight increases in muscle oxidative enzyme activity in semipalmated sandpipers at a migratory stopover (Maillet and Weber, 2006; Maillet and Weber, 2007). Nonetheless, it was unclear whether the observed increases in oxidative capacity were driven by dietary ω3 fatty acids or other processes of migratory preparation at stopover. More recent work showed that these changes in enzyme activity could be induced by controlled dietary

manipulation in sedentary quails (*Colinus virginianus*) (Nagahuedi et al., 2009). Still, it has yet to be shown that ω3 fatty acids improve whole-animal exercise performance in birds, and it is difficult to interpret these findings in light of previous works (Ayre and Hulbert, 1997; McKenzie et al., 1998) that show better exercise performance with low levels of dietary ω3 fatty acids in other vertebrates. Overall these studies indicate that the composition of dietary fat alone can affect exercise performance in birds, but the nature of this effect is still unclear. The lack of understanding of the mechanism by which dietary fatty acids hinder or enhance exercise limits synthesis and broader application of these results. Below, I discuss several of the proposed mechanisms by which fatty acids affect exercise performance.

Mechanisms of enhanced performance: the 'energy hypothesis'

Probably the oldest hypothesis for how dietary fatty acids affect avian exercise performance focuses on their role as oxidative substrates. Early observations of migration-related increases to unsaturation (see above discussion) led to the hypothesis that unsaturates benefit performance because they should be readily utilized during exercise due to their greater solubility in water (Blem, 1976; Conway et al., 1994; Egeler and Williams, 2000; Johnson and West, 1973; Johnston, 1973). Due to this increased solubility, unsaturated fatty acids should be more easily/quickly transported through the body for eventual oxidation in the flight muscles, potentially allowing higher rates of oxidation. In its entirety, this hypothesis involves the many steps in the fatty acid supply pathway, including lipolysis and mobilization of fat from adipocytes, systemic transport, muscular uptake, intramyocyte transport, and finally intramyocyte oxidation for

production of ATP. The transport and utilization of fat have been studied at several steps along this pathway in migratory birds, e.g., adipocyte lipolysis (Ramenofsky et al., 1999; Vaillancourt and Weber, 2007), circulatory transport (George and John, 1993; Jenni-Eiermann and Jenni, 1992), muscular uptake (McFarlan et al., 2009; Ramenofsky et al., 1999; Savard et al., 1991), intramyocyte transport and oxidation (Driedzic et al., 1993; Guglielmo et al., 2002a; Lundgren and Kiessling, 1985; Marsh, 1981; Pelsers et al., 1999). However, only one of these studies (Vaillancourt and Weber, 2007) has considered the ramifications of different types of fatty acids being involved in these processes. Thus, the hypothesis that greater unsaturation of fatty acids leads to higher rates of fat mobilization and utilization during flight remains largely untested.

Nonetheless, there is substantial evidence from other taxa that would suggest an important role for fatty acid type in mobilization and utilization. Leyton et al. (1987) demonstrated differential rates of whole-organism oxidation for a series of fatty acids fed to rats. Their study, however, is not necessarily comparable to avian migratory flight due to the higher rates of lipid utilization in birds and because migratory flight is powered mostly by fats mobilized from adipose tissue, not directly from the gut. Other work in various mammals (Florant et al., 1990; Herzberg and Skinner, 1997; Nieminen et al., 2006; Raclot and Groscolas, 1993; Soppela and Nieminen, 2002; Wheatley et al., 2008), has demonstrated that adipose fatty acid mobilization is a selective process, with both unsaturation and chain length affecting patterns of preferential mobilization (Raclot, 2003). Data from fasting penguins also support this finding (Groscolas, 1990). Fatty acid uptake and utilization in muscles is also apparently a preferential process in fish (Egginton, 1996; Sidell et al., 1995).

An important consideration when evaluating the substrate supply hypothesis is whether fatty acid supply is limiting to avian exercise during migration. Other factors, such as oxygen supply or oxidative capacity (e.g. mitochondrial density) may limit maximal exercise in migrating birds (Jenni and Jenni-Eiermann, 1998; Marsh, 1981; Suarez, 1996; Weber, 1992). If so, fatty acid selectivity at points along this pathway may not have any effect on maximal performance. Although fatty acid transport proteins increase in abundance during migration (Driedzic et al., 1993; Guglielmo et al., 2002a; McFarlan et al., 2009), this is also true of oxidative enzymes such as citrate synthase (CS) (Evans et al., 1992; Guglielmo et al., 2002a; Lundgren and Kiessling, 1985), indicating that either fatty acid or oxygen supply could be limiting to maximal performance during migration. Further, oxygen supply has been considered limiting to aerobic performance in homeothermic vertebrates (Suarez, 1996), although this has not been thoroughly investigated in migrant birds (Jenni and Jenni-Eiermann, 1998). Even when fatty acid supply is limiting, certain steps in the supply pathway may be more important than others. In particular, circulatory transport and especially muscular uptake are thought to be limiting in the substrate supply pathway (Guglielmo et al., 2002a; Jenni-Eiermann and Jenni, 1992; McFarlan et al., 2009; Vock et al., 1996; Weber, 1992).

Another important consideration is whether metabolic limitations are normally reached during migratory flight. Conway et al. (1994) noted that readily mobilized fat stores should aid migrants in unpredictable weather, presumably because inclement weather could force migrants to approach maximal metabolic rates in order to fly in heavy winds. Pierce et al. (2005) found that dietary fatty acid composition could affect peak metabolic rates in red-eyed vireos, attributing this finding primarily to the

composition of adipose stores, although other mechanisms (such as membrane composition; see below) could have been involved. In fact, they found that birds fed a highly unsaturated diet achieved lower peak metabolic rates than those with moderately unsaturated diets, indicating that indeed another mechanism is at work, or that selectivity of the substrate supply pathway is more complex than the simple dichotomy of saturated vs. unsaturated fatty acids. Moreover, birds may not often achieve maximal metabolic rates during migration. Instead, increased endurance or energy efficiency may be of greater importance during migration, allowing flights of greater distance between stopovers, particularly during flights that cross geographical barriers. Given the higher solubility of unsaturates, one can imagine a mechanism by which birds using stored unsaturates expend less energy on maintenance of fat transport proteins (e.g. H-FABP, FAT), thereby achieving greater efficiency during flight. McWilliams and Pierce (2006) found that dietary fatty acid composition affected the efficiency of 6 hour wind tunnel flights, with birds fed a diet enriched with polyunsaturates expending 13% less energy than those fed a diet enriched with monounsaturates. They attributed this finding to the change in adipose composition, but did not test other mechanisms.

In summary, although unsaturated fatty acids have long been hypothesized to affect exercise via their increased solubility and therefore increased supply to flight muscles, there is little experimental evidence to corroborate this mechanism.

Mechanisms of enhanced performance: the 'phospholipid hypothesis'

Another hypothesis that has gained recent interest focuses on muscle membrane PL composition and its effect on exercise (Guglielmo et al., 2002b; Weber, 2009).

Specifically, this hypothesis states that 1) dietary fatty acids are incorporated into the 'tail' groups of PL in cellular or subcellular membranes, and 2) because membranes are involved in many metabolic processes, altered fatty acid composition of the membrane affects these processes, and this somehow affects exercise performance at the whole-organism level. The first part of the hypothesis, i.e., that dietary fatty acids are incorporated into membrane PL, is supported by strong experimental evidence in birds and other vertebrates (Ayre and Hulbert, 1996; Guderley et al., 2008; Maillet and Weber, 2006; Nagahuedi et al., 2009; Thil et al., 2003; Turner et al., 2004). The second part of the hypothesis has its basis in various loosely connected lines of evidence. Membrane-bound enzyme activity is affected by the fatty acid composition of the membrane (Guo et al., 2005; Infante et al., 2001; Murphy, 1990). Elements of membrane fatty acid composition vary with metabolic rate and membrane-bound enzyme activity across species (Hulbert and Else, 1999; Hulbert and Else, 2005), although this relationship may not be strong in birds (Turner et al., 2005). Membrane fatty acid composition is also associated with rate of mitochondrial leak (Gerson et al., 2008; Guderley et al., 2008; Hulbert and Else, 1999; Pan et al., 1994). While these observations provide evidence that membranes can play an important role in cellular energetics, it has been harder to connect these observations to higher levels of organization, and particularly to an exercising organism.

Exercise has been shown to cause changes in membrane fatty acid composition in mammals (Andersson et al., 1998; Helge et al., 1999; Nikolaidis and Mougios, 2004; Turner et al., 2004) and it is tempting to predict that the reverse should also be true, i.e., that membrane fatty acid composition should affect exercise. In one of the first papers to

apply the 'phospholipid hypothesis' to migratory birds, Guglielmo et al. (2002b) demonstrated seasonal changes in membrane composition, specifically finding a drop in the ω6/ω3 ratio during the migratory season of western sandpipers. Reviewing the mammal literature, they concluded that membrane ω6 fatty acids were beneficial to exercise performance but were consumed during exercise, such that availability of ω6 in the membranes could limit exercise performance (Guglielmo et al., 2002b). Ruf et al. (2006) similarly concluded that ω6 fatty acids were beneficial to maximal running speed based on their comparative analysis of mammals. However, the effects of exercise on membrane composition have been varied (Helge et al., 1999; Helge et al., 2001; Nikolaidis and Mougios, 2004), and other authors have come to opposite conclusions (Infante et al., 2001; Weber, 2009). Klaiman et al. (In press) found elevated levels of ω6 fatty acids of white-throated sparrow pectoralis muscle PL during the migratory season, and concluded that seasonal changes in membrane fatty acids are likely driven more by diet than exercise. Infante et al. (2001) found that the highly aerobic pectoralis muscles of hummingbirds have high levels of membrane 22:6ω3.

Some experiments in birds and other species have suggested a role of PL in exercise performance, but the link to PL has generally been associative and not based on independently manipulated PL composition. Ayre and Hulbert (1997) found that high ω6 fatty acid diets improved the treadmill running endurance performance of rats compared to those fed high ω3 diets, and they suggested this was due to diet-induced changes to muscle PL composition, although other hypotheses might also explain their result. Maillet and Weber (2006) showed that semipalmated sandpipers fattening at a stopover site in the Bay of Fundy ingest large amounts of ω3-rich invertebrate prey, causing an

increase in muscle PL ω3 content. In a follow-up study (Maillet and Weber, 2007), they showed that the fattened sandpipers also had higher activities of CS and 3-hydroxyacyl-CoA-dehydrogenase (associated with β-oxidation) in flight muscles, an association they termed 'natural doping'. This evidence was strengthened by a study in quail, where dietary fat manipulation resulted in changes to oxidative enzyme activity, although not always in simple ways (Nagahuedi et al., 2009). For example, diets enriched with 20:5ω3 or 22:6ω3 increased 3-hydroxyacyl dehydrogenase activity, but the combination of the two fatty acids did not (Nagahuedi et al., 2009). Further, it has yet to be shown that these changes in oxidative enzyme activity result in improved exercise performance. Part of the reason for the failure of previous studies to demonstrate a direct effect of PL composition on exercise performance is the difficulty of manipulating membrane composition *in vivo* independently of other tissues. Manipulation of membrane composition has generally been accomplished by diet manipulation, a procedure which can affect the composition of other tissues, including adipose triacylglycerol (see above). This methodological difficulty makes it problematic to tease apart the energy and phospholipid hypotheses.

Further investigation of this hypothesis should also examine the mechanisms in more detail and, in particular, seek to connect tissue-level traits to organismal-level ones. For example, an increase in HOAD activity could represent a higher capacity to oxidize fatty acids. But it might also indicate that fatty acids causing this change are more slowly oxidized, and so HOAD activity could reflect upregulated expression in an attempt to offset this handicap. Studies directly linking these enzyme activities and particularly PL

composition to whole-organism performance are needed, and these studies must distinguish between the 'PL hypothesis' and other proposed mechanisms.

Other mechanisms of enhanced performance

Several alternate mechanisms have been proposed for how dietary fatty acids affect exercise performance. Most of these have received little attention from ornithologists to date; I discuss two of these possibilities below.

Activation of peroxisome proliferator-activated receptors (PPARs)

Maillet and Weber (2007) and Weber (Weber, 2009) proposed that fatty acids could increase oxidative capacity and exercise performance by interacting with PPARs, another mechanism that they termed 'natural doping'. Many saturated and unsaturated fatty acids are ligands for these receptors, which, after binding with the fatty acid, can then interact with the promoter regions of genes associated with oxidation and increase transcription rates of these genes (Desvergne and Wahli, 1999). Only one study to date has attempted to investigate this possibility in birds (Nagahuedi et al., 2009). However, this study examined the upregulation of expression of PPARs themselves by dietary fatty acids, which does not test the primary mechanism by which dietary fatty acids are hypothesized to work (i.e. by upregulation of expression of oxidative enzyme genes). This hypothesis thus remains largely untested.

Future work on this hypothesis should investigate the ability of the many fatty acids to act as PPAR ligands, and to determine their effect on migration. PPAR activation could increase peroxisome activity, which can increase fatty acid throughput,

but could also waste energy during peroxisomal oxidation, which yields less ATP than mitochondrial β-oxidation. Increased mitochondrial and peroxisomal activity might also be beneficial during flight, but detrimental during stopover when birds are trying to gain fat mass.

Incorporation into eicosanoids

PUFA serve as the basic building block for synthesis of eicosanoids (prostaglandins, prostacyclin, thromboxanes, and leukotrienes), hormones that have varied functions associated with inflammation, immunoregulation, cell proliferation, oviposition, thermoregulation, and other functions (Craig-Schmidt et al., 1987; Klasing, 1998; Watkins, 1991). The composition of dietary PUFA can affect the activity of these hormones, because ω3 fatty acids can inhibit eicosanoid production, and because eicosanoids constructed from ω3 vs. ω6 fatty acids have different activities (Craig-Schmidt et al., 1987; Watkins, 1991). Some studies have suggested a possible role of dietary fatty acids acting through eicosanoids to affect exercise or exercise recovery in mammals (Ayre and Hulbert, 1997; Mickleborough et al., 2006; Simopoulos, 2008; Toft et al., 2000), but this has not been studied in birds.

Measurements of eicosanoids in wild migrants are, to my knowledge, non-existent. Klaiman et al. (In press) proposed that observed decreases in 20:4ω6 in sparrow muscle PL during migration could be adaptive endogenous changes to prevent the conversion of this fatty acid to prostaglandins, which would increase inflammation and impair muscle recovery after migratory flights. However, in exercising humans, Toft et

al. (2000) found no effect of dietary ω3 fatty acids on inflammatory response or muscle damage. There has been no experimental test of this hypothesis in birds.

An introduction to the experiments

The following four chapters describe experiments I performed to investigate how dietary fatty acids affect avian exercise performance. My objective was to determine the plausibility of the 'energy' and 'PL' hypotheses for explaining diet effects on exercise performance in birds. I begin by investigating the 'energy hypothesis' in Chapter 2, studying the preference of the fatty acid substrate supply pathway. Specifically, I demonstrate selectivity of avian adipocytes with regard to fatty acid lipolysis and mobilization. In Chapter 3, I look at the 'PL hypothesis' by examining the effects of exercise and migratory disposition on muscle PL composition. I find no evidence that birds prepare for migration by endogenously altering muscle PL composition. In Chapter 4, I attempt to tease apart the 'energy hypothesis' from the 'phospholipid hypothesis'. By using a complex dietary manipulation, I alter membrane composition independently of adipose composition. The results indicate that membrane PL composition is not likely playing an important role in exercise performance, but that stored triacylglycerol composition may indeed affect exercise in birds. In Chapter 5 I return to the 'energy hypothesis', this time focusing on the selectivity of the intramuscular portion of the fatty acid supply pathway. I find that the mitochondrial membrane transporter carnitine palmitoyl transferase (CPT) is selective with respect to fatty acyl-CoA substrate. In Chapter 6, I summarize my findings and develop a framework for studying avian fat storage.

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CHAPTER 2
SELECTIVE MOBILIZATION OF FATTY ACIDS FROM ADIPOSE TISSUE IN MIGRATORY BIRDS[1]

Introduction

During times of fasting or high energy demand, fatty acids are mobilized from adipocyte triacylglycerol stores and exported to the circulatory system. Inside the adipocytes, this process involves cleavage of triacylglycerol by hormone-sensitive lipase (HSL) into constituent fatty acids and glycerol moieties. In recent studies it has become clear that the proportions of the specific fatty acid species mobilized differ somewhat from their proportions in the triacylglycerol from which they were released (Herzberg and Skinner, 1997; Hollenberg and Angel, 1963; Raclot and Groscolas, 1993; Soppela and Nieminen, 2002). This fatty acid selectivity has been demonstrated both *in vivo* (Herzberg and Skinner, 1997; Nieminen et al., 2006; Soppela and Nieminen, 2002) and *in vitro* (Raclot and Groscolas, 1993; Raclot et al., 1995), and has led to adaptive explanations regarding the functions of the specific fatty acids that are selectively retained or mobilized (Falkenstein et al., 2001; Florant et al., 1990; Nieminen et al., 2006; Soppela and Nieminen, 2002). However, in a recent review, Raclot (2003) concluded that differential mobilization of fatty acids occurs primarily due to the molecular structure of each fatty acid species; fatty acids that are shorter, more unsaturated, and have their double bonds closer to the methyl end are more polar and are preferentially mobilized. This may occur due to a combination of enzyme specificity, access of HSL to polar lipids at the lipid/aqueous

[1] A version of this chapter has been published (Price et al., 2008) and is adapted with permission from the Journal of Experimental Biology.

interface, and other lipid transport processes within the adipocyte (Raclot, 2003; Raclot et al., 2001).

During migratory flights, birds engage in endurance (often 8+ hours) exercise, achieving maximal rates of oxygen consumption that are more than double those achieved by similarly sized mammals, all while fasting (Butler and Woakes, 1990). Stored adipose serves as the primary fuel source during these flights (McWilliams et al., 2004; Odum et al., 1964; Ramenofsky, 1990), and birds undergo seasonal changes in physiology which allow them to utilize fat at high rates (Guglielmo et al., 2002a; Jenni-Eiermann and Jenni, 1992; Pelsers et al., 1999). The mobilization of lipids from adipocytes is an important step in this process (Johnston, 1973). The selective mobilization of particular fatty acids may also be important given that the fatty acid composition of adipose stores can affect migratory performance (Johnston, 1973; Pierce et al., 2005). Nonetheless, the phenomenon of selective fatty acid mobilization from the adipose tissue has not been studied in birds except in fasting penguins (Groscolas, 1990; Johnson and West, 1973). Moreover, to my knowledge, selective fatty acid mobilization has not been investigated *in vitro* for any animal during its different life history stages and periods of energy demand. The goals of this study, therefore, were to characterize the selective mobilization of fatty acids *in vitro* for two migratory bird species, and to investigate any changes in selective fatty acid mobilization associated with the migratory period in one of the species. Specifically, I was interested in whether migratory state would result in an increase in mobilization of 18:2ω6 (Soppela and Nieminen, 2002) or a selective retention of 18:2ω6, perhaps

to conserve this fatty acid for use during breeding (Florant et al., 1990; Mostafa et al., 1994).

Materials and Methods

Animals

Six ruffs (*Philomachus pugnax* Linnaeus) were obtained from a captive colony maintained at Simon Fraser University by Dr. David Lank and housed communally at the University of Montana. They were maintained on a diet of trout pellets (Purina Mills) supplemented occasionally with hard boiled eggs and supplied with water *ad libitum*. This diet contained a broad diversity of fatty acids which allowed me to examine how patterns of relative mobilization vary with chain length and unsaturation. Ruffs were maintained on a 12L: 12D light cycle (light 0600-1800) for the duration of the experiment, with a nightlight provided (<1 lux). The ruffs were weighed weekly and sex (3 males/3 females) was determined by size dimorphism and plumage.

Seventeen white-crowned sparrows (*Zonotrichia leucophrys gambelii* Nuttall) were captured during fall migration in Missoula, Montana in September 2004. Another 5 sparrows were captured in fall 2003 at Sunnyside, Washington and used for other diet experiments until April 2004 (Cerasale and Guglielmo, 2006), after which they were kept in an outdoor aviary in Missoula until my experiment. All sparrows were thus exposed to natural light conditions for an extended period before experiments and had completed feather molt and the autumn migration period before entering the short-day photoperiod conditioning. Sparrows were individually housed

indoors in 38 cm x 43 cm x 41 cm cages at 22 °C for the duration of the study. Sparrows were weighed in the morning before feeding every 2 – 3 days throughout the experiment. Ten sparrows were aged as "hatch year" and the rest as "after hatch year" according to Pyle (1997). Sex was determined after the experiment by inspection of the gonads. Sparrows were fed a diet of black oil sunflower seeds and water was provided *ad libitum*, with supplemental grit and vitamins offered weekly. This limited diet ensured that sparrows on all treatments would have similar adipose stores so that relative mobilization rates would be more easily comparable. A nightlight (< 1 lux) was provided to encourage nightly migratory behavior. Experimental protocols were approved by the University of Montana Institutional Animal Care and Use Committee and appropriate permits for collection, export/import and possession were obtained from the US Fish and Wildlife Service, Canadian Wildlife Service, and Montana State Department of Fish Wildlife and Parks.

Photoperiod Manipulation

All sparrows were initially kept on a short day light cycle (8L:16D) for 58 days to break photorefractoriness and simulate winter. Sparrows were then randomly assigned to treatment groups of long days (migrant = MIG; n = 10; 16L:8D), short days (winter = WIN; n = 7; 8L:16D), and short days with exercise (winter plus exercise = WEX; n=5; 8L:16D). All sparrows were then maintained another 22 – 26 days before experiments were initiated. This manipulation of the light cycle in MIG sparrows induced zugunruhe, a captive analog of the migratory condition in which

birds undergo nightly hopping and wing fluttering (Breuner et al., 1999; King and Farner, 1963). As evidence of this, nightly hopping activity, as measured by counters attached to the perches, was significantly elevated in the MIG sparrows compared to WIN sparrows ($P < 0.05$). In the last 2 weeks before the experiments WEX birds were exercised every 1 – 2 days for approximately 1 h. These birds were temporarily moved to a separate room and the experimenter reached into the cages, stimulating the birds to fly back and forth as normally occurs when birds are captured for weighing. Visibly fatigued or panting birds were allowed to rest.

Adipocyte Incubation and Fatty Acid Analysis

The protocol for determining relative mobilization from adipocytes generally followed that described by Raclot and Groscolas (1993). Birds were euthanized between 1000h-1430h (2-6.5 h after lights on) by pentabarbitol overdose. Approximately 400 mg adipose tissue was removed immediately from the claviculo-corocoid depot and placed in Krebs Ringer buffer (made with 1.26 g/l $NaHCO_3$, 0.368 g/l CaCl, and 4% fatty acid free Bovine Serum Albumin; Krebs Ringer and Albumin both from Sigma, St. Louis MO) (KRBA). Tissue was minced with scissors and washed with KRBA 3 times. Washed tissue was then transferred to a polypropylene flask containing 4 ml fresh KRBA warmed to 37 °C. I added epinephrine (Sigma, St. Louis MO) to a final concentration of 100 μM and the flask was flushed with 95%/5% O_2/CO_2 and capped. The flask was incubated 90 min at 37 °C in a shaking water bath. The contents were then filtered under vacuum with a glass microfiber filter (VWR cat # 28297-978). A sample of this filtrate was taken

for glycerol analysis (described below). I added 210 µl heptadecanoic acid (17:0; 31 mg/100 ml in hexane) as an internal standard. Three 1 ml aliquots of the incubation medium were each added to vials containing 15 ml chloroform:methanol and shaken vigorously. For analysis of adipose lipids, 5 - 10 mg subcutaneous adipose tissue was removed from the claviculo-corocoid depot, added to 15 ml 1:1 chloroform:methanol and homogenized at high speed 3 x 10 s with a 1 cm generator (Polytron). Total lipid extracts from incubation medium and from adipocytes were then centrifuged 15 min at 2056 x g and filtered (Whatman no. 1), adding 10 ml of 2:1 chloroform:methanol to rinse. I added 6 ml 0.25 % KCl to partition and remove aqueous solutes. The aqueous layer was vacuum pipetted off and the organic solvent was evaporated (Rotovapor, Buchi, Switzerland). Lipids were resuspended in 100 µl chloroform for loading onto Supelclean solid phase extraction tubes (Supelco, LC-NH2, 100 mg). Neutral lipids (NL, primarily triacylglycerol) were eluted with 1.8 ml chloroform:isopropanol (2:1 v/v). Non-esterified fatty acids (NEFA) were then eluted with 1.6 ml isopropyl ether:acetic acid (49:1 v/v). Columns were washed between samples with hexane and methanol. I added an internal standard (200 µl of 17:0 at 30mg/100ml hexane) to the adipocyte NL fraction, and then evaporated each lipid fraction to dryness under N_2. NEFA were methylated at room temperature for 30 min following addition of 100 µl methanol, 1 ml dimethoxypropane, and 40 µl concentrated HCl. The sample was dried under N_2, and resuspended in 40 µl isooctane before injection in the gas chromatograph column. The NL fraction was transesterified with 2 ml acetyl chloride in methanol (1M), heated for 2 h at 90 °C.

