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Mimicking the natural doping of migrant sandpipers in sedentary quails: effects of dietary n-3 fatty acids on muscle membranes and PPAR expression

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SUMMARY

Wild semipalmated sandpipers (*Calidris pusilla*) eat n-3 fatty acids to prime their muscles for long migrations. Sedentary bobwhite quails (*Colinus virginianus*) were used as a model to investigate the mechanisms for this natural doping. Our goal was to characterize the stimulating effects of n-3 eicosapentaenoic acid (EPA) and n-3 docosahexaenoic acid (DHA) on oxidative capacity. Mechanisms linked to changes in membrane composition and in gene expression for peroxisome proliferator-activated receptors (PPAR) were investigated. Dietary n-3 fatty acids stimulated the activities of oxidative enzymes by 58–90% (citrate synthase, cytochrome oxidase, carnitine palmitoyl transferase and hydroxyacyl dehydrogenase), and sedentary quails showed the same changes in membrane composition as sandpipers preparing for migration. EPA and DHA have the same doping effect. The substitution of n-6 arachidonic acid by n-3 EPA 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). The fatty acid composition of mitochondrial membranes and sarcoplasmic reticulum can be monitored by measuring total muscle phospholipids because all phospholipids are equally affected by diet. Only extreme regimes of endurance training can lead to increments in oxidative capacity matching those induced here by diet. As they prepare for long migrations, semipalmated sandpipers improve their physical fitness by eating! Choosing n-3 fatty acid doping over endurance training strikes us as a better strategy to boost aerobic capacity when rapid storage of energy is critical.

Key words: lipid metabolism, membrane phospholipid, peroxisome proliferator-activated receptor gene expression, mitochondrial enzyme, sarcoplasmic reticulum, *Colinus virginianus*.

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; Maillet and Weber, 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 (Maillet and Weber, 2007). 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; Maillet and Weber, 2007). The observed increase in muscle oxidative capacity may not be entirely caused by diet but by other seasonal effects of migration such as exercise training and hormonal changes. Here, we carried out controlled laboratory experiments to focus on the effects of diet and to attempt 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; Maillet and Weber, 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 (Gerson et al., 2008; Guderley et al., 2008; Murphy, 1990; Stillwell et al., 1997). They include enzymes from oxidative pathways, ATPases, hormone receptors and ion channels [carnitine palmitoyl transferase (CPT) (Guo et al., 2005; Power and Newsholme, 1997); 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 with distinct tissue distributions and functions have been identified. 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 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: (1) whether dietary n-3 fatty acids alone can stimulate capacity for aerobic metabolism in the flight muscle of a non-migratory bird, (2) whether the activation of oxidative enzymes is caused by changes in the composition of membrane phospholipids and/or PPAR gene expression, (3) whether dietary EPA and DHA have different effects and (4) whether 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

Tamed adult bobwhite quails of both sexes (*Colinus virginianus* L.; 224±27 g, *N*=40) were obtained from a local supplier (Sainte-Hyacinthe, QC, Canada) and fed a commercial starter turkey diet (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 to minimize stress. They were banded for individual identification and housed in groups of 20. Each group was kept in a windowless room (4×1.5×2 m) with wood chips as substrate. They were held at 22°C and 50% humidity under a 12 h:12 h L:D 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; Maillet and Weber, 2007) was mimicked by supplementing the food of bobwhite quails with oils administered by gavage. The birds were randomly divided into five groups of eight individuals: 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^{-1} for 6 weeks) or GEM (5 mg in 0.7 ml of corn oil per 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 1. After the gavage period, the animals were euthanized with CO_2 followed by cervical dislocation. Pectoral muscle was rapidly excised by dissection. Approximately 5 g of muscle was freeze-clamped in liquid N_2 to measure enzyme activities and the fatty acid composition of membrane phospholipids. Approximately 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.

Table 1. Fatty acid composition of quail food and oil supplements used for gavage: corn, EPA-enriched and DHA-enriched

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

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.

