1993

Dietary fats and exercise

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DIETARY FATS AND EXERCISE

A thesis submitted in fulfilment of the requirements

for the award of the degree of

Doctor of Philosophy

from

THE UNIVERSITY OF WOLLONGONG

by

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1993

Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the requirements of the degree of Doctor of Philosophy. The work described in this thesis was carried out by me and has not been submitted to any other university or institution.

Kerry Ayre

June, 1993

ABSTRACT

Despite the great importance of lipids in many biological functions, little is known about the effects of different types of fats on muscle composition and physical performance. In this study, the levels of essential polyunsaturated fatty acids in the diets of male rats were manipulated in order to determine whether the composition of skeletal muscle membranes would be altered and if so, the function of isolated skeletal muscles and whole animal performance.

Three isocaloric diets (providing 10% fat), were developed. An essential fatty acid deficient (EFAD) diet contained 1% n - 6 and 0.2% n - 3 fatty acids; a polyunsaturated fatty acid diet (PUFA) enriched diet contained 35% n - 6 and 21% n - 3 fatty acids and a Control diet contained 51% n - 6 and 9% n - 3 fatty acids.

Weanling litter-mates were assigned at random to dietary groups and maintained on their test diets for nine weeks, followed by zero, two or six weeks recovery on normal rat pellets. Groups of rats were tested at each of these stages and results were analysed by 2-way ANOVA, with diet and litter as factors.

Membrane fatty acid analysis revealed that the total proportions of saturated and unsaturated fatty acids remained constant between the three dietary groups. However, the proportions of unsaturated fats in the membranes reflected the proportions of unsaturated fats in the diets. In the EFAD group, essential fatty acid deficiency was verified using the triene-tetraene ratio (> 0.5 in both soleus and EDL). Effects on membrane composition in soleus muscles ("slow-twitch") and

extensor digitorum longus (EDL) muscles ("fast-twitch") were similar, but the two muscles varied in their rates of recovery. After nine weeks, there was a significant increase, in both muscles, in n - 9 fatty acids in the EFAD group, compared with the Control and PUFA groups. There was also a significant increase in n - 3 fatty acids in the PUFA group, compared with the Control and EFAD groups. After two weeks recovery, the difference in the level of n - 9 fatty acids in the EFAD group was no longer was apparent in soleus, but persisted in EDL. After six weeks recovery, it was no longer apparent in EDL. However, the increased proportion of n - 3 fatty acids in the PUFA group was present in both soleus and EDL muscles, even after six weeks recovery. Thus, the fatty acid composition of muscle membranes recovered from the effects of the EFAD diet (although at different rates), but did not return to control levels following the PUFA diet.

Effects of changes in membrane fatty acid composition on skeletal muscle function were determined in isolated soleus and EDL muscles from groups of five to seven rats. Muscles were isolated and stimulated electrically after nine weeks on the test diets, followed by zero, two or six weeks recovery. Parameters measured included tensions generated, response times (latent time, contraction time and half-relaxation time), fatigue time and endurance. Overall, the EFAD diet resulted in significantly lower tensions and reduced response times compared with the Control and PUFA diets. Results for muscles from the Control and PUFA groups were very similar, as was the composition of the two diets. Although muscles from the EFAD rats had significantly reduced tensions and response times after nine

weeks on the test diets, they recovered from these effects, although again, soleus recovered within two weeks and EDL within six weeks.

Effects of changes in dietary fatty acids on whole animal physical performance were investigated in groups of nine to 11 rats. A series of performance-related tests was conducted including treadmill endurance, grip strength, basal oxygen consumption ($\dot{V}O_{2\ basal}$) and peak oxygen consumption ($\dot{V}O_{2\ peak}$). Each rat was tested twice; after nine weeks on the test diets and after six weeks recovery. Changes in dietary fatty acids had no effect on grip strength, $\dot{V}O_{2\ basal}$ or $\dot{V}O_{2\ peak}$, but had a highly significant effect on endurance. In the rats on the PUFA diet, endurance was 44% less than for the the Control rats after nine weeks, and there was no sign of recovery six weeks later.

Effects of changes in dietary fatty acids on Na^+,K^+-ATPase (in the sarcolemma) and Ca^{2+}-ATPase (in the sarcoplasmic reticulum membrane) were examined in groups of nine rats since these two membrane-bound enzyme systems are very important for muscle contraction and relaxation. Concentration of Na^+,K^+-ATPase was estimated using a vanadate-facilitated [^{3}H]ouabain binding assay and activity of Na^+,K^+-ATPase was measured by the production of phosphate by muscle homogenate during the hydrolysis of ATP in the presence and absence of ouabain. Activity of Ca^{2+}-ATPase was measured by the hydrolysis of ATP in the presence of low and high concentrations of calcium. Changes in dietary fatty acids had no significant effect on activity of either enzyme. Although the concentration of Na^+,K^+-ATPase was also unaffected, there was a significant effect of diet on affinity of Na^+,K^+-ATPase for ouabain;

affinity appeared to be reduced in muscles from rats on the PUFA diet.

Summary

Changes in the composition of dietary fatty acids had the following effects in rats:

1. The composition of skeletal muscle membrane phospholipid fatty acid composition was altered. The pattern of unsaturated fatty acids reflected the unsaturated fatty acid composition of the diets. Muscle membrane composition of EFAD rats recovered to Control levels, but EDL muscles took longer to recover than soleus muscles. Muscle membrane composition of PUFA rats did not recover to Control levels, since they retained a high level of n -3 fatty acids after the recovery period.

2. The function of isolated muscles from rats on the EFAD diet was altered. Although all changes in isolated muscle function recovered to control levels, EDL muscles took longer to recover than soleus muscles.

3. Endurance was markedly reduced in whole rats which had been on the PUFA diet and there was no sign of recovery after six weeks.

4. Activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase were unaffected, as was the concentration of Na^+,K^+-ATPase, but affinity for ouabain in muscles from rats on the PUFA group appeared to be decreased.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Associate Professor Tony Hulbert (Biological Sciences), for suggesting and developing this project, for help and financial support throughout the study and with preparation of the thesis; and Dr Karen Chad (Biomedical Science), for help and support during the early stages of the project.

These people are thanked for valuable discussion and critical comment on procedures: John Booth, Patrice Couture, Josette Dangain, Peter Jørgensen, Keld Kjeldsen, Otto Meyer and Len Storlien. The following people read parts of my thesis and I am immensely appreciative of their efforts and comments: David Allen, David Ayre, Denis Calvert, Patrice Couture, Jim Cotter, Paul Else, Graeme Maw, Mike McKenna, Peter Milburn, Stephen Phinney, Len Storlien and Nigel Taylor.

Special thanks to Paul Else (Deakin University) who was always willing to talk about ouabain binding and who kindly showed me how to measure sodium pump activity. A very big thankyou goes to Dr Martin Brand (Cambridge University) who discussed all aspects of my study and kept on asking questions that made me think about what I was doing.

Professors Rob Whelan (Biological Sciences) and Dennis Calvert (Public Health and Nutrition) were always willing to be a sounding board and were very supportive at all stages of my work.

Several people from the University of California at Davis provided much appreciated opportunities for learning new techniques and refining others. These include: Dick Carlsen (Human Physiology), Kristin Eiffert (Nutrition), Rick Grosberg (Zoology), Dory Harold (Veterinary Science), Joel Pickar (Clinical Endocrinology), Mark Wineinger (Human Physiology), Professor Judy Stern (Nutrition) and all the other people on the campus at Davis who provided a lot of help and insight into my project including Sarah Gray, Roger MacDonald, Isaac Pessah and Stephen Phinney. I would especially like to thank the Nutrition laboratory group, particularly Alison Dimond and Kristin Eiffert for friendship, valuable discussions and help during my time in Davis.

Many people provided valuable technical assistance. In particular, I would like to thank John Forrest, Kerry Withers and Dominic Fanning - oxygen consumption in rats; Wojciech Mantaj - fatty acid analysis; Roger Coles - isolated muscle assay; Rachel Standish - photography; Mick Gregory and Rob Whelan - word processing as well as all the technical staff of Biological Sciences. Trevor Gould and Alison Hunt inclined my treadmill beautifully. Ken Russell was never too busy and was never stumped for a method of statistical analysis. Geoff Hurt (technical workshop) and John Reay (electronics workshop) no longer need hide whenever they see me coming.

I am deeply indebted to Meadowlea Foods, and R.P. Scherer Pty. Ltd. for the provision of sesame oil and Max EPA oil, respectively. They were always willing to provide more whenever I ran out. In

addition, Roche Products and Cenovis very generously provided vitamins for the rat diets.

Thanks to my friends - Jennie, Julie, Sue and especially Alison - for helping to keep me sane and not take myself too seriously and for all the child care. Thanks to the other gel-runners, especially Jane, Mandy, Sandra, Rachel, Tanya, and Martin - there was always someone in the Genetics lab. willing to have a chat.

Finally, I would like to thank my two boys and Molly (my ever-faithful companion during these last six months). Justin made his presence known on the day this Ph.D. was started, and probably grew up thinking it was normal for mothers to go to work at 4 a.m. At 4 years old he was practising talks to give at pre-school, and at 5 he was drawing graphs. He always kept me in touch with reality, even if I didn't do the same for him. I am indebted to Diana, Rhys and Peter Williams, who not only cared for Justin for five years, but were like another family for him.

Last, but most importantly, I would like to thank David, who kept me going. He gave vast advice and criticism as well as many many times being a sole parent overnight and in the early hours of the mornings. This would never have been finished without you.
Now let's get on with life!

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1.1 Introduction

It is now well established that body composition reflects dietary intake. The composition of at least some cell membranes reflects the proportional intake of distinct types of dietary fats. There are three commonly recognised classes of fatty acids (saturated, monounsaturated and polyunsaturated) and each class is represented by a range of fatty acids with carbon chains of different lengths. The unsaturated fatty acids are distinguished by the location of one or more carbon to carbon double bonds. Interconversion of these molecules is often possible but some types of polyunsaturated fatty acids (hereafter referred to as PUFAs) are essential as they cannot be synthesized by animals and therefore must be consumed in the diet. These are the "essential fatty acids" (hereafter referred to as EFAs - see section 1.3.4). Many "fad" diets, for the purposes of weight loss, centre on a restriction of dietary fats, since these are the food type with the highest caloric content. However, diets low in fats are likely to be low in EFAs.

The ratio of saturated to unsaturated fatty acids in membranes appears to be homeostatically controlled and remains remarkably constant despite dietary changes (Abeywardena *et al.*, 1984; McMurchie *et al.*, 1983; 1984; 1986; Chapter 2 of this study). Nevertheless the proportions of the different classes of unsaturated fatty acids can change in response to dietary change. Not surprisingly, animals on diets low in EFAs show reduced levels of these groups in their

membranes and the symptoms commonly attributed to deficiencies of the EFAs are actually related to membrane dysfunction (Mead, 1984).

Membranes are vital, but structurally complex components of all cells. Processes such as the trans-membrane movement of nutrients, metabolic wastes, circulating ions and hormones may be changed if the membrane itself changes. For example, if membrane structure becomes more rigid or more flexible, both active and passive transport of ions may be affected, as hypothesized by Else & Hulbert (1987).

Changes in membrane lipids have also been shown to affect the functioning of intrinsic proteins which are partially embedded in the lipid bilayer (Kimelberg, 1976; Sandermann, 1978; Quinn, 1981; Stubbs & Smith, 1984; Carruthers & Melchior, 1986; Kleinfeld, 1987; Yeagle, 1989; Murphy, 1990; Skou, 1992; Christon *et al.*, 1992). For example, some enzymes are unable to function fully unless particular phospholipids are present (Sandermann, 1978) and variations in chain length and degree of unsaturation of phospholipids appear to affect membrane-bound enzyme activity (Kimelberg & Papahadjapoulos, 1974). Membrane lipids are thus able to affect cellular function (including skeletal muscle function) in many ways.

Within muscles, the cell membrane (sarcolemma) and the intracellular membranes of organelles play an essential role in both contraction and relaxation. If the structure of muscle membranes is altered, this may alter the capacity of the sarcolemma to transmit and propagate action potentials and affect the process of excitation-contraction coupling

(release of calcium (Ca^{2+}) from sarcoplasmic reticulum) essential for crossbridge cycling and the generation of force.

The effects of dietary lipid intake on cardiac muscle function have received vast attention in the last 20 years in relation to cardiovascular disease which is the leading cause of death in Australia (National Heart Foundation, 1991) (for review of cardiovascular disease, see Kinsella *et al.*, 1990a). Besides the large volume of research which has focused on the relationship between nutrition and coronary heart disease, the studies of Charnock *et al.* (1982; 1985b; 1987) and McLennan *et al.* (1985; 1987a;b; 1989; 1990) have also shown that dietary lipids can directly affect cardiac muscle function by affecting contractile properties of cardiac muscle.

Although it has been demonstrated in marmosets that the lipid composition of skeletal muscle membranes and cardiac muscle membranes are almost identical (Charnock *et al.*, 1989) and that this similarity is maintained following dietary lipid changes (Charnock *et al.*, 1992), few studies have examined the effects of dietary lipids on the function of skeletal muscles.

Many of the studies documenting effects of changes in dietary lipid composition have not used isocaloric diets, thus making it difficult to determine whether effects are due to differences in the types of lipids, or to the differences in fat levels of the diet (for review, see Jeejeebhoy, 1986). In fact, most of the evidence on the effects of dietary lipid imbalances on muscle function comes from studies of malnourished subjects. Patients with gastrointestinal disorders, obesity and anorexia show increased fatigue and altered patterns of

contraction and relaxation (Russell *et al.*, 1984b; Berkelhammer *et al.*, 1985) and these parameters recover to normal levels following refeeding on a normal diet (Lopes et al., 1982; Russell *et al.*, 1983a; b; Chan *et al.*, 1986 . Similar results have been found in rats (Russell *et al.*, 1984a; Lewis *et al.*, 1986; Dureuil *et al.*, 1989; Nishio & Jeejeebhoy, 1991). However, the particular dietary components responsible have not been investigated. Many of the factors affecting muscle function have been elucidated from studies of patients with malfunctioning muscles, e.g. those with particular metabolic enzymes missing (Wiles *et al.*, 1981), or from conditions such as muscular dystrophy (Dangain & Vrbová, 1990; Dangain & Neering, 1993). However, since the muscles of these patients are already malfunctioning, it may not be appropriate to extrapolate such results to normal subjects.

The objective of this study was to determine whether dietary-induced changes in the fatty acid composition of skeletal muscle membranes affects muscle function and physical performance.

Diets were formulated which were isocaloric and appeared to be equally palatable and they differed only in their type of fat. A series of specific questions were investigated.

1. (a) Does the fatty acid composition of skeletal muscle membranes respond to changes in dietary fatty acids?
   If so:
   (b) Do different types of muscles respond differently to changes in dietary fatty acids?

(c) Does the fatty acid composition of skeletal muscle membranes recover from these dietary-induced changes?

2. (a) Is the function of isolated muscles affected by changes in dietary fatty acids?
   If so:
   (b) Does the function of different types of muscle respond differently to changes in dietary fatty acids?
   (c) Does isolated muscle function recover from the effects of changes in dietary fatty acids?

3. (a) Is physical performance in whole animals, measured by aerobic power, endurance and strength, affected by changes in dietary fatty acids?
   If so:
   (b) Does whole animal performance recover from the effects of changes in dietary fatty acids?

4. (a) Is the activity of membrane-bound enzymes, vital for muscle function, affected by changes in dietary fatty acids?

Since the EFAs are known to play such important roles in cardiovascular disease and development, and may also be involved in other prevalent conditions, it was decided to investigate their effects on skeletal muscle membrane composition and function and whole animal performance, with particular emphasis on the effects of enrichment and deficiency. Three isocaloric diets were used. The three diets were: (a) Control; (b) Essential Fatty Acid Deficient

(hereafter referred to as EFAD) and (c) enriched with Polyunsaturated Fatty Acids (hereafter referred to as PUFA). Effects of these diets on physical performance were unpredictable; it was not known whether they would be beneficial, detrimental or non-existent.

1.2 Experimental Design

This study consisted of four related experiments in which the effects of manipulating the composition of essential fatty acids in the diets of rats were investigated. The experimental design is presented in Figure 1.1.

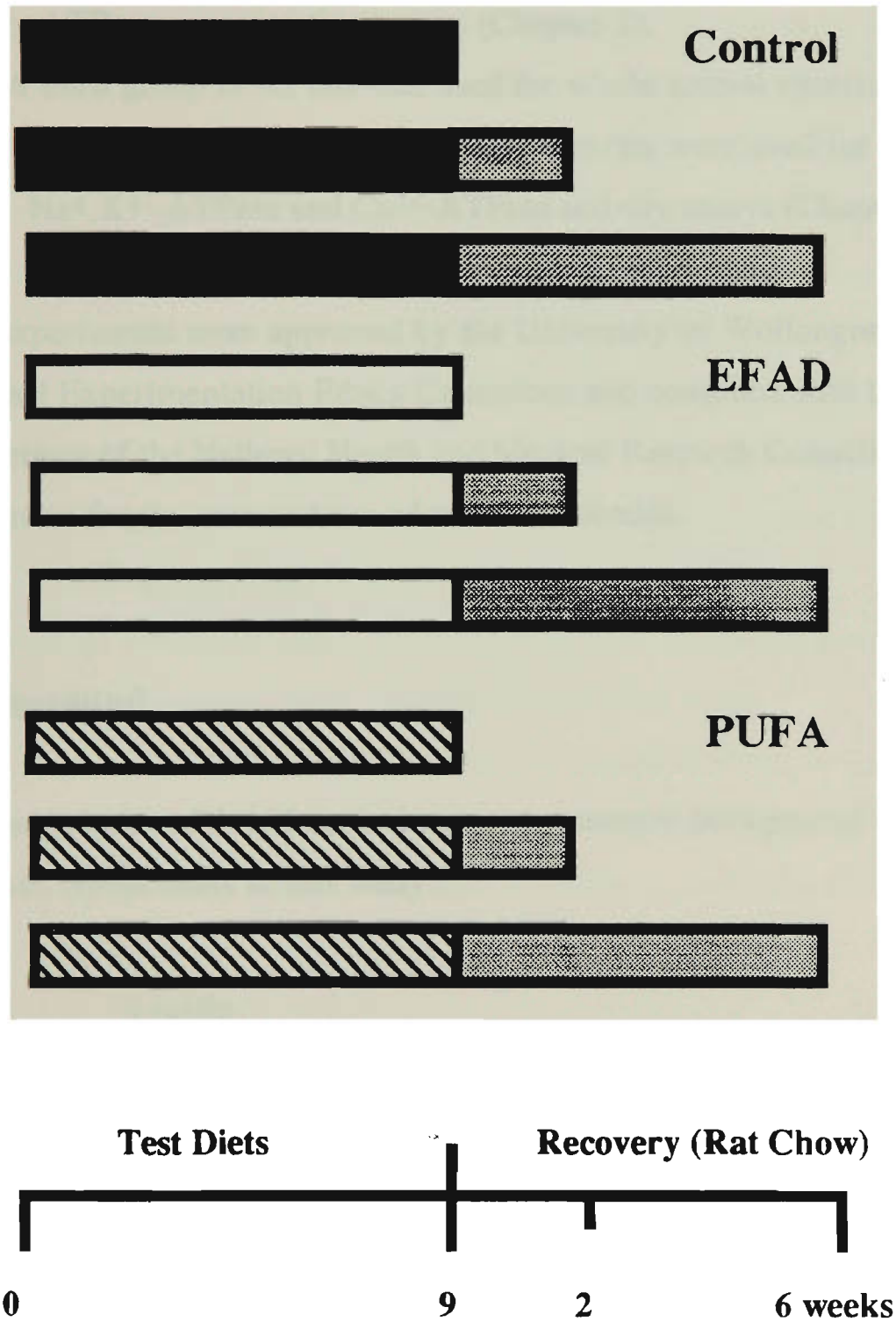
Three diets were tested. They each contained 10% fat (by weight) and were identical except for the type of fat. The three diets were: (a) a Control; (b) essential fatty acid deficient (EFAD) diet and (c) polyunsaturated fatty acid enriched (PUFA). Details of the diets are provided in Chapter 2.

All rats were on their test diet for nine weeks, followed by zero, two or six weeks recovery on normal rat pellets. In Chapters 2 and 3, there were two recovery periods of two and six weeks. In Chapter 4, there was one recovery period of six weeks In Chapter 5 there was no recovery period.

Three groups of rats were bred for this study.

(i) An initial group of 73 rats was used for dietary trials (Chapter 2) and trials of the Na^+,K^+ -ATPase concentration assay (Chapter 5).

Figure 1.1 Experimental design used to test the effects of changes in dietary fatty acids on membrane fatty acid composition (Chapter 2), isolated muscle function (Chapter 3), whole animal physical performance (Chapter 4) and enzyme concentration and activity (Chapter 5). At three weeks of age, male Wistar rat litter-mates were randomly assigned to one of three diets: Control, EFAD (essential fatty acid deficient) or PUFA (enriched with polyunsaturated n - 3 fatty acids) for nine weeks, followed by zero, two or six weeks recovery on normal rat pellets. This study was designed to be analysed by ANOVA, with diet and litter as the factors. Two-way ANOVAs were performed after nine weeks on the test diets, and after two weeks and six weeks recovery. In Chapter 4, there was only one recovery period of six weeks. In Chapter 5, there were no recovery periods.


Control
EFAD
PUFA
Test Diets
Recovery (Rat Chow)
0
9
2
6 weeks

(ii) A second group of 63 rats was used for studies on isolated muscles (Chapter 3). Muscles from these same rats were used for phospholipid fatty acid analysis (Chapter 2) and Na^+,K^+ -ATPase concentration assays (Chapter 5).

(iii) A third group of 42 rats was used for whole animal exercise experiments (Chapter 4) and their muscles were used for Na^+,K^+ -ATPase and Ca^{2+}-ATPase activity assays (Chapter 5).

All experiments were approved by the University of Wollongong Animal Experimentation Ethics Committee and complied with the guidelines of the National Health and Medical Research Council of Australia for the care and use of research animals.

Background

The remainder of this chapter documents essential background to the various components of this study.

1.3 Lipids

Most dietary fatty acids are in the form of triglycerides. After ingestion, triglycerides are emulsified and hydrolysed in the small intestine. The resulting fatty acids and monoglycerides are resynthesized into triglycerides and incorporated into chylomicrons which enter capillaries. The fatty acid composition of chylomicrons reflects dietary fatty acid intake. In capillaries, chylomicrons are hydrolysed and fatty acids pass into tissues where they are used directly or stored as triglycerides (Brindley, 1985).

1.3.1 Nomenclature

Fatty acids are named by the number and position of double bonds between carbon atoms. Saturated fatty acids have no double bonds whereas unsaturated fatty acids have at least one. In unsaturated fatty acids, the position of the double bond(s) with respect to the terminal methyl group of the molecule determines the class of fatty acid. The nomenclature used in this thesis is: "A:B, n - x", where A denotes the number of carbon atoms, B denotes the number of double bonds and x denotes the position of the first double bond counting from the terminal methyl end, and hence the class of that particular fatty acid (Sinclair *et al.*, 1992).

1.3.2 Membrane Structure

Membranes are composed of a "fluid" bilayer of lipids and proteins (Singer & Nicholson, 1972). The main lipid class is the phospholipids, but plasma membranes and the membranes of the Golgi complex and erythrocytes also contain significant amounts of sphingomyelin and cholesterol (Hadley, 1989). Phospholipids are amphipathic, i.e. they consist of a phosphate-containing "head" which is polar and hydophilic (water-soluble), and usually two non-polar fatty acyl chains (the "tail") which are hydrophobic (water-insoluble). Within the membrane, they are oriented with the head towards the outside and the fatty acid tails towards the centre (Cullis & Hope, 1985). Both the amount and type of lipid varies greatly among membranes, but the fact that they are all amphipathic molecules (Yeagle, 1989) enables them to form a bilayer. It is this arrangement which makes membranes largely impermeable to hydrophilic substances.

The fatty acid chains consist of a number of carbon atoms (predominantly ranging from 14 - 24) which are either saturated or unsaturated with hydrogen atoms. It has been shown that differences in the ratio of unsaturated to saturated fatty acyl chains and in the length of the chains can influence the fluidity of membranes, their permeability characteristics and the activity of membrane-bound enzymes (Hadley, 1989).

In addition to the lipid component of membranes, there are also membrane proteins which are either integral (embedded in the bilayer among the fatty acid tails) or peripheral (loosely bound and easily separated). Specific proteins allow the selective passage of certain types of ions and enable the cell membrane to regulate ion permeability (for review, see Murphy, 1990).

1.3.3 Effects of Dietary Lipids on Membrane Lipids

The composition of membrane lipids can be changed by altering ingestion of particular groups of fats (Grundy, 1986; 1987; Spady & Dietschy, 1988). All dietary fats contain a number of fatty acids, so when dietary lipid patterns are altered, any resulting effect is due to the balance of fatty acids in the diet (Gurr, 1992). Although it appears that there is a homeostatic mechanism which prevents a drastic change occurring in the ratio of saturated fatty acid to unsaturated fatty acid composition (McMurchie *et al.*, 1983; 1984; 1986; Abeywardena *et al.*, 1984), the proportions of PUFAs can be markedly altered in response to dietary changes.

Patients with coronary heart disease are commonly advised to lower total dietary fat, cholesterol and saturated fatty acids (MRFITRG,

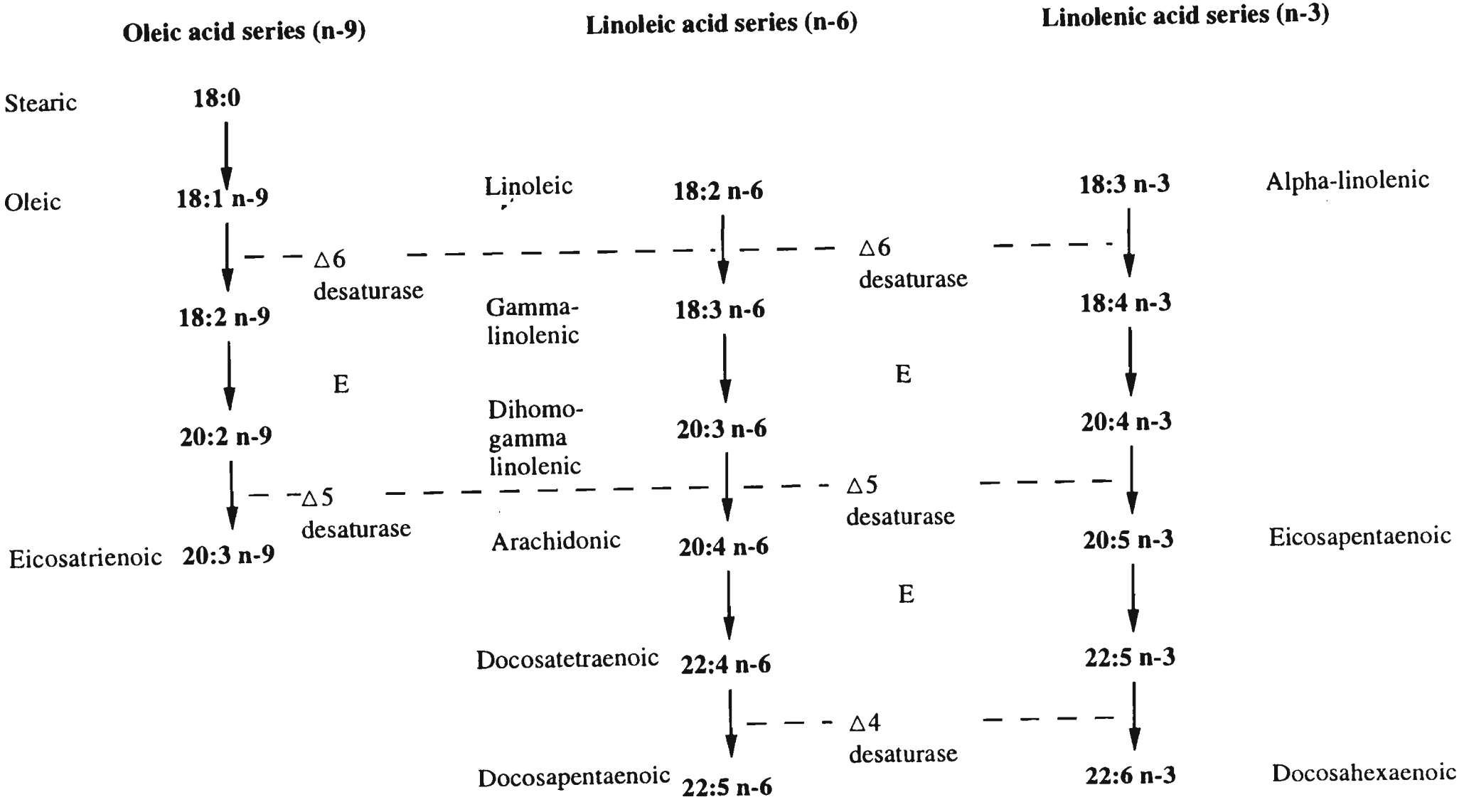
1982; Burr *et al.*, 1989). At the same time, a common dietary recommendation is increased consumption of long-chain PUFAs (the n - 6 and n - 3 PUFAs). These long-chain PUFAs have been shown to have beneficial cardiovascular effects and are also likely to alter membrane function (for reviews, see Kinsella *et al.*, 1990a; Simopoulos, 1991; Sinclair, 1991; Gurr, 1992; Norum, 1992). However, it is common for people wishing to reduce body weight, increase physical fitness or improve general health to either modify the types and amounts of fats consumed or eliminate fats altogether. In doing so, a reduction in consumption of fats may lead to a deficiency of essential fatty acids and potential severe metabolic consequences (Guarnieri & Johnson, 1970; Holman, 1970; Neuringer *et al.*, 1988).

1.3.4 Essential Fatty Acids

Most of the fatty acids in phospholipids can be derived endogenously from other shorter chain fatty acids with the aid of desaturase and elongase enzymes. There are two families of fatty acids however (known as the n - 6 and n - 3 series, Figure 1.2), which are essential for the health of mammals and must be consumed as part of the diet (for reviews, see Holman, 1986; Neuringer *et al.*, 1988; Connor *et al.*, 1992). These fatty acids are important components of most membranes and are essential because animals lack the enzymes necessary to desaturate at certain positions. They are called "essential fatty acids".

That dietary fats are essential was first recognized about 60 years ago by Burr & Burr (1929) by feeding rats fat-free diets. Their rats typically developed growth retardation, infertility, hair loss, skin

Figure 1.2 Outline of pathways of desaturation and elongation of dietary unsaturated fatty acids. The enzymes used for desaturation and elongation are denoted by Δ and E, respectively. Each of the three fatty acid classes: n - 9; n - 6 and n - 3, compete for the same enzymes and the order of competitive capability is n - 3 > n - 6 > n - 9 (Holman, 1964).


Oleic acid series (n-9)
Linoleic acid series (n-6)
Linolenic acid series (n-3)
Stearic
18:0
Oleic
18:1 n-9
Linoleic
18:2 n-6
18:3 n-3
Alpha-linolenic
Δ6 desaturase
Δ6 desaturase
18:2 n-9
Gamma-linolenic
18:3 n-6
18:4 n-3
E
E
Dihomo-gamma linolenic
20:2 n-9
20:3 n-6
20:4 n-3
Δ5 desaturase
Δ5 desaturase
Eicosatrienoic
20:3 n-9
Arachidonic
20:4 n-6
20:5 n-3
Eicosapentaenoic
E
Docosatetraenoic
22:4 n-6
22:5 n-3
Δ4 desaturase
Docosapentaenoic
22:5 n-6
22:6 n-3
Docosahexaenoic

lesions, increased water ingestion and increased skin permeability to water. Later, Burr & Burr (1930) showed that a particular fatty acid, linoleic acid (18:2, n - 6), was able to alleviate the symptoms of EFA deficiency and that the concentrations of fatty acids in various tissues could be altered by changing the dietary intake of dietary fats (Rieckehoff *et al.*, 1949; Widmer & Holman, 1950). Other early studies on the effects of EFA deficiency on membrane lipid composition also made use of fat-free diets (Burr & Burr, 1929; Aaes-Jörgensen & Hölmer, 1969; Galli *et al.*, 1970; White *et al.*, 1971; Bloj *et al.*, 1973; Lin *et al.*, 1979). However, as pointed out by Alling *et al.* (1974), a total withdrawal of fat leads to altered fatty acid metabolism and the observed effects might have been partly due to the reduced fat intake. Also, in some studies, the caloric composition of diets differed markedly because sucrose was substituted for fat (Burr & Burr, 1929, 1930; Pudelkewicz *et al.*, 1968; Galli *et al.*, 1970; White *et al.*, 1971).

Although alpha-linolenic acid (18:3, n - 3) was also shown to alleviate some of the symptoms of EFAD (Holman, 1968), and studies were done to determine requirements (Pudelkewicz *et al.*, 1968), until recently, the n - 6 fatty acid, linoleic acid, was thought to be the only EFA (Tinoco, 1981). However, epidemiological studies appeared suggesting that dietary fish containing relatively high proportions of n - 3 PUFAs could possible prevent coronary heart disease. Bang & Dyerberg (1981) showed a low incidence of death from coronary heart disease among Greenland Eskimos and Kromhout *et al.* (1985) showed an inverse dose-reponse between fish consumption and death from coronary heart disease over a twenty year period. Nevertheless, the acceptance that the n - 3 fatty acids are also essential was hindered

since there are no overt, easily recognizable, deficiency symptoms. It was not until the 1980s when case studies appeared detailing linolenic acid deficiencies (Holman *et al.*, 1982; Bjerve *et al.*, 1987a; b), as well as studies of experimental n - 3 deficiency (Neuringer *et al.*, 1984), that interest developed in PUFAs, particularly the n - 3 fatty acids, due to their reported beneficial effects (Norum & Drevon, 1986; Leaf & Weber, 1988). They were then finally recognized as also being essential (Neuringer & Connor, 1986; Neuringer *et al.*, 1988; Anderson & Connor, 1989). Since then, there has been an explosion of studies on heart disease and its association with nutrition (McMurchie, 1988).

The n - 3 fatty acids are now accepted as playing a vital role in the retina and nervous system (Neuringer *et al.*, 1988; Connor *et al.*, 1992). Also, they are considered as being very important in cardiovascular disease, often as precursors of n - 3 eicosanoids (which have similar, but less potent, functions than the n - 6 eicosanoids) (Kinsella *et al.*, 1990b; Sardesai, 1992).

Of the two EFAs, linoleic acid is the metabolic precursor for arachidonic acid (AA; 20:4, n - 6) and alpha-linolenic acid is used to form eicosapentaenoic acid (EPA; 20:5, n - 3) and docosahexaenoic acid (DHA; 22:6, n - 3) (Widmer & Holman, 1950) (Figure 1.2).

1.3.5 Role of Essential Fatty Acids

Coronary heart disease is a condition in which the main coronary arteries supplying the heart are no longer able to supply blood and sufficient oxygen to the myocardium, which then quickly dies (for review, see Gurr, 1992) and it is closely associated with

atherosclerosis. Dietary fatty acids have been associated with conditions such as atherosclerosis and thrombosis in humans. Studies have shown that dietary fats, particularly those containing saturated fats and cholesterol, may induce atherosclerosis by increasing plasma lipids which are very atherogenic (Grundy, 1987; McNamara, 1987), but that diets containing monounsaturated and polyunsaturated fatty acids have beneficial anti-thrombotic and anti-arrhythmogenic effects in the cardiovascular system (Eisenberg, 1984; Shaefer & Levy, 1985; Grundy & Vega, 1988; Kinsella *et al.*, 1990a; Simopoulos, 1991; Sinclair, 1991; Gurr, 1992; Norum, 1992). Also, Katz & Messineo (1981) suggested that the incidence of cardiac arrhythmias may be related to changes in the phospholipid composition of cardiac muscle membranes.

The n - 6 and n - 3 polyunsaturated fatty acids are thought to influence atherogenesis and thrombosis via their conversion to eicosanoids (prostaglandins, thromboxanes and leukotrienes) (Kinsella *et al.*, 1990b). In addition to modulating triglyceride and cholesterol metabolism, the n - 6 and n - 3 PUFAs, arachidonic acid and alpha-linolenic acid, are used to form longer chain fatty acids which are metabolized to eicosanoids (Kinsella *et al.*, 1990b; Simopoulos, 1991). Both 20:4, n - 6 and 20:5, n - 3 form eicosanoids which have ameliorative effects on blood pressure, viscosity, hypolipidemia, capillary permeability, inflammatory reactions and platelet functions (Drevon, 1992). The relationship between the n - 6 and n - 3 derived eicosanoids appears to be that those formed from 20:5, n - 3 are less potent than corresponding compounds from 20:4, n - 6 (Drevon, 1992), but that they compete more efficiently for the desaturase enzymes (Holman, 1964; Jeffcoat & James, 1984).

Besides the studies that have shown that saturated fats are an important risk factor in the development of atherosclerosis, a number of studies have demonstrated that dietary fatty acids exert direct effects on the contractility of cardiac muscle. Studies of cardiac muscle have shown that the fatty acid composition of cardiac muscle membrane can be altered by the type of fat ingested by rats (Abeywardena *et al.*, 1984; 1987; 1991; Charnock *et al.*, 1983; 1984; 1985c; 1986; McMurchie *et al.*, 1984) and by marmosets (Charnock *et al.*, 1985a; 1992; McMurchie *et al.*, 1984; 1986). In long-term feeding studies (> 12 months), they showed that PUFAs can protect against arrhythmias and infarctions in cardiac muscle, while saturated fats increased arrhythmogenesis (Charnock *et al.*, 1985c; McLennan *et al.*, 1985; 1987a; b; 1989; 1990). Similarly, mechanical performance of heart muscle in marmosets was improved when the animals were fed increased PUFAs (Charnock *et al.*, 1987; McLennan *et al.*, 1987b).

