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EFFECTS OF DIETARY N-3 FATTY ACIDS ON OXIDATIVE METABOLISM OF BOBWHITE QUAILS

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Master of Science (2008) University of Ottawa

(Biology)

Effects of dietary n-3 fatty acids on oxidative metabolism of Bobwhite quails

Author: Simba Nagahuedi

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EFFECTS OF DIETARY N-3 FATTY ACIDS ON OXIDATIVE METABOLISM OF BOBWHITE QUAILS

SUMMARY

Lipids are believed to be the major fuel used during non-stop flights in migrant bird species, and potential mechanisms that enhance the capacity for fatty acid utilization have been identified in migrant birds. One of these adjustments is the use of dietary lipids, such as n-3 eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), as performance-enhancing substances. Recently, these fatty acids have been found to improve the oxidative capacity of wild semipalmated sandpipers (*Calidris pusilla*) just prior to their migration to South America, by increasing the activity of the Krebs cycle and β-oxidation enzymes. However, these experiments were conducted in a "natural context" and consequently it is impossible to control for a number of confounding variables (such as seasonal migration effects) that could also affect oxidative enzymes independent of diet. Therefore, this thesis aimed to investigate the effects of the diet on Krebs cycle and β-oxidation enzymes using captive bobwhite quails (*Colinus virginianus*) as a model, while also attempting to elucidate the potential mechanisms of action. My goal was to characterize the roles of EPA and DHA in stimulating oxidative capacity, either through changes in membrane composition or through activation of peroxisome proliferator-activated receptors (PPARs). Birds received the following dietary treatments for 6 weeks: EPA, DHA or EPA+DHA. Following the experimental treatments, the activity of several enzymes was measured in quail flight muscles including Krebs cycle enzymes such as citrate synthase and cytochrome oxydase (respectively CS, COX); and markers for β-oxidation such as carnitine palmitoyl transferase and 3-hydroxyacyl dehydrogenase (respectively CPT and HOAD), as well as the expression of PPAR genes. Results reveal that dietary n-3 fatty acids stimulated the activity of oxidative enzymes by 58-90%; surprisingly increases such as these are found only in extreme regimes of endurance training. Moreover, sedentary quails showed the same changes in membrane composition as

refueling sandpipers. EPA and DHA have shown similar doping effects, possibly because the two fatty acids are easily interconverted. The substitution of n-6 arachidonic acid (ARA) by dietary n-3 fatty acids in membrane phospholipids plays an important role in mediating the metabolic effects of the diet, but results provide no significant support for the involvement of PPARs (as determined by changes in gene expression). This study also demonstrates that modifications in fatty acid composition of mitochondrial and sarcoplasmic reticulum membranes can be assessed by monitoring total muscle phospholipids, because all phospholipids are equally affected by diet. Overall the dietary n-3 fatty acids stimulate the capacity for aerobic metabolism in quail flight muscles, thereby mimicking the natural doping effects previously reported in wild sandpipers. However, the absence of associations between the activity of some enzymes and membrane composition is strong evidence that PPARs could be involved in the stimulation of oxidative capacity.

RESUMÉ

Il a été démontré que les oiseaux migrateurs utilisent les réserves lipidiques comme carburant principal durant leur migration. Différents mécanismes utilisés, afin de maximiser l'utilisation de ces acides gras, ont été identifiés chez certaines espèces d'oiseaux. Ainsi l'une de ces ajustements est l'utilisation des acides gras diététiques du type n-3 comme substances améliorant la performance physique, tels que l'acide eicosapentaenoïque (EPA) et l'acide docosahexaenoïque (DHA). Récemment, il a été observé que les bécasseaux semipalmés (*Calidris pusilla*) se nourrissent de ces acides gras afin d'augmenter leur capacité oxydative en stimulant les enzymes du cycle de Krebs et de la β-oxydation, ce juste avant leur vol sans-arrêt vers l'Amérique du Sud. Toutefois, ces expériences ont été réalisées en milieu naturel où il est impossible de contrôler certaines variables (associées à la migration saisonnière) pouvant affecter l'activité enzymatique. Ainsi donc, cette thèse avait pour objectif d'investiguer les effets de la diète sur l'activité des enzymes du cycle de Krebs et de la β-oxydation. D'où le colin de Virginie (*Colinus virginianus*) a été utilisé comme model afin d'identifier les mécanismes potentiels qui y sont impliqués. Les rôles spécifiques d'EPA et DHA dans la stimulation de la capacité aérobic ont été analysés, ce en explorant deux mécanismes d'action : le changement dans la composition membranaire ou la stimulation des récepteurs activés par les inducteurs de la prolifération des peroxysomes (PPARs). Ainsi donc, les colins de Virginie ont été séparés en groupe et ont reçu les diètes suivantes durant 6 semaines: EPA, DHA, EPA+DHA. Après ce traitement, l'activité des enzymes suivants a été mesurée dans les muscles pectoraux: citrate synthase, cytochrome oxydase (CS et COX respectivement, enzymes du cycle de Krebs) et carnitine palmitoyl transférase, 3-hydroxyacyl-déshydrogénase (CPT et HOAD respectivement, index de la β-oxydation). L'activation des PPARs au niveau du gène a été aussi analysée.

Mes résultats démontrent qu'EPA et DHA stimulent la capacité oxydative de 58-90%, et ce genre d'augmentation est seulement observé durant un régime extrême d'exercice d'endurance. De plus, le changement observé au niveau des phospholipides des muscles pectoraux chez les cailles domestiques, reflète ce qui a été observé chez les bécasseaux migrateurs. EPA et DHA ont démontré le même effet dopant, possiblement parce qu'ils ont la capacité de s'interconvertir. La substitution membranaire de l'acide gras arachidonique du type n-6 (ARA) par les acides gras diététiques du types n-3 EPA joue un rôle important dans l'effet métabolique de la diète, cependant aucun résultat n'a supporté l'implication des PPARs (en analysant leurs expressions au niveau du gène). Étant donné que les résultats ont démontré que la diète affecte tous les types de membrane de la même manière, il a été conclu que le changement membranaire au niveau du muscle est représentatif de ce qui se passe au niveau des phospholipides des mitochondries et des réticulums sarcoplasmiques. Cette étude démontre que les acides gras de type n-3 stimulent la capacité aérobic dans les muscles pectoraux du colin de Virginie, ce en imitant le dopage naturel qui a été observé auparavant chez les bécasseaux semipalmés. Le fait qu'il n'y ait pas eu d'association entre l'activité enzymatique et la composition membranaire indique la contribution des PPARs dans la stimulation de la capacité oxydative ne peut être négligée.

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LIST OF ACRONYMS

ARA= Arachidonic Acid

COX= Cytochrome Oxydase

CPT= Carnitine palmitoyltransferase

CS= Citrate synthase

DHA= Docosahexaenoic Acid

DU= Degree of unsaturation

EPA= Eicosapentaenoic Acid

GEM= Gemfibrozil

HOAD= 3-Hydroxyacyl CoA dehydrogenase

MUFA: Monounsaturated fatty acids

PPARs= Peroxisome proliferator-activated receptors

PUFA: Polyunsaturated fatty acids

SFA: Saturated fatty acids

SR= Sarcoplasmic reticulum

FATTY ACIDS TERMINOLOGY

20:5[1]= Eicosapentaenoic Acid

22:6= Docosahexaenoic Acid

16:0= Palmitic Acid

16:1= Palmitoleic Acid

18:0= Stearic Acid

18:1= Oleic Acid

18:2= Linoleic Acid

20:4= Arachidonic Acid

CHAPTER 1. GENERAL INTRODUCTION

Selection of fuel during endurance flight: Their storage, supply and oxidation

Birds respond to extreme environmental conditions in a variety of ways including migration. This spatial movement helps to ensure a sufficient food supply and nesting sites for survival and/or reproduction (Blem, 1990). Many species of migrating birds, particularly shorebirds, are known to undertake long-distance transoceanic flights for thousands of kilometers during which they are unable to feed. These nonstop flights are exceptional for three reasons: 1) they may last for more than 100 h (Battley et al., 2000; Piersma and Baker, 2000); 2) they are performed without any intake of food or water, and thus, the animals must rely exclusively on body energy stores as well as metabolic water; 3) the birds must maintain a very high metabolic rate well above the maximum sustainable rate observed in small exercising mammals (Butler and Woakes, 1990).

Even though the metabolism of birds during endurance flight is still poorly understood for technical reasons (Butler and Bishop, 2000), general patterns of premigratory energy storage are well established (Blem, 1990). One of the most critical 'choices' faced by a migrating bird is to select the appropriate types and proportions of fuels to be used during flight. These include carbohydrates, proteins or lipids that can be stored for later use during endurance exercise. Amongst different possible fuel sources, glycogen represents the main carbohydrate storage in liver and muscle tissues (Hazelwood, 1986). Glycogen is usually stored in conjunction with an extensive amount of water (as much as 3-5 g water per gram carbohydrate) which does substantially increase its transportation cost (Schmidt-Nielsen, 1979). Furthermore, the amount of energy available from these stores is fairly limited, because the energy content of these compounds is only 16.7-18.8 KJ/g compared to 37.6 KJ/g for lipids.

Glycogen stores are most probably the first to be mobilised as a rapid source of energy when migratory flight is initiated (Schwilch et al., 1996). However, its role in fueling long-distance flight is poorly understood and the total amount stored by birds preparing for migration is relatively small (Dawson et al., 1983; Marsh, 1983). Indeed, glycogen is the primary fuel used during very high exercise intensities, such as hovering, burst of short flights and take-off (Suarez et al., 1990). Consequently, the storage of large carbohydrate reserves prior to migration is probably not needed, but cannot be neglected.

Unlike carbohydrates, there are no specific forms of protein reserves. Instead, proteins are a principal constituent of muscle tissue where they serve a functional purpose. Consequently, the use of muscle protein as a source of energy only occurs under extreme conditions (Schmidt-Nielsen, 1979). However, it does represent an excellent fuel which provides up to 15 % of all the energy used for migration. It can be metabolised to amino acids which are then used as gluconeogenic precursors which are metabolized in the liver. Furthermore they may also act as intermediates to counteract the depletion of Krebs cycle intermediates during the oxidative metabolism of fatty acids (Jenni and Jenni-Eiermann, 1998; Klaassen et al., 2000). A drawback of protein catabolism is the production of toxic by-products which require removal or incorporation into less toxic compounds. In addition, a large decrease in protein reserves may lead to a reduction in mass of metabolically costly tissues (i.e. liver, pectoral muscle) even prior to the onset of flight (Piersma and Gill, 1998; Battley et al., 2000), suggesting that protein can be catabolised from any tissue including working muscles, inevitably leading to functional loss.

If protein catabolism is restricted to certain organs, it remains to be determined whether a reduction in organ size is advantageous (as it relates to reducing the size of energetically costly organs that are not needed during flight) or whether it is a means of minimizing

overall damage (in terms of obtaining protein from temporarily less important organs) (Piersma and Lindström, 1997). Alternatively, the hypertrophy of muscles that occurs prior to migration, may represent a compensation of the flight engine in order to cope with the larger body mass resulting from fat stores (Lundgren et al., 1995; Piersma and Gill, 1998) as well as an adjustment to the expected protein loss during endurance flight (Davidson and Evans, 1988).

During long flights, lipids that are sequestered mainly as triglycerides in adipose tissues, but also to a lesser extent in muscle and other organs, are believed to be the major fuel during this period of high energy demand (Dawson et al., 1983). With regards to energy density, lipids are more than seven times denser than glycogen or protein, and the amount of lipids stored may equal or slightly exceed lean wet body mass (Piersma and Gill, 1998). Therefore many possible mechanisms have been identified to enhance the capacity for fatty acid utilization in migratory birds (Weber et al., 1996; Hochachka and Somero, 2002). In many birds species, studies have shown that several factors have been evolved, one of these being the use of dietary fatty acids as performance-enhancing substances. Dietary fatty acids are known to modify energy metabolism by increasing the activity of Krebs cycle and β-oxidation enzymes (Driedzic et al., 1993; Bishop et al., 1995; Sanz et al., 2000). Their effect on energy metabolism is discussed in the following section.

Implication of dietary fatty acids in energy metabolism

Successful, long distance migration does not only depend on intrinsic characteristics of muscles, but on the nature of the oxidative fuels available. Dietary fatty acids are well known to affect metabolism and there are several types of fatty acids, many of which can be synthesized endogenously. However, vertebrates are unable to synthesize either the n-6 or n-3 classes of fatty acids, also called essential fatty acids. These must be ingested in the diet. In the past, fats have largely been regarded as being all similar in their influence on metabolism. It is now obvious, that not all dietary fats should be treated equally. Dietary n-3 fatty acids have well known effects on performance in vertebrates and also have many beneficial health effects in humans (Ruxton et al., 2004). There are several mechanisms by which these fatty acids can affect lipid metabolism. They can either alter membrane composition by getting incorporated into phospholipids (Hulbert et al., 2005), and/or they can bind to nuclear receptors that regulate gene expression of key enzymes involved in energy metabolism (Desvergne and Wahli, 1999; Feige et al., 2006). The following section of this thesis will only focuses on these two mechanisms.

Effects of the incorporation of n-3 fatty acids in membrane phospholipids

Multiple lines of evidence have revealed that the phospholipids composition of membranes can be altered by the inclusion of dietary n-3 fatty acids (Hulbert et al., 2005). The incorporation of dietary n-3 fatty acids increases the degree of unsaturation in muscle membrane phospholipids of birds (Maillet and Weber, 2006), rats (Turner et al., 2004) and humans (Andersson et al., 2002). Many reports have indicated that membranes from mitochondria and other organelles are susceptible to dietary manipulation (Awad, 1986; Guderley et al., 2008), but to our knowledge no study has evaluated the response of different

membrane types to the diet simultaneously. Several studies have shown that dietary n-3 fatty acids can alter membrane fluidity and permeability (Stillwell et al., 1997; Stillwell and Wassall, 2003) and this functional change may facilitate transmembrane lipid transport needed during high energy demands. This physiological response of membrane phospholipids is known to alter the fatty acids surrounding key membrane proteins involved in energy metabolism (Murphy, 1990), including enzymes of oxidative metabolism, ion channels and hormone receptors. In turn, such changes can affect the activity of these proteins. Recent *in vivo* and *in vitro* studies in fish, birds and mammals have shown that n-3 fatty acids stimulate the oxidative capacity of hepatocytes, cardiomyocytes and adipocytes. These fatty acids trigger mitochondrial and peroxisomal proliferation (Froyland et al., 1996; Totland et al., 2000), thereby increasing the activities of β-oxidation and Krebs cycle enzymes (Miyasaka et al., 1996; Froyland et al., 1997; Sanz et al., 2000; Moya-Falcon et al., 2004; Guo et al., 2005). Moreover, Maillet and Weber (2007) found a correlation between the incorporation of dietary n-3 fatty acids and Krebs cycle enzymes activities in the pectoral muscle of semipalmated sandpipers (Maillet and Weber, 2007). The local molecular environment of membrane–bound proteins such as $Na^{+}K^{+}$-ATPase and Ca^{2+}-ATPase pumps, located in the sarcolemma and the sarcoplasmic reticulum (SR), respectively, are influenced by dietary fatty acids (Swanson et al., 1989; Hulbert, 2003; Turner et al., 2005). Interestingly, an elevated proportion of n-3 docosahexaenoic acid (DHA) within the membrane phospholipids of birds increases the $Na^{+}K^{+}$-ATPase activity in mitochondria, suggesting that n-3 DHA content and membrane packing may have profound effects on the aerobic capacity of pectoral muscles typically used for sustained flight (Infante et al., 2001; Else et al., 2003).

