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# Docosahexaenoic acid varies in rat skeletal muscle membranes according to fibre type and provision of dietary fish oil

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## Abstract

**Background** Dietary fish oil provides polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) and is associated with modified oxygen consumption, contractile fatigue and physiological responses to ischaemia or hypoxia in striated muscle. This study systematically investigated the membrane incorporation of fatty acids, with a focus on DHA, into skeletal muscle in relation to functional/metabolic differences and their responsiveness to fish oil doses. **Methods** Male Sprague-Dawley rats were randomised to isoenergetic diets (10% fat by weight). Human Western-style diets were simulated with 5.5% tallow, 2.5% n-6 PUFA sunflower seed oil and 2% olive oil (Control). High-DHA tuna oil exchanged for olive oil provided a Low (0.32%) or moderate (Mod) (1.25%) fish oil diet. Membrane phospholipid fatty acid composition was analysed in samples of five skeletal muscles selected for maximum variation in muscle fibre-type. **Results** Concentrations of DHA varied according to muscle fibre type, very strongly associated with fast oxidative glycolytic fibre population ($r^2 = 0.93$; $P < 0.01$). No relationship was evident between DHA and fast glycolytic or slow oxidative fibre populations. Fish oil diets increased membrane incorporation of DHA in all muscles, mainly at the expense of n-6 PUFA linoleic and arachidonic acid. **Conclusion** The exquisite responsiveness of all skeletal muscles to as little fish oil as the equivalent of 1-2 fish meals per week in a human diet and the selective relationship to fatigable muscle fibre-types supports an integral role for DHA in muscle physiology, and particularly in fatigue resistance of fast-twitch muscles.



## Keywords

docosahexaenoic, fish, according, dietary, membranes, provision, muscle, skeletal, rat, type, varies, fibre, acid, oil

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**Highlights:**

- Mapping muscle membrane fatty acid and fibre type correlations within the rat.
- DHA incorporation strongly associated with fast oxidative glycolytic muscle fibres.
- DHA is incorporated at the expense of n-6 PUFA linoleic and arachidonic acid.
- Preferential incorporation highlights integral role of DHA in contractile cells.

# Docosahexaenoic acid varies in rat skeletal muscle membranes according to fibre type and provision of dietary fish oil

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## ABSTRACT

*Background:* Dietary fish oil provides polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) and is associated with modified oxygen consumption, contractile fatigue and physiological responses to ischaemia or hypoxia in striated muscle. This study systematically investigated the membrane incorporation of fatty acids, with a focus on DHA, into skeletal muscle in relation to functional/metabolic differences and their responsiveness to fish oil doses.

*Methods:* Male Sprague-Dawley rats were randomised to isoenergetic diets (10% fat by weight). Human Western-style diets were simulated with 5.5% tallow, 2.5% n-6 PUFA sunflower seed oil and 2% olive oil (Control). High-DHA tuna oil exchanged for olive oil provided a Low (0.32%) or moderate (Mod) (1.25%) fish oil diet. Membrane phospholipid fatty acid composition was analysed in samples of five skeletal muscles selected for maximum variation in muscle fibre-type.

*Results:* Concentrations of DHA varied according to muscle fibre type, very strongly associated with fast oxidative glycolytic fibre population ($r^2$ = 0.93; $P$ < 0.01). No relationship was evident between DHA and fast glycolytic or slow oxidative fibre populations. Fish oil diets increased membrane incorporation of DHA in all muscles, mainly at the expense of n-6 PUFA linoleic and arachidonic acid.

*Conclusion:* The exquisite responsiveness of all skeletal muscles to as little fish oil as the equivalent of 1-2 fish meals per week in a human diet and the selective relationship to fatigable muscle fibre-types supports an integral role for DHA in muscle physiology, and particularly in fatigue resistance of fast-twitch muscles.



*Key words:*
Polyunsaturated fatty acids; omega-3; contractile fatigue; fast oxidative glycolytic; fast-twitch muscle

*Summary:*

Skeletal muscle fibres vary according to structural, metabolic and neurological characteristics and ultimately influences contractile function. This study sort to determine if the composition of phospholipid polyunsaturated fatty acids (PUFA), incorporated in their membranes, might also differ according to fibre type and when omega-3 PUFA are made available in the diet. We systematically demonstrated that the omega-3 PUFA, docosahexaenoic acid (DHA), incorporated into skeletal muscle membranes well above its provision in the diet and without competitive influence of high omega-6 PUFA concentrations, typical to the Western-style human diet. Notably, incorporation preferentially occurred according to metabolic characteristics of each muscle, supporting the notion that DHA plays an integral role in fast oxidative glycolytic muscle fibres.

## 1. INTRODUCTION

Skeletal muscles vary according to their different contractile roles, physiological and metabolic characteristics which enables a vast array of tasks [1]. For that reason, based upon contractile function, skeletal muscle fibres are often termed slow oxidative (SO), fast oxidative glycolytic (FOG) or fast glycolytic (FG) fibres, reflecting the range of contractile fatigue resistance [2]. Comparative physiology studies have demonstrated that certain animals have a propensity for rapid and powerful force production or prolonged endurance capacity [3]. Of primary interest to the current investigation, is that the composition of phospholipid polyunsaturated fatty acids in skeletal muscle cell membranes seem to be associated with contractile function [4, 5]. However, until now, there has been no comprehensive within species study that has sought to determine if membrane phospholipid fatty acid composition of skeletal muscle is related to the fibre type.