1.3.6 Essential Fatty Acid Deficiency

The enzymes used to produce longer chain fatty acids appear to be used competitively by the different classes of fatty acids and the order of competitive capabilities is 18:3, n - 3 > 18:2, n - 6 > 18:1, n - 9 (Holman, 1964; Jeffcoat & James, 1984). Under normal conditions, desaturase and elongase enzymes are used to convert linoleic and alpha-linolenic acids to their longer chain derivatives. However, if a mammal's diet is lacking in either linoleic acid or alpha-linolenic acid, then the same enzymes are used to produce longer chain fatty acids of the n - 9 series and there will be a higher than normal proportion of n - 9 fatty acids (Mead, 1970). This causes an increased ratio of 20:3, n - 9 to 20:4, n - 6, known as the "triene-tetraene ratio". Essential fatty acid deficiency is most often diagnosed by analysis of this ratio,

which, if greater than 0.4, is considered as being indicative of EFAD (Holman, 1960).

The effects of dietary EFA deficiency or enrichment on membrane phospholipid composition have been investigated in a variety of mammalian tissues including brain (Galli *et al.*, 1970; White *et al.*, 1971; Sun, 1972; Sun & Sun, 1974; Alling *et al.*, 1972; Alam & Alam, 1983; Gerbi, 1993), liver (Alling *et al.*, 1972; Brivio-Haugland *et al.*, 1976; Lin *et al.*, 1979; Christon *et al.*, 1992; Muriana & Ruiz-Gutierrez, 1992); kidney (Lin *et al.*, 1979), and many studies on cardiac muscle (Alam *et al.*, 1987; Charnock *et al.*, 1985a; b; c; 1986; Swanson *et al.*, 1989) but fewer studies (Neudoerfer & Lea, 1967; Alling *et al.*, 1972; Charnock *et al.*, 1989, 1992) have looked at effects on skeletal muscle.

1.3.7 Recovery of Membranes

Few studies have investigated whether dietary induced changes in membrane phospholipids are reversible. With a longitudinal cross-over feeding design, Innis & Clandinin (1981) showed that the fatty acid profile of rat heart mitochondria rapidly and reversibly responds to changes in dietary fat composition within 12 days. In long-term studies, Abeywardena *et al.* (1987) have shown that cardiac muscle membrane phospholipid content induced by prolonged ingestion of saturated fats can be reversed by feeding polyunsaturated fats. The associated adverse effects on the incidence of cardiac arrhythmias can also be reduced (McLennan *et al.*, 1990).

In young rats, Youyou *et al.* (1986) and Bourre *et al.* (1989) have shown that it takes several months for the composition of brain cells

and organelles to recover from a lack of n - 3 fatty acids (for discussion, see Bourre *et al.*, 1993). Anderson *et al.* (1992) showed that if chicks from fish-oil fed hens were first fed fish oil diets and then fed a control soybean oil diet, their brain tissue maintained a high level of 22:6, n - 3 if the cross-over occurred after three weeks of age. They concluded that at least in the short term, high levels of 22:6, n - 3 are not reversible. It is probably not surprising that the brain tenaciously retains 22:6, n - 3, since a growing body of evidence has shown that n - 3 fatty acids are essential for normal retinal and brain development (Neuringer *et al.*, 1988; Simopoulos, 1991; Connor *et al.*, 1992). These studies show that reversibility of the effects of dietary lipids can occur rapidly, at least in the heart, but the process may take much longer in the brain.

1.3.8 Effects of Dietary Lipids on Membrane Proteins

A crucial protein-mediated function of membranes, which is dependent on the composition and/or fluidity of the membrane, is the maintenance of ion gradients (Korenbrot, 1977; Stubbs & Smith, 1984; Spector & Yorek, 1985; Murphy, 1990). In skeletal muscle, ion transport is regulated by enzyme systems which are intimate components of cell membranes including the sarcolemma and sarcoplasmic reticulum. In particular, the sarcolemma contains Na^+,K^+-ATPase ("sodium pumps") and the sarcoplasmic reticulum membrane contains Ca^{2+}-ATPase ("calcium pumps"), both of which play major roles in contraction and relaxation of muscle.

1.3.8.1 Na^+-K^+-ATPase

The importance of the fatty acids surrounding membrane enzymes is evident from studies showing that specific phospholipids are required

before some membrane-bound enzymes can function fully (Sandermann, 1978). For example, the role of Na^+,K^+-ATPase in maintaining Na^+ and K^+ concentrations has been shown to be influenced by membrane phospholipids (Tanaka & Strickland, 1965; Kimelberg & Papahadjopoulos, 1972; Stahl, 1973; Wheeler *et al.*, 1975; Wheeler & Walker, 1975; Charnock & Simonson, 1977; Keeffe *et al.*, 1979) and diet-induced changes in membrane lipids have been correlated with changes in the activity of Na^+,K^+-ATPase in a variety of tissues (Sun & Sun, 1974; Lin *et al.*, 1979; Bloj *et al.*, 1973; Alam & Alam, 1983). Essential fatty acid deficiency has been shown to increase the activity of Na^+,K^+-ATPase in kidneys (Lin *et al.*, 1979; Alam & Alam, 1983), liver (Brivio-Haugland, 1976; Lin *et al.*, 1979), brain (Sun & Sun, 1974; Hannah & Campagnoni, 1987) and salivary glands (Alam & Alam, 1983).

The continued propagation of action potentials via the sarcolemma is dependent upon Na^+,K^+-ATPase (Berne & Levy, 1988). However, Abeywardena *et al.* (1984) investigated the effects of a PUFA-enriched diet on both Na^+,K^+-ATPase and Ca^{2+}-ATPase in cardiac muscle and found no effect on activity. Since cardiac and skeletal sarcolemmal lipid compositions have been shown to be very similar, it might be hypothesized that effects on contractility and enzyme function are also similar. The effects of dietary PUFAs on contractility of skeletal muscle have not previously been investigated.

1.3.8.2 Ca^{2+}-ATPase

Calcium plays an important role in the regulation of cardiac muscle contraction and relaxation and Ca^{2+}-ATPase is a key enzyme in Ca^{2+} regulation (Shamoo, 1985). Since it is known that dietary lipid

manipulation can affect the function of many membrane enzymes (Sandermann, 1978), it is possible that reported protective effects of dietary fish oil on cardiac function may be related to dietary-lipid induced modification of the physical and functional properties of cardiac membranes.

Studies have shown that dietary lipid manipulation can alter transport of Ca^{2+} and activity of Ca^{2+}-ATPase (Martonosi *et al.*, 1968; Hidalgo *et al.*, 1976; 1978; Messineo *et al.*, 1984) and that Ca^{2+}-ATPase has specific phospholipid requirements for optimal activity (Hidalgo *et al.*, 1976; 1978; Messineo *et al.*, 1984).

A number of studies have examined the effects of PUFA supplementation on Ca^{2+} transport by Ca^{2+}-ATPase in cardiac muscle. Abeywardena *et al.* (1984) examined the effects of dietary lipid manipulation on the composition and physical properties on cardiac muscle sarcolemma and sarcoplasmic reticulum membrane, as well as activity of sarcolemmal Na^{+},K^{+}-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase in rats. Although they observed changes in membrane composition (increased n - 6 / n - 3 ratio in rats on an n - 6 PUFA enriched diet) and fluidity (decreased fluidity in the sarcoplasmic reticulum membrane in rats on a saturated fat diet), the different diets had no effect on the activity of either enzyme. Karmazyn *et al.* (1987) showed that increased dietary n - 3 fatty acids, due to cod liver oil supplementation, increased Ca^{2+} uptake in rat cardiac myocytes but did not affect Ca^{2+} release.

The effects of changes in dietary fatty acids on Ca^{2+}-ATPase are unclear or potentially complex and may be influenced by both the

overall percentage of dietary fat and the proportion of different types of fatty acids. Swanson *et al.* (1989), using diets comprised of 12% fat by weight, found that a fish oil diet decreased both calcium pump activity and Ca^{2+} transport in cardiac sarcoplasmic reticulum vesicles of mice. However, they later showed, using a lower fat diet (2%), that Ca^{2+} uptake was decreased but calcium pump activity was unaffected by an n - 3 enriched diet (Croset *et al.*, 1989). They suggested that changes in the structural integrity of the membrane were responsible for the altered Ca^{2+} transport in the low fat diet. In contrast, Stubbs & Kisielewski (1990), used 12% fat diets and examined the effects of increasing n - 3 fatty acids on Ca^{2+}-ATPase in unidentified skeletal muscle from rats. They found no effect of fish oil on Ca^{2+}-ATPase, although the fatty acid composition of the membrane altered in response to the dietary changes and they suggested that Ca^{2+}-ATPase is insensitive to unsaturation changes.

Since the PUFAs have been shown to have such important and wide-ranging effects on animals, this study investigated the effects of both deficiency (the EFAD diet) and enrichment of PUFAs (the PUFA diet) on skeletal muscle membrane composition, muscle function and whole animal performance using isocaloric diets.

1.4 Muscles

1.4.1 Classification

Most skeletal muscles contain varying proportions of three fairly distinct muscle fibre types that differ widely in structural, metabolic, electrophysiological and histochemical properties. These are the red "SO" fibres (slow-twitch oxidative or type I) which are considerably

resistant to fatigue, red "FOG" fibres (fast-twitch oxidative or type IIA) which are fairly resisistant to fatigue and white "FG" fibres (fast-twitch glycolytic or type IIB) which are highly fatiguable (Close, 1972; Ariano *et al.*, 1973; Burke *et al.*, 1973; Essén *et al.*, 1975; Armstrong & Phelps, 1984; Kelso *et al.*, 1987). The different fibre types are recruited selectively according to the intensity and duration of exercise.

There are large physiological differences between muscle types. Since the effects of dietary lipid manipulation may differ between fibre types and effects on one fibre type may be hidden in a heterogeneous muscle by opposite effects on another type, in this study two fairly homogeneous muscles were chosen for determination of fatty acid composition (Chapter 2), isolated muscle function (Chapter 3) and biochemical analysis (Chapter 5). These were the hindlimb muscles, Soleus (which has 87% type I fibres and 13% type IIA fibres) and Extensor Digitorum Longus (EDL) (which has 98% "fast-twitch" fibres (including 42% type IIA and 56% type IIB) (Armstrong & Phelps, 1984). The EDL muscle closely resembles the overall muscle mass of the rat hindlimb, which is made up of 76% type IIB fibres, 19% type IIA fibres and 5% type I fibres (Armstrong & Phelps, 1984). Since both of these muscles are thin, can be dissected intact and maintained *in vitro* for several hours (Goldberg *et al.*, 1975), they are ideally suited for examination *in vitro* , as in Chapter 3.

1.4.2 Physiological Differences between Muscle Types

All three fibre types have similar glycogen contents. The triglyceride content, on the other hand, is two to three times larger in type I fibres

compared to fast-twitch fibres and type IIA fibres contain more lipid than type IIB (Essén, 1977). The rate of glycogen utilization by each fibre type is a function of the duration and intensity of the exercise. For prolonged exercise performed at 30 - 85% of maximum aerobic power, the first fibre type that shows glycogen loss is the type I. As exercise is continued, a progressive depletion of glycogen occurs in the type II fibres (Gollnick *et al.*, 1973). As the exercise intensity starts to exceed the capacity of the oxidative type II fibres, more glycolytic type II fibres are activated; usually, the type IIA fibres are depleted of glycogen before the type IIB. (Essén, 1977).

The two muscles investigated in Chapter 2, soleus and EDL, have very different fibre compositions and physiological characteristics. Such major differences in the fibre composition of these individual muscles means that the whole muscle responds very differently to stimulation. Soleus muscles take two to three times longer to contract to peak tension than EDL muscles and the length of time for tension to halve following a muscle twitch is considerably longer in soleus than in EDL (Close, 1965; 1967). Therefore, soleus muscles are not only slower to contract than EDL muscles, they are also more resistant to fatigue.

1.4.3 Measures of Muscle Function

Muscle function has been measured in a variety of ways. In mammals, physical exercise tests of whole organisms, single working limbs *in vivo*, individual muscles stimulated *in vivo* (e.g. the adductor pollicis of the thumb), both directly and indirectly via a nerve, isolated muscles with an intact blood flow (perfused) and without an intact blood flow (incubated), single muscle fibres and biopsies of

muscle segments have been used. Each of these approaches has its own limitations and advantages.

Studies of whole animal performance (even though many variables are involved) may detect overall changes in physical performance. Ultimately, it is these changes at the whole animal level which may be of greatest interest but they may have a complex basis and some effects may be completely masked. It is often difficult to pinpoint the cause of whole animal effects for a variety of reasons and repeatability may be low due to varying motivation of the subjects. Furthermore, if the diet does exert a direct physiological effect on muscle function, it may be that different muscle fibre types are affected differently by the same dietary treatment. A particular diet may have a beneficial effect on one fibre type and a detrimental effect, or none at all, on another fibre type. This may result in no overall effect on performance in the whole organism. There may also be indirect effects on the organism. For example, if an animal on a particular diet shows decreased endurance, this could be due to an indirect effect on energy stores. One of the main disadvantages of using whole animals in studies of physical performance is that they may appear to be fatigued before they really are, or refuse to give their "best" performance.

To avoid the problems of whole animals, isolated muscles or single muscle fibres can be used. I have elected to use whole isolated muscles since this may provide a more realistic test of the effects of muscle function. Studies of isolated muscles enable precise manipulation of stimulation protocols, temperature and the surrounding medium, and the effect on different muscle types can be

determined. Isolated muscles can be either perfused or incubated. Perfused muscle may be preferable in some situations to incubated muscle, since nutrients, hormones and oxygen are still available to the muscle and wastes can be removed via its capillaries, whereas incubated muscle relies almost entirely on diffusion through the muscle.

In this study, I was interested in effects of dietary changes on overall muscle function and isolated, whole, incubated muscles were used. The rationale behind this decision was based on a number of factors. I was interested in effects on overall muscle function so the use of isolated muscles meant that I could be sure there would be no confounding effects of blood flow or nerve impulses. More precise information about the responses of the different fibre types could be gained by stimulating single fibres, but since almost all muscles are heterogeneous, it would be difficult to extrapolate to whole muscle function. If I had stimulated muscles indirectly via an intact nerve, it would not be possible to tell whether any observations of fatigue were the result of muscle fatigue, or fatigue at the neuromuscular junction. The use of isolated muscles also meant that environmental factors could be controlled precisely. Since all muscle were treated identically, any significant differences observed between the dietary groups could only be due to the changes in dietary lipids.

Since the effects of dietary fatty acids on muscle function is an unexplored area, I decided to adopt a broad-scale approach and examine a wide variety of muscle contraction parameters. For this reason, I devised a specific protocol which covered a suite of responses. It is not always possible to determine whether an effect is

beneficial or detrimental. Some responses can easily be interpreted as improved muscle function, e.g. decreased contraction times, increased endurance and heightened peak tensions generated during muscle twitches, tetanus and fatigue, but others such as twitch relaxation times and post-tetanic potentiation are harder to interpret.

Isometric contractions were investigated by direct stimulation of isolated rat muscles. A battery of tests was established to cover both single and multiple isometric contractions (muscle twitch and tetanus, respectively), as well as fatigue during continual and intermittent stimulation. In isometric contractions there is minimal shortening of the muscle. The length remains nearly the same, but the tension on the muscle increases greatly. Unlike isotonic contractions, where one muscle shortens and pulls on another structure such as a bone to produce movement and tension remains constant, isometric contractions do not result in movement (Tortora & Anagnostakos, 1987).

1.4.4 Fatigue

Fatigue is an important determinant of performance in both individual muscles and whole organisms. It has been defined as "an inability to maintain the required or expected force" (Edwards, 1981; 1986). This definition is useful for describing whole animal performance but it suggests that fatigue is a sudden phenomenon, occurring suddenly, after a delay. To account for the reduction in force which occurs soon after the onset of contractile activity, Bigland-Ritchie & Woods (1984) defined fatigue as "any reduction in the force-generating capacity of the entire neuromuscular system, regardless of the force required".

As can be seen from the definition, fatigue has traditionally been thought of as a failure of normal physiological function (Edwards, 1981). Perhaps it should be thought of as a protective mechanism for survival, since under stressful conditions, the firing rate of motor neurons is limited so that performance may be optimized in the limb muscles at the same time as preventing contractile failure in the respiratory muscles (Bigland-Ritchie *et al.*, 1986). When the rate of energy consumption by working muscle exceeds its rate of production or importation, the stores of available ATP may be decreased. Since ATP is necessary for muscle relaxation following contraction, some process must ensure that ATP stores do not become so depleted as to allow the onset of rigor mortis (non-relaxation of muscle) and associated respiratory failure.

A chain of events within the central nervous system and skeletal muscle system regulates muscle contraction and relaxation and includes neural motor drive (motivation), motor unit recruitment by peripheral nerves, neuro-muscular transmission, propagation of action potentials along the sarcolemma and T-tubules and effective excitation-contraction coupling. Availability of oxygen and energy supplies and accumulation of metabolites may also affect both metabolic and electrical processes. Changes may occur at any of these steps and be manifested as fatigue. For excellent reviews on fatigue see Jones, 1981; Jones & Bigland-Ritchie, 1986; MacLaren *et al.*, 1989 and Sahlin, 1992)

The mechanisms of fatigue are still not entirely clear and depend to a large extent on the type of muscle contraction involved. The response of muscles to stimulation can vary depending on (i) the type of

stimulation (voluntary or electrical; intermittent or sustained); (ii) the type of contraction (static / isometric or dynamic / isotonic); (iii) characteristics of the stimulus (frequency, intensity, duration); (iv) the type of muscle being stimulated (slow-twitch; fast-twitch oxidative; fast-twitch glycolytic) as well as the presence of metabolites and availability of an energy supply and oxygen.

Factors associated with the central nervous system such as motivation and the ability to ignore pain can exert substantial influence on physical performance. However, early experiments by Merton (1954) showed no difference in muscle fatigue, following electrical stimulation or maximal voluntary contraction, suggesting that fatigue has its roots in factors within the muscle itself (for review, see Bigland-Ritchie & Woods, 1984).

1.4.4.1 Metabolic Factors in Fatigue

Many studies have shown that muscle fatigue is associated with a low level of phosphocreatine, accumulation of lactic acid and a concomitant increase in H^+ concentration (Hermansen & Osnes, 1972; Dawson *et al.*, 1978; Donaldson *et al.*, 1978; Edman & Mattiazzi, 1981; Sahlin, 1983; Sahlin *et al.*, 1983) and that this is associated with decreased endurance (Hultman *et al.*, 1985; Jones *et al.*, 1977; Sutton *et al.*, 1981). However, studies of recovery time courses after fatiguing contractions have shown that maximal force can return more rapidly (half-time ($t_{1/2}$) of about 15 s) than the recovery of lactate and muscle pH ($t_{1/2}$ of several minutes) or creatine phosphate (PCr) ($t_{1/2}$ of about 30 seconds) (Sahlin & Ren, 1989), demonstrating that maximum force can be attained during acidic conditions. Acidosis may still be an important indirect factor in fatigue. Also, patients

with McArdle's syndrome (a glycogenolysis enzyme defect characterized by myophosphorylase deficiency) produce no lactic acid but their muscle fatigue more rapidly than normal muscle (Wiles *et al.*, 1981). For reviews of metabolic factors in fatigue, see Vøllestad & Sejersted (1988) and Sahlin (1992).

In the past it was often suggested that a lack of one or more of the energy sources (ATP, PCr or glycogen) was responsible for the lack of force during fatigue. However, both biochemical studies (Karlsson & Saltin, 1970; Knuttgen & Saltin, 1972; Katz *et al.*, 1986) and non-invasive nuclear magnetic resonance (NMR) studies (Dawson *et al.*, 1978; Wilkie, 1981; Miller *et al.*, 1988) have shown only small changes in total ATP, ADP and AMP levels after exercise, which demonstrates that fatigue can occur while high energy stores are adequate (Edwards, 1981a; b). Even though the level of PCr can be severely depleted after one to two minutes of maximal work (Hultman *et al.*, 1967; Karlsson, 1971), it has been shown that there is no proportional relationship between force development and PCr level (Dawson *et al.*, 1978).

During short periods of high intensity exercise, there is a rapid breakdown of glycogen but only about half of the total muscle stores are depleted (Hermansen & Vaage, 1977). There may be an associated decline in force since there would be early depletion of glycogen in the fast-twitch glycolytic fibres (Gollnick *et al.*, 1974). However, during prolonged periods of exercise at lower intensities, energy is provided by glucose and free fatty acids, stored as glycogen and triglycerides, respectively (Newsholme, 1981). Although all three fibre types have similar glycogen contents, the rate of glycogen

utilization by each fibre type is a function of the duration and intensity of the exercise (Essén *et al.*, 1977). For prolonged submaximal exercise, the first fibre type that demonstrates glycogen loss is the slow-twitch fibres. As exercise is continued, a progressive depletion of glycogen occurs in the fast-twitch fibres (Gollnick *et al.*, 1973). As the exercise intensity starts to exceed the capacity of the oxidative fast-twitch fibres, more glycolytic fast-twitch fibres are activated. (Essén *et al.*, 1977). Although the total store of glycogen may be depleted within two hours, there is no evidence that fat deposits are depleted during submaximal work (Essén *et al.*, 1977) and in fact should be sufficient to sustain five days of continuous running in normal humans (Newsholme, 1981).

1.4.4.2 Electrical Factors in Fatigue

Although energy metabolism is considerably important in fatigue, alterations in the excitation and activation of muscle contraction may be of even greater importance in determining the onset of fatigue (Edwards, 1981). Studies of isolated, curarized rat muscle have shown that fatigue cannot be due simply to a lack of energy. The force of contraction of fatigued muscle can be increased immediately by altering the excitation conditions, either by reducing the stimulation frequency or increasing pulse duration (Jones *et al.*, 1979; Jones, 1981).

Two types of fatigue have been described in response to electrical stimulation and manifest themselves as a reduction of force, although they appear to be the result of different fatiguing processes. These are the results of high-frequency tetanic stimulation (high frequency fatigue or HFF) and low-frequency tetanic stimulation (low

frequency fatigue or LFF) (Edwards *et al.*, 1977; Edwards, 1981; Jones, 1981). Following the onset of fatigue as a result of high frequency stimulation, force can be rapidly restored by decreasing stimulus intensity or by increasing stimulus duration (Krnjevik & Miledi, 1958; Jones, 1979; Edwards, 1981). However, following prolonged low frequency stimulation, there is long-term (several hours) loss of force (Edwards *et al.*, 1977). For review of the role of ionic processes in fatigue, see McKenna (1992).

Calcium has been shown to be the intracellular link between membrane depolarization and activation of the contractile proteins. When an action potential is propagated along the sarcolemma and into the central regions of the fibres along the T-tubules, it triggers the release of Ca^{2+} from the sarcoplasmic reticulum. Released Ca^{2+} then combines with the protein, troponin, so that actin and myosin combine and form cross-bridges and contraction occurs. Relaxation occurs when Ca^{2+} is pumped out of the sarcoplasm by Ca^{2+}-ATPase and the cross-bridges detach. Although the details of the mechanism of Ca^{2+} release from the sarcoplasmic reticulum are still not fully determined (Ríos & Pizarró, 1988; Caswell & Brandt, 1989; Dulhunty, 1991; Lamb & Stephenson, 1992), it follows that any factor which causes a decrease in the amount of intracellular Ca^{2+} will also cause a decline in force. Studies by Vergara *et al.* (1978) have shown that the amount of Ca^{2+} released from sarcoplasmic reticulum depends on the amplitude of the action potential. Reduction in the amplitude of action potentials from -125 mV to -70 mV can reduce the release of Ca^{2+} by about 50%.

During exercise, substantial amounts of potassium (K^+) are lost from contracting muscles (Tibes *et al.*, 1974; Hnik *et al.*, 1976; Hazeyama & Sparks, 1979; Hirche *et al.*, 1980; Medbø *et al.*, 1982; Vyskocil *et al.*, 1983; Sjøgaard *et al.*, 1985; 1986; Sjøgaard, 1986; Juel, 1986; Hirche, 1989; Sahlin & Broberg, 1989). This is true for isolated muscles, voluntary contractions of muscle groups and whole animal exercise (for reviews, see Saltin *et al.*, 1987; Sjøgaard, 1991). Both HFF and LFF may be related to this loss of intracellular K^+ and its effect on intracellular Ca^{2+} levels, although in different ways. Since intracellular and extracellular ionic compositions directly affect resting membrane potential, membrane depolarization, action potential propagation, size of the action potential, membrane transport properties, intracellular enzyme function and strength of the muscle contraction, this increase in the K^+ concentration ($[K^+]$) of the interstitial fluid or plasma may constitute an important limiting factor for physical performance, as has been suggested for HFF (Jones, 1981; Hermansen *et al.*, 1984).

During each action potential there is a Na^+ influx followed by a K^+ efflux. Under normal conditions, the role of the membrane-bound "sodium pump", (Na^+,K^+-ATPase) is to oppose these ion fluxes and restore ion concentrations to resting levels (Clausen, 1986; 1989). Adrian & Peachey (1973) estimated that a single action potential would increase the $[K^+]$ of the T-tubules by 0.28 mM and decrease the $[Na^+]$ by 0.5 mM. The rate of loss of K^+ has been shown to be related to the intensity of the exercise so that during high intensity exercise, these fluxes in Na^+ and K^+ will be greatly increased and can cause large changes in intracellular and extracellular concentrations of Na^+ and K^+. The sodium pump therefore plays a vital role in maintaining

the ion concentrations and muscle membrane potential. It has been suggested that the large fluxes in Na^+ and K^+ that occur during intense exercise may exceed the capacity of the sodium pumps to maintain constant ionic balance (Clausen, 1986; Medbø & Sejersted, 1990).

Since the membrane of the T-tubules contains few sodium pumps (Venosa & Horowicz, 1981) and the rate of diffusion through the opening of the tubules is restricted (Almers, 1980), the T-tubules have been suggested as being the site of greatest accumulation of extracellular K^+ (Adrian & Peachey, 1973; Bigland-Ritchie *et al.*, 1978). As resting membrane potential is dependent on the intracellular and extracellular concentrations of K^+, large fluxes of K^+ across the muscle membrane may lead to impaired excitation of the sarcolemma and in particular, the T-tubules (Sjøgaard, 1990; Sjøgaard *et al.*, 1985). Both an increase in extracellular K^+ and a decrease in intracellular K^+ will reduce the membrane potential and lead to depolarization (Adrian, 1956). This will result in inactivation of the Na^+ channels and a decline in the amplitude of the action potential (Hodgkin & Huxley, 1952). This membrane depolarization would be more pronounced in the T-tubules, resulting in impairment of the action potentials in the central region of the muscle fibres (Jones, 1981; Jones & Bigland-Ritchie, 1986). Prolonged membrane depolarization may result in a reduction in the release of Ca^{2+} and subsequently, a reduced tension developed by the muscle.

Three possible ways in which changes in intracellular Ca^{2+} could cause a decline in muscle tension are (i) a decline in the amount of intracellular Ca^{2+} (by failure of the sarcolemmal or T-tubule action

potential or decreased release of Ca^{2+} from the sarcoplasmic reticulum or decreased sequestration of Ca^{2+} following contraction) (ii) decreased sensitivity of the contractile proteins to Ca^{2+} (if the Ca^{2+} has to compete with other ions, e.g. H^+ for binding sites on troponin) and (iii) decreased tension at saturating [Ca^{2+}] (for discussion, see Allen *et al.*, 1992).

1.4.4.3 High Frequency Stimulation

During high frequency stimulation, failure of action potential propagation can occur (Bigland-Ritchie *et al.*, 1979). Since accumulation of extracellular K^+ is likely to be greatest in the T-tubules (as discussed above) a "conduction block" at the T-tubular level has been suggested by several investigators as a possible cause of HFF (Bigland-Ritchie *et al.*, 1979; Jones, 1981). This will contribute to greater depolarization of the T-tubule membrane (i.e. becoming more positive), impaired propagation of action potentials, a reduction in Ca^{2+} release from the sarcoplasmic reticulum and reduced muscular tension. If the stimulus rate is reduced, the sodium pumps would be able to return excess extracellular K^+ to the cells. The same authors showed that increasing the duration of muscle stimulations from 0.02 ms to 0.2 ms also resulted in increased tension development following sustained tetany. They interpreted this effect to be the result of an increase in the action potential amplitude (Jones *et al.*, 1979; Jones, 1981).

In fatigue due to prolonged high frequency stimulation, the decline of tension has been shown to be associated with a Ca^{2+} gradient which is high close to the outer parts of the fibre but falls towards the centre (Westerblad *et al.*, 1990). This pattern is what would be expected if

high frequency stimulation caused failure of conduction of the action potential to central parts of the fibre. In agreement with this is the rapid recovery of tension seen when the frequency of stimulation was lowered. This recovery, which occurred in two to three seconds, is too fast to be caused by metabolic recovery, which takes several minutes (for review, see Sahlin, 1986) but has a time course similar to the diffusion of ions in or out of the T-tubules (Nakajima *et al.*, 1973). Westerblad *et al.* (1990) concluded that HFF is due to reduced Ca^{2+} release from the sarcoplasmic reticulum as a result of either impaired conduction of action potentials into the T-tubules or metabolic factors.

1.4.4.4 Low Frequency Stimulation

During low frequency stimulation, force generation can decrease and remain depressed for hours (Edwards *et al.*, 1977). Low frequency fatigue has been attributed to impaired excitation/contraction coupling (Edwards, 1981). It is thought that each action potential fails to release the normal amount of Ca^{2+} so that fewer cross bridges are available for force generation.

Allen *et al.* (1989) showed a decreased concentration of Ca^{2+} at fatigue which, although it recovered sufficiently to enable maximum tension to be generated, was still less than the control level. They later demonstrated that during recovery from fatigue due to intermittent tetanic stimulation, the concentration of intracellular Ca^{2+} remained uniform across all fibres, unlike the situation in HFF where there was a Ca^{2+} gradient across fibres (Westerblad *et al.*, 1990). The cause of this relatively long-term reduction of Ca^{2+} release is unclear (Westerblad *et al.*, 1990) but since Sjøgaard (1988) showed

that recovery of plasma [K^+] following prolonged low-level exercise is also a long term process due to the high blood flow surrounding the muscle, it is possible that these two factors are related through the exercise hyperkalemia which is dissipated by high blood flow.

1.4.4.5 Proposed Causes of High Frequency and Low Frequency Fatigue

Most studies investigating the effects of exercise on ionic shifts in skeletal muscle have examined changes during dynamic exercise (Medbø & Sejersted, 1985; 1990; Sjøgaard *et al.*, 1985; Juel, 1986; Sjøgaard, 1986; Juel & Sjøgaard, 1987; Fosha-Dolezal & Fedde, 1988; Lindinger & Heigenhauser, 1988; Sejersted & Medbø, 1989). Extracellular K^+ levels have been shown to rise sharply during the exercise and fall away rapidly during recovery. During high intensity isometric contractions, blood flow to the contracting muscles is insufficient or even totally blocked (Saltin *et al.*, 1981) due to compression of blood vessels. It would therefore be expected that not only would energy stores be rapidly consumed, but K^+ lost from cells would accumulate locally, both of which could lead to fatigue. Following contraction, the excess K^+ could be taken up again rapidly by muscle cells, as reported.

During low intensity isometric contractions, a relatively high blood flow is maintained which supplies the muscle with oxygen and glucose and removes metabolites before they can accumulate (Sjøgaard *et al.*, 1986; 1987). Both Sjøgaard (1988) and Vøllestad & Sejersted (1989) demonstrated that during low-level prolonged static contraction, although there is a continuous loss of K^+ from muscles and the level of plasma [K^+] remains constant, the [K^+] in the muscles is not

restored within at least five minutes of rest. Sjøgaard (1988) suggested that K^+ is being taken away by the blood during contraction and immediately taken up during recovery, but that long resting periods are needed following prolonged low intensity exercise in order to restore K^+ homeostasis. This may account for the long period of recovery required after LFF (Edwards, 1981). Sjøgaard *et al.* (1987) compared K^+ loss from muscles during continuous and intermittent contractions at the same workload and calculated that, due to the increased blood flow during intermittent contractions, there was a much greater loss of muscle K^+ during the intermittent contractions. The high circulating blood flow to the muscles would remove any build-up of extracellular K^+, which would alter trans-sarcolemmal ion gradients and the membrane potential and may thus also decrease the release of Ca^{2+} from the sarcoplasmic reticulum. If the level of K^+ takes some time to be restored, as shown by Sjøgaard (1988; 1989), this could also explain the decrease in tension evident for some time following prolonged low level stimulation.

1.4.5 Cardiotoxicity

In most animals, normal values for serum $[K^+]$ range from 3.5 to 4.6 mM (Whang, 1976). When large groups of muscles are engaged in heavy dynamic exercise, the extracellular $[K^+]$ of the whole body may double within a few seconds (Kjeldsen, 1991). Medbø & Sejersted (1990) reported that plasma $[K^+]$ reached more than 8 mM during one minute of exhausting exercise and McKechnie *et al.* (1967) have reported levels of up to 10 mM in marathon runners. According to Whang (1976), clinical hyperkalemia, in which serum $[K^+]$ exceed 6 mM, constitutes a medical emergency and it is accepted that cardiac arrest threatens when plasma $[K^+]$ are greater than 7 - 8 mM

(Kjeldsen, 1991). Cardiac muscle faces the same problems as skeletal muscle when excess K^+ is lost from the interior of cells. Prominent electrocardiogram (ECG) changes occur when plasma $[K^+]$ reaches values of 6-7 mM. Rapid and prolonged depression of myocardial conductance occurs progressively as the plasma $[K^+]$ exceeds 8 mM and the ECG reveals "peaking" T waves. As $[K^+]$ exceeds 10 mM, asystole is a predominant mechanism of cardiac failure (Whang, 1976). It is possible that the cardiotoxic effect of hyperkalemia could explain certain cases of sudden death occurring in connection with heavy exercise in untrained subjects. Beef cattle are sedentary animals that generally do not exercise voluntarily at heavy workloads but are often forced into exertion during handling and shipping procedures (Fosha-Dolezal & Fedde (1988). Their serum $[K^+]$ was shown to increase by 60% during maximal exercise which could alter depolarization of the cell membrane and reduce conduction velocity. This could eventually degenerate to ventricular fibrillation and death. Such hyperkalemic effects on cardiac function could occur in any organism placed under unaccustomed physical stress.

1.4.6 Relaxation

Relaxation rate is a basic property of skeletal muscle and muscle fatigue is associated with a slowing of the rate of relaxation. (Dawson *et al.*, 1980; Jones, 1981; MacLaren *et al.*, 1989). However, its cause is not yet clear. Relaxation of force from an isometric contraction has a well-defined time course which is characteristic of the "type" of muscle, the type of contraction, the species it comes from, and the degree of fatigue of the muscle (Jones, 1981). In sustained voluntary contractions of human muscle (i.e. tetanic contractions) this slowing of contraction is thought to preserve force during isometric

contractions (Jones & Bigland-Ritchie, 1986) and thus increase resistance to fatigue.

Two main mechanisms thought to determine the time course of relaxation are the time course of dissociation of actin-myosin cross-bridges after the activating Ca^{2+} has been removed (Edwards *et al.*, 1975; Jones, 1981; de Haan *et al.*, 1989) and the rate of Ca^{2+} uptake by the sarcoplasmic reticulum (Briggs *et al.*, 1977, Stein *et al.*, 1982; Dulhunty, 1990). Relaxation from an isometric contraction requires the dissociation of myosin cross-bridges, which requires ATP to bind to ATP-binding sites on the myosin. However, using NMR, Dawson *et al.* (1980) found no change in the rate of ATP turnover per unit force in fatiguing frog muscles, indicating that cross-bridge turnover was not affected. Dawson *et al.* (1980) found though, that the rate of relaxation may be related to the rate Ca^{2+} uptake into the sarcoplasmic reticulum and the results of Dulhunty (1990) supported this by showing that tetanic relaxation is closely correlated with the density of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes.

1.4.7 Fatigue in Isolated Muscles

Two main mechanisms have been proposed to explain fatigue in isolated muscle :

(i) effects of metabolic products on the contractile proteins, causing a reduction in maximum tension-generating capacity (Fabiato & Fabiato, 1978; Cooke & Pate, 1985; Kentish, 1986; Godt & Noesk, 1989), and

(ii) failure of Ca^{2+} release, causing reduced activation of the contractile proteins (Eberstein & Sandow, 1963; Grabowski *et al.*, 1972; Lännergren & Westerblad, 1989).

However, since caffeine (which releases Ca^{2+} from the sarcoplasmic reticulum) has been shown to overcome the reduced tension of fatigued muscle (Eberstein & Sandow, 1963; Grabowski *et al.*, 1972; Lännergren & Westerblad, 1989) by a "sparing effect" on glycogen (Spriet *et al.*, 1992) and intracellular Ca^{2+} release during a tetanus is known to be reduced in fatigued muscle (Allen *et al.*, 1989), it would appear that failure of Ca^{2+} release is the most likely cause.

Reduced Ca^{2+} release in fatigued muscle could be due to many different mechanisms but would certainly be expected to arise from (i) mechanisms that cause failure of inward conduction of the action potential, resulting in reduced Ca^{2+} release in the central part of the muscle fibre (such as impaired conduction of action potentials into the T-tubules) and (ii) mechanisms that cause a spatially uniform failure of Ca^{2+} release (such as metabolic factors).

