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Journal

Physiological Research, 63(1)

ISSN

0862-8408

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Publication Date

2014-02-28

DOI

10.33549/physiolres.932529

Peer reviewed

Published in final edited form as:
Physiol Res. 2014 ; 63(1): 57–71.

The Influence of dietary lipid composition on skeletal muscle mitochondria from mice following eight months of calorie restriction

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Summary

Calorie restriction (CR) has been shown to decrease reactive oxygen species (ROS) production and retard aging in a variety of species. It has been proposed that alterations in membrane saturation are central to these actions of CR. As a step towards testing this theory, mice were assigned to 4 dietary groups (control and 3 CR groups) and fed AIN-93G diets at 95% (control) or 60% (CR) of ad libitum for 8 months. To manipulate membrane composition, the primary dietary fats for the CR groups were soybean oil (also used in the control diet), fish oil or lard. Skeletal muscle mitochondrial lipid composition, proton leak, and H_2O_2production were measured. Phospholipid fatty acid composition in CR mice was altered in a manner that reflected the n-3 and n-6 fatty acid profiles of their respective dietary lipid sources. Dietary lipid composition did not alter proton leak kinetics between the CR groups. However, the capacity of mitochondrial complex III to produce ROS was decreased in the CR lard compared to the other CR groups. The results of this study indicate that dietary lipid composition can influence ROS production in muscle mitochondria of CR mice. It remains to be determined if lard or other dietary oils can maximize the CR-induced decreases in ROS production.

Keywords

Dietary lipids; Energy restriction; Mitochondria; Phospholipids; Reactive oxygen species

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Conflict of Interest
There is no conflict of interest.

Introduction

Calorie restriction (CR) without malnutrition has been shown to delay the onset of age-related diseases and increase mean and maximum life spans in a variety of species (Weindruch and Sohal 1997). However, its underlying mechanisms are not fully known. It has been proposed that alterations in mitochondrial membrane lipid composition may be a key contributory factor in the age-delaying effects conferred by CR (Yu 2005). This idea is consistent with the membrane theory of aging which proposes that the number of double bonds in membrane phospholipids is inversely correlated with life span (Hulbert *et al.* 2007, Pamplona *et al.* 2002, Pamplona *et al.* 1998, Portero-Otin *et al.* 2001) and CR has been reported to alter membrane composition in a manner that decreases long chain n-3 polyunsaturated fatty acid (PUFA) content and decreases the degree of unsaturation of membranes (Faulks *et al.* 2006, Laganiere and Yu 1993). This decrease in membrane unsaturation is hypothesized to favor longevity by increasing the resistance of membranes to lipid peroxidation (Pamplona *et al.* 2002, Yu *et al.* 2002). However, alterations in membrane lipid composition can also influence the function of membrane proteins (Lee 2004). The biochemical functions of mitochondria strongly depend on phospholipids (Daum 1985) whose fatty acid side chains are important contributory factors to membrane structure. Thus, alterations in membrane lipid composition can change membrane structure and influence the functions of proteins that are embedded in the specific lipid medium (Lee 2004).

The inner mitochondrial membrane is one of the primary cellular sites for reactive oxygen species (ROS) production as well as the primary target for oxidative damage. Specifically, the mitochondrial electron transport chain complexes I and III, which reside in the inner mitochondrial membrane, have been identified as major sites of ROS production (Andreyev *et al.* 2005, Lambert and Brand 2009, Murphy 2009). It is conceivable that alterations in membrane lipid composition could influence aging by modulating ROS production from these complexes. A variety of experimental evidence has confirmed that CR decreases mitochondrial ROS production in skeletal muscle (Bevilacqua *et al.* 2004, 2005), liver (Gredilla *et al.* 2001, Hagopian *et al.* 2005, Lambert and Merry 2004), heart (Judge *et al.* 2004, Sohal *et al.* 1994), kidneys (Sohal *et al.* 1994) and brain (Sanz *et al.* 2005, Sohal *et al.* 1994). Dietary intervention studies also suggest that alterations in membrane lipid composition may influence mitochondrial ROS production (Hagopian *et al.* 2010, Ramsey *et al.* 2005). However, it is not clear if changes in membrane lipid composition contribute to CR-induced alterations in ROS production.

CR-related changes in membrane lipid composition could also impact membrane permeability. It has been reported that mitochondrial proton leak shows a positive correlation with membrane unsaturation index and n-3 PUFAs (Brookes *et al.* 1998, Porter *et al.* 1996). Also, it has been demonstrated that CR alters mitochondrial proton leak in skeletal muscle (Asami *et al.* 2008, Johnson *et al.* 2006). However, it is not entirely known whether CR-induced alterations in membrane composition influence changes in mitochondrial proton leak.

We previously investigated the influence of dietary lipid composition on mitochondrial fatty acid composition, ROS production and mitochondrial proton leak with short-term(1 month)

CR in mice (Chen *et al.* 2012b). The objective of the current study was to determine if dietary lipid source (fish oil, soybean oil or lard) altered skeletal muscle mitochondrial membrane composition, ROS production, and proton leak with chronic CR (eight months) in mice. Skeletal muscle, a post-mitotic tissue, is a major contributor to whole animal oxygen consumption/energy expenditure (Ramsey *et al.* 2000) and there is considerable evidence that muscle shows increases in oxidative damage with aging (Aoi and Sakuma 2011, Cortopassi and Wong 1999, Marzetti *et al.* 2009, Sastre *et al.* 2003). Mitochondrial membrane fatty acid composition may play an important role in determining the magnitude of age-related changes in ROS production and oxidative damage in skeletal muscle. In particular, PUFA-enriched membranes are more susceptible to oxidative damage than those containing primarily saturated and monounsaturated fatty acids (MUFAs) (Hulbert 2005). CR has been shown to mitigate the accumulation of oxidative damage in skeletal muscle with aging (Lass *et al.* 1998), and it is possible that this may be at least partly due to the CR-induced changes in mitochondrial fatty acid composition (Hulbert 2005, Yu *et al.* 2002). This study will help indicate if a specific change in membrane fatty acid composition is required for mitochondrial changes commonly reported with CR and it will determine the influence of dietary lipid composition on skeletal muscle mitochondrial membrane fatty acid composition, ROS production and proton leak in CR mice.

Methods

Chemicals

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO), except for protein assay kit (BioRad, Hercules, CA) and bovine serum albumin (MP Biochemicals, Santa Ana, CA).