In animals, membrane unsaturation has been reported to vary according to skeletal muscle contractile function. For example, in the European hare, with a fast-maximal running speed, the skeletal muscle membranes contain a high degree of unsaturation [6]. In fact, across species the maximal running speed of an animal has been positively associated with omega-6 polyunsaturated fatty acid (n-6 PUFA)[1] membrane content [4]. However, in contrast, there is a high concentration of long chain omega-3 polyunsaturated fatty acid (LCn-3PUFA) docosahexaenoic acid (22:6n-3; DHA) found in the rattlesnake tail and hummingbird wing skeletal muscles, each with the specialised high-speed characteristics and the additional capacity for repeated force production over protracted durations [5]. Most notably, this same

[1] Abbreviations: **n-6 PUFA,** omega-6 polyunsaturated fatty acids; **LCn-3PUFA,** long chain omega-3 polyunsaturated fatty acids; **16:0,** palmitic acid; **18:0,** stearic acid; **18:1n-9,** oleic acid; **18:2n-6,** linoleic acid (LA); **18:3n-3,** α-linolenic acid (ALA); **20:4n-6,** arachidonic acid (AA); **20:5n-3,** eicosapentaenoic acid (EPA); **22:5n-3,** docosapentaenoic acid (DPA); **22:6n-3,** docosahexaenoic acid (DHA); **SFA,** saturated fatty acid; **MUFA,** monounsaturated fatty acid; **SO,** slow oxidative; **FG,** fast oxidative; **FOG,** fast oxidative glycolytic.

propensity for higher concentration of membrane DHA is found within skeletal muscles of rats that have enhanced contraction speed requirements, such as in mixed fast oxidative glycolytic and fast glycolytic fibre types gastrocnemius muscle [7-9]. Yet, surprisingly there has been limited within species fibre type comparisons of membrane phospholipid composition, where rats are the ideal model given that the skeletal muscle fibre type profile of the rat hindlimb is well-established [10, 11]. Therefore, the first objective of the current study was to sample a wide range of skeletal muscles from the rat hindlimb and determine if certain fatty acids are more likely to concentrate according to fibre type.

Notwithstanding the potential of the contractile stimuli to modify skeletal muscle membrane phospholipids, it is the modification of fatty acids in the diet that produces the most effective changes [12]. Skeletal muscle membrane phospholipids maintain relatively constant saturated fatty acid and mono-unsaturated fatty acid concentrations, independent to dietary manipulation. Additionally, when small changes are made to dietary n-6 PUFA content, membrane phospholipid concentrations remain unperturbed [13] and large increases in dietary linoleic acid (18:2n-6; LA) only produce small changes in membrane n-6 PUFA [14]. In contrast, when pre-formed eicosapentaenoic acid (20:5n-3; EPA) and DHA are added to the diet via fish oil, membrane phospholipids are very responsive, particularly DHA concentrations [13]. This suggests that in most dietary studies, tissue requirements for n-6 PUFA are fully met whereas those for LCn-3PUFA are not. Therefore, the second objective of the current study was to determine if the provision of DHA-rich dietary achievable fish oil doses, could augment the incorporation of DHA into a range of skeletal muscles and according to fibre type distribution. Collectively, this study tested the hypothesis that membrane phospholipid, in particular DHA

incorporation into skeletal muscle, is related to fibre type of the muscle and can be further manipulated by the provision of a DHA-rich fish oil.

## 2. MATERIALS AND METHODS

### *2.1. Ethical standards:*

Experiments were approved by the Animal Care and Ethics Committee from the University of Wollongong and were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

### *2.2. Animals and study design.*

Thirty-two *Sprague Dawley* rats (Male; 8-10 weeks of age; baseline body mass: 428 ± 10 g) were used for the study. Animals were housed two per cage in the institution's animal facility with a room temperature maintained at 23$^{o}$C-25$^{o}$C and a 12-hour light-dark cycle. Animals were allowed *ad libitum* access to regular lab chow for a minimum of 1 week prior to being placed on the pre-fabricated diets in a randomised manner and allowed *ad libitum* access to the diet and water for 4-5 weeks. The animals in this study were a subgroup of animals that underwent *in vivo* cardiac physiological experiments under anaesthetic (pentobarbital: 60 mg/kg I.P). As such, tissue collection occurred immediately following physiological experiments and animal euthanasia (rapid exsanguination and removal of the heart).

### 2.3. *Diet Composition.*

Three diets were carefully prepared. A control diet (Control) and two experimental diets that provided a low dose of fish oil (Low) and a moderate dose of fish oil (Mod). The pre-fabricated diets were developed for use in similar feeding studies [13]. All diets contained 10% fat, 50% carbohydrate and 20% protein by weight, plus minerals and vitamins based on the American Institute of Nutrition AIN-93M diet [15], differing only in the fatty acid composition making up the total fat. Four sources of fats and oils were used to provide the total 10% of fat in the diet. The specific mix of oils (5.5% beef tallow, 2.5% n-6 PUFA rich sunflower seed oil, 2.5% olive oil) aimed to

replicate the high amounts of saturated fatty acids and n-6 PUFA found in a typical Western-style diet. Extra light (refined) olive oil was provided, therefore free of most antioxidant polyphenols commonly present in less refined oils. The Low and Mod fish oil diets were prepared by substituting 0.31% and 1.25% fish oil, respectively, for olive oil using DHA-rich tuna oil (26% DHA and 7% EPA) (Nu-Mega Ingredients Pty Ltd, Sydney, Australia). The final fat composition of each diet is outlined in Table 1. The prefabricated diets were prepared using purified ingredients and stored at -20ºC.

2.4. *Tissue collection.*

A pre-determined selection of skeletal muscles with varied populations of slow oxidative, fast oxidative glycolytic and fast glycolytic fibre types, according to Armstrong *et al.,* (1984), were carefully dissected from the left thigh and lower limb. The soleus was chosen as a muscle with a predominance of slow oxidative fibres, the rectus femoris $_{white}$ and extensor digitorum longus were chosen as examples of muscles with predominantly fast glycolytic fibres and the rectus femoris $_{red}$ and gastrocnemius $_{red}$ were chosen as examples of muscles with predominantly fast oxidative glycolytic fibres. Each dissection was weighed to the nearest milligram, freeze-clamped using liquid nitrogen and stored (-80$^{o}$C) until fatty acid analysis. Individual hindlimb skeletal muscles were anatomically referenced to the upper hindlimb or lower hindlimb and categorised into fibre type groups (slow oxidative, fast glycolytic, fast oxidative glycolytic) to allow calculations of total mass.