The effects of dietary n-3 fatty acids on Ca^{2+}-ATPase activity are quite ambiguous. Some studies have shown that no change in the Ca^{2+}-ATPase activity were detected in the skeletal muscle of rabbits and rats fed with a supplement of fish oil (Gould *et al.*, 1987; Stubbs and Kisielewski, 1990). Whereas in pig skeletal muscles an increase in the ratio of n-3/n-6 in the SR membranes was associated with an increase in Ca^{2+}-ATPase activity (Nurnberg et al., 1998).

Furthermore, the impaired insulin binding is closely associated with insulin-resistant states, such as those associated with feeding on a high-fat diet , as well as obesity (Corcoran et al., 2007).The binding properties of the insulin receptor can be altered by the membrane lipid environment, especially the degree of unsaturation of membrane phospholipids (Gould et al., 1982; Storlien et al., 2000). In cultured (Ginsberg et al., 1981) and isolated cells (Field et al., 1988) membranes enriched in unsaturated fatty acids tend to increase the binding affinity for insulin than membranes enriched in saturated fatty acids.

Peroxisome proliferator-activated receptors (PPARs) and energy metabolism

Dietary n-3 fatty acids can regulate enzyme activity by other mechanisms involving the activation of peroxisome proliferator-activated receptors (PPARs), transcription factors that regulate the expression of target genes in response to natural or synthetic ligands (i.e. n-3 fatty acids, fibrates). After being activated, they recognize and bind to specific nucleotide sequences known as peroxisome proliferator response elements (PPREs) located in the promoter region of target genes (Feige et al., 2006). Three PPAR isoforms (α, β and γ), have been identified encoded by three different genes. PPARα and β are expressed in the tissues with high rates of fatty acid catabolism such as skeletal muscle, which have high mitochondrial and peroxisomal ß-oxidation activity, whereas PPARγ is a major regulator of lipid storage in adipose tissue where it regulates adipocyte differentiation by sensing lipid

availability (Feige et al., 2006). The identification of n-3 fatty acids as PPAR ligands provides firm evidence that at least part of the PPAR-dependent transcriptional activity of fatty acids results from a direct interaction of the nuclear receptors with lipids.

Known effects of dietary n-3 fatty acids

Dietary fatty acids are known to stimulate the capacity for aerobic metabolism through changes in membrane phospholipids and/or through PPAR activation (Hulbert et al., 2005; Feige et al., 2006). It has been shown that they also clearly affect function from organelles to the whole animal, irrespective of their exact mechanism of action. Therefore, dietary intake of EPA and DHA stimulates mitochondrial and peroxisomal proliferation (Froyland et al., 1996; Totland et al., 2000), as well as increase the activities of Krebs cycle and β-oxidation enzymes in mammalian muscles (Power and Newsholme, 1997), liver (Froyland et al., 1996) adipose (Guo et al., 2005) and lymphoid tissue (Miyasaka et al., 1996).

In addition, *in vivo* studies have shown that the capacity for endurance exercise is affected by dietary n-3 fatty acids in mammals, birds and fish. Whole-animal performance of rats increased with a supplement of dietary n-6 fatty acids fatty acids during treadmill exercise (Ayre and Hulbert, 1997). Furthermore, an experiment conducted on Atlantic salmon suggests that dietary n-3 fatty acids increase swimming performance, while another study came to the opposite conclusion, suggesting that swimming performance is increased by dietary n-6 fatty acids (McKenzie et al., 1998; Wagner et al., 2004). Similarly, Pierce et al. have shown that the aerobic performance of a migrant bird species, the red-eyed vireo (*Vireo olivaceous*), was influenced by the fatty acid composition of its diet (Pierce et al., 2005). Overall, therefore, there are disagreements amongst in vivo studies as to whether n-3 fatty acids have a positive or negative impact on aerobic performance.

Doping effects of n-3 fatty acids in semipalmated sandpipers (Calidris pusilla)

Before its fall migration to South America, semipalmated sandpipers (*Calidris pusilla*), a long-distance migrant bird, stops in the Bay of Fundy (east coast of Canada) for two weeks, where it doubles it body mass (~20 g to 40 g) by feeding on a an amphipod, *Corophium volutator*. This diet contains record amounts of n-3 fatty acids (eicosapentaenoic (EPA) and docosahexaenoic acid (DHA)) (Ackman *et al.*, 1979; Maillet and Weber, 2006). It has been recently suggested that this migrant bird uses n-3 fatty acids as a performance-enhancing agent to prime its flight muscles for endurance exercise (Maillet and Weber, 2006, 2007). During refueling, the double bond index of muscle phospholipids (PL) increased by 25% and the fatty acid profiles of muscle PL converged with that of the diet. Moreover, this unusual diet appears to increase the activities of key oxidative enzymes of lipid metabolism (citrate synthase (CS), 3-hydroxyacyl CoA dehydrogenase (HOAD)) in muscle, and some correlations were found relating the abundance of n-3 fatty acids in muscle PL and enzyme activities: implying a functional relationship between diet and energy metabolism.

Maillet and Weber (2007) suggest that EPA and DHA may have different effects and act through changes in membrane composition [%DHA in phospholipids is correlated with CS activity and %EPA with HOAD activity or %EPA in triacylglycerol reserves is correlated with CS], as well as through membrane-independent mechanisms (Maillet and Weber, 2007). Thus, the incorporation of dietary n-3 PUFA from marine invertebrates in muscle membrane PL alters membrane fluidity and the local molecular environment of membrane proteins (Murphy, 1990; Stillwell and Wassall, 2003). This physiological change may improve the capacity for fat intake and oxidation, which in turn can affect muscle performance. An alternative strategy to improve energy metabolism is the binding of n-3

fatty acids to peroxisome proliferator-activated receptors (PPARs) that modulate the expression of genes involved in lipid metabolism (Feige et al., 2006). This mechanism supports the idea that dietary n-3 fatty acids may be used as molecular signals to prime flight muscles for extreme exercise in some long-distance migrants.

Given that these experiments were carried out in a "natural context", it was not possible to control for a number of confounding variables, such as seasonal migration effects like exercise training (see appendices, Tables 2.5) or hormonal changes that could also affect oxidative enzymes independently of diet. Furthermore, it is not clear whether : 1) EPA or DHA is responsible for the doping effects because both are present in the diet of semipalmated sandpipers; 2) PPARs are involved in the up-regulation of genes important for lipid metabolism; 3) all membranes are equally affected by the diet: effects of dietary n-3 fatty acids on aerobic metabolism can be associated more specifically to mitochondrial membranes and may be more relevant to this kind of study. Only total tissue phospholipids were measured in previous studies. Maillet and Weber have used indirect evidence to propose a mechanistic link between the presence of particular fatty acids and the induction of key enzymes of lipid metabolism. Their study was an important step towards identifying potential mechanisms of action of specific fatty acids on specific enzymes involved in energy metabolism. In this thesis, I have carried out controlled lab experiments focusing on the effects of the diet on aerobic performance in birds, and to investigate the roles of EPA and DHA independently. In order to evaluate the effects of dietary n-3 fatty acids and to characterize their mechanism of action, a non migrant birds species, Bobwhite quails (*Colinus virginianus*) were selected as a model organism because the diet of this domesticated bird can easily be manipulated in captivity with minimal stress.

Objectives of the study

Previous studies on semipalmated sandpipers (*Calidris pusilla*) only provided indirect evidence that dietary n-3 fatty acids are involved in increasing aerobic metabolism. The mechanisms of action for the induction of particular enzymes by specific fatty acids have not been characterized. Consequently, the goals of this study were to use captive Bobwhite quails as a model to determine: 1) whether dietary n-3 fatty acids can, on their own, stimulate the capacity for aerobic metabolism in the flight muscle of a non-migratory bird, 2) whether the activation of Krebs cycle enzymes [CS, and Cytochrome oxidase (COX)] and β–oxidation enzymes [HOAD and Carnitine palmitoyl transferase(CPT)] is caused by changes in membrane phospholipids composition and/or changes in PPAR gene expression, 3) whether dietary EPA and DHA have different effects, and 4) whether total muscle phospholipids, mitochondrial membranes and sarcoplasmic reticulum show the same pattern of fatty acid incorporation from the diet. I anticipated that the natural doping effects of n-3 fatty acids previously reported in migrant sandpipers could be replicated in quails, and are mediated by membrane- and PPAR-related mechanisms. It was hypothesized that EPA and DHA would have different effects and that all muscle membranes would be equally affected by diet.

CHAPTER 2. MIMICKING THE NATURAL DOPING OF MIGRANT SANDPIPERS IN SEDENTARY QUAILS:EFFECTS OF DIETARY N-3 FATTY ACIDS ON MUSCLE MEMBRANES AND PPAR EXPRESSION

Introduction

The semipalmated sandpiper (*Calidris pusilla*) uses natural doping to prime its flight muscles for transatlantic migration from Canada to South America (Maillet and Weber, 2006, 2007). In preparation for this non-stop flight, body mass is doubled by feeding frantically on marine invertebrates containing record amounts of n-3 eicosapentaenoic acid (EPA) and n-3 docosahexaenoic acid (DHA) (Maillet and Weber, 2006). This unusual diet causes the rapid incorporation of n-3 fatty acids in tissue lipids, thereby augmenting unsaturation, and it increases the activities of oxidative enzymes in muscle. The observed correlations between the abundance of n-3 fatty acids in muscle lipids and enzyme activities imply an exciting functional relationship between diet and energy metabolism. Previous studies suggest that EPA and DHA have different effects and do not only act through changes in membrane composition, but also through membrane-independent mechanisms. However, current evidence for natural doping is mainly based on indirect support (Maillet and Weber, 2006, 2007). The observed increase in muscle oxidative capacity may not be entirely caused by diet, but by other seasonal effects of migration like exercise training and hormonal changes. Here, we carried out controlled laboratory experiments to focus on the effects of diet and to investigate the roles of EPA and DHA independently. Bobwhite quail (*Colinus virginianus*) was selected as a model to characterize the mechanisms of action of n-3 fatty acids because the diet of this domesticated bird can easily be manipulated in captivity. In previous studies, it was assumed that the fatty acid composition of total tissue phospholipids reflects the composition of mitochondrial membranes (Maillet and Weber, 2006, 2007). However, we could find no published data demonstrating that all membranes are equally affected by diet.

EPA and DHA are well known to influence metabolism *in vivo* and *in vitro*, either by getting incorporated into membranes (Hulbert et al., 2005) or through binding to nuclear receptors that regulate gene expression (Feige et al., 2006). Diet and *in vitro* manipulations have been used to alter the fatty acid composition of phospholipids. The resulting changes in membrane fluidity, permeability, n-3/n-6 ratio, and local molecular environment play important roles in modulating the activities of key membrane proteins (Murphy, 1990; Stillwell et al., 1997; Gerson et al., 2008; Guderley et al., 2008). They include enzymes from oxidative pathways, ATPases, hormone receptors and ion channels [carnitine palmitoyl transferase (CPT) (Power and Newsholme, 1997; Guo et al., 2005); citrate synthase (CS)(Miyasaka et al., 1996); Na^+K^+ ATPase; insulin receptor (Corcoran et al., 2007); Na^+ and Ca^{2+} channels (Leaf et al., 2005)]. EPA and DHA are also natural ligands for peroxisome proliferator-activated receptors (PPARs), and these transcription factors regulate the expression of genes orchestrating fundamental aspects of lipid metabolism. Three PPAR isoforms have been identified with distinct tissue distributions and functions. PPARα and β are mostly involved in stimulating fatty acid oxidation, whereas PPARγ modulates lipid storage and adipocyte differentiation (Berger and Moller, 2002; Feige et al., 2006).

Membrane changes (Hulbert et al., 2005) and/or PPAR-induced mitochondrial and peroxisomal proliferation (Froyland et al., 1996; Totland et al., 2000) can stimulate capacity for aerobic metabolism. It is clear that n-3 fatty acids affect function from organelles to the whole organism, even though exact mechanisms are still unknown. The physiological response to n-3 fatty acids is characterized by increases in the activities of Krebs cycle and β-oxidation enzymes in mammalian muscle (Power and Newsholme, 1997), liver (Totland et al., 2000) adipose tissue (Guo et al., 2005) and lymphoid tissue (Miyasaka et al., 1996). *In*

vivo capacity for endurance exercise is also affected by dietary fatty acids in mammals (Ayre and Hulbert, 1997), fish (McKenzie et al., 1997; Wagner et al., 2004) and birds (Pierce et al., 2005). However, these whole-organism studies fail to agree as to whether n-3 fatty acids have a positive or negative impact on aerobic performance.

The goals of this study were therefore to use domestic bobwhite quails as a model to determine whether: 1) dietary n-3 fatty acids alone can stimulate capacity for aerobic metabolism in the flight muscle of a non-migratory bird; 2) the activation of oxidative enzymes is caused by changes in the composition of membrane phospholipids and/or PPAR gene expression; 3) dietary EPA and DHA have different effects; and 4) phospholipids from total muscle, mitochondria and sarcoplasmic reticulum show the same pattern of fatty acid incorporation from the diet. We anticipate that the doping effects of n-3 fatty acids previously observed in migrant sandpipers can be replicated in non-migratory quails and are mediated by membrane-related as well as PPAR-related mechanisms. We hypothesize that EPA and DHA have different effects on oxidative metabolism and that all membranes are equally affected by the diet.