Enzymes

Muscle homogenates were prepared in Hepes buffer (40 mmol l^{-1}, pH 7.3). The activities of 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) and cytochrome oxidase (COX; E.C. 1.9.3.1) were measured at 37°C using a Spectramax spectrophotometer (Gemini XS, Molecular devices, Sunnyvale, CA, USA) and 96-well flat-bottom microplates (Thermo Fisher Scientific, Nepean, ON, Canada). Enzyme activity was measured in triplicate and mean values were used for calculations. Preliminary measurements were performed to determine homogenate concentrations yielding maximum reaction velocities. Detailed procedures for enzyme assays have been described previously [CS, CPT and HOAD (Maillet and Weber, 2007); COX (Moyes et al., 1997)].

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 in GenBank (accession numbers shown in Table 2). Changes in the expression of PPARα, 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

Table 2. Sequences of the real-time RT-PCR primers for quail 18S and for the PPARα, β and γ genes

Gene	GenBank accession no.	Real-time RT-PCR (5′ to 3′)	
		Forward	Reverse
18S	EU847817	GAAACGGCTACCACATCCAA	CACCAGACTTGCCCTCCA
PPARα	EU159428	ATCCCTGGCTTCTCCAATCT	CAGCATCCCGTCTTTGTTC
PPARβ	EU847819	GCTCCTCTCCCTCCCTGT	TCCGCTCACACTTCTCATACTC
PPARγ	EU847820	CCTCCTTCTCCACCCTATTTTT	AGCCCTTACAACCCTCACAC

performed using 1 μg RNA in 11 μl RNase-/DNase-free water, and primed with 1 μl random hexamer primers. The mixture was incubated at 65°C for 10 min, quickly chilled on ice, and briefly spun (13,750 ***g***). Four microliters of 5× reaction buffer, 2 μl 0.1 mol l^{-1} DTT, 1 μl 10 mmol l^{-1} dNTPs, and 1 μl RNase inhibitor were added, gently mixed, and the solution was incubated at 42°C for 2 min. One microliter of SuperScript™ II RNase H- Reverse Transcriptase or 1 μl 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 Table 2). Primers of 18–22 nucleotides with optimal annealing temperature between 59 and 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, 1× QPCR, 2.5 mmol l^{-1} $MgCl_2$, 100–400 nmol l^{-1} gene-specific primer (depending on the primer set used), 0.25× SYBR green, 200 μmol l^{-1} dNTPs, 1.25 U HotStarTaq (Qiagen, Mississauga, ON, Canada) and 100 nmol l^{-1} ROX reference dye, in a 25 μl 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 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 ml^{-1}. 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 (Mississauga, Ontario, Canada) equipped with a fused silica capillary column (Supelco DB-23, 60 m×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 (μmol 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 in μmol^{-1} ml (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. 1), PPAR gene expression (Fig. 2), % individual fatty acids in membrane phopholipids (Figs 3–5) and n-3/n-6 ratio (Fig. 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) and on the difference in n-3/n-6 ratio between lean and fat semipalmated sandpipers (Fig. 7). Relationships between enzyme activities and % contribution of individual fatty acids in membrane phospholipids (Table 4) or between enzyme activities and n-3/n-6 ratio (Fig. 8) were assessed by linear regression. 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

Fig. 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 two 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 and DHA increased the activity