The solvent was evaporated under N_2 and fatty acid esters were resuspended in 60 µl isooctane for injection onto the gas chromatograph column.

Fatty acids were separated on a Hewlett Packard HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) with a J&W Scientific High Resolution Gas Chromatography Column (DB-225ms, Agilent Technologies, Palo Alto, CA) and flame ionization detector. The carrier gas was N_2. The temperature program was 2 min 120 °C, then increase at 5 °C per min for 16 min, hold at 200 °C for 5 min, increase at 5 °C per min for 4 min, then hold 13 min at 220 °C. Fatty acids with fewer than 16 carbons were excluded from statistical analysis because precautions to minimize volatization were not taken. Only fatty acids constituting more than 0.25% of the total neutral lipids in adipose tissue were considered in the analysis. The identities of fatty acids were determined by comparison of retention times to standard mixtures of fatty acid methyl esters (Supelco 37 component mix, Supelco PUFA No. 3, Sigma, St. Louis MO)

Glycerol concentration was measured in the medium after adipose incubation as a measure of overall lipolysis rate. Glycerol was assayed on a microplate spectrophotometer (BioTek Powerwave X340) in 400 µl flat-bottomed microplates with an endpoint assay (SIGMA, Trinder reagent A, 5 µl medium, 300 µl reagent). Glycerol is reported as (concentration in medium)/(mass of adipose incubated).

Statistical analyses

For each bird, relative mobilization for each fatty acid was calculated as (its mass % in NEFA from the incubation) divided by (its mass % in NL from adipose).

Only fatty acids which were detected in both NL and NEFA were included in the analysis (infinite and zero relative mobilizations were excluded). The 18:1ω9 and 18:2ω6 chromatograph peaks were inseparable in one WIN sparrow's adipose NL. These fatty acids were removed from analysis for this animal.

In comparing the relative mobilization rates of different fatty acid species, normal statistical approaches are inappropriate due to the non-independence of data. For my analyses I used a permutation approach aided by scripts written in S-Plus (Insightful Corp, Seattle, WA). To test for a general effect of chain length, I measured a least squares regression "observed slope" of relative mobilization vs. chain length on the relative mobilizations of all fatty acids for all individuals. I then performed a random permutation of the relative mobilizations within an individual and within a degree of unsaturation (i.e., the relative mobilizations for 16:0, 18:0, and 20:0 were permuted for each animal, the relative mobilizations for 16:1 and 18:1 were permuted for each animal, etc.). A new slope was determined for all the permuted data. This process was repeated 1000 times to obtain a distribution of slopes. I considered chain length to have a significant effect if the observed slope was as or more extreme than the most extreme 5% (two-sided) of the slopes in the permuted distribution. A similar process was conducted for analyzing the effect of degree of unsaturation (# of double bonds), permuting within individual and within a given chain length. While I recognize that the effects of these factors may not be linear and these slopes may not have biological meaning, these tests give a general indication of the effects of chain length and unsaturation on relative mobilization.

To compare mobilization rates of 2 different fatty acids I performed a standard permutation test. To evaluate the effects of sex, age, and photoperiod on the relative mobilization of a particular fatty acid and glycerol I used Mann-Whitney tests. Results are presented as mean ± s.e.m.

Results

Ruff Adipocyte Relative Mobilization

There was no difference in relative mobilization between males and females for any of the fatty acids ($P > 0.1$) so data were pooled. The pattern of relative mobilization observed in ruffs generally followed the order of mobilization of fatty acids that was found previously in mammals and penguins (Figure 2.1). The relative mobilization of fatty acids in ruffs increased with degree of unsaturation (Figure 2.2; $P < 0.001$) and decreased with carbon length, particularly for lengths 18 and greater (Figure 2.2; $P < 0.001$). The only pair of positional isomers that I measured was 18:3ω3 and 18:3ω6. There was no significant difference between the relative mobilizations of these two fatty acids ($P > 0.9$).

Sparrow Adipocyte Relative Mobilization

Due to the limited fatty acid composition of the sparrow diet, only 4 fatty acids were found in substantial amounts in adipocytes. These four fatty acids (16:0, 18:0, 18:1ω9, and 18:2ω6) constituted over 97% of the total present in adipose triacylglycerol and only two others (20:0 and 20:1ω9) constituted more than 0.25% of the total. This limited variety of fatty acids precluded analysis of the effects of chain length and degree of unsaturation on relative mobilization in sparrows. However, the pattern of relative mobilization of fatty acids was similar to the pattern previously reported for mammals and penguins (Figure 2.3), and was similar to the pattern seen

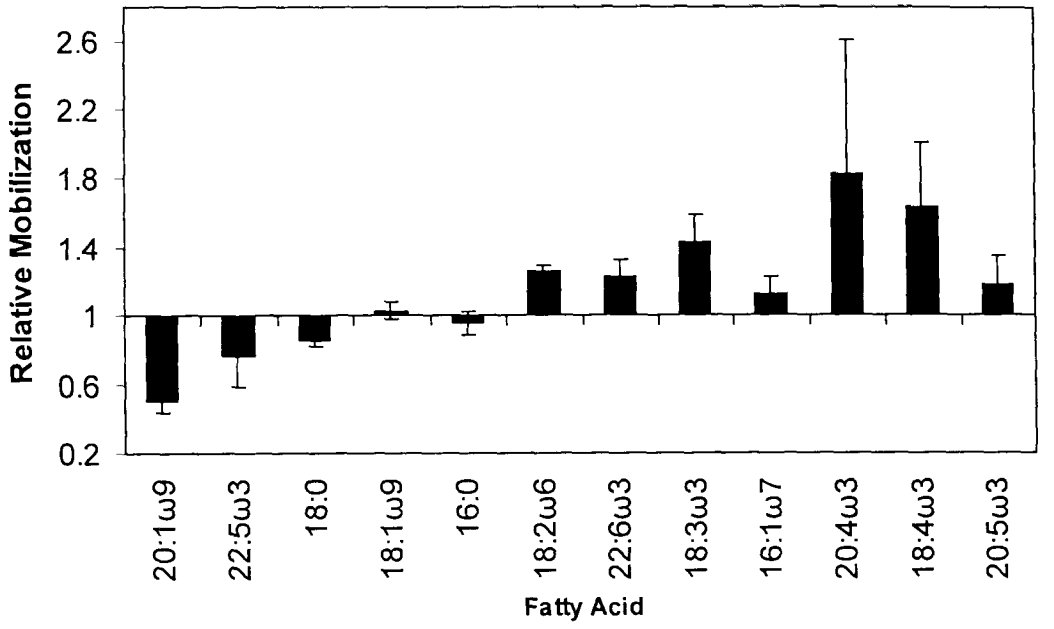

Figure 2.1. Relative mobilization of fatty acids from ruff adipocytes. Fatty acids are ordered from least to most mobilized according to Raclot's (2003) studies in rats. Data are means + SE; n=6.

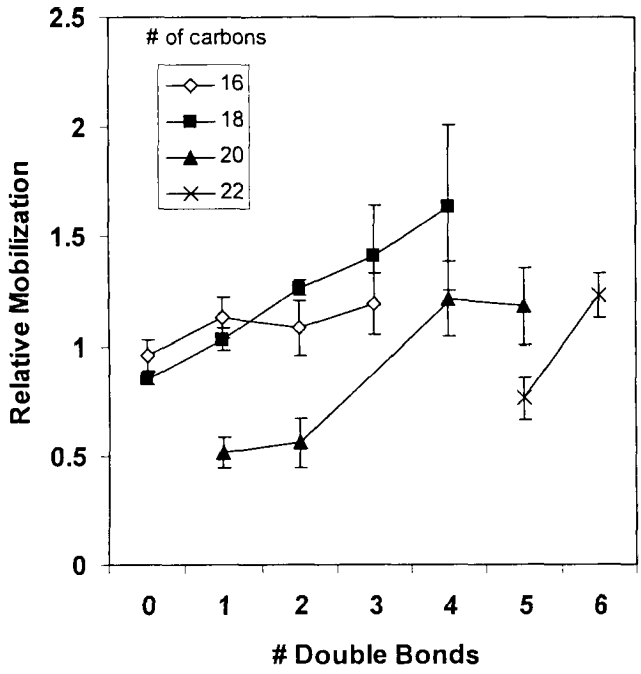

Figure 2.2. Relative mobilization of fatty acids from ruff adipocytes in relation to the number of double bonds. Data are means + SE; n=6. The value for 18:3 is the mean of 18:3ω3 and 18:3ω6. The value for 20:4 is for 20:4ω6. The number of double bonds and chain length both had significant effects on relative mobilization ($P < 0.001$). See text for statistical details.

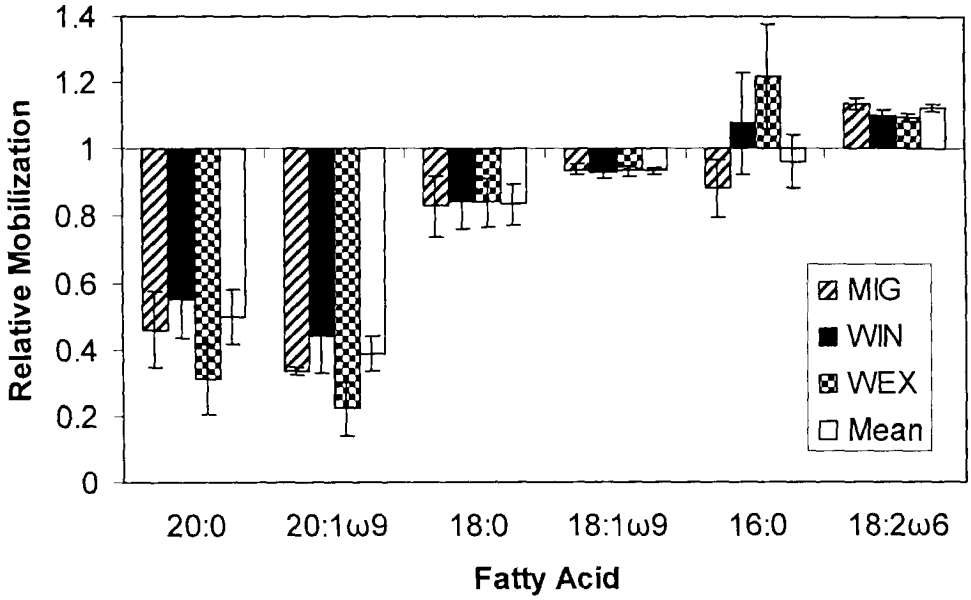

Figure 2.3. Relative mobilization of fatty acids from white-crowned sparrow adipocytes. Fatty acids are ordered from least to most mobilized according to Raclot's (2003) studies in rats. Data are means ± SE. There are no significant differences in relative mobilization of any fatty acid according to migratory state or exercise. MIG=migrant, n=10; WIN=winter, n=7; WEX=winter with exercise, n=5.

in ruffs (this study). Sex did not affect the relative mobilization of any of the fatty acids measured ($P > 0.1$), but age did have a significant effect on mobilization of 18:0 ($P = 0.033$) with relative mobilization of this fatty acid being higher for hatch year birds. Relative mobilization was not affected by migratory state or exercise for the 6 most common fatty acids ($P > 0.133$).

Treatment did not have a significant effect on glycerol concentration ($P = 0.070$) although there was a trend toward higher glycerol in migrant birds (Figure 2.4). Neither age ($P = 0.536$) nor sex ($P = 0.601$) had significant effects on glycerol concentration. The ratio of moles fatty acids released to moles glycerol was 2.28:1.

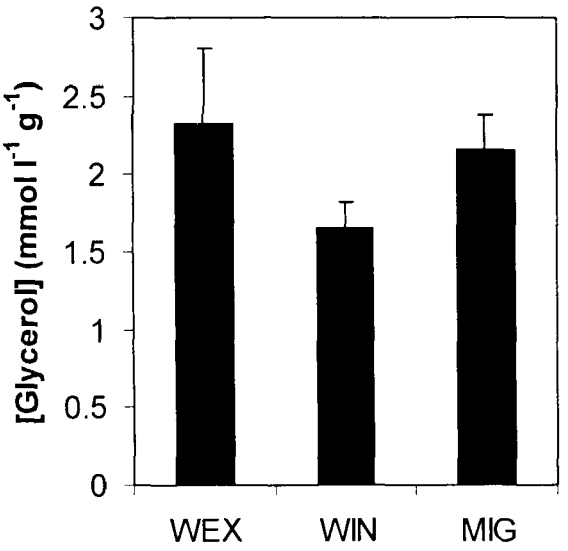

Figure 2.4. Glycerol concentration of incubation medium at the end of incubation (per gram adipose tissue incubated). MIG=migrant, n=10; WIN=winter, n=7; WEX=winter with exercise, n=5. There were no significant differences between treatment groups (P = 0.070).

Discussion

Fatty acid relative mobilization rates have not previously been measured *in vitro* in birds. The current study demonstrates that in ruffs and white-crowned sparrows, adipose fatty acid relative mobilization rates are largely affected by structural factors such as chain length and the number of double bonds. This finding agrees with previous *in vitro* studies in mammals (Raclot, 2003), as well as *in vivo* measurements from fasting penguins (Groscolas, 1990). Although I have not studied subcellular processes such as hydrolysis of triacylglycerol by HSL, this agreement generally suggests that the biochemical processes resulting in selective mobilization of fatty acids are similar between mammals and birds. These mechanisms may include specificity of HSL for certain triacylglycerol-bound fatty acids as well as access of HSL to more polar fatty acids at the lipid droplet interface (Hazel and Sidell, 2004; Raclot, 2003).

I used a photoperiodic manipulation to investigate the effects of migratory state on relative mobilization rates in sparrows. My results indicate that the patterns of mobilization rates of individual fatty acids relative to the overall mobilization rate do not vary with migratory state. While I did not find a significant effect of migratory state on overall lipolysis, the trend I observed can fuel further hypotheses. Specifically, it appears that exercise and migratory state may alter adipose tissue such that it responds more strongly to stimulation by epinephrine. Similar results were found by Savard et al. (1991) in dark-eyed juncos (*Junco hyemalis*). This could occur by an increase in receptor density (Breuner et al., 2003; Landys et al., 2004a).

Additional findings include the observation that 18:2ω6 was not selectively retained in the birds, contrary to the predictions of Mostafa et al. (1994) based on work in a congeneric species. Interestingly, young sparrows mobilized 18:0 at higher relative rates than their older counterparts, although the importance of this finding is unknown.

Traditional use of photoperiod-manipulated captive birds as models for migration in endocrinology and orientation studies have relied on zugunruhe as evidence for migratory condition (Able and Cherry, 1986; Landys et al., 2004b). Data on bird zugunruhe indicate that the MIG sparrows did respond to the photoperiodic treatment by increasing nightly activity. This treatment may not, however, perfectly mimic the biochemical changes that occur in adipocytes during true migration. Using a captive protocol, however, provides the opportunity to study birds with very similar adipose stores by controlling diet. Additionally, it is possible that no change in relative mobilization was observed with migration due to the limited number of fatty acids available in abundance for this experiment, although this limited fatty acid composition may be typical for wild passerines (Blem, 1976; Conway et al., 1994). Further experiments could evaluate relative mobilization rates in free-living migrants across seasons, particularly in shorebirds which eat a varied diet and whose adipose depots would be expected to have a variable fatty acid composition.

Selective mobilization and seasonal changes in energetic demand

Several investigators have studied changes in adipose tissue fatty acid composition under conditions of fasting, nutritional stress, and/or hibernation (Falkenstein et al., 2001; Florant et al., 1990; Groscolas, 1990; Nieminen et al., 2006; Soppela and Nieminen, 2002). When selective mobilization of fatty acids has been observed, it has often been interpreted as adaptive (for example to maintain metabolism and functions of essential fatty acids or to maintain fluidity of fat reserves) (Falkenstein et al., 2001; Nieminen et al., 2006; Soppela and Nieminen, 2002). Given the apparent ubiquity and consistency in the pattern of selective mobilization of fatty acids (Groscolas, 1990; Groscolas and Herzberg, 1997; Hazel and Sidell, 2004; Raclot, 2003; Raclot et al., 1995, current study), I recommend that such interpretations should be made in light of "background" selective mobilization. For example, Soppela and Nieminen (2002) infer functional importance from the observation that 18:2ω6 was highly mobilized from malnourished reindeer, but high mobilization should not be unexpected because in general, 18:2ω6 is preferentially mobilized relative to the other fatty acids that make up reindeer triacylglycerol. On the other hand, the selective retention of 18:2ω6 in hibernating marmots (Florant et al., 1990) and hibernating echidnas (Falkenstein et al., 2001) is notable as it demonstrates an exception to the general pattern. Valuable information about lipid metabolism might be gained from these and other species by comparing *in vitro* selective mobilization from adipocytes excised at different stages of the animals' life histories.

Importance of selective mobilization to migratory birds

Because birds use extramuscular lipids as their primary fuel during migratory flights (McWilliams et al., 2004), the composition of stored fats has long been of interest to physiologists. A particular focus has been on the amount of unsaturated fats stored (Conway et al., 1994; Egeler and Williams, 2000; Johnston, 1973; Yom-Tov and Tietz, 1978). Although unsaturated fats are less energy dense than saturates, this energetic difference is relatively small (Bower and Helms, 1968), and whole-organism oxidation rates of unsaturates are higher than those of saturated fats in fed rats (Leyton et al., 1987). Thus it might be advantageous for birds to store unsaturated fats because they are more easily transported from adipose stores to the muscle during energetically demanding flights. Johnston (1973) observed that migratory species tend to have more unsaturated fatty acids in adipose stores than non-migratory birds, although Blem (1976; 1980) found no trend in saturation for migratory and non-migratory birds. Additionally some researchers have noted a change in fatty acid composition leading up to migration, although this observation has not been consistent (Egeler and Williams, 2000; Hicks, 1967; McGreal and Farner, 1956), and does not seem to be the direct result of selective metabolism (Pierce and McWilliams, 2005). Many authors have noted that adipose fatty acid composition tends to reflect dietary composition, and changes in bird diets leading up to migration can alter adipose stores (Bower and Helms, 1968; Conway et al., 1994; Egeler and Williams, 2000; Morton and Liebman, 1974; Pierce and McWilliams, 2005; West and Meng, 1968). Experiments with sandpipers have indicated that diet is not solely responsible for changes to increased adipose unsaturation leading up to

migration, but that endogenous modification, preferential deposition, and *de novo* synthesis can increase adipose unsaturation prior to and during migration (Egeler et al., 2003; Egeler and Williams, 2000; Egeler et al., 2000).

During a migratory flight, the utilization rate for fatty acids will depend on 4 processes: the rates of 1) mobilization from adipocyte, 2) transport through the blood, 3) uptake at the muscles, and 4) intracellular oxidation (Egeler and Williams, 2000). Despite the importance of fat utilization to avian flight, none of these processes have previously been studied in migratory birds with respect to fatty acid composition. Leyton et al. (1987) have often been cited as reporting that oxidation rates of unsaturated fatty acids are high. However, their study reported rates of whole-organism oxidation from fed rats and, therefore, their results could be due to preferential deposition of saturated dietary fatty acids rather than selective transport, uptake, and oxidation of unsaturates. As such, their results may not be easily comparable to the process of fat utilization in fasting, migrating birds. My results indicate that mobilization of fatty acids from adipocytes is generally most rapid for highly unsaturated fatty acids (as well as for short fatty acids). This could have adaptive significance for the preferential storage of unsaturated fatty acids, but only if adipose mobilization is limiting (which does not appear to be the case; McWilliams et al., 2004) or may be limiting under certain conditions (e.g. increased energetic demands due to adverse weather (Conway et al., 1994)). Other processes may limit the supply of fats to fuel migratory flight, although it seems likely that unsaturated fatty acids would also be transported and utilized more quickly in these processes as well due to their greater solubility in water. Additionally, the preferential

mobilization of unsaturated fatty acids compared to saturates may not indicate substantially increased rates of overall lipolysis. My results indicate a trend toward increased lipolysis in migratory-stage birds without a change in the pattern of selective mobilization of fatty acids.

Although many researchers have speculated that the higher mobility of unsaturated fatty acids is beneficial to migratory performance, Pierce et al. (2005) found that very high concentrations of dietary and adipose 18:1 and total unsaturates resulted in poorer exercise performance in captive vireos. Conversely, vireos with higher dietary 18:2ω6 had improved performance. This finding has been replicated in rats (Ayre and Hulbert, 1997) and fish (McKenzie et al., 1998), and may be due to diet-influenced changes in phospholipid (PL) fatty acid composition (Ayre and Hulbert, 1997; Guglielmo et al., 2002b). Thus, the composition of adipose stores may not be the only mechanism by which dietary lipids can affect performance. The relative importance of dietary, adipose, and PL fatty acid composition to performance in migratory birds deserves further study.

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CHAPTER 3
PREPARING FOR MIGRATION? THE EFFECTS OF PHOTOPERIOD AND EXERCISE ON MUSCLE OXIDATIVE ENZYMES, LIPID TRANSPORTERS, AND PHOSPHOLIPIDS IN WHITE-CROWNED SPARROWS[1]

Introduction

Migratory birds undergo many physiological changes prior to and during the migratory journey. These can include changes in nutrient stores, organ sizes, hematocrit, muscle and liver biochemistry, resistance to sleep deprivation, and endocrine function (Berthold, 1975; Breuner et al., 1999; Egeler et al., 2000; Evans et al., 1992; Fusani and Gwinner, 2004; George and Vallyathan, 1964; Guglielmo et al., 2002a; Landys et al., 2004b; McWilliams et al., 2004; Piersma and Everaarts, 1996; Rattenborg et al., 2004). In the laboratory, a captive analog of migratory condition known as zugunruhe can be induced in birds by alteration of the photoperiod. Zugunruhe, or 'migratory restlessness', is generally indicated by nocturnal wing fluttering and hopping in nocturnally migrating passerines. Studies of zugunruhe have been particularly fruitful for understanding migratory orientation (Emlen, 1967; Emlen, 1970), genetics (Berthold and Querner, 1981), and sleep/wake cycles (Rattenborg et al., 2004) . However, the zugunruhe model hasn't been broadly applied to many other aspects of physiology. In regard to muscle physiology in particular, it is unclear the extent to which endogenous cycles (often cued by external factors, mainly photoperiod) can prepare muscles for high intensity exercise, or if muscle "training" via high intensity exercise is required to induce the changes in muscle physiology typical of migrants (Guglielmo et al., 2002a). In this experiment I

[1] A version of this chapter has been accepted for publication in Physiological and Biochemical Zoology (Price et al., Accepted).

tested the effects of photoperiod and exercise on muscle enzyme activity, lipid transporter expression, and phospholipid fatty acid composition in white-crowned sparrows (*Zonotrichia leucophrys gambelii* Nuttall).