1.4.8 Dietary Manipulation and Isolated Muscles

A number of studies have demonstrated that poor nutrition can alter skeletal muscle function but published results on the effects of malnutrition on muscle function are contradictory. These studies have usually been done by indirect electrical stimulation of the adductor pollicis muscle, the force of which is recorded by a strain gauge connected to a loop around the interphalangeal joint of the thumb (Merton, 1954; Edwards *et al.*, 1977b). Studies of malnourished humans (Lopes *et al.*, 1982; Russell *et al.*, 1983a; b; 1984b; Chan *et al.*, 1986) and rats (Choong *et al.*, 1983; Russell *et al.*, 1984a; Nishio & Jeejeebhoy, 1991) have shown that their skeletal muscles generate less power, fatigue more easily and relax more slowly than muscles

from controls. In the diaphragm, Lewis *et al.* (1986) and Dureuil *et al.* (1989) observed no effect on isometric twitch and tetanic tensions but they reported prolonged endurance in muscle strips and reduced endurance in intact muscle, respectively. In later studies, Sieck *et al.* (1989) and Lewis & Sieck (1990) showed reduced endurance after chronic malnutrition but little effect following acute nutritional deprivation in adult rats. However, in acute deprivation in adolescent rats, Lewis & Sieck (1992) reported prolonged endurance. Other studies however, (McCarter *et al.*,1978; Gardiner *et al.*, 1980; Lennmarken & Larsson, 1986) have reported no effect of malnutrition on muscle function. Unfortunately, these studies have employed a variety of experimental conditions, have varied in duration and types of diets and have measured a variety of parameters all of which makes reliable and consistent comparisons difficult.
In addition, different muscle fibre types have been shown to respond differently to malnutrition (Li & Goldberg, 1976; Gardiner *et al.*, 1980; Fong *et al.*, 1987; Nishio & Jeejeebhoy, 1991). Although muscle atrophy and altered contractile responses have been shown to be induced by dietary changes (particularly the amount of total food and types of carbohydrate), the specific dietary components which are responsible have not been investigated.

1.5 Whole Animal Physical Performance

1.5.1 Dietary Manipulation and Whole Animal Performance

1.5.1.1 Human Studies

Human physical performance is known to be affected by the composition of food ingested. In studies which have demonstrated this, either diets have been hypocaloric, in overweight (Phinney *et al.*, 1980) or obese (Davis & Phinney, 1989) patients (for review, see Phinney, 1992), or diets have been manipulated by boosting or restricting particular types of foods, such as carbohydrates, fats or proteins. Protein catabolism is normally only a minor source of metabolic energy. Plante & Houston (1984) estimated that it provides no more than 5% of total energy even for a marathon runner. The effects of restricted protein intake have not been experimentally investigated.

Most studies have concentrated on the effects of changed levels of carbohydrate (for review, see Coggan & Swanson, 1992), but in doing so, the levels of ingested fat have also been changed (Hughson & Kowalchuk, 1981; Yoshida *et al.*, 1984; Aitken & Thompson, 1989; Greenhaff *et al.*, 1991). Diets designated as "low carbohydrate" could equally be called "high fat" as in Quirion *et al.*'s (1988) study comparing effects of high carbohydrate and high fat diets on maximal exercise. Results have not typically been interpreted in terms of fat metabolism, even though fats have been shown to make a large contribution to energy metabolism. In a study of marathon runners, Callow *et al.* (1986) showed that the mean proportion of total energy

output accounted for by carbohydrate, lipid and protein catabolism during marathon running was 59%, 40% and 1% respectively. The contribution of carbohydrates started at about 75% and dropped to about 55% at fatigue, whereas the contribution of lipids started at about 25% and rose to about 45% at fatigue. In studies manipulating dietary composition, the energy value of different food groups must be taken into consideration. Since each gram of lipid produces more than twice the kilocalories of a gram of carbohydrate (Tortora & Anagnostakos, 1987), it may be difficult to ensure that caloric intake is similar between groups.

In some dietary manipulation studies, diets have been isocaloric (Yoshida, 1984; Quirion *et al.*, 1988; Greenhaff *et al.*, 1991), but others have not (e.g. Aitken & Thompson, 1989) or the calorific value was unspecified (e.g. Hughson & Kowalchuk, 1981). In these latter studies, it is difficult to determine whether observed effects were a result of the change in dietary composition, or energy availability. Even though these different diets have resulted in altered physical performance, effects of dietary manipulation on specific organ systems, which also may affect performance, have not been identified.

1.5.1.2 Animal Studies

Animal studies of dietary effects on physical performance, have used either hypocaloric diets to examine isolated skeletal muscle function in different types of muscles (McCarter *et al.*, 1978; Gardiner *et al.*, 1980; Russell *et al.*, 1984; Lewis *et al.*, 1986; Pichard *et al.*, 1988; Dureuil *et al.*, 1989; Sieck *et al.*, 1989; Lewis & Sieck, 1990; 1992; Nishio & Jeejeebhoy, 1991; Barclay & Loiselle, 1992), or have manipulated the levels of carbohydrates or fats and examined whole

animal performance (Miller *et al.*, 1984). In rats, Miller et al. (1984) found increased endurance in rats on a high fat diet compared with rats on a normal diet and suggested this was due to an improved ability to oxidize fats and spare glycogen.

Although maximal oxygen consumption was found to be 10% greater in subjects who had been on a "low-carbohydrate" (and thus "high-fat") diet (Hughson & Kowalchuk, 1981), later studies found no difference between groups on diets with different total levels of fat (Yoshida, 1984; Quirion et al., 1988; Aitken & Thompson, 1989; Greenhaff *et al.*, 1991). In a cycling test, Yoshida (1984) found that endurance time to exhaustion was 5% longer in subjects who had been on a "high-carbohydrate"(i.e. "low-fat") diet than subjects who had been on a "low-carbohydrate" diet. Quirion *et al.* (1988), in another cycling test, also found a non-significant 7% increase in endurance for subjects on a "high-carbohydrate" diet. It is difficult to make comparisons between these studies, however, since the levels of dietary fat varies between groups (e.g. the total amount of fat in "low-carbohydrate" diets varies from 50 to 70%) as well as the types of exercise tests used (cycling or treadmill running) and exercise protocols.

1.5.2 Measures of Performance

Physical performance, *in vivo*, can be measured in terms of speed, strength or endurance, but the most common parameter used to measure the capacity of humans to perform prolonged exercise is the increase in amount of oxygen consumed per unit time (i.e. maximal oxygen uptake or $VO_{2\ max}$ (Sutton, 1992). It is a measure of maximal aerobic power. In humans, performance can be assessed during a

variety activities; such as running, swimming, cycling and weight-lifting but VO_{2max} is less easy to measure in rats.

Maximal oxygen uptake has most commonly been measured while rats run on a treadmill (e.g. Bedford *et al.*, 1979; Patch & Brooks, 1980; Baldwin *et al.*, 1982; Gleeson *et al.*, 1983; McDonald *et al.*, 1988; Musch *et al.*, 1988; 1991; Hilty *et al.*, 1989; Cortez *et al.*, 1991), but also during swimming (McArdle, 1967; Ghaemmaghami *et al.*, 1987), on a laddermill (Russell *et al.*, 1980), free running on an activity wheel (Shepherd & Gollnick, 1976) and weight lifting combined with isometric exercise (Gordon *et al.*, 1967; Exner *et al.*, 1973; Jaweed *et al.*, 1977). For a review of common exercise programs for rats, see Harpur (1980). However, although values of $VO_{2\ max}$ have been reported for rats in a number of studies, there not only appears to be considerable variation in reported values, but comparisons between studies are difficult because of different experimental methods. Factors such as strain, age, sex and state of training all affect $VO_{2\ max}$ (Bedford *et al.*, 1979).

Besides measuring $VO_{2\ max}$, most studies have also measured basal oxygen consumption as an estimate of basal metabolic rate (BMR). Another parameter used to describe physical performance is work capacity (endurance) which is measured by the length of time that a rat can run or swim before exhaustion (Baldwin *et al.*, 1982; Gleeson *et al.*, 1983). Endurance has been shown to be longer at low work intensities since stored fat can provide the total energy required (for discussion of endurance, see Sahlin, 1992).

1.6 Biochemistry of Membrane-Bound Enzymes

In skeletal muscle, ion transport is regulated by enzyme systems which are integral components of the sarcolemma and sarcoplasmic reticulum. As discussed earlier (see Section 1.4.4.2), Na^+,K^+-ATPase in the sarcolemma and Ca^{2+}-ATPase in the sarcoplasmic reticulum membrane both play major roles in contraction and relaxation of muscle. Changes in the properties of these ion pumps could alter the distribution of ions within the intracellular and extracellular compartments and affect contractility.

1.6.1 Na^+,K^+-ATPase

The integral membrane protein, Na^+,K^+-ATPase, found in almost all animal cells, catalyzes the active coupled electrogenic transport of Na^+ and K^+ across cell membranes (Skou, 1957; 1960). In most cells, the internal environment is high in K^+ and low in Na^+, whereas the external environment is usually low in K^+ and high in Na^+. In order to maintain an electrical potential of about -70 mV, both Na^+ and K^+ ions must be moved against a concentration gradient and the Na^+ moved against an electrical gradient because the inside of the cell is negative with respect to the outside. The energy for the transport of these ions is provided by the hydrolysis of ATP.

In skeletal muscles, Na^+,K^+-ATPase units are located primarily in the sarcolemma, however, transverse tubules are also known to contain Na^+,K^+-ATPase, but at a lower density (Narahara *et al.*, 1979; Venosa & Horowicz, 1981 Seiler & Fleischer, 1982). Na^+,K^+-ATPase is inhibited by a class of drugs, known as cardiac glycosides, for which it is the only known receptor. The enzyme spans the membrane and has an outer binding site for ouabain, and an inner binding site for

phosphorylation. An important characteristic of Na^+,K^+-ATPase is its sensitivity to the cardiac glycoside, ouabain which is frequently used to distinguish this enzyme from other membrane-bound ATPases with which it is closely associated.

The activity of the Na^+/K^+ transport system is acutely regulated by catecholamines & insulin (for reviews, see Clausen, 1986; Clausen & Everts, 1989). These hormones increase active transport of Na^+ and K^+ across the plasma membrane and result in hyperpolarization, an increased intracellular ratio of K^+ : Na^+ and improved clearance of K^+. Long-term control of Na^+,K^+-ATPase concentration is provided by thyroid hormones which stimulate the synthesis of Na^+,K^+-ATPase. Diet and exercise are also known to influence Na^+,K^+-ATPase in skeletal muscle. Physical training has been shown to increase the concentration of Na^+,K^+-ATPase and K^+ deficiency leads to a marked decrease in the concentration of Na^+,K^+-ATPase as well as the capacity for performing Na^+-K^+ transport.

1.6.1.1 Isoforms of Na^+,K^+-ATPase

There are three known isozymes of Na^+,K^+-ATPase (for review, see Sweadner, 1989). These are specific for species, tissue and developmental stage (Orlowski & Lingrel, 1988) and their specific roles are not well understood (Jewell *et al.*, 1992). In rat skeletal muscles, the high-affinity isozyme of Na^+,K^+-ATPase appears most common and there are lower amounts of a low-affinity binding site (Lytton *et al.*, 1985; Young & Lingrel, 1987; Kjeldsen *et al.*, 1985; 1988; Sweadner, 1989).

1.6.1.2 Quantification of Na^+,K^+-ATPase

The quantification of Na^+,K^+-ATPase can be based on measurements of enzyme activity or concentration. However, in measuring Na^+,K^+-ATPase activity in membrane fractions of skeletal muscle, as developed by Jørgensen (1974) for other tissues, the high background level of unspecific ATPases and difficulties associated with the separation of plasma membrane from other cell structures mean that the recovery of Na^+,K^+-ATPase is usually very low (Kjeldsen, 1986; Hansen & Clausen, 1988). Clausen (1986) pointed out that reported estimates of Na^+,K^+-ATPase activity from purified membranes of rat heart and skeletal muscle were only a minor fraction (0.2 to 9%) of total Na^+,K^+-ATPase activity so it is difficult to determine whether reported values of Na^+,K^+-ATPase activity are representative of the total population of Na^+,K^+-ATPase molecules.

The concentration of Na^+,K^+-ATPase (i.e. the number of sodium pumps) can be quantified with accuracy and precision by measuring the uptake of labelled cardiac glycosides (specifically [^{3}H] ouabain) by intact muscle tissue since each Na^+,K^+-ATPase unit has one only such binding site (Clausen & Hansen, 1974). Results of studies relating [^{3}H]ouabain binding and $^{42}K^+$ uptake indicate that measurements of [^{3}H]ouabain binding accurately quantify the number of functional sodium pumps (Kjeldsen *et al.*, 1985). According to Everts & Clausen (1988) and Jørgensen (personal communication), this method of [^{3}H]ouabain binding probably only detects the high-affinity form of Na^+,K^+-ATPase in muscles (apparent dissociation constant (K_D) for ouabain around 0.1 μM) and not the low affinity form (apparent K_D for ouabain around 10 μM). However, recent studies on the distribution of the mRNAs encoding for the isoforms of Na^+,K^+-

ATPase have shown that 90 - 95% of the mRNA in muscle encodes the high affinity isoform whereas the remaining 5 - 10% encodes the low affinity isoform (See Young & Lingrel, 1987; Schneider *et al.*, 1988). Clausen *et al.* (1987) showed that induced changes in [^{3}H]ouabain binding site concentration are associated with proportional changes of the maximum transport rate of the sodium pump. Therefore measurement of [^{3}H]ouabain binding provides a useful approach for the quantification of sodium pumps in skeletal muscle.

In using [^{3}H]ouabain binding to measure the concentration of Na^+,K^+-ATPase, ouabain inhibits the binding of ATP to Na^+,K^+-ATPase from the outside of the cell and vanadate inhibits the binding from the cytoplasmic side. Ouabain is a specific inhibitor of Na^+,K^+-ATPase, but not other ATPases at the outside of the membrane. Under normal conditions, it appears to act by inhibiting the extrusion of Ca^{2+} by the cell, thereby increasing intracellular Ca^{2+}. This in turn triggers the actin-myosin mediated contraction of heart muscle. This interaction is intensified by decreased extracellular K^+ (hypokalemia) and is inhibited by increased extracellular K^+ (hyperkalemia). Vanadate, in low concentrations, facilitates [^{3}H]ouabain binding (Hansen, 1979) which appears to inhibit Na^+,K^+-ATPase by competing for the ATP phosphorylation site on the cytoplasmic side of the membrane. This inhibition is increased when external $[K^+]$ is > 5 mM or when external $[Na^+]$ is lowered. It also inhibits other ATPases.

In this study, both the concentration of Na^+,K^+-ATPase in intact muscle and the activity of Na^+,K^+-ATPase in muscle homogenates and purified muscle were measured.

1.6.1.3 Importance of Na^+,K^+-ATPase in Exercise

During exercise, the sodium pump is mainly stimulated by a rise in intracellular Na^+, but it is also stimulated by increased extracellular K^+ and by epinephrine and norepinephrine (Clausen, 1986). The large increases in extracellular K^+ (known as "exercise hyperkalemia") and intracellular Na^+ during intense exercise indicate that the capacity of the Na^+,K^+-ATPase to maintain ionic balance can be exceeded .

Endurance training has been shown to reduce the exercise-induced increase in plasma [K^+] (exercise hyperkalemia) in humans (Tibes *et al.*, 1974; 1976; Hazeyama & Sparks, 1979; Kiens & Saltin, 1986; Kjeldsen *et al.*, 1990a), dogs (Knochel *et al.*, 1985) and calves (Fosha-Dolezal & Fedde, 1988). This may be related to either increased activity or increased concentration of Na^+,K^+-ATPase in the muscles, since both have been shown to occur following intense training. Also, during periods of inactivity, the concentration of Na^+,K^+-ATPase has been found to be decreased by up to 30% in skeletal muscle (Clausen *et al.*, 1981; Kjeldsen *et al.*, 1988).

Moderate training in dogs has been shown to increase the activity of Na^+,K^+-ATPase by 165% (Knochel *et al.*, 1985) and several studies have shown effects of training on the concentration of Na^+,K^+-ATPase. Intense training has been shown to increase the concentration of [^{3}H]ouabain binding sites in rats (Kjeldsen *et al.*, 1986) and humans (Klitgaard & Clausen, 1989; McKenna *et al.*, in press). An increase of 22-43% in the total concentration of Na^+,K^+-ATPase (quantified by [^{3}H]ouabain binding sites) has been demonstrated in the hindlimb skeletal muscles of rats after six weeks

of intensive swim training (endurance) (Kjeldsen *et al.*, 1986). Although these increases were reversed during deconditioning, it was interesting to note that in EDL muscles, the return to the control level was still incomplete even three weeks after the cessation of training. In elderly men who had been training for between 12 and 17 years, a 30-40% increase in [^{3}H]ouabain binding site concentration was demonstrated compared to untrained controls (Klitgaard & Clausen, 1989). Recently, McKenna *et al.* (in press) using 7 weeks of intense sprint training, showed a 16% increase in [^{3}H]ouabain binding concentration after training and a decrease in exercise hyperkalemia. Kjeldsen *et al.* (1990b) showed no effect of moderate endurance training on [^{3}H]ouabain binding site concentration in skeletal muscle of military conscripts, but still demonstrated decreased exercise hyperkalemia. There may have been no associated decrease in Na^+,K^+-ATPase concentration because the training period (10 weeks) was too short or the intensity of training was too low. It is not known what causes this increased concentration of Na^+,K^+-ATPase, but it appears (Kjeldsen *et al.*, 1986) that it is restricted to the exercising muscles. This effect of muscle activity on either concentration or activity of Na^+,K^+-ATPase probably explains the reduced exercise hyperkalemia observed following training.

1.6.2 Ca^{2+}-ATPase

Ca^{2+}-ATPase of the sarcoplasmic reticulum, which transports Ca^{2+}, plays a vital role in muscle relaxation (Gillis, 1985). It is the major transport system responsible for the removal of Ca^{2+} ions from the sarcoplasm during relaxation. Relaxation rate and the concentration of Ca^{2+}-ATPase are known to be affected by fibre type (Close, 1964; Dulhunty *et al.*, 1987; Everts *et al.*, 1989). Both Everts *et al.* (1989)

and Dulhunty (1990) have shown in rats, that the concentration of Ca^{2+}-ATPase in EDL muscles is about five to eight times greater than in soleus muscles.

A number of studies have suggested that changes in the total concentration or activity of Ca^{2+}-ATPase may be involved in disorders affecting muscle performance and metabolism, such as malignant hyperthermia (Cheah & Cheah, 1981; Dulhunty *et al.*, 1991) and muscular dystrophy (Hanna *et al.*, 1981).

1.6.2.1 Quantification of Ca^{2+}-ATPase

Methods of accurately quantifying Ca^{2+}-ATPase are still being developed. In purifying and isolating sarcoplasmic reticulum vesicles, relatively large amounts of muscle are required and studies have shown that the recovery is only about 10-20% (Simonides & van Hardeveld, 1985; 1990). Everts *et al.* (1989) used measurements of the Ca^{2+}-dependent phosphorylation in crude muscle homogenates to quantify the concentration of Ca^{2+}-ATPase. Simonides & van Hardeveld (1990) developed a method of measuring the activity of Ca^{2+}-ATPase in muscle homogenates using spectrophotometry. In their assay, the rate of total ATP hydrolysis, in the presence of a low and high levels of calcium, is followed spectrophotometrically from recordings of the oxidation of NADH. Although Ca^{2+} at low concentrations (0.5 - 0.8 mM) is necessary for the measurement of Ca^{2+}-ATPase activity, very high Ca^{2+} concentrations (about 20 mM) inhibit the activity of this enzyme (Simonides & van Hardeveld, 1990).

1.6.2.2 Role of Calcium in Exercise

Calcium ions (Ca^{2+}) play a very important role in the function of muscle cells, both in contraction and relaxation (Gillis, 1985), as well as in responses to hormones and drugs. The level of cellular Ca^{2+} is regulated mainly by the sarcolemma and the sarcoplasmic reticulum (Winegrad, 1982; Shamoo, 1985) which contains a large pool of Ca^{2+}.

The resting level of free Ca^{2+} in sarcoplasm is about 100 nM (Lamb & Stephenson, 1992). In order for contraction to occur, this level of Ca^{2+} must increase. Thus, contraction is controlled by the level of sarcoplasmic Ca^{2+}, which in turn is controlled by the sarcoplasmic reticulum. When the sarcolemma and T-tubules are depolarized, channels in the sarcoplasmic reticulum open up to release Ca^{2+} which binds to troponin (Endo, 1977) and leads to activation of the contractile filaments, myosin and actin. Following contraction, Ca^{2+} is released from the actomyosin complex and cleared from the sarcoplasm by the Ca^{2+} pump of the sarcoplasmic reticulum (Ebashi *et al.*, 1969). Slow skeletal muscles have been found to contain less sarcoplasmic reticulum (Kim *et al.*, 1981; Everts *et al.*, 1989) and to exhibit reduced Ca^{2+} uptake (Fitts *et al.*, 1980; Kim *et al.*, 1981) compared to fast skeletal muscles, which is not surprising given the longer relaxation times in slow muscles (Close, 1964; 1972).

There is much controversy about the way in which the T-tubules initiate Ca^{2+} release from the sarcoplasmic reticulum (Endo, 1985; Shamoo, 1985; Ríos & Pizarró, 1988; Caswell & Brandt, 1989). Evidence suggests that in cardiac muscle, it is a calcium-induced calcium release (Shamoo, 1984; Shamoo *et al.*, 1985), whereas in skeletal muscle, it is a depolarization-induced release (Endo, 1985;

Melzer *et al.*, 1986; Bers, 1991). The three main hypotheses of muscle "T-tubule to sarcoplasmic reticulum communication" include (i) direct electrical communication, (ii) depolarization-released neurotransmitter and (iii) direct mechanical communication (Caswell & Brandt, 1989). The current favoured mechanism is direct mechanical coupling via junctional "feet" of the sarcoplasmic reticulum (Caswell & Brandt, 1989; Dulhunty, 1989). Ríos & Pizarró (1988) suggested that besides mechanical communication, there is also a calcium-induced stimulus for Ca^{2+} release.

1.6.2.3 Importance of Ca^{2+}-ATPase in Exercise

Calcium has been implicated as a cause of fatigue since muscle contraction relies on the release of Ca^{2+} from the sarcoplasmic reticulum and because relaxation and subsequent contractions rely on the re-uptake of Ca^{2+} by Ca^{2+}-ATPase. Sembrowich & Gollnick (1977) suggested that the changes in contractile activity observed at fatigue, such as a slowing of relaxation and a decline in tension, might be related to changes in excitation-contraction coupling since these could be influenced by the amount of Ca^{2+} released and the rate of Ca^{2+}uptake. They found that the rate of Ca^{2+} uptake by the sarcoplasmic reticulum was inversely related to the duration of exercise. Studies of isolated muscles have shown that decreased sarcoplasmic reticulum Ca^{2+}-ATPase activity, increased relaxation time and decreased uptake of Ca^{2+} are associated with both chronic low frequency stimulation (Leberer *et al.*, 1987) and high frequency stimulation (Byrd *et al.*, 1989; Gollnick *et al.*, 1991) and are thus related to muscle fatigue. Matsushita & Pette (1992) suggested that the observed inactivation of sarcoplasmic reticulum calcium pumps during low frequency stimulation was due to a structural alteration of

the enzyme. Westerblad *et al.* (1990) showed that in isolated *Xenopus* fibres, the Ca^{2+} concentration was reduced during both prolonged and intermittent fatiguing tetanic contractions. Gross fibre necrosis, known as "exercise-induced muscle damage" may be related to alterations in Ca^{2+} handling by the sarcoplasmic reticulum (for review, see Byrd, 1992)

As discussed earlier, (see Section 1.4.7), two main mechanisms proposed for fatigue in isolated muscles are (a) detrimental effects of metabolic products on the contractile proteins and (b) failure of Ca^{2+} release (Westerblad *et al.*, 1990). Intracellular Mg^{2+} is likely to play a significant role in muscle metabolism because it (i) inhibits Ca^{2+} release from the sarcoplasmic reticulum; (ii) inhibits the sarcoplasmic reticulum Ca^{2+}-ATPase (Westerblad & Allen, 1992). Recent evidence suggests that Mg^{2+} may be the key to explaining how specialized "voltage sensors" of the T-tubules control the Ca^{2+} release channels (for discussion, see Lamb & Stephenson, 1992).

Westerblad & Allen (1992) showed that a high level of Mg^{2+} can inhibit the depolarization-induced Ca^{2+} release from the sarcoplasmic reticulum. They found that when the concentration of Mg^{2+} in skeletal muscle fibres was increased three to four fold, tetanic force decreased by about 50%. At rest, the most important binding site for Mg^{2+} is ATP, so a possible source of the increased $[Mg^{2+}]$ at fatigue is the breakdown of ATP. Westerblad & Allen (1992) suggested that the increased $[Mg^{2+}]$ reflects a breakdown of ATP and contributes to the tension decline seen in fatigue.

The declining intracellular ATP may indirectly be a cause of fatigue since both Na^+,K^+-ATPase of the sarcolemma and Ca^{2+}-ATPase of the sarcoplasmic reticulum require ATP for maintaining their respective ionic balances. A decline in ATP would limit the functioning of Na^+,K^+-ATPase which may cause failure of action potentials, leading to reduced release of Ca^{2+} from the sarcoplasmic reticulum.

Chapter 2 Effects of Changes in Dietary Fatty Acids on Skeletal Muscle Membrane Composition

2.1 Introduction

Recent studies have used isocaloric diets to show that diet-induced changes in membrane fatty acids can affect a wide range of cellular activities in tissues other than skeletal muscle. In particular, studies have focused on the essential polyunsaturated fatty acids, the n - 6 and n - 3 classes, as they have been shown to exert both direct and indirect effects throughout the body. Since they act as precursors for the eicosanoids, the n - 6 and n - 3 fatty acids are particularly important for the cardiovascular system in their roles in atherogenesis, thrombosis and coronary infarction and the n - 3 fatty acids in particular, are vital for brain and retinal development (for excellent reviews, see Innis, 1991; Simopoulos, 1991; Connor *et al.*, 1992; Drevon, 1992). Although the studies of Charnock *et al.* (1982; 1985b; 1987) and McLennan *et al.* (1985; 1987a; b; 1989; 1990) investigated the effects of dietary fatty acid manipulation on cardiac muscle function and later studies from the same laboratory showed that the membrane fatty acid compositions of cardiac and skeletal muscles in marmosets were very similar (Charnock et al., 1989, 1992), there are no comparable studies on the effects of dietary fatty acid manipulation on skeletal muscle function and very little is known about their effects on physical performance.

This part of the study aimed to:

(1) trial a number of diets in order to determine the levels and types of essential polyunsaturated fatty acids which would be approximately equally palatable and therefore consumed at a similar rate as a Control diet;

(2) confirm that the chosen diets do alter the fatty acid composition of skeletal muscle membranes;

(3) determine whether skeletal muscle membrane composition recovers from such diet-induced changes, and if so, how long the recovery process takes; and

(4) compare the effects of dietary lipid changes on representatives of two major types of skeletal muscle ("slow twitch" and "fast twitch" muscle), specifically the soleus muscle and the extensor digitorum longus muscle.

2.2 Materials and Methods

2.2.1 Rats

2.2.1.1 Maintenance

Male albino Wistar rats were used for all experiments. Male rats have been used for many comparable studies and this avoids any confounding effects of estrous cycles in females. Young rats were used since they have small fat reserves and were thus considered more likely to be responsive to dietary changes.

Rats were bred in the University of Wollongong's Animal House and were kept in environmentally controlled conditions with a 12 hour

light cycle (lights on at 05.00). The mean temperature was 22°C (range 19°C - 24°C) and the mean relative humidity was 57 ± 2%. All rats were weaned at 21 - 23 days of age and thereafter housed in standard plastic cages (17cm by 25cm by 40cm) which were cleaned two to three times per week. In initial dietary trials, one to three rats were housed in each cage. In later studies, rats were housed individually. Rats were numbered and marked for later identification with coded picric acid stains.

Rat weights were recorded throughout all experiments in order to monitor growth and general well-being of the rats. It was necessary to establish whether any observed differences between the dietary groups could be explained by the effects of the diets, or simply by differences in size of either rats or muscles.

2.2.1.2 Diets

In the trial tests, rats were fed normal rat pellets for three weeks after weaning, followed by two weeks or six weeks on the trial diets, as described later. Mean weight three weeks after weaning was 158 ± 7g.

Once the diets were established, male rats weighing 54 ± 1g at weaning were assigned at random, one per litter, to each of the three dietary groups: Control, Essential Fatty Acid Deficient (EFAD) or high Polyunsaturated Fatty Acid (PUFA) enriched. Throughout the study, rats were maintained on these diets for six weeks (trial tests only) or nine weeks, followed by a recovery period of zero, two or six weeks on normal rat pellets. Diets were supplied in 100 ml glass

jars which were cleared of sawdust and re-filled every one to two days. Food and water were available *ad libitum*.

All diets were identical except for their lipid composition. They each contained 10% fat by weight, but varied in their type of fat (Table 2.1). The diet composition was based on that of the rat basal diet plan of the Association of Official Agricultural Chemists, Washington D.C. (1960) which is regarded as a complete diet for rats. Each diet contained (w/w) 56% carbohydrate (sucrose, CSR); 22% protein (casein, Murray Goulburn Pty. Ltd., Melbourne, Australia and H.E. Cottee Corporation, Sydney, Australia); 10% fat (see below), 5% water, 5% salt mix (Sigma Chemical Co., St. Louis, U.S.A. and BDH, Melbourne, Australia), 1% vitamin mix (Roche Products, Sydney, Australia), and 1% cellulose (Sigma, St. Louis, U.S.A), by weight (Table 2.1). Diets were prepared fortnightly in one kg lots using a Kenwood Chef Food Processor, and stored sealed, at -20 ^{o}C until required. Max EPA oil (R.P. Scherer Pty. Ltd., Melbourne, Australia) was stored under nitrogen and all diets contained additional Vitamin E as an anti-oxidant. Rat pellets were obtained from Fielders' Agricultural Products (Tamworth, Australia). Their composition (w/w) is shown in Table 2.2 and their total fatty acid composition is presented in Table 2.3.

The Control diet contained 10% sesame oil (Meadowlea Foods, Sydney, Australia). Trial studies were conducted with EFAD diets (containing 10% stearic acid (BDH, Melbourne, Australia) and PUFA diets (containing varying quantities of Max EPA oil and sesame oil, respectively. Final EFAD diets contained 10% coconut oil (ETA Food Services, Wollongong, Australia) and final PUFA diets

Table 2.1 Composition of experimental diets. All diets were identical except for the type of fat. The Control diet contained 10% sesame oil; the EFAD diet contained 10% coconut oil and the PUFA diet contained 7% sesame oil and 3% Max EPA oil. Dashes denote zero amount.

Ingredient	**Amount (% by weight)**		
	Control	**EFAD**	**PUFA**
Protein (casein)	22	22	22
Sesame oil	10	-	7
Coconut oil	-	10	-
MaxEPA oil	-	-	3
Salts mix†	5	5	5
Vitamin mix††	1	1	1
Cellulose	1	1	1
Water	5	5	5
Sucrose	56	56	56

† The salts mixture consisted of NaCl (139.3 g); KI (0.79 g): KH_2PO_4 (389.0 g);$MgSO_4.7H_2O$ (57.3 g);$CaCO_3$ (381.4 g); $FeSO_4.7H_2O$ (27.0 g); $CoCl_2$ (0.023 g).

†† The vitamin mixture consisted of menadione (vitamin K) (0.0175 g); choline Cl (7 g); p-amino benzoic acid (0.3500 g); inositol (0.3500 g); nicotinic acid (0.1400 g); Ca pantothenate (0.1400 g); riboflavine (0.0280g); thiamine HCl (0.0175g); pyridoxine HCl (0.0175 g); folic acid (0.0070 g); biotin (0.0014 g); vitamin B12 (0.000105 g); and dextrose (up to 35 g). Vitamins A, D and E were added separately to provide 2000 i.u. vitamin A, 200 i.u. vitamin D and 30 i.u. vitamin E per 100g diet.

Table 2.2 Composition of rat pellets (% by weight) used during trial dietary tests and during recovery periods. Fat content (% by weight) of each ingredient is in parentheses. Information provided by Fielders' Agricultural Products.

Ingredient	**Amount** (% by wt)
Cereals, bran, pollard	55 - 60 (2 - 2.5)
Vegetable protein (cottonseed/canola/soybean meals)	15 - 20 (2 - 2.5)
Meat and bone meal	15 (10)
Beef tallow	1.5 - 2.0 (99)
Whey powder	4 - 5 (0.5)
Salts, vitamins, minerals	3 (0)

Table 2.3 Fatty acid composition (%) of Control, essential fatty acid deficient (EFAD), polyunsaturated fatty acid (PUFA) enriched diets and rat pellets. Values represent the mean ± SE of three determinations.

	Dietary Group			Rat
Fatty Acid	**Control**	**EFAD**	**PUFA**	**Pellets**
8:0	0.0	4.1 ± 1.3	0.0	0.0
10:0	0.0	7.2 ± 0.3	0.0	0.0
12:0	0.0	56.9 ± 0.6	0.0	0.0
14:0	0.0	16.9 ± 0.4	2.8 ± 0.1	1.71 ± 0.0
15:0	1.38 ± 0.05	0.46 ± 0.04	1.4 ± 0.1	1.37 ± 0.01
16:0	10.3 ± 0.05	6.9 ± 0.2	12.9 ± 0.1	20.3 ± 0.2
16:1	0.0	0.0	3.33 ± 0.05	1.58 ± 0.03
16:4, n-3	0.0	0.0	0.95 ± 0.03	0.0
18:0	4.09 ± 0.02	2.4 ± 0.1	3.7 ± 0.1	8.6 ± 0.2
18:1, n-9	28.6 ± 0.2	4.1 ± 0.2	22.1 ± 0.2	31.3 ± 0.3
18:1, n-7	1.01 ± 0.02	0.0	1.69 ± 0.02	1.30 ± 0.04
18:2, n-6	50.12 ± 0.04	1.15 ± 0.07	35.2 ± 0.1	29.8 ± 0.3
18:3, n-3	3.94 ± 0.04	0.0	3.00 ± 0.03	2.15 ± 0.02
18:4, n-3	0.0	0.0	1.34 ± 0.02	0.0
20:0	0.38 ± 0.01	0.0	0.2 ± 0.1	0.38 ± 0.01
20:1, n-9	0.0	0.0	0.38 ± 0.05	0.33 ± 0.01
20:4, n-6	0.0	0.0	0.19 ± 0.09	0.18 ± 0.02
20:5, n-3	0.0	0.0	6.23 ± 0.08	0.0
22:1, n-9	0.0	0.0	0.0	0.0
22:5, n-3	0.12 ± 0.12	0.0	0.55 ± 0.01	0.0
22:6, n-3	0.0	0.0	3.45 ± 0.03	0.0
% Saturated	16.2	94.9	21.3	33.2
% Unsaturated				
% n-9	28.6	4.1	22.5	31.6
% n-6	50.1	1.2	35.4	30.0
% n-3	4.1	0.0	15.5	2.2
Unsaturation Index	142	6	172	101

contained 3% Max EPA oil and 7% sesame oil. Relative proportions of classes of fatty acids in the final diets are shown in Figure 2.1 and total fatty acid composition is provided in Table 2.3.

2.2.1.3 Dietary Trials

One main difficulty of assessing the effects of modified diets is that they may vary in palatability, caloric content or energy availability. Initial trial studies were conducted to establish diets that were isocaloric and equally palatable and thus consumed in approximately equal quantities. A fat level of 10% was chosen, as recommended by the Association of Agricultural Chemists (1960) and this was similar to fat levels used in other studies (5%- Brivio-Haugland, 1976; 7% - Huang *et al.*, 1984; Christon *et al.*, 1992, 10% - Alling *et al.*, 1974, 16% - Charnock *et al.*, 1984).

In formulating a Control diet, sesame oil was used as the fat component. This diet contained 84% unsaturated fat, including 50% n - 6 fatty acids and 4% n - 3 fatty acids, giving an n - 6 to n - 3 ratio of 12 (Figure 2.1). This diet was considered to contain a sufficient amount of both essential fatty acids since the optimal dietary ratio of n - 6 to n - 3 fatty acids is between 4 and 10 (James, 1992). Coconut oil was used for the final EFAD diet. This diet contained 96% saturated fats and only 0.9% n - 6 fatty acids and 0.2% n - 3 fatty acids. A mixture of 7% sesame oil and 3% Max EPA oil was used for the final PUFA diet. Max EPA oil contains 18% eicosapentaenoic acid (EPA - 20:5, n - 3) and 12% docosahexaenoic acid (DHA - 22:6, n - 3) . The PUFA diet contained 78% unsaturated fat, including 35% n - 6 fatty acids and 14% n - 3 fatty acids).