Materials and methods

Animals

Seven-week-old tamed bobwhite quails of both sexes (*Colinus virginianus*; 224 ±27g, N=40) were obtained from a local supplier (Sainte-Hyacinthe, QC, Canada) and fed a commercial diet used for turkey (21% protein, 3.5% lipids, 4% fibre; LaBonté Belhumeur, St. Bonaventure, QC, Canada) with water *ad libitum*. The animals were handled daily from hatching by the supplier to familiarize them with humans and minimize stress. They were banded for individual identification and housed in groups of 20. Each group was kept in a windowless room (4 x 1.5 x 2 m) with wood chips as substrate. They were held at 22°C and 50% humidity under a 12h:12h (light:dark) photoperiod, and were acclimated to these conditions for at least 1 month before experiments.

Dietary treatments and tissue sampling

The natural diet of migrating semipalmated sandpipers (Maillet and Weber, 2006, 2007) was mimicked by supplementing the food of bobwhite quails with oils administered by gavage. The birds were divided into 5 groups of 8 individuals (a sex ratio of 50/50 was maintained in each treatment group): control (corn oil), EPA (oil enriched with n-3 eicosapentaenoate), DHA (oil enriched with n-3 docosahexaenoate), EPA+DHA (EPA and DHA oils were given on alternate days), and GEM (gemfibrozil, a hypolipidemic drug and PPAR agonist).EPA and DHA oils were a generous gift from Ocean Nutrition (Dartmouth, NS, Canada). The oils (0.7 ml/day for 6 weeks) or GEM (5 mg in 0.7 ml of corn oil/day for 4 days) were administered using a 14 G gavage needle made of stainless steel. For each animal, gavage was performed daily in ~30 s with minimal stress because all the birds were habituated to the procedure. The fatty acid composition of the food and of the oil supplements is presented in Table 2.1 (see appendices). After the gavage period, the animals

were euthanized with CO_2 followed by cervical dislocation. Pectoral muscle was rapidly excised by dissection. About 5 g of muscle was freeze-clamped in liquid N_2 to measure enzyme activities and the fatty acid composition of membrane phospholipids. About 0.5 g of muscle was snap frozen in liquid N_2 to measure PPAR expression. All samples were stored at –80°C for a maximum of 3 months before analyses.

Homogenate preparation

Approximately 200 mg of frozen pectoral muscle samples were combined with an ice-cold homogenization buffer (40 mM Hepes, pH 7.3) at a 9:1 ratio (10% mass/volume). Samples were homogenized on ice using a ground-glass homogenizer. Homogenate was centrifuged at 12,400 rpm for 10 minutes at 4°C.

Enzyme assays

The activities of the following enzymes were performed in triplicate at 37°C using a Spectramax Plus 384 and Clear 96-weel flat bottom (Costar): citrate synthase (CS; E.C. 2.3.3.1), total carnitine palmitoyl transferase (CPT I + CPT II; E.C. 2.3.1.21), 3-hydroxyacyl CoA dehydrogenase (HOAD; E.C. 1.1.1.35) ((Maillet and Weber, 2007); Guglielmo et al., 2002) and cytochrome oxidase (COX; E.C. 1.9.3.1) (Moyes et al,1997,1998). Activities were determined by changes in absorbance at 412 nm (CS and CPT), 340 nm (HOAD) and 550 nm (COX). Preliminary measurements were performed to determine homogenate concentrations yielding maximum reaction velocities..

For CS, assay conditions contained solution A [0.15 mM DTNB (5,5_-dithiobis 2-nitrobenzoic acid), 0.15 mM acetyl CoA), 0.5 mM oxaloacetate (substrate)] and 1:9 diluted homogenate that were added to Tris buffer (50 mM, pH 8.0). For CPT, assay conditions contained solution A [0.15 mM DTNB, 0.035 mM, palmitoyl CoA, 1.9 mM EDTA), 5 mM carnitine (substrate)] and 1:9 diluted homogenate that were added to Tris buffer (50 mM, pH·8.0). For HOAD, assay conditions contained solution A (1 mM EDTA, $2.5x\ 10^{-6}$ mM KCN) , Solution B [0.2 mM NADH (ß-nicotinamide adenine dinucleotide, reduced form)], 0.1 mM acetoacetyl CoA (subtrate) and 1:9 diluted homogenate that were added Imidazole buffer (50 mM, pH 7.4). For COX, assay conditions contained Tris buffer (20 mM, pH 8.0), 0.5% Tween 20, 1:1 diluted homogenate and 0.05 mM reduced cytochrome *c*, which was reduced by using ascorbate (Sigma, St Louis, MO, USA), dialyzed exhaustively against 20 mM Tris (pH 8.0), and frozen in aliquots at −80°C. Sample homogenates were preincubated for 6 min at 37°C with buffer and detergent before cytochrome *c* addition. Shorter incubations reduced activities. Substrates and homogenates were omitted in controls, and background activity was subtracted from measurements in the presence of substrate and homogenate. The reaction was started by addition of substrate.

PPAR expression

Partial coding sequences for quail PPARα, β, and γ, as well as for 18S were cloned from quail pectoral muscle and the sequence information was deposited to GenBank (see appendix for accession numbers shown in Table 2.2). Changes in the expression of PPAR α, β and γ were measured in pectoral muscle. All reagents were obtained from Invitrogen (Burlington, ON, Canada) unless otherwise indicated. Total RNA was isolated from ~0.1 g frozen muscle using TRIzol®reagent (Gibco BRL, Burlington, ON, Canada). RNA concentration and quality were verified using NanoDrop 1000 (Thermo Fisher Scientific).

Isopropanol and linear acrylamide (Ambion, Austin, TX, USA) were added to aid RNA precipitation. The samples were placed for 30 min on dry ice and centrifuged to collect the RNA pellet (12,000 g, 5 min, 4 °C). Total RNA was DNase-treated (DNase I, Amplification Grade) and First-strand cDNA synthesis was performed using 1 ug RNA in 11 ul RNase-/DNase-free water, and primed with 1 ul random hexamer primers. The mixture was incubated at 65 °C for 10 min, quickly chilled on ice, and briefly spun (13,750 g). Four ul of 5X reaction buffer, 2 ul 0.1 M DTT, 1 ul 10 mM dNTPs, and 1 ul RNase inhibitor were added, gently mixed, and the solution was incubated at 42 °C for 2 min. One ul SuperScript™ II RNase H- Reverse Transcriptase or 1 ul of water (NoRT) was added and the reaction was allowed to continue for 50 min at 42 °C. The reaction was inactivated at 70°C for 15 min and stored at -20 °C until use. The genes of interest were cloned and sequenced, and Primer3 (http://frodo.wi.mit.edu) was used to design primers based on the gene sequence of bobwhite quail (see appendix Table 2.2). Primers of 18-22 nucleotides with optimal annealing temperature between 59-61°C were designed to amplify sequences of 150-250 base pairs (bp). Primers were initially tested using quail muscle cDNA and the resultant amplicons were sequenced to confirm specificity.

Real-time RT-PCR analysis of gene expression was carried out on first-strand cDNA derived from DNase-treated RNA samples from control and treatment groups. Each PCR mixture contained ~25 ng first-strand cDNA template, 1X QPCR, 2.5 mM $MgCl_2$, 100-400 nM gene-specific primer (depending on the primer set used), 0.25X SYBR green, 200 uM dNTPs, 1.25 U HotStarTaq (Qiagen, Mississauga, ON, Canada), and 100 nM ROX reference dye, in a 25 ul total reaction volume. The primer sets used in this study are reported in Table 2. Thermal cycling parameters were as follows: initial 1 cycle Taq activation at 95°C for 10 min, 40 cycles at 95°C for 15 s, 58 °C for 5 s, 72 °C for 54 s, and a detection step at 80 °C for 22 s.

Real-time RT-PCR was assayed on a MX3005® Multiplex Quantitative PCR system (Stratagene, Mississauga, ON, Canada) and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data were analyzed using the MxPro Software Package (Stratagene). The relative expression of the PPAR genes was normalized to the expression of 18S RNA, which was not affected by the experimental treatments.

Lipid analysis

The fatty acid composition of membrane phospholipids was measured in total muscle, isolated mitochondria and isolated sarcoplasmic reticulum. Chloroform-methanol (2:1 v/v) (Folch et al., 1957) was used for double extraction of total lipids from 0.5 g muscle homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland), or from 10 mg protein of isolated mitochondria or isolated sarcoplasmic reticulum. These organelles were purified by ultracentrifugation as published previously (Ashour and Hansford, 1983), with the following modifications: 0.5 g of frozen tissue was used and protein concentration was adjusted to ~5 mg of protein/ml. After filtration, 0.25% KCl was added and the mixture was placed at 60°C to separate the organic phase containing the lipids. This phase was dried on a rotating evaporator (Büchi Rotavapor, Flawil, Switzerland). The same procedure was used to extract total lipids from the food and oil supplements. Phospholipids in total muscle, mitochondria or sarcoplasmic reticulum were separated from total lipids using Supelclean LCNH2 solid-phase extraction tubes (Sigma, St Louis, MO, USA). The fatty acid composition of the phospholipids was measured using gas chromatography after transesterification as previously (Maillet and Weber, 2006). Fatty acid methyl esters were analyzed on an Agilent Technologies 6890N gas chromatograph equipped with a fused silica capillary column (Supelco DB-23, 60 m x 0.25 mm i.d., 0.25 μm film thickness) using

hydrogen as carrier gas. The system was equipped with an automatic injection system (Agilent Technologies 7683B Series). Detailed conditions of analysis have been described previously (Magnoni and Weber, 2007).

Calculations and statistical analyses

Enzyme activities ($\mu mol.min^{-1}.g^{-1}$) were calculated as follows:

$$\text{Activity} = \frac{(\Delta Abs / \Delta t) \times V_f}{(\varepsilon \times V_h)} \times D$$

Where ΔAbs is the change in absorbance at 340, 412, or 550 nm, Δt is the reaction time in min, V_f is the final cuvette volume in µl, V_h is the volume of added homogenate in µl, ε is the extinction coefficient ($\mu mol^{-1}.ml$, mM^{-1}) (13.6 for DNTB, 6.22 for NADH, and 28.5 for cytochrome c) and D is the dilution factor of the homogenate. Fatty acids accounting for less than 1% of total fatty acids in phospholipids were not included in the analysis.

Statistical analyses were performed using SYSTAT 8.0 or SigmaStat 3.1 (Systat Software, Chicago, IL, USA). Principal component analysis was used to identify which fatty acids from membrane phospholipids were affected by the treatments. The effects of the diets on enzyme activities (Fig. 2.1), PPAR gene expression (Fig. 2.2), % individual fatty acids in membrane phopholipids (Figs. 2.3-2.5), and n-3/n-6 ratio (Fig. 2.7) were analyzed using one way non-parametric ANOVA on ranks and Bonferroni *post-hoc* test. Non-parametric t-tests on ranks were performed to examine the effects of GEM on PPAR gene expression (Fig. 2.2) and on the difference in n-3/n-6 ratio between lean and fat semipalmated sandpipers (Fig. 7). Non-parametric tests where used when the assumption of normality or homoscedasticity was not met.

Relationships between enzyme activities and % contribution of individual fatty acids in membrane phospholipids (Table 2.4) or between enzyme activities and n-3/n-6 ratio (Fig. 2.8) were assessed by linear regression. No gender differences were found in all the analyses that were performed in this study. Statistical significance was set at $P<0.05$ and all the values presented are means ± S.E.M.

Results

Dietary n-3 fatty acids and enzyme activities

Figure 2.1 shows the changes in the activities of quail pectoral muscle enzymes caused by dietary EPA, DHA or a combination of both fatty acids (EPA+DHA). Two Krebs cycle enzymes (CS and COX) and 2 β-oxidation enzymes (CPT and HOAD) were monitored. By themselves, EPA or DHA caused increases in CS and HOAD ($P<0.05$), but these diets had no effect on COX ($P>0.05$). DHA was the only dietary treatment that stimulated CPT ($P<0.05$). The combined administration of EPA+DHA increased the activity of the Krebs cycle enzymes CS and COX ($P<0.05$), but had not effect on the β-oxidation enzymes CPT and HOAD ($P>0.05$).

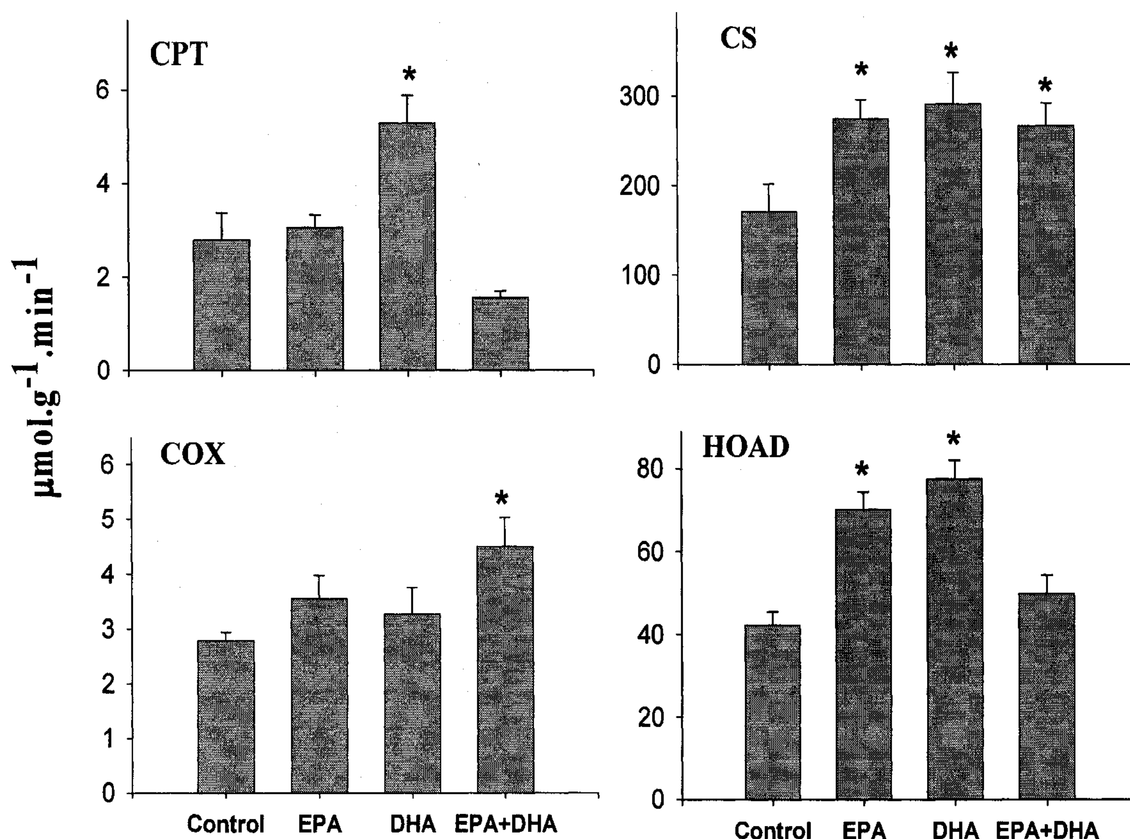

Figure 2.1: Changes in the activities (μmol.g^{-1} .min^{-1}) of Krebs cycle enzymes (CS and COX) and β-oxidation enzymes (CPT and HOAD) in quail pectoral muscle for the different treatment groups (EPA, DHA and EPA+DHA). Values are means ± S.E.M. (N=8, except for DHA where N=7). * indicates differences from control ($P < 0.05$). CS: Citrate synthase; HOAD: 3-hydroxyacyl CoA dehydrogenase; CPT: Carnitine palmitoyl transferase; COX: Cytochrome oxidase. EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

Effects of dietary n-3 fatty acids and GEM on PPAR gene expression

The effects of EPA, DHA, EPA+DHA, and of the fibrate drug GEM on the expression of genes coding for PPAR α, β and γ in pectoral muscle are reported in Fig. 2.2. Overall, the n-3 fatty acid supplements had no significant effects on the relative expression of any PPAR gene ($P>0.05$). GEM caused minor but statistically significant 1.5-fold and 2-fold increases in the expression of PPAR α and PPAR γ ($P<0.05$), respectively.