Given the high energetic demand during flight, it is expected that oxidative capacity and fatty acid oxidation potential should increase during migration. Migrating birds to have higher levels of citrate synthase (CS), 3-hydroxyacyl-CoA dehydrogenase (HOAD), and carnitine palmitoyl transferase (CPT) than wintering birds of the same species (Evans et al., 1992; Guglielmo et al., 2002a; Lundgren and Kiessling, 1985). Some of these have also been shown to increase in preparation for migration or during a stopover between migratory flights (Driedzic et al., 1993; Evans et al., 1992; Lundgren and Kiessling, 1986; Maillet and Weber, 2007; Marsh, 1981). Nonetheless, the degree to which birds can modulate their lipid oxidative capacity in advance of migration and the mechanisms by which they do so are poorly known. This study examines the effect of photoperiod on the activity of CS, HOAD, and CPT.

The high energetic demand of flight also necessitates an exceptionally high fat transport capacity. Fat transporter mRNA or protein expression increases in shorebirds and white-throated sparrows (*Zonotrichia albicollis* Gmelin) during the migratory period (Guglielmo et al., 2002a; McFarlan et al., 2009), although it is unclear whether this is a response to exercise, or occurs in advance of migration. Here I test whether mRNA expression of several fat transport proteins can be increased by photoperiodic cues alone.

Because of the potential for membrane fatty acids to affect cellular and higher level physiology, the relationship between the fatty acid composition of muscle phospholipids (PL) and exercise performance has been the subject of recent investigation, although few generalities have emerged from these studies (reviewed in Nikolaidis and Mougios, 2004). A major problem encountered in exercise studies is interpreting the changes observed in PL fatty acid composition. An exercise-induced decrease in the ω6:ω3 ratio may represent an adaptive acclimation (or acclimatization) to exercise, a negative outcome of exercise (a type of muscle damage, perhaps via consumption of essential n6 fatty acids), or a performance-neutral consequence of exercise. For example, Infante et al. (2001) found high levels of docosahexaenoic acid (22:6ω3) associated with high performance muscles of rattlesnakes and hummingbirds and suggested that high 22:6ω3 was important for calcium transport in these muscles, although high levels could also be a negative consequence of high performance exercise. Ayre and Hulbert (1997) showed that rats with high ω6/ω3 in muscle PL had greatly increased endurance running ability. In birds, Guglielmo et al. (2002b) found migration-related changes in pectoralis muscle PL fatty acid composition, including decreases in 20:4ω6, ω6/ω3 ratio, and an increase in 22:6ω3. They suggested that ω6 fatty acids were depleted during exercise and that the observed changes to PL composition represented a cost of migration, although they could not exclude possible effects of seasonal variation in diet or endogenous adaptive modulation of membrane fatty acids (Guglielmo et al., 2002b). In contrast, Maillet and Weber (2007) suggested that migrating shorebirds use dietary ω3 fatty acids to alter their muscle membranes favorably in preparation for migration.

In this experiment, I sought to determine whether sparrows endogenously modify the fatty acid composition of muscle PL in preparation for migration and whether exercise affects fatty acid composition of PL. By examining these measures of oxidative capacity, fat transporter expression, and muscle phospholipid composition, this experiment investigates several ways in which bird muscles might prepare for migration.

Materials and Methods

Animals

I studied the Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelli* Nuttall), a common North American long distance short-bout migrant. It has been used extensively in studies of zugunruhe (King and Farner, 1963; Landys et al., 2004b; Ramenofsky et al., 2003), making it an ideal model species for this study. Seventeen sparrows were captured during fall migration in Missoula, Montana in September 2004. Another 6 sparrows were captured in fall 2003 at Sunnyside, Washington and used for other diet experiments until April 2004 (Cerasale and Guglielmo, 2006), after which they were kept in an outdoor aviary until my experiment. All sparrows were individually housed indoors in 38 cm x 43 cm x 41 cm cages at 22 °C for the duration of the study. Birds were weighed in the morning before feeding every 2 – 3 days throughout the experiment. Fourteen birds were aged as "hatch year" and the rest as "after hatch year" according to Pyle (1997). Sex was determined after the experiment by inspection of the gonads (Table 3.1). Birds were fed a diet of black oil sunflower seeds and water was provided *ad libitum*, with supplemental grit and vitamins offered weekly. A nightlight (<1 lux) was provided for both rooms. Experimental protocols were approved by the University of Montana Institutional Animal Care and Use Committee (Protocol #04404CGDBS121304) and appropriate permits for collection were obtained from the US Fish and Wildlife Service.

Table 3.1. Mass, sex, age, and nighttime activity of white-crowned sparrows

	Treatment		
	WEX	**WIN**	**MIG**
N	5	8	10
# Males/Females	3/2	3/5	3/7
# Hatch Year	3	5	6
Mass (g)	25.30 ± 1.41	25.03 ± 1.50	24.61 ± 0.89
Hops per night	6 ± 4	58 ± 57	375 ± 137*

Note.- Data presented are means ± SE; WEX = Winter with exercise; WIN = Winter (short days); MIG = Migratory (long days).

* Significantly different from WIN and WEX ($P < 0.05$).

Photoperiod and exercise

Initially, all birds were kept on a short day light cycle (8L:16D) for 58 days to break photorefractoriness and simulate winter (Agatsuma and Ramenofsky, 2006). Sparrows were then randomly assigned to treatment groups of long days (migrant = MIG; 16L:8D), short days (winter = WIN; 8L:16D) and short days with exercise (winter plus exercise = WEX; 8L:16D). All birds were then maintained another 22 – 26 days before being euthanized (Landys et al., 2004b). In the last 2 weeks before euthanasia, WEX birds were exercised every 1 – 2 days for approximately 1 h. These birds were temporarily moved to a separate room and the experimenter reached into the cages, stimulating the birds to fly back and forth as normally occurs when birds are captured for weighing. Visibly fatigued or panting birds were allowed to rest.

Bird masses were monitored throughout captivity, measured in the morning before feeding, as well as just before euthanasia. Migratory restlessness, or zugunruhe, was measured using laboratory counters with perches attached to the counter switch (King and Farner, 1963). Each time the animal hopped on the perch it was recorded by the counter. Hopping activity was recorded for 7 hours, beginning 30 min after the lights were turned off. Sparrows were euthanized by pentobarbital overdose and a central portion of the pectoralis muscle was immediately dissected out and stored at -80 °C.

Lipid analysis

Muscle lipids were extracted as in Guglielmo et al. (2002b). Briefly, pectoralis muscle tissue (300 – 400 mg) was removed and homogenized 3×10 s in 15 ml chloroform:methanol (1:1 by vol; all chemicals from Sigma, St. Louis, MO, USA, unless noted otherwise) with a high-speed stainless steel homogenizer to extract total lipids. This was centrifuged for 15 min at 2056 g. The extract was then filtered (Whatman no. 1 filters, Whatman Inc., Piscataway, NJ) with the addition of 10 ml chloroform:methanol (2:1 by vol). I then added 6 ml 0.25% KCl to partition and remove hydrophilic solutes. The solvent was evaporated (Rotovapor, Buchi, Switzerland) and lipids were resuspended in benzene:methanol (2:1 by vol) for storage at -20 °C. Later, the benzene:methanol was evaporated and lipids were resuspended in 100 µl chloroform for loading onto Supelclean solid phase extraction tubes (Supelco, LC-NH2, 100 mg). Neutral lipids (NL) were eluted with 1.8 ml chloroform:isopropanol (2:1 by vol). Non-esterified fatty acids (NEFA) were then eluted with 1.6 ml isopropyl ether:acetic acid (49:1 by vol), and PL were eluted with 3 ml methanol. I added heptadecanoic acid (17:0; 200 µl, 30 mg/100ml in hexane) as an internal standard, and then evaporated the PL fraction to dryness under N_2. The PL fraction was transesterified with 2 ml acetyl chloride in methanol (1M), heated for 2 h at 90 °C. The solvent was evaporated under N_2 and fatty acid methyl esters were resuspended in 60 µl isooctane for injection onto the (GC) column.

Fatty acid methyl esters were separated on an HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) with a J&W Scientific High Resolution Gas Chromatography Column (DB-225ms, Agilent Technologies, Palo Alto, CA) and

flame ionization detector. The carrier gas was N_2. The temperature program was 2 min 120 °C, then increase at 5 °C per min for 16 min, hold at 200 °C for 5 min, increase at 5 °C per min for 4 min, then hold 13 min at 220 °C. Fatty acids with fewer than 16 carbons were excluded from statistical analysis because precautions to minimize volitization were not taken. One MIG bird's muscle PL was not successfully extracted, leaving a sample size of 9 for PL analysis in the MIG treatment.

Enzyme assays

Approximately 100 mg pectoralis muscle was combined with 9 volumes of a homogenization buffer (20 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% defatted BSA, 50% glycerol [Caledon Laboratories, Georgetown, ON], 0.1% Triton x-100, and Aprotinin at 50 μg/ml). This was then homogenized 3 x 10 sec, waiting 30 sec between bouts while keeping the sample on ice. Samples were sonicated 3 x 10 sec, again waiting 30 sec between bouts with samples on ice. The wattage was set low enough to prevent foaming of the samples. Samples were then stored again at -80 °C until conducting enzyme assays. All enzyme assays were performed in duplicate at 39 °C in 1 ml reaction volume in disposable cuvettes on a Cary 100 Bio Spectrophotometer (Varian Inc., Palo Alto, CA).

CPT (EC 2.3.1.21) was assayed in 50 mM Tris buffer, pH 8.0, with 5 mM carnitine, 0.15 mM DTNB, 0.035 mM palmitoyl CoA, and 10 μl of homogenate diluted 1:5. CS (EC 2.3.3.1) was assayed in the same tris buffer, with 0.5 mM oxaloacetic acid, 0.15 mM DTNB, 0.3 mM acetyl CoA, and 10 μl of homogenate

diluted 1:20. HOAD (EC 1.1.1.35) was assayed in 50 mM imidazole buffer, pH 7.4, with 0.2 mM NADH, 1 mM EDTA, 0.1 mM acetoacetyl CoA, and 10 µl of the homogenate diluted 1:10. Lactate dehydrogenase (LDH; EC 1.1.1.27) was assayed in the same imidazole buffer with 8 mM pyruvate, 0.3 mM NADH, 5 mM DTT, and 10 µl homogenate diluted 1:100. Activities were calculated from ΔA_{412} for CS and CPT and from ΔA_{340} for HOAD and LDH.

mRNA expression

Expression of two membrane fatty acid transporters, fatty acid translocase (FAT/CD36) and plasma membrane fatty acid binding protein (FABPpm), the cytosolic transporter H-FABP, and two housekeeping genes (β-actin and GAPDH) were measured using real-time PCR according to the methods of McFarlan (2009), which were developed for the congeneric white-throated sparrow (*Zonotrichia albicollis*). Briefly, RNA was extracted from ~100 mg pectoralis muscle using TRIzol (Invitrogen, Burlington, ON) in a glass homogenizer following the manufacturer's procedure, with the addition of 2 chloroform extractions and 2 ethanol washes. RNA was then eluted with autoclaved ddH_2O and quantified spectrophotometrically at λ=260 in a Tris-EDTA buffer (absorbance of 1 is equal to 40 ug/ml RNA). Only samples with $A_{260}/A_{280} > 1.8$ were used. Five µg of RNA were digested with 2 U DNase I (New England Biolabs, Ipswich, MA) for 15 min at 37 °C (then inactivated at 75 °C for 10 min) to remove any contaminating DNA. RNA samples were then reverse transcribed to create complementary DNA (cDNA). The conditions were 20 µl reaction volume including 0.5 µg RNA, 0.5 µg oligo-$dT_{12\text{-}18}$ primer, 0.5 mM dNTP

for each dNTP, 1x FS Buffer, 10 mM DTT, 1 μl RNase OUT, and 200 U SuperScript II Reverse Transcriptase (all from Invitrogen), incubated 90 min at 42 °C and then stopped with 15 min at 70 °C. I prepared negative controls using water instead of RNA. Samples were stored at -80 °C until PCR analysis.

Real-time PCR was run for each gene separately with a Rotor-Gene 6000 Real-Time Rotary Thermocycler (Corbett Life Science, Concorde, NSW, Australia). The reaction conditions for FAT/CD36 and FABPpm were 1x Reaction Buffer, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM primers, 0.75 U Platinum Taq polymerase, 0.7x SYBR-Green I (all from Invitrogen). The conditions for the remaining genes differed only in having 4 μM $MgCl_2$. In all cases I used 1 μl of cDNA (first diluted 1:5 with water) in a total reaction volume of 20 μl. I used the following primers supplied by Invitrogen (5'-3'): (FAT/CD36 A): ACA CCT TGA CCG TCC TCA AC; (FAT/CD36 B): TGA AGG CCT CAC AAG AAG GT; (FABPpm A): TTC CAG AGA AGA GCA TCA TCC; (FABPpm B): GTC AGT GAT GTG CTG CCA GT; (H-FABP A): CCC ACC ACC ATC ATC GAG; (H-FABP B): GCC CAT GGT GAG AGT CAG AA; (β-Actin A): CCC TGA AGT ACC CCA TTG AA; (β-Actin B): GGG GTG TTG AAG GTC TCA AA; (GAPDH A): CAG CAA TGC TTC CTG CAC TA; (GAPDH B): CCT CTG CCA TCT CTC CAA AG (McFarlan et al., 2009). The cycling conditions were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C 10 sec, 56 °C for 15 sec, 72 °C for 20 sec, 83 °C for zero sec, and finally a melt curve analysis from 72-95 °C. Fluorescence of the samples was measured with excitation at 470 nm and detection at 510 nm at the end of both the 72 °C and 83 °C points of each cycle.

Samples were run in duplicate and the cycle threshold of each gene was compared to a single calibrator on every run. The calibrator was created from a pool of several sparrow cDNA samples. Using a serial dilution of the calibrator I also calculated reaction efficiency for each gene. Expression in each sample was calculated as $\text{Efficiency}^{\Delta Ct}$, where ΔCt is the cycle threshold of the calibrator minus the cycle threshold of the sample. For each fatty acid transporter gene, I calculated an expression ratio, which was the expression of the transporter gene divided by the geometric average of the 2 housekeeper genes' expression (Vandesompele et al., 2002). For each transporter gene I normalized the expression ratio to the WEX treatment group arithmetic mean. mRNA was not successfully extracted from two samples, leaving sample sizes of 4 in the WEX treatment group and 9 in the MIG treatment group for mRNA expression.

Statistical analyses

Differences in enzyme activity and mRNA expression were determined by ANOVA with Tukey's post-hoc test. In cases where there was heterogeneity of variance (detected using Levine's test), mRNA data were log transformed and retested for heterogeneity of variance prior to ANOVA (Zar, 1999). To reduce variability due to background noise, only PL fatty acids above 1% mass of the total PL fatty acids were considered in the statistical analyses. Fatty acids are presented as proportions in order to standardize my results to a given amount of membrane. For proportions of individual fatty acids, $\omega 6/\omega 3$, mass, and hopping data, Kruskal-Wallis tests were used to detect differences between treatment groups. Dunn's post-hoc test was used for

multiple comparisons (Zar, 1999). Mann-Whitney tests were employed to test the effects of sex and age on fatty acid proportions. Fatty acid proportions were arcsin-transformed before utilizing linear regressions. To measure changes in the overall composition in PL fatty acids, I employed a principal component (PC) analysis with varimax rotation to obtain PC scores for each bird. All individual fatty acids that comprised more than 1 % of muscle PL as well as ω6/ω3 were used as variables in this analysis. I then used Kruskal-Wallis tests on the resultant PC scores to determine differences between treatments. Pearson correlation coefficients were calculated for several variables and I conducted tests of significance for differences from zero. Statistical significance was determined at $P < 0.05$. All analyses were performed in SPSS 11.0 (SPSS Inc., Chicago, IL).

Results

There were no significant differences in mass between treatment groups at the end of the treatment period ($P > 0.8$, Table 3.1). Sex had no effect on the proportions of any of the PL fatty acids, enzymes, or mRNA expression ($P > 0.1$). The proportion of 16:0 in PL increased with body mass ($P = 0.016$); however, no other fatty acids varied significantly with mass. HOAD increased significantly with mass ($P=0.004$) and LDH decreased significantly with mass ($P=0.005$). Birds aged "after hatch-year" had significantly higher proportions of the fatty acid 16:0 in muscle phospholipids ($P = 0.024$); this was offset primarily by decreased 18:1ω9 in these birds ($P = 0.035$). Birds aged "after hatch-year" also had significantly higher HOAD ($P=0.047$). Photoperiod had a significant effect on nighttime hopping activity of the sparrows (Table 3.1), indicating that the MIG birds were indeed experiencing a captive analog of migratory state.

Enzymes

There was no difference between treatment groups in activity of CS ($P = 0.467$), HOAD ($P= 0.746$), or LDH ($P=0.832$) (Figure 3.1). Treatment affected CPT activity ($P=0.044$), with post-hoc tests revealing greater activity in MIG than in WEX (Figure 3.1).

mRNA Expression

The treatments had no effect on the expression of either of the housekeeping genes (GAPDH, $P = 0.166$; β-Actin, $P= 0.152$), nor the geometric mean of the two

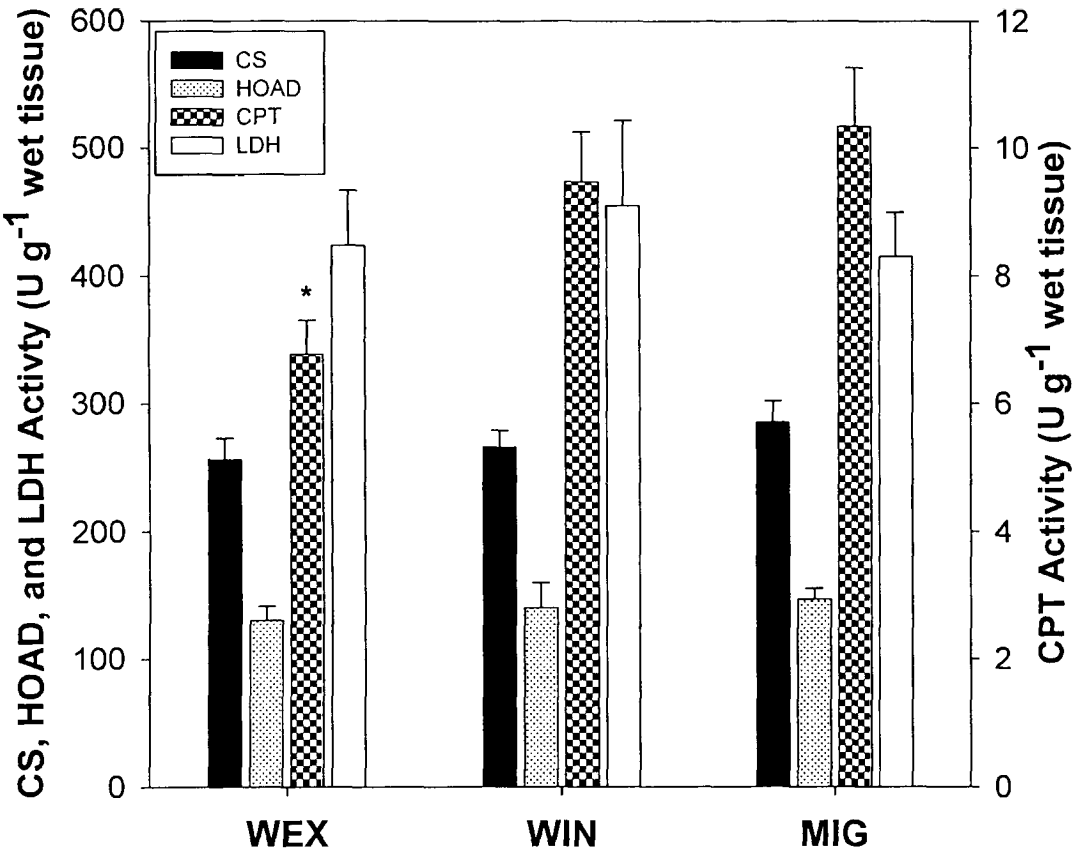

Figure 3.1. Enzyme activity of citrate synthase (CS), 3-hydroxyacyl-CoA dehydrogenase (HOAD), carnitine palmitoyl transferase (CPT), and lactate dehydrogenase (LDH) in white-crowned sparrow pectoralis muscle. Data are means + SE. WEX = winter with exercise, n=5; WIN = winter, n=8; MIG = migratory, n=10. *There is a significant difference in CPT activity between the WEX and MIG treatments ($P < 0.05$).

housekeeping genes (P=0.139), validating their use in standardizing the fat transport genes. Using these genes in a housekeeping index, there was no difference in the plasma membrane fatty acid transporters FAT/CD36 (P = 0.327) and FABPpm (P = 0.188) between treatment groups (Figure 3.2). Although there was a noticeable trend for increased cytosolic fatty acid transporter H-FABP in the MIG group (Figure 3.2), the data were highly variable such that the difference was not significant (P=0.181). There was no effect of either treatment on RNA/g muscle (P = 0.812).

Phospholipids

Photoperiod did not affect the fatty acid composition of pectoralis muscle PL, although notable trends were observed in the predicted directions for 22:6ω3 and the ratio of ω6 fatty acids to ω3 fatty acids (ω6/ω3) (Table 3.2). Additionally, of all birds measured, the 3 highest levels of ω6/ω3 and lowest levels of 22:6ω3 were measured in MIG birds. Nonetheless, there was a great deal of variability in the MIG group. This raises the possibility that not all MIG birds responded similarly to the photoperiod treatment. Specifically, birds with higher activity might have exercised enough during nightly restlessness to counteract an effect of endogenous modulation (see below). To investigate this, I examined the relationship between nightly hopping activity and ω6/ω3 and 22:6ω3 (Figure 3.3). Although there is a downward trend in ω6/ω3 with activity, the relationship is not significant (P > 0.1).