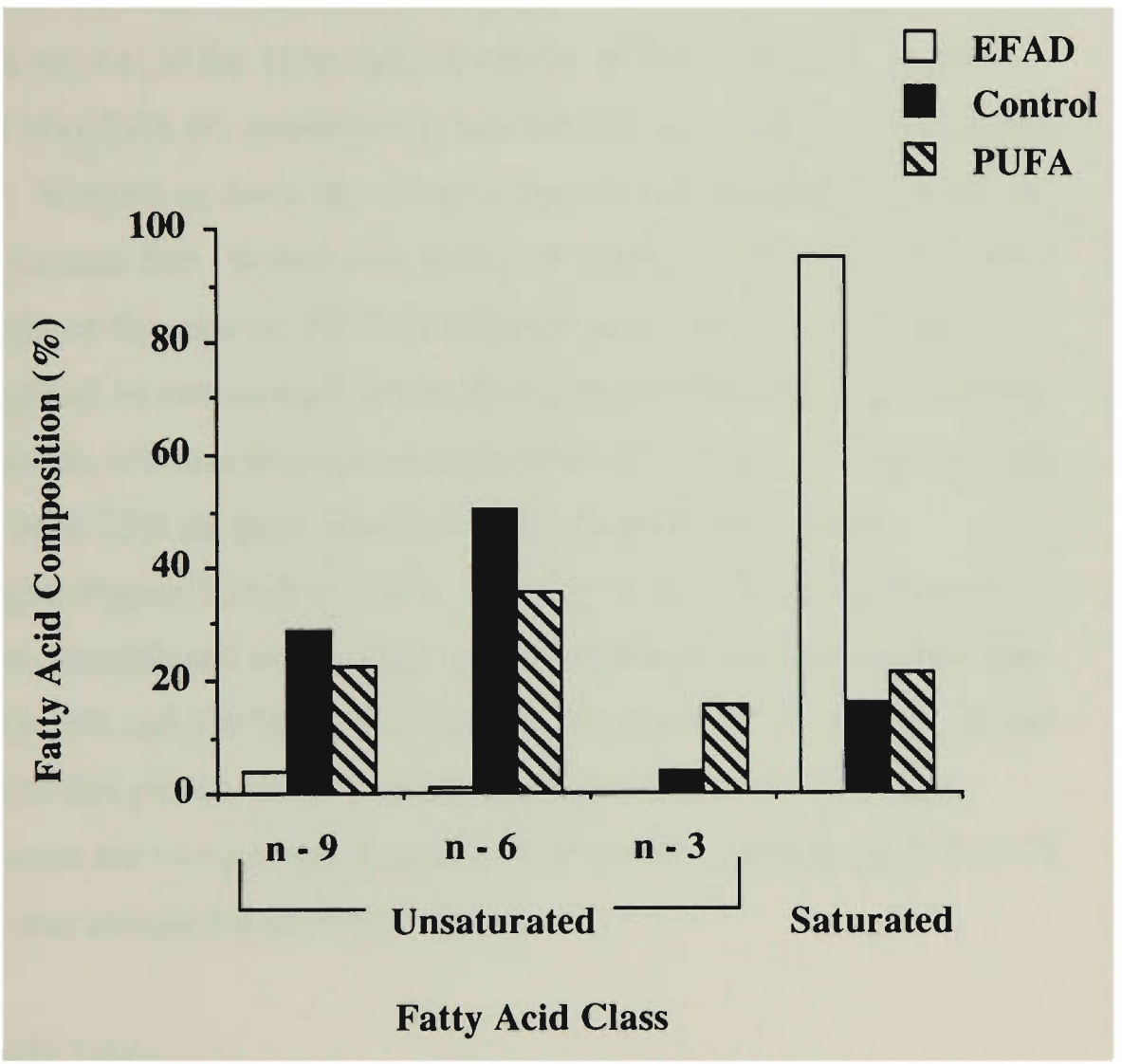


Figure 2.1
Fatty acid composition (%) of the three test diets:Control (containing 10% sesame oil), "essential fatty acid deficient" (EFAD) (containing 10% coconut oil) and "polyunsaturated fatty acid enriched" (PUFA) (containing 7% sesame oil and 3% MaxEPA oil). Values represent the mean of three determinations.

PUFA Trials

Trial tests were conducted to determine the optimum proportion of polyunsaturated fats in the PUFA diet. Groups of five to six rats, six weeks old, were assigned to one of three diets (2%, 3% and 5% Max EPA oil, i.e. of the 100g lipid in one kg of PUFA food; 2, 3 or 5 g was Max EPA oil, respectively, and the rest was made up with sesame oil). Weights of these rats were compared with weights of 14 rats on the Control diet. Within two weeks of starting on the diets, the mean weight of five rats on 5% Max EPA oil was only 72% of the mean weight of 14 rats on the Control diet (consumption was also markedly reduced), whereas the mean weight of six rats on each of the 2% and 3% Max EPA oil diets was 96% of the Control rats' mean weight.(Figure 2.2) ($P < 0.01$). The rats on the 5% Max EPA oil were discontinued at this stage. The remaining rats were maintained on the 2% and 3% Max EPA oil diets for a total of six weeks. At the end of this period, there was no significant difference in weight between the two groups (Figure 2.2) ($P > 0.5$) so a Max EPA level of 3% was chosen for all future studies.

EFAD Trials

In an initial study, an EFAD diet was developed which contained 10% stearic acid. However, the rats on this diet ate significantly more than the rats on the PUFA diet (Figure 2.3 A) and gained significantly less weight than the rats on either the Control or PUFA diets (Figure 2.3 B). Since other work being done in this laboratory at the same time indicated that rats on an EFAD diet containing stearic acid were unable to efficiently utilize the fuel in the diet (Withers, 1990, personal communication), as evidenced by a large amount of energy in their faeces, it was decided to change to 10% coconut oil as the

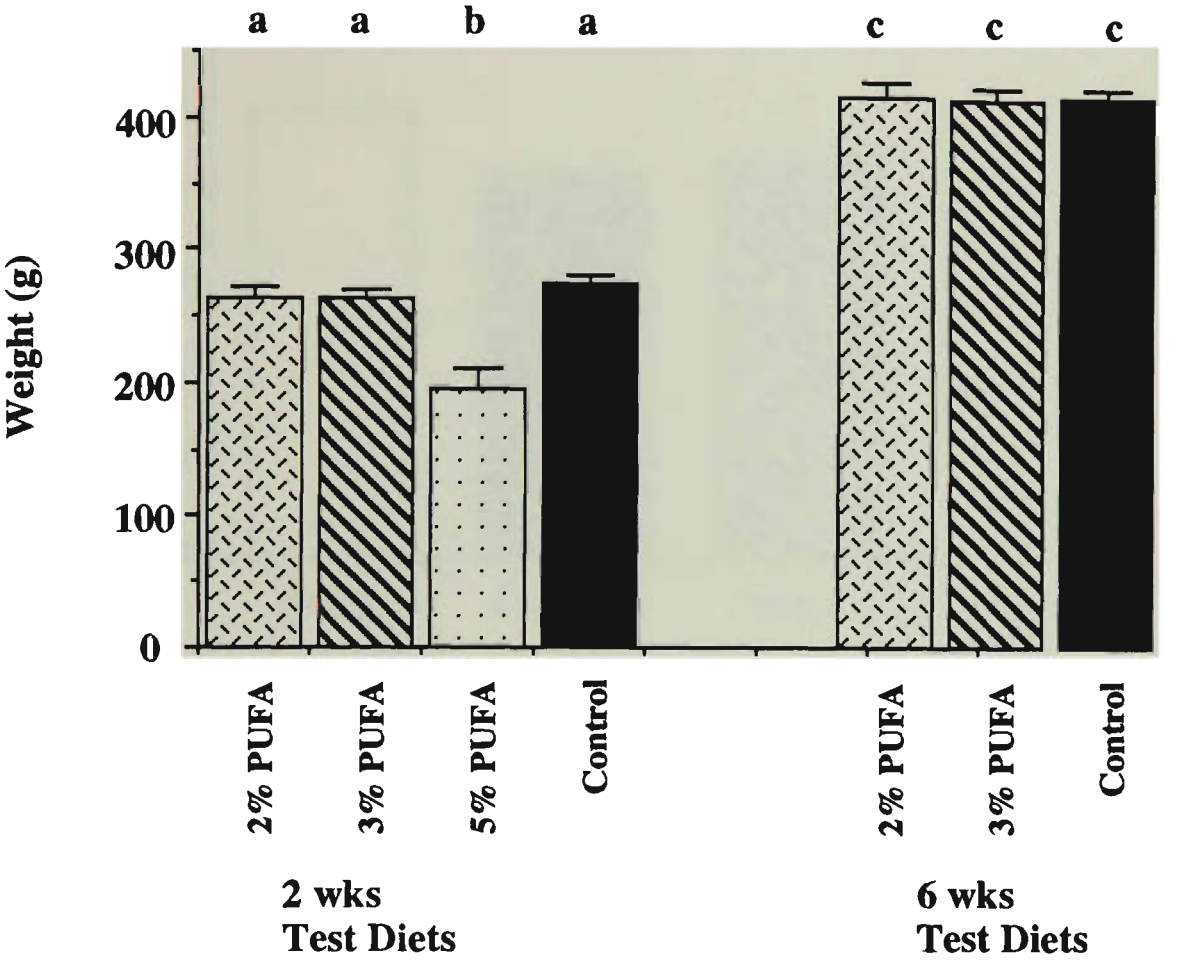


Figure 2.2
Weights (g) of groups of five rats following two weeks and six weeks on test diets (mean ± SE). Initially there were four dietary groups: a Control diet (containing 10% sesame oil) and three trial PUFA diets (containing 2%, 3% or 5% Max EPA oil and 8%, 7% and 5% sesame oil, respectively. After two weeks, weight was significantly heterogeneous ($P < 0.01$); rats on 5% PUFA diets were lighter than rats on 2% PUFA, 3% PUFA and Control diets. These rats were then deleted, leaving the Control group and the 2% and 3% Max EPA oil groups to continue for a further four weeks weeks. After a total of six weeks, weight did not differ significantly ($P > 0.5$). Significantly different treatment means are denoted by different superscripts.

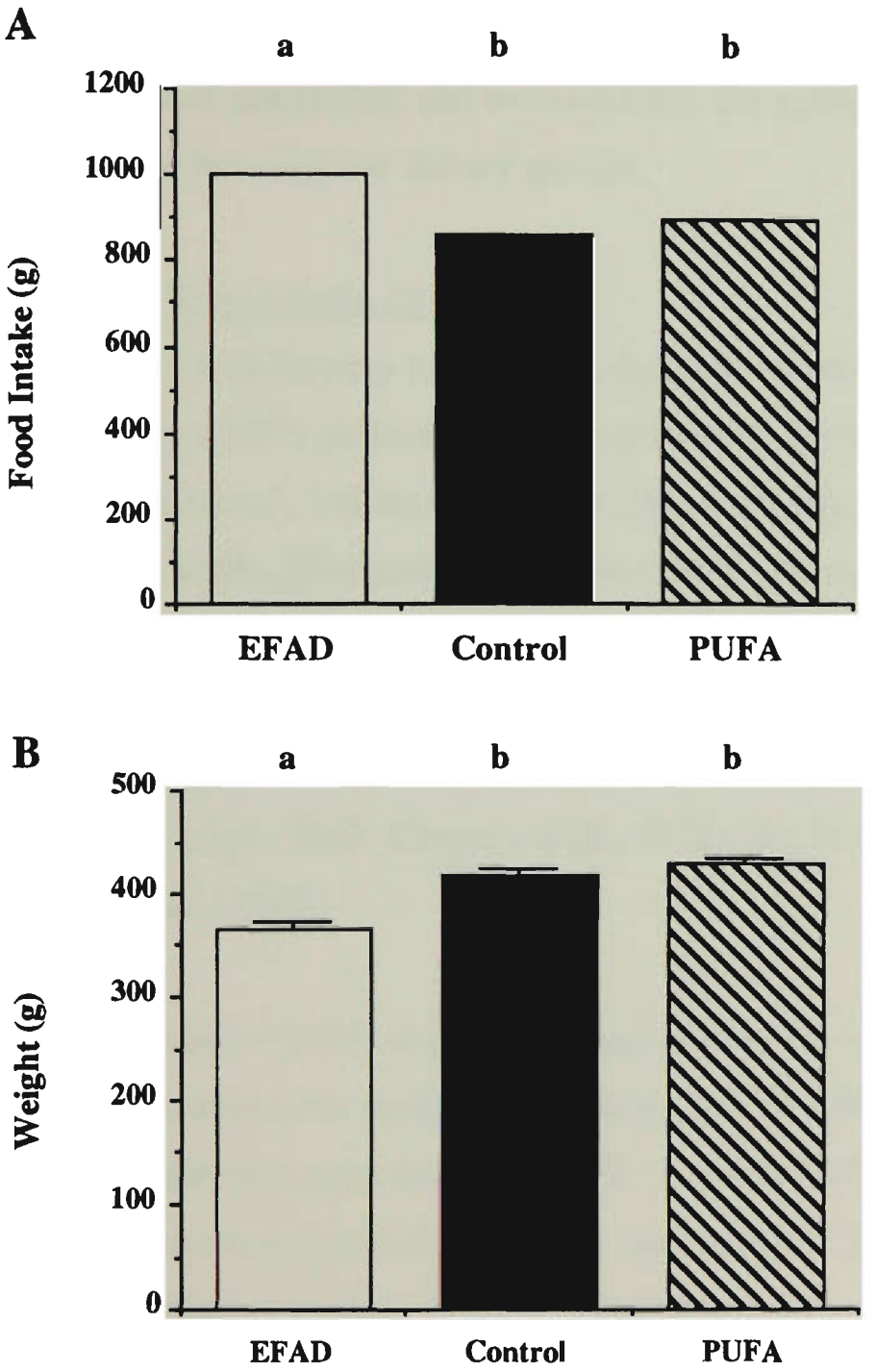


Figure 2.3
Total food intake (g) (Fig. 2.3 A) and weights (g) (Fig. 2.3 B) of groups of 14 twelve-week old rats (mean ± SE) following six weeks on each of three diets: a Control diet (containing 10% sesame oil), an EFAD diet (containing 10% stearic acid) and a PUFA diet (containing 3% Max EPA oil and 7% sesame oil). Both intake ($P < 0.001$; 1-way ANOVA) and weight ($P < 0.001$; 2-way ANOVA) were significantly heterogeneous. Significantly different treatment means are denoted by different superscripts.

lipid source in all future EFAD diets. When coconut oil was used in EFAD diets, there were no differences between the groups in either rate of growth or food intake (See Results).

Daily food consumption for seven litters for nine weeks was recorded as a measure of palatability and to ensure that the amount consumed did not differ between the dietary groups.

2.2.1.4 Time Course of Study

Studies of EFA deficiency have shown the time course required for development of EFA deficiency symptoms in rats varies from four weeks (Rafael *et al.*, 1984) to six weeks (Åaes-Jørgensen & Hølmer, 1969) to 12 weeks (Guarnieri & Johnson, 1970). In studies investigating EFA deficiency, the length of time of test diets varies widely. For example, the duration of studies has been six weeks (Brivio-Haugland *et al.*, 1976), 12 - 14 weeks (Holman, 1960; Pudelkewicz *et al.*, 1968; Christon *et al.*, 1992) and 20 weeks (Phinney *et al.*, 1993).

Innis & Clandinin (1981) used a longitudinal cross-over feeding design to document the rapidity of membrane fatty acid turnover. They estimated that, after feeding rats for 12 days, the half-life of the major cardiac mitochondrial membrane fatty acids is two to three days. In long-term feeding studies, Abeywardena *et al.* (1987) showed that after nine months on a saturated fat diet, the effects of saturated fats on cardiac membrane fatty acid profiles can be significantly modified by cross-over to PUFA-supplemented diets and that the pro-arrhythmogenic effects associated with a diet high in saturated fats can also be reversed by cross-over to a PUFA diet (McLennan *et al.*, 1990).

In this study, a period of nine weeks on the test diets was used. This length of time seemed appropriate based on the findings of Innis & Clandinin (1981). It was chosen to ensure that dietary manipulation did indeed alter membrane composition, to allow sufficient time for physical testing of animals in the later experiments (see Chapters 3 and 4), and so that variations of one to two days in the length of the dietary test period would be unlikely to affect the results.

2.2.1.5 Dissections

In order to remove muscles for weighing and fatty acid analysis (and for enzyme assays - see Chapter 5) rats were firstly killed by concussion and decapitation. Anaesthesia was not used in case muscle function was affected. To remove the soleus and EDL muscles, the skin covering the hind limbs was reflected back. The calcaneal (Achilles) tendon was located, cut with a scalpel and the tendon of soleus muscle grasped with a haemostat clamp. It was then pulled backwards to display the soleus and the proximal tendon was cut. The muscle was separated from underlying muscles and frozen immediately in liquid nitrogen. To remove the EDL muscle, skin from the plantar surface of the foot was removed. The EDL tendon was located and separated from underlying tissue and traced backwards to the proximal tendon. It was separated from other muscles, cut free and frozen in liquid nitrogen. All muscles were then stored at -80^{o}C pending analysis.

2.2.2 Lipid Composition of Diets and Muscles

Lipid analysis was used firstly to confirm the composition of the diets, and secondly, to document changes in muscle membrane lipid composition over time. Analyses of both diets and muscles were done as double-blind trials.

An accepted criteria for the presence of EFAD in mammals is an increased triene-tetraene ratio (i.e. 20:3, n - 9 to 20:4, n - 6), which expresses in one term, the changes in the principal compensatory n - 9 fatty acid (20:3, n - 9) and the principal n - 6 fatty acid (20:4, n - 6) (Holman, 1960). If the rats on the EFAD diet were indeed deficient in essential fatty acids, this would be indicated by an increased level of n - 9 fatty acids (i.e. 16:1, 18:1, n - 9, 20:2, n - 9 and particularly, 20:3, n - 9) and a decreased level of 20:4, n - 6, compared to rats on Control and PUFA diets (Holman *et al.*, 1981).

In the case of the PUFA diet, an indication of altered fatty acid composition in the muscle membranes would be given by increased levels of 20:5, n - 3 and 22:6, n - 3 (as supplied in the PUFA diet) and decreased levels of 20:4, n - 6 (due to the higher competitive ability of the n - 3 fatty acids in the diet) (Holman, 1964; Jeffcoat & James, 1984) and a correspondingly lower n - 6 : n - 3 ratio compared to rats on the Control diets.

The lipid composition of both soleus and EDL muscles was determined in groups of rats at three times: (a) after nine weeks on the diets, and following a recovery period of either (b) two weeks or (c) six weeks on normal rat pellets. To enable comparisons to be

made between various groups of fatty acids, total amounts of saturated and unsaturated fatty acids are presented (Figures 2.6 and 2.7 in Results), as well as a break-down of the unsaturated fatty acids and the Unsaturation Index (U.I.) (Tables 2.4 to 2.9 in Results). The Unsaturation Index provides an approximate estimate of the mean number of double bonds per 100 fatty acid molecules. It is calculated by summing the mole per cent of the individual members of the different unsaturated classes and multiplying the total by the number of double bonds present in the class (White, 1971).

2.2.2.1 Lipid Analysis

All chemicals were analytical grade. Methanol, NaCl, Na_2SO_4, florisil and petroleum ether were from BDH (Melbourne, Australia); chloroform and diethyl ether from Mallinckrodt (Sydney, Australia); BHT (2,6-ditert-butyl-p-cresol) and silicic acid from Sigma (St. Louis, U.S.A.); boron tetrafluoride from Merck (Darmstadt, Germany); and hexane from AJAX (Sydney, Australia).

Lipid Extraction

Total lipids were extracted with chloroform : methanol (2 : 1) using the method of Folch *et al.* (1957). Approximately 100 mg of muscle tissue was homogenised (IKA Ultra Turrax T 25 with S25N 8G dispersing rotor) with 20 ml chloroform / methanol (2:1, v/v) at 2400 rpm for 45 s. Both chloroform and methanol contained 0.01% of the antioxidant BHT. After filtering (Whatman #1), 5 ml of 0.73% NaCl was added to the homogenate. Lipids were extracted in the chloroform phase using 3 x 1 ml additions of chloroform. Small amounts of anhydrous Na_2SO_4 were added to dry the combined chloroform extracts, excess chloroform was removed under vacuum

on a rotovaporator at 35 ^{o}C and samples were dried overnight in a desiccator.

Separation of Phospholipids and Neutral Lipids

The neutral lipids were separated from the phospholipids using silicic acid chromatography using the method of Borgstrom *et al.* (1952). Silicic acid columns (8 cm long, 0.62g, 325 mesh) were prepared in tubes (bottom i.d. 5 mm, length 100 mm; top i.d. 10 mm, length 100 mm) which had a flow rate of 0.1 ml.min^{-1}. Lipid samples were washed onto the adsorbent with 2 ml chloroform and neutral lipids and free fatty acids were eluted from the column with 10 ml chloroform. Phospholipids were then eluted with 12 ml methanol and collected in 15 ml teflon-lined screw cap test tubes (Crown Corning, Sydney, Australia).

Methylation of Fatty Acids

Phospholipid samples were evaporated to dryness under nitrogen. Boron trifluoride (14%) in methanol (2 ml) was added and the samples heated at 75 ^{o}C for 60 minutes before being cooled in ice. Methylated fatty acids were extracted with 3 x 3 ml petroleum ether (BP 40 - 60 ^{o}C). Extracts were combined and dried over anhydrous Na_2SO_4 and then evaporated down to approximately 3 ml under nitrogen (Morrison & Smith, 1964).

Purification of Methylated Fatty Acids

Methylated fatty acids were purified on Florisil chromatography columns using the method of Carrol *et al.* (1961). Florisil (60 - 100 mesh) was washed with chloroform, methanol and water and dried overnight at 110^{o} C. After cooling, water (7 ml per 93 g Florisil) was added and allowed to equilibrate for 24 hours. Florisil columns (10 cm) were

prepared in tubes (i.d. 6mm) with a flow rate of 0.5 ml . min^{-1}. Samples were applied to the column head, washed with petroleum spirit (8 ml) and methyl esters were eluted with 5% diethyl ether (7 ml) in petroleum ether (7 ml). Methyl esters were collected in teflon-lined screw cap vials and evaporated to dryness under nitrogen. The gas liquid chromatographic solvent, hexane, was added and samples were stored at 0° C until analysis.

Gas Chromatography

Methylated fatty acids were quantitatively analysed using gas chromatography (Hewlett Packard 427 gas chromatograph) and a flame ionization detector. Methylated fatty acids (1 µl) were injected onto a fused silica WCOT column (SGE) with the temperature maintained at 170 °C for 20 minutes and then raised 1° each minute until 195 °C. Results were recorded on an electronic integrator (Shimadzu CR-3) which determined peak areas and retention times and calculated the mole percent of each fatty acid. Individual fatty acids were identified by comparison with retention times of standard methyl esters (Sigma, St. Louis, U.S.A.; Larodan, Sweden and Activon, Sydney, Australia).

2.2.2.2 Dietary Lipids

Lipid analysis revealed the predicted differences in fatty acid composition (Table 2.3). The proportions of total saturated and unsaturated fatty acids were relatively similar in the Control and PUFA diets (ratio of unsaturated to saturated fatty acids was 5.2 and 3.5, respectively), but the EFAD diet was at least 4 times more saturated (ratio of unsaturated to saturated fatty acids was 0.05). Although the total level of unsaturation was similar for the Control and PUFA diets, the proportions of n - 6 and n - 3 polyunsaturated fatty acids differed between the two groups since

the PUFA diet contained 3% Max EPA oil which contains a high proportion of n-3 fatty acids (Shils & Young, 1988). In the rat pellets, the ratio of unsaturated to saturated fatty acids was 2.0. As expected, the U.I. was greatest in the PUFA diet (172). The U.I. in the Control diet and the rat pellets was 142 and 101, respectively. These were in great contrast with the EFAD diet where the U.I. was 6.

2.2.3 Statistical Methods

All measurements are expressed as mean ± SE. The level of statistical significance chosen for this experiment was $P < 0.05$.

One-way ANOVA was used to test for heterogeneity of weight gain and food intake in the trial diet studies. (Since some litters contributed different numbers of rats to the treatment groups, the statistical package, SAS, was used to estimate "variance components". This was done using a technique called Restricted Maximum Likelihood Estimation in which the variability between litters and the intrinsic variability (between rats of the same litter on the same treatment) was estimated. These "variance components" were then used to calculated "adjusted means" for each treatment and appropriate standard errors. The standard errors are estimates of the standard deviations of the differences between the various "adjusted means". A Least Squares analysis of variance-covariance matrix of these estimates was then performed using the statistical package, Minitab. (The litter effect was treated as a "random" factor and the dietary group was treated as a "fixed" effect). Once the diets were established and equal numbers of rats per litter were assigned to each

dietary group, 2-way ANOVA with diet and litter as the factors was used to test for heterogeneity of total food intake and rat weights. All data and matching statistical analyses for the trial studies are presented in Appendix 1.

Correlation coefficients between individual membrane phospholipid fatty acids were calculated to test for independence, since it would be expected that if the percentage of one particular muscle membrane fatty acid changed as a result of dietary lipid manipulation, then others would also be changed. There were in fact several pairs of fatty acids which were highly correlated. In soleus at each of the three testing times (i.e. after nine weeks on the diet and following two or six weeks recovery), examples of highly correlated fatty acids included:
22:5, n - 3 and 22:6 n - 3 ($r = 0.78$ after the dietary period, $r = 0.70$ after two weeks recovery; $r = 0.48$ after six weeks recovery)
22:5, n - 6 and 22:6, n - 3 ($r = 0.61, 0.86, 0.95$)
22:4, n - 6 and 22:6, n - 3 ($r = 0.65, 0.92, 0.82$)
20:5, n - 3 and 22:6, n - 3 ($r = 0.71, 0.76, 0.22$)
20:5, n - 3 and 22:4, n - 6 ($r = 0.56, 0.78, 0.45$)
22:4, n - 6 and 20:5, n - 3 ($r = 0.68, 0.89, 0.48$)
18:2, n - 6 and 20:3, n - 9 ($r = 0.82, 0.59, 0.39$).
Despite this lack of independence, there was no alternative method for analysing percentages of individual fatty acids in the three dietary groups and the results were tested for heterogeneity by ANOVA.

An analysis of variance by ranks (Kruskal-Wallis test) was used to test for heterogeneity of fatty acid composition in muscles, since these data were not normally distributed. In cases where there was a significant difference between the dietary groups, in order to determine which of

the samples were significantly different, a non-parametric multiple comparison (Student-Newman-Keuls mutiple range test of means) was used when sample sizes were equal, with a modified standard error (Zar, 1984) for unequal sample sizes.

2.3 Results

2.3.1 Food Consumption and Rat Weights

Rats on all three diets showed similar patterns of food consumption throughout the nine week test period (Figure 2.4). Furthermore, total food consumption over nine weeks was not significantly heterogeneous ($P = 0.76$) and ranged from 967 ± 42g on the Control diet to 1004 ± 34g on the PUFA diet.

Rat weights did not differ significantly between the three dietary groups after nine weeks on the diets, nor during the two and six week recovery periods when rats were fed normal rat pellets (Figure 2.5). Body weight increased about 8-fold during the nine week period on the test diets, but there was only a further 10 - 12% increase during the two week recovery period. Body weight appeared to decrease during the six week recovery period, but it must be remembered that different groups of rats were used at each stage.

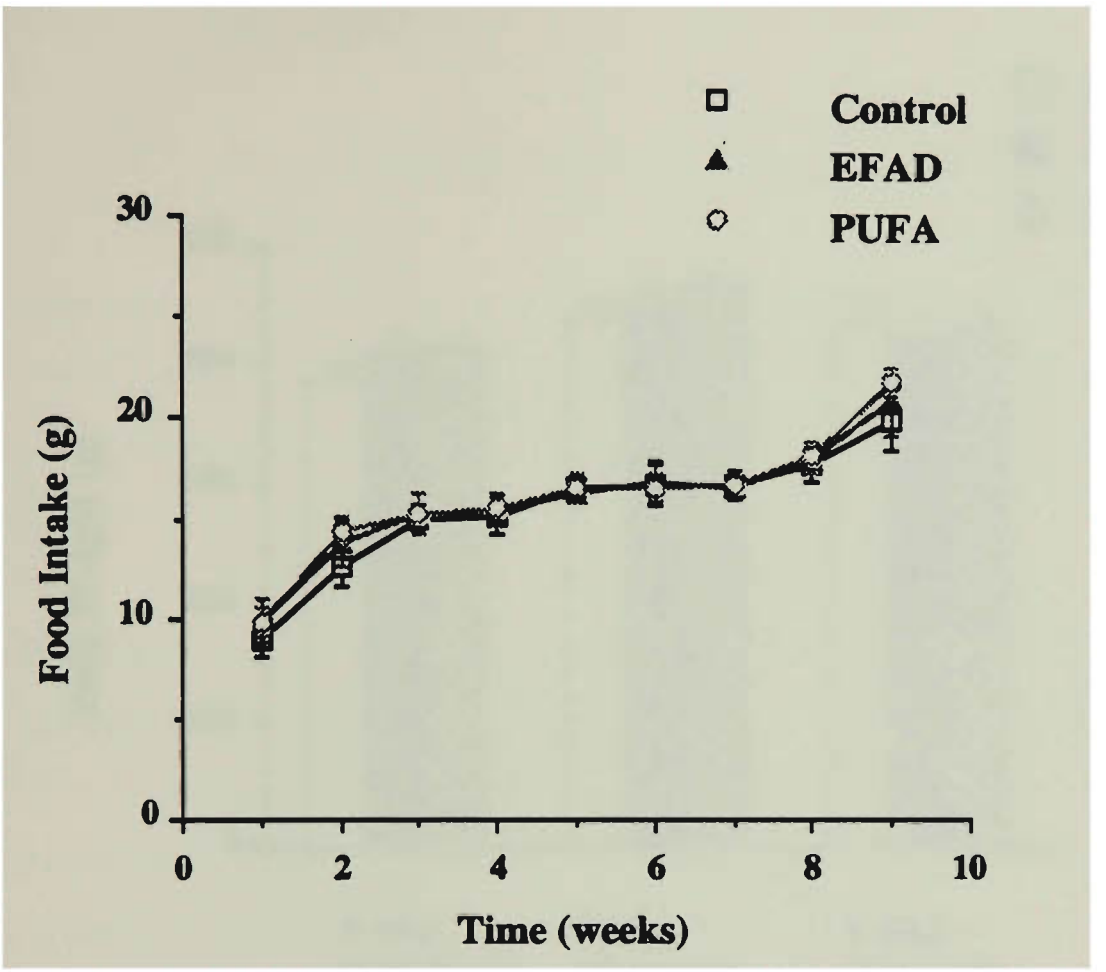


Figure 2.4

Mean daily intake (g) of groups of seven rats (mean ± SE) during nine weeks on each of three test diets: Control, EFAD and PUFA diet . Values shown represent daily consumption averaged for the preceding week.

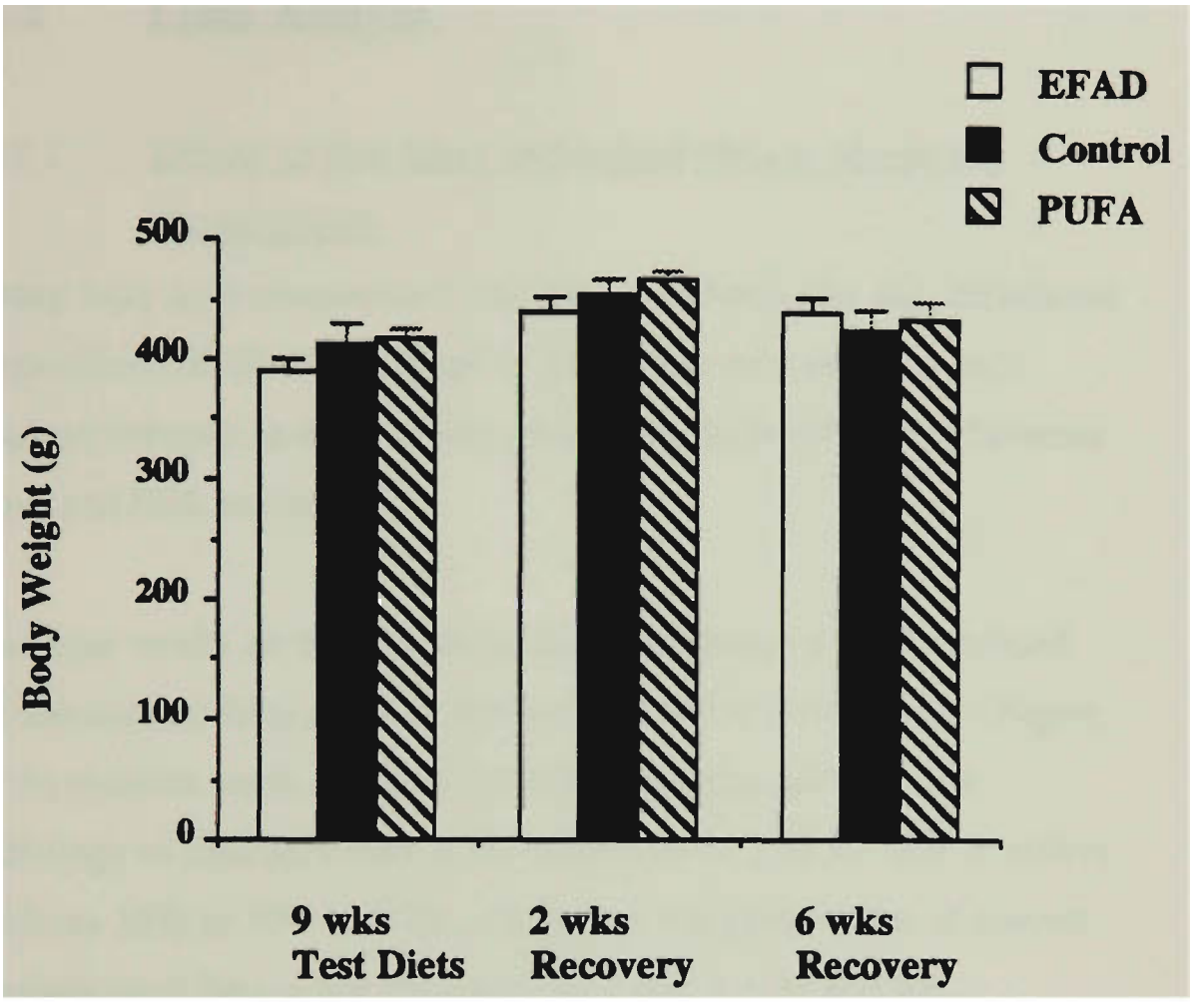


Figure 2.5
Weights (g) of groups of five to seven rats (mean ± SE) after nine weeks on the three test diets:Control, EFAD and PUFA, followed by a recovery period of zero, two or six weeks on normal rat pellets. Diet did not affect body weight at any stage ($P > 0.5$ at each time).

2.3.2 Lipid Analysis

2.3.2.1 Effects of Test Diets on Skeletal Muscle Membrane Phospholipids

Dietary fatty acid composition was clearly reflected by the membrane compositions of all three groups of rats, however there was little variation between animals within groups and little difference between soleus and EDL muscles.

After nine weeks on the test diets, the proportions of total saturated and unsaturated fatty acids in soleus (Figure 2.6 A) and EDL (Figure 2.7 A) muscles were similar for all three dietary groups. The percentage of saturated fatty acids ranged from 32% to 34% in soleus and from 38% to 39% in EDL. However, the proportions of classes of unsaturated fatty acids (Figures 2.6 A and 2.7 A) and the Unsaturation Index (U.I.) differed significantly between dietary groups (Tables 2.4 and 2.5). Importantly, the U.I. for the EFAD group was lowest and the U.I. for the PUFA group was greatest for both soleus and EDL muscles.