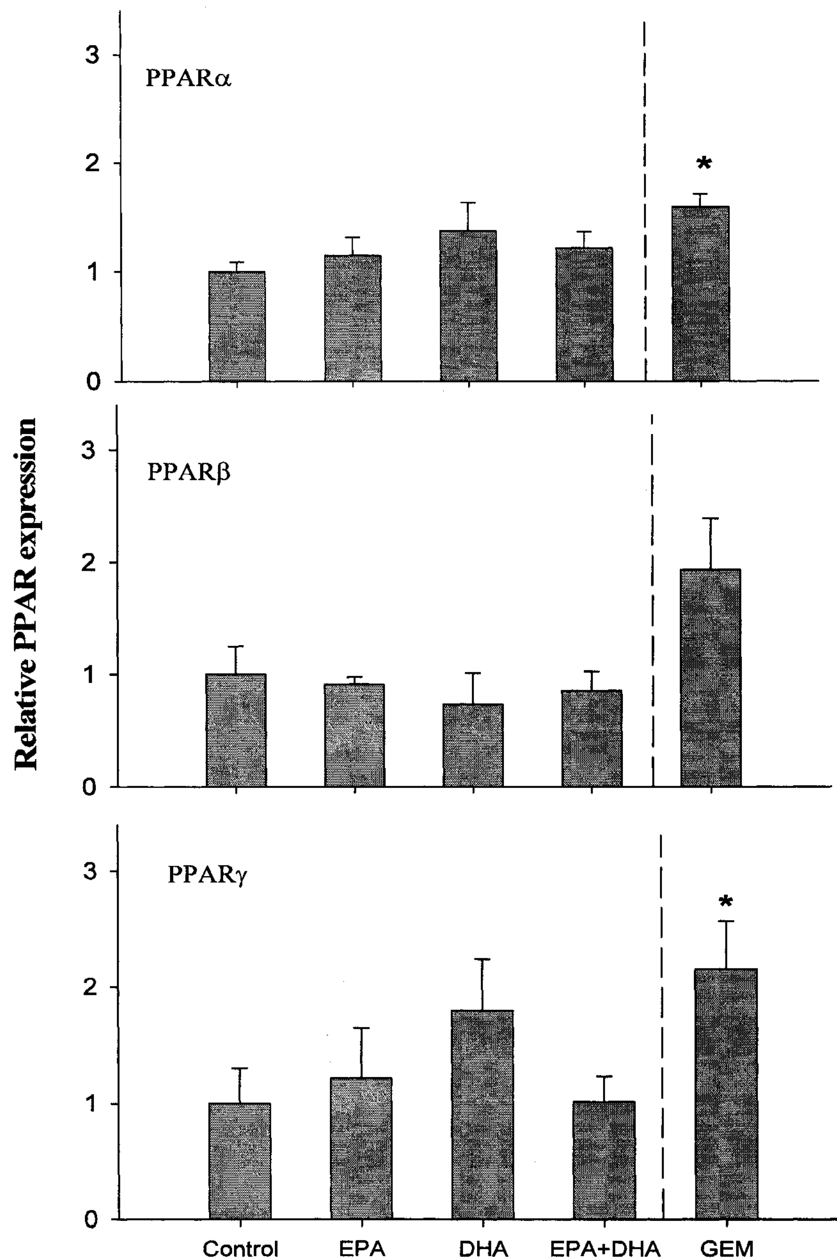

Figure 2.2: Effects of dietary n-3 fatty acids and Gemfibrozil (GEM) on the mRNA levels of peroxysome proliferator-activated receptors (PPAR) α, β and γ, in quail pectoral muscle. The expression of PPAR genes is normalized to the expression of 18S. Values are means ± S.E.M. (N=8, except for DHA where N=7) and * indicates differences from control (P<0.05).

Incorporation of dietary n-3 fatty acids in muscle membranes

Table 2.3 shows the detailed fatty acid composition of flight muscle membranes and how it is affected by dietary EPA and DHA. Principal component analysis reveals that only 3 fatty acids of membrane phospholipids were modified by the diets; they were arachidonic acid (ARA), EPA and DHA, and they are shown with grey highlights in Table 2.3. Dietary supplements had no significant effect on the relative membrane abundance of saturated, monounsaturated and polyunsaturated fatty acids ($P>0.05$). However, the degree of unsaturation was increased by the EPA and DHA diets ($P<0.05$), and the n-3/n-6 ratio was increased by all diets ($P<0.05$). The fatty acids whose relative abundance was affected significantly by the diets in different muscle membranes are reported in Figs. 2.3-2.5. For total muscle membranes, all the diets caused an increase in %EPA and %DHA, as well as a decrease in %ARA (Fig. 2.3; $P<0.05$). More importantly, these dietary effects were the same in mixed membranes from total muscle tissue (Fig. 2.3), in membranes specifically isolated from muscle mitochondria (Fig. 2.4), and in sarcoplamic reticulum (Fig. 2.5) ($P>0.05$).

Table 2.3: Fatty acid composition of flight muscle membranes in bobwhite quails from control, EPA, DHA and EPA+DHA treatment groups. Values are percentages of total fatty acids. Principal component analysis identified that EPA, DHA and ARA were the only fatty acids significantly modified by the diets and they are highlighted in grey. Statistical differences in SFA, MUFA, and PUFA, as well as DU and n-3/n-6 ratio in muscle phospholipids are indicated by * (one way ANOVA, $P<0.05$). Values are means ± S.E.M (N=8, except for DHA treatment group where N=7). See list of acronyms for abbreviations.

Fatty acid	*Control*	*EPA*	*DHA*	*EPA+DHA*
16:0	24.0 ±0.6	22.7 ± 0.6	21.0 ± 1.1	20.0 ± 1.2
18:0	16.8 ± 0.5	16.1 ± 0.6	16.6 ± 0.9	17.3 ± 2.3
18:1	14.2 ± 0.5	11.0 ± 0.7	11.6 ± 1.8	12.9 ± 1.6
n-6 18:2	9.4 ± 0.7	5.4 ± 0.3	6.5 ± 0.7	6.1 ± 0.6
n-6 20:4 (ARA)	20.9 ± 1.2	10.7 ± 0.5	6.3 ± 0.6	9.3 ± 0.6
n-3 20:5 (EPA)	0.1 ± 0.1	9.4 ± 0.8	8.1 ± 0.7	9.1 ± 0.6
n-3 22:5	0.6 ± 0.3	1.7 ± 0.1	3.0 ± 2.5	0.5 ± 0.2
n-3 22:6 (DHA)	14.1 ± 0.8	17.3 ± 0.6	22.0 ± 0.7	17.8 ± 0.5
SFA	40.8 ± 0.6	44.5 ± 2.6	42.4 ± 3.7	44.2 ± 2.1
MUFA	14.2 ± 0.5	11.7 ± 0.7	11.6 ± 1.8	12.9 ± 1.6
PUFA	45.1 ± 0.7	44.5 ± 0.7	45.9 ± 1.8	42.9 ± 1.0
n-3/n-6	0.5 ± 0.1	1.8 ± 0.1*	2.1 ± 0.2*	1.7 ± 0.1*
DU	2.0 ± 0.0	2.3 ± 0.0*	2.4 ± 0.1*	2.2 ± 0.0

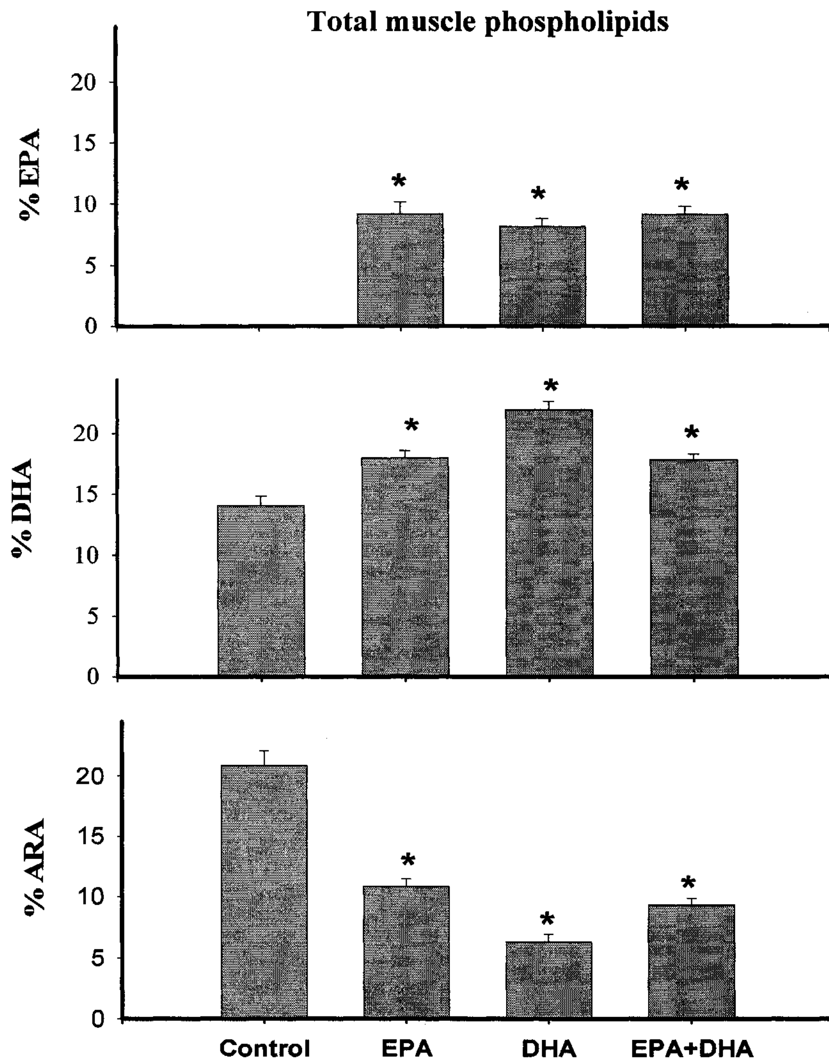

Figure 2.3: Changes in the % contribution of individual fatty acids in total phospholipids from muscle for the different treatment groups (Control, EPA, DHA, and EPA+DHA). Values are means ± S.E.M. (N=8, except for DHA where N=7) and * indicates differences from control (P<0.05). See list of acronyms for abbreviations.

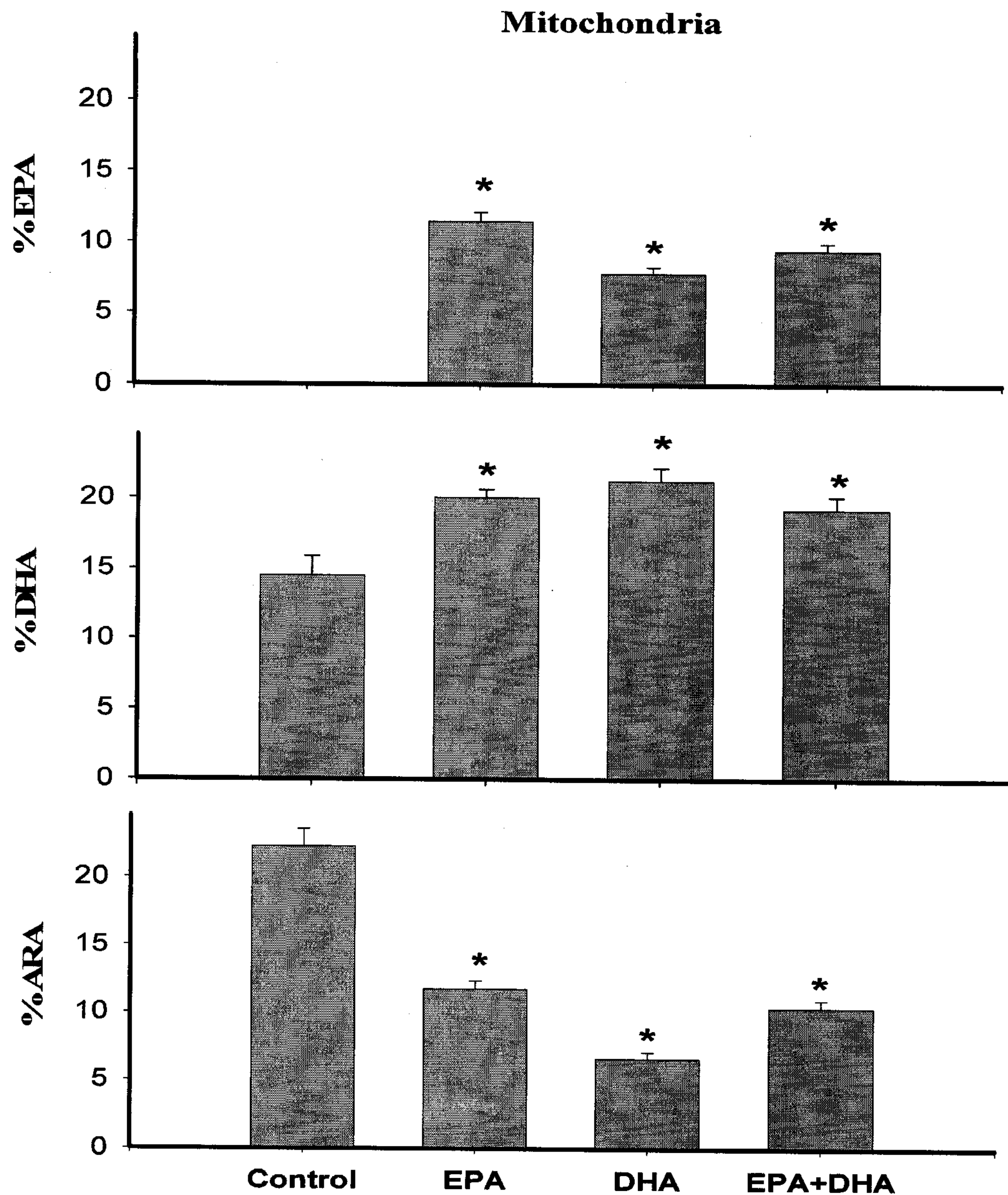

Figure 2.4: Changes in the % contribution of individual fatty acids in membranes from isolated muscle mitochondria for the different treatment groups (Control, EPA, DHA, and EPA+DHA). Values are means ± S.E.M. (N=8, except for DHA where N=7) and * indicates differences from control (P<0.05). See list of acronyms for abbreviations.