Animals and Diets

Male C57BL/6 mice were purchased from the Jackson Laboratory (West Sacramento, CA) at 14-wk of age. After habituation to a commercial rodent chow diet (Harlan Teklad #7012, Madison, WI) for 14 days, the mice were randomly assigned to 4 dietary groups and fed a modified AIN-93G purified diet (Chen *et al.* 2012b). The control group was fed 95% of a predetermined ad libitum intake (13.6 kcal/d). This slight restriction in food intake was initiated to prevent excessive weight gain during the study. The three CR dietary groups were maintained on 60% of the daily allowance of the ad libitum intake (8.6 kcal/d), and these diets were identical except for dietary lipid sources. The modified AIN-93G diets (% total Kcal/d) contained 20.3% protein, 63.8% carbohydrate, and 15.9% fat. The dietary fat for the control group was soybean oil. The dietary fats for the three CR group were soybean oil (high in n-6 PUFAs, Super Store Industries, Lathrop, CA), fish oil (high in n-3 PUFAs: 18% eicosapentaenoic acid, 12% docosahexaenoic acid, Jedwards International, Inc. Quincy, MA), or lard (high in saturated and monounsaturated fatty acids, ConAgra Foods, Omaha, NE). To prevent linoleic acid deficiency, the diet for the CR-fish group contained some soybean oil (~55% linoleic acid) to insure that the requirement for linoleic acid was met. The mice were fed the control or CR diets (modified AIN-93G diets) until they reached 6 months of age after which all mice were switched to a modified AIN-93M diet (Reeves *et al.* 1993) containing the same primary dietary lipids they were consuming previously (Table

1). The switch from the AIN-93G to AIN-93M diets reflected the protocol used for a parallel life span study. For this study, we wished to maintain the adult mice on a diet that did not contain high amounts of protein. Thus, the AIN-93M diet was selected for long-term maintenance of the mice since the level of protein in this diet was closer to the protein requirement than the AIN-93G diet. The daily energy intake (Kcal/d) remained the same when switching to the AIN-93M diet. To insure linoleic acid requirement, both the CR-fish and the CR-lard groups were supplemented with soybean oil (Table 1). The modified AIN-93M diets (% total Kcal/d) contained 14.8% protein, 75.7% carbohydrate, and 9.5% fat. The fatty acid composition of the dietary lipids has been previously reported (Chen *et al.* 2012b). All mice were housed individually in a vivarium maintained at 22–24°C and 40–60% relative humidity with a 12-hour light-dark cycle and free access to water. All experimental procedures were approved by the University of California Institutional Animal Care and Use Committee.

Isolation of Mitochondria from Skeletal Muscle

At the end of 8-mo CR, mice were euthanized by cervical dislocation after an overnight fast. Muscle mitochondria were isolated as previously described (Seifert *et al.* 2010) with some modifications. Briefly, muscle from the hindlimb was rapidly dissected and placed in ice-cold basic medium containing 140 mM KCl, 20 mM Hepes, 5 mM $MgCl_2$ and 1 mM EGTA (pH 7 at 25 °C). Muscle was washed and trimmed of connective tissue and fat, and minced into small pieces in 5 vol of ice-cold homogenization medium containing basic medium with 1% BSA, 1mM ATP, and two units of Subtilisin A (protease) per gram muscle wet weight. Muscle was incubated in the homogenization media for 2 min with constant swirling. The muscle solution was then diluted six fold (v/v) using homogenization media without Subtilisin A. The mixture was centrifuged at 10,000 g (Beckman Coulter Model J2-21M) for 10 min to remove the Subtilisin A. The supernatant was discarded and the pellet was homogenized (10% w/v in homogenization medium) in an ice cold glass-Teflon motor-driven homogenizer. The homogenate was centrifuged at 500 g for 10 min after which the pellet was discarded and the supernatant was centrifuged at 10,000 g for 10 min. The resulting pellet was re-suspended in wash medium (basic medium with 1% BSA) and incubated on ice for 5 min (myofibrillar repolymerization). Samples were then centrifuged at 500 g for 10 min in the above wash medium. The retained supernatant was centrifuged at 10,000 g for 10 min and the pellet was retained. This pellet was re-suspended in basic medium and centrifuged at 10,000 g for 10 min. The final pellet was re-suspended in a minimal volume of the basic medium. A portion of this final pellet was used for hydrogen peroxide, proton leak, and lipid peroxidation assays. The rest was stored under liquid nitrogen for lipid analysis.

Purity of the mitochondria was tested by measuring the activity of marker enzymes for lysosomes (acid phosphatase), microsomes (glucose-6-phosphatase) and peroxisomes (urate oxidase) as previously described (Hagopian *et al.* 2011). The activities of all enzymes were negligible indicating that the mitochondria were free from contamination with other organelles. This was the case for all four dietary treatments.

Protein assays

Protein concentrations of mitochondrial samples were determined using the BioRad (Hercules, CA) protein assay method, with BSA as the standard.

Organ weight measurements

Internal organs (brain, liver, heart, kidneys, spleen, and lungs) and fat pads (subcutaneous, epididymal, perirenal, visceral, and interscapular areas) were removed from the mice immediately after sacrifice. All tissues were cleaned of connective tissue and weighed.

Lipid extraction and fatty acid analysis

Hindlimb muscle mitochondria were used to confirm that dietary lipid manipulations were influencing fatty acid composition of total phospholipids. The isolated mitochondria were shipped on dry ice to Lipid Technologies LLC (Austin, MN) and analyzed for membrane fatty acid composition. Lipids were extracted from the mitochondrial membranes using the Bligh-Dyer extraction (Bligh and Dyer 1959). A mixture of mitochondrial membranes, chloroform/methanol (2:1 v/v) and water were prepared in order to recover the lipid in a chloroform layer. Lipid classes were separated by preparative thin layer chromatography and the desired corresponding lipid fractions were scraped from the thin layer plates and methylated with boron trifluoride (10%) in excess methanol in an 80°C water bath for 90 minutes. The resulting fatty acid methyl esters were extracted with petroleum ether and water and stored frozen for separation and quantification by capillary gas chromatographic analysis (Forsythe *et al.* 2010). Results were expressed as the percentage of each individual fatty acid in relation to the total fatty acids.