A. Essential Fatty Acid Deficiency

Muscle membrane phospholipids from both soleus and EDL muscles of the EFAD rats contained a significantly higher proportion of n - 9 fatty acids than either the Control and PUFA rats (ranging from three to six times more) ($P < 0.005$ for soleus and $P < 0.02$ for EDL) (Figures 2.6 A and 2.7 A). In particular, the EFAD group contained significantly higher levels of 20:3, n - 9, ranging from 60 to 100 times the level found in muscles from Control and PUFA rats ($P < 0.001$ for both soleus and EDL) (Tables 2.4 and 2.5). As a result,

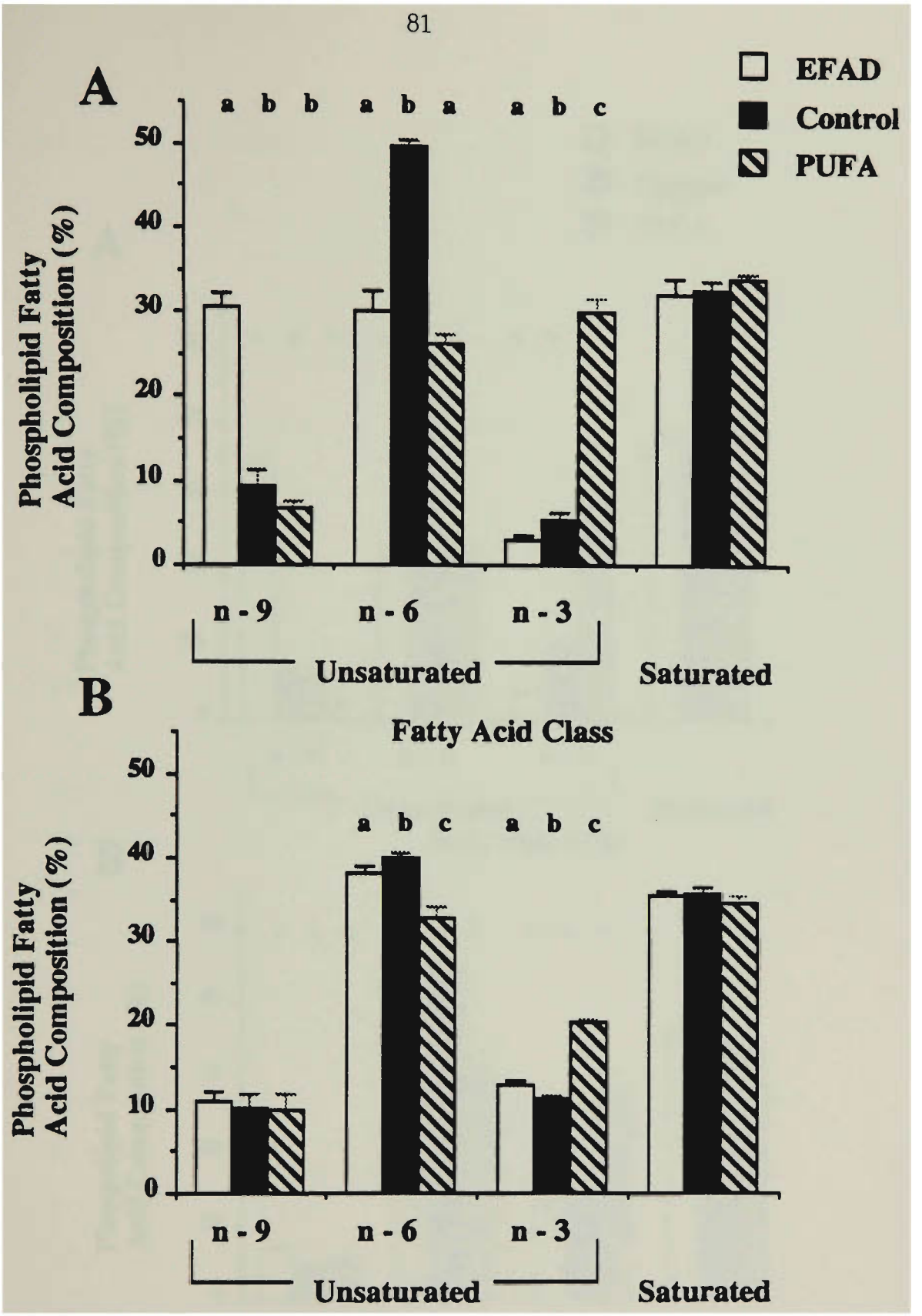


Figure 2.6
Phospholipid fatty acid composition (%) of soleus muscle membranes after nine weeks on the three test diets: Control, EFAD and PUFA (Fig. 2.6 A) followed by six weeks recovery on normal rat pellets (Fig. 2.6 B). Diet had a significant effect on the proportions of n - 9 fatty acids ($P < 0.005$); n - 6 fatty acids ($P < 0.01$) and n - 3 fatty acids ($P < 0.001$) after nine weeks as well as a significant effect on n - 6 fatty acids ($P = 0.001$) and n - 3 fatty acids ($P < 0.02$) after six weeks, as judged by 1-way ANOVA. Significantly different treatment means are denoted by different superscripts

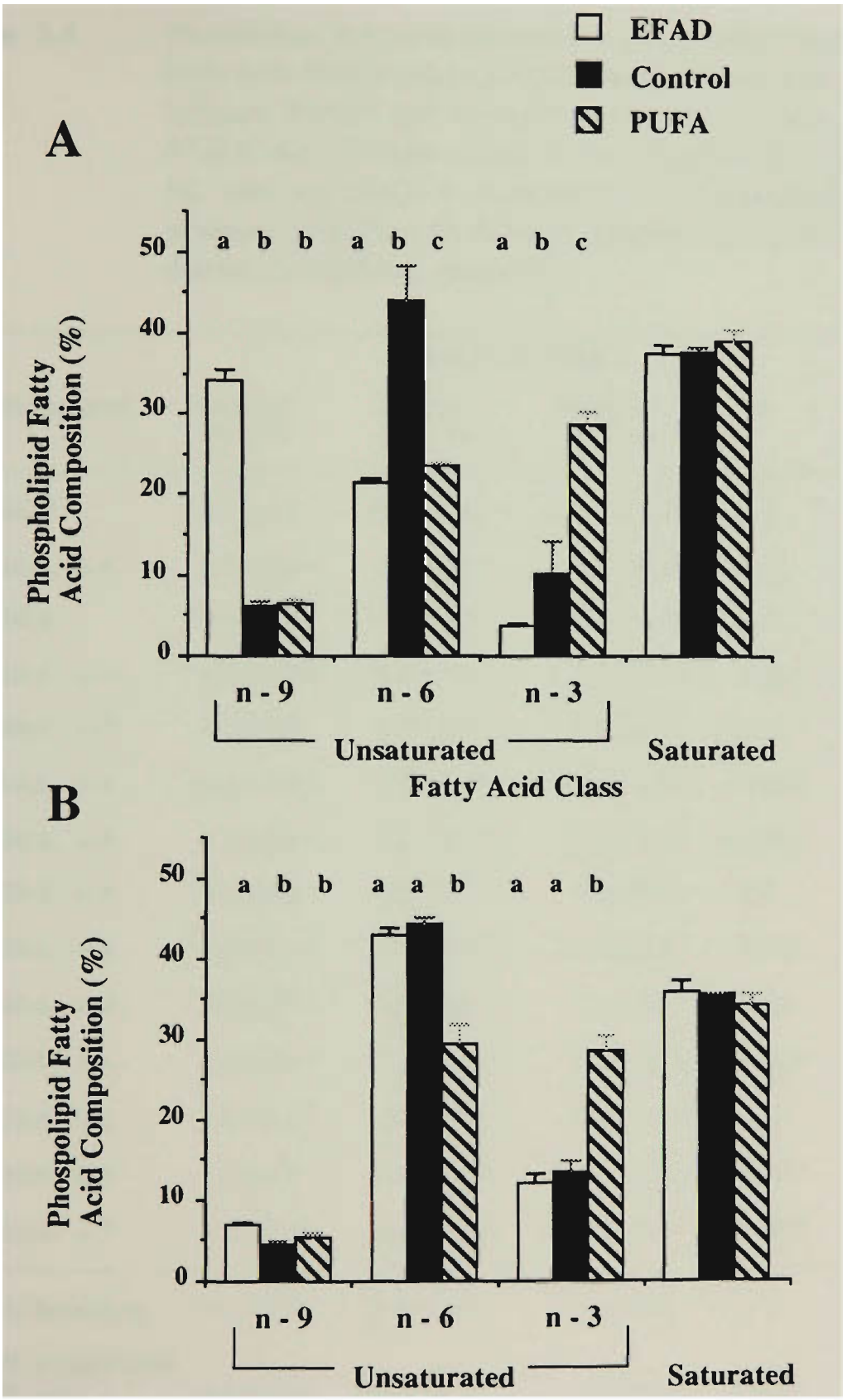


Figure 2.7
Phospholipid fatty acid composition (%) of EDL muscle membranes after nine weeks on the three test diets: Control, EFAD and PUFA (Fig. 2.7 A) followed by six weeks recovery on normal rat pellets (Fig. 2.7 B). Diet had a significant effect on the proportions of n - 9 fatty acids ($P < 0.02$); n - 6 fatty acids ($P < 0.001$) and the n - 3 fatty acids ($P < 0.001$) after nine weeks, as well as significant effect on n - 9 fatty acids ($P < 0.002$), n - 6 fatty acids ($P < 0.005$) and n - 3 fatty acids ($P < 0.02$) after six weeks, as judged by 1-way ANOVA. Significantly different treatment means are denoted by different superscripts.

Table 2.4 Phospholipid fatty acid composition (%) of soleus muscles from male Wistar rats on Control, essential fatty acid deficient (EFAD) and increased polyunsaturated fatty acid (PUFA) diets for nine weeks. Values represent the mean ± SE. One-way ANOVAs were used to test for heterogeneity of means. Significantly different treatment means are denoted by different superscripts.

	Dietary Group			
Fatty Acid	**Control (n = 4)**	**EFAD (n = 4)**	**PUFA (n = 4)**	**P**
16:0	13.2 ± 0.6	10.2 ± 1.8	14.2 ± 1.0	N.S.
16:1, n-9	0.9 ± 0.4 [a]	5.3 ± 0.5 [b]	0.7 ± 0.4 [a]	< 0.02
18:0	19.4 ± 1.6	21.9 ± 1.8	20.0 ± 1.3	N.S.
18:1, n-9	8.4 ± 1.7 [a]	15.6 ± 1.4 [b]	6.0 ± 0.5 [a]	0.001
18:1, n-7	3.1 ± 0.2	4.2 ± 0.7	2.9 ± 0.3	N.S.
18:2, n-6	20.1 ± 1.4 [a]	9.2 ± 1.0 [b]	15.4 ± 1.0 [a]	< 0.002
20:3, n-9	0.1 ± 0.1 [a]	9.6 ± 0.3 [b]	0.0 ± 0.0 [a]	< 0.001
20:3, n-6	0.4 ± 0.2	0.9 ± 0.1	0.5 ± 0.2	N.S.
20:4, n-6	23.0 ± 1.2 [a]	17.7 ± 2.1 [a]	10.4 ± 0.4 [b]	< 0.002
20:5, n-3	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.1 ± 0.4 [b]	< 0.02
22:4, n-6	2.4 ± 0.6 [a]	1.1 ± 0.2 [a]	0.0 ± 0.0 [b]	< 0.002
22:5, n-6	3.8 ± 0.5 [a]	1.2 ± 0.7 [b]	0.0 ± 0.0 [b]	< 0.01
22:5, n-3	1.6 ± 0.9 [a]	0.2 ± 0.1 [b]	3.5 ± 0.2 [a]	< 0.005
22:6, n-3	3.7 ± 0.3 [a]	2.6 ± 0.5 [a]	25.5 ± 1.7 [b]	< 0.002
% Saturated	32.6 ± 1.3	32.1 ± 1.9	34.2 ± 0.5	N.S.
% Unsaturated				
% n-9	9.4 ± 2.0 [a]	30.5 ± 1.6 [b]	6.6 ± 0.9 [a]	< 0.005
% n-6	49.6 ± 0.8 [a]	30.0 ± 2.5 [b]	26.3 ± 1.1 [b]	< 0.01
% n-3	5.4 ± 0.9 [a]	2.9 ± 0.5 [b]	30.1 ± 1.6 [c]	< 0.001
Unsaturation Index	205 ± 6 [a]	175 ± 8 [b]	259 ± 7 [c]	0.001

Table 2.5 Phospholipid fatty acid composition (%) of EDL muscles from male Wistar rats on Control, essential fatty acid deficient (EFAD) and increased polyunsaturated fatty acid (PUFA) diets for nine weeks. Values represent the mean ± SE. One-way ANOVAs were used to test for heterogeneity of means. Significantly different treatment means are denoted by different superscripts.

	Dietary Group			
Fatty Acid	**Control (n = 4)**	**EFAD (n = 4)**	**PUFA (n = 4)**	**P**
16:0	21.8 ± 1.0	23.9 ± 0.7	21.6 ± 2.2	N.S.
16:1, n-9	0.2 ± 0.2 ^{a}	6.4 ± 0.6 ^{b}	0.3 ± 0.2 ^{a}	< 0.005
18:0	15.9 ± 0.3 ^{a}	13.6 ± 0.7 ^{b}	17.6 ± 1.0 ^{a}	< 0.005
18:1, n-9	5.9 ± 0.3 ^{a}	21.6 ± 0.5 ^{b}	6.2 ± 0.3 ^{a}	< 0.002
18:1, n-7	21. ± 0.1 ^{a}	3.3 ± 0.1 ^{b}	2.2 ± 0.2 ^{b}	< 0.01
18:2, n-6	19.1 ± 1.3 ^{a}	6.8 ± 0.5 ^{b}	16.3 ± 0.6 ^{a}	< 0.002
20:3, n-9	0.1 ± 0.1 ^{a}	6.1 ± 0.7 ^{b}	0.0 ± 0.0 ^{a}	< 0.001
20:3, n-6	0.2 ± 0.2 ^{a}	0.5 ± 0.2 ^{b}	0.0 ± 0.0 ^{ab}	< 0.05
20:3, n-3	1.7 ± 1.2	0.6 ± 0.1	0.8 ± 0.0	N.S.
20:4, n-6	19.9 ± 2.0 ^{a}	11.3 ± 0.7 ^{b}	7.2 ± 0.4 ^{c}	< 0.001
20:5, n-3	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	N.S.
22:4, n-6	2.0 ± 0.6 ^{a}	0.9 ± 0.1 ^{a}	0.0 ± 0.0 ^{b}	< 0.005
22:5, n-6	2.8 ± 0.8 ^{a}	2.0 ± 0.1 ^{a}	0.0 ± 0.0 ^{b}	< 0.005
22:5, n-3	1.2 ± 0.3 ^{a}	0.5 ± 0.0 ^{b}	3.1 ± 0.2 ^{c}	< 0.001
22:6, n-3	7.2 ± 3.9 ^{a}	2.3 ± 0.2 ^{b}	24.5 ± 1.3 ^{c}	< 0.001
% Saturated	37.7 ± 0.7	37.5 ± 1.1	39.1 ± 1.3	N.S.
% Unsaturated				
% n-9	6.1 ± 0.5 ^{a}	34.0 ± 1.4 ^{b}	6.5 ± 0.5 ^{a}	< 0.02
% n-6	43.9 ± 4.5 ^{a}	21.5 ± 0.4 ^{b}	23.5 ± 0.4 ^{c}	< 0.001
% n-3	10.2 ± 4.1 ^{a}	3.7 ± 0.3 ^{b}	28.7 ± 1.6 ^{c}	< 0.001
Unsaturation Index	203 ± 8 ^{a}	143 ± 3 ^{b}	237 ± 9 ^{c}	0.001

muscles from the EFAD rats also had a high triene-tetraene ratio (Figure 2.8). After nine weeks on the EFAD diet, the triene-tetraene ratio was 0.56 ± 0.06 for soleus and 0.54 ± 0.08 for EDL. Triene-tetraene ratios greater than 0.4 are considered indicative of essential fatty acid deficiency (Holman, 1960). This compensatory increase in n - 9 fatty acids occurs during EFA deficiency (Holman, 1981) since the desaturase and elongase enzymes cannot preferentially produce fatty acids of the n - 6 and n - 3 classes. Consequently, both muscles from the EFAD rats contained a significantly lower proportion of n - 6 fatty acids (40% lower in soleus and 51% lower in EDL) and n - 3 fatty acids (47% lower in soleus and 64% lower in EDL) than the Control rats (Figures 2.6 A and 2.7 A), since the diet lacked the essential precursors, linoleic acid and linolenic acid (Table 2.3) (for soleus, $P < 0.01$ for n - 6 and $P < 0.001$ for n - 3 fatty acids; for EDL, $P < 0.001$ for n - 6 and $P < 0.001$ for n - 3 fatty acids).

B. Enrichment with n - 3 Polyunsaturated Fatty Acids

In the PUFA rats, membrane phospholipids from both soleus and EDL muscles contained a significantly higher proportion of n - 3 fatty acids than either the Control or EFAD rats (ranging from 3 - 10 time more) ($P < 0.001$ for both soleus and EDL) and a significantly lower proportion of total n - 6 fatty acids than the Control rats (47% less in both muscles) ($P < 0.01$ for soleus and $P < 0.001$ for EDL) (Figures 2.6 A and 2.7 A). There was no difference in the proportions of n - 9 fatty acids in muscles from Control and PUFA rats.

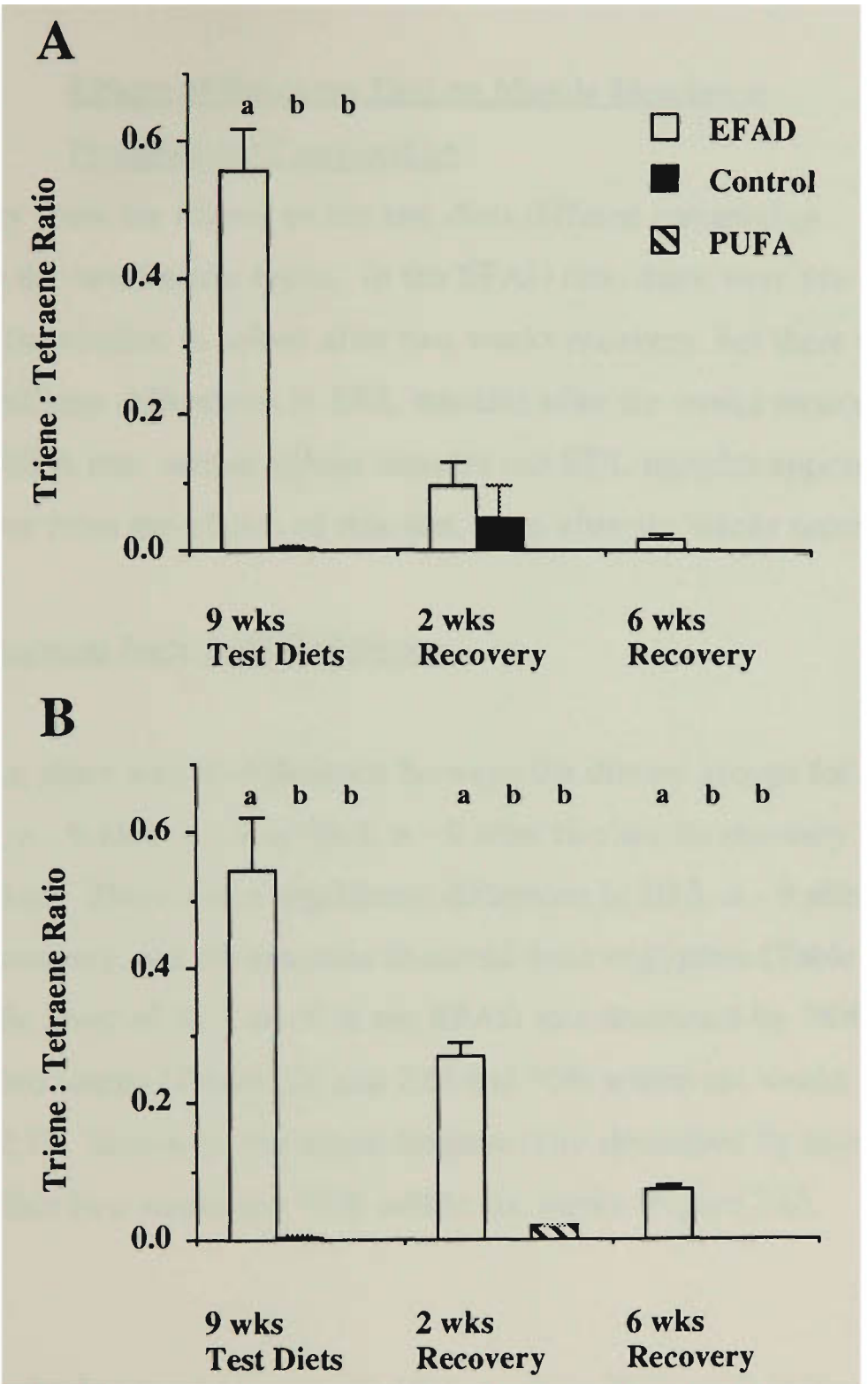


Figure 2.8
Triene : Tetraene ratio for soleus muscles (Fig. 2.8 A) and EDL muscles (Fig. 2.8 B) muscles after nine weeks on the three test diets: Control, EFAD and PUFA, followed by zero, two or six weeks recovery on rat pellets. This is a ratio between the amounts of 20:3, n - 9 and 20:4, n - 6 and provides an indication of the level of adequacy of essential fatty acids. If the ratio is greater than 0.4, then the organism is deficient in essential fatty acids (Holman, 1960). In soleus, there was a significant effect of diet on the triene : tetraene ratio in soleus muscles after nine weeks ($P < 0.001$) and in EDL, there was a significant effect of diet after nine weeks ($P < 0.001$), after two weeks recovery ($P < 0.001$) and after six weeks recovery ($P < 0.001$, as judged by 1-way ANOVA. Significantly different treatment means are denoted by different superscripts.

2.3.2.2. Effects of Recovery Diet on Muscle Membrane Phospholipid Composition

Recovery from the effects of the test diets differed remarkably between the two muscle types. In the EFAD rats, there were few differences evident in soleus after two weeks recovery, but there were still significant differences in EDL muscles after six weeks recovery. In the PUFA rats, neither soleus muscles nor EDL muscles appeared to recover from the effects of this diet, even after six weeks recovery.

A. Essential Fatty Acid Deficiency

Soleus

In soleus, there was no difference between the dietary groups for any of 16:1, n - 9,18:1, n - 9 or 20:3, n - 9 after two weeks recovery (Table 2.6). There was a significant difference in 20:3, n - 9 after six weeks recovery, but the amounts involved were negligible (Table 2.7). The level of 20:3, n - 9 in the EFAD rats decreased by 78% within two weeks (Tables 2.4 and 2.6) and 96% within six weeks (Table 2.7). Similarly, the triene-tetraene ratio decreased by about 83% within two weeks and 97% within six weeks (Figure 2.8).

EDL

In EDL, the levels of 16:1, n - 9, 18:1, n - 9 or 20:3, n - 9 in the EFAD group were still significantly higher than in Control and PUFA groups after two weeks recovery (Table 2.8). After six weeks, only the proportion of 20:3, n - 9 remained significantly elevated but the difference was less marked (Table 2.9). In fact, the level of 20:3, n - 9 decreased by 31% within two weeks (Tables 2.5 and 2.8) and 75% within six weeks (Table 2.9). Also, the triene-tetraene ratio decreased by 50% within two weeks and 87% within six weeks (Figure 2.8).

Table 2.6 Phospholipid fatty acid composition (%) of soleus muscles from male Wistar rats on Control, essential fatty acid deficient (EFAD) and increased polyunsaturated fatty acid (PUFA) diets for nine weeks, followed by two weeks recovery on normal rat pellets. Values represent the mean ± SE. One-way ANOVAs were used to test for heterogeneity of means. Significantly different treatment means are denoted by different superscripts.

	Dietary Group			
Fatty Acid	**Control (n = 4)**	**EFAD (n = 4)**	**PUFA (n = 3)**	**P**
16:0	10.2 ± 1.8 ^{ab}	8.3 ± 1.5 ^{a}	13.8 ± 0.1 ^{b}	< 0.05
16:1, n-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N.S.
18:0	24.0 ± 2.0	25.8 ± 0.8	22.1 ± 0.8	N.S.
18:1, n-9	6.1 ± 0.3	5.3 ± 0.3	5.2 ± 0.2	N.S.
18:1, n-7	2.6 ± 0.4	2.6 ± 0.4	3.2 ± 0.3	N.S.
18:2, n-6	14.7 ± 1.0	13.7 ± 0.5	16.4 ± 1.1	N.S.
20:3, n-9	1.0 ± 1.0	2.1 ± 0.7	0.0 ± 0.0	N.S.
20:3, n-6	0.7 ± 0.1	0.4 ± 0.2	0.3 ± 0.3	N.S.
20:4, n-6	21.1 ± 2.3	22.8 ± 1.1	20.4 ± 3.3	N.S.
20:5, n-3	0.3 ± 0.1	0.0 ± 0.0	0.2 ± 0.2	N.S.
22:4, n-6	2.2 ± 0.8	2.4 ± 0.4	2.0 ± 1.0	N.S.
22:5, n-6	3.9 ± 1.3 ^{ab}	6.1 ± 0.3 ^{a}	2.8 ± 1.4 ^{b}	< 0.05
22:5, n-3	2.7 ± 0.2	2.1 ± 0.1	1.8 ± 1.0	N.S.
22:6, n-3	10.8 ± 3.2	8.5 ± 0.6	11.8 ± 6.1	N.S.
% Saturated	34.2 ± 0.3	34.1 ± 0.9	35.9 ± 0.7	N.S.
% Unsaturated				
% n-9	7.1 ± 1.1	7.4 ± 0.9	5.2 ± 0.2	N.S.
% n-6	42.5 ± 3.6	45.4 ± 0.9	41.9 ± 6.3	N.S.
% n-3	13.7 ± 3.5	10.6 ± 0.7	13.7 ± 7.1	N.S.
Unsaturation Index	235 ± 3	236 ± 6	226 ± 16	N.S.

Table 2.7 Phospholipid fatty acid composition (%) of soleus muscles from male Wistar rats on Control, essential fatty acid deficient (EFAD) and increased polyunsaturated fatty acid (PUFA) diets for nine weeks, followed by six weeks recovery on normal rat pellets. Values represent the mean ± SE. One-way ANOVAs were used to test for heterogeneity of means. Significantly different treatment means are denoted by different superscripts.

	Dietary Group			
Fatty Acid	**Control (n = 4)**	**EFAD (n = 4)**	**PUFA (n = 4)**	**P**
16:0	16.2 ± 0.5	15.8 ± 0.6	15.2 ± 0.8	N.S.
16:1, n-9	1.4 ± 0.3	1.9 ± 0.2	1.7 ± 0.4	N.S.
18:0	19.4 ± 0.6	19.7 ±0.2	19.5 ± 0.5	N.S.
18:1, n-9	8.9 ± 1.5	8.7 ± 0.8	8.4 ± 1.3	N.S.
18:1, n-7	2.7 ± 0.3	2.4 ± 0.8	2.1 ± 0.7	N.S.
18:2, n-6	14.8 ± 0.5	14.3 ± 0.3	14.6 ± 0.0	N.S.
20:3, n-9	0.0 ± 0.0 [a]	0.4 ± 0.1 [b]	0.0 ± 0.0 [a]	< 0.02
20:3, n-6	0.1 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	N.S.
20:4, n-6	21.4 ± 0.7 [a]	20.1 ± 0.5 [a]	17.3 ± 0.8 [b]	< 0.005
20:5, n-3	0.2 ± 0.2	0.0 ± 0.0	0.5 ± 0.5	N.S.
22:4, n-6	1.5 ± 0.1 [a]	1.2 ± 0.1 [a]	0.4 ± 0.2 [b]	< 0.01
22:5, n-6	2.3 ± 0.0 [a]	2.3 ± 0.1 [a]	0.1 ± 0.1 [b]	< 0.005
22:5, n-3	2.4 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	N.S.
22:6, n-3	8.6 ± 0.4 [a]	10.6 ± 0.3 [b]	17.3 ± 0.4 [c]	< 0.001
% Saturated	35.6 ± 0.9	35.4 ± 0.5	34.7± 0.7	N.S.
% Unsaturated				
% n-9	10.3 ± 1.6	11.0 ± 1.0	10.0 ± 1.7	N.S.
% n-6	40.1 ± 0.5 [a]	38.2 ± 0.6 [b]	32.8 ± 1.2 [c]	0.001
% n-3	11.2 ± 0.4 [a]	13.0 ± 0.4 [b]	20.5 ± 0.2 [c]	< 0.02
Unsaturation Index	211 ± 3 [a]	216 ± 4 [a]	233 ± 4 [b]	< 0.02

Table 2.8 Phospholipid fatty acid composition (%) of EDL muscles from male Wistar rats on Control, essential fatty acid deficient (EFAD) and increased polyunsaturated fatty acid (PUFA) diets for nine weeks, followed by two weeks recovery on normal rat pellets. Values represent the mean ± SE. One-way ANOVAs were used to test for heterogeneity of means. Significantly different treatment means are denoted by different superscripts.

	Dietary Group			
Fatty Acid	**Control (n = 4)**	**EFAD (n = 3)**	**PUFA (n = 4)**	**P**
16:0	17.4 ± 1.8	21.8 ± 2.0	21.0 ± 1.4	N.S.
16:1, n-9	0.3 ± 0.3 ^{a}	1.5 ± 0.2 ^{b}	0.9 ± 0.3 ^{ab}	< 0.05
18:0	20.5 ± 1.4	17.3 ± 1.4	16.6 ± 1.1	N.S.
18:1, n-9	8.1 ± 0.6 ^{ab}	10.5 ± 0.4 ^{a}	7.2 ± 0.9 ^{b}	< 0.05
18:1, n-7	1.6 ± 0.1	2.1 ± 0.0	1.8 ± 0.1	N.S.
18:2, n-6	14.2 ± 0.8	15.9 ± 1.0	14.6 ± 0.5	N.S.
20:3, n-9	0.0 ± 0.0 ^{a}	4.2 ± 0.2 ^{b}	0.2 ± 0.0 ^{a}	< 0.001
20:3, n-6	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	N.S.
20:3. n-3	0.4 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	N.S.
20:4, n-6	21.3 ± 0.8 ^{a}	15.8 ± 1.0 ^{ab}	10.0 ± 0.3 ^{b}	< 0.01
20:5, n-3	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.1	N.S.
22:4, n-6	3.1 ± 0.3 ^{a}	1.2 ± 0.1 ^{ab}	0.1 ± 0.1 ^{b}	< 0.005
22:5, n-6	5.5 ± 0.6 ^{a}	3.1 ± 0.6 ^{ab}	0.2 ± 0.1 ^{b}	< 0.01
22:5, n-3	1.6 ± 0.1 ^{ab}	1.0 ± 0.1 ^{a}	2.7 ± 0.2 ^{b}	< 0.01
22:6, n-3	5.8 ± 0.4 ^{ab}	3.9 ± 0.6 ^{a}	23.3 ± 1.1 ^{b}	< 0.02
% Saturated	37.9 ± 0.7	39.1 ± 0.7	37.6 ± 0.6	N.S.
% Unsaturated				
% n-9	8.4 ± 0.7 ^{a}	16.3 ± 0.7 ^{b}	8.3 ± 1.2 ^{a}	< 0.05
% n-6	44.1 ± 0.7 ^{a}	36.1 ± 0.8 ^{b}	25.1 ± 0.6^{c}	< 0.001
% n-3	8.1 ± 0.8 ^{ab}	6.3 ± 0.6 ^{a}	27.3 ± 1.1 ^{b}	< 0.05
Unsaturation Index	209 ± 3 ^{ab}	176 ± 8 ^{a}	240 ± 5 ^{b}	< 0.002

Table 2.9 Phospholipid fatty acid composition (%) of EDL muscles from male Wistar rats on Control, essential fatty acid deficient (EFAD) and increased polyunsaturated fatty acid (PUFA) diets for nine weeks, followed by six weeks recovery. Values represent the mean ± SE. One-way ANOVAs were used to test for heterogeneity of means. Significantly different treatment means are denoted by different superscripts.

	Dietary Group			
Fatty Acid	**Control (n = 4)**	**EFAD (n = 4)**	**PUFA (n = 4)**	**P**
16:0	18.0 ± 1.3	19.8 ± 1.5	20.3 ± 0.8	N.S.
16:1, n-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N.S.
18:0	17.6 ± 1.3	16.0 ± 0.2	14.1 ± 1.4	N.S.
18:1, n-9	4.7 ± 0.2	5.7 ± 0.0	5.4 ± 0.4	N.S.
18:1, n-7	1.8 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	N.S.
18:2, n-6	13.7 ± 0.4 ^{a}	18.1 ± 0.3 ^{b}	14.2 ± 1.4 ^{a}	< 0.02
20:3, n-9	0.0 ± 0.0 ^{a}	1.4 ± 0.2 ^{b}	0.0 ± 0.0 ^{a}	< 0.001
20:3, n-6	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	N.S.
20:3, n-3	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	N.S.
20:4, n-6	22.7 ± 1.1 ^{a}	19.0 ± 0.5 ^{b}	14.4 ± 0.8 ^{c}	< 0.001
20:5, n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N.S.
22:4, n-6	2.4 ± 0.2 ^{a}	1.6 ± 0.1 ^{b}	0.6 ± 0.1 ^{c}	< 0.001
22:5, n-6	5.5 ± 0.3 ^{a}	3.8 ± 0.3 ^{b}	0.4 ± 0.0 ^{c}	< 0.001
22:5, n-3	3.1 ± 0.3	2.5 ± 0.1	2.7 ± 0.2	N.S.
22:6, n-3	10.0 ± 0.9 ^{a}	9.0 ± 0.8 ^{a}	25.2 ± 1.7 ^{b}	< 0.002
% Saturated	36.6± 0.1	35.8 ± 1.3	34.3 ± 1.2	N.S.
% Unsaturated				
% n-9	4.7 ± 0.2 ^{a}	7.0 ± 0.1 ^{b}	5.4 ± 0.4 ^{a}	< 0.002
% n-6	44.2 ± 0.7 ^{a}	42.9 ± 0.8 ^{a}	29.5 ± 2.2 ^{b}	< 0.005
% n-3	13.6 ± 1.2 ^{a}	12.1 ± 1.1 ^{a}	28.7 ± 1.8 ^{b}	< 0.02
Unsaturation Index	239± 4 ^{a}	219 ± 7 ^{a}	265 ± 5 ^{b}	< 0.002

During the recovery period, the EFAD rats were receiving the essential precursor n - 6 and n - 3 fatty acids in rat pellets and their enzymes were preferentially producing these longer chain fatty acids. This is evidenced by the decreased proportion of n - 9 fatty acids and the increased proportions of n - 6 and n - 3 fatty acids in both muscles from EFAD rats after recovery (Figures 2.6 A and B and 2.7 A and B).

A. Enrichment with n - 3 Polyunsaturated Fatty Acids

Soleus

In the PUFA rats, the differences in the proportions of both n - 6 and n - 3 fatty acids between the dietary groups were not significantly different after two weeks (Table 2.6), but they were significantly different after six weeks (Table 2.7). Given the large standard errors for both n - 6 and n - 3 fatty acids (specifically 20:4, n - 6 and 22:6, n - 3) after two weeks recovery (Table 2.6), and the results for EDL muscles after two and six weeks recovery (see below), it is likely that the value recorded for 22:6, n - 3 is abnormally low.

After six weeks, the level of n - 3 fatty acids was still significantly higher in the PUFA group than in both the Control and EFAD groups (58 - 83% higher) (Figure 2.6 B).

B. EDL

After two weeks recovery, the PUFA rats still had a significantly higher proportion of n - 3 fatty acids than either the Control or EFAD groups (three to four times higher). After six weeks recovery, there was still a two-fold difference (Figure 2.7 B). The level of n - 3

fatty acids in the PUFA rats was 2.1 and 2.4 times higher than in the Control and EFAD rats, respectively.

2.3.2.3 Variation in Fatty Acid Analysis

As a check on the variability inherent in measuring levels of fatty acids, muscles from both sides of seven rats (five soleus and two EDL) were tested in a double-blind trial. The average percentage difference from the mean for both sides of each rat for 15 fatty acids was 17% ± 3, but results were highly repeatable (i.e. an average percentage difference from the mean of 7% ± 1) for the 8 most common fatty acids (which included 16:0, 16:1, n - 9, 18:0, 18:1, n - 9, 18:2, n - 6, 20:3, n - 9, 20:4, n - 6, 22:5, n - 3 and 22:6, n - 3).

2.4 Discussion

This study provides a rigorous investigation of the dynamics of diet-induced EFA deficiency and heightened PUFA levels in fast and slow skeletal muscles. Both diets produced striking and predictable changes in membrane fatty acids after nine weeks exposure. These findings are supported by the limited set of earlier studies in skeletal muscles which show, separately, the dietary induction of similarly increased PUFA levels in unspecified turkey (Neudoerffer & Lea, 1967), and marmoset (Charnock *et al.*, 1989; 1992) skeletal muscles, sartorius muscles of chicks (Olomu & Baracos, 1991a; b) and Alling *et al.'s* (1972) study of quadriceps (a mixed fibre muscle) in EFAD weanling rats. Recovery of the phospholipid fatty acid composition of the EFAD group to Control levels was typically rapid (i.e. occurring within two weeks) in soleus muscles, but was not complete in EDL

muscles after six weeks. The composition of the muscle phospholipids from the PUFA group did not recover to Control levels since they still contained a high level of n - 3 fatty acids after the six week recovery period. This retention appears comparable to similar findings in retina and brain (Anderson et al., 1992).

Although many studies of dietary manipulation have used experimental periods of several weeks, months or even years, the present study and the earlier work of Innis & Clandinin (1981) shows clearly that major changes in lipid composition can occur within a few days or weeks. It is likely that changes in muscle phospholipid composition during the nine week test period may reflect the creation of much new membrane since body mass increased about 8-fold in that time. However, importantly, there was little additional growth during the recovery period (only 10 - 12% in all dietary groups), but there were equally major changes in fatty acid proportions during the return to Control levels. For example, the total proportion of n - 9 fatty acids in soleus muscles from the EFAD groups decreased by 75% during the two week recovery period. Similar changes occurred in EDL muscles from the EFAD group, but these changes required the six week recovery period. Also, the proportion of n - 3 fatty acids in soleus muscles from the Control group increased 2.5 times during the 2 week recovery period. On this basis, about 100% turnover of muscle membrane phospholipid seems able to occur in less than 14 days (i.e. the two week recovery period).

2.4.1 Effects of Changes in Dietary Lipids on Growth

An important outcome of the preliminary trials was the creation of equally caloric and palatable Control, EFAD, and PUFA diets. This was achieved by trialling various levels and types of fats to ensure that weight gain and intake intake did not differ between dietary groups. Thus changes in membrane lipid composition, including EFA deficiency, were induced under conditions of similar growth rates. In this study, none of the commonly reported symptoms of EFA deficiency (Holman, 1968) were present. Despite this however, it is important to note that these rats were clearly deficient in essential fatty acids as judged by a triene-tetraene ratio greater than 0.4 (Holman, 1960).

In contrast, many previous studies have used a wide array of symptoms, including reduced growth rate, as criteria for EFA deficiency (Holman, 1968; Guarnieri & Johnson, 1970). Other reported symptoms include skin lesions, infertility and increased water uptake and loss. However, diets used in early studies investigating EFA deficiency were often either completely fat-free (Burr & Burr, 1929; Åaes-Jörgensen & Hölmer, 1969; Galli *et al.*, 1970; Sun, 1972; Sun & Sun, 1974; Lin *et al.*, 1979; for review see Guarnieri & Johnson, 1970) and therefore showed obvious caloric differences, and / or specially prepared EFAD diets were compared with commercial Control diets which thus differed in composition (Huang *et al.*, 1984; Sun, 1972; Sun & Sun, 1974). Therefore the claimed effects of EFA deficiency may have reflected reduced caloric intake or altered dietary composition and not solely EFA deficiency.

Recent investigations into the effects of EFA deficiency have controlled food intake and employed more carefully prepared diets. The vast majority of these studies still report a range of symptoms in EFAD animals (Alling et al., 1972, 1974; Nedergaard *et al.*, 1983; Rafael *et al.*, 1984; Dvorak & Stepankova, 1992). However, at least one other recent study which controlled both caloric content and food intake reported no effect of EFA deficiency on growth in pigs (Christon *et al.*, 1992).

It has been suggested that skin lesions (Brown & Burr, 1936) and in fact, the whole set of symptoms, are more pronounced in low rather than high relative humidity (Mead, 1984). Alling *et al.* (1974), using a high and constant relative humidity (60%) found minor skin lesions in only 30% of EFAD rats. Rafael *et al.* (1984) suggested that increased basal metabolism, which is also symptomatic of EFAD (Holman, 1968), may be related to reduced growth rate and increased water consumption. If permeability of the skin increased, as a result of EFA deficiency, there would be greater heat loss through evaporation. In order to maintain a constant body temperature, metabolic rate may increase and if the rate of water loss was too great, there could be growth retardation.