Figure 2.5: Changes in the % contribution of individual fatty acids in isolated sarcoplasmic reticulum for the different treatment groups (Control, EPA, DHA, and EPA+DHA). Values are means ± S.E.M. (N=8, except for DHA where N=7) and * indicates differences from control ($P<0.05$). See list of acronyms for abbreviations.

Changes in membrane composition of bobwhite quails: comparison with refueling semipalmated sandpipers

Figures 2.6 and 2.7 compare the changes in membrane phospholipids observed in captive quails fed artificial diets under controlled conditions (this study) and in wild semipalmated sandpipers refueling naturally on marine invertebrates just before a long migratory flight (Maillet and Weber, 2006). The artificial diets of bobwhite quails (top panel Fig. 2.6) and the natural diet of semipalmated sandpipers (bottom panel Fig. 2.6) had the same qualitative effects on flight muscle membrane composition: an increase in %EPA and %DHA, accompanied by a decrease in %ARA. However, the increase in EPA and decrease in ARA were quantitatively larger in quails than in sandpipers. Figure 2.7 shows that the artificial diets of quails and natural diet of sandpipers caused a significant increase in the n-3/n-6 ratio of muscle membranes ($P<0.05$).

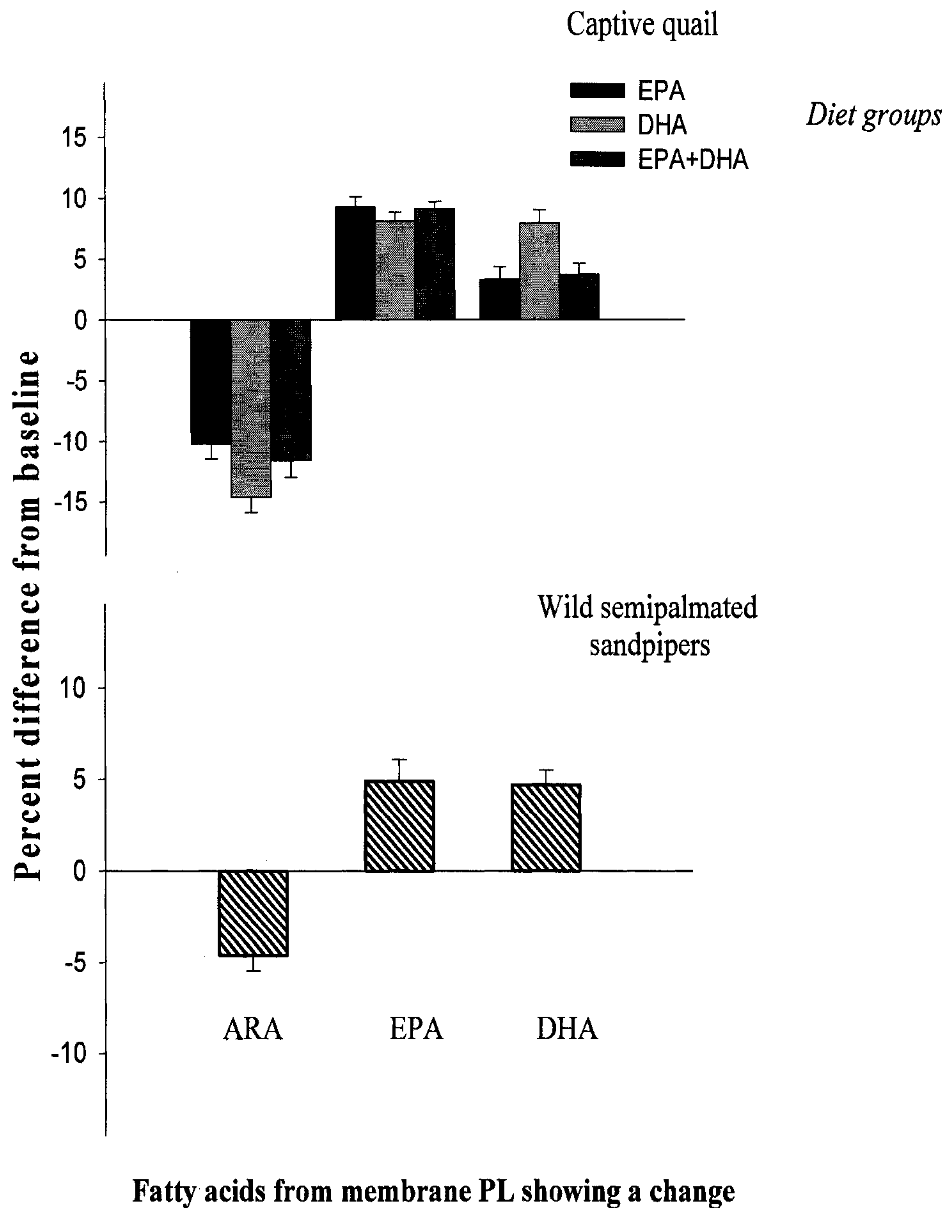

Figure 2.6: Relative changes in the fatty acid composition of muscle membrane phospholipids in captive quails fed different diets (Control, EPA, DHA, EPA+DHA)(top panel) and in wild semipalmated sandpipers during pre-migration refueling (bottom panel). Values are means ± S.E.M. See list of acronyms for abbreviations.

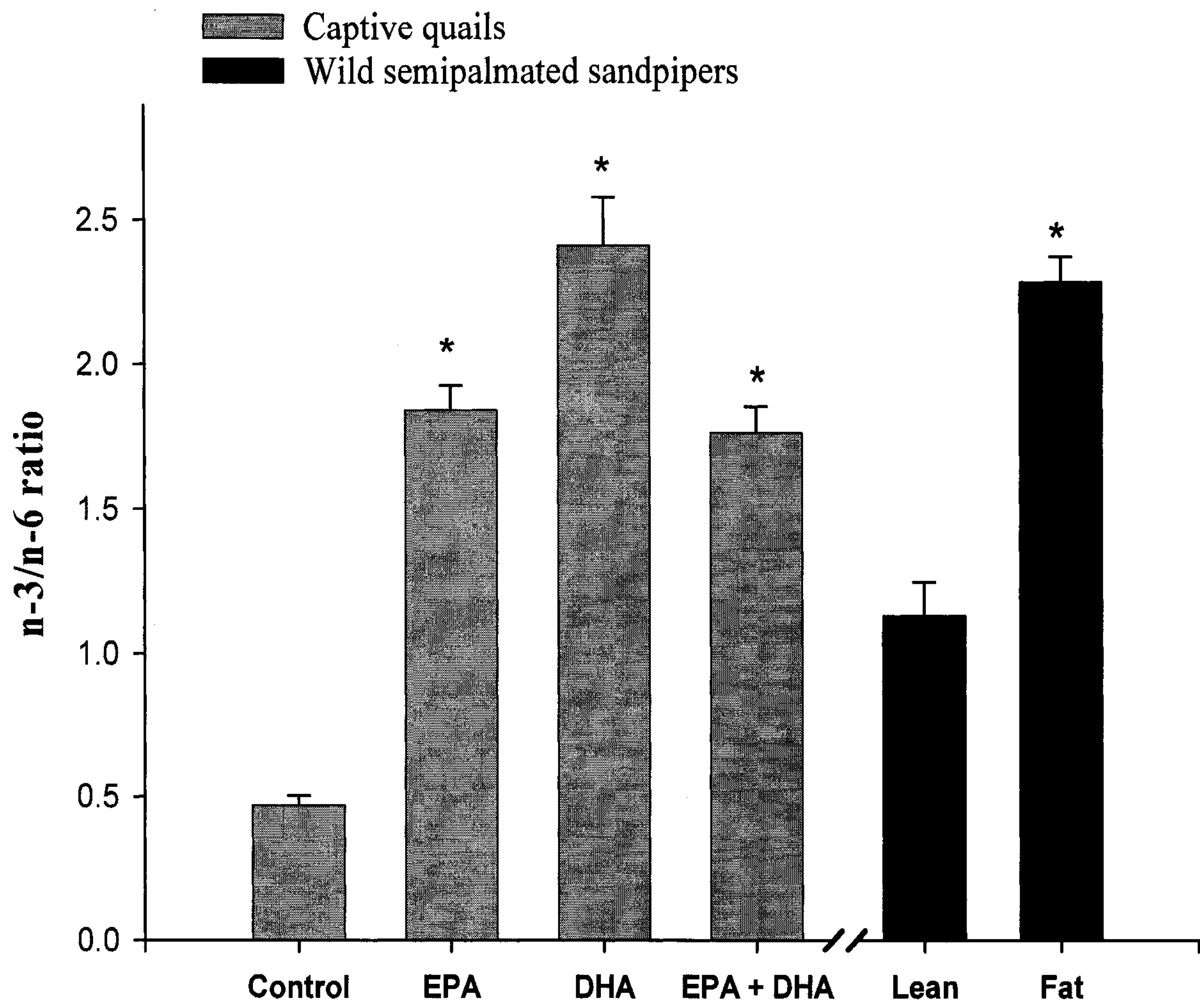

Figure 2.7: Changes in the n-3/n-6 ratio of membrane phospholipids caused by the incorporation of dietary n-3 fatty acids. Captive quails fed different diets are indicated in grey and migrating semipalmated sandpipers feeding on marine invertebrates in black . Values are means ± S.E.M. * indicates differences from control in quails, and a difference between lean and fat birds in sandpipers ($P<0.05$).

Relationship between enzyme activities and membrane composition

Possible relationships between the relative abundance of variable membrane fatty acids and oxidative enzyme activities in pectoral muscle were investigated using linear regression (Table 2.4). Flight muscle CS activity was positively associated with %EPA, but no significant relationship was found for %DHA and %ARA. However, no significant relationships were found between CPT, COX and HOAD activities and %EPA, %DHA and %ARA. Regression analyses between the n-3/n-6 ratio of membrane phospholipids and enzyme activities are presented in Fig. 2.8. This ratio was positively associated with CS , but no relationship was found for CPT ,COX and HOAD.

Table 2.4: Linear regression analyses of the relationships between the relative abundance of variable membrane fatty acids (%) and oxidative enzyme activities (μmol g^{-1} min^{-1}) in the flight muscle of bobwhite quails. The analysis was performed on group means (N=4) and values given in the table are the probabilities that the slopes are different from 0. Whether the slope is positive or negative is indicated in parenthesis. CS: Citrate synthase; HOAD: 3-hydroxyacyl CoA dehydrogenase; CPT: Carnitine palmitoyl transferase; COX: Cytochrome oxidase; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; ARA: Arachidonic acid.

	% EPA	% DHA	% ARA
CS	**<0.001(+)**	0.164 (+)	0.072 (-)
HOAD	0.317 (+)	0.153 (+)	0.247 (-)
CPT	0.98 (+)	0.58 (+)	0.72 (-)
COX	0.28 (+)	0.63 (+)	0.57 (-)

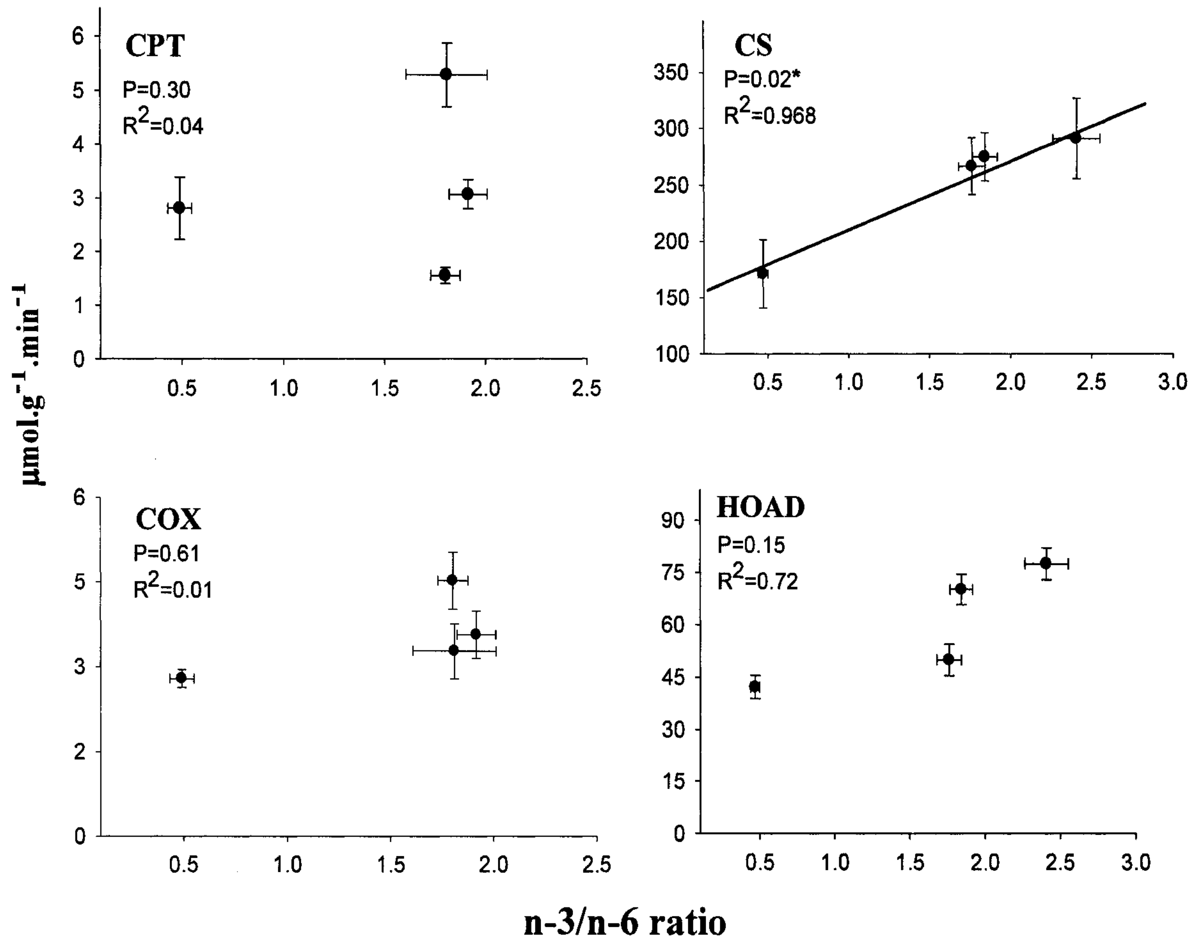

Figure 2.8: Relationships between the n-3/n-6 ratio of muscle membrane phospholipids and the activities of Krebs cycle enzymes (CS and COX) or β-oxidation enzymes (CPT and HOAD). Lines were fitted by linear regression on group means (N=4) and are only indicated when the slope was significantly different from 0. Values are means ± S.E.M. (N=8, except for DHA where N=7). See list of acronyms for abbreviations.