H_2O_2 Production by skeletal muscle mitochondria

The rate of mitochondrial H_2O_2 production was determined fluorimetrically (excitation 320 nm, emission 400 nm) as previously described (Hyslop and Sklar 1984), with modifications, at 37°C in a final volume of 3 ml containing (final concentrations) 10 mM potassium phosphate buffer, pH 7.4, 154 mM KCl, 0.1 mM EGTA, 3 mM $MgCl_2$, *p*-hydroxyphenylacetate (PHPA; 500 μg), horseradish peroxidase (4 units), superoxide dismutase (100 U/mL), and mitochondria (0.1 mg). Fluorescence was measured using a Perkin-Elmer LS 55 luminescence spectrometer equipped with a peltier water heating system and a magnetic stirring sample compartment. Measurements were completed with substrates alone (10 mM succinate or 10 mM pyruvate/5 mM malate), substrate plus rotenone (5 μM), or substrate plus antimycin A (5 μM). Rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) maintain complexes I and/or III in reduced state. The rates of H_2O_2 production were expressed as pmol H_2O_2 min^{-1} mg^{-1} protein. A standard curve generated over a range of H_2O_2 concentrations was used to determine the amount of H_2O_2 produced.

Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption (nmol O/min/mg protein) was measured using a previously described method (Venditti *et al.* 2006) with modifications. Mitochondrial respiration was monitored using a Clark-type oxygen electrode (Hansatech, Norfolk, UK).

All measurements were completed at 30°C using mitochondria (0.25 mg mitochondrial protein/mL) in air-saturated incubation medium (145 mM KCl, 5 mM KH_2PO_4, 30 mM Hepes, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4). Respiration was initiated by the addition of 5 mM succinate (plus 5 μM rotenone) in the absence (state 4) and in the presence (state 3) of 500 μM ADP. Respiratory control ratios (RCR) were calculated as state 3 divided by state 4 respiration rates. The RCR values for the four experimental groups were: Control, 7.0 ± 0.3; CR-soy, 6.7 ± 0.4; CR-fish, 6.4 ± 0.5; and CR-lard, 6.3 ± 0.3. These values were not statistically different from each other.

Measurement of proton leak kinetics

Mitochondrial membrane potential (Δ Ψm) in non-phosphorylating skeletal muscle was assessed simultaneously with oxygen consumption measurements using a methyl-triphenyl-phosphonium ($TPMP^+$)-sensitive electrode. All measurements were completed using mitochondria (0.25 mg/mL) in the above mentioned incubation medium. A $TPMP^+$ (0 – 2.5 μM) standard curve was generated in each sample prior to the initiation of respiration and membrane potential measurements. Proton leak kinetics were determined by titrating the electron transport chain with incremental additions of malonate (0.1 mM – 2.4 mM), an inhibitor of complex II, in the presence of 5 mM succinate, 5 μM rotenone, oligomycin (8 μg/mL) and nigericin (0.08 μg/mL) (Ramsey *et al.* 2004). Membrane potentials were calculated using a modified Nernst equation (Ramsey *et al.* 2004). A $TPMP^+$ binding correction for skeletal muscle of 0.35 $(\mu l/mg\ protein)^{-1}$ was used for membrane potential calculations (Cadenas *et al.* 2002).

Measurement of lipid peroxidation levels

Lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) test (Buege and Aust 1978), except 0.07 mM per assay of butylated hydoxytoluene (BHT) was added to prevent artificial lipid peroxidation during the boiling step (Guerrero *et al.* 1999). To 1 mL of muscle mitochondrial sample (0.25 – 0.50 mg protein), BHT was added, followed by the addition of 2 mL of TCA-TBA-HCl reagent (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25N hydrochloric acid) and mixed thoroughly. The solution was heated for 15 min in a boiling water bath, then cooled and centrifuged at 1000 g for 10 min to remove the precipitate. The absorbance of the supernatant was determined at 535 nm ($\varepsilon = 1.56\times10^5\ M^{-1}\ cm^{-1}$) against a blank that contained all the reagents minus the mitochondria. Results were expressed as nmol TBARS per mg protein.

Statistical analysis

All results were expressed as the mean ± S.E. Normality of the data distribution was determined using the Shapiro-Wilk test. Comparisons between the control and CR-soy group were used to determine the influence of CR on each variable of interest while comparisons among the three CR groups were used to determine the influence of dietary lipidson the variables of interest. Wilcoxon/Kruskal-Wallis tests or ANOVA were performed as appropriate using JMP software (SAS Institute Inc. Cary, NC). A post-hoc Tukey-Kramer test was performed to correct for multiple comparisons.

Results

Organ and body weights

Organ and body weights for each of the 4 diet groups are summarized in Table 2. Eight months of CR (Control vs. CR-soy) produced a decrease ($P < 0.05$) in body weight (34.2%), liver (38.3%), kidney (36.6%), heart (21.4%), lung (16.7%), spleen (37%), brain (9.3%), and hindlimb muscle (18%) weights. CR strikingly decreased the weights of all fat pads ($P < 0.05$), including subcutaneous (75%), epididymal (71.7%), perirenal (85.7%), visceral (68.9%), and interscapular (66.7%), as shown in Table 3.

Within the CR groups, the CR-lard mice had higher ($P < 0.05$) muscle weights than the CR-fish group. There were no other differences in organ or fat pad weights between the CR groups.

Mitochondrial total phospholipid fatty acid composition

Hindlimb muscle mitochondrial total phospholipid fatty acid composition is presented in Table 4. Lipid analysis showed that muscle mitochondrial membrane lipids changed in a manner which reflected the dietary polyunsaturated fatty acids.

When investigating the effect of CR (CR-soy vs. control) on mitochondrial phospholipid fatty acid composition, CR was observed to increase ($P < 0.05$) total n-6 fatty acid content. This was primarily due to a 32.1% increase in 18:2n-6 content in the CR-soy versus control mitochondria. CR also resulted in a decrease in monounsaturated fatty acid (MUFA) content due to decreases in the content of 16:1n-7 and 18:1n-7. However, CR did not alter unsaturation index (UI), saturated fatty acids (SFA), PUFA, total n-3, and total HUFAs (highly unsaturated fatty acids, three or more double bonds).