The exercise protocol caused a significant (P < 0.05) decrease in ω6/ω3 in pectoralis muscle PL (Table 3.2). This change occurred via a significant increase (P

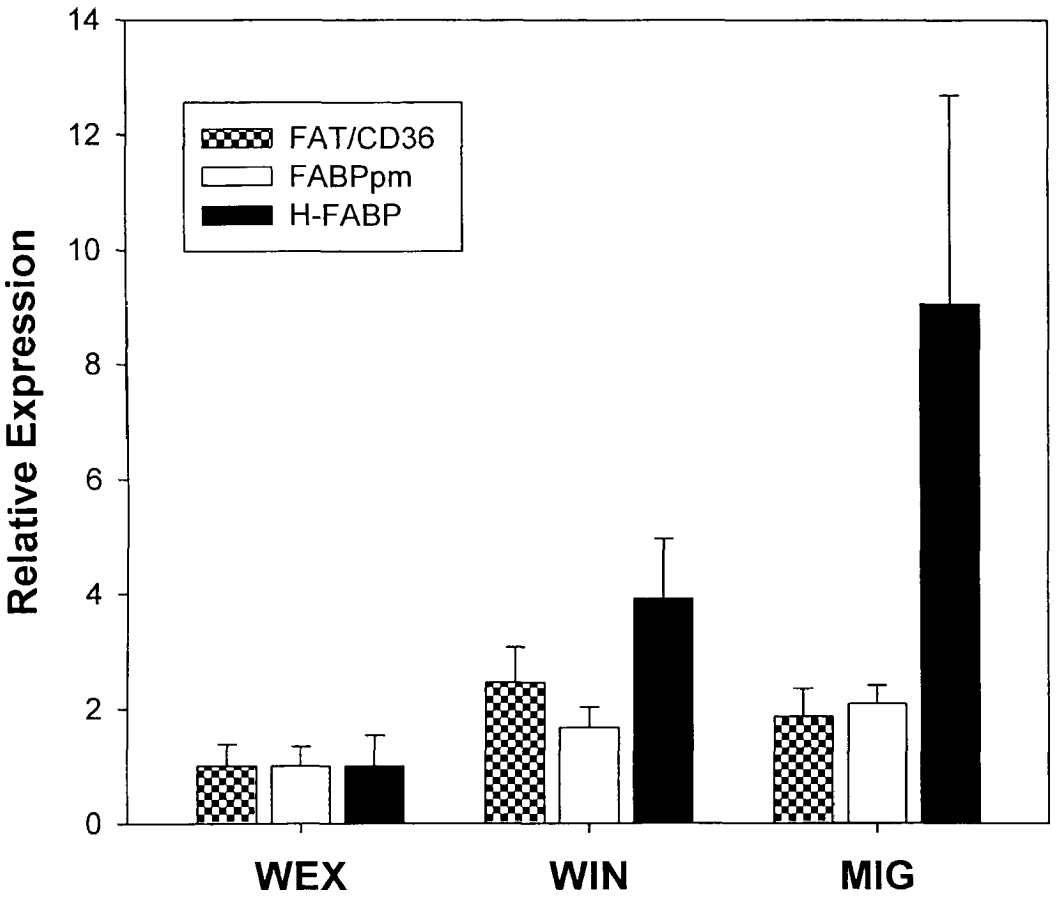

Figure 3.2. Relative mRNA expression of fatty acid transporter proteins in white-crowned sparrow pectoralis muscle. For each gene, the expression ratio (target gene expression/housekeeper gene expression) is normalized to the WEX average (which was set to 1). Data are means + SE. WEX = winter with exercise, n=4; WIN = winter, n=8; MIG = migratory, n=9. There were no significant differences between treatment groups.

Table 3.2. Percent fatty acid composition of pectoralis muscle phospholipids in white-crowned sparrows.

	Treatment		
	WEX	**WIN**	**MIG**
	(n=5)	**(n=8)**	**(n=9)**
Fatty Acid:			
16:0	19.56 ± 0.73	20.10 ± 0.48	20.37 ± 0.48
16:1ω7	0.03 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
18:0	22.45 ± 1.00	21.73 ± 0.29	21.52 ± 0.35
18:1ω9	16.67 ± 0.67	17.49 ± 0.52	17.44 ± 0.34
18:1ω7	0.90 ± 0.04	0.90 ± 0.02	0.89 ± 0.11
18:2ω6	22.24 ± 0.42	23.62 ± 0.43	23.89 ± 0.57
18:3ω6	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.01
20:0	0.37 ± 0.02	0.40 ± 0.04	0.48 ± 0.06
20:1ω9	0.17 ± 0.02	0.19 ± 0.01	0.26 ± 0.06
20:2ω6	0.10 ± 0.03	0.13 ± 0.01	0.16 ± 0.05
20:3ω6	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.02
20:4ω6	7.97 ± 0.27	8.18 ± 0.35	8.04 ± 0.31
20:5ω3	0.07 ± 0.06	0.05 ± 0.02	0.03 ± 0.02
22:0	0.43 ± 0.06	0.29 ± 0.05	0.34 ± 0.05
22:2ω6	0.13 ± 0.09	nd	0.05 ± 0.04
22:5ω3	0.15 ± 0.07	0.16 ± 0.02	0.11 ± 0.05
22:6ω3	8.38 ± 0.43*	6.40 ± 0.27	5.92 ± 0.52

24:0	0.23 ± 0.03	0.18 ± 0.02	0.25 ± 0.07
24:1ω9	0.05 ± 0.03	0.06 ± 0.02	0.12 ± 0.03
ω6/ω3[a]	3.55 ± 0.21*	4.86 ± 0.26	5.78 ± 0.76
Σ Unsaturated[a]	56.96 ± 0.33	57.29 ± 0.31	57.04 ± 0.38
Σ Monounsaturated[a]	17.81 ± 0.73	18.66 ± 0.53	18.74 ± 0.44
Σ Polyunsaturated[a]	39.15 ± 0.43	38.63 ± 0.49	38.30 ± 0.40
Longer Chain (20-24 carbon)[a]	17.48 ± 0.34	15.61 ± 0.37	15.31 ± 0.61
Double Bond Index[a]	137.47 ± 1.10	130.89 ± 1.46	128.31 ± 2.29

Note.- Data are mean mass percent ± SE; nd: not detected. Only identifiable peaks are reported.

[a]These indices were computed using only fatty acids that constituted more than 1 mass % of the total. Results of significance tests are unchanged by using all the fatty acids measured.

*Significantly different from WIN treatment group.

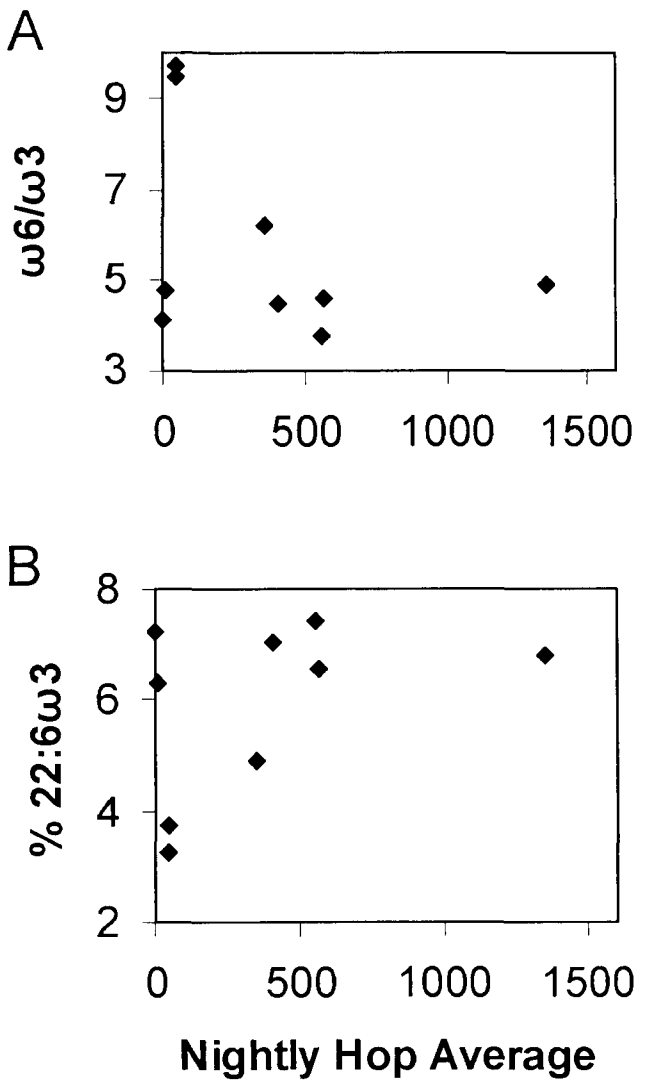

Figure 3.3. The ratio of ω6 to ω3 fatty acids (A) and the proportion of 22:6ω3 (B) in the phospholipids of MIG sparrow pectoralis muscle vs. nightly hop average. Nightly hop average was calculated for each MIG individual after the switch to long days. There is no significant trend for either variable.

< 0.01) in the proportion of 22:6ω3 as well as a trend toward decreased 18:2ω6 in exercised birds ($0.05 < P < 0.1$). There were no notable changes observed in any other fatty acids, nor in the total proportion of unsaturated fatty acids ($P > 0.5$), although there was a trend to increase in the total proportion of long-chain fatty acids ($C > 20$) with exercise (Table 3.2).

The first 2 principal components explained 68.1 % of the variation in the data (Table 3.3). PC1 can be interpreted as primarily an ω6/ω3 axis, with high loadings from ω6/ω3, 18:2ω6, and negative loading from 22:6ω3. Exercise caused a significant decrease in PC1 ($P < 0.05$, data not shown), but there was no effect of photoperiod on PC1 scores. PC2 is less easily interpreted, and did not vary by treatment group ($P > 0.8$).

Correlations

I summarized the correlations between enzyme activity and both PL fatty acid composition and mRNA expression in Table 3.4. Most fatty acids did not correlate significantly with enzyme activity. However, 22:6ω3 was significantly negatively correlated with CS and CPT activity (and near significance for HOAD; P=0.059). Similarly, the ω6/ω3 ratio was positively correlated with CS, and nearly significant for HOAD (P=0.055). There was no correlation between the total unsaturates and enzyme activity, but the total percentage of long-chain fatty acids (20 carbons or more) correlated negatively with HOAD (and near significance for both CS [p=0.056] and CPT [p=0.054]). All the measured enzymes correlated most strongly with 16:0, and inversely with 18:0 (the later significantly for CPT and LDH).

Table 3.3. Principal component (PC) loadings and percent variance explained by major components for pectoralis muscle phospholipid fatty acids.

	PC1	PC2
Fatty acid:		
16:0	0.41	-0.56
18:0	-0.30	0.84
18:1ω9	-0.05	-0.75
18:2ω6	0.85	0.13
20:4ω6	0.21	0.60
22:6ω3	-0.94	0.13
ω6/ω3	0.96	-0.09
Variance explained:	41.8 %	26.3%

Table 3.4. Correlations between enzyme activity and pectoralis muscle phospholipid fatty acid composition and mRNA expression.

	Pearson Correlation Coefficient			
	CS	**HOAD**	**CPT**	**LDH**
Enzyme:				
CS		0.674**	0.718**	-0.529**
HOAD			0.813**	-0.761**
CPT				-0.533**
Fatty Acid:				
16:0	0.599**	0.618**	0.636**	-0.565**
16:1ω7	-0.095	-0.239	-0.382	0.393
18:0	-0.095	-0.247	-0.455*	0.451*
18:1ω9	0.052	-0.162	0.179	0.120
18:1ω7	-0.048	0.137	-0.046	0.347
18:2ω6	0.152	0.407	0.173	-0.233
18:3ω6	0.048	0.245	-0.960	-0.306
20:0	0.196	0.018	0.170	-0.112
20:1ω9	0.367	0.244	0.306	-0.298
20:2ω6	0.354	0.255	0.500*	-0.130
20:3ω6	0.097	0.105	0.284	-0.309
20:4ω6	0.163	0.258	0.075	-0.153
20:5ω3	0.013	0.060	-0.112	-0.138
22:0	-0.393	-0.394	-0.214	0.094

22:2ω6	-0.091	-0.133	-0.166	0.242
22:5ω3	-0.281	-0.119	0.019	0.017
22:6ω3	-0.470*	-0.409	-0.438*	0.380
24:0	-0.468*	-0.328	-0.103	-0.058
24:1ω9	-0.178	-0.127	0.144	-0.147
ω3	-0.477*	-0.401	-0.448*	0.364
ω6	0.226	0.493*	0.210	-0.283
ω6/ω3[a]	0.474*	0.414	0.334	-0.418
Total Unsaturated[a]	-0.186	0.114	0.036	0.111
Longer Chain (20-24 carbon)[a]	-0.413	-0.501*	-0.416	0.332
Gene:				
FAT/CD36	-0.317	-0.300	-0.209	0.517*
$FABP_{pm}$	0.134	0.258	0.042	0.100
H-FABP	-0.071	0.108	0.125	-0.046

[a]These indices were computed using only fatty acids that constituted more than 1 mass % of the total. Results of significance tests are unchanged by using all the fatty acids measured.

*P<0.05; **P<0.01.

In general, mRNA expression did not significantly correlate with membrane PL fatty acid composition (Table 3.5). FABPpm expression did significantly correlate with a few fatty acids, notably 18:2ω6.

Table 3.5. Correlations between mRNA expression and pectoralis muscle fatty acid composition.

	Pearson Correlation Coefficient		
	FAT/CD36	**FABPpm**	**H-FABP**
Gene:			
FAT/CD36		0.535**	0.330
FABPpm			-0.055
Fatty Acid:			
16:0	-0.156	0.167	0.379
16:1ω7	0.257	0.458*	0.095
18:0	0.263	0.092	-0.005
18:1ω9	0.201	-0.015	-0.210
18:1ω7	0.104	0.026	0.093
18:2ω6	0.034	0.505*	0.230
18:3ω6	0.119	0.133	0.168
20:0	-0.183	0.120	-0.220
20:1ω9	-0.151	0.028	-0.147
20:2ω6	-0.134	-0.453*	-0.020
20:3ω6	-0.089	-0.380	-0.081
20:4ω6	-0.366	0.093	-0.336
20:5ω3	-0.096	-0.302	0.263
22:0	-0.206	-0.180	-0.228
22:2ω6	-0.260	-0.282	-0.232

22:5ω3	-0.035	-0.230	0.011
22:6ω3	-0.003	-0.307	-0.059
24:0	-0.282	-0.133	-0.084
24:1ω9	0.114	-0.217	0.197
ω6/ω3[a]	-0.127	0.248	-0.028
Total	-0.041	0.313	-0.184
Unsaturated[a]			
Longer Chain	-0.095	-0.487*	-0.249
(20-24 carbon)[a]			

[a]These indices were computed using only fatty acids that constituted more than 1 mass % of the total. Results of significance tests are unchanged by using all the fatty acids measured.

*P<0.05; **P<0.01.

Discussion

Birds undergo various physiological changes associated with endurance exercise during the migratory stage of their life histories. At least some of these changes can occur in advance of migratory flight and without exercise training, a phenomenon evidenced by migratory hyperphagia (Ramenofsky et al., 2003) and muscular hypertrophy (Dietz et al., 1999; Driedzic et al., 1993; Jehl, 1997). However, the degree to which birds can prepare their muscles for migration without training is largely unknown (Guglielmo et al., 2002a), and was the focus of this study. As photoperiod is the major cue stimulating migratory behavior (Ramenofsky et al., 2003), it makes sense to use captive birds stimulated by photoperiod to investigate these potential changes. I chose a study species that has been used extensively in zugunruhe studies, but is a short-bout migrant. It is possible that my results would have been different with a migrant species that must pass a long distance barrier such as an ocean. My data on nightly activity indicate that my birds did indeed respond to the photoperiodic manipulation with typical migratory behaviors. But I note that there was substantial variability in nightly activity within the MIG treatment group, and this may indicate that some birds were not responsive to the manipulation, although removal of two low-hopping MIG birds did not substantially change the results of this study (data not shown). Further, nightly activity, i.e. hopping and wing-fluttering, may serve as a type of exercise training, an artifact of the manipulation that cannot be avoided (Landys et al., 2004a) except perhaps by a migratory control group with complete darkness at night (M. Ramenofsky, personal comm.). Additionally, I note that my exercise treatment may not be representative of

the endurance exercise associated with migration. Endurance exercise simulating flight is notoriously difficult to achieve in the lab without a high quality wind tunnel. The exercise treatment available was more of a burst exercise, and this may have resulted in the limited changes in enzymes and mRNA seen in the WEX treatment group.

Effects of 'migratory' photoperiod on oxidative and lipid catabolic pathways

Aerobic capacity, measured as mitochondrial volume density or by CS, is generally greater in birds in the migratory season than during winter (Evans et al., 1992; Guglielmo et al., 2002a; Lundgren and Kiessling, 1985). Nonetheless, it may remain constant or decrease during a migratory stopover (Driedzic et al., 1993; Evans et al., 1992; Marsh, 1981). This latter finding may be explained by the present study in which CS did not change, indicating that overall oxidative capacity doesn't change in response to photoperiod and so high oxidative capacity may only be induced by long-distance flight. On the other hand, CPT and HOAD, enzymes associated with fat metabolism, have generally been found to increase both seasonally and during stopover refueling (Driedzic et al., 1993; Guglielmo et al., 2002a; Lundgren and Kiessling, 1985; Marsh, 1981). This would indicate that fat catabolic enzymes can increase in anticipation of a migratory flight. However, this contrasts with the current study, in which neither CPT nor HOAD were higher in birds in the WIN treatment compared to those in the MIG treatment, although CPT did increase in the MIG group compared to the winter/exercised (WEX) treatment.

In addition to catabolic capacity as measured by CPT or HOAD, the utilization of lipids to fuel migration also necessitates an abundance of fatty acid transporters. mRNA expression of the membrane fat transporters FAT/CD36 and FABPpm increases ~2 to 4 fold during natural migration in white-throated sparrow pectoralis muscles (McFarlan et al., 2009). mRNA expression of the cytosolic fat transporter H-FABP increases ~9-12 times in white-throated sparrows (McFarlan et al., 2009), and H-FABP protein increases from ~6 to ~10% of cytosolic protein during migration in western sandpipers (*Calidris mauri* Cabanis) (Guglielmo et al., 2002a). In this latter study, there was no difference between the H-FABP levels of wintering and premigratory sandpipers, and those authors suggested that endurance flight may be necessary for induction of H-FABP expression. The present results tend to support that conclusion, as none of the mRNA of fat transporter proteins increased significantly in the MIG treatment group, although there was a strong trend for increased H-FABP.

The role of muscle phospholipids in exercise

Although there has been much recent interest in the subject, there is still little understood about how muscle phospholipid FA composition affects exercise. This is in part due to the difficulty in independently manipulating membrane composition *in vivo*. Some studies have manipulated membrane composition by manipulating diet and then measuring the effects on exercise (Ayre and Hulbert, 1996; Ayre and Hulbert, 1997; McKenzie et al., 1998; Pierce et al., 2005; Turner et al., 2004). However, these studies are unable to distinguish between the exercise effects arising

from membrane changes, and those arising from other changes caused by the diet, such as changes in adipose composition (Chapter 2) or eicosanoid production (Craig-Schmidt et al., 1987; Li et al., 1994). Other studies have manipulated exercise and examined the effects on PL acyl composition (Andersson et al., 2000; Andersson et al., 1998; Helge et al., 1999; Petridou et al., 2005). It is unclear, however, what inferences can be made from these studies about how PL acyl composition can in turn affect exercise. Still others have examined the muscle PL of 'high performance' muscles in athletic animals (Infante et al., 2001; Valencak et al., 2003). In these cases, it can be difficult to determine whether the PL FA profiles observed represent adaptations to exercise or perhaps negative consequences of exercise. In the current study, I attempted to circumvent these difficulties using the photoperiodic manipulation. Specifically, if FA composition had changed in anticipation of migration due to photoperiod, I would conclude that the change observed is beneficial to exercise. The inverse does not hold however; the lack of change in membrane composition could indicate either that PL FA composition was unrelated to exercise, or that the birds were unable to endogenously modify PL in the absence of exercise activity.

A previous study observed seasonal differences in muscle PL in migratory sandpipers (Guglielmo et al., 2002b), but could not separate the effects of diet and exercise from any endogenously produced adaptive changes to muscle PL fatty acid composition. As there were no significant differences in muscle PL between MIG and WIN birds, the results of the current study argue against endogenous adaptive modulation of muscle PL composition in preparation for migration. However, trends

in ω6:ω3 and 22:6ω3 that oppose the significant differences observed due to exercise leave open a possibility of a pre-migratory effect which deserves closer study with larger sample sizes and control of nighttime activity. In my study MIG birds may have been hopping and fluttering enough to cause an exercise effect that counteracted any preparatory modulation, resulting in the non-significant trend. As exercise had an effect on muscle PL fatty acid composition, the present study reinforces the possibility that muscle PL composition may be important to migratory birds, although the results do not allow conclusions as to which specific fatty acids are beneficial or detrimental to exercise performance.

To my knowledge, the direct effect of exercise on muscle PL has never been studied previously in birds. Results of mammalian studies have been divergent, and due at least in part to differences in species and exercise protocol. For example, linoleic acid (18:2ω6) decreased as a proportion of muscle PL in lean rats after 2 wk training (Ayre et al., 1998) and in humans after 6 wk training (Andersson et al., 1998). In other studies, however, the same fatty acid increased in the muscle PL content of rats after exercise (Helge et al., 1999; Petridou et al., 2005; Turner et al., 2004). Similarly, arachidonic acid (20:4ω6) was found to increase consistently in muscle PL of rats due to eccentric contractions (Helge et al., 2001a), and humans after training (Helge et al., 2001b), yet the same authors previously reported a decrease in 20:4ω6 with exercise in rats (Helge et al., 1999). Muscle type, genetic background, and diet may account for some of these differences between studies (Ayre et al., 1998; Turner et al., 2004).

Docosahexaenoic acid (22:6ω3) has been associated with high performance endurance muscles such as rattlesnake rattle muscles and hummingbird pectoralis muscles (Infante et al., 2001). This fatty acid also increased in human muscle PL after training (Andersson et al., 2000) although it decreased in trained rats (Helge et al., 1999) and was unchanged by exercise in a number of other studies. Guglielmo et al. (2002b) found 22:6ω3 to be higher in muscle PL of migratory western sandpipers during a fall stopover compared to winter birds. Maillet and Weber (2007) found correlations between membrane 22:6ω3 and oxidative enzymes in sandpipers that were eating 22:6ω3-rich amphipods at a stopover site. This correlation, however, may be driven by the time spent at stopover; 22:6ω3 increased as the sandpipers approached departure (Maillet and Weber, 2006), a time when oxidative enzyme activity may also be independently ramping up (see previous discussion). In fact, I generally found 22:6ω3 to be negatively correlated with oxidative enzyme activity. The major effect of exercise in the current study was to increase the proportion of 22:6ω3 in the pectoralis muscle PL of white-crowned sparrows. Unresolved, however, is the functional importance of this change and the association between 22:6ω3 and high-performance muscles, as 22:6ω3 did not change significantly with photoperiod in the present study. In my study, increased 22:6ω3 could also be associated with stress from the exercise protocol, as catecholamines were found to increase 22:6ω3 content in rat hearts (Gudbjarnason, 1989). This requires further experimental study.

Correlation analysis

As found by Guglielmo et al. (2002a), I found significant positive correlations among CS, HOAD, and CPT, indicating that their expression may involve similar control elements. Additionally all of these enzymes correlated negatively with LDH. Similarly, the expressions of FAT/CD36 and FABPpm mRNA were positively correlated with each other, duplicating the finding of McFarlan (2009) in migrating sparrows, although the expression of neither gene was correlated with that of H-FABP. Recent studies in mammals indicate that these two membrane fatty acid transporters collaborate to increase fatty acid transport (Chabowski et al., 2007; Holloway et al., 2008). Interestingly, there was no significant correlation between the lipid catabolism enzymes' activities and the expression of the lipid transporter mRNA.

Some of the strongest correlations between enzyme activity and pectoralis muscle PL fatty acids were with 16:0. It is unclear why this should have occurred, as saturates are not generally proposed to affect enzyme activity. I note that 'hatch year' birds had less 16:0 than birds aged 'after hatch year', and that the correlations between 16:0 and enzyme activity were generally offset by opposite correlations in 18:0 (although less significantly so). The most notable relationship was a negative correlation between 22:6ω3 and CS and CPT. The correlation with CPT remained significant even when examined only within the WIN treatment group. These findings seem to counter the hypothesis that 22:6ω3 can be used as a 'natural doping' agent by migratory birds (Maillet and Weber, 2007), at least in their capacity as parts of muscle membranes. Fatty acid transporter mRNA expression seems to be

relatively independent of membrane fatty acids, with the possible exception of 18:2ω6, which was positively correlated with FABPpm.

In conclusion, I found little effect of migratory photoperiod on muscle oxidative enzymes, lipid transporters, or phospholipids in the white-crowned sparrow. Seasonal change in these factors is typical of wild migrants. My findings therefore indicate that factors besides photoperiod (perhaps migratory flight itself) are needed to induce these changes, or that the zugunruhe model may not successfully replicate the wild condition with regard to these aspects of muscle physiology.