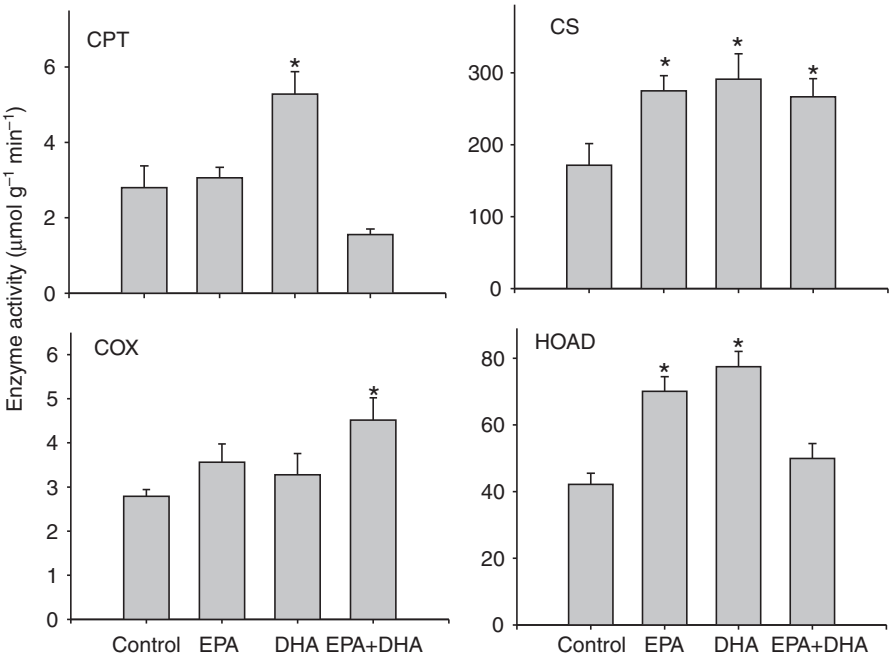

Fig. 1. Changes in the activities (μmol g^{-1} min^{-1}) of Krebs cycle enzymes (CS and COX) and β-oxidation enzymes (CPT and HOAD) in quail pectoral muscle for the different treatment groups (EPA, DHA and EPA+DHA). Values are means ± s.e.m. (*N*=8, except for DHA where N=7). Asterisks indicate differences from control (*P*<0.05). Abbreviations: CS, citrate synthase; HOAD, 3-hydroxyacyl CoA dehydrogenase; CPT, carnitine palmitoyl transferase; COX, cytochrome oxidase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

of the Krebs cycle enzymes CS and COX (*P*<0.05) but had no effect on the β-oxidation enzymes CPT and HOAD (*P*>0.05).

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

The effects of EPA, DHA, EPA+DHA and the fibrate drug GEM on the expression of genes coding for PPARα, β and γ in pectoral muscle are reported in Fig. 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 PPARα and PPARγ (*P*<0.05), respectively.

Incorporation of dietary n-3 fatty acids in muscle membranes

Table 3 shows the fatty acid composition of flight muscle membranes and how it is affected by dietary EPA and DHA. Principal component analysis revealed that only three fatty acids from membrane phospholipids were modified by the diets; they were arachidonic acid (ARA), EPA and DHA, and are shown with grey highlights in Table 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 the different membranes from muscle are reported in Figs 3–5. For total muscle membranes, all the diets caused an increase in %EPA and %DHA, as well as a decrease in %ARA (Fig. 3) (*P*<0.05). These dietary effects were the same in mixed membranes from total muscle tissue (Fig. 3), in membranes isolated from muscle mitochondria (Fig. 4) and in sarcoplamic reticulum (Fig. 5) (*P*>0.05).

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

Figs 6 and 7 compare the changes in membrane phospholipids observed in sedentary quails fed artificial diets under controlled