In this study, the fairly high relative humidity (about 60%) may account for the lack of symptoms usually reported in other studies. Obviously it is possible such symptoms of EFA deficiency are related and it may be that all of their effects are diminished in conditions of high relative humidity, as in this study.

It is also possible that strain differences between rats may account for some of the observed effects of EFA deficiency in other studies since most have used Sprague-Dawley rats, and this study employed Wistar rats. However, Sun (1972) used female Wistar rats and reported the usual symptoms, and Phinney *et al.* (1993) used Sprague-Dawley rats and found none of the effects in high humidity.

2.4.2 Effects of Changes in Dietary Lipids on Muscle Phospholipids

Many studies have now established that dietary induced changes in membrane lipid composition in both cells and organelles are integral to changes in biological function. This has been demonstrated in a variety of tissues and clear differences have been shown between organs in their response to changes in dietary fatty acids (Burns *et al.*, 1983; Abeywardena *et al.*, 1991; Charnock *et al.*, 1992). Few studies (Neudoerffer & Lea, 1967; Alling *et al.*, 1972; Charnock *et al.*, 1989; 1992; Storlien *et al.*, 1991; Pan & Storlien, 1993; in Press) however, have examined the effects of changes in dietary fatty acids in skeletal muscle tissue.

The proportions of the different classes of unsaturated fatty acids in the muscle membranes of both EFAD and PUFA groups were as predicted for these particular diets. Studies have shown in mammals that there is no interconversion between n - 9, n - 6 and n - 3 fatty acids (Holman, 1964) and that the types of dietary fats can affect activity of the desaturase enzymes of membranes (Muriana & Ruiz-Gutierrez, 1992). Pan & Storlien (1993) recently showed that fatty acids compete with each other for the desaturation enzymes. The

competitive abilities of the fatty acids appear to act to favour n - 3 fatty acids first, followed by n - 6 and n - 9 fatty acids (Holman, 1964; Jeffcoat & James, 1984). If the diet does not contain adequate amounts of linoleic acid (18:2, n - 6) and linolenic acid (18:3, n - 3), then an animal cannot form longer chain n - 6 and n - 3 fatty acids and concurrently, there will be an increased proportion of n - 9 fatty acids.

For the EFAD rats, there was an increased proportion of 20:3, n - 9 and a reduced proportion of 20:4, n - 6 in muscle phospholipids, as expected for animals on such a diet. Therefore, not only was the triene-tetraene ratio significantly greater than 0.4 (the level considered to be indicative of EFA deficiency (Holman, 1960)), but there were also increased levels of 16:1, n - 9 and 18:1, n - 9 fatty acids compared to the Control rats. These changes are all indicative of EFA deficiency and were no longer apparent following recovery.

For the PUFA rats, the observed increase in n - 3 fatty acids can be explained by the greater competitive capabilities of the n - 3 fatty acids for the elongation and desaturation enzymes (Holman, 1964, Jeffcoat & James, 1984). Since the PUFA diet contained a much high proportion of n - 3 fatty acids than the Control and EFAD diets, these rats were able to manufacture more n - 3 fatty acids, whereas muscles from Control and EFAD groups contained higher proportions of n - 6 and n - 9 fatty acids, respectively.

2.4.3 Recovery from the Effects of Changes in Dietary Lipids

Essential Fatty Acid Deficiency

This study showed some interesting differences between soleus and EDL muscles with respect to recovery from the effects of the EFAD diet. It demonstrated that although the different dietary lipids had similar effects on membrane lipid composition in soleus and EDL and that they both recovered to Control levels, the two muscles differed in their rates of recovery. Most differences in soleus muscle phospholipid fatty acid composition between the dietary groups were no longer apparent after two weeks recovery. However, the EDL muscles took longer to recover to Control levels. In most cases however, recovery was complete within six weeks. The finding in this study that the two types of muscles differed in their responses to recovery was not surprising since in a comparison of several mouse tissues following dietary lipid manipulation, Burns *et al.* (1983) showed significant selectivity between tissues in fatty acid alteration. It is possible that since the soleus muscle is responsible for posture as well as flexing the foot, it may be in use for more of the time than EDL, especially in a caged rat and the fatty acid turnover rate may normally be greater in such muscles. These factors certainly suggest that future investigations into the effects of dietary manipulation on skeletal muscle structure and function need to specify the types of muscles being tested.

Results from the EFAD group suggest that turnover of fatty acids in muscle phospholipids must be very rapid, possibly a matter of just a

few days, as suggested by the results of Innis & Clandinin (1981) in cardiac mitochondrial lipids.

Enrichment with n - 3 Polyunsaturated Fatty Acids

The phospholipid fatty acid composition of muscles from the PUFA group did not appear to recover to normal levels since the level of n - 3 fatty acids remained high throughout the recovery period. It appears that both muscles tenaciously retained the high level of n - 3 phospholipid fatty acids, as has been demonstrated in chick brain and retina (Anderson *et al.*, 1992).

Although the proportion of n - 3 fatty acids in soleus muscles in the PUFA group was not significantly greater than in the Control and EFAD groups after two weeks recovery, the difference after six weeks recovery was highly significant. Given the large standard error in soleus after two weeks recovery and the significantly greater proportion of n - 3 fatty acids in EDL after both two and six week recovery periods, it is likely that experimental error contributed to the abnormally low level of 22:6, n - 3 in soleus after two weeks.

Recent preliminary studies by von Au *et al.* (1988) and Brändle *et al.* (1990) examined the effects of n - 3 fatty acids on cardiac parameters in hypertensive rats. They found that a fish oil diet decreased blood pressure, increased heart rate and non-significantly reduced the development of eccentric hypertrophy of the heart. Also, the nutritional benefits of increased dietary PUFA in enhancing the mechanical performance of cardiac muscle has been demonstrated in marmosets (Charnock *et al.*, 1987; McLennan *et al.*, 1987b) and rats (Charnock et al., 1985b; McLennan et al., 1985; 1987a; 1989a; 1990).

Further to this, Charnock *et al.* (1989; 1992) have shown that the fatty acid composition of marmoset cardiac muscle and skeletal muscle are almost identical, and that this similarity is still present when the marmosets are fed a variety of diets. Combining these two observations of improved mechanical performance of cardiac muscle and extremely similar phospholipid content in cardiac and skeletal muscles, it is possible that a diet boosted with polyunsaturated fatty acids may also result in improved mechanical performance in skeletal muscle. Charnock *et al.* (1989; 1992) have suggested that this relationship between cardiac and skeletal muscles may be of important clinical value since a skeletal muscle biopsy may serve as a reliable index of the fatty acid composition of heart muscle.

Since this study showed that manipulation of dietary lipids can affect the composition of skeletal muscle membranes and that different types of muscles respond to dietary changes at different rates, the next step was to investigate whether these changes are related to the function of individual muscles and physical performance of the whole animal. Effects on whole animals are clearly harder to predict since they will involve a host of different muscles and must reflect changes throughout the body including the capacity for oxygen consumption and overall metabolism.

Chapter 3 Effects of Changes in Dietary Fatty Acids on Isolated Fast and Slow Skeletal Muscles of Rats

3.1 Introduction

As discussed in Chapter 1, several studies have demonstrated (i) that poor nutrition can alter skeletal muscle function both in humans (Lopes *et al.*, 1982; Russell *et al.*, 1983a;b; Russell *et al.*, 1984b; Lennmarken & Larsson, 1986) and rats (Russell *et al.*, 1984a; Lewis *et al.*, 1986; Pichard *et al.*, 1988; Dureuil *et al.*, 1989; Sieck *et al.*, 1989; Lewis & Sieck, 1990; 1992; Nishio & Jeejeebhoy, 1991) and (ii) that different muscle fibre types respond differently to malnutrition (Li & Goldberg, 1976; Gardiner *et al.*, 1980; Fong *et al.*, 1987; Nishio & Jeejeebhoy, 1991). However, the specific dietary components which are responsible have not been investigated.

In Chapter 2, I manipulated the ratio of saturated to polyunsaturated fats in the diets of rats and showed that it was possible to produce marked and reversible changes in the fatty acid composition of skeletal muscle membranes and that recovery from these effects was different in fast and slow muscle types.

Since the sarcolemma of muscle fibres plays an integral role in contraction and relaxation, any changes to its structure may also alter the way the muscle functions. It is possible that the changed muscle function observed in cases of malnutrition and hypocaloric feeding tests are the result of changes in amounts or types of dietary lipids and their effects on muscle membranes.

In this part of the study, effects of changes in dietary lipids on the function of isolated skeletal muscles were determined. Since all muscle were treated identically, any significant differences observed between the dietary groups could only be due to the dietary differences. Whole incubated muscles were stimulated directly and properties of single isometric contractions, sustained isometric contractions and repetitive intermittent contractions were investigated.

This part of the study aimed to:

(1) investigate whether changes in dietary fatty acids can modify a range of skeletal muscle contractile functions;

(2) determine whether different types of skeletal muscles respond differently to changes in dietary fatty acids and

(3) determine whether changes in skeletal muscle function can recover from the effects of changes in dietary fats, and if so, how long the reversal process takes.

3.2 Materials and Methods

3.2.1 Experimental Design

Male albino Wistar rats, weighing 54 ± 1g at weaning, were used, as described in Chapter 2. Litter-mates were fed one of the Control, EFAD or PUFA diets for nine weeks, followed by recovery periods of zero, two or six weeks on normal rat pellets. Muscles from groups of rats were tested at each of these three times. On each testing day, one rat per litter from each dietary group was tested to ensure that all

groups were exposed to similar day-to-day environmental changes (see Figure 1.1).

3.2.2 Anaesthesia

Anaesthesia was used so that while the soleus muscle from each rat was being tested, the EDL muscle from the same rat had a continual blood supply until it was later removed for testing. In a few cases (three out of a total of 120 muscles tested), the initial soleus or EDL muscle was abandoned due to unforseen problems or equipment error and the intact blood flow in the contralateral muscle ensured its availability for testing.

The standard anaesthetics used in tests of isolated muscles are halothane (Burke *et al.*, 1973; Krarup, 1981; Fong *et al.*, 1987; Enoka *et al.*, 1988; Cope *et al.*, 1991) and sodium pentobarbital (Close, 1964; 1967; 1968a; Gardiner *et al.*, 1980). Halothane was the anaesthetic of choice since pentobarbital is known to depress skeletal muscle force and slow the rate of contraction (Taylor *et al.*, 1984) and with halothane, there is good control over the level of unconsciousness of the rats. In this study, rats were anaesthetized with halothane (Fluothane, ICI, Australia) in oxygen for removal of muscles. The anaesthetic was delivered via a mask at an initial concentration of 5% and thereafter at a level of 1 - 2 % as necessary. There were no deaths from anaesthesia.

3.2.3 Dissections

To remove the muscles, the skin covering the left lower hind limb was reflected back, exposing the calcaneal (Achilles) tendon. This tendon was slit and the cut end looped over a steel rod and tied securely with #2.0 silk suture thread. The complete soleus muscle was carefully removed and any adherent fat removed. The muscle was initially incubated in 100 ml freshly prepared, carbogenated, curarized (tubocurarine chloride; 2.9 x 10^{-2}mM) Krebs solution (4.74 mM KCl, 1.19 mM KH_2PO_4, 120 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 5 mM glucose), pH 7.4, at room temperature for 15 minutes to ensure that neuromuscular transmission was blocked by the tubocurarine chloride. After removal of the soleus muscle, the hind limb of the rat was lightly covered with a mixture of mineral oil and petroleum jelly and plastic film to prevent desiccation while the soleus was tested. Following stimulation of the soleus muscle, the lower tendons of the EDL muscle of the same leg were exposed. These tendons were slit and tied securely to the wire rod used previously for soleus and the muscle was removed and prepared in the same way as soleus.

All chemicals were analytical grade. Tubocurarine chloride came from Sigma (St. Louis, USA) and all other chemicals came from BDH Chemicals (Melbourne, Australia).

3.2.4 Muscle Chamber

Each curarized muscle, tied to the steel rod, was attached to a force-displacement transducer (Grass, type FT 03 C, Quincy, U.S.A.). At

the other end of the muscle, the tendon of origin was held fixed with a modified hemostat clamp. The whole muscle was lowered into an experimental chamber containing curarized Krebs solution, maintained at 33 - 34 ^{o}C by a temperature controlled circulating water bath. The solution in the chamber was continuously bubbled with carbogen and was replaced at the rate of one drop per second to minimise any possible change in pH or a build-up of any substances released by the muscle. The chamber surrounding the muscle contained two platinum sheet electrodes, 3 cm long by 1 cm wide. These extended along the entire length of the muscle on two sides and produced a transverse electrical field when stimulated by a Grass stimulator (type SD 9, Quincy, U.S.A.) and constant current supplier. The force transducer was calibrated with known weights.

The output from the force transducer was relayed through a physiograph (Norco Instruments, model DMP-4B, Texas, U.S.A.) and was then recorded by a MacLab (Analog Digital Instruments, Sydney, Australia) and Apple Macintosh SE computer. The MacLab is a peripheral hardware device which is controlled by the computer and the software package, Chart. In combination with Chart (version 3.1), the MacLab interface allows the Macintosh computer to operate as a chart recorder. Data can be stored and analysed at a later date.

3.2.5 Electrical Stimulation

3.2.5.1 Optimal Muscle Length and Stimulus Voltage

Once in position, the length of each muscle was increased by 1 mm increments with a micromanipulator (# 1241, Norishige, Japan) and stimulated with single, square-wave pulses until further increases in

length elicited no further increase in twitch tension. This length was assumed to be the optimal length (L_O), i.e. the length at which peak twitch tension was maximal (Close, 1964). The stimulus intensity was also increased incrementally until maximum twitch force was obtained; supra-maximal stimulation was then set at this value (generally 24 volts). This voltage was checked for each muscle to ensure that a further increase in voltage did not result in greater tension.

3.2.5.2 Stimulation Trials

Preliminary measures were made of muscle twitch characteristics to ensure that values were repeatable. In pilot trials involving soleus and EDL muscles from four rats, contraction time (mean ± SE) was 60 ± 1 ms and 35 ± 2 ms, respectively and mean half-relaxation time was 41 ± 1 ms and 24 ± 1 ms, respectively. These values were higher than some reported in the literature (Close, 1964; 1965; 1967; Krarup, 1981; Carlsen & Gray, 1987) but comparable with others (Gardiner *et al.*, 1980; Sahlin *et al.*, 1981). However, values for peak tension (30 ± 4g and 39 ± 6 g for soleus and EDL, respectively) were very similar to those reported elsewhere. Further pilot trials in groups of four rats from the three experimental dietary groups revealed very similar and repeatable values (Table 3.1), but they were still higher than expected. Since coefficients of variation for all results in this study were calculated to be typically less than 15% and were very similar to those in all other studies, the results obtained provide a valid comparison of the effects of the different diets.

Table 3.1 Preliminary twitch response times (ms) recorded for soleus and EDL muscles from groups of five rats in each of three dietary groups: Control, EFAD and PUFA

	Dietary Group		
	Control	**EFAD**	**PUFA**
Soleus			
Contraction Time (T_c)	72 ± 2	62 ± 3	71 ± 2
Half-Relaxation Time ($T_{1/2\ R}$)	46 ± 1	42 ± 1	47 ± 1
EDL			
Contraction Time (T_c)	43 ± 1	39 ± 2	42 ± 3
Half-Relaxation Time ($T_{1/2\ R}$)	24 ± 1	23 ± 1	24 ± 1

3.2.5.3 Tests Conducted

A six-step protocol was established and the same procedure was followed for each muscle. The following responses were elicited:

(i) single muscle "twitches",

(ii) force-frequency relationship (used to determine optimal frequency),

(iii) sustained tetanic contractions,

(iv) post-tetanic potentiation (only in EDL muscles),

(v) fatigue during high frequency stimulation (Jones *et al.*, 1979) and

(vi) fatigue during low frequency stimulation (Edwards *et al.*, 1977).

Since post-tetanic potentiation (PTP), is a phenomenon normally found only in adult fast muscles (Close & Hoh, 1968a), it was not tested in soleus muscles.

Each of these tests is discussed in the following section.

Twitch and Tetanic Contractions

Different methods of stimulation were used to elicit isometric twitch and tetanic contractions of whole muscles. A twitch contraction is the response of a muscle to one single stimulus. When multiple stimuli are applied to a muscle, far enough apart to avoid the previous refractory period, the muscle maintains a sustained contraction and the result is a smooth "fused" tetanus.

Twitch parameters were determined for 10 twitches. The relationship between stimulation frequency and force generated was then examined in order to determine optimal frequency (see section 3.2.6 below).

This value was used to set the stimulating frequency used for the low frequency fatigue test (see below).

Post-Tetanic Potentiation (PTP)

Repetitive stimulation of fast-twitch skeletal muscle has been shown to cause potentiation of twitch tension (Close & Hoh, 1968a; Krarup, 1981) but this effect is poorly understood. Carlsen & Walsh (1987) observed a decline in PTP in aging fast twitch muscle which they suggested is due to changes in the release of Ca^{2+} from the sarcoplasmic reticulum. PTP is usually absent in neonatal EDL and soleus muscles and adult soleus muscles, but appears in adult EDL muscles during the first weeks after birth (Close & Hoh, 1968a).

Fatigue

Fatigue, defined as a reduction in force generating capacity (Bigland-Ritchie & Woods, 1984), can occur either very rapidly after high frequency stimulation, or after an extended period of time following submaximal stimulation. Both levels of stimulation result in reduction of tension generated by the muscle. Responses to high and low frequency stimulations were measured in soleus and EDL muscles.

(a) High Frequency Fatigue

Studies have shown that the frequency and duration of repetitive stimulation largely determines the rate of fatigue (for review, see MacLaren, 1989). Decreasing the frequency of stimulation and/or increasing the duration of pulses can result in an immediate increase in tension (Jones *et al.*, 1979; Jones, 1981). In this study, the effects of high frequency stimulation during pulses of different duration were examined. During high frequency stimulation, the duration of

individual pulses was initially 0.02 ms (for a 20 s period for soleus and a 10 s period for EDL). The pulse duration was then increased to 0.2 ms, which is the same pulse duration used in all other tests (again, for a 20 s period for soleus and a 10 s period for EDL).

(b) Low Frequency Fatigue

During lower intensity exercise, it is unlikely that there will be a build-up of metabolites, due to the high blood flow through muscles (Sjøgaard *et al.*, 1986) or a significant reduction in energy stores, due to stores of glycogen and fat (for review, see Sahlin, 1992). However, there can be a long-term loss of force which rapidly returns to normal after high frequency stimulation (Edwards *et al.*, 1977a). To examine fatigue at submaximal intensity, the muscles in this study were each stimulated at the same relative work rate (60% of the frequency required for maximal force generation, i.e. optimal frequency, Carlsen & Gray; 1987).

Details of the protocol (i.e. frequencies, pulse durations and lengths of stimulation) were modelled on methods from published studies and are provided in Table 3.2. Each series of tests in the protocol was followed by a rest period of five minutes, with a 10 minute rest period between the high frequency fatigue test and the final low frequency fatigue test.

Following testing, the length and weight of each muscle were determined and cross-sectional surface area (CSSA) was estimated by dividing muscle mass by muscle length. Contralateral soleus and EDL muscles were removed and the rat was decapitated. The muscles from rats used in this study were immediately frozen in liquid nitrogen and

Table 3.2 Details of stimulation (frequency, pulse duration, length) for each isometric contraction and fatigue test conducted on isolated soleus and EDL muscles from rats which have been on one of three diets, Control, EFAD or PUFA for nine weeks, followed zero, two or six weeks recovery on rat pellets.

Response	Stimulus	Protocol		Variables		Reference
Isometric Contractions						
Twitch	Single Pulses	**Pulse duration:**	0.2 ms	Peak tension	(P_t)	Close & Hoh, 1968
		Frequency:	0.1 Hz	Latent time	(T_L)	
		Total # pulses:	10	Contraction time	(T_C)	
				Half-relaxation time	($T_{1/2R}$)	
Optimal Frequency	Repeated stimulations	**Pulse duration:**	0.2 ms	Peak tension	(f_6)	Carlsen & Gray, 1987
		Frequency:	50-130 Hz for 500 ms 1 every 100s	The frequency which produced greatest peak tension was used during the low frequency fatigue test		
		Total # pulses:	9			
Tetanus	Repeated stimulations	**Pulse duration:**	0.2 ms	Peak tension	(P_o)	Close, 1968
		Frequency:	300 Hz for 500 ms	Tetanic relaxation Time	(TRT)	
		Total # pulses:	1			

Table 3.2 ctd.

Response	Stimulus	Protocol	Variables		Reference
Post-Tetanic Potentiation (EDL only)	Single pulses, tetanic trains, single pulses	**Pulse duration:** 0.2 ms **Frequency:** 0.1 Hz for 30s 30 Hz for 350 ms, each 1 s for 25 s, 0.1 Hz for 30 s	Pre-tetanus twitch tension Post-tetanus twitch tension	(PTP)	Carlsen & Walsh, 1987
Fatigue					
High Frequency Fatigue	Repeated stimulations	**Pulse duration:** 0.02 ms **Frequency:** 300 Hz for 20s (soleus) 10s (EDL), followed by	Peak tension 1 Fatigue time 1	(HF_1P_o) ($HF_1T_{1/2T}$)	modified from Jones, 1981
		Pulse duration: 0.2 ms **Frequency:** 300 Hz for 20s (soleus) 10s (EDL)	Peak tension 2 Fatigue time 2	(HF_2P_o) ($HF_2T_{1/2T}$)	
Low Frequency Fatigue	Intermittent tetanic trains	**Pulse duration:** 0.2 ms **Frequency:** 60% of optimal frequency, stimulated for 330 ms every 1 s for 4 mins	Peak tension Fatigue time	(LF P_o) (LF $T_{1/2T}$)	Carlsen & Walsh, 1987

stored at -20 °C pending analysis of their fatty acid composition (see Chapter 2). Contralateral muscles were used to measure the concentration of Na^+,K^+-ATPase (see Chapter 5).

3.2.6 Parameters Measured

The following properties of isometric contraction were measured:

1. In muscle twitches (see Figure 3.1):

(a) maximum isometric twitch tension (g) (P_t) - peak twitch tension at the optimal length (L_o)

(b) latent time (ms) (T_L) - the time between a stimulus and the beginning of contraction

(c) contraction time (ms) (T_c) - time from onset of contraction to the peak of the isometric twitch

(d) half-relaxation time (ms) ($T_{1/2R}$) - time for decay of tension from the peak of the isometric twitch to one half of the peak tension (Close,1964; Close & Hoh, 1968a).

2. In muscle tetanus:

(a) optimal frequency (Hz) (f_o) - the lowest frequency of repetitive stimulation at which maximal tension is developed 500 ms after the onset of contraction (Close, 1964), i.e. the lowest frequency for maximal response.

(b) maximum tetanic tension (g) (P_o) - maximum tension generated at L_o during repetitive stimulation. at 300 Hz for 500 ms (Close & Hoh, 1968a)

(c) tetanic relaxation time (ms) (TRT) - time for decay of tension from the peak of isometric tetanic tension to one half of the peak tension

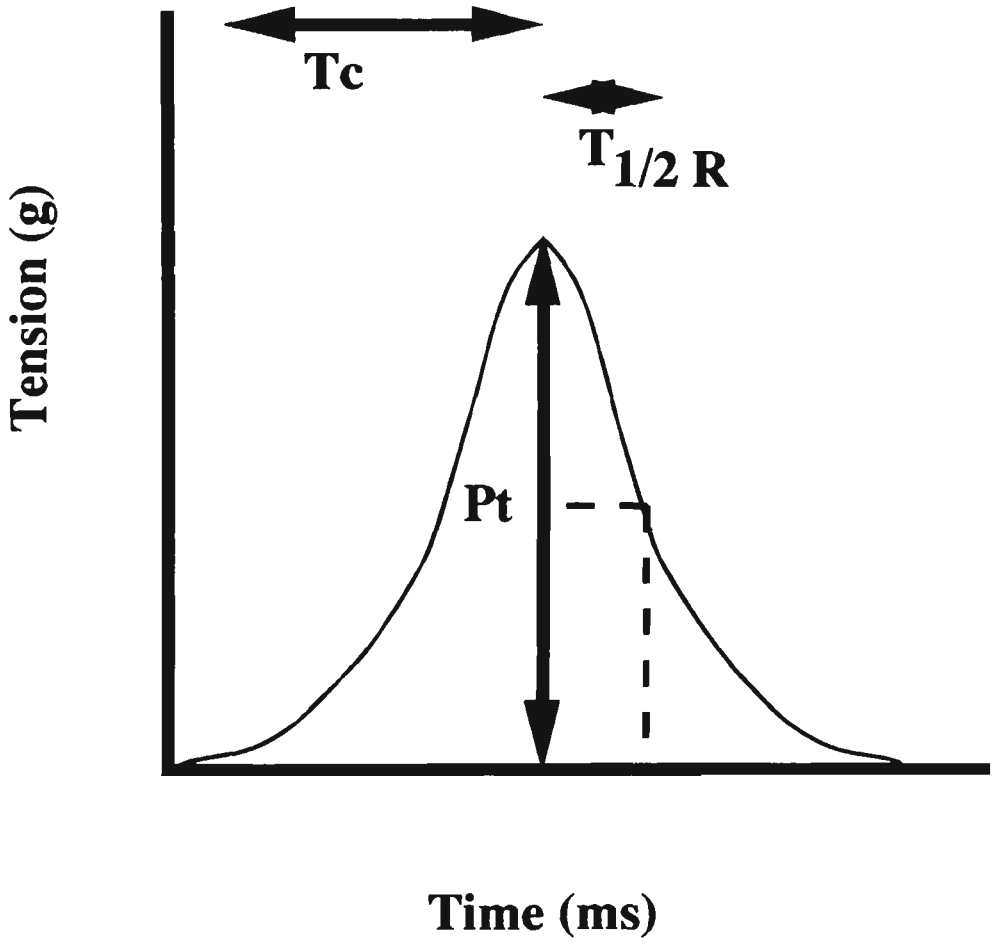


Figure 3.1
Diagrammatic representation of a muscle twitch which is the response to one single stimulus. Variables measured for all twitches included:
peak tension (Pt) - peak twitch tension at the optimal length
latent time (T L) - the time between a stimulus and the beginning of contraction
contraction time (Tc) - time from onset of contraction to peak tension;
half-relaxation time (T 1/2R) - time for decay of tension from peak tension to one half of peak tension

(d) ratio of tetanic peak tension to twitch peak tension (P_o/P_t) as an indication of the increase of tetanic tension over twitch tension.

3. Post-tetanic potentiation (PTP) -the degree of potentiation in EDL muscles was expressed as a ratio of post-tetanic twitch tension to the mean tension of three pre-tetanic twitches (Close & Hoh, 1968a).

Muscle fatigue was assessed under two different conditions of stimulation

4. In high frequency stimulation:

(a) peak tetanic tension (g) and

(b) fatigue time(ms) (time for peak tension to decline to half peak tension)

were determined at both low pulse duration (0.02 ms) and high pulse duration (0.2 ms) (modified from Jones *et al.*, 1979; Jones, 1981).

5. In low frequency stimulation:

(a) peak tension (g) and

(b) endurance (s) (time for decline of tension to half-peak tension)

were determined, as well as 2 indices of fatigue:

(c) total tension fatigue index (TTFI) and

(d) active tension fatigue index (ATFI).

Muscles were subjected to interrupted tetanic trains of stimulations for four minutes at a frequency equal to 60% of their optimal frequency (Burke *et al.*, 1973; Carlsen & Gray, 1987). In this study, mean optimal frequency ranged from 99 Hz to 111 Hz. Therefore, mean frequency for the low frequency fatigue test ranged from 60 Hz to 67 Hz.

As reported in other muscles (Carlsen & Walsh, 1987), the soleus developed a progressive increase in baseline tension during stimulation. To take the different baselines into account, the fatigue index was calculated in 2 ways. TTFI is the ratio of final train tension above the original baseline / initial train tension) and the ATFI is the ratio of final train tension above the new baseline / initial train tension (Carlsen & Walsh, 1987).

3.2.7 Statistical Methods

All measurements are expressed as mean ± SE. The level of statistical significance chosen for this experiment was $P < 0.05$.

For all measures of tension and response times, a general linear models procedure was used to perform multiple analysis of variance (MANOVA) among the dietary groups at each of three times - after nine weeks on the test diets, and following two weeks and six weeks recovery. The statistical package, SAS (SAS Institute, 1979), was used to perform Model lll two-factor ANOVAs without replication; the fixed factor was diet and the random factor was litter. Wherever ANOVA revealed significant effects of diet, Tukey's studentized range test was used to determine which dietary groups produced significantly different responses. All results expressed as ratios were $\log_{10}$ transformed prior to analysis to normalize the variance. Coefficients of variation were calculated in preliminary data as a measure of relative variability to aid interpretation of results (Zar, 1984).

3.3 Results

All figures show mean ± SE from groups of five to seven rats after nine weeks on one of the three diets: Control, EFAD or PUFA, followed by zero, two or six weeks recovery on normal rat pellets. Significantly different dietary treatment means are denoted by different superscripts. Variation between groups is exaggerated in these plots since the effect of variation among litters is not factored out. Differences between dietary groups may therefore appear less marked than the "P" values suggest.

In all the measures of muscle function recorded, significant statistical heterogeneity after six weeks recovery was seen in only one instance so it will generally not be mentioned.

The following analyses were performed on the "whole muscle" values and where appropriate, on normalized values (i.e. adjusted for both estimated muscle cross-sectional surface area (CSSA) and muscle weight). However, since neither muscle CSSA nor muscle weight differed significantly between dietary groups, these adjustments did not generally affect other differences between the groups. Since the aim of the experiment was to determine whether dietary manipulation affected performance in the whole muscles, the whole muscle values are presented. Normalized data and matching statistical analyses are presented in Appendix 2.

3.3.1 Morphology

As described in Chapter 2, final rat weights were not affected by diet, either after nine weeks on the test diets, or following two or six weeks recovery (Table 3.3). As discussed, although body weight in these rats increased about eight-fold during the nine week period on the test diets, there was only a small increase during the recovery period. From about 12 weeks of age (which coincided with the end of the period on the test diets), the growth rate had slowed markedly to about 4% per week. Similarly, none of muscle weight, length or CSSA of soleus or EDL were affected by diet (Tables 3.4 - 3.6). All groups displayed very similar mean muscle weights at all times, however, after six weeks recovery, there was a significant litter effect on the length of EDL muscles ($P < 0.05$).

3.3.2 Muscle Function

Muscle function results are presented for contractile properties for soleus and EDL, followed by fatigue properties for soleus and EDL. As can be seen from the typically small size of the standard error bars in the results, there was great similarity amongst rats in the same dietary groups for almost all measures of muscle function, notable exceptions being peak tension and fatigue time for both soleus and EDL during high frequency fatigue with short pulses, as will be discussed later.

Table 3.3 Weights (g) of groups of five to seven male Wistar rats (mean ± SE) after nine weeks on one of three diets: Control, EFAD diet and PUFA, followed by zero, two or six weeks recovery on normal rat pellets. Numbers of rats per group are in parentheses. Diet did not affect body weight after the nine week test period ($P < 0.1$) or after two weeks ($P < 0.5$) or six weeks ($P > 0.5$) recovery

	Time Since Test Diet		
	0 wks	**2 wks**	**6 wks**
Control	413 ± 16 (7)	454 ± 13 (7)	425 ± 17 (6)
EFAD	390 ± 9 (7)	438 ± 14 (7)	438 ± 13 (6)
PUFA	417 ± 8 (7)	465 ± 8 (7)	433 ± 17 (6)

Table 3.4 Weights (mg) of soleus and EDL muscles from groups of five to seven male Wistar rats (mean ± SE) after nine weeks on one of three diets: Control, EFAD diet and PUFA, followed by zero, two or six weeks recovery on normal rat pellets. Numbers of rats per group are in parentheses. Diet did not affect soleus muscle weight at any stage ($P > 0.5$ at each stage) or EDL muscle weight after nine weeks on the test diets ($P > 0.5$) or after two or six weeks recovery ($P < 0.5$ for both).

	Time Since Test Diet		
	0 wks	**2 wks**	**6 wk**
Soleus			
Control	190 ± 10 (7)	197 ± 7 (7)	196 ± 8 (6)
EFAD	179 ± 6 (7)	201 ± 7 (7)	202 ± 9 (6)
PUFA	183 ± 7 (7)	203 ± 8 (7)	198 ± 10 (6)
EDL			
Control	188 ± 6 (7)	203 ± 7 (6)	187 ± 11 (6)
EFAD	185 ± 6 (7)	183 ± 7 (7)	192 ± 1 (6)
PUFA	189 ± 1 (5)	203 ± 4 (7)	202 ± 7 (6)

Table 3.5 Lengths (cm) of soleus and EDL muscles from groups of five to seven male Wistar rats (mean ± SE) after nine weeks on one of three diets: Control, EFAD diet and PUFA, followed by zero, two or six weeks recovery on normal rat pellets. Numbers of rats per group are in parentheses. Diet did not affect soleus muscle length after nine weeks on the test diets ($P > 0.5$), or after two weeks ($P > 0.5$) or six weeks ($P < 0.2$) recovery. Diet also had no effector EDL muscle length after nine weeks on the test diets ($P < 0.5$) or after two or six weeks recovery ($P > 0.5$ for both).

	Time Since Test Diet		
	0 wks	**2 wks**	**6 wks**
Soleus			
Control	2.81 ± 0.06 (7)	2.83 ± 0.06 (7)	2.96 ± 0.10 (6)
EFAD	2.75 ± 0.03 (7)	2.94 ± 0.07 (7)	2.89 ± 0.07 (6)
PUFA	2.82 ± 0.04 (7)	2.97 ± 0.07 (7)	2.74 ± 0.17 (6)
EDL			
Control	3.34 ± 0.04 (7)	3.43 ± 0.06 (6)	3.47 ± 0.11 (6)
EFAD	3.43 ± 0.04 (7)	3.45 ± 0.06 (7)	3.46 ± 0.06 (6)
PUFA	3.28 ± 0.10 (5)	3.46 ± 0.05 (7)	3.58 ± 0.10 (6)

Table 3.6 Cross-sectional surface area (mm^2) (CSSA) of soleus and EDL muscles from groups of five to seven male Wistar rats (mean ± SE) after nine weeks on one of three diets: Control, EFAD diet and PUFA, followed by zero, two or six weeks recovery on normal rat pellets. Numbers of rats per group are in parentheses. Diet did not affect soleus muscle weight at any stage ($P > 0.5$ at each stage) or EDL muscle weight after nine weeks on the test diets ($P > 0.5$) or after two weeks ($P < 0.5$) or six weeks recovery ($P > 0.5$).

	Time Since Test Diet		
	0 wks	**2 wks**	**6 wks**
Soleus			
Control	67.8 ± 3.8 (7)	64.9 ± 2.5 (7)	64.8 ± 2.5 (6)
EFAD	70.1 ± 3.1 (7)	68.5 ± 3.0 (7)	68.3 ± 1.7 (6)
PUFA	66.4 ± 2.6 (7)	69.9 ± 3.4 (7)	75.5 ± 9.6 (6)
EDL			
Control	56.3 ±2.2 (7)	52.7 ± 1.4 (6)	57.9 ± 1.9 (6)
EFAD	59.4 ± 2.6 (7)	54.5 ± 2.2 (7)	58.5 ± 1.1 (6)
PUFA	53.7 ± 1.7 (5)	55.0 ± 0.6 (7)	58.1 ± 1.8 (6)

3.3.2 1 Contractile Properties

Overview

After the nine week test period, both diet and litter affected peak tensions and response times during isometric contractions in soleus muscles. Typically, the EFAD diet impaired function whereas the PUFA group showed unaltered or improved contractile performance. However, following recovery, results were generally not significantly heterogeneous after two weeks and were almost identical after six weeks.

Isometric contractions in EDL muscles were not significantly affected by either diet or litter during the nine week test period. However, in contrast with soleus, some non-significant differences between the dietary groups, present at nine weeks, were still apparent after two weeks recovery and were statistically significant. Like soleus, tensions generated by the EFAD rats were less than for the Control rats.

A. Contractile Properties Related to Strength

(i) Twitch Tension (P_t)

Representative traces of the response to a single stimulus by soleus muscles and EDL muscles are shown in Figure 3.2.