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CHAPTER 4

THE EFFECT OF MUSCLE PHOSPHOLIPID FATTY ACID COMPOSITION ON EXERCISE PERFORMANCE: A DIRECT TEST IN THE MIGRATORY WHITE-THROATED SPARROW (*ZONOTRICHIA ALBICOLLIS*)[1]

Introduction

Dietary polyunsaturated fatty acids (PUFA) are essential nutrients that play pivotal roles in animal health (Simopoulos, 1999) and metabolism (Hulbert and Else, 1999). Although PUFA can be oxidized for energy, they also serve as essential structural components of various biological molecules. They can be incorporated into eicosanoids, signaling molecules that play roles in inflammation, platelet aggregation, and other processes. As part of phospholipids (PL) in membranes, they have been implicated in controlling metabolic rate (Hulbert and Else, 1999). They are also known to regulate gene expression, particularly in hepatic and adipose tissues through peroxisome proliferator-activated receptors (PPARs) (Sessler and Ntambi, 1998). These varied effects of PUFA acting as structural, energy, regulatory, and signaling molecules can in turn affect processes at higher levels of organization.

Many recent studies have focused on the effects of PUFA on exercise performance in humans and other animals (Infante et al., 2001; Maillet and Weber, 2007; McKenzie et al., 1998; Nikolaidis and Mougios, 2004). The mechanism by which dietary PUFA affect exercise performance is unknown, but it has often been ascribed to the associated changes in muscle PL composition. For example, Ayre and

[1] A version of this chapter has been published in the American Journal of Physiology (Price and Guglielmo, 2009) and is used with permission from the American Physiological Society.

Hulbert (1997) demonstrated that treadmill running endurance in rats could be affected by dietary PUFA, a result they concluded was due to changes in the composition of muscle PL fatty acids. Various other studies have demonstrated an effect of exercise on muscle PL fatty acid composition or vice versa (Andersson et al., 1998; Infante et al., 2001; Turner et al., 2004; Chapter 3). Further, a comparative analysis of maximal running speed in mammals revealed a positive correlation with the proportion of muscle PL ω6 fatty acids (Ruf et al., 2006). Nonetheless, while membrane PL composition is often proposed as a mechanism to explain the performance differences observed in these studies, this hypothesis has not been thoroughly tested with manipulative experiments. For example, dietary manipulations intended to change PL fatty acid composition can also affect adipose fatty acid composition, which itself may affect exercise performance (Johnston, 1973; Pierce et al., 2005; Chapter 2). The failure to tease apart these potential mechanisms by which PUFA affect exercise is in large part due to the difficulty of directly and independently manipulating membrane composition *in vivo*.

Because of their extreme athletic accomplishments, migratory birds have recently received attention as models for studying the effects of dietary fatty acids on exercise (Pierce et al., 2005; Chapter 3). For example, Maillet and Weber (2006; 2007) demonstrated correlations between oxidative capacity and muscle membrane PL composition in shorebirds eating a diet rich in ω3 fatty acids at a migratory stopover. Aside from their athletic prowess, migratory birds also provide an animal model that can rapidly change the amount of stored adipose triacylglycerol. In the present study, I take advantage of this trait, as well as the difference in turnover rates

between adipose triacylglycerol and muscle PL. By employing a dietary composition manipulation and a food restriction, I test whether muscle membrane fatty acid composition is driving exercise performance differences. Further, I examine the effects of the diets rich in either ω3 or ω6 PUFA on muscle enzyme activities and fatty acid transporter mRNA expression.

Materials and Methods

Animals

Fifty four white-throated sparrows (*Zonotrichia albicollis*) were captured during migration through southwest Ontario, Canada. White-throated sparrows were used because they are common long-distance short-bout migrants that are relatively easy to keep in captivity and will readily eat synthetic diets. Sparrows were individually housed in 40 cm (height) by 45 cm by 45 cm cages for the duration of the study. Sex was determined after the experiment by inspection of the gonads. Experimental protocols were approved by the University of Western Ontario Animal Use Subcommittee (Protocol #2005-060-08) and a collection permit was obtained from the Canadian Wildlife Service (CA 0170).

Diets and dietary manipulation

Two isocaloric synthetic diets were used in the experiment, modified from the "grain" diet of Pierce and McWilliams (2004)(Table 4.1) and differing from each other only in the type of oils used. The 'high ω6' diet contained 10% Loriva extra virgin sesame oil (nSpired Natural Foods, San Leandro, CA) and 3% refined menhaden oil (MP Biomedicals, Solon, OH) by mass. The 'high ω3' diet contained 10% menhaden oil and 3% sesame oil. These diets should supply more than the minimum dietary requirements of essential fatty acids for birds (National Research Council Board on Agriculture, 1994; Stevens, 1995). An 'intermediate' diet contained 6.5% of both oils. All sparrows were provided water *ad libitum* throughout

Table 4.1. Composition of semi-synthetic diets.

Ingredients		Percent Mass
Corn starch[a]		61.53
Casein (high N)[a]		10.0
Amino acid mix[b]		2.77
Vitamin and minerals mix (AIN-76)[c]		1.0
Salt mix (Briggs-N salt mixture)[c]		5.5
Cellulose (Celufil)[a]		5.0
Sodium bicarbonate[d]		1.0
Choline chloride[a]		0.2
Oil Mixture[e]		13.0
Fatty Acid[f]:	High ω3	High ω6
16:0	20.6	16.8
16:1ω7	6.1	1.7
18:0	13.0	15.5
18:1ω9	12.8	26.3
18:1ω7	2.6	1.2
18:2ω6	11.2	29.3
18:3ω3	1.4	0.7
20:4ω6	1.7	0.6
20:5ω3	10.2	2.4
22:5ω3	2.8	0.6
22:6ω3	11.6	2.7

[a]U .S. Biochemicals Corp, Cleveland, Ohio.

[b]Amino acid mix from Murphy and King (1982), all amino acids supplied by U. S. Biochemicals except methionine (supplied by Sigma).

[c]MP Biomedicals, Solon, Ohio.

[d]BDH Chemicals, Poole, England.

[e]See text for details of oil mixture.

[f]Major fatty acids components are listed here as percent of total fatty acids as measured by gas chromatography of fat extracted from the diets.

all experiments. The first experiment tested whether dietary fatty acids can alter exercise performance. Thirteen sparrows were fed high ω6 diet *ad libitum* ('Hω6' treatment), and 13 sparrows were fed high ω3 diet *ad libitum* ('Hω3' treatment). Exercise performance was assessed (see below) at the conclusion of 3 weeks on the diet.

The second experiment tested whether the effects on exercise observed in the first experiment were caused by changes in muscle membrane fatty acid composition, or another change such as composition of adipose triacylglycerol stores. In this experiment I employed a restriction of food followed by feeding the intermediate diet (thus these treatments were named the 'Hω6RI' and 'Hω3RI' treatments). Specifically, 14 sparrows were fed the high ω6 diet (Hω6RI) and 14 sparrows were fed the high ω3 diet (Hω3RI) *ad libitum* for 3 weeks, (similar to the Hω6 and Hω3 treatments). This altered the fatty acid composition of both muscle membrane fatty acids and adipose fatty acids (Figure 4.1). I then restricted the total amount of diet provided to the birds each day, causing them to utilize triacylglycerol from their adipose stores. Birds store most extramuscular lipids in the furcular hollow and abdomen; these stores are visible through the translucent skin (Kaiser, 1993). Thus, I was able to determine when the sparrows had essentially exhausted their extramuscular lipid stores. I found that the sparrows could generally maintain weight on 7 g of food, and that providing less than 3 g of food each day successfully reduced fat stores within a few days. The amount of food given was tailored to each bird's response to the restriction; I attempted to exhaust visible fat stores in four days unless an individual started with exceptionally large fat stores. This timeline was generally

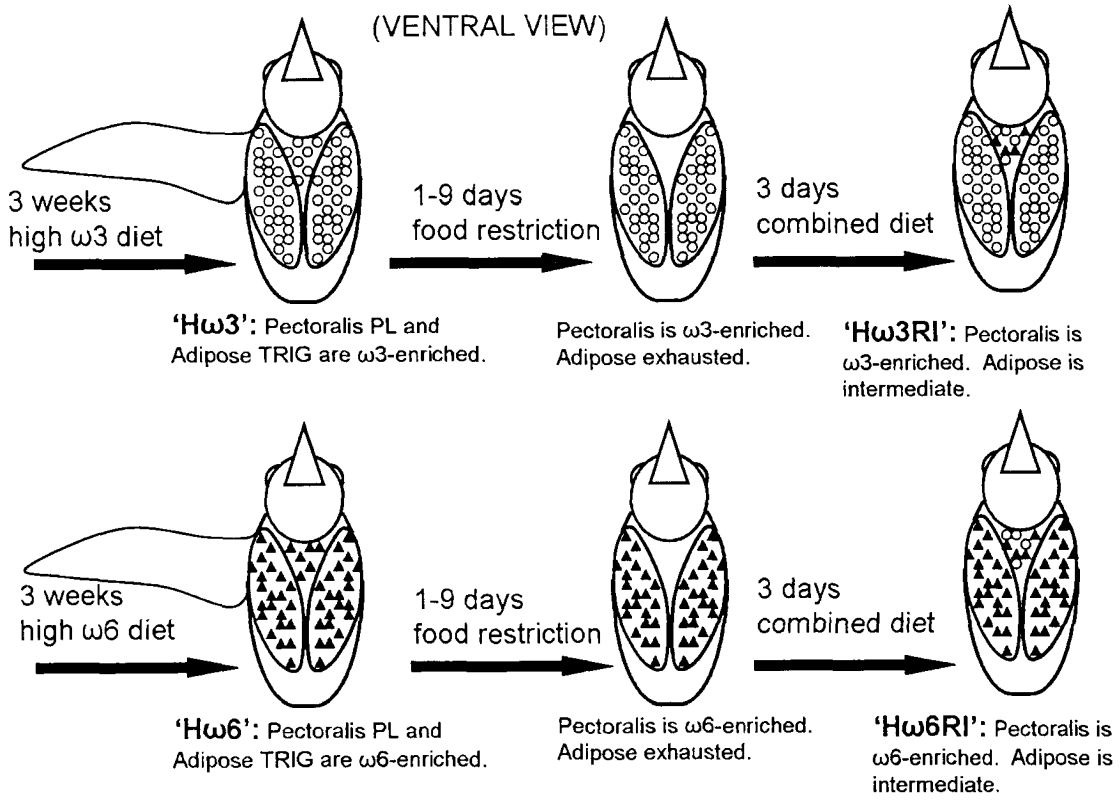

Figure 4.1. Schematic of the diet manipulation. A ventral view of sparrows is depicted, showing the fatty acid composition of the pectoralis PL and adipose tissue triacylglycerol (the main avian adipose depot lies in the furcular hollow between the paired pectoralis muscles). Triangles denote tissues enriched with ω6 PUFA; circles denote those enriched with ω3 PUFA. Twenty-six sparrows (13 Hω3 and 13 Hω6) only underwent the first step in this protocol before the exercise assessment and euthanasia for tissue sampling. A distinct group of 28 sparrows (14 Hω3RI and 14 Hω6RI) underwent the entire diet manipulation, food restriction, and fattening before exercise assessment and euthanasia for tissue sampling.

successful, although this process varied from 1-9 days. At that point, I provided the intermediate diet *ad libitum* so that both groups of sparrows would store adipose fat of the same composition (Figure 4.1). Turnover of fatty acids in muscle PL was expected to be slower than adipose triacylglycerol (Thil et al., 2003), such that muscle PL differences would persist between diet groups, while adipose differences would disappear. The success of this manipulation was verified by post-mortem analysis of tissue fatty acid composition (see Results). On the 3rd day of eating the intermediate diet, birds were assessed for exercise performance.

Exercise performance

I assessed exercise performance in an enclosed 'flight wheel', similar to that used by Chappell et al. (1999) and others (Buttemer et al., 2008; Pierce et al., 2005). The rubber-lined wheel (16 cm width, 24 cm diameter) was continuously supplied with air (3.5 l min^{-1} STP), that had been dried (Drierite, W.A. Hammond Drierite Company, Xenia, OH) and scrubbed of CO_2 (Ascarite, Thomas Scientific, Swedesboro NJ). Air exiting the chamber was subsampled at ~150 ml min^{-1} and dried before entering a CO_2 analyzer (Sable Systems, model CA-2A, Las Vegas, NV), passed through ascarite to remove CO_2, and then drawn through an O_2 analyzer (Sable Systems, model FC-1B). Analyzers were calibrated daily with nitrogen and a span gas (21% O_2/2% CO_2/77% N_2). Analyzer output (6 readings min^{-1}) was recorded and analyzed on a laptop computer with Datacan software (Sable Systems).

On the day of exercise assessment, food was removed from the birds' cages 3 hours before exercise to ensure that the animals were in a post-absorptive state. Prior

to assessing exercise, ~75 µl blood was drawn from the alar vein and spun down in a hematocrit centrifuge. The bird was placed in the wheel which was then covered to induce the bird to rest. After 15 min, I removed the cover and began spinning the wheel for 30 min. The spinning induces the bird to flutter and hop, generally achieving a high metabolic rate early which then decreases. One experimenter performed the tests for all birds, and the wheel was spun at the maximum speed that the bird could maintain. I calculated a peak metabolic rate (PMR) from the maximum average V_{O2} achieved over a 5-min interval. Immediately following the exercise bout, sparrows were euthanized by cervical dislocation under isofluorane anesthesia. Pectoralis muscle and adipose tissue were immediately dissected out and stored at -80 °C for later analysis.

Lipid extraction and quantification

Lipids were extracted as in Guglielmo et al. (2002) from a subset of Hω3 and Hω6 birds and from nearly all Hω3RI and Hω6RI birds. Briefly, pectoralis (50-100 mg) or adipose (5-10 mg) was homogenized 3 x 10 s in 15 ml chloroform:methanol (1:1 v/v; all solvents supplied by Caledon Laboratories, Georgetown, ON) with a high-speed stainless homogenizer to extract all lipids. This was centrifuged for 15 min at 2056 g. I filtered this (Whatman no. 1) and added 10 ml chloroform/methanol (2:1 v/v). I then added 6 ml 0.25% KCl to partition and remove hydrophilic solutes. The solvent was evaporated (Rotovapor, Buchi, Switzerland) and lipids were resuspended in benzene/methanol (2:1 v/v) for storage at -20 °C. Later the benzene:methanol was evaporated and lipids were resuspended in 100 µl chloroform

for loading onto Supelclean solid phase extraction tubes (Supelco, LC-NH2, 100 mg, Sigma-Aldrich Canada, Oakville, ON). Neutral lipids (NL; mostly triacylglycerol) were eluted with 1.8 ml chloroform:isopropanol (2:1 v/v). Non-esterified fatty acids (NEFA) were then eluted with 1.6 ml isopropyl ether:acetic acid (49:1 v/v). PL were eluted with 3 ml methanol. I added heptadecanoic acid (17:0; 200 µl, 30 mg/100 ml in hexane, Sigma) as an internal standard, and then evaporated the PL fraction to dryness under N_2. The PL fraction was transesterified with 2 ml acetyl chloride in methanol (1M), which was heated for 2 h at 90 °C. The solvent was evaporated under N_2 and fatty acid methyl esters were resuspended in 1 ml dichloromethane. This was then diluted 1:10 in dichloromethane for injection into the gas chromatograph.

Fatty acids were separated on a 6890N gas chromatograph (Agilent Technologies Santa Clara, CA) with a J&W Scientific high resolution gas chromatography column (DB-23, Agilent Technologies) and flame ionization detector. The carrier gas was He. The temperature program was 2 min at 80 °C, increase by 5 °C/min to 180 °C, then hold 3 min, increase by 1.5 °C/min to 200 °C, then increase 10 °C/min to 240 °C, then hold 3 min. Fatty acids with fewer than 16 carbons were excluded from statistical analysis because precautions to minimize volalitzation were not taken. Only fatty acids constituting more than 0.5 % mass of the total fatty acids in pectoralis PL or adipose were considered in the analyses. Results are presented as mass percents of the fatty acid methyl esters. The identities of fatty acids were determined by comparison of retention times to those of standard mixtures of fatty acid methyl esters (Supelco 37 component mix FAME mix, PUFA No. 3 from Menhaden Oil, F.A.M.E. Mix C8-C24, Sigma).

To better understand the ability of the birds in different treatment groups to mobilize fatty acids, I used the double bond index (DBI, the number of double bonds per 100 fatty acids), as well as 2 indexes I created based on my previous work (Chapter 2). The Mobilizability Index is an index of expected grams of fatty acid mobilized per time, assuming relative mobilization values measured previously (Chapter 2) are proportional to mobilization rates. This was calculated as Σ[(Mass % FA_i)(Relative Mobilization of FA_i)], where Relative Mobilization of FA_i comes from values for *Philomachus pugnax* (Chapter 2). Rates for *P. pugnax* were used because the diversity of fatty acids in their adipose (Chapter 2) allowed for construction of this index using all the adipose fatty acids observed in the current study. Similar patterns of mobilization were also observed in the current study species (ERP, unpublished results) and in the congener *Z. leucophrys* (Chapter 2), but with a much more limited set of fatty acids. The ATP Mobilization Index was the same index converted from g to expected ATP production from β-oxidation of the mobilized fatty acids. It was calculated as Σ[(Mobilizability Index)(mol ATP/g FA_i)]. As these indices were created based on mobilization rates measured in another species, I do not present them as quantitative rates, but rather as qualitative indices of mobilization.

Enzyme activity

Approximately 100 mg pectoralis samples were used for enzyme assays. Carnitine palmitoyl transferase (CPT; EC 2.3.1.21), citrate synthase (CS; EC 2.3.3.1), 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35), and lactate dehydrogenase (LDH; EC 1.1.1.27) were assayed as previously reported (Chapter 3).

mRNA expression

Expression of two membrane fatty acid transporters, fatty acid translocase (CD36/FAT) and plasma membrane fatty acid binding protein (FABPpm), the cytosolic transporter H-FABP, and two housekeeping genes (β-actin and GAPDH) were measured using real-time PCR according to the methods of (2009) and are previously described (Chapter 3). Briefly, pectoralis muscle was ground in a glass homogenizer with TRIzol (Invitrogen, Burlington, ON). RNA was extracted and then reverse transcribed to create complementary DNA (cDNA). I prepared negative controls using water instead of RNA. Real-time PCR was run for each gene separately with a Rotor-Gene 6000 Real-Time Rotary Thermocycler (Corbett Life Science, Concorde, NSW, Australia). Fluorescence of the samples was measured at the end of each cycle.

Samples were run in duplicate and the cycle threshold of each gene was compared to a single calibrator on every run. The calibrator was created from a pool of several sparrow cDNA samples. Using a serial dilution of the calibrator I also calculated reaction efficiency for each gene. Expression in each sample was calculated as $\text{Efficiency}^{\Delta Ct}$, where ΔCt is the cycle threshold of the calibrator minus the cycle threshold of the sample. For each fatty acid transporter gene, I calculated an expression ratio, which was the expression of the transporter gene divided by the geometric average of the 2 housekeeper genes' expression. For each transporter gene I normalized the expression ratio to the Hω3 treatment group arithmetic mean.

Statistical analyses

Sex had no effect on performance variables, so I combined sexes for further statistical analysis. The effect of body mass was not significant when included as a covariate. For analysis for fatty acid percentages, I performed an arcsine square root transformation prior using t-tests to determine differences between the Hω3 and Hω6 groups and differences between the Hω3RI and Hω6RI treatment groups. ANOVA were used with Tukey's post-hoc tests to determine differences in performance, enzyme activity, and gene expression. mRNA data were log transformed prior to ANOVA where there was heterogeneity of variance. Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago IL). Data in the text are presented as means ± SE.

Results

Fatty acid analysis

The diets caused significant changes in both adipose triacylglycerol composition (Table 4.2) and muscle PL fatty acid composition (Tables 4.3). The adipose tissue of the Hω3 and Hω6 birds became distinct in fatty acid composition, with Hω3 adipose enriched with 20:5ω3 and 22:6ω3, and the Hω6 adipose enriched with 18:2ω6. Notably, proportions of nearly all of the fatty acids differed between the Hω3 and Hω6 treatments (Table 4.2). Pectoralis PL fatty acid composition was also affected by diet, although the effects were more restricted. The PL fatty acid composition differed only between the Hω3 and Hω6 groups for certain unsaturates, i.e., 18:1ω9, 18:2ω6, 20:4ω6, and 22:6ω3 (Table 4.3).

After the food restriction and then feeding on the intermediate diet, differences in adipose composition of the Hω3RI and Hω6RI groups largely disappeared, with only 16:0 and 18:2ω6 remaining significantly different between groups (Table 4.2). The proportions of pectoralis PL fatty acids were significantly different between the Hω3RI and Hω6RI groups, and a few fatty acids were significantly different between these groups that had not been so prior to the restriction (Table 4.3).

In the adipose, DBI was significantly greater in Hω3 than Hω6 birds, but significantly greater in Hω6RI than Hω3RI birds. Both the Mobilizability Index and ATP Mobilizability Index were significantly greater in Hω6 than Hω3 birds and greater in Hω6RI than Hω3RI birds (Table 4.2).

Table 4.2. Fatty acid composition of adipose triacylglycerol from white-throated sparrows in different dietary treatments.

	Hω3 (n=7)	Hω6 (n=5)	Hω3RI (n=14)	Hω6RI (n=13)
Fatty acids:				
16:0	27.16 ± 0.68	19.70 ± 0.40**	24.73 ± 0.54	22.22 ± 0.44**
16:1ω7	7.10 ± 0.29	2.42 ± 0.19**	5.54 ± 0.16	5.40 ± 0.15
18:0	9.66 ± 0.26	8.46 ± 0.34*	9.21 ± 0.39	8.27 ± 0.39
18:1ω9	25.90 ± 1.21	39.08 ± 0.92**	30.85 ± 0.40	31.64 ± 0.61
18:1ω7	2.33 ± 0.05	1.19 ± 0.02**	1.79 ± 0.04	1.82 ± 0.03
18:2ω6	14.43 ± 0.37	26.53 ± 0.90**	18.89 ± 0.34	22.09 ± 0.37**
18:3ω3	0.82 ± 0.03	0.30 ± 0.08**	0.57 ± 0.05	0.68 ± 0.07
20:4ω6	0.05 ± 0.05	n.d.	0.08 ± 0.04	0.26 ± 0.05
20:5ω3	2.42 ± 0.20	0.53 ± 0.08**	1.94 ± 0.14	2.02 ± 0.12
22:5ω3	2.03 ± 0.20	0.41 ± 0.06**	1.14 ± 0.07	0.99 ± 0.06
22:6ω3	5.55 ± 0.54	1.01 ± 0.172**	3.10 ± 0.18	3.08 ± 0.18
Summary Metrics[a]				
Total unsaturated	63.19 ± 0.83	71.71 ± 0.48**	65.67 ± 0.79	69.12 ± 0.82**
Total ω3	11.65 ± 0.40	2.26 ± 0.28**	7.32 ± 0.33	7.57 ± 0.26
Total ω6	14.49 ± 0.40	26.53 ± 0.90**	18.98 ± 0.35	22.25 ± 0.37**
ω6:ω3	1.28 ± 0.09	12.40 ± 1.39**	2.67 ± 0.14	2.99 ± 0.12
Total PUFA	26.14 ± 1.24	28.78 ± 0.97	26.30 ± 0.55	29.82 ± 0.40**

DBI[b]	120.32 ± 4.46	105.34 ± 1.29*	110.83 ± 2.05	118.49 ± 1.26**
Mobilizability Index[c]	104.81 ± 0.28	106.55 ± 0.17**	105.13 ± 0.29	106.87 ± 0.18**
ATP Mobilizability Index[d]	41.04 ± 0.09	41.89 ± 0.06**	41.23 ± 0.11	41.90 ± 0.07**

Data are mean mass percent composition ± SE. n.d. = not detected. Hω3 and Hω6 birds were fed high-ω3 and high-ω6 PUFA diets, respectively. Hω3RI and Hω6RI birds were fed the same diets, lost mass via restricted intake, and then switched to an intermediate diet. Statistical tests detect differences between Hω3 and Hω6 or between Hω3RI and Hω6RI; *P < 0.05, **P<0.01.