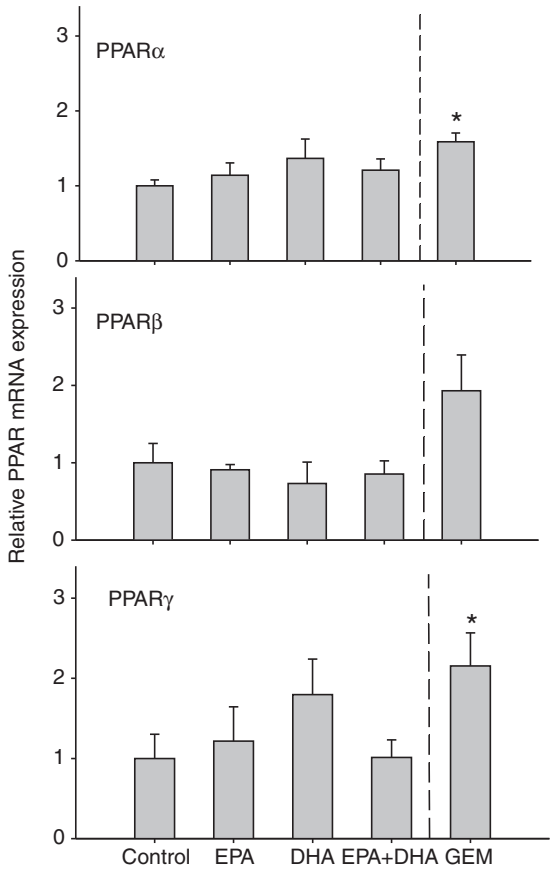

Fig. 2. Effects of dietary n-3 fatty acids and gemfibrozil (GEM) on the mRNA levels of peroxisome proliferator-activated receptors (PPAR) α, β and γ, in quail pectoral muscle. The expression of PPAR genes is normalized to the expression of 18S. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are means ± s.e.m. (*N*=8, except for DHA where *N*=7) and asterisks indicate differences from control (*P*<0.05).

Table 3. Fatty acid composition of flight muscle membranes in bobwhite quails from control, EPA, DHA and EPA+DHA treatment groups

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	38.8±1	37.6±1.3	37.7±1.5
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

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 asterisks (one-way ANOVA, $P<0.05$). Values are means ± s.e.m. ($N=8$, except for DHA treatment group where $N=7$). See Table 1 for abbreviations.

conditions (present 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 (Fig. 6A) and the natural diet of semipalmated sandpipers (Fig. 6B) had the same effects on flight muscle membrane composition: increases in %EPA and %DHA accompanied by a decrease in %ARA. However, the changes in EPA and ARA were quantitatively larger in quails than in sandpipers. Fig. 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 ($P<0.05$).

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 4). Flight muscle CS activity was positively associated with %EPA, but no significant relationship was found for %DHA and %ARA. HOAD activity was positively associated with %EPA and %DHA and was negatively associated with %ARA. The relationship between %EPA and muscle COX activity had a significant positive slope. However, %EPA, %DHA and %ARA showed no relationships with CPT

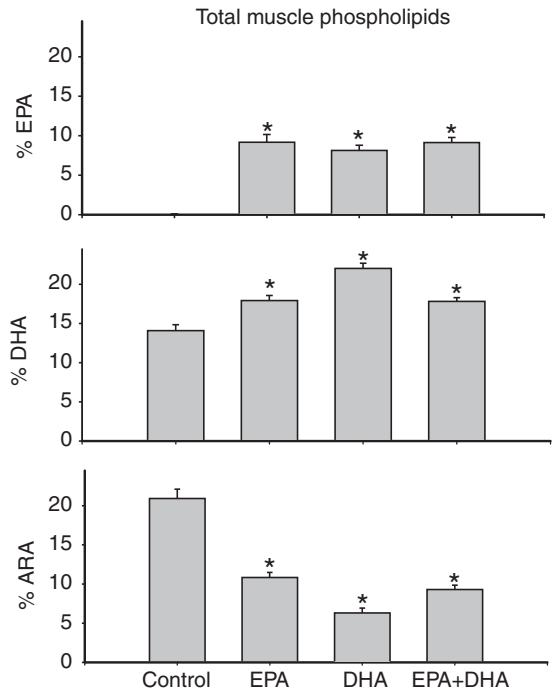

Fig. 3. Changes in the % contribution of individual fatty acids in total phospholipids from muscle for the different treatment groups (Control, EPA, DHA and EPA+DHA). Values are means ± s.e.m. ($N=8$, except for DHA where $N=7$) and asterisks indicate differences from control ($P<0.05$). See Fig. 1 for abbreviations.