*2.5. Membrane phospholipid fatty acid analysis.*

The methods used for membrane fatty acid analysis have been described previously [16-18]. In brief, 100–150 mg of tissue was weighed and homogenised in a chloroform–methanol mixture (2:1, v/v) (analytical grade, Thermo-Fisher Scientific, North Ryde, Australia; Sigma-Aldrich, Castle Hill, Australia). Steps were taken for

acidified total lipid extraction using 1 M - $H_2SO_4$ (Sigma-Aldrich), solid phase phospholipid separation via silica Sep-Pak® columns (Waters, Sutton, MA, USA), transesterification of phospholipid fatty acids using 14% boron trifluoride in methanol ([Sigma-Aldrich] stored at 0–4°C) heated at 85°C for 1 h, purification via Sep-Pak® Florisil columns (Waters) using diethyl ether (5%) (Fluka; Sigma-Aldrich) in petroleum spirit (7 mL) (Fluka; Sigma-Aldrich) and finally GC (Shimadzu GC-17A, 30 m × 0·25 mm internal diameter capillary column, total run time 23 min; Shimadzu, Rydalmere, Australia). All solvents were freshly prepared at the time of analysis and contained 0·01% (w/v) butylated hydroxytoluene (Sigma-Aldrich). Individual fatty acids were identified by comparison with the known standards in the laboratory. The relative amount of each fatty acid was determined by integrating the area under the peak and dividing by the result for all fatty acids detected.

### *2.6. Statistical analysis.*

For investigation of correlations between membrane fatty acid content and the population of muscle fibre type, we used published data on mean muscle fibre composition, in which contents of the same set of muscles from the same strain of rats as investigated in our current study was provided [10]. To investigate relationships independent of diet, correlations were conducted within the Control group only. Pearson's *r* was used to assess the strength and direction of relationships between membrane phospholipid fatty acid concentration and population of muscle fibre type. Membrane phospholipid fatty acid composition data were analysed using a one-way ANOVA. When an effect of dietary treatment was detected, *post-hoc* comparisons were completed with the Bonferroni procedure. All statistical analyses were performed using Statistix for Windows (Statistix for Windows; Analytical Software). For all comparisons, statistical significance was

accepted for Type I error of $P < 0.05$. Data was reported as mean ± standard error of the mean (SEM).

## 3. RESULTS

### *3.1. Membrane phospholipid fatty acid correlation with fibre type population.*

When correlations were calculated according to the population of fast oxidative glycolytic fibre type typical for each muscle (Fig. 1), there was an extremely strong positive correlation with the concentration of DHA . In contrast, arachidonic acid (AA) demonstrated a moderate negative correlation with the population of fast oxidative glycolytic muscle fibres . No other relationships were evident for any other fatty acids measured in correlation to fast oxidative glycolytic fibre type population. When correlations were calculated according to the population of fast glycolytic fibre type (Fig. 2), LA demonstrated a very strong negative correlation . In contrast, α-linolenic acid (18:3n-3; ALA) demonstrated a strong positive and docosapentaenoic acid demonstrated a moderate positive correlation with fast glycolytic muscle fibres. No other relationships were evident for any other fatty acids measured in correlation to fast glycolytic fibre type population. When correlations were calculated according to the population of slow oxidative fibre type (Fig. 3), LA demonstrated a strong positive correlation . No other relationships were evident for any other fatty acids measured in correlation to slow oxidative fibre type population. Correlations for EPA were unable to be calculated for any fibre type as it was not detected in enough samples.

### *3.2. Effect of diet on muscle mass and membrane phospholipid fatty acids.*

There was no effect of dietary treatment on the mean mass of any of the individual muscles measured (Table 1) including when muscle mass was analysed according to total upper hindlimb (Control, 2.97 ±0.15 g; Low, 3.08 ±0.19 g; Mod, 3.20 ±0.17 g; $P > 0.05$) or lower hindlimb weight (Control, 3.39 ±0.12 g; Low: 3.31 ±0.07 g; Mod: 3.33 ±0.27 g; $P > 0.05$). Across all tissues following supplementation, the fish oil diets produced several marked differences in membrane phospholipid

fatty acid concentrations (Table 1). In all muscles, total LCn-3PUFA concentration were significantly higher in a dose-related manner in the Low and Mod fish oil groups compared to Control. Elevated LCn-3PUFA concentrations were predominantly attributable to significantly greater DHA incorporation across all muscles compared to Control. In the Control diet, the lowest concentrations of DHA were observed in the soleus and the greatest concentrations were observed in the gastrocnemius $_{red}$. With fish oil diets, DHA increased in all muscles in a dose-related manner, the linear relationship with the population of fast oxidative glycolytic fibre type remained evident but it became progressively weaker (Fig. 4). Increasing DHA concentrations were associated with a dose-related lower total n-6 PUFA concentration with significantly lower concentrations of AA across all tissues of fish oil fed groups. The concentration of LA was lower in all muscles of the Mod fish oil group compared to the Control group. These changes in muscle phospholipid composition were reflected in significantly lower n-6:n-3 ratios across all muscles in the Low and Mod fish oil groups.

## 4. DISCUSSION

This study confirmed DHA as the principal LCn-3PUFA and AA as the principal n-6 PUFA, which together contribute a major proportion of the total PUFA present in all skeletal muscles. However, for the first time we demonstrate that while DHA is highly incorporated into membrane phospholipid of all skeletal muscle, it is preferentially integrated into muscles of the rat hindlimb that have a greater population of fast oxidative glycolytic fibres. Furthermore, supplementing DHA-rich fish oil caused skeletal muscle membrane phospholipids to avidly incorporate even higher concentrations of DHA (in exchange for n-6 PUFA: AA & LA). The other marine derived LCn-3PUFA in muscle membrane, docosapentaenoic acid (DPA), was consistently present in higher concentrations than EPA in Control animals and showed a moderate positive correlation with fast glycolytic fibres but no consistent pattern of change with fish oil feeding. In combination, the preferential incorporation of DHA into skeletal muscle, and further into muscles with high populations of fast oxidative glycolytic fibres, provides novel structural evidence of an integral role for DHA in contractile cells [5] including the cardiomyocyte [19].