Discussion

This study demonstrates that dietary n-3 fatty acids stimulate the capacity for aerobic metabolism in avian muscle. The consumption of EPA and DHA increases the activities of Krebs cycle and β–oxidation enzymes in captive quails, thereby mimicking the natural doping effects previously reported in wild sandpipers (Maillet and Weber, 2006, 2007). On its own, the diet is able to increase the activities of oxidative enzymes by 58-90% in the flight muscle of a non-migratory bird (Fig.2.1): an increase in aerobic capacity normally only observed after prolonged endurance training. We have used a quail model to show that changes in membrane composition play an important role in mediating the metabolic effects of the diet. However, results provide no significant support for the involvement of PPARs as determined by changes in gene expression. All membranes are equally affected by diet because total tissue phospholipids, mitochondrial membranes and sarcoplasmic reticulum show the same changes in fatty acid composition. Presumably, birds can interconvert EPA and DHA since the consumption of either fatty acid has the same effects on membrane composition. However, EPA and DHA appear to upregulate oxidative metabolism through different pathways because their pattern of enzyme stimulation is specific.

Effects of n-3 fatty acids on oxidative enzymes

The stimulation of oxidative capacity by n-3 fatty acids has been demonstrated in a variety of mammalian tissues including muscle (Power and Newsholme, 1997), liver (Totland et al., 2000), adipose (Guo et al., 2005) and lymphoids (Miyasaka et al., 1996). The fatty acid composition of the diet is also known to affect whole-organism aerobic capacity (VO_2 max) in rats (Ayre and Hulbert, 1997), salmon (Wagner et al., 2004) and one species of migrant bird (Pierce et al., 2005). Previous studies on semipalmated sandpipers could not eliminate the possibility that their pre-migration increase in oxidative capacity could be caused by hormonal changes or exercise training (Maillet and Weber, 2006, 2007). Here, through controlled laboratory experiments, we show that diet alone can strongly stimulate flux capacity through the Krebs cycle and β-oxidation in a sedentary bird. Therefore, dietary n-3 fatty acids are responsible for the metabolic changes observed in refueling sandpipers, not other factors associated with migration. Six weeks of n-3 fatty acid supplements were sufficient to increase enzyme activities by 58-90% in quail flight muscle (Fig. 2.1). This effect of the diet is impressive because such a strong response can only be obtained through prolonged endurance training in mammals. A survey of the literature shows that aerobic exercise training can stimulate enzyme activities by up to 42% in rats (after 8 weeks of training (Siu et al., 2003; Leandro et al., 2007)), 38-76% in humans (7 weeks (Carter et al., 2001)), and 41-72% in horses (10 weeks (Kim et al., 2005)). Therefore, the increases in oxidative enzyme activities observed in birds eating n-3 fatty acids can surpass those reported for mammals after endurance training and they occur more rapidly.

The largest increments in enzyme activities reported for training mammals were smaller than in the present study and they were accompanied by improvements of 10-26% in mass-specific $VO_{2\,max}$ (Bedford et al., 1979; Kim et al., 2005; Carter et al., 2001). Therefore, dietary n-3 fatty acids were probably able to boost the aerobic capacity of quails by more than 20%, although their $VO_{2\,max}$ could not be quantified in our study. The effect of diet on enzyme activities was stronger in quails (Fig. 2.1) than in refueling sandpipers (Maillet and Weber, 2007). Potential reasons for this difference include: 1) sedentary quails may have more scope for improvement because their baseline activities are low; 2) the n-3 fatty acid content of the diets were different (59 *vs* 31% EPA and 70 *vs* 14% DHA for quails *vs* sandpipers); and 3) increased consumption of n-3 fatty acids lasted longer for quails than sandpipers (6 *vs* 2 weeks). The oxidative capacity of flight muscle is known to be much lower in sedentary than migrant birds (Lundgren and Kiessling, 1986), and bobwhite quails are no exception to this pattern. Surprisingly, the effects of dietary n-3 fatty acids were strong enough to activate the enzymes of sedentary quails to levels only normally observed in migrants (Driedzic et al., 1993; Bishop et al., 1995; Guglielmo et al., 2002; Maillet and Weber, 2007).

In one of the experimental treatments, EPA and DHA were administered together. It was selected to mimic the natural diet of semipalmated sandpipers and to test whether birds take advantage of possible synergistic effects. Overall, the combined effects of the two dietary fatty acids do not provide a metabolic advantage, except maybe for COX whose activity was only increased in the EPA+DHA group (Fig. 2.1). The variable responses obtained between dietary treatments support the notion that EPA and DHA act through different pathways, and two potential mechanisms of action were investigated: incorporation in membrane phospholipids and activation PPAR gene expression.

Incorporation of n-3 fatty acids in muscle membranes

The fatty acid composition of membrane phospholipids can be altered by the diet (Awad, 1986; Maillet and Weber, 2006; Guderley et al., 2008), but no previous study had established whether all membranes respond similarly. Results reveal that the consumption of n-3 fatty acids modifies all membranes equally (Figs. 2.3-2.5), and, therefore, that compositional changes of total tissue phospholipids mirror those of mitochondrial and sarcoplasmic membranes. Consumption of the experimental diets only caused changes in the abundance of 3 membrane fatty acids (increases in %EPA and %DHA were compensated by a decrease in %ARA; Table 2.3, Figs. 2.3-2.5). This response closely mimics the changes in fatty acid composition and n-3/n-6 ratio observed in the muscle membranes of sandpipers during pre-migration fattening (Figs. 2.6 and 2.7). Therefore, the bobwhite quail is a useful experimental model to investigate the mechanisms responsible for the doping effects of n-3 fatty acids. Although qualitatively identical between quails and sandpipers, compositional changes were quantitatively stronger in quails. In future studies, the experimental procedure could be adjusted by reducing gavage time to less than 6 weeks to obtain exactly the same changes in composition between quails and sandpipers. Alternately, the enhanced membrane response elicited here may amplify the doping mechanisms under investigation, thereby making them easier to study.

The fatty acid composition of membrane phospholipids can also be altered through regular exercise and some of the diet-induced changes found in quails match those observed in mammals after endurance training. Of particular interest is the fact that the muscles of humans subjected to 8 weeks of aerobic training show significant increases in %DHA (+31%) and n-3/n-6 ratio (+80%)(Andersson et al., 2000; Helge et al., 2001). By eating n-3 fatty acids for 6 weeks, quails were able to exaggerate these aspects of the human

training response. They showed remarkable increases of 35-69% in % DHA and of 240-320% in their n-3/n-6 ratio (Table 2.3, Figs. 2.3-2.5, Fig. 2.7). The large change in %DHA is particularly interesting because the membrane abundance of this fatty acid seems to play a significant role in modulating the sensitivity of CPT to malonyl-CoA: its natural inhibitor (Morash et al., 2008).

Quails may have the capacity for EPA-DHA interconversion because the same changes in membrane composition are observed after the consumption of either fatty acid (% DHA is increased by eating EPA and % EPA is increased by eating DHA; Figs. 3-5). However, this possibility should be taken with caution because our DHA diet also contains significant amounts of EPA and the EPA diet contains some DHA (Table 1). Tracer studies have shown that rats and humans have limited capacity for retroconversion of DHA to EPA when their diet includes normal DHA levels (only 1.4% of dietary DHA is retroconverted to EPA (Brossard et al., 1996)). In contrast, the pathway is strongly stimulated when humans consume DHA supplements for 6 weeks (up to 12% of dietary DHA is retroconverted (Conquer and Holub, 1997; Arterburn et al., 2006)). Unfortunately, actual conversion capacities in either direction have never been measured in birds, and this obscurs our ability to distinguish specific effects of EPA or DHA..

Finally, membranes can act as a reservoir for signalling molecules like anti-inflammatory n-3 fatty acids and pro-inflammatory n-6 fatty acids that can be recruited to regulate the inflammation response (Surette, 2008). Therefore, increasing the n-3/n-6 ratio of membrane phospholipids causes chronic inhibition of inflammation pathways. In migrant birds, a reduced capacity for inflammation caused by eating large quantities of n-3 fatty acids could be greatly beneficial because long-distance flights are known to cause muscle damage (Guglielmo et al., 2001).

Relationship between membrane composition and oxidative metabolism

The local molecular environment of proteins affects their function, and for many enzymes, it can be altered by modulating the fatty acid composition of membrane phospholipids. Therefore, the changes in membrane fluidity, permeability, and n-3/n-6 ratio that result from n-3 fatty acid incorporation are known to influence the activities of key oxidative enzymes and ATPases (CS, CPT, $Na^{+}K^{+}$ATPase and Ca^{2+}ATPase among others) (Swanson et al., 1989; Miyasaka et al., 1996; Power and Newsholme, 1997; Turner et al., 2005). In an attempt to explore possible functional links, we have identified several associations between enzyme activities and particular characteristics of membrane composition (n-3/n-6 ratio and relative abundance of long-chain polyunsaturated fatty acids)(Fig. 2.8, Table 2.4). However, these regressions should be interpreted with caution because the membrane parameters selected for this analysis may not reflect compositional characteristics that really affect enzyme function. Therefore, the absence of significant relationships cannot be used to eliminate membrane-related mechanisms of enzyme regulation. Conversely, the presence of a significant regression does not prove a mechanistic link, but merely suggests its possibility. Keeping these important limitations in mind, we have observed that only CS activity was correlated with the n-3/n-6 ratio (Fig. 2.8) as well as with %EPA (Table 2.4). It is important to mention that changes in membrane composition may not only affect enzymes located within them (such as COX), but matrix enzymes as well. A potential explanation for this interesting observation is that many so-called "matrix enzymes" are preferentially placed in very close proximity to inner mitochondrial membranes(D'Souza and Srere, 1983b) or even actually bound to them like CS (D'Souza and Srere, 1983a). The observed correlation is very useful because it allow the design of further

experiments to investigate specific mechanisms of enzyme activation via changes in membrane.

Overall, direct substitution of n-6 ARA by n-3 EPA appears to play the most prominent role in activating the enzymes examined in this study. The absence of associations between the activity of some enzymes and membrane composition suggests that membrane-independent mechanisms of activation could also be at play.

Expression of PPAR genes

PPARs are transcription factors activated by natural and synthetic ligands like long-chain polyunsaturated fatty acids and some hypolipidemic drugs (Feige et al., 2006). PPARα, β and γ mRNAs are present in quail flight muscle. Expression levels were not affected by the consumption of n-3 fatty acids (Fig. 2.2), but they did respond to GEM: a synthetic PPAR agonist. Previous studies had failed to show any changes in PPARα expression in adult hen livers and in isolated hepatocytes from chicken embryos after they were treated with the other fibrate drugs clofibrate and fenofibrate (Konig et al., 2007; Cwinn et al., 2008). Reasons for this discrepancy are unclear, but may reflect differences between drugs, tissues, exposure times or doses. That dietary n-3 fatty acids did not stimulate the expression of PPAR genes (Fig. 2.2) is insufficient evidence to eliminate the involvement of PPAR-related mechanisms in enzyme activation. This is because the recruitment of PPAR pathways may only occur with early activation of gene expression (i.e. earlier than 6 weeks after starting the diets) or without significant changes in mRNA levels if turnover is high. In birds, the upregulation of some target genes (e.g. CPT, lipopotein lipase) can be triggered by fibrate drugs in the absence of changes in the expression of PPAR genes (Konig et al., 2007; Cwinn et al., 2008). More experiments are needed to establish whether PPAR-related mechanisms

are activated by dietary n-3 fatty acids in bird muscle. Measuring whether PPAR protein expression is stimulated or if PPAR modulators can affect the doping response are promising avenues for future work.