[a]These metrics include all fatty acids detected, including minor ones not listed above.

[b]DBI: Double bond index = Σ[(Molar % FA_i)(number of double bonds per FA_i)]

[c]Mobilizability Index = Σ[(Mass % FA_i)(Relative Mobilization of FA_i)], where Relative Mobilization of FA_i comes from values for *Philomachus pugnax* in Chapter 2.

[d]ATP Mobilizability Index = Σ[(Mobilizability Index)(mmol ATP/g FA_i)], where mol ATP/g was calculated using non-integer values for ATP production (Berg et al., 2002).

Table 4.3. Fatty acid composition of pectoralis phospholipids from white-throated sparrows in different dietary treatments.

	Hω3 (n=7)	Hω6 (n=6)	Hω3RI (n=14)	Hω6RI (n=14)
Fatty Acids				
16:0	26.01 ± 0.69	25.61 ± 0.44	26.72 ± 0.47	27.54 ± 0.40
18:0	20.81 ± 0.37	20.22 ± 0.25	19.71 ± 0.42	18.49 ± 0.23*
18:1ω9	2.20 ± 0.18	4.58 ± 0.19**	4.37 ± 0.35	6.27 ± 0.31**
18:1ω7	1.16 ± 0.08	1.15 ± 0.07	1.17 ± 0.07	1.22 ± 0.05
18:2ω6	1.40 ± 0.13	4.07 ± 0.45**	2.58 ± 0.23	5.93 ± 0.35**
20:4ω6	1.18 ± 0.03	1.67 ± 0.14**	1.17 ± 0.06	1.80 ± 0.12**
20:5ω3	1.97 ± 0.19	1.76 ± 0.20	2.06 ± 0.17	3.16 ± 0.20**
22:5ω3	1.62 ± 0.09	1.89 ± 0.09	2.11 ± 0.09	1.92 ± 0.07
22:6ω3	44.62 ± 0.37	38.64 ± 0.87**	40.14 ± 1.02	33.74 ± 0.64**
Summary Metrics[†]				
Total unsaturated	53.11 ± 0.69	53.77 ± 0.50	53.42 ± 0.32	53.48 ± 0.42
Total ω3	47.70 ± 0.37	42.30 ± 0.85**	44.16 ± 0.89	38.32 ± 0.67**
Total ω6	2.58 ± 0.13	5.74 ± 0.47**	3.66 ± 0.27	7.47 ± 0.40**
ω6:ω3	0.054 ± 0.003	0.14 ± 0.014**	0.084 ± 0.008	0.20 ± 0.014**
Total PUFA	49.92 ± 0.69	48.04 ± 0.47	47.82 ± 0.68	45.79 ± 0.48*

Data are mean mass percent composition ± SE. Hω3 and Hω6 birds were fed high-ω3 and high-ω6 PUFA diets, respectively. Hω3RI and Hω6RI birds were fed the

same diets, lost mass via restricted intake, and then switched to an intermediate diet. Statistical tests detect differences between Hω3 and Hω6 or between Hω3RI and Hω6RI; *P<0.05, **P<0.01.

[†]These metrics include all fatty acids detected, including minor ones not listed above.

Exercise performance

Body mass was not significantly different between the Hω6 (25.68 ± 1.38 g) and Hω3 (24.59 ± 0.78 g) groups nor the Hω6RI (22.66 ± 0.43 g) and Hω3RI (22.16 ± 0.26 g) groups. Neither liver mass nor pectoralis mass were significantly affected by treatment ($P>0.3$).

PMR was significantly greater in the Hω6 group compared to the Hω3 group ($P=0.037$; Figure 4.2). There was no significant difference in PMR between the Hω3RI and Hω6RI groups ($P=0.849$), but PMR was lower in both of these groups than the Hω3 and Hω6 groups, respectively ($P<0.01$). Respiratory exchange ratio during PMR did not differ significantly ($P>0.5$) between the Hω3 (0.79 ± 0.01) and Hω6 (0.80 ± 0.01) groups, although it did differ significantly ($P=0.046$) between the Hω3RI (0.81 ± 0.03) and Hω6RI (0.91 ± 0.04) groups. Hematocrit was significantly elevated ($P=0.046$) in the Hω6 group (47 ± 0.7 %) compared to the Hω3 group (44 ± 1.0 %). There was no difference in hematocrit between the Hω3RI (42 ± 0.8) and Hω6RI (43 ± 1.1) groups. Hematocrit was not significantly correlated with PMR, either within or across treatments.

Enzyme activity

Between the Hω6 and Hω3 groups, there were no significant differences in the activities of any of the measured enzymes ($P > 0.5$ for all, Figure 4.3). Between the Hω6RI and Hω3RI groups, there were no significant differences in CPT, HOAD, or LDH ($P >0.4$ for all). However, CS was significantly greater in the Hω3RI group when compared to the Hω6RI group ($P=0.014$).

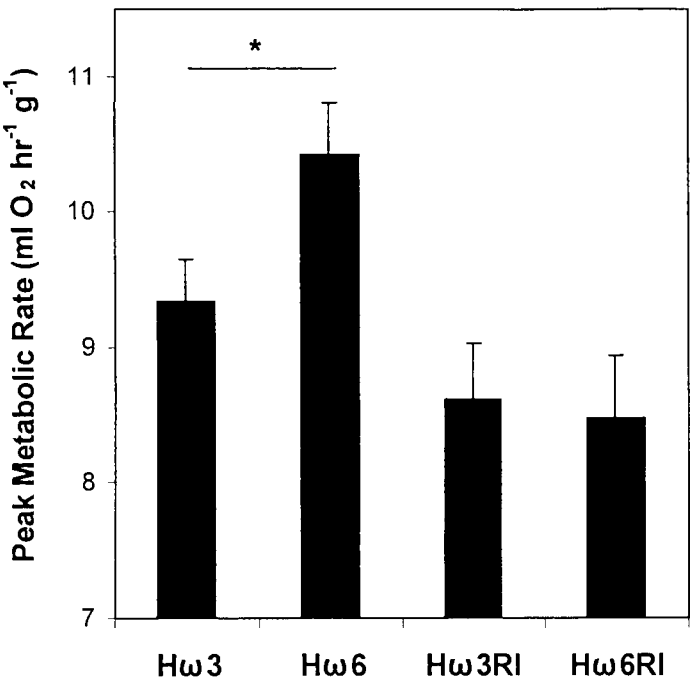

Figure 4.2. Peak metabolic rate (PMR) in white-throated sparrows. Hω3 (n=13) and Hω6 (n=13) birds were fed high-ω3 (n=13) and high-ω6 (n=14) PUFA diets, respectively. Hω3RI and Hω6RI birds were fed the same diets, lost mass via restricted intake, and then switched to an intermediate diet. There was a significant difference in PMR between the Hω3 and Hω6 groups, but no difference between the Hω3RI and Hω6RI groups. Data are means + SE.

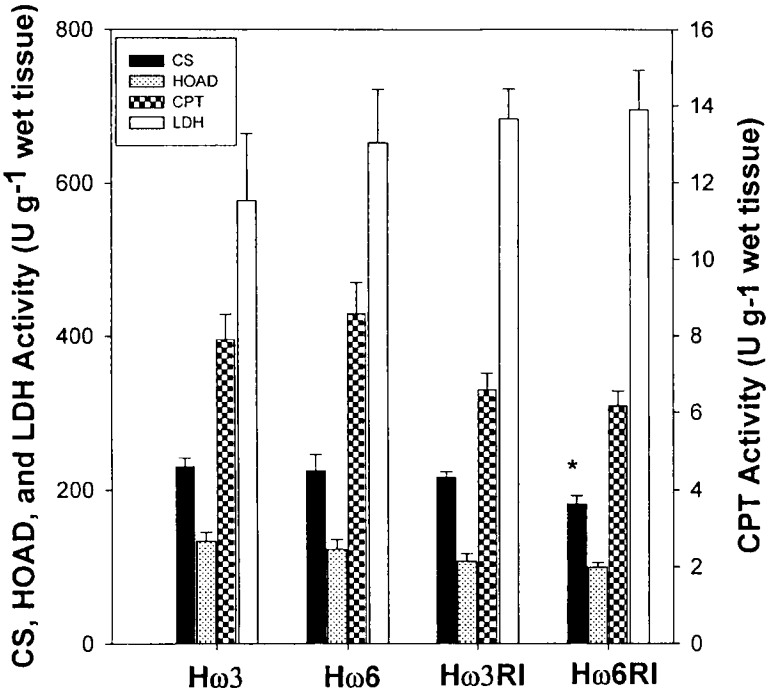

Figure 4.3. Enzyme activity of citrate synthase (CS), 3-hydroxyacyl-CoA dehydrogenase (HOAD), carnitine palmitoyl transferase (CPT), and lactate dehydrogenase (LDH) in white-throated sparrow pectoralis muscle. Hω3 (n=7) and Hω6 (n=7) birds were fed high-ω3 and high-ω6 PUFA diets, respectively. Hω3RI (n=14) and Hω6RI (n=14) birds were fed the same diets, lost mass via restricted intake, and then switched to an intermediate diet. There were no significant differences in enzyme activity between the Hω3 and Hω6 groups, however, CS was significantly elevated in Hω3RI compared to Hω6RI. Data are means + SE.

mRNA expression

The treatments had no effect on the expression of either of the housekeeping genes (GAPDH, P = 0.359; β-Actin, P= 0.898), nor the geometric mean of the two housekeeping genes (P=0.565), validating their use in standardizing the fat transport genes. Using these genes in a housekeeping index, there was no difference in the membrane fatty acid transporters FABPpm (P = 0.999) and CD36/FAT (P = 0.548), nor the cytosolic H-FABP (P=0.895) between treatment groups (Figure 4.4).

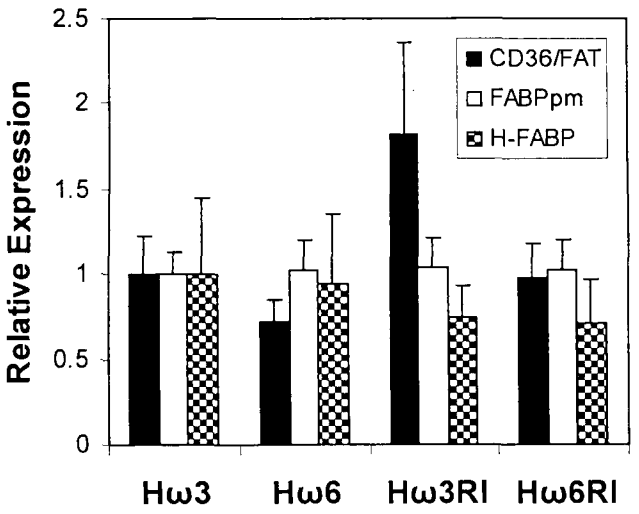

Figure 4.4. Relative mRNA expression of fatty acid transporter proteins in white-throated sparrow pectoralis muscle. For each gene, the expression ratio (target gene expression/housekeeper gene expression) is normalized to the Hω3 average (which was set at 1). Hω3 (n=6) and Hω6 (n=7) birds were fed high-ω3 and high-ω6 PUFA diets, respectively. Hω3RI (n=14) and Hω6RI (n=13) birds were fed the same diets, lost mass via restricted intake, and then switched to an intermediate diet. There were no significant differences between treatment groups. Data are means + SE.

Discussion

Effects of dietary fatty acids on exercise performance

Dietary fat manipulations have had variable effects on exercise performance (Ayre and Hulbert, 1997; McKenzie et al., 1998; Oostenbrug et al., 1997; Pierce et al., 2005; Wagner et al., 2004). These studies have been performed on a wide range of vertebrate taxa with a variety of diet components, and this may account for some of this inconsistency. Furthermore, dietary fatty acids could have different effects on different types of exercise. Previous work in birds has demonstrated performance differences due to dietary fat composition, in both PMR and energy efficiency (McWilliams and Pierce, 2006; Pierce et al., 2005). My results show a clear effect of dietary fatty acid profile on exercise performance in sparrows. In particular, a diet enriched in ω6 fatty acids resulted in higher peak metabolic rate achieved by the sparrows. This also agrees with several previous findings that rats and fish had greater running endurance and maximal swimming speed, respectively, when fed diets enriched with ω6 fatty acids (Ayre and Hulbert, 1997; Chatelier et al., 2006; McKenzie et al., 1998).

Maillet and Weber proposed the natural doping hypothesis, which states that some birds are able to increase their migratory performance by feeding on ω3-rich amphipod prey immediately prior to a long flight (Maillet and Weber, 2007; Weber, 2009). This could occur via changes to muscle phospholipids or via upregulation of fatty acid oxidation capacity through PPAR mediated pathways (Weber, 2009). The hypothesis is based on correlations between muscle oxidative capacity and ω3 fatty acids in muscle PL of semipalmated sandpipers (*Calidris pusilla*) refueling at a

migratory stopover (Maillet and Weber, 2007). I have previously found a negative correlation between muscle PL ω3 and oxidative capacity in sparrows (Chapter 3), and in the current study, dietary and tissue PUFA composition had no effect on oxidative enzymes or fatty acid transporters, many of which are known to be controlled by PPAR (Weber, 2009). The general effects of PUFA on exercise are still a matter of debate. Based on the current study and previous studies that directly manipulated diet in birds and other animals (Ayre and Hulbert, 1997; McKenzie et al., 1998; Pierce et al., 2005), the evidence suggests that consumption of diets enriched with ω6 fatty acids, more so than ω3 fatty acids, could lead to better migratory performance. ω3 fatty acids, particularly via their presence in membranes, could still play beneficial roles in exercise performance, but such effects appear small compared to other factors affected by fatty acids in the diet.

The role of phospholipid composition in performance

Many explanations have been proposed for why dietary fatty acids should affect performance. These include alteration of muscle PL fatty acids (Andersson et al., 2000; Ayre and Hulbert, 1997; Guglielmo et al., 2002; Helge et al., 2001; Infante et al., 2001; Valencak et al., 2003; Chapter 3), alteration of adipose stores (Conway et al., 1994; Johnston, 1973; Pierce et al., 2005; Chapter 2), PPAR activation resulting in increased mitochondrial and peroxisomal proliferation (Weber, 2009), changes in red blood cell deformability (Oostenbrug et al., 1997), alteration of cardiovascular function (Chatelier et al., 2006; Simopoulos, 2008), and other hypotheses. I was specifically interested in the possibility that muscle PL could affect whole-organism

exercise performance. Membrane fatty acid composition can alter membrane-bound enzyme activity (Infante et al., 2001; Murphy, 1990), mitochondrial proton leak and oxidative capacity (Gerson et al., 2008; Guderley et al., 2008; Pan et al., 1994), and insulin sensitivity (Borkman et al., 1993), properties that could potentially result in exercise performance differences. 'High performance' muscles from hummingbirds and rattlesnakes have high proportions of ω3 in their PL (Infante et al., 2001). In addition, changes in muscle PL have been associated with migration (Guglielmo et al., 2002; Maillet and Weber, 2006). In controlled studies, however, manipulation of muscle PL fatty acid composition has generally been accomplished via a simple diet switch, a protocol which does not allow the 'PL hypothesis' to be distinguished from alternative hypotheses about how dietary fatty acids affect exercise.

My protocol allowed me to manipulate PL composition while maintaining a common adipose composition and (recent) diet, the first study I am aware of to thus independently manipulate PL fatty acid composition. Given the lack of a performance difference between the Hω3RI and Hω6RI sparrows, it appears that the differences in muscle PL fatty acid composition observed in these groups do not cause a performance difference. I also observed an overall decrease in PMR in both Hω3RI and Hω6RI sparrows relative to the other two treatments. The cause of this decline is unknown, but was associated with the food restriction and therefore the disappearance of the performance difference could be an artifact of this aspect of the methodology. I suspect that the decrease in PMR is related to a loss of some oxidative enzymes during the food restriction protocol; this trend was observed for CS, CPT, and HOAD (Figure 4.3). Birds are known to decrease specific basal or

resting metabolic rate in the early response to fasting (Battley et al., 2001; Boismenu et al., 1992; Cherel et al., 1988), and the food restriction of my protocol may have triggered this response. While I did not measure basal metabolic rate in this study, this could explain my results assuming aerobic scope remained constant. This problem might be alleviated by allowing greater recovery time after the food restriction, but greater recovery might also allow higher turnover of membrane phospholipids. Future studies could examine this possibility further by employing the opposite protocol (i.e., starting birds on a similar diet, restricting food, and then feeding them distinct diets). However, given the similarity of muscle PL between the Hω3 and Hω3RI and between the Hω6 and Hω6RI treatments, I conclude that the performance differences seen with a simple diet manipulation are not being driven by differences in muscle PL.

Other mechanisms of enhanced performance

The fatty acid composition of triacylglycerol stores has also been implicated in altering exercise performance (Egeler and Williams, 2000; Johnston, 1973; McKenzie et al., 1998; Pierce et al., 2005; Chapter 2). This explanation is generally supported by my results, as performance differences were associated with differences in adipose composition. In birds, which rely heavily on extra-muscular fats to fuel migratory flight, it has been suggested that adipose stores enriched with more unsaturates could increase performance because they are more readily mobilized from adipose tissue (Egeler and Williams, 2000; Johnston, 1973; Chapter 2). My results do not lend strong support to that mechanism. While both the Mobilizability Index and

ATP Mobilizability Index were significantly greater in the Hω6 than Hω3 groups, this was also true for the for the Hω6RI group compared to the Hω3RI group, where no performance difference was seen. These indices were based on observed relative mobilizations from a different bird species fed a different diet, and may not describe well the current study subjects. Nonetheless, they certainly provide no support for the hypothesis that performance is enhanced via a mechanism based on faster mobilization rates of certain fatty acids from adipocytes. Furthermore, although the respiratory exchange ratios indicate that a mixture of primarily fat and carbohydrates were oxidized, and a previous study of ruff sandpipers showed that respiratory exchange ratios did not change during shivering (Vaillancourt and Weber, 2007), it is unclear if adipocyte triacylglycerol (as opposed to intramuscular stores) is the main source of that fat during such early stages of exercise.

Although adipocyte fatty acid mobilization does not appear to explain the performance results in my study, other aspects of triacylglycerol composition could still play a role in affecting performance. Intramuscular and adipose triacylglycerol composition are substantially similar in this species (Klaiman et al., In press). While mobilization of fatty acids from adipocytes is known to be related to chain length and unsaturation (Raclot et al., 1995; Chapter 2), other aspects of the lipid oxidative pathway have yet to be examined with regard to substrate preference in birds. Sarcolemmal uptake and intramyocyte transport and oxidation are thought to be the important limiting steps in lipid oxidation (McWilliams et al., 2004), yet fatty acid selectivity has not been studied for these processes in birds. In fish, the activity of CPT, an enzyme critical to the translocation of fatty acids into mitochondria, is

strongly influenced by fatty acid substrate (Egginton, 1996; Sidell and Driedzic, 1985). In particular, CPT activity is much greater with 18:2ω6 as a substrate than with longer chain fatty acids like 22:1 and 24:1 (Egginton, 1996). Given the large differences between my Hω6 and Hω3 birds with respect to the adipose composition of 18:2ω6 and longer chain fatty acids (20:5ω3, 22:5ω3, and 22:6ω3), I speculate that the preferential use of particular fatty acids by CPT or intramyocyte fatty acid transporters drives the performance pattern in my results.

The mixture of fatty acids in the diet could also affect performance via their influence on PPARs. PPAR activity increases gene expression of key lipid metabolic proteins, such as CPT, HOAD, H-FABP, CD36/FAT, and others (Weber, 2009). 20:5ω3 and 22:6ω3 are ligands for PPARs, and have been suggested to thus be able to increase fuel oxidative capacity (Weber, 2009). I did find a non-significant trend for increased CD36/FAT expression in the Hω3 and Hω3RI birds, a finding that is worth further investigation. Nonetheless, this trend did not result in enhanced performance in these treatment groups.

Hematocrit can be affected by dietary fats (Foitzik et al., 2002), and hematocrit was greater in the Hω6 group than the Hω3 group. If this difference is great enough to limit oxygen supply to muscles, it is possible that this is the source of the performance difference between these groups. Hematocrit did not, however, correlate with PMR either within or across treatments. Fuel selection also does not appear to explain my performance results, as respiratory exchange ratio was similar between Hω3 and Hω6 groups.

Limitations

The diets fed to all of the birds were heavily enriched in total % fat and total PUFA. My results may therefore only apply to similar diets, and ω3 and ω6 fatty acids may have different effects on performance at lower dietary levels. Notably, my sparrows had much higher levels of PL 22:6ω3 than the sandpipers studied by Maillet and Weber (Maillet and Weber, 2006). Further, I used sparrows that normally encounter dietary 18:2ω6, but probably have lower exposure to longer chain PUFA fatty acids in their natural diets. Species-level differences may play a role in determining how dietary fatty acids affect performance. Finally, it is possible that birds with higher PMR are also less efficient during endurance flights, such that there is a tradeoff between maximal and endurance performance. Further experimentation will be necessary to determine the effects of dietary fats on performance generally in birds.

Perspectives and Significance

Previous studies of dietary fatty acids and avian exercise have focused on essentially two mechanisms: 1) rates of utilization of fatty acids oxidized for fuel, and 2) membrane fatty acids and their effects on enzymes and other processes. My results indicate that future work should be directed primarily at the first mechanism, with a particular focus on the rates at which various fatty acids are utilized by muscles. I also encourage investigation of other hypotheses, such as the effects of PPAR activation and eicosanoid production on avian exercise. Elucidation of these mechanisms will allow a broader understanding of the impact of diet on migration,

the choice of migratory stopover habitats and food selection, and how dietary fats affect exercising animals in general.

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CHAPTER 5
FATTY ACID SUBSTRATE PREFERENCE OF CARNITNE PALMITOYL TRANSFERASE FROM PECTORALIS MUSCLE OF A MIGRATORY BIRD

Introduction

Birds primarily use fat to fuel their migratory flights, and their capacity to use fat at high rates is unequaled among vertebrates. Supporting this high lipid oxidation capacity in the muscle are fatty acid transport proteins in the membranes, cytosol, and mitochondrial matrix which are known to increase during the migratory season (Guglielmo et al., 2002a). These include muscle membrane fatty acid transporters (McFarlan et al., 2009), cytosolic transporters (Guglielmo et al., 2002a; Guglielmo et al., 1998; McFarlan et al., 2009), the mitochondrial membrane-bound enzyme carnitine palmitoyl transferase (CPT) (Driedzic et al., 1993; Guglielmo et al., 2002a), and enzymes involved in β-oxidation (Lundgren and Kiessling, 1985; Marsh, 1981).

Of interest to ornithologists has been the effect of dietary lipids on exercise and the mechanism by which dietary lipids affect flight performance (Blem, 1976; Conway et al., 1994; Johnston, 1973; Pierce et al., 2005; Weber, 2009). Although it has been suggested that dietary fats can influence exercise performance by incorporation into membranes (Guglielmo et al., 2002b; Maillet and Weber, 2007; Weber, 2009), independent manipulation of both membrane lipids and stored triacylglycerol has pointed to the later as more likely mechanism (Chapter 4). Unsaturated fatty acids have been thought to improve exercise performance because they are more hydrophilic, and could therefore be transported more quickly (Conway et al., 1994; Pierce et al., 2005). This pattern generally holds for mobilization of fat

from avian adipocytes, where unsaturation and chain length both affect mobilization rates (Groscolas, 1990, Chapter 2). However, muscle FA transport and oxidation are protein-mediated processes, and it is possible that these enzymes and transporters have other patterns of preferred substrates that do not depend on hydrophilic properties of the fatty acid substrate. Moreover, overall rates of transport from adipocytes to mitochondria may depend on critical limiting steps that are protein-mediated. In particular, CPT1 is thought to exert a large amount of control over fatty acid flux (Eaton et al., 2001; Spurway et al., 1997).