To examine the effects of dietary lipids on the mitochondrial membrane fatty acid composition of CR mice, comparisons were made between the three CR dietary groups. The fish oil group had the highest levels of total n-3 fatty acids, total HUFA, and n-3 HUFA content and concomitantly they had the lowest n-6 fatty acids and n-6 HUFA content compared to the soy and lard groups. This was due to fish oil induced increases in 20:5n-3, 22:5n-3 and 22:6n-3 content and concomitant decreases in 18:2n-6, 20:3n-6, 20:4n-6 and 22:5n-6 content. The fish oil group also had increased unsaturation index compared to the other diet groups. The lard group had the lowest total n-3 fatty acids and n-3 HUFA content and concomitantly they had the highest total n-6 fatty acid and n-6 HUFA content. This was due to lard induced decreases in 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3 content and concomitant increases in 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 content. The soybean oil group had the highest 18:2n-6 and 18:3n-3 content.

Mitochondrial H_2O_2 production

The influence of CR and dietary lipid composition on mitochondrial H_2O_2 production is summarized in Figure 1. To examine the effects of CR on H_2O_2 production, comparisons were made between the control and CR-soy groups. Overall, our data indicated that under all

conditions tested, there were no differences between the two groups in H_2O_2 production regardless of substrates and/or inhibitors used (Fig. 1A–C).

To examine the effects of dietary lipids on H_2O_2 production in CR mice, comparisons were made between the 3 CR dietary groups. Mitochondria respiring on pyruvate/malate (a Complex I-linked substrate) plus rotenone (Fig. 1A) showed similar H_2O_2 production irrespective of the dietary fats consumed. Rotenone blocks electron transport to coenzyme Q and maintains Complex I in a reduced state, producing a condition which maximizes ROS production from Complex I. This observation indicated that the ROS producing capacity from complex I was not altered by dietary fats within the CR regimens. However, H_2O_2 production was decreased in the lard compared to fish oil ($P < 0.05$) or soybean oil ($P < 0.05$) CR groups when mitochondria were respiring on pyruvate/malate plus antimycin A. Antimycin A blocks electron flow at Complex III and maintains both Complexes I and III in a reduced state, thus producing a condition of maximal ROS production. However, since the rotenone results showed no differences between CR groups in capacity to produce ROS from complex I, the pyruvate/malate plus antimycin A results suggest that the CR-lard group had decreased ROS producing capacity from complex III. There was also a clear trend towards decreased H_2O_2 production in the CR-lard compared to other CR groups ($P = 0.07$: CR-lard vs. CR-soy; $P = 0.08$: CR-lard vs. CR-fish) for pyruvate/malate/succinate (Complex I and II-linked substrates) plus antimycin A (Fig. 1C). There were no differences in H_2O_2 production between any of the CR groups when mitochondria were respiring on succinate (Complex II-linked substrate) (Fig. 1B and 1C). Succinate was used as a substrate to investigate backflow into complex I. The lack of significant differences between CR groups in mitochondria respiring on succinate suggests that dietary lipids had little influence on ROS produced from backflow into complex I.

Mitochondrial proton leak kinetics

Mitochondrial proton leak kinetics curves are summarized in Figure 2. To examine the effects of CR on proton leak kinetics, comparisons were made between the CR-soy and the control groups. Our data showed that state 4 oxygen consumption and membrane potential (furthest points to the right on the proton leak kinetics plot) were not significantly different between the CR-soy and control groups. When assessing the effects of dietary fats on proton leak, comparisons were made between the 3 CR diet groups. The results indicated that dietary lipid composition did not markedly influence mitochondrial proton leak in skeletal muscle of CR mice.

Lipid peroxidation in skeletal muscle mitochondria

TBARS were measured to assess lipid peroxidation in skeletal muscle mitochondria from the four groups of mice (Fig. 3). Comparisons between the control and CR-soy groups indicated no change in TBARS levels following eight months of CR. Comparisons between the CR groups indicated that dietary lipids did not markedly influence the levels of TBARS.

Discussion

It has been proposed that alterations in mitochondrial membrane fatty acid composition may play a central role in the actions of CR (Yu *et al.* 2002). To test this idea, membrane fatty acid composition was manipulated in CR mice by feeding the animals diets that differed in lipid composition. The objective of the study was to determine if mitochondrial phospholipid fatty acid composition influenced mitochondrial ROS production and proton leak with CR. To determine the efficacy of using this approach, it was important to determine if mitochondrial fatty acids are sensitive to dietary lipid composition in mice maintained on CR for 8 months. In ad libitum fed animals, it has been shown that dietary lipids can alter the fatty acid profile of mitochondrial membranes in multiple tissues, including liver (Quiles *et al.* 2002, Ramsey *et al.* 2005, Tahin *et al.* 1981), heart (Quiles *et al.* 2002, Tahin *et al.* 1981, Yamaoka *et al.* 1988), brain (Tahin *et al.* 1981), and skeletal muscle (Quiles *et al.* 2002). However, there is little information to indicate if these same changes in mitochondrial fatty acids are observed in CR animals. We have previously shown that mitochondrial fatty acid composition is altered to reflect dietary polyunsaturated fatty acid composition in mice maintained on CR for 1 month (Chen *et al.* 2012b). The results of the present study indicate that dietary lipid composition also has a substantial influence on mitochondrial phospholipid fatty acids at 8 months of CR. Specifically, in CR mice fish oil markedly increased n-3 fatty acids and soybean oil increased 18:2n-6 levels in mitochondrial phospholipids. Our results demonstrate that muscle mitochondrial fatty acid composition is sensitive to dietary lipid composition in mice maintained on long-term (8 months) CR.

It has been reported that CR increases the content of 18:2n-6 and decrease long chain polyunsaturated fatty acids of membrane phospholipids in liver (Laganiere and Yu 1989, 1993), spleen (Venkatraman and Fernandes 1992) and heart (Lee *et al.* 1999). The results of the present study are consistent with the idea that CR increases the level of 18:2n-6 in mitochondrial phospholipids, however, we found no evidence that CR decreases long chain polyunsaturated fatty acids or unsaturation index in skeletal muscle mitochondria. Thus, it does not appear that a sustained decrease in membrane unsaturation is a mechanism contributing to the actions of CR in skeletal muscle mitochondria. However, it is possible that additional changes in fatty acid composition may occur with age and/or duration of CR. In support of this idea, it has been reported that both level of CR and length of CR influence skeletal muscle phospholipid fatty acid composition (Faulks *et al.* 2006). Similarly, CR-induced changes in mitochondrial phospholipid fatty acid composition were not entirely uniform between our 1 month (Chen *et al.* 2012b) and 8 month CR studies. In particular, a significant CR-induced increase in mitochondrial 18:2n-6 level was only observed at 8 months of CR. Thus, additional studies at multiple time points are likely needed to completely characterize the influence of CR and lipid composition on mitochondria phospholipid fatty acids.