Conclusions

The changes in membrane composition previously observed in refueling sandpipers were replicated here in sedentary quails that served as a model to characterize mechanisms of natural doping. Results show that the substitution of n-6 ARA by n-3 EPA in membrane phospholipids plays an important role in mediating the metabolic effects of the diet. EPA and DHA have the same stimulating effect on oxidative metabolism, possibly because the two fatty acids are easily interconverted. Changes in the fatty acid composition of mitochondrial membranes and sarcoplasmic reticulum can be assessed by monitoring total muscle membranes because all phospholipids are equally affected by diet. The oxidative capacity of bird muscle is very strongly stimulated by dietary EPA and DHA and this physiological response occurs rapidly. Only extreme regimes of endurance training can lead to increments in oxidative capacity matching those induced here by diet in domestic quails. In preparation for long migrations, some birds improve their physical fitness by eating! When maximal energy storage is critical, chosing n-3 fatty acid doping over endurance training strikes us as the better strategy to increase aerobic capacity

CHAPTER 3. GENERAL CONCLUSION

Major conclusions

Wild semipalmated sandpipers (*Calidris pusilla*) use n-3 fatty acids to prime their flight muscles for migration. For this thesis I have used captive Bobwhite quails (*Colinus virginianus*) as an experimental model to investigate the mechanisms for this natural doping. My goal was to characterize the roles of dietary EPA and DHA in the stimulation of oxidative capacity in quail flight muscle, effectively mimicking the physiological response observed in refueling sandpipers. The main objectives of this thesis were to determine if the aerobic metabolism of quail flight muscles are stimulated by dietary n-3 fatty acids alone and to investigate whether the activation of oxidative enzymes are mediated by changes in the composition of membrane phospholipids and/or in PPAR gene expression. It was hypothesized that EPA and DHA would have different effects on oxidative metabolism but will equally affect membranes from total muscle, mitochondria and sarcoplasmic reticulum (SR). Results reveal that the consumption of EPA and DHA increases the activities of Krebs cycle and β-oxidation enzymes by 58-90% in quail flight muscles (Fig. 2.1). This supports the role of the diet in mediating the metabolic changes observed in migrant sandpipers (Maillet and Weber, 2006, 2007), and not other factors associated with migration like hormonal fluctuations or training effects. In addition, previous studies have reported that the oxidative capacity of migrant flight muscles is known to be much higher than sedentary birds (Lundgren and Kiessling, 1986), and captive quails are no exception to this pattern . Interestingly, I observed that the effect of these dietary n-3 fatty acids was stronger in quail muscle enzymes compared to what was previously observed in refueling sandpipers. However, a review of the literature indicates that the level of increment occurred in quail oxidative capacity is normally observed in migrant birds (Driedzic et al., 1993; Bishop et al., 1995; Guglielmo et al., 2002; Maillet and Weber, 2007). It is also important to mention that

the aerobic metabolism stimulation found here is remarkable as only extreme regimes of endurance training in mammals are known to produce a comparable response (see Appendices, Table 2.5).

Diet can alter the fatty acid composition of membrane phospholipids. The results of this thesis reveal that the consumption of n-3 fatty acids modifies all membrane types equally (Total muscle, mitochondria and SR, Figs. 2.3-2.5); consequently, changes in mitochondria and SR membranes can be assessed by monitoring the membrane alteration of total muscle. Moreover, these compositional modifications in muscle membranes are reflected by changes in the abundance of three fatty acids in particular: (increase in %EPA and DHA, with a decrease in % n-6 ARA). The alteration in the percent abundance of these fatty acids is reflected by an increase of the n-3/n-6 ratio which is an indication of the substitution of n-6 by n-3 fatty acids. The same response was also observed in the muscles of refueling sandpipers (Maillet and Weber, 2006, 2007). Therefore, this new experimental model is useful to investigate the mechanisms responsible for the doping effects of n-3 fatty acids. In addition, EPA and DHA have shown the same effects in stimulating enzyme activities in quail flight muscles. This metabolic response might be due to the interconversion occurring between these two fatty acids in the membrane phospholipids (% DHA was increased by consuming EPA and % EPA was increased by consuming DHA) (Figs. 2.3-2.5), and this might be one reason why the identification of specific effects for either EPA or DHA was difficult or impossible. Several associations between enzyme activities and particular characteristics of membrane composition were investigated in this study (Fig. 2.8, Table 2.4). The membrane parameters selected for the regression analyses may not reflect compositional characteristics that affect enzyme function *in vivo*.

However, we observed that only CS activity was correlated with the n-3/n-6 ratio as well as with %EPA. This information is very useful because it allows the design of further experiments to investigate specific mechanisms of enzyme activation via changes in membrane composition. Furthermore, the absence of associations between the activity of some enzymes and membrane composition suggests that membrane-independent mechanisms of activation could also be present.

Consequently, I have investigated the presence and expression of PPAR in quail flight muscle and I was able to identify the three isoforms of this transcription factor (PPARα, β and γ). No activation of PPAR genes (α, β and γ) was observed after the consumption of dietary n-3 fatty acids; however they did respond to GEM treatment: a synthetic PPAR agonist (Fig. 2.2). The fact that no PPAR gene stimulation was observed with the administered fatty acid diet is insufficient evidence to eliminate the implication of PPAR-related mechanisms in enzyme activations. This is because the activation of gene expression may occur earlier than 6 weeks after starting the diets or occur without significant changes in mRNA levels if turnover is high.

Overall, the variable physiological responses in enzyme activities obtained between dietary treatments support the notion that EPA and DHA act through different pathways and, due to this variation, further investigations are needed to fully elucidate the exact contribution of the membrane-related and the PPAR-related mechanisms of action that are investigated here.

Future work

This thesis supports the role of diet mediating the metabolic changes observed in quail flight muscles; however more studies can clarify unresolved questions. Whole-animal experiments could be carried out to determine how dietary n-3 fatty acids can affect the endurance performance of captive quails during sustained exercise [eg. By the use of a flight wheel (Chappell, 1999; Pierce et al., 2005)]. Moreover, experiments are also required to determine whether PPAR-related mechanisms are activated by dietary n-3 fatty acids, because the activation of PPAR gene expressions may only occur earlier than 6 weeks after starting the diets (Woods et al., 2007) or without significant changes in mRNA levels if turnover is high. Therefore, these experiments require that samples be taken throughout the treatment period. In addition, the analysis of the PPAR protein levels may also be used to address this issue. Furthermore, the further use of PPAR modulators like Protein kinase C can be considered (Gray et al., 2005; Burns and Vanden Heuvel, 2007), to investigate if there is an alteration of the doping response observed after n-3 fatty acids treatments. Finally, another promising avenue for future work is to analyse the direct effect of these fatty acids on the expression of oxidative enzymes that contain PPAR response elements in the promoter sequence of their genes (i.e. CPT1 in mammals).

APPENDICES

Table 2.1: Fatty acid composition of quail food and oil supplements used for gavage: corn, EPA-enriched, and DHA-enriched. Individual fatty acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n-6 and n-3 fatty acids are expressed as a percentage of total fatty acids. Degree of unsaturation (DU) is also indicated. ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

Fatty acids	*Food*	*Corn oil*	*EPA oil*	*DHA oil*
16:0	16	11	-	-
18:0	4	2	5	-
18:1	20	29	9	2
n-6 18:2	53	58	-	-
n-3 18:3	7	-	4	2
n-6 20:4 (ARA)	-	-	4	-
n-3 20:5 (EPA)	-	-	59	13
22:1	-	-	2	1
n-3 22:5	-	-	-	11
n-3 22:6 (DHA)	-	-	18	70
SFA	20	13	5	-
MUFA	20	29	11	4
PUFA	60	58	84	96
n-6	53	58	4	-
n-3	7	-	81	96
DU	1.3	1.4	4.4	5.5

Table 2.2: Sequences of the real-time RT-PCR primers for quail 18S and for the PPAR α, β and γ genes.

Gene	GenBank	Real-time RT-PCR (5' to 3')	
		Forward	Reverse
18S	EU847817	GAAACGGCTACCACATCCAA	CACCAGACTTGCCCTCCA
PPARα	EU159428	ATGAACAAAGACGGGATGCT	ATGATGGCAGCGACAAAAA
PPARβ	EU847819	CGCTACCAGCTTCCTCTTCT	AGGGCTGCAAGGGTTTCT
PPARγ	EU847820	TTGAATGTCGTGTGTGTGGA	TTCTCCTTCTCCGCTTGTG

Table 2.5: Percent changes in oxidative capacity of muscle tissue : Effect of dietary n-3 fatty acids *vs* endurance training

Enzymes	Species	Oxidative capacity	Diet or training	Duration (weeks)	References
CS	Human	45%	Bicycle exercise training	16	(Short et al., 2003)
	Rat	42%	Treadmill exercise training	8	(Siu et al., 2003; Leandro et al., 2007)
	Rat	38%	Treadmill exercise training	10	(Powers et al., 1994)
	Horse	41%	Treadmill exercise training	10	(Kim et al., 2005)
	Sandpiper	39%	N-3 fatty acids supplement	2	(Maillet and Weber, 2007)
	Quail	58%-72%	N-3 fatty acids supplement	6	This study
HOAD	Human	38%	Bicycle exercise training	7	(Carter et al., 2001)
	Horse	72 %	Treadmill exercise training	10	(Kim et al., 2005)
	Sandpiper	43%	N-3 fatty acids supplement	2	(Maillet and Weber, 2007)
	Quail	67%-90%	N-3 fatty acids supplement	6	This study
COX	Human	76%	Bicycle exercise training	7	(Carter et al., 2001)
	Quail	61%	N-3 fatty acids supplement	6	This study
V_{O2}max	Human	10 %	Bicycle exercise training	7	(Carter et al., 2001)
	Rat	26%	Treadmill exercise training	10	(Bedford et al., 1979)
	Horse	22%	Treadmill exercise training	10	(Kim et al., 2005)

REFERENCES

Ackman, R. G., Nash, D. M. and McLachlan, J. (1979). Lipids and fatty acids of Corophium volutator from Minas Basin. *Proc. N. S. Inst. Sci.* **29**, 501 -516.

Andersson, A., Nalsen, C., Tengblad, S. and Vessby, B. (2002). Fatty acid composition of skeletal muscle reflects dietary fat composition in humans. *Am. J. Clin. Nutr.* **76**, 1222-1229.

Andersson, A., Sjödin, A., Hedman, A., Olsson, R. and Vessby, B. (2000). Fatty acid profile of skeletal muscle phospholipids in trained and untrained young men. *Am. J. Physiol.* **279**, 744-751.

Arterburn, L. M., Hall, E. B. and Oken, H. (2006). Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am. J. Clin. Nutr.* **83**, 1467-1476.

Ashour, B. and Hansford, R. G. (1983). Effect of fatty acids and ketones on the activity of pyruvate dehydrogenase in skeletal-muscle mitochondria. *Biochem. J.* **214**, 725-736.

Awad, A. B. (1986). Effect of dietary fat on membrane lipids and functions. *J. Env. Pathol.Toxicol. Oncol.* **6**, 1-14.

Ayre, K. J. and Hulbert, A. J. (1997). Dietary fatty acid profile affects endurance in rats. *Lipids* **32**, 1265-1270.

Battley, P. F., Piersma, T., Dietz, M. W., Tang, S., Dekinga, A. and Hulsman, K. (2000). Empirical evidence for differential organ reductions during trans-oceanic bird flight. *Proc. R. Soc. Lond.* **267**, 191 -195.

Bedford, T. G., Tipton, C. M., Wilson, N. C., Oppliger, R. A. and Gisolfi, C. V. (1979). Maximum oxygen consumption of rats and its changes with various experimental procedures. *J. Appl. Physiol.* **47**, 1278-1283.

Berger, J. and Moller, D. E. (2002). The mechanisms of action of PPARs. *Ann. Rev. Med.* **53**, 409-435.

Bishop, C. M., Butler, P. J., Eggington, S., El Haj, A. J. and Gabrielsen, G. W. (1995). Development of metabolic enzyme activity in locomotor and cardiac muscles of the migratory barnacle goose. *Am. J. Physiol.* **269**, R64-R72.

Blem, C. R. (1990). Avian energy storage. *Curr. Ornithol.* **7**, 59-113.

Brossard, N., Croset, M., Pachiaudi, C., Riou, J. P., Tayot, J. L. and Lagarde, M. (1996). Retroconversion and metabolism of [13C]22:6n-3 in humans and rats after intake of a single dose of [13C]22:6n-3-triacylglycerols. *Am. J. Clin. Nutr.* **64**, 577-586.

Burns, K. A. and Vanden Heuvel, J. P. (2007). Modulation of PPAR activity via phosphorylation. *Bioch.et Biophy. Acta (BBA) - Mol.and Cell Biol. of Lipids* **1771**, 952.

Butler, P. J. and Woakes, A. J. (1990). The physiology of bird flight. *In Bird Migration (ed. E. Gwinner)*, 300-318.

Butler, P. J. and Bishop, C. M. (2000). Flight. *In Sturkie's Avian Physiology (ed. G. C. Whittow)*, 391- 435.

Carter, S. L., Rennie, C. D., Hamilton, S. J. and Tarnopolsky, M. A. (2001). Changes in skeletal muscle in males and females following endurance training. *Can. J. Physiol. Pharmacol.* **79**, 386-392.

Chappell, M. A., Bech, C. and Buttemer, W. A. (1999). The relationship of central and peripheral organ masses to aerobic performance variation in house sparrows. *J. Exp. Biol.* **202**, 2269-2279.

Conquer, J. A. and Holub, B. J. (1997). Dietary docosahexaenoic acid as a source of eicosapentaenoic acid in vegetarians and omnivores. *Lipids* **32**, 341-345.

Corcoran, M. P., Lamon-Fava, S. and Fielding, R. A. (2007). Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. *Am. J. Clin. Nutr.* **85**, 662-677.

Cwinn, M. A., Jones, S. P. and Kennedy, S. W. (2008). Exposure to perfluorooctane sulfonate or fenofibrate causes PPAR-[alpha] dependent transcriptional responses in chicken embryo hepatocytes. *Comp. Biochem. Physiol.* **148**, 165-171.

D'Souza, S. F. and Srere, P. A. (1983a). Binding of citrate synthase to mitochondrial inner membranes. *J. Biol. Chem.* **258**, 4706-4709.

D'Souza, S. F. and Srere, P. A. (1983b). Cross-linking of mitochondrial matrix proteins in situ. *Biochem. Biophys. Acta* **724**, 40-51.

Davidson, N. C. and Evans, P. R. (1988). rebreeding accumulation of fat and muscle protein by arctic-breeding shorebirds. *P. In Acta XIX Congressus Internationalis Ornithologici (ed. H. Ouellet)*, 342-352. Ottawa: University of Ottawa Press.

Dawson, W. R., Marsh, R. L. and Yacoe, M. E. (1983). Metabolic adjustments of small passerine birds for migration and cold. *Am. J. Physiol.* **245**, R755-R767.

Desvergne, B. and Wahli, W. (1999). Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocr.Rev.* **20**, 649-688.

Driedzic, W. R., Crowe, H. L., Hicklin, P. W. and Sephton, D. H. (1993). Adaptations in pectoralis muscle, heart mass and energy metabolism during premigratory fattening in semipalmated sandpipers *(Calidris pusilla)*. *Can. J. Zool.* **71**, 1602- 1608.

Else, P. L., Wu, B. J., Storlien, L. H. and Hulbert, A. J. (2003). Molecular activity of Na+,K+-ATPase relates to the packing of membrane lipids. *Ann. N. Y. Acad.Sc.* **986**, 525-526.

Feige, J. N., Gelman, L., Michalik, L., Desvergne, B. and Wahli, W. (2006). From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Progr.Lipid Res.* **45**, 120-159.