CPT1 catalyzes the conversion of a fatty acyl-CoA to fatty acyl-carnitine. The resultant molecule is then translocated across the mitochondrial membranes where CPT2 catalyzes the reconstitution of fatty acyl-CoA prior to β-oxidation. Given the importance of CPT in the lipid supply pathway, its substrate preference has the potential to affect maximal rates of oxidation when various fatty acids are supplied to the muscle (Gavino and Gavino, 1991; Stonell et al., 1997). Previous work in fish has shown CPT activity to be highest when 16:1 was a substrate, with lower activity when 16:0 and 18:1 were supplied, and much lower activity when 18:2 and 20:4 were supplied (Egginton, 1986; Egginton, 1996; Sidell et al., 1995). However, this pattern of substrate preference can vary substantially among taxa (Power et al., 1997; Stonell et al., 1997); in rat liver for example, CPT activity with 18 carbon polyunsaturates and 20:4 as substrates exceeded that obtained with 18 carbon monounsaturates (Gavino and Gavino, 1991). Here, I seek to expand those previous results to a migratory bird in an attempt to explain previously observed diet-induced differences in performance (Chapter 4).

Materials and Methods

Animals

Twelve white-throated sparrows (*Zonotrichia albicollis*) were captured during autumnal migration near Longpoint, Ontario. The white-throated sparrow is a long-distance short-bout migrant of eastern North America. Sparrows were kept in captivity at the University of Western Ontario in 40 cm (height) by 45 cm by 45 cm cages for the duration of the study. Sparrows were first provided standard birdseed and then quickly weaned onto a commercial diet (ground Mazuri Small Bird diet, PMI Nutrition International, Brentwood MO, USA) which was provided *ad libitum*. The birds were immediately placed on a 'short-day' (winter) light cycle (8L:16D). After 55 days, 6 sparrows were placed on a 'long-day' light cycle (16L:8D) to stimulate migratory behavior (Agatsuma and Ramenofsky, 2006; Landys et al., 2004) while the other 6 sparrows remained on the winter light cycle. The increased nightly activity (zugunruhe) that is characteristic of migratory condition was observed in the 'long-day' group, verified by infrared video cameras (D. Cerasale, D. Zajac, and C. Guglielmo, unpublished data). Twenty-eight days after the light cycle switch, sparrows from both groups were euthanized by cervical dislocation under isofluorane anesthesia. Pectoralis muscle was immediately dissected out and frozen at -80 °C. Experimental protocols were approved by the University of Western Ontario Animal Use Subcommittee (Protocol #2005-060-08) and a collection permit was obtained from the Canadian Wildlife Service (CA 0170).

Enzyme assay

Approximately 100 mg pectoralis muscle was combined with 9 volumes of a homogenization buffer (20 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% defatted BSA, 50% glycerol [Caledon Laboratories, Georgetown, ON], and Aprotinin at 50 μg/ml, pH 7.4 (all chemicals from Sigma, St. Louis, MO, USA, unless noted otherwise). This was then homogenized 3 x 10 sec, waiting 30 sec between bouts while keeping the sample on ice. Assays were performed in duplicate at 39 °C in 1 ml reaction volume in disposable cuvettes on a Cary 100 Bio Spectrophotometer (Varian Inc., Palo Alto, CA). CPT (EC 2.3.1.21) was assayed in 50 mM Tris buffer, pH 8.0, with 5 mM carnitine (omitted for control), 0.15 mM DTNB, 0.016 mM fatty acyl CoA, and 10 μl of muscle homogenate diluted 1:10 with the homogenization buffer. This concentration of palmitoyl CoA was found to give maximal activity in preliminary experiments, and the pattern of CPT preference did not change when run at 0.032 mM or 0.08 mM fatty acyl CoA (data not shown). Activity was calculated by measuring the change in absorbance over 2 min at λ=412 nm. Fatty acyl CoAs were purchased from Sigma (16:0, 16:1ω7, 18:0, and 18:1ω9) and Avanti Polar Lipids, Inc (Alabaster, AL, USA) (16:0, 18:2ω6, 18:3ω3, 18:3ω6, 20:0, 20:4ω6).

The measurement of CPT by this assay includes activity of both CPT 1 and CPT 2 (the latter enzyme run in reverse of the direction normal for fatty acid oxidation). To determine the amount of activity in my samples that was due to CPT1 and CPT2, I assayed 2 samples with 0.016 mM palmitoyl CoA (16:0) and in the presence or absence of malonyl CoA (0.2 mM; Sigma) which is known to inhibit CPT1, but not CPT2, although sensitivity of CPT 1 to malonyl CoA inhibition can

depend on various factors including diet, membrane fluidity, and temperature (Kolodziej and Zammit, 1990).

Statistical analysis

For each sparrow muscle sample, CPT activity was measured for the panel of different fatty acyl CoAs. To compare activities among samples, the activities with the various fatty acyl CoAs were normalized by dividing by the activity measured for 16:0 in the same muscle sample. Activity was normalized using the 16:0 CoA supplied by the same manufacturer (e.g. 16:1 was normalized to 16:0 supplied by Sigma, while 20:4 was normalized to 16:0 supplied by Avanti Polar Lipids). This relative activity was used for statistical analysis. Relative activities of fatty acyl CoAs were compared within a chain length using ANOVA and Tukey's post-hoc test. Comparisons of relative activities of fatty acid pairs were made using t-tests.

Results

Malonyl CoA decreased CPT activity by approximately 33% (Figure 5.1). Doubling the concentration of malonyl CoA did not result in any higher inhibition (result not shown).

CPT showed strong selectivity with respect to fatty acyl CoA substrate (Figure 5.2, Table 5.1). The relative activities did not differ significantly with photoperiod for any of the fatty acyl CoA substrates ($P > 0.15$ for all) so both photoperiod treatments were combined for further analyses. Unsaturated fatty acyl-CoAs tended to display more activity than their saturated counterparts of the same chain length ($P<0.001$), and the 16 carbon fatty acyl CoAs displayed more activity than the 18 carbon fatty acyl-CoAs ($P<0.001$ for all comparisons). The activity of 20:0 was so low as to be undetectable by this assay. In the large group of 18 carbon fatty acyl CoAs, activity trended non-significantly higher from 18:0 to 18:1 ($P=0.149$) and significantly from 18:1 to 18:2 ($P=0.001$), but did not increase substantially from 18:2 to 18:3. The activities of the 2 isomers 18:3ω3 and 18:3ω6 were similar.

To understand the potential ATP production differences arising from the differences in CPT activity, I calculated 'relative ATP activity' as the product of relative activity and the ATP produced per fatty acid (ATP production values based on Berg et al., 2002), normalized to the value for 16:0 (Figure 5.3). Although the values for the 18 carbon fatty acids were slightly larger due to the higher amount of ATP produced per fatty acid, the pattern remained the same as for relative activity, and statistical differences remained the same.

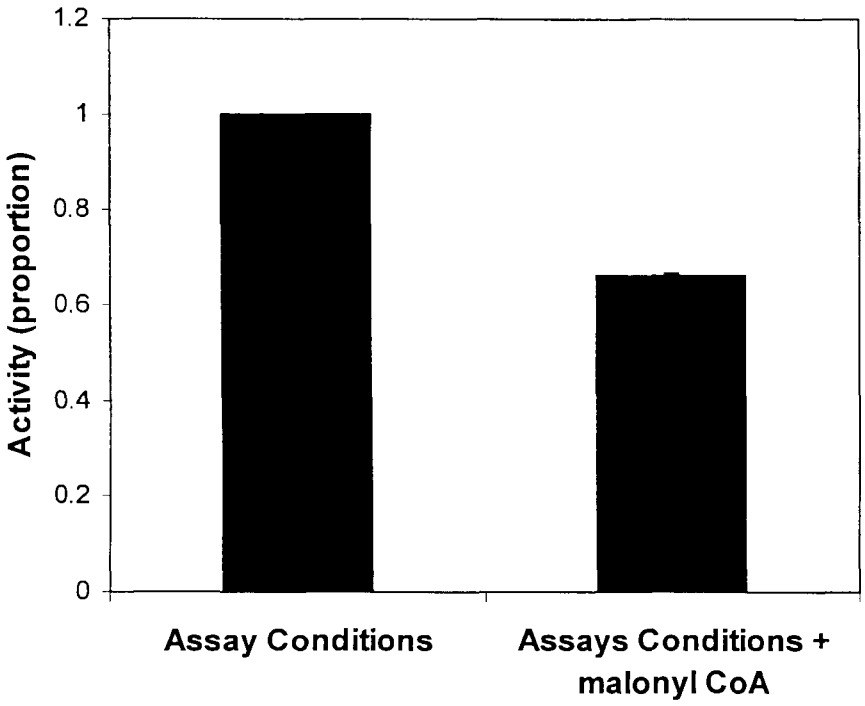

Figure 5.1. Inhibition of CPT activity by malonyl CoA. Enzyme activity is presented as a proportion of uninhibited activity. Addition of 0.2 mM malonyl CoA decreased CPT activity by approximately 33%. Data are means + SE, n=2.

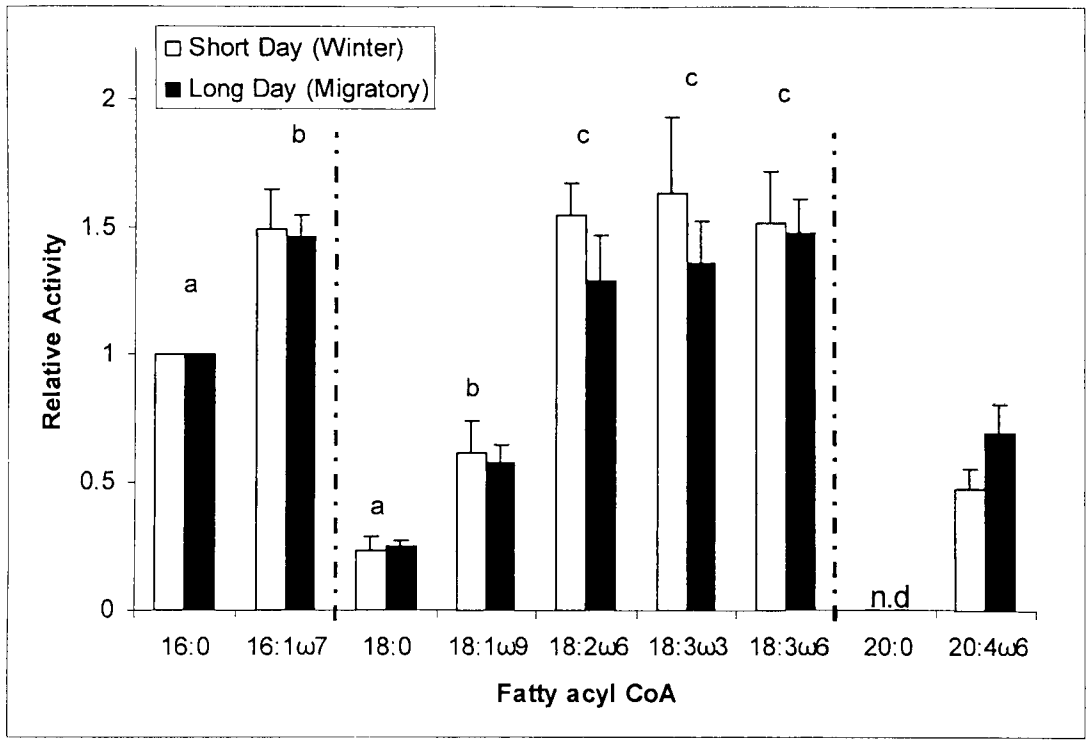

Figure 5.2. Relative activity of CPT with various fatty acyl CoA substrates. For each sample, activity of the various fatty acyl CoAs was normalized to the activity of that sample with 16:0 as a substrate. Data are presented as means + SE; n=6 per photoperiod treatment. 'n.d.'= no activity detected. Dashed lines separate fatty acyl CoAs by chain length. The relative activities did not vary significantly by photoperiod treatment for any fatty acyl CoA so both treatments were combined for further analyses. Different letters indicate statistically significant differences *within a chain length.*

Table 5.1. CPT activity (U/g muscle) with various fatty acyl substrates.

	16:0[s]	16:0[a]	16:1	18:0	18:1	18:2ω6	18:3ω3	18:3ω6	20:0	20:4ω6
Short	4.25	3.09	6.10	1.06	2.57	3.87	2.99	3.83	n.d.	1.77
Day	±1.06	±0.81	±1.44	±0.26	±0.74	±0.68	±0.53	±0.80		±0.21
Long	3.14	2.40	4.79	0.70	2.10	3.49	2.99	3.29	n.d.	1.19
Day	±0.30	±0.40	±0.68	±0.16	±0.47	±0.49	±0.54	±0.60		±0.23

Data are means ± SE. n.d. indicates no detected activity.

[s]Supplied by Sigma.

[a]Supplied by Avanti.

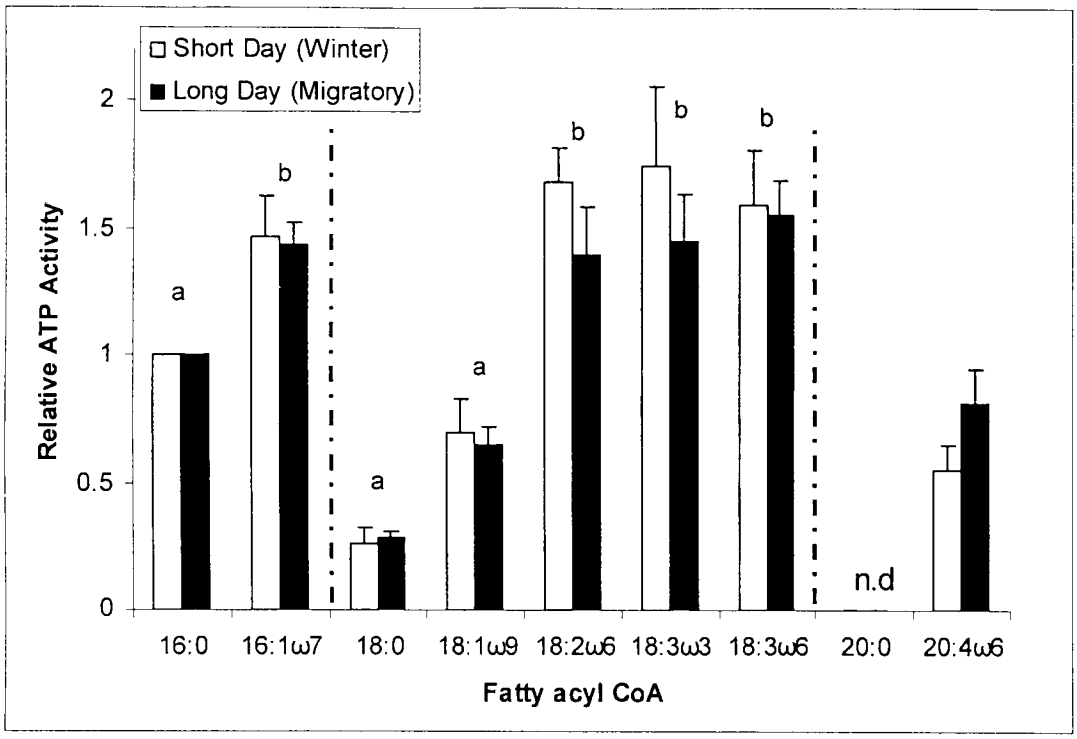

Figure 5.3. Relative ATP activity of CPT with various fatty acyl CoA substrates. Relative ATP activity is calculated as the relative activity multiplied by the ATP produced per molecule of fatty acid during β-oxidation (values based on Berg et al., 2002), and normalized to 16:0. Data are presented as means + SE; n=6 per photoperiod treatment'n.d.'= no activity detected. Dashed lines separate fatty acyl CoAs by chain length. The relative activities did not vary significantly by photoperiod treatment for any fatty acyl CoA so both treatments were combined for further analyses. Different letters indicate statistically significant differences *within a chain length.*

Discussion

Pattern of CPT selectivity

My results showed a clear pattern of substrate selectivity, with increased activity observed with monounsaturates compared to saturates. In this respect, the pattern seen in the present study is similar to that found previously in muscles from several species of fish (Egginton, 1986; Egginton, 1996; Sidell et al., 1995), and mammals (Stonell et al., 1997). Although the pattern of CPT selectivity varies significantly by species and by tissue (Gavino and Gavino, 1991; Power et al., 1997; Stonell et al., 1997), in general, monounsaturates outperform their saturated counterparts of similar chain length (Cake et al., 1998). In other respects, the pattern of CPT selectivity has been variable among species. For example, 18:2ω6 was preferred to 18:1ω9 in the present study, in agreement with results from rat muscle and liver (Gavino and Gavino, 1991; Stonell et al., 1997), but differing from the pattern observed in muscle and liver of several species of fish, eels, and lamprey (Cake et al., 1998; Crockett and Sidell, 1993; Egginton, 1996; Sidell et al., 1995; Stonell et al., 1997). Similarly, I found 18:3ω3 to be relatively preferred as a substrate in sparrows; in rat livers it displays even higher activity than 18:2ω6 (Gavino and Gavino, 1991), while in fish muscles it results in barely detectable activity (Crockett and Sidell, 1993). Thus, differences in CPT selectivity appear to be split between (fishes + lamprey) and (mammals + birds) (see also Egginton, 1996). In both rats and fishes, CPT has been shown to have lower activity with 20:4 than with 16:0 (Egginton, 1996; Gavino and Gavino, 1991; Sidell et al., 1995), as was found in the current study. Other long chain polyunsaturates 20:5ω3 and 22:6ω3

have demonstrated variably low or high activity relative to 16:0 (Cake et al., 1998; Crockett and Sidell, 1993; Gavino and Gavino, 1991; Stonell et al., 1997). The selectivity of CPT can also vary by diet (Power et al., 1997) and physiological state (Kolodziej and Zammit, 1990), although migratory condition did not change the pattern of selectivity in the current study. Future work in this direction could investigate the effect of diet on avian CPT selectivity.

Implications for migratory birds and fat storage

The composition of stored adipose triacylglycerol is of interest to ornithologists because birds rely nearly exclusively on fat utilization to fuel their long distance migrations. The storage of unsaturated fatty acids has received particular attention, because it has been thought that unsaturates should be mobilized more quickly than saturated fatty acids (Blem, 1976; Conway et al., 1994; Egeler and Williams, 2000; Johnson and West, 1973; Johnston, 1973; Pierce et al., 2005). Mobilization of fatty acids from avian adipocytes is indeed selective toward unsaturated fatty acids (Groscolas, 1990, Chapter 2). However, the process of fatty acid transport from adipose fat droplets to intramuscular sites of oxidation involves several processes, including hydrolysis of triacylglycerol, mobilization from adipose, circulatory transport, muscular uptake, and intramuscular transport (Egeler and Williams, 2000). The latter processes have been proposed to be limiting to overall lipid flux through the system (Vock et al., 1996; Weber, 1992), and the importance of these processes is evidenced by seasonal, migration-related increases in muscle membrane and intramuscular fatty acid transport proteins including CPT (Driedzic et

al., 1993; Guglielmo et al., 2002a; McFarlan et al., 2009). Nonetheless, these processes have not previously been studied with respect to fatty acid type in birds. My results demonstrate that there is selectivity of avian CPT with respect to fatty acid substrate. Unsaturates demonstrated more activity than saturates, with 16:1 being a particularly preferred substrate. Polyunsaturated 18 carbon fatty acids did have higher activity than 18:1, although there was not a substantial increase from 18:2 to 18:3. It appears that carbon chain length can be more important than a single double bond in determining flux rates across mitochondria; activity was higher with 16:0 than the 18:1 fatty acyl CoA. The current study suggests no importance of positional isomers (ω3 or ω6) to fatty acid oxidation in birds.

In a study of red-eyed vireos (*Vireo olivaceous*), Pierce et al. (2005) found that higher peak metabolic rates were achieved with birds fed a diet containing a moderate amount of unsaturated fatty acids (58%) than those fed a diet with high unsaturated content (82%). This counterintuitive result might be explained by the findings of the current study. The highly unsaturated diet of the vireo study contained ~70% 18:1, whereas the moderately unsaturated diet had ~30% 18:1 with similar amounts of 16:0 and 18:2. The monounsaturate 18:1 delivers 33% less 'ATP flux' than 16:0 through CPT, and less than half that of 18:2 (Figure 5.3). If CPT is the limiting step in this pathway, their moderately unsaturated group may have achieved higher maximal performance by having more 16:0 and 18:2 in stored triacylglycerol. Similarly, this may explain the differences in PMR exhibited by my sparrows in Chapter 4, although it is difficult to predict the relative activity of long chain polyunsaturates such as 22:5 and 22:6.

Given the strong preference of CPT for 16:1 among the *de novo* synthesizable fatty acids (16:0, 16:1, 18:0, 18:1, 20:0), it is notable that this fatty acid does not make up a large proportion of bird fat reserves, instead comprising around 8% for migrants (Blem, 1980). Birds store much more 18:1 (~40%; Blem, 1980). This discrepancy may indicate that the major limitation on muscle fatty acid flux occurs upstream of CPT1. This was the conclusion reached by Sidell et al. (1995) working with fish, who found selectivity for 18:1 over 16:0 in whole muscle preparations, but no such selectivity was exhibited by CPT alone. It could also suggest that achieving maximal lipid oxidation rate is not a strong selective pressure on migratory birds. Maximizing energy storage, via longer chain saturates could be of greater importance to migrants. Future research should investigate fatty acid selectivity at upstream points along this pathway in birds, including intramuscular transport, muscular uptake, and circulatory transport.

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CHAPTER 6
A THEORETICAL FRAMEWORK FOR AVIAN FAT STORAGE

Summary of the experiments

The preceding chapters have taken a focused look at the role of fatty acids in adipose stores and muscle membranes. Taken as a whole, they provide scant evidence for the hypothesis that muscle membrane fatty acid composition affects migratory performance. Birds do not endogenously alter membrane composition in preparation for migration (Chapter 3). Further, direct alteration of muscle membranes, when done independently of adipose composition, does not result in changes to maximal exercise performance (Chapter 4). However, these experiments do lend support for a role of adipose composition in affecting exercise performance via the energy substrate supply pathway. Selectivity with respect to fatty acid occurs at multiple points in the lipid supply pathway, including the adipocytes and intramuscular CPT (Chapter 2, Chapter 5), creating the possibility that the composition of stored fatty acids can affect maximal exercise performance.

Nonetheless, this field is open and there remains a great deal of work in further evaluating competing hypotheses. One worthwhile avenue would be better determination of which fatty acids affect performance. Various authors have focused on ω3 fatty acids (Maillet and Weber, 2006; Nagahuedi et al., 2009; Weber, 2009), ω6 fatty acids (Ayre and Hulbert, 1996; Guglielmo et al., 2002b; McKenzie et al., 1998), and total unsaturated fatty acids (Pierce et al., 2005). The 'best' diet for exercise may be some optimal combination of these or others, and may well vary by

species and type of exercise. Further elucidation of how dietary fatty acids affect exercise in birds could aid understanding of the mechanisms involved.

A theoretical framework for stored fat composition in migratory birds

Previous studies of avian fat storage have focused almost exclusively on unsaturated fatty acids and their mobilization from adipose (Blem, 1990). But the requirement of rapid mobilization is not the only, or even necessarily most important, selection pressure on the composition of migratory fat storage. A full enumeration of these pressures is absent from the literature, and yet this seems a necessary beginning to understanding migratory fat storage. To that end, I present the following framework based on first principles and discuss several major strategies that migratory birds may use in response to these selective pressures.