One way that mitochondrial membrane fatty acids could influence aging is by altering mitochondrial ROS production. CR has been reported to decrease mitochondrial ROS production in a variety of tissues (Gredilla and Barja 2005, Sohal and Weindruch 1996). Studies in rats have reported that skeletal muscle mitochondrial ROS production is decreased with long-term (1 year or longer) CR (Bevilacqua *et al.* 2005, Drew *et al.* 2003),

and either decreased (Bevilacqua *et al.* 2004) or not changed (Gredilla *et al.* 2004) with short-term CR. In mice, it has been shown that muscle ROS production is not altered with short-term (6 months or less) CR (Chen *et al.* 2012b, Faulks *et al.* 2006) in mitochondria respiring on substrates in the absence of electron transport chain inhibitors. The results of the present study are consistent with these studies in mice and indicate that CR and control animals show similar levels of skeletal muscle mitochondrial ROS production at 8 months of CR. Thus, the CR-induced changes in mitochondrial fatty acid composition in the CR-soy group alone were not sufficient to induce alteration in ROS production at 8 months of CR. However, there is evidence that CR prevents age-related increases in skeletal muscle ROS production in mice (Lass *et al.* 1998) and it is possible that studies of longer duration are needed to consistently see changes in muscle ROS production. The reason(s) for the differences between studies in ROS production in response to CR is not clear. It does not appear to be due to species differences since similar responses to CR have been observed in both rats and mice (Walsh *et al.* 2013). The differences may be influenced by the level of CR, age or diet composition. Studies do indicate that lipid (Chen *et al.* 2012a, Chen *et al.* 2012b, Ramsey *et al.* 2005) and protein (Caro *et al.* 2009, Gomez *et al.* 2011) composition of diets can influence ROS production. However, additional studies are needed to determine the extent to which diet composition, age or magnitude of CR may influence CR-related changes in ROS production.

The primary sites of ROS production in mitochondria are complexes I and III of the ETC (Gredilla *et al.* 2001). Since ROS are produced by these membrane-bound proteins, it is conceivable that the rate of ROS production could be influenced by mitochondrial phospholipid fatty acid composition. Changes in lipids can influence mitochondrial membrane structure and stability and affect the function of individual enzymes (Daum 1985). In particular, fatty acid composition of cardiolipin has been shown to influence the activities of ETC enzymes (Hoch 1992). However, little is known about the influence of membrane lipid composition on mitochondrial ROS production. Previous investigations (Hagopian *et al.* 2010, Ramsey *et al.* 2005) have shown that increased n-3 HUFA content in liver mitochondrial membrane phospholipids is associated with decreased ROS production. However, it is unclear whether fatty acid composition of mitochondrial phospholipids influence muscle mitochondrial ROS production in a manner similar to that of liver. The results from our one month CR study indicate that dietary lipid composition did not alter skeletal muscle H_2O_2 production in CR mice, despite producing major changes in mitochondrial phospholipid fatty acid composition (Chen *et al.* 2012b). The results of the present study suggest that length of CR and/or age may induce changes in mitochondrial ROS production between dietary lipid groups. In particular, H_2O_2 production was decreased in the CR-lard compared to other CR groups when maximal ROS production was induced by adding the Complex III inhibitor antimycin A to mitochondria respiring on pyruvate/malate or pyruvate/malate/succinate. Since no decrease in H_2O_2 production was observed in this group with rotenone (a complex I inhibitor), these results suggest that capacity for ROS production from Complex III is decreased in the CR-lard versus other CR groups. The use of inhibitors allows chemical dissection of the electron transport chain (ETC) and identification of the specific complexes responsible for ROS production. The inhibitors maintain specific components of the ETC in a reduced state and produce conditions which maximize ROS

production. They may identify changes in capacity for ROS production that would be missed with the use of substrates alone and may provide an indication of ROS production under physiological conditions which limit ETC activity (such as decreased oxygen or ADP levels). Nonetheless, the results of the current study provide support for the idea that dietary lipid composition does influence ROS production in skeletal muscle mitochondria of CR animals.

It is also possible that mitochondrial fatty acid composition could influence mitochondrial proton leak. Proton leak is a process where protons bypass the ATP synthase and passively cross the mitochondrial inner membrane. It has been shown that basal proton leak increases with age (Hagen *et al.* 1997, Harper *et al.* 1998, Lal *et al.* 2001) and it has been proposed that CR may oppose this age-related increase and improve mitochondrial efficiency by inducing a sustained decrease in proton leak (Ramsey *et al.* 2000). Factors affecting the rates of proton leak include uncoupling proteins (UCP 2 and 3) (Brookes 2005), ANT (Brookes 2005), and possibly phospholipids (Brand 2005). It has been shown in mice that skeletal muscle proton leak is either not altered or decreased with CR depending on duration of CR and/or animal age (Asami *et al.* 2008). We previously showed that there was a trend towards a decrease in skeletal muscle proton leak at one month of CR (Chen *et al.* 2012b), although any difference had disappeared by 8 months of CR. Existing skeletal muscle data supports the idea that CR does not produce uniform changes in proton leak kinetics across time when compared to control animals. It has previously been shown that these changes in proton leak kinetics are primarily due to age-related changes in control rather than CR mice (Asami *et al.* 2008). Thus, it is possible that time –related changes in proton leak kinetics between CR and control mice reflect the fact that CR mitigates age-related changes in proton leak.

Unsaturation index and level of n-3 PUFA in mitochondrial inner membrane phospholipids has been shown to be positively correlated with proton leak in comparative studies using liver mitochondria (Brookes *et al.* 1998, Porter *et al.* 1996) or liposomes prepared from liver mitochondria (Brand *et al.* 1994). However, the influence of dietary lipids and mitochondrial membrane lipid composition on proton leak in skeletal muscle is not entirely clear. The results of our studies indicate that dietary lipids and mitochondrial phosholipid composition have little influence on skeletal muscle mitochondrial proton leak in CR animals. This reflects the fact that only a slight decrease in skeletal muscle mitochondrial proton leak was observed in the CR-lard compared to other CR groups at one month of CR (Chen *et al.* 2012b) and no differences in proton leak were observed between the CR groups at eight months of CR. This lack of change in skeletal muscle mitochondrial proton leak occurred despite the fact that diet produced large changes in mitochondrial phospholipid fatty acid composition. These results indicate that basal proton leak in skeletal muscle mitochondria is primarily a function of membrane proteins and is largely insensitive to membrane fatty acid composition.