Peak twitch tensions in soleus muscles were significantly affected by both diet ($P < 0.001$) and litter ($P < 0.001$) after nine weeks (Figure 3.3 A). The EFAD group generated about 20% less tension than either the Control group or the PUFA groups. However, after two

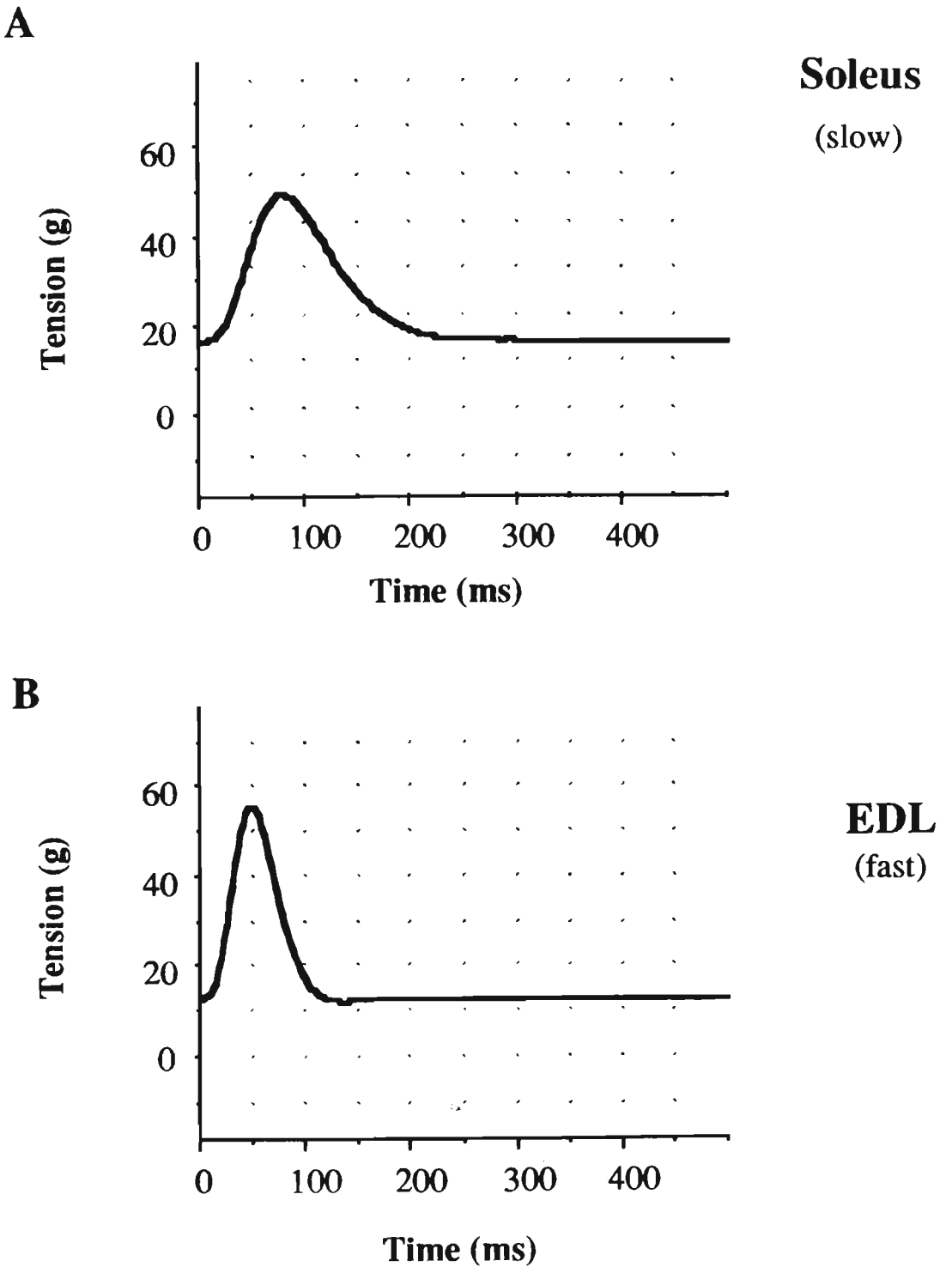


Figure 3.2
Representative single twitches from soleus muscles (Fig. 3.2 A) and EDL (Fig. 3.2 B) muscles from male Wistar rats in response to 0.2 ms pulses at a frequency of 0.1 Hz .

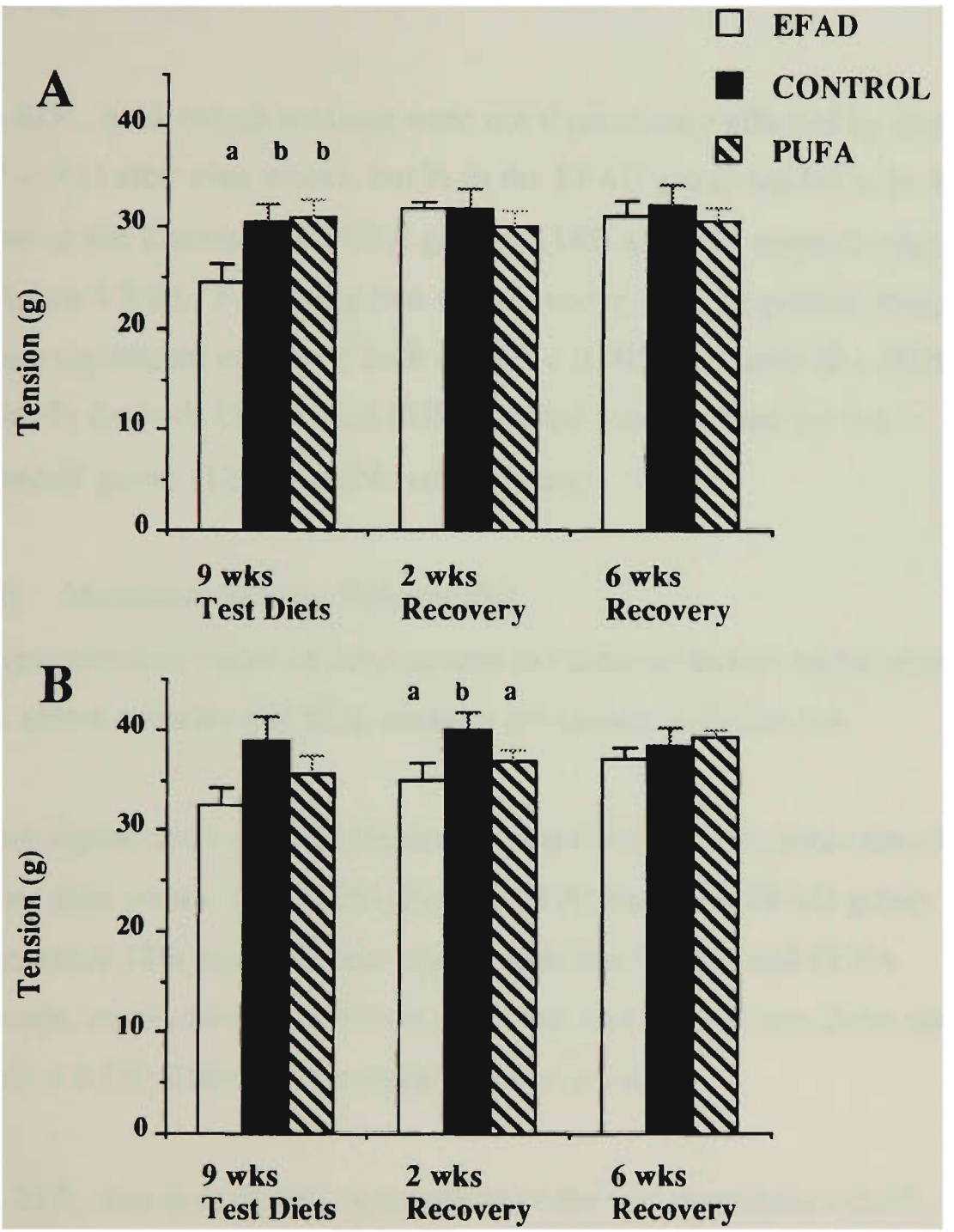


Figure 3.3

Peak tension (g) generated by a single twitch in isolated soleus muscles (Fig.3.3 A) and EDL muscles (Fig. 3.3 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 0.1 Hz with 0.2 ms square-wave pulses. Diet had a significant effect on soleus after nine weeks ($P < 0.001$). Although diet had no effect on EDL after nine weeks ($P < 0.1$), there was a significant effect of diet after two weeks recovery ($P < 0.005$). Significantly different treatment means are denoted by different superscripts.

weeks on normal rat pellets, all groups generated similar tensions during a twitch.

In EDL, peak twitch tensions were not significantly affected by diet ($P < 0.1$) after nine weeks, but P_t in the EFAD group tended to be less than in the Control and PUFA groups, (16% and 8%, respectively). (Figure 3.3 B). Following two weeks recovery on rat pellets, there were significant effects of both diet ($P < 0.005$) and litter ($P < 0.05$), with P_t for both EFAD and PUFA groups was less than for the Control group (12% and 8%, respectively).

(ii) Maximum Tetanic Tension (P_o)

Representative traces of the response to continual tetanic stimulation by soleus muscles and EDL muscles are shown in Figure 3.4.

Diet significantly affected maximum tetanic tensions in soleus muscles after nine weeks ($P < 0.05$) (Figure 3.5 A) since the EFAD group generated 12% and 10% less tension than the Control and PUFA groups, respectively. However, after two weeks recovery, there was only a 0.5% difference between all three groups.

In EDL, diet did not have a significant effect on maximum tetanic tension after nine weeks ($P < 0.1$) (Figure 3.5 B) (however P_o was less for the EFAD (10%) and PUFA (5%) groups than for the Control group). As with twitch tension, after two weeks recovery, P_o was significantly affected by diet ($P < 0.05$) since P_o for the EFAD group was less than for the PUFA group.

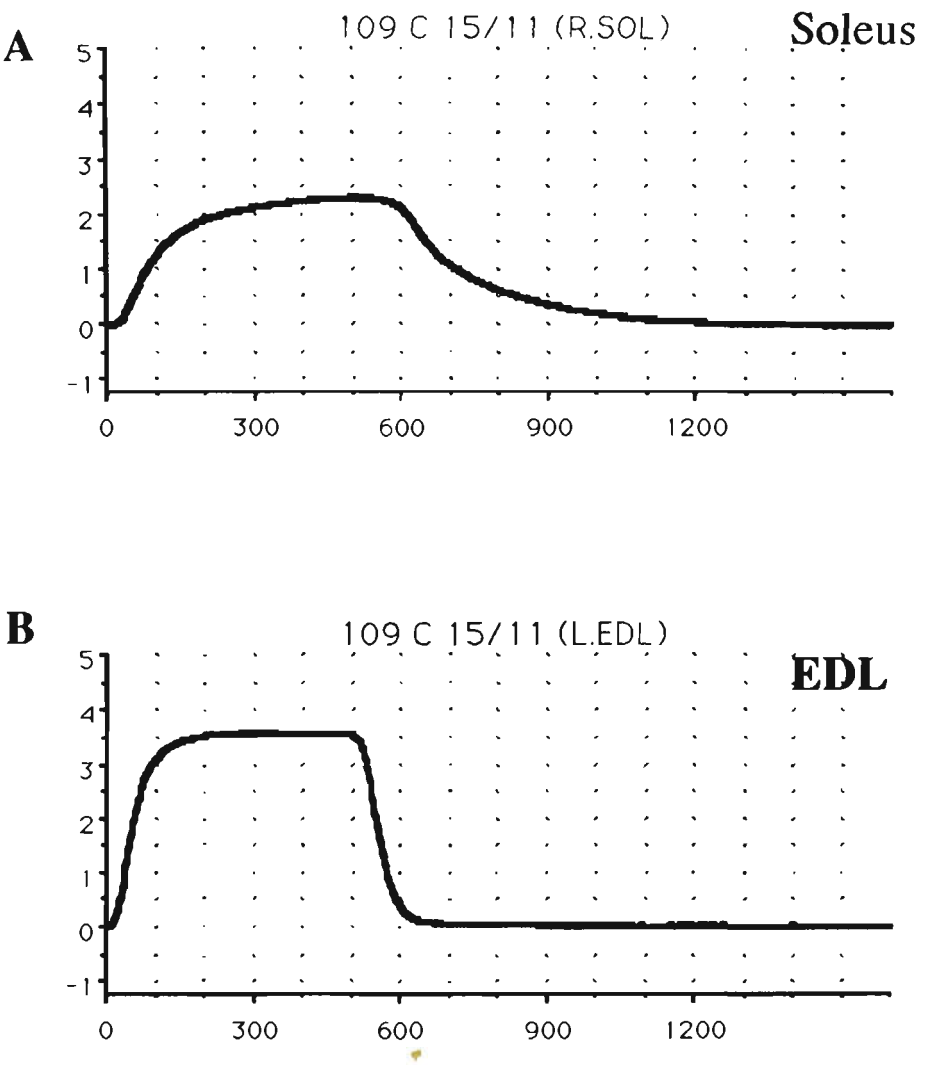
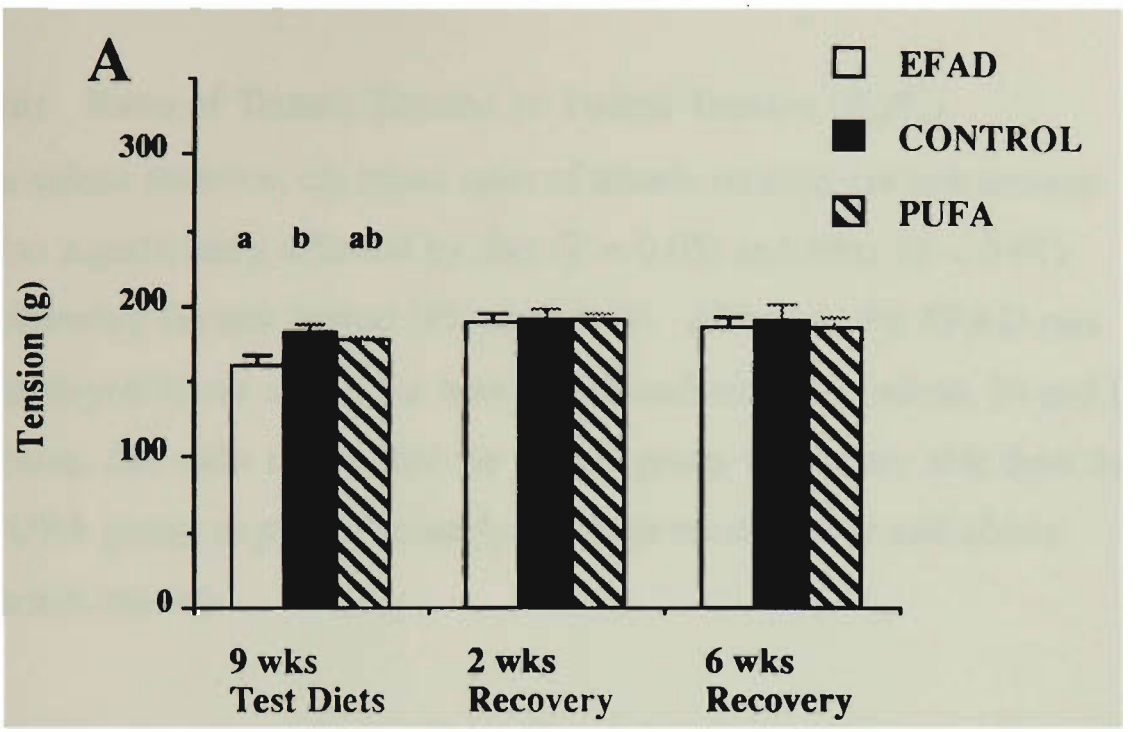


Figure 3.4

Representative traces of maximum tetanic tension generated during 500 ms stimulation at 300 Hz from soleus (A) and EDL (B) muscles from male Wistar rats. A and B are tracings recorded by a MacLab.



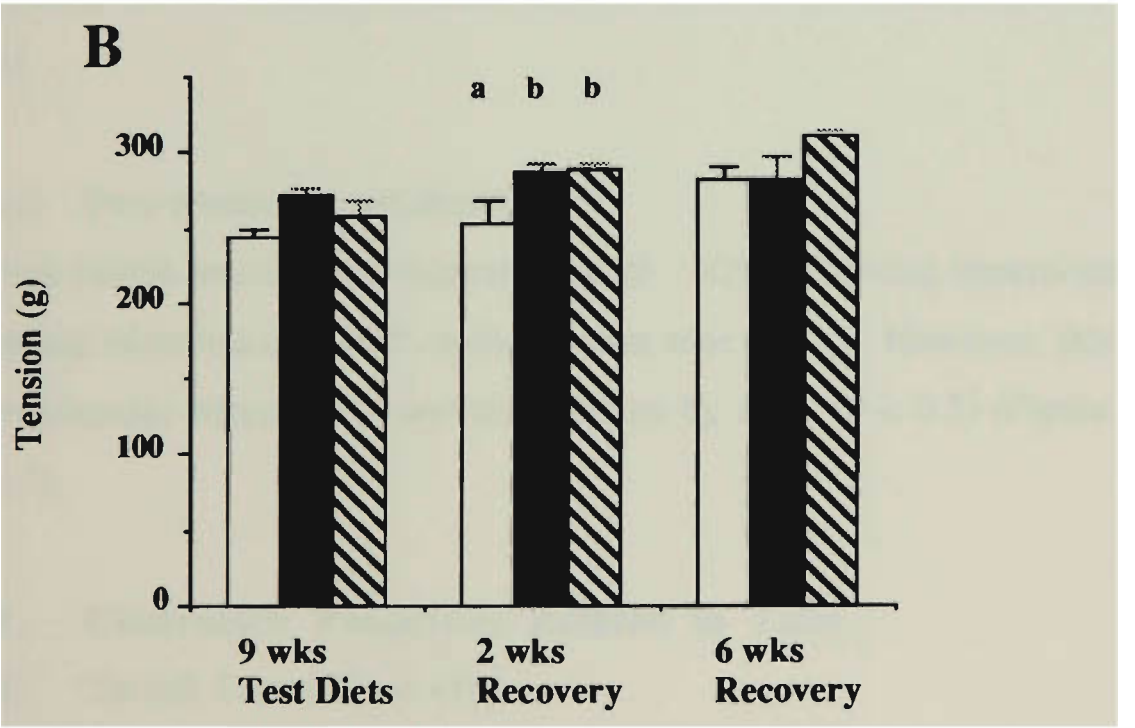


Figure 3.5
Peak tension (g) generated during maximum tetanic stimulation in isolated soleus muscles (Fig. 3.5 A) and EDL muscles (Fig. 3.5 B) from groups of five to seven male Wistar rats . Muscles were stimulated at 300 Hz for 500 ms with 0.2 ms square-wave pulses. Diet had a significant effect on soleus after nine weeks ($P < 0.05$). Although diet had no effect on EDL after nine weeks ($P < 0.1$), there was a significant effect of diet after two weeks recovery ($P < 0.05$). Significantly different treatment means are denoted by different superscripts.

(iii) Ratio of Tetanic Tension to Twitch Tension (P_o/P_t)

In soleus muscles, the mean ratio of tetanic tension : twitch tension was significantly affected by diet ($P = 0.05$) and litter ($P < 0.01$) following the test period (Figure 3.6 A). Although the EFAD rats displayed lower values for both twitch and tetanic tensions, (i) and (ii) above, this ratio shows that the EFAD group was better able than the PUFA group to proportionately increase tension over and above twitch tension.

Neither diet ($P > 0.5$) nor litter ($P > 0.5$) affected the ratio of tetanic tension : twitch tension in EDL muscles after nine weeks (Figure 3.6 B).

(iv) Post-tetanic Potentiation (PTP)

Peak twitch tension was increased by 23 - 30% following intermittent tetanic stimulation in EDL muscles after nine weeks. However, this post-tetanic potentiation was not affected by diet. ($P < 0.5$) (Figure 3.7).

B. Contractile Properties Related to Time

(i) Twitch Latent Time (T_L)

Latent times for both soleus and EDL muscle twitches displayed greater variation than many other parameters, but they were not significantly affected by diet ($P < 0.5$ for soleus and $P > 0.5$ for EDL) (Figure 3.8). In soleus, there was a significant litter effect at nine weeks ($P < 0.001$) and again after six weeks recovery ($P < 0.0001$) and variation among litters accounted for a considerable portion of the variation (Figure 3.8 A). In EDL, there was also a significant litter effect after six weeks recovery ($P < 0.0001$) (Figure 3.8 B).

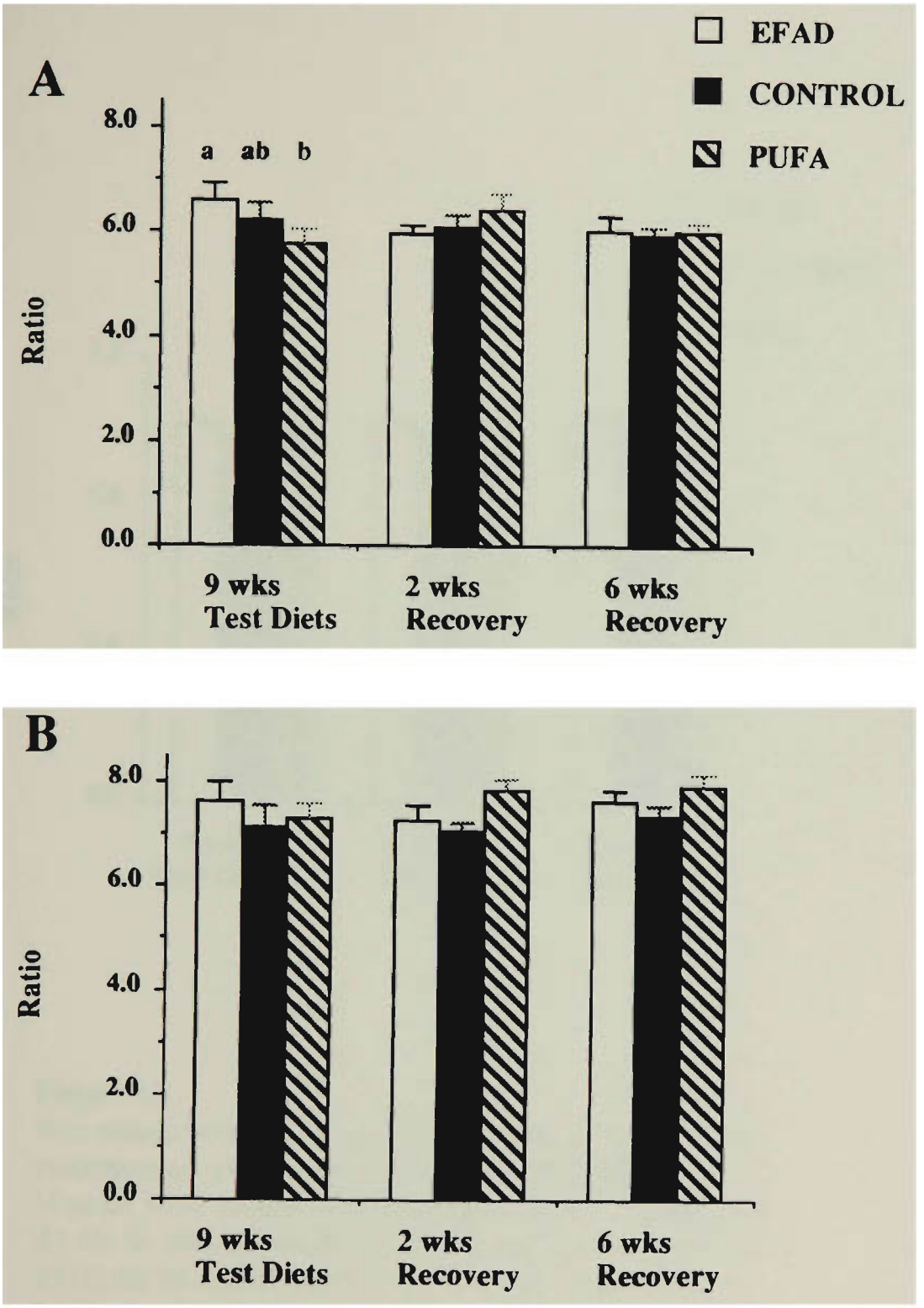


Figure 3.6
Ratio of maximum tetanic tension to peak twitch tension in isolated soleus muscles (Fig. 3.6 A) and EDL muscles (Fig 3.6 B) from groups of five to seven male Wistar rats. Diet had a significant effect on soleus after nine weeks ($P = 0.05$), but had no effect on EDL ($P > 0.5$). Significantly different treatment means are denoted by different superscripts.

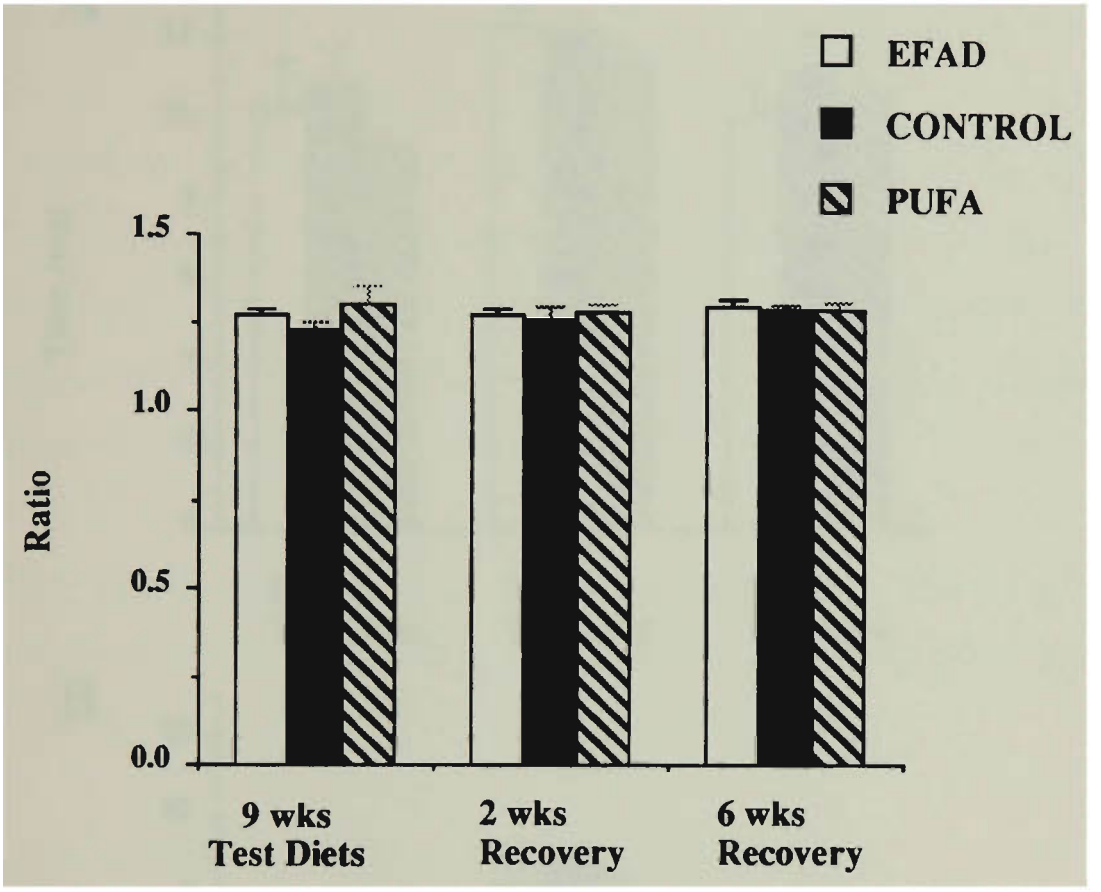


Figure 3.7
Post-tetanic potentiation of single twitches in isolated EDL muscles from groups of five to seven male Wistar rats. Muscles were stimulated with 0.2 ms square-wave pulses at 0.1 Hz for 30s, followed by 350 ms square-wave pulses at 25 Hz for 25 s, followed by 0.2 ms square-wave pulses at 0.1 Hz for 30s. Diet had no effect on EDL muscles after nine weeks ($P = 0.5$).

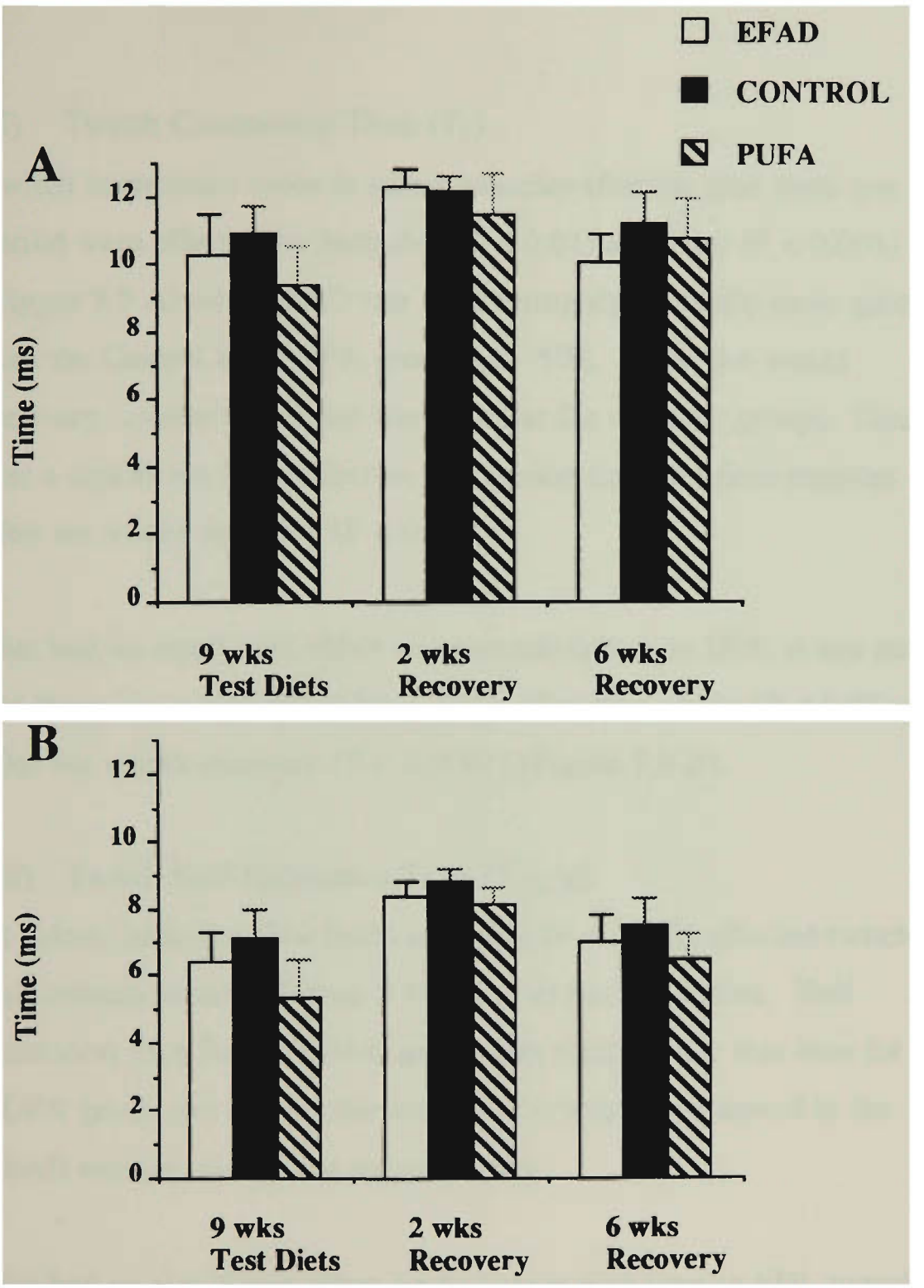


Figure 3.8
Latent time (ms) during a single twitch in groups of five to seven isolated soleus muscles (Fig. 3.8 A) and EDL muscles (Fig. 3.8 B) from male Wistar rats. Muscles were stimulated at 0.1 Hz with 0.2 ms square-wave pulses. Diet did not affect latent time in soleus muscles ($P < 0.5$) or EDL muscles ($P > 0.5$).

(ii) Twitch Contraction Time (T_c)

Twitch contraction times in soleus muscles after the nine week test period were affected by both diet ($P < 0.01$) and litter ($P < 0.005$) (Figure 3.9 A) with EFAD rats contracting significantly more quickly than the Control and PUFA groups (4 - 5%). After two weeks recovery, contraction times were similar for all three groups. There was a significant litter effect on contraction time in soleus muscles after six weeks recovery ($P < 0.05$).

Diet had no significant effect on contraction time in EDL at any stage, but there was a significant litter effect after nine weeks ($P < 0.01$) and after six weeks recovery ($P < 0.0001$) (Figure 3.9 B).

(iii) Twitch Half-Relaxation Time ($T_{1/2\ R}$)

In soleus, both diet ($P < 0.01$) and litter ($P < 0.002$) affected twitch half-relaxation time (Figure 3.10 A) after the test period. Half-relaxation time for the EFAD group was significantly less than for the PUFA group and follows the much lower tension generated in the EFAD muscles during the muscle twitch.

Diet had no significant effect on half-relaxation time in EDL muscles at any stage (Figure 3.10 B).

(iv) Tetanic Relaxation Time (TRT)

Tetanic relaxation times in soleus muscles at nine weeks also tended to be shorter in rats on the EFAD diet than in rats on the Control and PUFA diets (16% and 7%, respectively). This difference was not significant ($P > 0.5$) (Figure 3.11 A) and again follows a lower

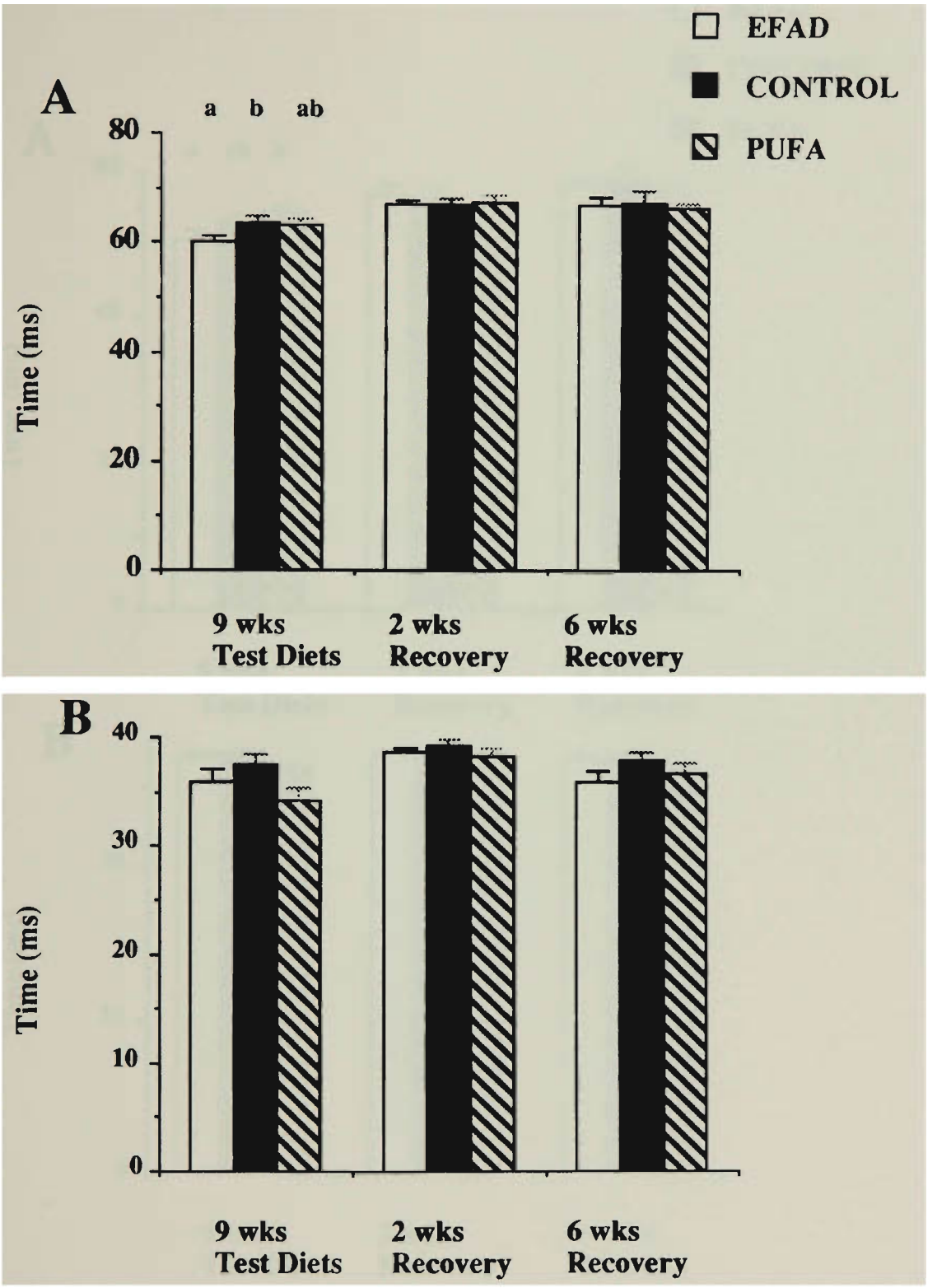


Figure 3.9
Contraction time (ms) during a single twitch in isolated soleus muscles (Fig. 3.9 A) and EDL muscles (Fig. 3.9 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 0.1 Hz with 0.2 ms square-wave pulses. Diet had a significant effect on soleus muscles after nine weeks ($P = < 0.01$), but had no effect on EDL ($P > 0.5$). Significantly different treatment means are denoted by different superscripts.