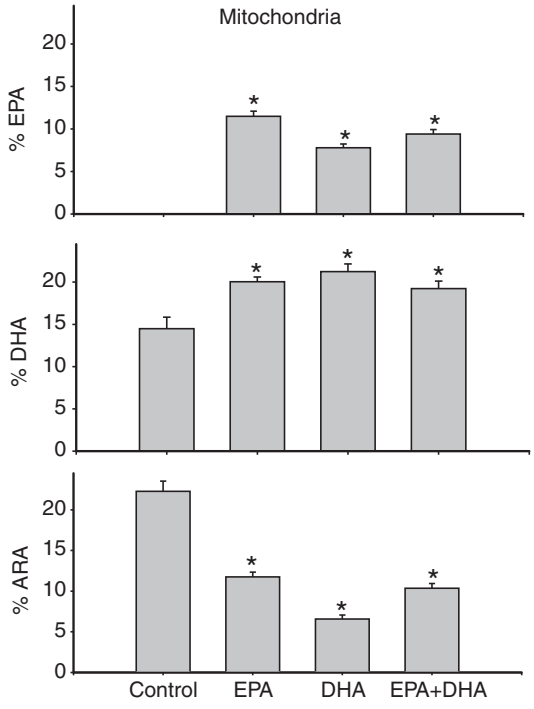

Fig. 4. Changes in the % contribution of individual fatty acids in membranes from isolated muscle mitochondria for the different treatment groups (Control, EPA, DHA and EPA+DHA). Values are means ± s.e.m. ($N=8$, except for DHA where $N=7$) and asterisks indicate differences from control ($P<0.05$). See Fig. 1 for abbreviations.

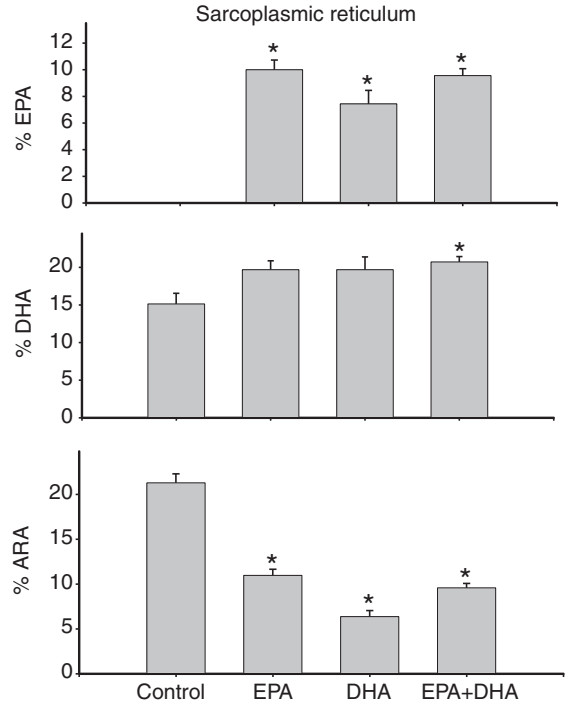

Fig. 5. Changes in the % contribution of individual fatty acids in isolated sarcoplasmic reticulum for the different treatment groups (Control, EPA, DHA, and EPA+DHA). Values are means ± s.e.m. (*N*=8, except for DHA where *N*=7) and asterisks indicate differences from control (*P*<0.05). See Fig. 1 for abbreviations.

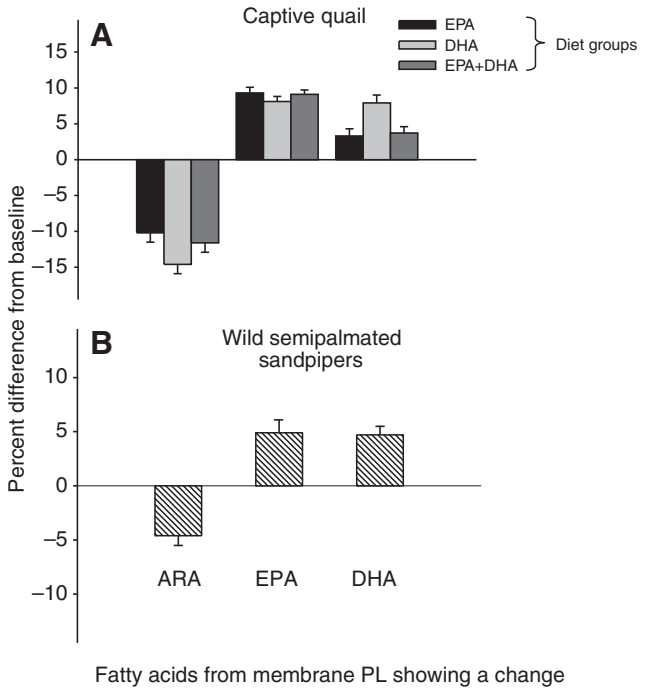

Fig. 6. Relative changes in the fatty acid composition of muscle membrane phospholipids in (A) captive quails fed different diets (Control, EPA, DHA, EPA+DHA) and (B) wild semipalmated sandpipers during pre-migration refueling. Values are means ± s.e.m. See Fig. 1 for abbreviations.