### *4.1. Relationship between membrane fatty acids and skeletal muscle fibre type.*

In the current study, DHA was increasingly incorporated into muscle membrane phospholipid according to relative populations of fast oxidative glycolytic fibre type, characteristic of fast-twitch fatigable muscle. Despite reports of a relationship between omega-6 PUFA content of skeletal muscle and maximum running speed across species [4], this study did not reveal any similar relationship between omega-6 PUFA and muscle fibre type within the one species. Fast reacting muscles such as the rectus femoris $_{red}$, with high populations of fast oxidative glycolytic fibres, have an extensive network of sarcoplasmic reticulum for the rapid distribution and reuptake of

$Ca^{2+}$ by the sarcoplasmic reticulum $Ca^{2+}$-ATPase pump. Abnormalities of $Ca^{2+}$ regulation in skeletal muscle tissue causes contractile dysfunction [20]. Noteworthy, in the cardiomyocyte, both the sarcoplasmic reticulum $Ca^{2+}$-ATPase pump-mediated $Ca^{2+}$ reuptake and ryanodine receptor-mediated $Ca^{2+}$ release are modulated by membrane DHA [19]. Paradoxically, sarcoplasmic reticulum function is impaired in association with decreased omega-6 to omega-3 ratio when very low dietary intakes of omega-6 drives a small decrease in membrane linoleic acid and a simultaneous small increase in α-linolenic acid derived membrane DHA [21]. This contrasts to the effects of direct dietary inclusion of DHA on sarcoplasmic reticulum function [22]. As such, it is plausible that preferential incorporation of DHA into muscles with high populations of rapid-twitch fast oxidative glycolytic fibres is linked to the ability of DHA to modulate $Ca^{2+}$ regulation, leading to dietary-modulated fatigue resistance [7]. Indeed, intracellular $Ca^{2+}$ handling is implicated in DHA modulated cardiac function, oxygen consumption and arrhythmia vulnerability [23]. Thus, DHA incorporation would facilitate more rapid contractile function and fatigue resistance across all muscle types, including myocardium.

Comparative physiology studies support this notion with DHA concentrated in membrane phospholipids of skeletal muscles that frequently experience low oxygen environments [24, 25], and in rapid-twitch muscles [5]. Further evidence of the adaptive incorporation of DHA to facilitate activity of highly excitable cells comes from the contrasting effects of between-species membrane DHA differences and dietary induced membrane DHA differences within a species. Allometric analysis describes metabolic rate, oxygen consumption, and heart rate comparatively increased as species body size decreases. Membrane DHA increases as species body size decreases [26]. However, a causative link between DHA and metabolism

is contradicted by effects of dietary induced increases in DHA slowing heart rate and metabolism in many species [27]. Furthermore, dietary fish oil has been demonstrated to enhance oxygen efficiency in heart [23], mixed fibre skeletal muscle [8] and during whole body exercise [28, 29]. In heart, which is resistant to beat to beat fatigue and devoid of these highly fatigable fast oxidative glycolytic muscle fibres, the enhanced oxygen efficiency is expressed as functional improvement only when the heart is under stress, such as during or following myocardial ischaemia [23, 30] or in heart failure [31].

In skeletal muscle, the improved oxygen efficiency is expressed under less extreme conditions than the heart, during normal physiological function, as contractile fatigue resistance [7] and better recovery between bouts of contraction [8], especially in the fast responses attributable to fast oxidative glycolytic muscle fibres. Furthermore, when physiological stress is increased, as in skeletal muscle hypoxia, provision of DHA in the diet has been demonstrated to attenuate the reduction in contractile force [32]. Together, this suggests that phospholipid membrane incorporation of DHA from fish oil is integral to maintaining contractile cell performance (cardiac and skeletal), particularly during conditions where oxygen availability is challenged.

The differential incorporation of DHA and other fatty acids reflects the different membrane composition of muscle fibre types, with varying contributions of subcellular lipid membranes (SR content, mitochondrial content) according to their physiological / metabolic function. In turn, these organelles and the external sarcolemmal membrane have characteristic phospholipid make up. Fatty acid incorporation from dietary lipids vary markedly between phospholipid types, with DHA incorporated much more into phosphatidylethanolamine than into

phosphatidylcholine, and cardiolipin [33]. However, while diets alter fatty acid composition of individual and total phospholipids, diets do not affect the phospholipid distribution within a tissue [34].

*4.2. The effect of fish oil on skeletal muscle mass.*

There was no evidence of catabolic or anabolic properties associated with fish oil supplementation from the skeletal muscle mass measured in healthy animals from this study. Provision of LCn-3PUFA, DHA and EPA, are reported to modify skeletal muscle mass in healthy elderly subjects with [35] or without resistance training [36]. However, the evidence provided of an anabolic effect of fish oil supplementation in these studies is when the skeletal muscles are in a state of decay e.g., sarcopenia associated with ageing rather than a healthy state, which is likely why they contrast to the current findings of this study. This is in line with animal studies which demonstrate attenuation of muscle mass loss during cachetic cancer model [37], burn injury model [38] and an immobilisation model [39]. Several recent human studies have also come to similar conclusions that fish oil supplementation results in an anti-catabolic rather than an anabolic effect in skeletal muscle [40, 41], particularly in older individuals [42]. Notably, these observations of attenuated muscle mass loss, have been achieved with supra-therapeutic doses of fish oil which are not realistically achievable in the habitual human diet. Given the increased phospholipid membrane LCn-3PUFA incorporation demonstrated in the current study using much lower doses, further research is warranted to determine if a dietary achievable dose of DHA-rich fish oil can alleviate skeletal muscle atrophy.

*4.3. The effect of fish oil on skeletal muscle membrane phospholipid fatty acids.*

It is well established that feeding a DHA-rich fish oil to rats modifies skeletal muscle membranes to incorporate high DHA concentrations [7, 43]. The doses of

fish oil that generated such marked responses in this rat study, at 0.31% and 1.25% of the diet by weight, were equivalent in dietary energy terms to quantities that a 70 kg person on 8700 kJ daily energy intake could reasonably expect to obtain from the diet, by either habitual consumption of oily fish or supplement capsules (Low fish oil: 1-2 x ~125 g salmon portions per week; Mod fish oil: 6 fish oil capsules per day [13]). Muscle tissue with the highest proportion of fast oxidative fibre type were least responsive to dietary fish oil as fish oil intake increased. This most likely indicates that the high fast oxidative glycolytic, high DHA red muscle components of gastrocnemius and rectus femoris were approaching saturation point, in line with the maximum incorporation of DHA achieved using much higher fish oil doses [8].

Previous animal studies investigating the effects of fish oils have either used doses that are far beyond what could realistically be achieved in a human diet [8, 43] or used realistic doses but either much lower or much higher concentrations of n-6 PUFA in the background diet [7, 13]. Whereas, a typical Western-style diet contains high amounts of saturated fatty acids and n-6 PUFA and very little pre-formed LCn-3PUFA [44], as could also be implied from global erythrocyte EPA+DHA levels [45]. This study therefore confirms previous reports that the dose of LCn-3PUFA and not the dietary n-6:n-3 PUFA ratio is critical for LCn-3PUFA membrane phospholipid incorporation [13, 46].