Lipid peroxidation is one factor that could influence the comparisons in the present study. Previous research has demonstrated an inverse correlation in mammalian species between peroxidation index and lifespan (Hulbert 2008) and it has been proposed that CR may retard aging by making membranes more resistant to peroxidation (Yu *et al.* 2002). An increased degree of unsaturation in membrane phospholipids, as a result of fish oil consumption, may

deplete some antioxidants and augment membrane susceptibility to lipid peroxidation. Experimental manipulation of membrane composition allows us to assess the consequences of varying membrane compositions on oxidative damage. It should be noted that to prevent membrane oxidative damage, the fish oil fed CR group was provided with twice the amount of the dietary antioxidant t-butylhydroquinone than the other groups. Our TBARS measurements demonstrated there were no significant differences between any of the groups of mice in mitochondrial lipid peroxidation. This indicates that none of the mitochondrial comparisons were influenced by differences in level of lipid peroxidation between groups.

The present study indicates that skeletal muscle phospholipid fatty acid composition in mice following 8-month CR is altered in a manner that reflects the n-3 and n-6 fatty acid profiles of their respective dietary lipid sources. Despite these changes in mitochondrial fatty acid composition, dietary lipid composition had no influence on skeletal muscle mitochondrial proton leak at 8 months of CR. Dietary lipid composition did, however, have an influence on mitochondrial ROS production in the CR mice. In particular, capacity for ROS production from Complex III was decreased in the CR-lard compared to other CR groups. The results of this study indicate that dietary lipid composition can influence ROS production in mice maintained on CR for 8 months. It remains to be determined if lard or other mixtures of dietary oils can maximize decreases in skeletal muscle ROS production with long-term CR in older animals.

Acknowledgments

This work was supported by the National Institutes of Health grants R01 AG028125 and P01 AG025532.

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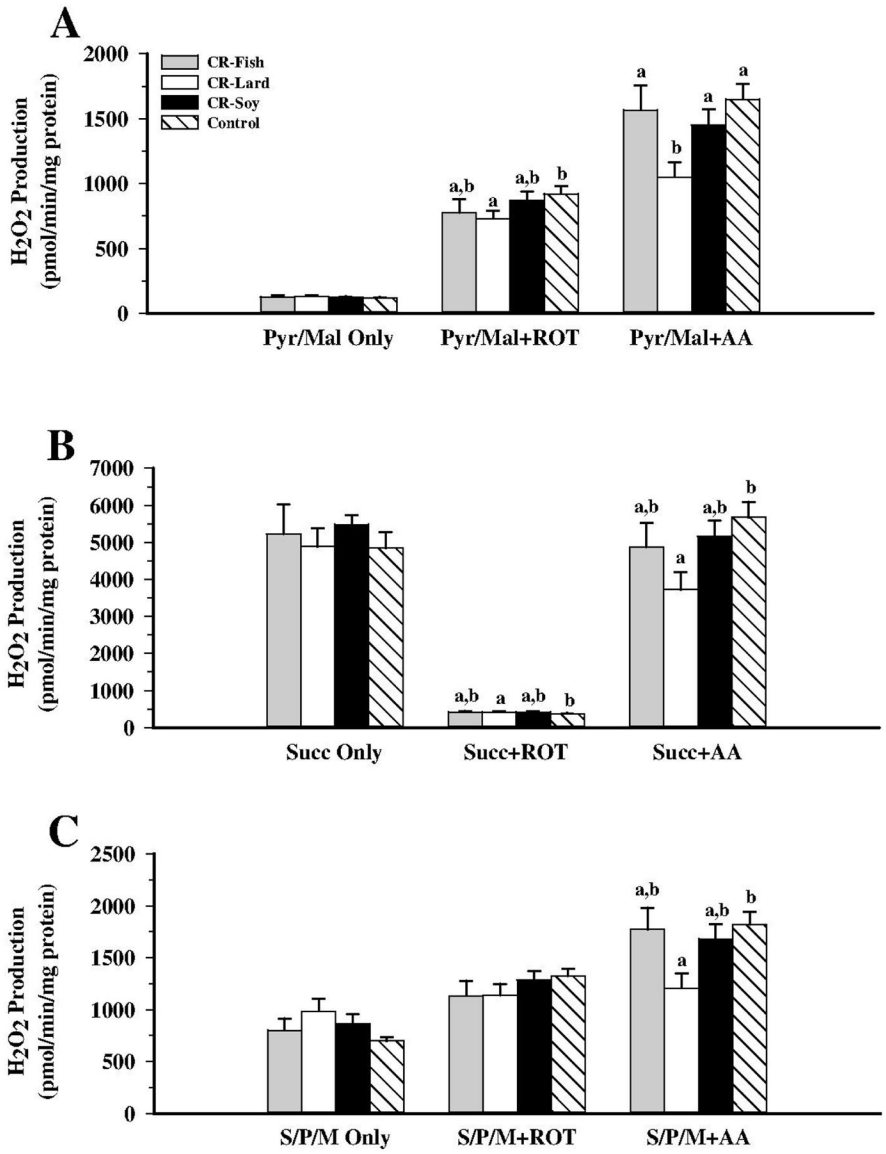

Fig. 1.

Hydrogen peroxide production in muscle mitochondria at the end of 8 mo CR from mice consuming a control diet (Control) or CR diets containing lard (CR-Lard), soybean oil (CR-Soy), or fish oil (CR-Fish). All measurements were completed on freshly isolated mitochondria. H_2O_2 production was monitored in mitochondria respiring on Pyruvate/Malate (Panel A), succinate (Panel B), or Succinate plus Pyruvate/Malate (Panel C). All comparisons were within substrate only or within the substrate and inhibitor combination. Bars that do not share a common letter indicate a significant difference ($P < 0.05$) between treatments. All values are mean ± SEM (n = 7 – 9). AA, Antimycin A; ROT, Rotenone.