Energetic properties of common fatty acids

As apparent from Table 6.1, longer fats are more energy-dense than shorter ones. This is most evident on a molar basis, increasing 40% from 16:0 to 22:0, but increasing only 5% over the same range on a per mass basis. The increase is slightly smaller when the dry mass and ATP generation from the glycerol backbone are considered, and presumably even smaller if wet mass is considered.

Saturated fats are more energy dense than unsaturated fats, on both a molar basis and mass basis. Introduction of one double bond introduces a decrease of about 1.5 ATP per fatty acid molecule (due to the loss of one $FADH_2$ production during β-oxidation), representing a 1-1.4% decrease in ATP production for a 16 - 22 carbon

Table 6.1. Energetic properties of some fatty acids stored by birds.

Common Name	Formula	ATP per fatty acid[a]	mmol ATP/g (dry)	mmol ATP/g TRIG (dry)[b]	Relative mobilization from adipocytes[c]	ATP mobilizability index[d]
Myristic	14:0	92	402.9	404.5		
Palmitic	16:0	106	413.4	414.3	0.959	397.36
Palmitoleic	16:1ω7	104.5	410.8	411.8	1.129	464.97
Stearic	18:0	120	421.8	422.3	0.851	359.40
Vaccenic	18:1ω7	118.5	419.5	420.1	1.029	432.32
Oleic	18:1ω9	118.5	419.5	420.1	1.029	432.32
Linoleic	18:2ω6	115	410.1	411.1	1.265	520.02
Linolenic	18:3ω3	113.5	407.6	408.8	1.43	584.56
γ-Linolenic	18:3ω6	111.5	400.5	401.9	1.392	559.46
Stearidonic	18:4ω3	110	398.0	399.5	1.633	652.43
Arachidic	20:0	134	428.8	429.0		
Eicosenoic	20:1ω9	132.5	426.7	427.0	0.511	218.19
Eicosatetraenoic	20:4ω3	124	407.3	408.3	1.829	746.82
Arachidonic	20:4ω6	124	407.3	408.3	1.216	496.52
Eicosapentaenoic	20:5ω3	122.5	405.0	406.2	1.182	480.10
Behenic	22:0	148	434.6	434.5		
Docosapentaenoic	22:5ω3	136.5	413.0	413.8	0.76	314.47
Docosahexaenoic	22:6ω3	133	404.9	406.0	1.23	499.33

[a]Mitochondrial ATP production based on non-integer values for ATP production from β-oxidation (10 ATP per acetyl-CoA, 2.5 per NADH, and 1.5 per $FADH_2$ produced, minus 2 for activation and minus 3.5 per NADPH when required) (Berg et al., 2002; Garrett and Grisham, 1995).

[b]These ATP values based on oxidation of 3 fatty acid chains plus glycerol (16.5 ATP).

[c]Relative mobilization values come from *Philomachus pugnax* (Chapter 2).

[d]ATP mobilizability values are calculated as in Chapter 4 from the ATP produced per triacylglycerol and the relative mobilization of that fatty acid.

fatty acid oxidized in mitochondria (Table 6.1). Measured per mass, the loss of ATP production from the introduction of one double bond is ~0.4 - 0.6%. The situation for polyunsaturates is different, however, because the location of the double bond is important in determining ATP yield. Because double bonds in polyunsaturates are separated by 3 carbons, polyunsaturates will always have double bonds at both even and odd numbers of carbons from the carboxyl end. Double bonds at an odd carbon position will entail a loss of ~1.5 ATP as above. Oxidation of double bonds at an even position does produce $FADH_2$, but requires input of NADPH to complete β-oxidation. NADPH comes from the pentose phosphate pathway, which is also responsible for producing ribose 5-phosphate, the precursor for nucleotide synthesis. If the need for NADPH outpaces that for nucleotide synthesis, some glucose could be required to supply this NADPH, representing another cost for a bird trying to spare glucose (although NADPH can also be produced by transhydrogenase or malic enzyme activity which transfers a hydrogen from NADH to NADPH with the cost of 1 extra ATP). The ATP equivalence of NADPH is difficult to calculate, but here I assign it a value of 3.5 ATP (Garrett and Grisham, 1995). The total loss of ATP from multiple double bonds can be substantial; oxidation of 22:6ω3 yields 10% less ATP than 22:0. On a per gram basis, the loss is near 7% (Figure 6.1).

Fatty acids can also be partially oxidized in peroxisomes, although this involves a significant reduction in ATP production compared to mitochondria (Berg et al., 2002; Reddy and Mannaerts, 1994). Very long chain fatty acids ($>C_{22}$) are preferred substrates in peroxisomes, and these organelles likely function, in part, to

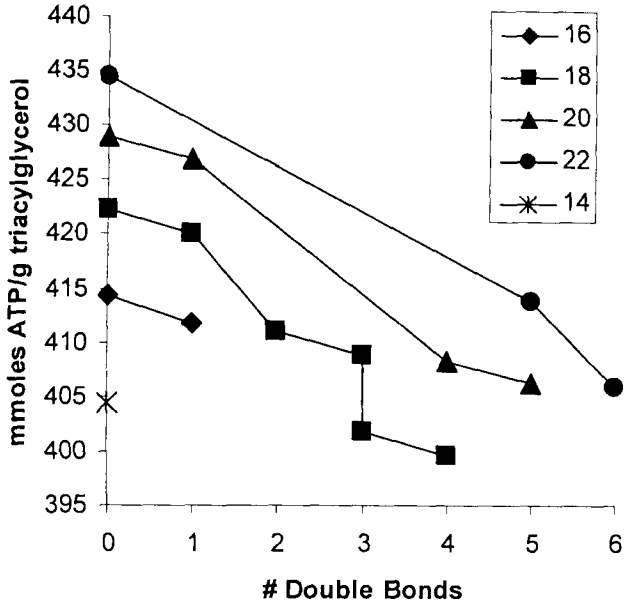

Figure 6.1. Relationship between ATP production and the number of double bonds per fatty acid. Only common fatty acids found in migrants are plotted.

shorten long and very long chain fatty acids before they enter mitochondria for further β-oxidation (Kunau et al., 1995; Reddy and Mannaerts, 1994).

Selection pressures on migrant fat storage

Proximately, triacylglycerol fatty acid composition is determined by diet, *de novo* synthesis and modification, and preferential use. Ultimately, however, patterns of lipid storage will also depend on birds' physiological and behavioral responses to various selection pressures, including the following:

1) Achieve a high maximal rate of fat oxidation during flight

Fat storage in this case will be biased towards those fats which can be moved most rapidly through the organism, from adipose to mitochondria. The rapidity of transport may be determined over this entire pathway, or at critical limiting steps. My research generates specific predictions for this pressure. If adipose mobilization is limiting, then short unsaturated fatty acids will be preferred, as I demonstrated in Chapter 2. If use in the muscles is limiting, as is likely (McWilliams et al., 2004; Vock et al., 1996), the short unsaturate 16:1 as well as 18:2 and 18:3 will be preferred, based on the CPT preference I observed in Chapter 5.

During sustained migratory flight, birds operate at 60 - 85% of maximal metabolic rates (Guglielmo et al., 2002a). While this represents relatively intense exercise, it still provides a reasonably large power buffer before maximal rates are reached. Preparing for high maximal rates would be beneficial when inclement weather is expected or when weather is unpredictable over the migratory route.

2) Store maximal energy while minimizing weight

Saturated and longer chain fatty acids are the most energetically dense (Table 6.1). Measured per gram triacylglycerol, elongation by 2 carbons adds ~7.4 ATP/g, the increase being largest at shorter lengths (converting 14:0 to 16:0 stores 9.8 more ATP per gram while converting 20:0 to 22:0 stores 5.5 mmol more ATP per gram). That is, the benefit of elongation, in terms of energy density, decreases as carbon chains lengthen. The energetic cost of double bonds varies depending on their location from the carboxyl end, with those at odd positions costing ~2.3 mmol ATP/g triacylglycerol. This cost decreases slightly as chain lengths increase. Because elongation stores more energy than is lost by desaturation, 18:1ω9 is more energy dense than 16:0.

Birds under selective pressure to minimize weight while maximizing energy storage should therefore store longer fatty acids, and should also prefer storage of saturates. This strategy should be most evident in birds that must cross barriers such as oceans, where long distance flights are unavoidable. Additionally, larger birds are limited in their extra weight-carrying capacity, more so than small birds (Pennycuick, 1972), such that they should be more likely to minimize weight by carrying saturated fatty acids. This strategy may also be important for capital breeding species that need to arrive at breeding grounds with substantial fat stores to last through the breeding season (Ankney and MacInnes, 1978; Ryder, 1970).

3) Rapidly gain weight at stopover

At a migratory stopover, rapid weight gain is considered beneficial (Alerstam and Lindström, 1990). Most energy during the migratory journey is used during stopover (Hedenström and Alerstam, 1998; Wikelski et al., 2003), and predation risk is high during stopovers (Lind and Cresswell, 2006; Sillett and Holmes, 2002). Rapid weight gain requires rapid absorption of nutrients and low energetic expenditure. These may not always coincide; for example, fruits containing easily assimilated fats might also contain secondary compounds that require energy expenditure to detoxify (Bairlein and Gwinner, 1994). This strategy is important to fat composition because birds can store certain fatty acids as a consequence of their benefit to stopover refueling rate rather than their effect on migratory flight physiology.

The importance of rapid mass gain is likely higher in vernal migration relative to the slower paced autumnal migration, and also higher during stopover relative to pre-migratory fattening. Food items that are abundant, easily obtained, have low toxicity, and have high caloric density will obviously be preferred when birds are under heavy selective pressure to refuel quickly. Below I discuss two additional considerations that relate to the type of fatty acids that are stored:

3a) Achieve high absorption rate of dietary fatty acids

Unsaturated fatty acids are generally absorbed more efficiently than saturated fats (Leeson and Summers, 2001; Place, 1996). Among common

dietary fatty acids encountered by overland migrants (16:0, 18:0, 18:1ω9), 18:1ω9 is most quickly and efficiently absorbed in the intestine (Pierce et al., 2004; Renner and Hill, 1961). Birds may therefore seek out food items that contain higher levels of 18:1ω9 and other unsaturates in order to increase rates of energy assimilation. Dietary fatty acids can of course be modified post-absorption; this process, however, decreases utilization efficiency because of the associated metabolic costs (Place, 1996). Birds under pressure to minimize energy expenditure should therefore deposit relatively more dietary lipids intact without hepatic modification.

3b) Achieve high conversion efficiency of other macromolecules to fat

Dietary carbohydrates and proteins can be converted to fat at stopover. This involves catabolism to acetyl-CoA units, followed by the activation to produce malonyl-CoA and then joining to produce a longer aliphatic chain. Once a long chain fatty acid such as 16:0 or 18:0 has been constructed, a desaturase enzyme may be employed to introduce double bonds (perhaps in accordance with strategy #1 above). However, the action of this desaturase involves not only the cost of reduced ATP potential from the resultant unsaturated fatty acid, it also uses cytosolic NADH (or NADPH) directly (equivalent to ~3 ATP per fatty acid, or about 3% of the energy of the resultant molecule)(Berg et al., 2002). This is an extra cost of unsaturated fatty acids when formed from saturates, and it is one that is incurred during

pre-flight fueling. Highest efficiency of glucose or protein conversion therefore results from production of saturated fatty acids.

4) Obtain dietary fatty acids that have important benefits beyond oxidation

These strategies are not directly linked to energetic demands, but will nonetheless affect the composition of fat stores and have the potential to affect energy expenditure during migratory flight.

4a) Arrive at the breeding grounds with reproductively important fatty acids

Capital breeding species are thought to accumulate the nutrient reserves used for egg formation and incubation during migratory stopovers (Ankney and MacInnes, 1978; Drent and Daan, 1980; Ryder, 1970), although some nutrients may also be obtained on the breeding grounds (Gauthier et al., 2003; Hobson et al., 2005). Essential fatty acids are particularly important in avian eggs (Watkins, 1991) and developing vertebrates in general (Xia et al., 1993). If limited in abundance at breeding grounds, essential fatty acids could be necessary to obtain during stopover, particularly for capital breeding species, although most natural foods meet nutritional requirements for essential fatty acids (Klasing, 1998).

4b) Consume dietary fatty acids with other beneficial non-oxidative functions (PPAR activators or membrane PL)

Birds can consume fatty acids for non-oxidative purposes, but doing so will result in the storage of some of these fatty acids in adipose tissue. Investigation of these mechanisms is nascent in birds. PPAR activators remain poorly studied, and eicosanoid production and its effects on avian exercise are completely unknown. The role of peroxisomes in exercise needs to be understood more fully; peroxisomes may be seen as wasting energy but required for oxidation of PUFAs, or they may be seen as alternative routes of oxidation, allowing for greater maximal rates. Although PL have been thought to be important for exercise performance (Maillet and Weber, 2006), my research (Chapter 4) suggests that the importance of muscle PL composition is minimal.

Conclusions

The selective pressures that drive the strategies described above are not mutually exclusive and can act simultaneously to affect migrant fat stores, pushing birds to some optimal composition or range of compositions (Pierce and McWilliams, 2005). Further, there may be limitations to the extent to which these pressures can operate. For example, as seen in Chapters 2 and 5, 18:2ω6 is highly mobilized from adipose and into mitochondria, and therefore might be desirable under selective pressure 1, but its abundance is dependent on the diet. Additionally, there are probably limitations to how saturated triacylglycerol stores can become before they solidify, such that a certain amount of unsaturation is necessary in adipose stores (see

discussion in Chapter 1). Lipid peroxide production is also a potential concern for the storage and oxidation of PUFA.

My research demonstrates the importance of storage of unsaturated fatty acids on maximal rates of fat oxidation. In terms of oxidation during flight, one can see that achieving high maximal rates and storing maximal energy are opposed; the former predicts storage of unsaturates, while the latter predicts storage of saturated fatty acids. There is a tradeoff between double bonds and energy storage (Figure 6.1). This is particularly apparent when one takes into account the increased mobilization of unsaturates, based on values I determined in Chapter 2. In figure 6.2 I have plotted 'ATP mobilizability', calculated as (relative mobilization) x (mmol ATP/g triacylglycerol), as a function of energy storage. This variable is meant to examine the amount of energy throughput during exercise, accounting for both how readily each fatty acid is mobilized and how much ATP it will yield during β-oxidation. Of course, this must come with the caveats that adipose mobilization may not be limiting to fatty acid supply and that the relative mobilization values found in Chapter 2 may not correspond to actual rates. Nonetheless, it provides an approximation of energy throughput. From figure 6.2 it can seen that as energy storage (ATP/g triacylglycerol) increases about 10%, the mobilizability of that stored energy decreases approximately 80%. This result is replicated if the limiting step in fatty acid transport is intramuscular. In figure 6.3 I have plotted relative ATP transport for CPT (based on values I determined in Chapter 5) as a function of energy storage. In a similar way, there is a severe ATP transport cost associated with higher energy storage. Thus, the energetic cost of unsaturation is small (less than 1% per

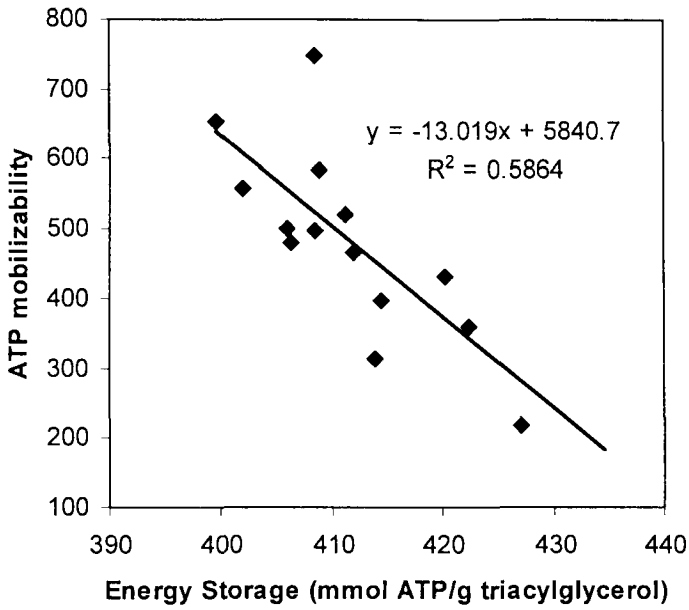

Figure 6.2. Energy mobilization plotted against energy storage for common fatty acids. ATP mobilizability is calculated as (relative mobilization) * (mmol ATP/g triacylglycerol), where relative mobilization values are provided from Chapter 2 in *Philomachus pugnax*.

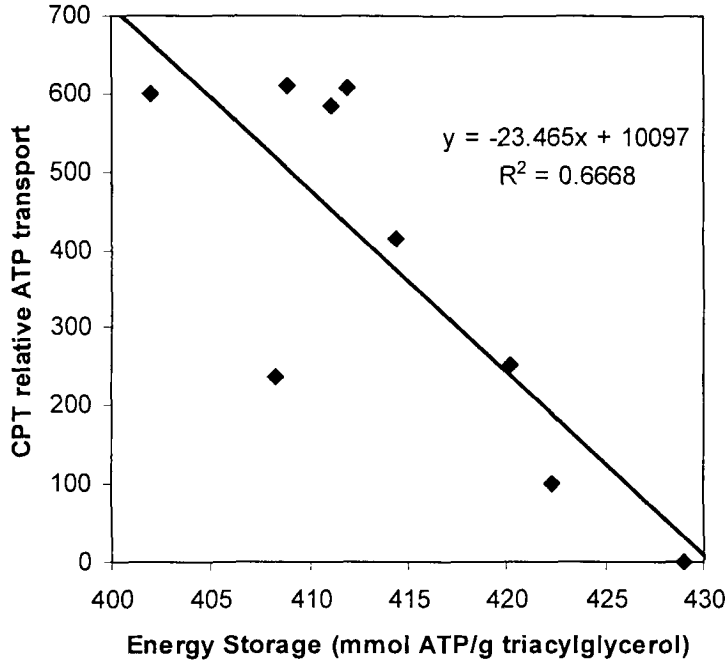

Figure 6.3 CPT relative ATP transport for various fatty acids as a function of energy storage. CPT relative ATP transport values are calculated as (mmol ATP/ g triacylglycerol) x (CPT relative activity), where CPT relative activity was determined in Chapter 5 for *Zonotrichia albicollis*.

double bond) in terms of potential oxidative energy production, and the cost of saturation is potentially a severe mobilization/transport cost. In a migrating bird the energetic cost of unsaturation is probably not biologically significant, except to extremely long-bout migrants. Evaluation of the importance of these tradeoffs will require a survey of adipose stores in long- and short-distance migrants.

The predicted fat storage patterns based on flight energetics (#1 and #2) are confounded by the need to gain mass rapidly at stopover (#3). Moreover, rapid mass gain itself does not entail a clear prediction; assimilation of fats is most efficient for unsaturates, but efficient conversion of carbohydrates to fat will result in the production of saturated fatty acids. Further, food item abundance will likely play a large role in determining these patterns. Migratory fattening is achieved by hyperphagia and increased assimilation efficiency, the latter occurring by both endogenous physiological changes and dietary choice mechanisms (reviewed by Bairlein and Gwinner, 1994). Given that migratory energy expenditure and mortality are highest during stopover, refueling speed may ultimately be more important than flight considerations in determining the fats that are stored by birds.

There are other questions yet to be answered regarding migrant fat storage and use. Notably, the selection pressures and fat storage strategies discussed above do not provide an obvious explanation for the altered fuel efficiency observed by McWilliams and Pierce (2006). The greater mobilizability and transport rates for unsaturates (as demonstrated in Chapters 2 and 5) may require fewer transport proteins to achieve the same metabolic rate during flight, thus decreasing the overall energetic cost. The possibility remains, however, that changes in efficiency are

caused by incorporation of dietary fats into other tissues, such as PPAR and eicosanoids. Phospholipids have also been considered a likely possibility for affecting efficiency, although my results demonstrate that birds don't endogenously alter muscle PL composition for migration (Chapter 3), nor does muscle PL composition affect maximal exercise performance (Chapter 4). Future experiments should focus specifically on this question of efficiency and endurance, and how they can be altered by dietary fats. Ω

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APPENDIX 1: ETHICS COMMITTEE APPROVALS

J. Douglas Coffin, Ph.D.
Associate Professor of Molecular Genetics
Department of Biomedical & Pharmaceutical Sciences
Center for Environmental Health Sciences
The University of Montana

Monday, December 13, 2004

Dr. Chris Guglielmo
Division of Biological SCiences
The University of Montana
Missoula, MT 59812

Dear Dr. Guglielmo,

Your animal care and use protocol titled "The Effects of Migratory Condition on Avian Muscle Phospholipid Composition " was approved by the University of Montana Institutional Animal Care and Use Committee (IACUC) effective December 13, 2004. The protocol identification number is 04404CGDBS121304. Please include your protocol number and the title in correspondence. Your protocol was received on 9/13/2004 and was not approved until now due to an administrative error on our part. Rest assured that the IACUC takes full responsibility for problems associated with that delay and we apologize for any inconveniences our error may have caused.

Please be advised of the following items regarding your approved protocol:

- Any substantial increase in the number of animals used (greater than 10%), changes in care procedures, euthanasia procedures, substantial changes in treatments (surgical, drug or otherwise), changes in personnel or substantial changes in the experimental plan could represent a possible change in animal care an use and you should amend you protocol according to IACUC guidelines.
- Approved animal care and use at the University of Montana is subject to you and the users listed on your protocol following IACUC and Department of Animal Resource guidelines, protocols and procedures. Violations could result in revocation of your approved animal care and use protocol or suspension/revocation of privileges.
- The principle investigator listed on the protocol is ultimately responsible for all activities associated with animal care and use under this protocol.
- Approved animal care and use protocols are public information and may be viewed by the public upon request. Therefore, they should not contain sensitive or proprietary information.
- The IACUC reserves the right to review (and ask for modifications to) your approved protocol at any time.
- You protocol is reviewed and renewed on an annual basis. It may require revisions before December 13, 2005. Your protocol will permanently expire (requiring a new application) on December 13, 2007. Please use the latter (permanent) expiration date for your grant proposals.

Congratulations and good luck with your experiments. The IACUC and Department of Animal Resources at the University of Montana are at your service and willing to provide assistance with any matters related to animal care and use. Please see the DAR web page http://www.umt.edu/research/ar.htm for more information. Thank you for your courtesy.

Sincerely,

J. Douglas Coffin, Ph.D.
Chair, Institutional Animal Care & Use Committee
The University of Montana
Department of BPS, SB 244
The University of Montana, Missoula, MT 59812-1552
Email:
Ph:

Western

August 11

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2009

Dear Dr. Guglielmo:

Your Animal Use Protocol form entitled:

The effects of dietary lipids on sacrolemmal phospholipids, fatty acid transporters and exercise performance in birds.
Funding Agency NSERC Discovery - Grant #311901-05

has been approved by the University Council on Animal Care. This approval is valid from **August 11, 2005 to August 31, 2006** . The protocol number for this project is **2005-060-08.**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR. **PAIN LEVEL Click Here**

Species	Strain	Other Detail	Animal # Total for 1 Year
Bird-Wild	SongBird - White Crowned Sparrow	Adult M/F	60

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post-Admission
320 Euthanasia
360 Blood Collection/Volumes/Multiple Species

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

1. Please ensure that all mandatory training for working on this protocol has been completed.

c.c. Approved Protocol - C. Guglielmo, J. Wasylenko-Weber, D. Cheshuk
Approval Letter - J. Wasylenko-Weber, D. Cheshuk

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Good luck with your thesis.

Best wishes,
Sue

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September 10, 2009

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Canada N6A 5B7

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