### *4.4. Conclusions.*

This study confirmed that DHA is avidly incorporated into all skeletal muscle, well above its provision in the diet, without competition from dietary n-6 PUFA or adverse influence of dietary saturated fat. Additionally, DHA was increasingly incorporated into muscle membrane phospholipid according to relative populations of fast oxidative glycolytic fibre type, characteristic of repeatedly powerful contractile

function. Incorporation occurred from doses of fish oil achievable in a human diet against background dietary saturated fatty acids and n-6 PUFA typical of a Western diet. The preferential incorporation of DHA into muscle, and further into muscles with high populations of fast oxidative glycolytic fibres provides evidence, for the first time, that links to the consistent physiological observations of an integral role for DHA in the attenuation of muscle fatigue [7, 32]. Further investigation is warranted to determine if fish oil modifies contractile function uniformly across striated muscles (including heart), regardless of fibre type proportion, or is more effective in the presence of a high proportion of a particular fibre type. The outcomes of such research could inform dietary translational studies, using DHA-rich fish oil, in populations experiencing augmented skeletal muscular fatigue and exercise intolerance.

## 5. ACKNOWLEDGEMENTS

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*Author contributions:*

All named authors contributed substantially to (a) the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting the article or revising it critically for important intellectual content; (c) final approval of the version to be published.

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*Tables:*

**Relative proportion (%) of phospholipid membrane fatty acids**

| | Fatty acid profile (% fat in diet) | | | Relative proportion (%) of phospholipid membrane fatty acids | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Soleus* | | | | *Rectus femoris (white)* | | | |
| | Diet | | | Fibre pop (%): SO: 87; FG: 0; FOG: 13 | | | | Fibre pop (%): SO: 1; FG: 74; FOG: 25 | | | |
| | ***Control*** | ***Low*** | ***Mod*** | ***Control*** | ***Low*** | ***Mod*** | ***P*** | ***Control*** | ***Low*** | ***Mod*** | ***P*** |
| **16:0** | 17.2 | 17.5 | 18.4 | 13 ± 0.2 | 14 ± 0.6 | 14 ± 0.5 | | 22 ± 0.4 | 22 ± 0.2 | 22 ± 0.1 | |
| **18:0** | 12.1 | 12.1 | 12.4 | 20 ± 0.1 | 19 ± 0.3 | 18 ± 0.2* | 0.03 | 14 ± 0.3 | 14 ± 0.5 | 14 ± 0.1 | |
| **18:1n-9** | 41 | 39.1 | 33.4 | 9.7 ± 0.2 | 9.9 ± 0.9 | 7.9 ± 0.2 | 0.07 | 8.7 ± 0.1 | 7.1 ± .09* | 7.6 ± 0.1* | <0.01 |
| **18:2n-6 (LA)** | 19.3 | 19.1 | 18.4 | 22 ± 0.5 | 19 ± 0.3 | 18 ± 0.7* | 0.01 | 15 ± 0.3 | 13 ± 0.6* | 12 ± .07* | < 0.01 |
| **18:3n-3 (ALA)** | 0.5 | 0.55 | 0.56 | 0.7 ± .02 | 0.6 ± .01* | 0.6 ± .02 | 0.01 | 0.9 ± .05 | 0.8 ± .03 | 0.8 ± .03 | |
| **20:4n-6 (AA)** | 0.02 | 0.08 | 0.24 | 17 ± 0.2 | 13 ± 0.4* | 11 ± 0.6*† | < 0.01 | 17 ± 0.6 | 15 ± 0.1* | 13 ± 0.3*† | < 0.01 |
| **20:5n-3 (EPA)** | - | 0.22 | 0.87 | ND | ND | 0.5 ± .09*† | < 0.01 | ND | 0.1 ± .01* | 0.4 ± .09*† | < 0.01 |
| **22:5n-3 (DPA)** | - | 0.03 | 0.14 | 1.7 ± 0.1 | 1.6 ± 0.1 | 1.7 ± 0.1 | | 2.5 ± 0.3 | 2.3 ± 0.1 | 1.7 ± 0.3* | 0.04 |
| **22:6n-3 (DHA)** | - | 0.89 | 3.61 | 8.3 ± 0.1 | 14 ± 0.2* | 19 ± 0.7*† | < 0.01 | 8.5 ± 0.2 | 14 ± 0.4* | 20 ± 0.3*† | < 0.01 |
| **SFA** | 32.2 | 32.7 | 34.3 | 37 ± 0.2 | 38 ± 0.4 | 38 ± 0.3 | | 42 ± 0.9 | 44 ± 0.6 | 41 ± 0.1 | |
| **MUFA** | 43.7 | 42.1 | 37 | 13 ± 0.3 | 13 ± 1.2 | 11 ± 0.3 | | 13 ± 0.2 | 11 ± 0.2* | 11 ± .09* | 0.02 |
| **PUFA** | 19.9 | 20.9 | 23.9 | 49 ± 0.4 | 48 ± 1.4 | 51 ± 0.6 | | 45 ± 0.9 | 45 ± 0.7 | 48 ± 0.2* | 0.03 |
| **n-6 PUFA** | 19.4 | 19.2 | 18.7 | 38 ± 0.4 | 32 ± 1.3* | 29 ± 1.1* | < 0.05 | 33 ± 0.6 | 28 ± 0.5* | 24 ± 0.2*† | < 0.01 |
| **n-3 PUFA** | 0.55 | 1.69 | 5.17 | 11 ± 0.1 | 16 ± 0.4* | 22 ± 0.7*† | < 0.01 | 12 ± 0.4 | 17 ± 0.4* | 23 ± 0.2*† | < 0.01 |
| **EPA + DHA** | - | 1.11 | 4.48 | 8.3 ± 0.1 | 14 ± 0.2* | 19 ± 0.7*† | < 0.01 | 8.5 ± 0.2 | 14 ± 0.4* | 21 ± 0.2*† | < 0.01 |
| **n-6:n-3** | 35.4 | 11.3 | 3.62 | 3.5 ± .07 | 2.0 ± .07* | 1.3 ± .09*† | < 0.01 | 2.7 ± .06 | 1.6 ± .04* | 1.0 ± .02*† | < 0.01 |
| **Tissue Mass (g)** | | | | 0.19 ± .03 | 0.18 ± .03 | 0.22 ± .04 | > 0.05 | 0.30 ± .02 | 0.29 ± .01 | 0.29 ± .03 | > 0.05 |