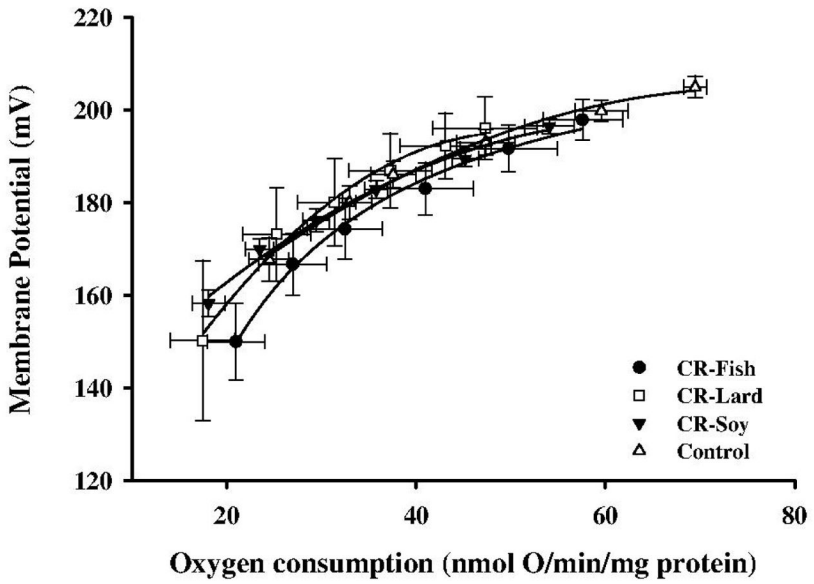

Fig. 2.

Proton leak kinetics curves in muscle mitochondria at the end of 8 mo CR from mice consuming a control diet (Control) or CR diets containing lard (CR-Lard), soybean oil (CR-Soy), or fish oil (CR-Fish). Leak-dependent respiration and membrane potential were measured simultaneously using a Clark-type electrode and a $TPMP^+$-sensitive electrode, respectively. The furthest point on the right in each panel represents state 4 respiration. All measurements were completed using mitochondria (0.25 mg/mL) in incubation medium (145 mM KCl, 5 mM KH_2PO_4, 30 mM Hepes, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4). A $TPMP^+$(0–2.5 μM) standard curve was generated in each sample prior to the initiation of respiration and membrane potential measurements. All assays were performed in the presence of 5mM succinate, 5 μM rotenone, oligomycin (8 μg/mL) and nigericin (0.08 μg/mL), and incremental additions of malonate (0.1 mM–2.4 mM). All values are mean ± SEM (n = 7 – 9).

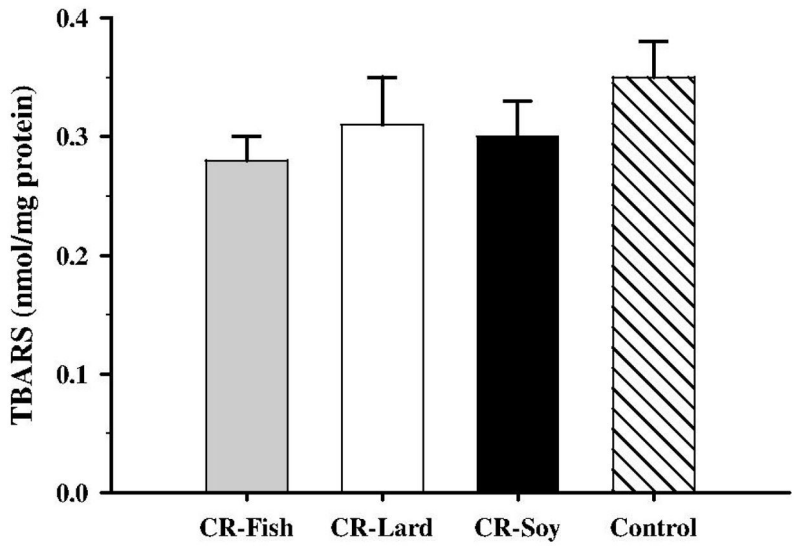

Fig. 3.

TBARS levels in hindlimb muscle mitochondria at the end of 8 mo CR from mice consuming a control diet (Control) or CR diets containing lard (CR-Lard), soybean oil (CR-Soy), or fish oil (CR-Fish). No differences were observed between mitochondria from different treatments. All values are expressed as nmol/mg protein and presented as mean ± SEM (n = 7 – 9).

Table 1

AIN-93M Diet composition[1]

	Dietary group		
Ingredients (g/100g Diet)	**Control/CR-Soy**	**CR-Fish**	**CR-Lard**
Corn starch	46.5	46.5	46.5
Casein	14	14	14
Maltodextrin	15.1	15.1	15.1
Sucrose	10	10	10
Soybean oil	4	1.2	0.7
Fish oil	0	2.8	0
Lard	0	0	3.3
Cellulose	5	5	5
Mineral mix	3.5	3.5	3.5
Vitamin mix	1	1	1
L-Cystine	0.18	0.18	0.18
Choline Bitartrate	0.25	0.25	0.25
t-Butylhydroquinone	0.0008	0.0016	0.0008

[1] All diets contained AIN-93M mineral mix and AIN-93 vitamin mix obtained from Dyets Inc. (Bethlehem, PA).

Table 2

Organ and Body weights (grams) at the end of 8 mo CR in mice consuming a control diet (Control) or CR diets containing either soybean oil (CR-Soy), fish oil (CR-Fish) or lard (CR-Lard).[1]

Body & Organs	Control (n = 12)	CR-Fish (n = 11)	CR-Lard (n = 9)	CR-Soy (n = 16)
Body weight	35.4 ± 0.7[a]	22.6 ± 0.3[b]	23.2 ± 0.3[b]	23.3 ± 0.2[b]
Liver	1.49 ± 0.06[a]	0.87 ± 0.02[b]	0.93 ± 0.04[b]	0.92 ± 0.02[b]
Muscle	1.39 ± 0.05[a]	1.05 ± 0.04[b]	1.27 ± 0.03[a,c]	1.14 ± 0.03[c]
Kidneys	0.41 ± 0.009[a]	0.26 ± 0.005[b]	0.25 ± 0.004[b]	0.26 ± 0.003[b]
Heart	0.14 ± 0.004[a]	0.10 ± 0.003[b]	0.11 ± 0.004[b]	0.11 ± 0.002[b]
Lungs	0.18 ± 0.006[a]	0.16 ± 0.002[b]	0.17 ± 0.002[a,b]	0.15 ± 0.003[b]
Spleen	0.081 ± 0.002[a]	0.052 ± 0.003[b]	0.046 ± 0.004[b]	0.051 ± 0.002[b]
Brain	0.43 ± 0.006[a]	0.40 ± 0.003[b]	0.40 ± 0.003[b]	0.39 ± 0.004[b]

[1] Values in a row that do not share a common letter indicate a significant difference ($P < 0.05$) between treatments.