Field, C. J., Ryan, E. A., Thomson, A. B. and Clandinin, M. T. (1988). Dietary fat and the diabetic state alter insulin binding and the fatty acyl composition of the adipocyte plasma membrane. *Biochem J.* **253(2)**, 417-424.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Froyland, L., Vaagenes, H., Asiedu, D., Garras, A., Lie, O. and Berge, R. K. (1996). Chronic Administration of Eicosapentaenoic Acid and Docosahexaenoic Acid as Ethyl Esters Reduced Plasma Cholesterol and Changed the Fatty Acid Composition in Rat Blood and Organs. *Lipids* **31**, 169-178.

Froyland, L., Madsen, L., Vaagenes, H., Totland, G. K., Auwerx, J., Kryvi, H., Staels, B. and Berge, R. K. (1997). Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. *J. Lipid Res.* **38**, 1851-1858.

Gerson, A. R., Brown, J. C. L., Thomas, R., Bernards, M. A. and Staples, J. F. (2008). Effects of dietary polyunsaturated fatty acids on mitochondrial metabolism in mammalian hibernation. *J Exp Biol* **211**, 2689-2699.

Ginsberg, B. H., Brown, T. J., Simon, I. and Spector, A. A. (1981). Effect of the membrane lipid environment on the properties of insulin receptors. *Diabetes* **30**, 773-780.

Gould, G. W., McWhirter, J. M., East, J. M. and Lee, A. G. (1987). Effects of diet on the function of sarcoplasmic reticulum. *Biochem. J.* **245**, 751-755.

Gould, R. J., Ginsberg, B. H. and Spector, A. A. (1982). Lipid effects on the binding properties of a reconstituted insulin receptor. *J. Biol. Chem.* **257**, 477-484.
Gray, J. P., Burns, K. A., Leas, T. L., Perdew, G. H. and Vanden Heuvel, J. P. (2005). Regulation of Peroxisome Proliferator-Activated Receptor α by Protein Kinase C. *Biochemistry* **44**, 10313-10321.
Guderley, H., Kraffe, E., Bureau, W. and Bureau, D. P. (2008). Dietary fatty acid composition changes mitochondrial phospholipids and oxidative capacities in rainbow trout red muscle. *J. Comp. Physiol.* **178**, 385-399.
Guglielmo, C. G., Piersma, T. and Williams, T. D. (2001). A sport-physiological perspective on bird migration: evidence fo flight-induced muscle damage. *J. Exp. Biol.* **204**, 2683-2690.
Guglielmo, C. G., Haunerland, N. H., Hochachka, P. W. and Williams, T. D. (2002). Seasonal dynamics of flight muscle fatty acid binding protein and catabolic enzymes in a migratory shorebird. *Am. J. Physiol.* **282**, R1405-1413.
Guo, W., Xie, W., Lei, T. and Hamilton, J. (2005). Eicosapentaenoic acid, but not oleic acid, stimulates β-oxidation in adipocytes. *Lipids* **40**, 815 -821.
Hazelwood, R. L. (1986). Carbohydrate metabolism. *In Avian Physiol. (P. D. Sturkie, Ed.)* **4th ed.**, 309-325.
Helge, J. W., Wu, B. J., Willer, M., Daugaard, J. R., Storlien, L. H. and Kiens, B. (2001). Training affects muscle phospholipid fatty acid composition in humans. *J. Appl. Physiol.* **90**, 670-677.
Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation - Mechanisms and Process in Physiological Evolution. New York: Oxford University Press.*
Hulbert, A. J. (2003). Life, death and membrane bilayers. *J Exp Biol* **206**, 2303-2311.
Hulbert, A. J., Turner, N., Storlien, L. H. and Else, P. L. (2005). Dietary fats and membrane function: implications for metabolism and disease. *Biol. Rev.* **80**, 155-169.
Infante, J. P., Kirwan, R. C. and Brenna, J. T. (2001). High levels of docosahexaenoic acid (22:6n-3)-containing phospholipids in high-frequency contraction muscles of hummingbirds and rattlesnakes. *Comp. Biochem. Physiol.* **130**, 291 -298.
Jenni, L. and Jenni-Eiermann, S. (1998). Fuel supply and metabolic constraints in migrating birds. *Avian Biol.* **29**, 521 -528.
Kim, J., Hinchcliff, K. W., Yamaguchi, M., Beard, L. A., Markert, C. D. and Devor, S. T. (2005). Exercise training increases oxidative capacity and attenuates exercise-induced ultrastructural damage in skeletal muscle of aged horses. *J. Appl. Physiol.* **98**, 334-342.
Klaassen, M., Kvist, A. and Lindstrom, A. (2000). Flight costs and fuel composition of a bird migrating in a wind tunnel. *The Condor* **102**, 444-451.
Konig, B., Kluge, H., Haase, K., Brandsch, C., Stangl, G. I. and Eder, K. (2007). Effects of Clofibrate Treatment in Laying Hens. *Poult. Sci.* **86**, 1187-1195.
Leaf, A., Xiao, Y.-F., Kang, J. X. and Billman, G. E. (2005). Membrane effects of the n-3 fish oil fatty acids, which prevent fatal ventricular arrhythmias. *J. Memb. Biol.* **206**, 129-139.
Leandro, C. G., Levada, A. C., Hirabara, S. M., ManhÃ£es-De-Castro, R., De-Castro, C. B., Curi, R. and Pithon-Curi, T. C. (2007). A program of moderate physical training for wistar rats based on maximal oxygen consumption. *J. Stren. Cond. Res.* **21**, 751.

Lundgren, B., Hedenström, A. and Pettersson, J. (1995). Correlation between some body components and visible fat index in the willow warbler *Phylloscopus trochilus*. *Ornis Svecica* **5**, 75-79.

Lundgren, B. D. and Kiessling, K. H. (1986). Catabolic enzyme activities in pectoralis muscle of premigratory and migratory juvenile Reed Warblers, *Acrocephalus scirpaceus* (Herm.). *Oecologia* **68**, 529-532.

Magnoni, L. and Weber, J.-M. (2007). Endurance swimming activates trout lipoprotein lipase: plasma lipids as a fuel for muscle. *J. Exp. Biol.* **210**, 4016-4023.

Maillet, D. and Weber, J.-M. (2006). Performance-enhancing role of dietary fatty acids in a long-distance migrant shorebird: the semipalmated sandpiper. *J. Exp. Biol.* **209**, 2686-2695.

Maillet, D. and Weber, J.-M. (2007). Relationship between n-3 PUFA content and energy metabolism in the flight muscles of a migrating shorebird: evidence for natural doping. *J. Exp. Biol.* **210**, 413-420.

Marsh, R. L. (1983). Adaptations of the gray catbird *Dumetella carolinensis* to long-distance migration: energy stores and subtrate concentrations in plasma. *Auk* **100**, 170-179.

McKenzie, D. J., Higgs, D. A., Dosanjh, B. S., Deacon, G. and Randall, D. J. (1998). Dietary fatty acid composition influences swimming performance in Atlantic salmon (Salmo salar) in seawater. *Fish Physiol. Bioch.* **19**, 111-122.

McKenzie, D. J., Piraccini, G., Papini, N., Galli, C., Bronzi, P., Bolis, C. G. and Taylor, E. W. (1997). Oxygen consumption and ventilatory reflex responses are influenced by dietary lipids in sturgeon. *Fish Physiol. Biochem.* **16**, 365-379.

Miyasaka, C. K., Azevedo, R. B., Curi, R., Mancini Filho, J. and Lajolo, F. M. (1996). Administration of fish oil by gavage increases the activities of hexokinase, glucose-6-phosphate dehydrogenase, and citrate synthase in rat lymphoid organs. *Gen. Pharm.* **27**, 991-994.

Morash, A. J., Kajimura, M. and McClelland, G. B. (2008). Intertissue regulation of carnitine palmitoyltransferase I (CPTI): Mitochondrial membrane properties and gene expression in rainbow trout (Oncorhynchus mykiss). *Biochem. Biophy. Acta (BBA)* **1778**, 1382-1389.

Moya-Falcon, C., Hvattum, E., Dyroy, E., Skorve, J., Stefansson, S. O., Thomassen, M. S., Jakobsen, J. V., Berge, R. K. and Ruyter, B. (2004). Effects of 3-thia fatty acids on feed intake, growth, tissue fatty acid composition, B-oxidation and Na^+, K^+-ATPase activity in Atlantic salmon. *Comp. Biochem. Physiol.* **139**, 657 -668.

Murphy, M. G. (1990). Dietary Fatty Acids and Membrane Protein Function. *J. Nutr. Biochem.* **1**, 68-79.

Nurnberg, K., Kuchenmeister, U., Ender, K., Nurnberg, G. and Hackl, W. (1998). Influence of dietary n-3 fatty acids on the membrane properties of skeletal muscle in pigs. *Fett-Lipid* **100**, 353-358.

Pierce, B. J., McWilliams, S. R., O'Connor, T. P., Place, A. R. and Guglielmo, C. G. (2005). Effect of dietary fatty acid composition on depot fat and exercise performance in a migrating songbird, the red-eyed vireo. *J. Exp. Biol.* **208**, 1277-1285.

Piersma, T. and Lindström, Å. (1997). Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends Ecol. Evol.* **12**, 134-138.

Piersma, T. and Gill, R. E. J. (1998). Guts don't fly: small digestive organs in obese bar-tailed godwits. *Auk* **115**, 196-203.

Piersma, T. and Baker, A. J. (2000). Life history characteristics and the conservation of migratory shorebirds. *In Behaviour and Conservation (ed. L. M. Gosling and W. J. Sutherland)*, 105-124.

Power, G. W. and Newsholme, E. A. (1997). Dietary Fatty Acids Influence the Activity and Metabolic Control of Mitochondrial Carnitine Palmitoyltransferase I in Rat Heart and Skeletal Muscle. *J. Nutr.* **127**, 2142-2150.

Powers, S. K., Criswell, D., Lawler, J., Ji, L. L., Martin, D., Herb, R. A. and Dudley, G. (1994). Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **266**, R375-380.

Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A. and Millington, K. J. (2004). The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J. Hum. Nutr.Diet.* **17**, 449-459.

Sanz, M., Lopez-Bote, C. J., Menoyo, D. and Bautista, J. M. (2000). Abdominal Fat Deposition and Fatty Acid Synthesis Are Lower and {beta}-Oxidation Is Higher in Broiler Chickens Fed Diets Containing Unsaturated Rather than Saturated Fat. *J. Nutr.* **130**, 3034-3037.

Schmidt-Nielsen, K. (1979). Animal physiology: adaptation and environment. *Cambridge University Press, Cambridge London New York Melbourne.*

Schwilch, R., Jenni, L. and Jenni-Eiermann, S. (1996). Metabolic responses of homing pigeons to flight and subsequent recovery. *J. Comp. Physiol. B* **166**, 77-87.

Short, K. R., Vittone, J. L., Bigelow, M. L., Proctor, D. N., Rizza, R. A., Coenen-Schimke, J. M. and Nair, K. S. (2003). Impact of Aerobic Exercise Training on Age-Related Changes in Insulin Sensitivity and Muscle Oxidative Capacity. *Diabetes* **52**, 1888-1896.

Siu, P. M., Donley, D. A., Bryner, R. W. and Alway, S. E. (2003). Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. *J. Appl. Physiol.* **94**, 555-560.

Stillwell, W. and Wassall, S. R. (2003). Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chemistry and Physics of Lipids* **126**, 1.

Stillwell, W., Jenski, L. J., Crump, F. T. and Ehringer, W. (1997). Effect of docosahexaenoic acid on mouse mitochondrial membrane properties. *Lipids* **32**, 497-506.

Storlien, L. H., Higgins, J. A., Thomas, T. C., Brown, M. A., Wang, H. Q., Huang, X. F. and Else, P. L. (2000). Diet composition and insulin action in animal models. *British Journal of Nutrition* **83**, S85-S90.

Stubbs, C. D. and Kisielewski, A. E. (1990). Effect of increasing the level of n-3 fatty acids on rat skeletal muscle sarcoplasmic reticulum. *Lipids* **25**, 553-558.

Suarez, R. K., Lighton, J. R. B., Moyes, C. D., Brown, G. S., Gass, C. L. and Hochachka, P. W. (1990). Fuel selection in rufous hummingbirds: Ecological implications of metabolic biochemistry. *Proc. Natl. Acad. Sci.* **87**, 9207-9210.

Surette, M. E. P. (2008). The science behind dietary omega-3 fatty acids. *Can. Medic. Ass. J.* **178**, 177-180.

Swanson, J. E., Lokesh, B. R. and Kinsella, J. E. (1989). Ca-2+-Mg-2+ Atpase of Mouse Cardiac Sarcoplasmic-Reticulum Is Affected by Membrane N-6 and N-3 Poly-Unsaturated Fatty-Acid Content. *J. Nutr.* **119**, 364-372.

Totland, G. K., Madsen, L., Klementsen, B., Vaagenes, H., Kryvi, H., Froyland, L., Hexeberg, S. and Berge, R. K. (2000). Proliferation of mitochondria and gene expression of carnitine palmitoyltransferase and fatty acyl-CoA oxidase in rat skeletal muscle, heart and liver by hypolipidemic fatty acids. *Biol.Cell* **92**, 317-329.

Turner, N., Haga, K. L., Hulbert, A. J. and Else, P. L. (2005). Relationship between body size, Na+-K+-ATPase activity, and membrane lipid composition in mammal and bird kidney. *Am. J. Physiol.* **288**, R301-310.

Turner, N., Lee, J. S., Bruce, C. R., Mitchell, T. W., Else, P. L., Hulbert, A. J. and Hawley, J. A. (2004). Greater effect of diet than exercise training on the fatty acid profile of rat skeletal muscle. *J. Appl. Physiol.* **96**, 974-980.

Wagner, G. N., Balfry, S. K., Higgs, D. A., Lall, S. P. and Farrell, A. P. (2004). Dietary fatty acid composition affects the repeat swimming performance of Atlantic salmon in seawater. *Comp. Biochem. Physiol.* **137**, 567-576.

Weber, J.-M., Roberts, T. J., Vock, R., Weibel, E. R. and Taylor, C. R. (1996). Design of the oxygen and substrate pathways. III. Partitioning energy provision from carbohydrates. *J. Exp. Biol.* **199**, 1659 -1666.

Woods, C. G., Kosyk, O., Bradford, B. U., Ross, P. K., Burns, A. M., Cunningham, M. L., Qu, P., Ibrahim, J. G. and Rusyn, I. (2007). Time course investigation of PPAR[alpha]- and Kupffer cell-dependent effects of WY-14,643 in mouse liver using microarray gene expression. *Tox. and Appl. Phar.* **225**, 267.