Table 1. *Fat sources and concentration of major fatty acids of experimental diets, and the effect of diet on phospholipid fatty acid composition and skeletal muscle mass.*

| | | *Extensor digitorum longus* | | | | *Rectus femoris* *(red)* | | | | *Gastrocnemius* *(red)* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Fibre pop (%): SO: 2; FG: 56; FOG: 42 | | | | Fibre pop (%): SO: 7; FG: 40; FOG: 53 | | | | Fibre pop (%) SO: 30; FG: 8; FOG: 62 | | | |
| | | ***Control*** | ***Low*** | ***Mod*** | ***P*** | **Control** | ***Low*** | ***Mod*** | ***P*** | ***Control*** | ***Low*** | ***Mod*** | ***P*** |
| | **16:0** | 20 ± 0.3 | 21 ± 0.5 | 20 ± 0.1 | | 15 ± 0.3 | 17 ± 0.5 | 16 ± 0.5 | | 16 ± 0.4 | 16 ± 0.2 | 17 ± 0.5 | |
| | **18:0** | 18 ± 0.5 | 17 ± 0.2 | 17 ± 0.2 | | 18 ± 0.4 | 18 ± 0.9 | 17 ± 0.2 | | 17 ± 0.4 | 16 ± 0.3 | 17 ± 0.2 | |
| | **18:1n-9** | 8.2 ± 0.2 | 7.0 ± .08 | 7.9 ± 0.2 | | 7.4 ± 0.2 | 8.0 ± 0.2 | 7.1 (0.3 | | 8.3 ± 0.2 | 7.6 ± .04 | 7.6 ± 0.2 | |
| | **18:2n-6 (LA)** | 18 ± 0.5 | 17 ± 0.7 | 15 ± 0.3 | | 20 ± 0.7 | 18 ± 0.1 | 18 ± 0.8* | 0.03 | 20 ± 0.8 | 18 ± 0.7 | 16 ± 0.4* | 0.01 |
| | **18:3n-3 (ALA)** | 0.7 ± .05 | 0.7 ± .04 | 0.7 ± .02 | | 0.6 ± .04 | 0.7 ± .03 | 0.5 ± .02* | 0.02 | 0.7 ± .03 | 0.6 ± .07* | 0.5± .07*† | < 0.01 |
| | **20:4n-6 (AA)** | 16 ± 0.2 | 13 ± 0.4* | 9.9 ± 0.2*† | < 0.01 | 16 ± 0.2 | 12 ± 0.3* | 9.3 ±0.5*† | < 0.01 | 16 ± 0.4 | 12 ± 0.1* | 9.4 ±0.3*† | < 0.01 |
| | **20:5n-3 (EPA)** | [b].03 ± .02 | 0.1 ± .04* | 0.5 ± .04*† | < 0.01 | ND | [a]0.3 ± .02 | 0.5 ± .06* | < 0.05 | 0.1 ± .01 | 0.3 ± .01* | 0.5± .06*† | < 0.01 |
| | **22:5n-3 (DPA)** | 1.6 ± .09 | 1.7 ± .05 | 1.6 ± .04 | | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.4 ± 0.1 | | 1.9 ± 0.1 | 1.6 ± 0.1 | 1.3 ± 0.1* | 0.02 |
| | **22:6n-3 (DHA)** | 11 ± 0.2 | 16 ± 1.1* | 21 ± 0.4*† | < 0.01 | 11 ± 0.3 | 16 ± 0.2* | 22 ± 0.6*† | < 0.01 | 13 ± 0.2 | 19 ± 0.2* | 23 ± 0.5*† | < 0.01 |
| | **SFA** | 41 ± 0.3 | 41 ± 0.4 | 40 ± 0.2 | | 38 ± 0.3 | 39 ± 0.6 | 38 ± 0.5 | | 38 ± 0.2 | 37 ± 0.3 | 39 ± 0.7 | |
| | **MUFA** | 12 ± 0.2 | 10 ± 0.2 | 11 ± 0.2 | | 11 ± 0.1 | 12 ± 0.4 | 10 ± 0.4 | | 11 ± 0.2 | 10 ± 0.1* | 10 ± 0.3* | < 0.01 |
| **Σ** | **PUFA** | 47 ± 0.5 | 49 ± 0.5 | 49 ± 0.4* | 0.05 | 50 ± 0.2 | 49 ± 0.5 | 51 ± 0.8† | 0.02 | 51 ± 0.3 | 52 ± 0.4 | 51 ± 0.7 | |
| | **n-6 PUFA** | 33 ± 0.3 | 30 ± 0.8* | 25 ± 0.2*† | < 0.01 | 37 ± 0.5 | 30 ± 0.3* | 27 ± 0.8*† | < 0.01 | 36 ± 0.5 | 30 ± 0.6* | 25 ± 0.7*† | < 0.01 |
| | **n-3 PUFA** | 13 ± 0.1 | 18 ± 1.0* | 23 ± 0.5*† | < 0.01 | 14 ± 0.4 | 18 ± 0.3* | 24 ± 0.5*† | < 0.01 | 15 ± 0.2 | 22 ± 0.1* | 25 ± 0.5*† | < 0.01 |
| | **EPA + DHA** | 11 ± 0.2 | 16 ± 1.1* | 21 ± 0.4*† | < 0.01 | 11 ± 0.3 | 16 ± 0.3* | 22 ± 0.6*† | < 0.01 | 13 ± 0.2 | 20 ± 0.2* | 24 ± 0.5*† | < 0.01 |
| | **n-6:n-3** | 2.5 ± .02 | 1.6 ± 0.1* | 1.1 ± .03*† | < 0.01 | 2.6 ± 0.1 | 1.6 ± .03* | 1.1 ± .05*† | < 0.01 | 2.3 ± .06 | 1.3 ± .04* | 1.0 ±.04*† | < 0.01 |
| **Tissue Mass (g)** | | 0.19 ± .01 | 0.17 ± .02 | 0.20 ± .01 | > 0.05 | 0.27 ± .03 | 0.25 ± .02 | 0.31 ± .03 | > 0.05 | 0.23 ± .03 | 0.24 ± .01 | 0.29 ± .04 | > 0.05 |

Table 1. cont.