activity. Regression analyses between the n-3/n-6 ratio of membrane phospholipids and enzyme activities are presented in Fig. 8. This ratio was positively associated with CS and HOAD, but no relationship was found for CPT and COX.

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; Maillet and Weber, 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. 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 ($\dot{V}_{O_2,max}$) 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; Maillet and Weber, 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. 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 (Leandro et al., 2007; Siu et al., 2003)], 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 $\dot{V}_{O_2,max}$ (Bedford et al., 1979; Carter et al., 2001; Kim et al., 2005). Therefore, dietary n-3 fatty acids were probably able to boost the aerobic capacity of quails by more than 20%, although their $\dot{V}_{O_2,max}$ could

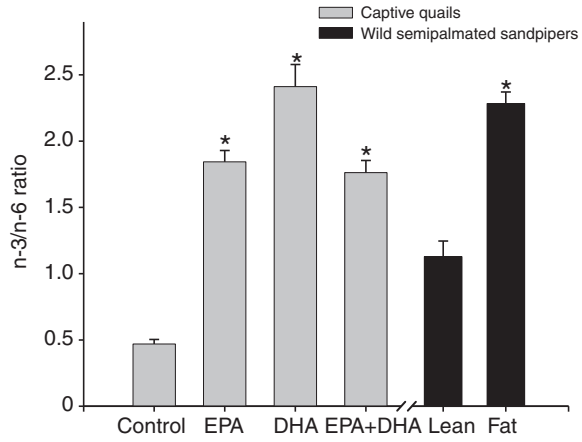

Fig. 7. Changes in the n-3/n-6 ratio of membrane phospholipids caused by the incorporation of dietary n-3 fatty acids. Captive quails fed different diets are indicated in grey, and migrating semipalmated sandpipers feeding on marine invertebrates in black. Values are means ± s.e.m. Asterisks indicate differences from control in quails, and a difference between lean and fat birds in sandpipers (P<0.05).

not be quantified in our study. The effect of diet on enzyme activities was stronger in quails (Fig. 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 in 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 (Bishop et al., 1995; Driedzic et al., 1993; Guglielmo et al., 2002; Maillet and Weber, 2007).

Table 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

	% EPA	% DHA	% ARA
CS	**0.03 (+)**	0.09 (+)	0.06 (–)
HOAD	**0.007 (+)**	**0.007 (+)**	**<0.001 (–)**
CPT	0.79 (+)	0.32 (+)	0.11 (–)
COX	**0.045 (+)**	0.95 (–)	0.46 (–)

All treatment groups were pooled for each analysis (N=31), and values are the probabilities that the slopes are different from 0. Whether the slope is positive or negative is indicated in parentheses. CS, citrate synthase; HOAD, 3-hydroxyacyl CoA dehydrogenase; CPT, carnitine palmitoyl transferase; COX, cytochrome oxidase; EPA, eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA, arachidonic acid. Bold values indicate when the slope of the relationship is significantly different from 0.