Table 3

Fat pad weights (grams) at the end of 8 mo CR in mice consuming a control diet (Control) or CR diets containing either soybean oil (CR-Soy), fish oil (CR-Fish) or lard (CR-Lard). Comparisons are between the treatments within a specific fat pad (row).[1]

Fat pads	Control (n = 12)	CR-Fish (n = 11)	CR-Lard (n = 9)	CR-Soy (n = 16)
subcutaneous	2.64 ± 0.14[a]	0.70 ± 0.05[b]	0.63 ± 0.04[b]	0.66 ± 0.05[b]
visceral	0.90 ± 0.06[a]	0.28 ± 0.02[b]	0.38 ± 0.02[b]	0.28 ± 0.02[b]
epididymal	1.87 ± 0.08[a]	0.57 ± 0.03[b]	0.58 ± 0.02[b]	0.53 ± 0.03[b]
perirenal	0.63 ± 0.047[a]	0.08 ± 0.006[b]	0.10 ± 0[b]	0.09 ± 0.008[b]
interscapular	0.30 ± 0.037[a]	0.11 ± 0.007[b]	0.11 ± 0.005[b]	0.10 ± 0.008[b]

[1] Values in rows that do not share a common letter indicate a significant difference ($P < 0.05$) between treatments.

Table 4

Fatty acid composition (%) of total phospholipids from muscle mitochondria of mice consuming a control diet (Control) or CR diets containing lard (CR-Lard) soybean oil (CR-Soy) or fish oil (CR-Fish) for a period of 8 months.[1]

Fatty acids	Control (n = 8)	CR-Fish (n = 8)	CR-Lard (n = 8)	CR-Soy (n = 7)
14:0	0.73 ± 0.07[a]	0.73 ± 0.06[a]	0.48 ± 0.03[b]	0.50 ± 0.03[b]
16:0	19.9 ± 0.7	20.3 ± 0.8	18.3 ± 0.4	18.2 ± 0.4
18:0	12.5 ± 0.4[a]	13.9 ± 0.5[a,b]	14.3 ± 0.3[b]	14.2 ± 0.4[b]
20:0	0.08 ± 0.01	0.06 ± 0.02	0.07 ± 0.01	0.11 ± 0.02
24:0	0.08 ± 0.003[a]	0.12 ± 0.012[b]	0.12 ± 0.006[b]	0.10 ± 0.005[a,b]
16:1n-7	3.4 ± 0.2[a]	3.0 ± 0.2[a,c]	2.6 ± 0.1[b,c]	2.0 ± 0.1[b]
18:1n-7	5.3 ± 0.3[a]	4.3 ± 0.3[b]	4.6 ± 0.2[a,b]	3.9 ± 0.1[b]
18:1n-9	5.8 ± 0.2	5.6 ± 0.5	6.7 ± 0.2	6.0 ± 0.5
18:2n-6	13.7 ± 0.5[a]	7.7 ± 0.4[b]	15.4 ± 0.3[a]	18.1 ± 0.7[c]
18:3n-6	0.21 ± 0.03	0.30 ± 0.06	0.17 ± 0.01	0.22 ± 0.04
20:3n-6	1.01 ± 0.03[a]	0.45 ± 0.03[b]	1.18 ± 0.04[c]	0.84 ± 0.04[d]
20:4n-6	8.0 ± 0.2[a]	2.6 ± 0.1[b]	10.9 ± 0.4[c]	8.5 ± 0.1[a]
22:4n-6	0.89 ± 0.07[a]	0.08 ± 0.01[b]	1.19 ± 0.04[c]	0.52 ± 0.15[a,b]
22:5n-6	1.33 ± 0.10[a]	0.43 ± 0.04[b]	3.24 ± 0.12[c]	1.11 ± 0.08[a]
18:3n-3	0.30 ± 0.01[a]	0.23 ± 0.01[b]	0.14 ± 0.01[c]	0.33 ± 0.01[a]
20:5n-3	0.11 ± 0.01[a]	1.33 ± 0.13[b]	0.09 ± 0.01[a]	0.16 ± 0.02[a]
22:5n-3	2.12 ± 0.13[a]	3.57 ± 0.24[b]	0.99 ± 0.03[c]	1.73 ± 0.12[a]
22:6n-3	20.7 ± 1.6[a]	31.4 ± 1.5[b]	15.5 ± 0.8[c]	19.3 ± 1.4[a,c]
Unsaturation Index	225.3 ± 10.1[a]	258.6 ± 10.0[b]	214.0 ± 5.5[a]	220.6 ± 6.6[a]
%SFA	33.5 ± 1.0	35.5 ± 1.2	33.6 ± 0.6	33.5 ± 0.6
%MUFA	15.3 ± 0.7[a]	13.7 ± 0.9[a,b]	14.7 ± 0.6[a,b]	12.5 ± 0.6[b]
%PUFA	48.7 ± 1.6	48.4 ± 2.0	49.4 ± 1.1	51.2 ± 0.9
%Total n-3	23.3 ± 1.8[a]	36.7 ± 1.8[b]	16.8 ± 0.8[c]	21.6 ± 1.4[a,c]
%Total n-6	25.4 ± 0.3[a]	11.7 ± 0.4[b]	32.6 ± 0.5[c]	29.6 ± 0.9[d]
HUFA	34.6 ± 2.0[a,b]	40.6 ± 1.7[a]	33.4 ± 1.2[b]	32.8 ± 1.3[b]
% n-3 HUFA[1]	66.7 ± 1.4[a]	90.4 ± 0.8[b]	50.1 ± 0.9[c]	67.2 ± 1.1[a]
% n-6 HUFA[1]	33.3 ± 1.4[a]	9.6 ± 0.8[b]	49.7 ± 0.9[c]	32.8 ± 1.1[a]

[1] Values are percent of total HUFA (highly unsaturated fatty acids, three or more double bonds) amount.

Comparisons are between the treatments within a row. Superscripts that do not share a common letter indicate a significant difference ($P < 0.05$) between treatments.