**Abbreviations: 16:0,** palmitic acid; **18:0,** stearic acid; **18:1n-9,** oleic acid; **18:2n-6,** linoleic acid (LA); **18:3n-3,** α-linolenic acid (ALA); **20:4n-6,** arachidonic acid (AA); **20:5n-3,** eicosapentaenoic acid (EPA); **22:5n-3,** docosapentaenoic acid (DPA); **22:6n-3,** docosahexaenoic acid (DHA); **SFA,** saturated fatty acid; **MUFA,** monounsaturated fatty acid; **PUFA,** polyunsaturated fatty acid; **pop,** population; **SO,** slow oxidative; **FG,** fast oxidative; **FOG,** fast oxidative glycolytic. Fatty acid concentrations as determined by gas chromatography. Muscle fibre composition previously published in Armstrong & Phelps (1984). Values are mean ± SEM (n = 4 – 5 per group). ND = Not Detected. [a] = Only detected in 3 samples. [b] = Only detected in 2 samples. Some individual fatty acids were not present "-" in dietary fats and oils. **P* < 0.05 vs Control diet within tissue. †*P* < 0.05 vs Low diet within tissue (One-way ANOVA with Bonferroni post-hoc test).

*Figure captions:*

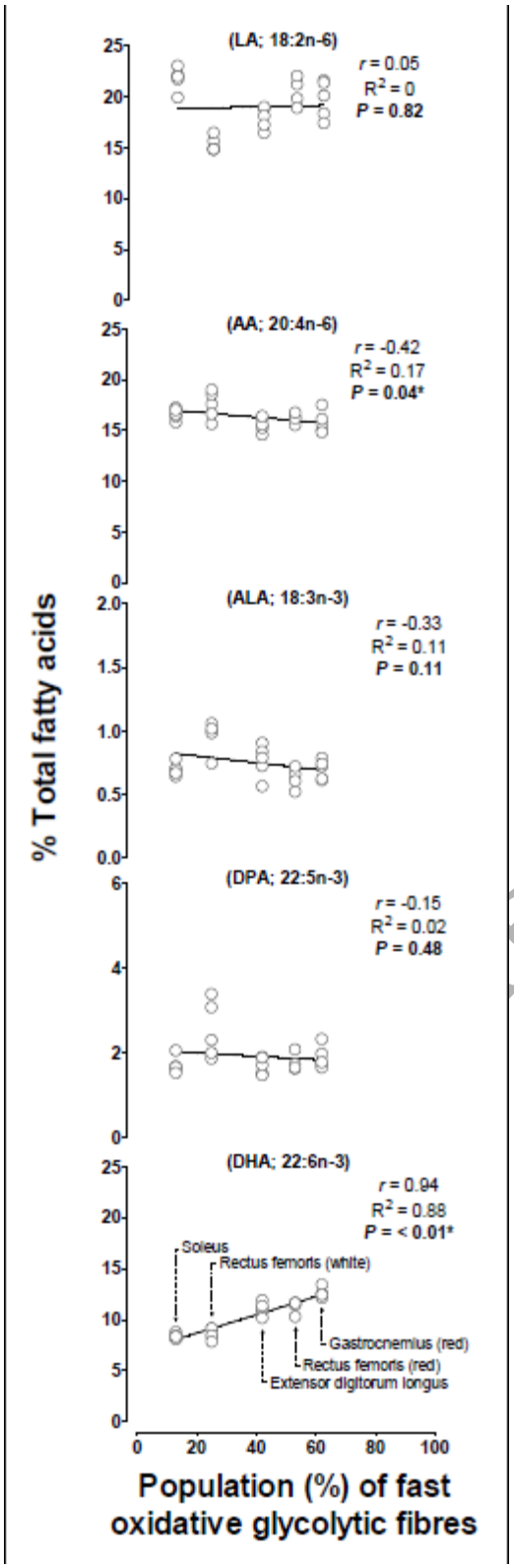




**Fig. 1.** Fatty acid correlations (*n*=4-6 per muscle) calculated according to the population of fast oxidative glycolytic fibre type typical for each muscle collected from

within the same animal. Correlations (Pearson's *r*) were completed using previously published data on mean muscle fibre composition, in which contents of the same set of muscles from the same strain of rats as investigated in our current study were used (Armstrong and Phelps, 1984). To investigate relationships independent of diet, correlation analysis was conducted within the Control group only. **Abbreviations: LA,** Linoleic acid; **AA,** Arachidonic acid; **ALA,** α-linolenic acid; **DPA,** Docosapentaenoic acid; **DHA,** Docosahexaenoic acid.

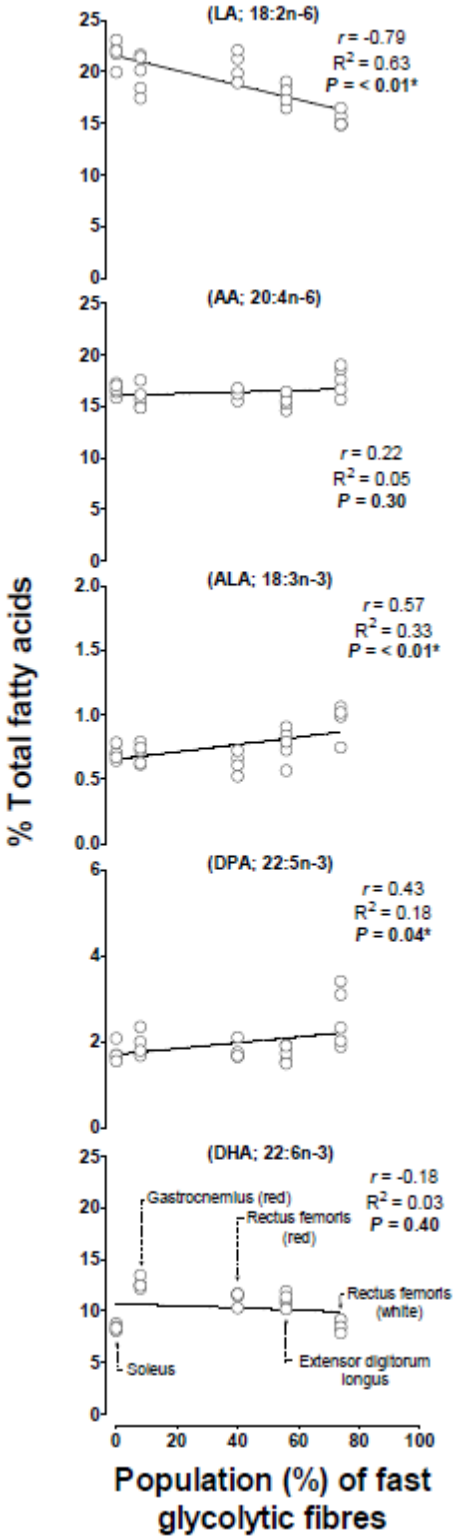




**Fig. 2.** Fatty acid correlations (*n*=4-6 per muscle) calculated according to the population of fast glycolytic fibre type typical for each muscle collected from within the same animal. Correlations (Pearson's *r*) were completed using previously