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. 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 of 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; Guderley et al., 2008; Maillet and Weber, 2006), 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 3–5) and, therefore, that compositional changes of total tissue phospholipids mirror those of mitochondrial and sarcoplasmic membranes. Consumption of the

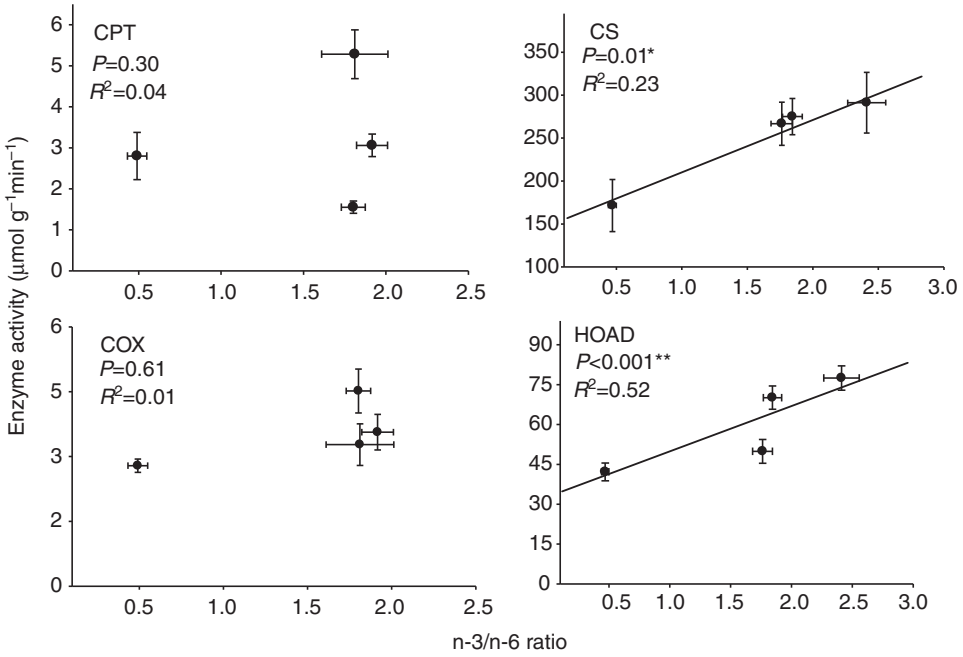

Fig. 8. Relationships between the n-3/n-6 ratio of muscle membrane phospholipids and the activities of Krebs cycle enzymes (CS and COX) or β-oxidation enzymes (CPT and HOAD). Lines were fitted by linear regression on individual values (N=31) and are only indicated when the slope was significantly different from 0. CS activity *vs* n-3/n-6 ratio: activity=(54.858×ratio)+162.244. HOAD activity *vs* n-3/n-6 ratio: activity=(16.812×ratio)+32.920. Values are means ± s.e.m. (N=8, except for DHA where N=7). See Fig. 1 for abbreviations.

experimental diets only caused changes in the abundance of three membrane fatty acids (increases in %EPA and %DHA were compensated by a decrease in %ARA) (Table 3; Figs 3–5). 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 6 and 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. Alternatively, 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 3; Figs 3–5, 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)]. By contrast, the pathway is strongly stimulated when humans consume DHA supplements for 6 weeks [up to 12% of dietary DHA is retroconverted (Arterburn et al., 2006; Conquer and Holub, 1997)]. Unfortunately, actual conversion capacities in either direction have never been measured in birds and this obscures our ability to distinguish specific effects of EPA or DHA.

Finally, membranes can act as a reservoir for signaling 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) (Miyasaka et al., 1996; Power and Newsholme, 1997; Swanson et al., 1989; 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. 8; Table 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 enzyme activities were correlated with the n-3/n-6 ratio (CS and HOAD), %EPA (CS, HOAD and COX), %DHA (HOAD) and %ARA (HOAD) (Fig. 8; Table 4). Therefore, 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 are even actually bound to them, e.g. CS (D’Souza and Srere, 1983a). The observed correlations are very useful because they allow the design of further experiments to investigate specific mechanisms of enzyme activation *via* changes in membrane composition. 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 such as 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) 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 (Cwinn et al., 2008; Konig et al., 2007). Reasons for this discrepancy are unclear but may reflect differences between drugs, tissues, exposure times or doses. The fact that dietary n-3 fatty acids did not stimulate the expression of PPAR genes (Fig. 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 (Cwinn et al., 2008; Konig et al., 2007). 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 inhibitors 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 birds can interconvert the two fatty acids. 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 muscles 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, choosing n-3 fatty acid doping over endurance training strikes us as the better strategy to increase aerobic capacity.

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