published data on mean muscle fibre composition, in which contents of the same set of muscles from the same strain of rats as investigated in our current study were used (Armstrong and Phelps, 1984). To investigate relationships independent of diet, correlation analysis was conducted within the Control group only. **Abbreviations: LA,** Linoleic acid; **AA,** Arachidonic acid; **ALA,** α-linolenic acid; **DPA,** Docosapentaenoic acid; **DHA,** Docosahexaenoic acid.

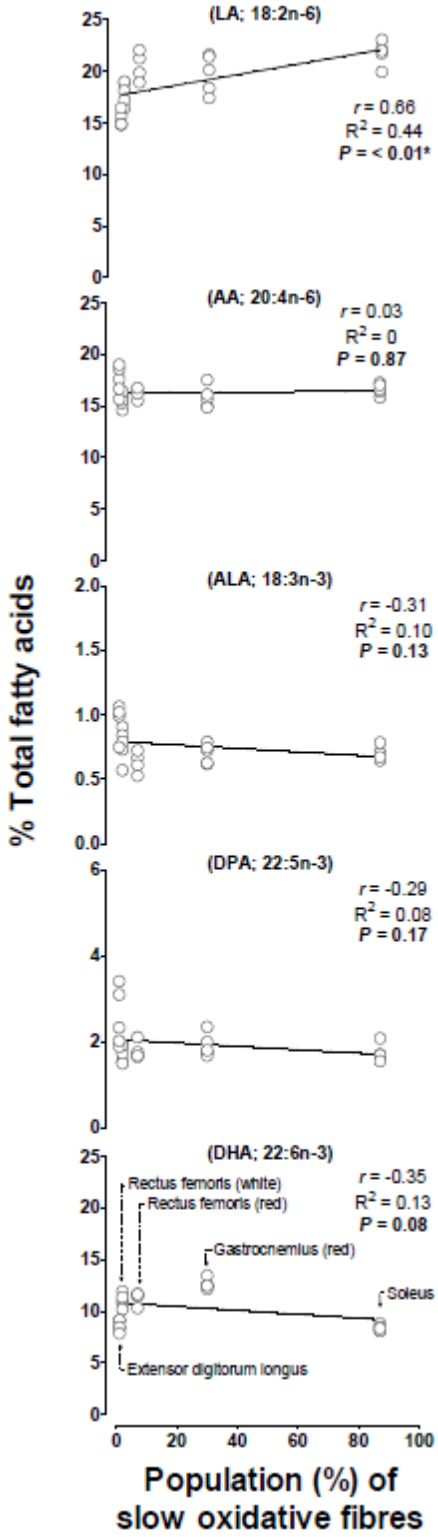




**Fig. 3**. Fatty acid correlations (*n*=4-6 per muscle) calculated according to the population of slow oxidative fibre type typical for each muscle collected from within the same animal. Correlations (Pearson's *r*) were completed using previously published data on mean muscle fibre composition, in which contents of the same set

of muscles from the same strain of rats as investigated in our current study were used (Armstrong and Phelps, 1984). To investigate relationships independent of diet, correlation analysis was conducted within the Control group only. **Abbreviations: LA,** Linoleic acid; **AA,** Arachidonic acid; **ALA,** α-linolenic acid; **DPA,** Docosapentaenoic acid; **DHA,** Docosahexaenoic acid.

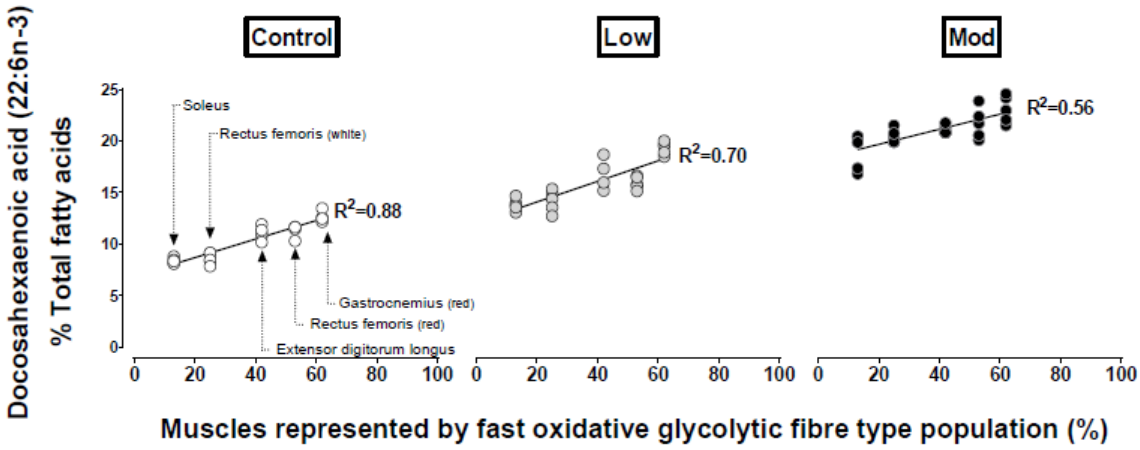


**Fig. 4**: Effect of fish oil diets on the relationship between DHA concentration and the population of fast oxidative glycolytic fibres in different muscles. Muscle fibre composition previously published in Armstrong & Phelps (1984). Soleus, 13%; Rectus femoris $_{(white)}$, 25%; Extensor digitorum longus, 42%; Rectus femoris $_{(red)}$, 53%; Gastrocnemius $_{(red)}$, 62%.