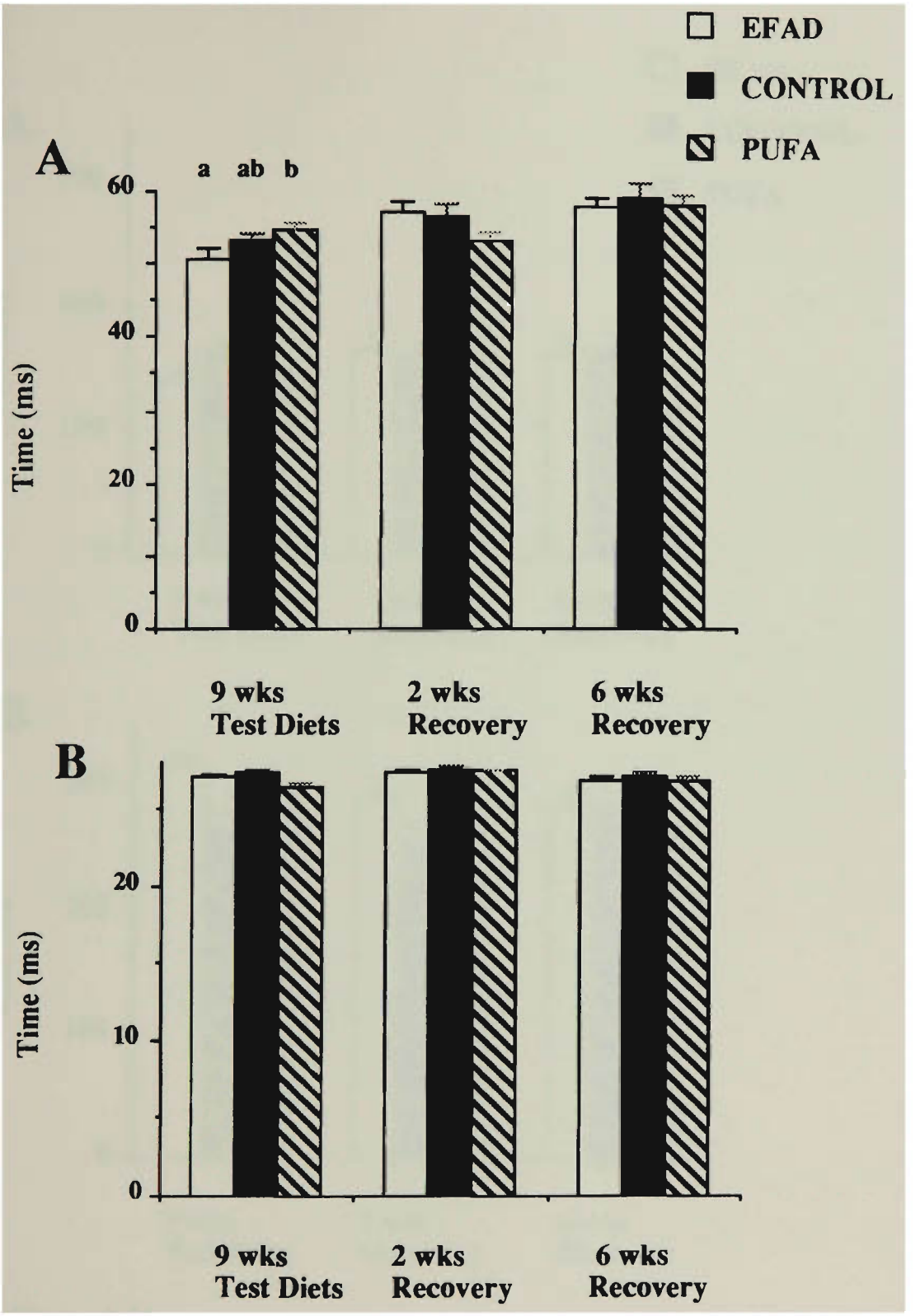


Figure 3.10

Half-relaxation time (ms) during a single twitch in isolated soleus muscles (Fig. 3.10 A) and EDL muscles (Fig. 3.10 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 0.1 Hz with 0.2 ms square-wave pulses. Diet had a significant effect on soleus after nine weeks ($P < 0.01$), but had no effect on EDL ($P < 0.1$). Significantly different treatment means are denoted by different superscripts.

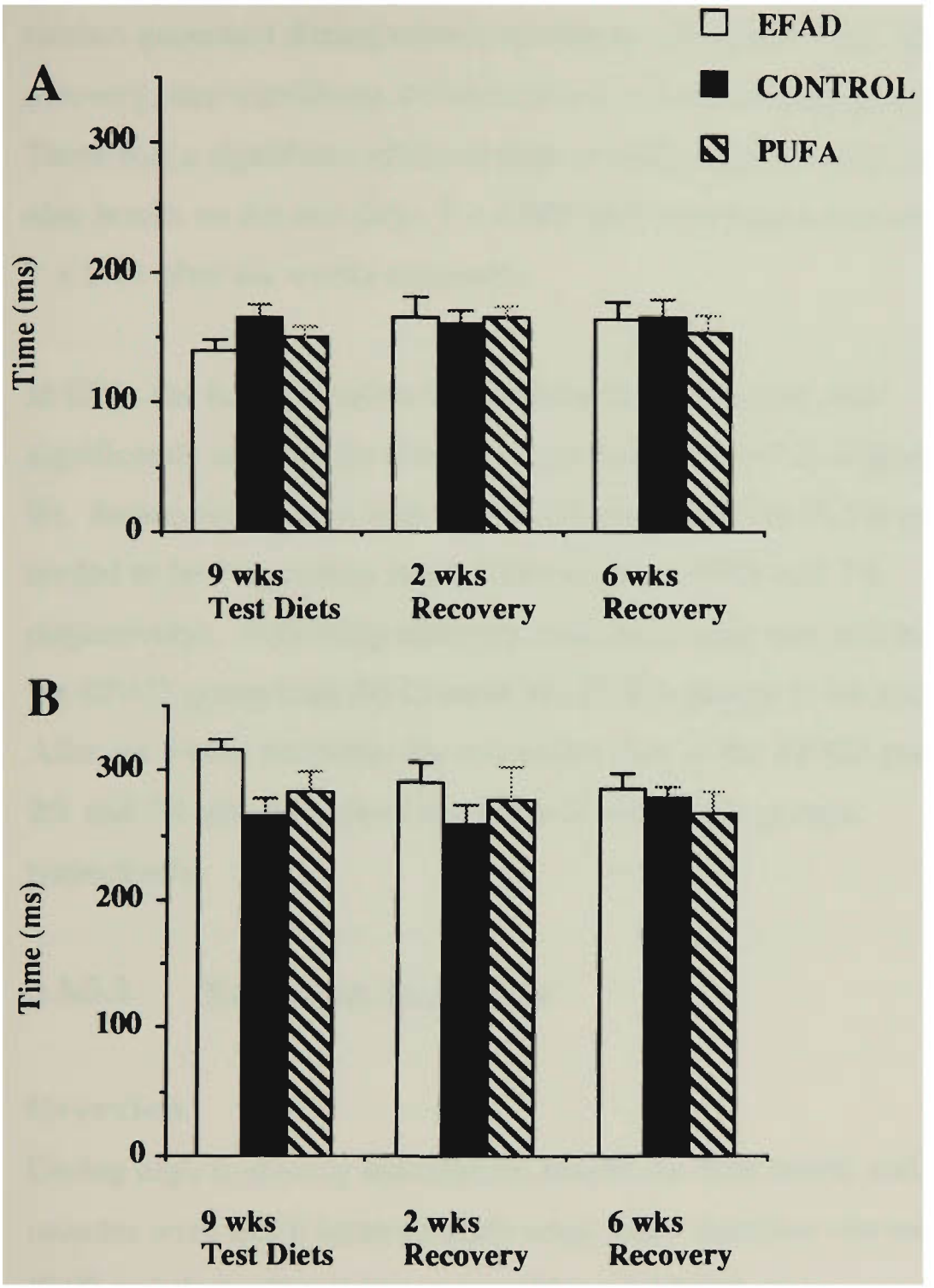


Figure 3.11
Tetanic relaxation time (ms) following maximal tetanic tension for 500 ms at 300 Hz in soleus muscles (Fig. 3.11 A) and EDL muscles (Fig. 3.11 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 300 Hz for 500 ms with 0.2 ms square-wave pulses. Diet did not significantly affect tetanic relaxation time in either soleus or EDL at any stage.

tension generated during tetanic stimulation (Figure 3.5 A). During recovery, non-significant differences were found among groups. There was a significant effect of litter at each stage ($P < 0.01$ after nine weeks on the test diets, $P < 0.005$ after two weeks recovery and $P < 0.01$ after six weeks recovery).

In EDL, the half-relaxation time following tetanus was non-significantly affected by diet after nine weeks ($P < 0.2$) (Figure 3.11 B). Relaxation time in both the EFAD group and the PUFA group tended to be longer than in the Control group (19% and 7%, respectively). Following recovery, relaxation time was still longer in the EFAD group than the Control and PUFA groups (13% and 5%). After six weeks recovery, the relaxation time in the EFAD group was 2% and 7% greater than in the Control and PUFA groups, respectively.

3.3.2.2 Resistance to Fatigue

Overview

During high frequency stimulation, results for both soleus and EDL muscles were much more variable when pulse duration was short (0.02 ms) than when it was increased ten-fold (0.2 ms).

Diet had no effect on tension generated or fatigue time during short duration stimulations at high frequency in soleus muscles. However, when the pulse duration was increased, diet affected both peak tension and fatigue time. In EDL, peak tension during high frequency stimulation was not significantly affected by nine weeks on the test diets, but was significantly modified following recovery. The fatigue

time was unaffected by diet during the first high frequency stimulation with short pulse durations, but there was a significant effect of diet during high frequency stimulations of longer pulse duration.

During low frequency stimulation, diet significantly affected peak tension in soleus, but had no effect on endurance, whereas in EDL, there was a significant dietary effect on endurance.

A. High Frequency Fatigue (HFF)

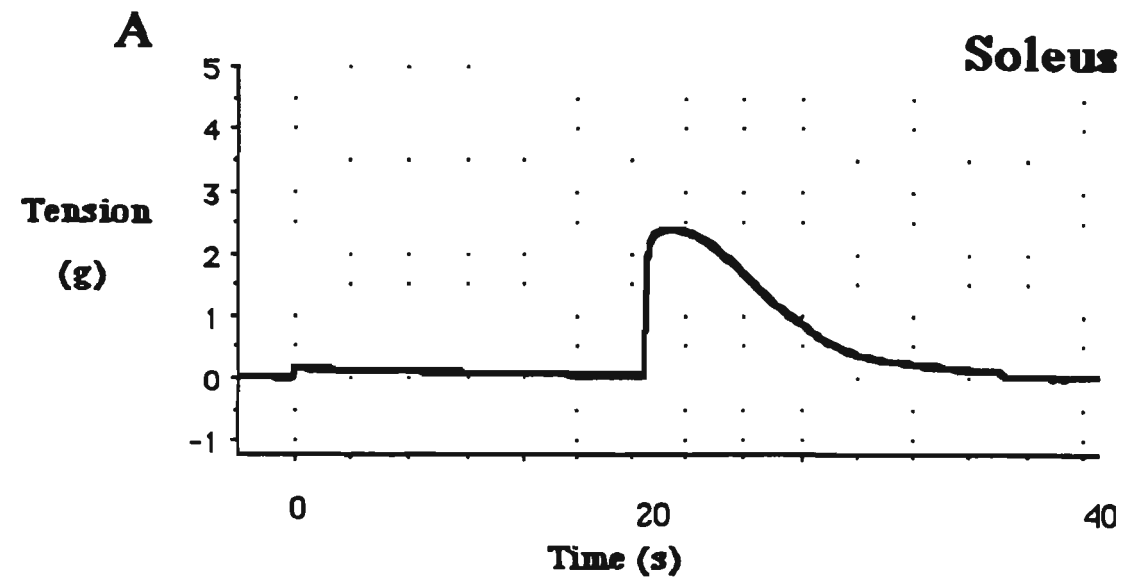
Representative traces of the response to high frequency stimulation by soleus and EDL muscles are shown in Figure 3.12.

(i) Short Pulses (0.02 ms duration)

During the first HFF test in soleus (i.e. 0.02 ms pulses), peak tension was not significantly affected by diet, either after the nine week test period ($P > 0.5$) or following recovery (Figure 3.13 A), but the results were very variable at each stage. However, the tension generated by the Control group was always the lowest. After two weeks on rat pellets there was a striking effect of litter ($P < 0.0001$) on peak tension. For example, in all three rats from one particular litter, peak tension generated was only 18 - 20% of the mean value in all dietary groups.

In EDL, diet did not significantly affect peak tension generated after nine weeks when pulse duration was short ($P < 0.5$) (Figure 3.13 B). However, following recovery, there were dramatic and significant effects of diet. Peak tension for the PUFA group was significantly

Figure 3.12 Representative traces of the responses of isolated soleus muscles (Fig. 3.12 A) and EDL muscles (Fig. 3.12 B) to high frequency stimulation. Muscles were stimulated at 300 Hz for 20 s (soleus) and 10 s (EDL) with 0.02 ms square-wave pulses, followed by 300 Hz for 20 s (soleus) and 10 s (EDL) with 0.2 ms square-wave pulses.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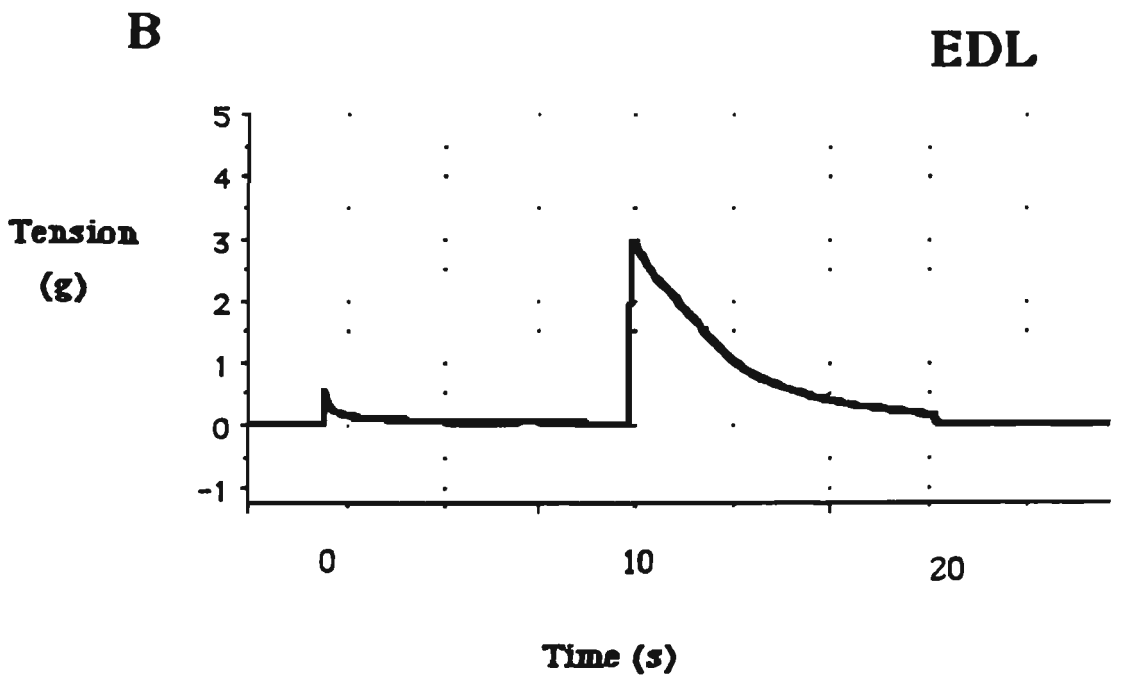
Soleus
Tension
(g)
5
4
3
2
1
0
-1
0
20
40
Time (s)


B
EDL
Tension
(g)
5
4
3
2
1
0
-1
0
10
20
Time (s)

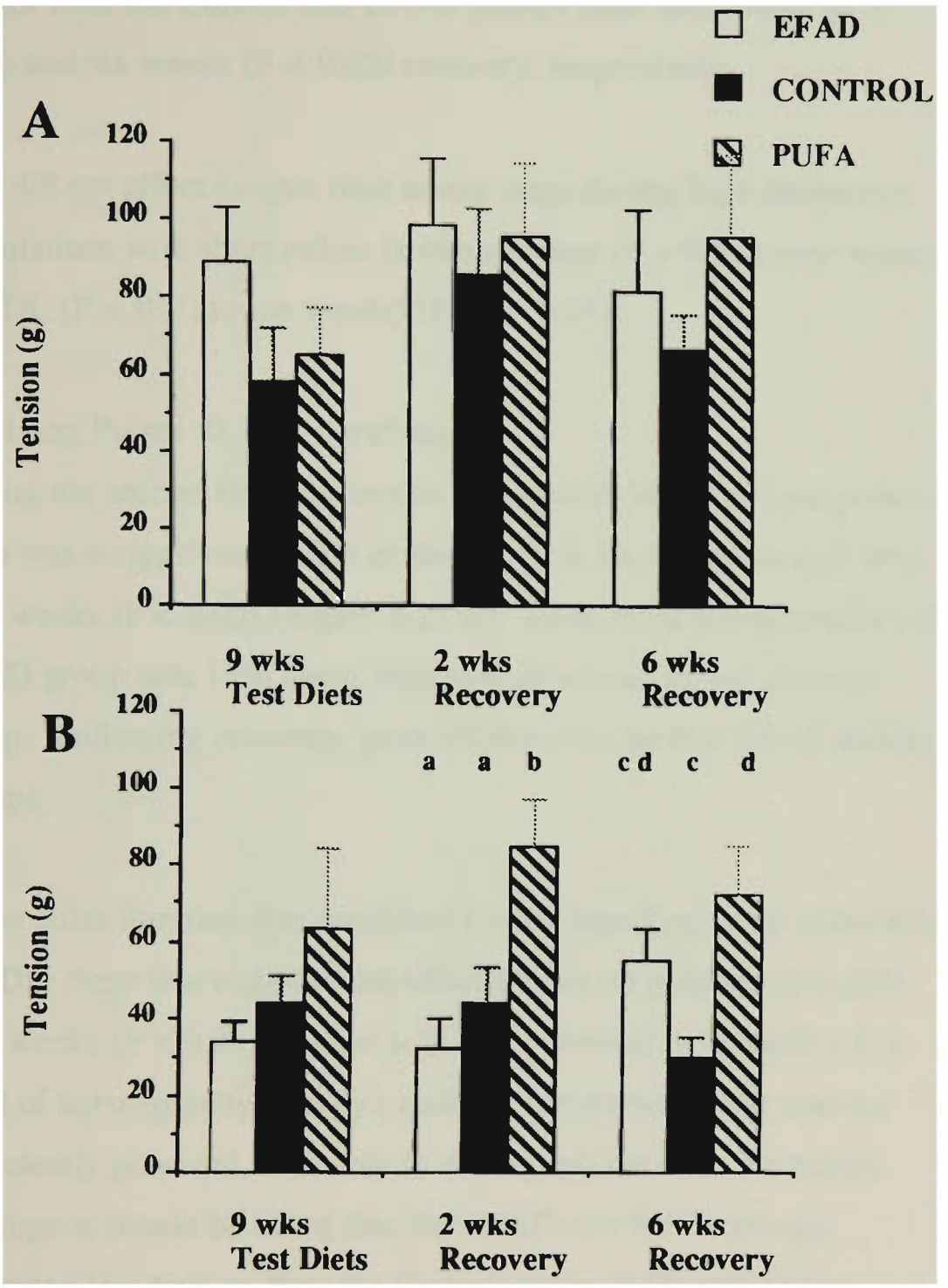


Figure 3.13
Peak tension (g) generated during high frequency stimulations of isolated soleus muscles (Fig. 3.13 A) and EDL muscles (Fig. 3.13 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 300 Hz for 20s with 0.02 ms square-wave pulses. Diet did not significantly affect peak tension in soleus ($P > 0.5$). Although diet had no significant effect in EDL after nine weeks ($P < 0.5$), there was a significant effect of diet after two weeks recovery ($P < 0.02$) and after six weeks recovery ($P < 0.02$). Significantly different treatment means are denoted by different superscripts.

greater than the Control and EFAD groups after two weeks ($P < 0.02$) and six weeks ($P < 0.02$) recovery, respectively.

Diet did not affect fatigue time at any stage during high frequency stimulations with short pulses in either soleus ($P > 0.5$ at nine weeks) or EDL ($P < 0.2$ at nine weeks) (Figure 3.14).

(ii) Long Pulses (0.2 ms duration)

During the second HFF test in soleus (i.e. with longer 0.2 ms pulses), there was a significant effect of diet on peak tension generated after nine weeks ($P < 0.05$) (Figure 3.15 A). Peak tension generated by the EFAD group was 15% lower than that generated by the Control group. Following recovery, peak tension was similar for all dietary groups.

When pulse duration was increased during high frequency stimulation in EDL, there was a significant effect of diet on peak tension after nine weeks ($P < 0.05$) (Figure 3.15 B). However, with such a low level of heterogeneity, Tukey's multiple comparisons test was not sufficiently powerful to be able to distinguish between the means although it should be noted that the EFAD and PUFA groups generated less tension than the Control group (15% and 10%, respectively).

Diet had no apparent effect on fatigue time in soleus after nine weeks on the test diets ($P > 0.5$) (Figure 3.16 A). There was a significant effect of litter ($P < 0.005$) on fatigue time after six weeks recovery.

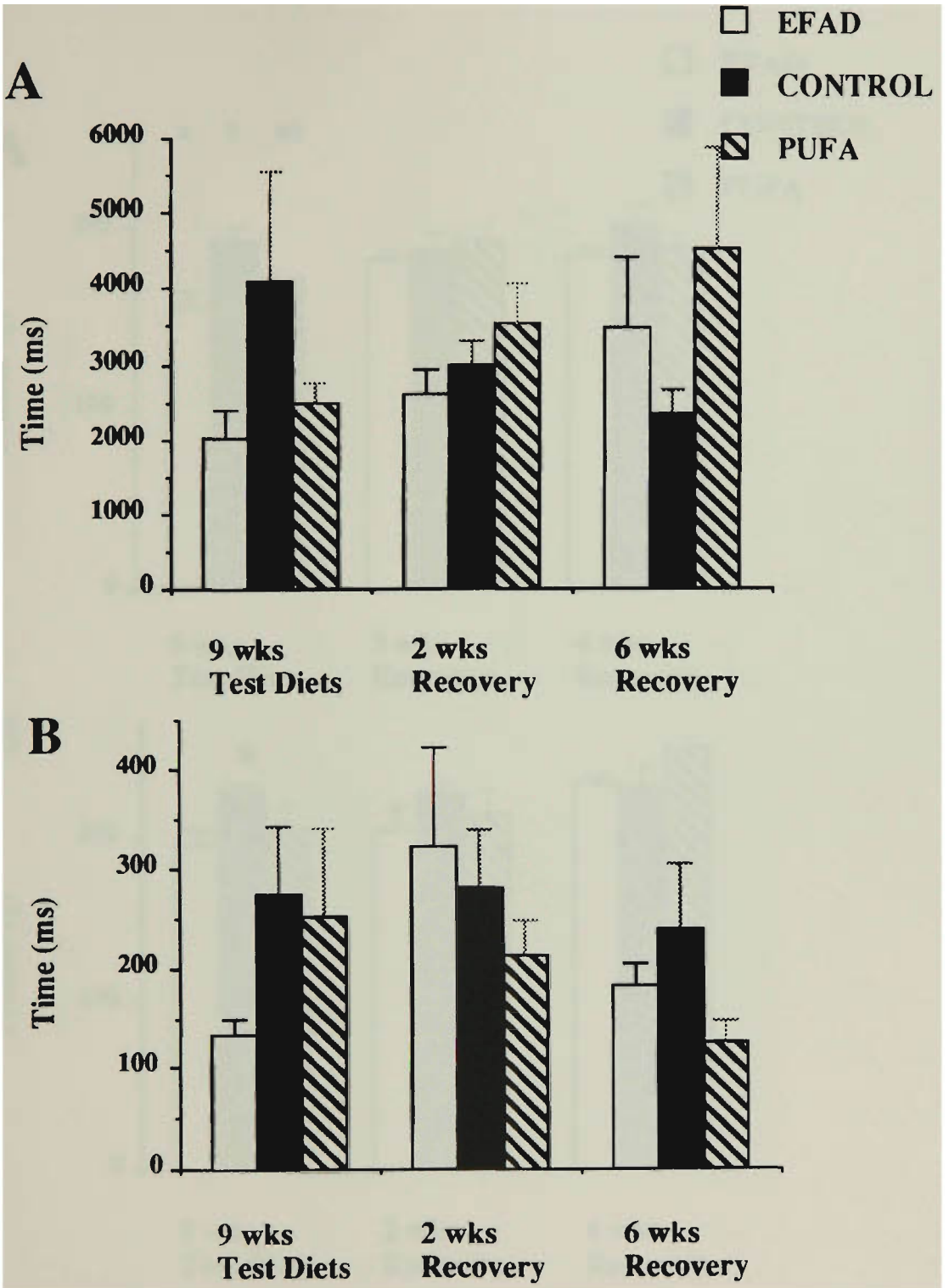


Figure 3.14
Fatigue time (ms) to half peak tension during high frequency stimulations of isolated soleus muscles (Fig. 3.14 A) and EDL muscles (Fig. 3.14 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 300 Hz for 20s with 0.02 ms square-wave pulses. Diet did not affect fatigue time in either soleus ($P > 0.5$) or EDL ($P < 0.2$).

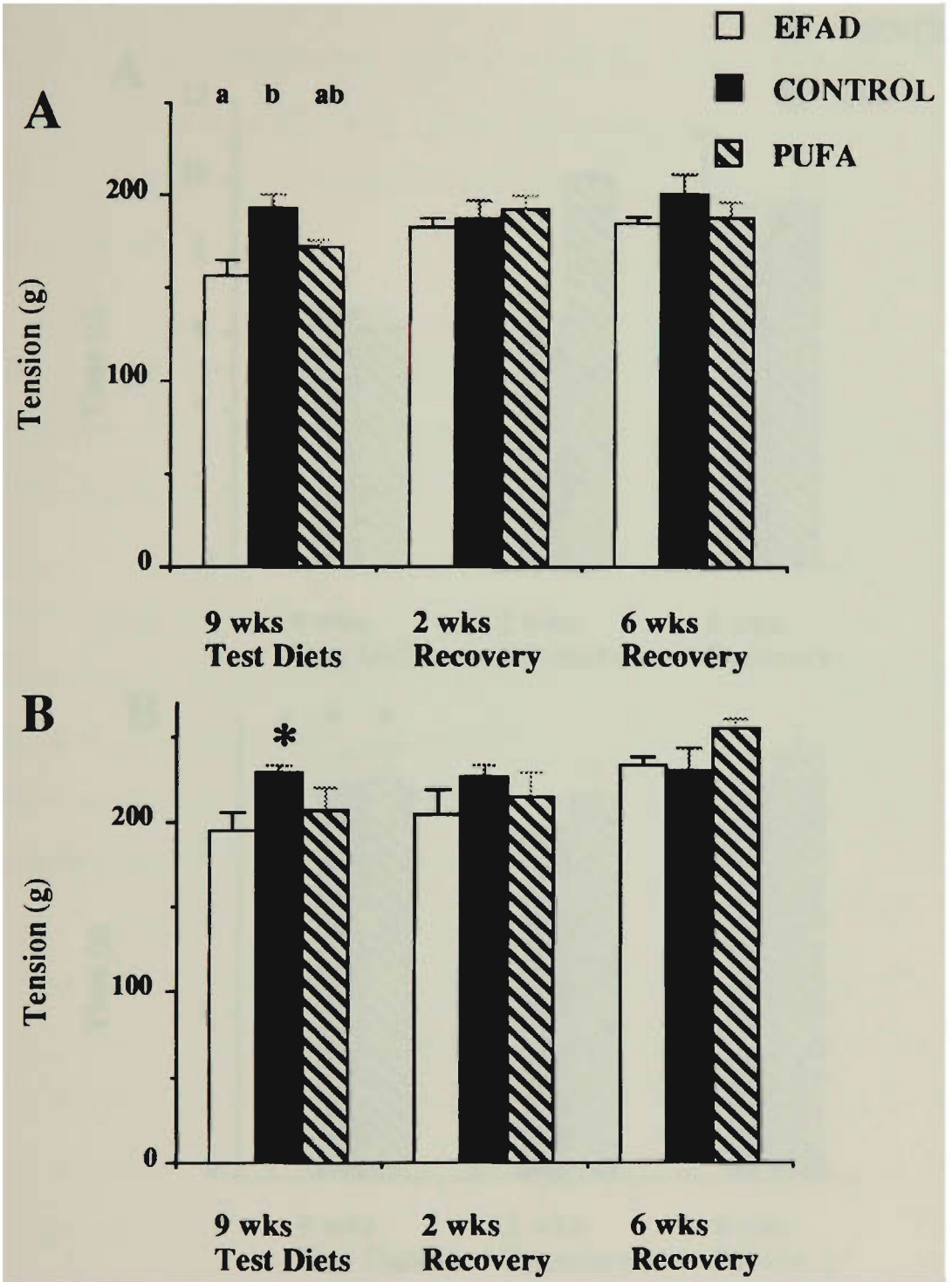


Figure 3.15

Peak tension (g) generated during high frequency stimulations of isolated soleus muscles (Fig. 3.15 A) and EDL muscles (Fig. 3.15 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 300 Hz for 20s with 0.2 ms square-wave pulses. Diet had a significant effect in both soleus ($P < 0.05$) and EDL ($P < 0.05$) after nine weeks. Significantly different treatment means are denoted by different superscripts.* In EDL, Tukey's multiple comparisons tests was unable to distinguish between the means.

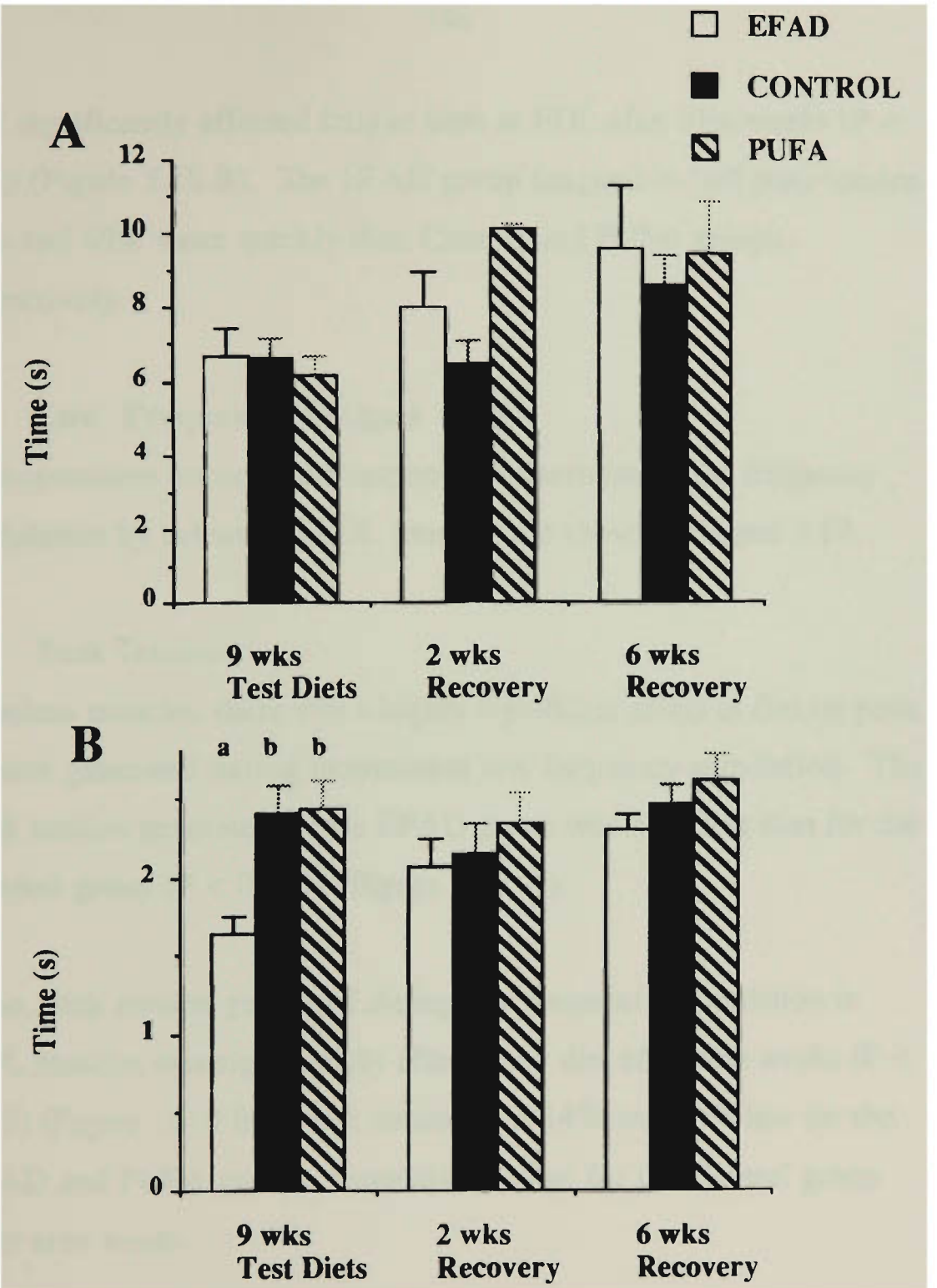


Figure 3.16

Fatigue time (ms) to half peak tension during high frequency stimulations of isolated soleus muscles (Fig. 3.16 A) and EDL muscles (Fig. 3.16 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 300 Hz for 20s with 0.2 ms square-wave pulses. Diet had no effect on soleus after nine weeks ($P > 0.5$) but did have a significant effect on EDL ($P < 0.01$). Significantly different treatment means are denoted by different superscripts.

Diet significantly affected fatigue time in EDL after nine weeks ($P < 0.01$) (Figure 3.16 B). The EFAD group fatigued to half peak tension 32% and 49% more quickly than Control and PUFA groups, respectively.

B. Low Frequency Fatigue (LFF)

Representative traces of the response to intermittent low frequency stimulation by soleus and EDL muscles are shown in Figure 3.17.

(i) Peak Tension

In soleus muscles, there was a highly significant effect of diet on peak tension generated during intermittent low frequency stimulation. The peak tension generated by the EFAD group was 30% less than for the Control group ($P < 0.005$) (Figure 3.18 A).

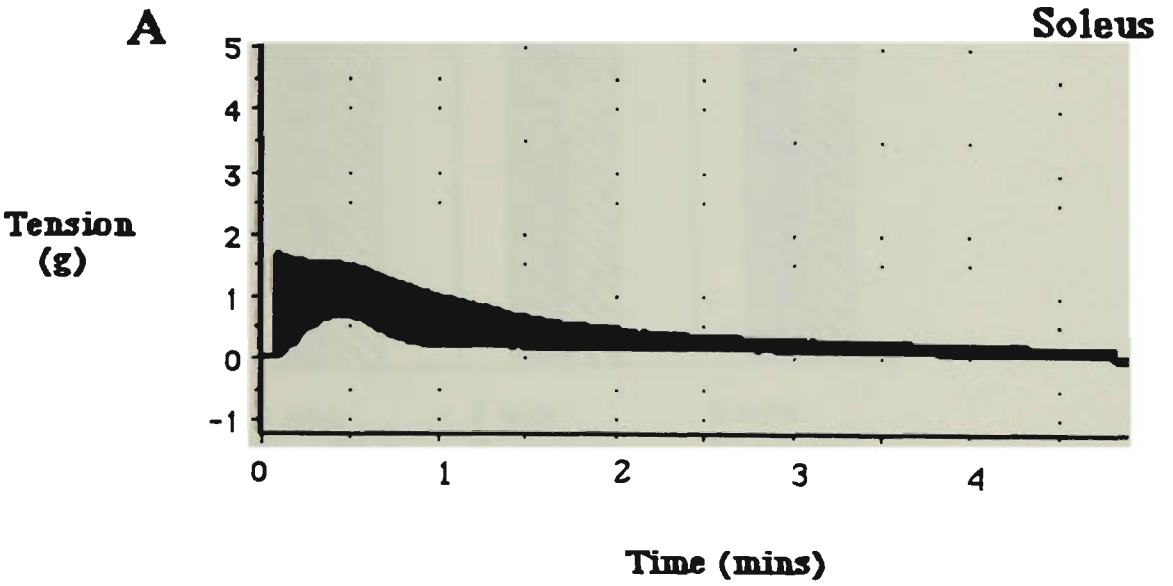
Also, peak tension generated during low frequency stimulation in EDL muscles was significantly affected by diet after nine weeks ($P < 0.02$) (Figure 3.18 B). Peak tension was 14% and 18% less for the EFAD and PUFA groups, respectively, than for the Control group after nine weeks.

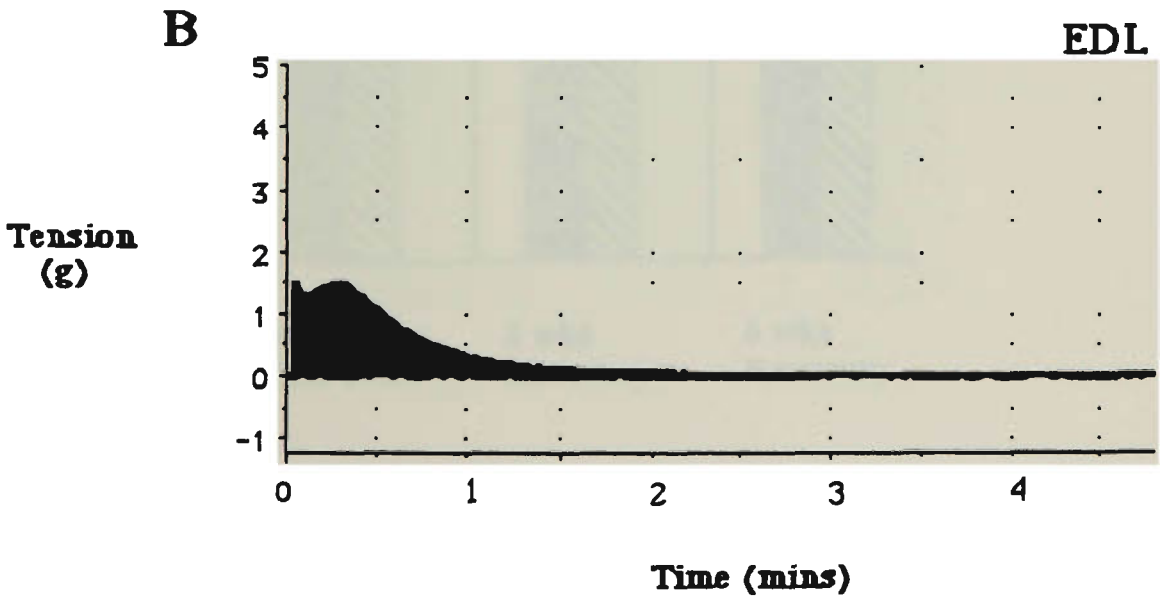
(ii) Endurance

Diet did not affect endurance (measured as fatigue time to half peak tension) in soleus during the LFF test ($P > 0.5$) (Fig 3.19 A).

However, there was a significant effect of diet on endurance in EDL muscles following nine weeks on the test diets ($P < 0.02$) (Figure 3.19 B). The time taken to return to half peak tension was 20% greater for

Figure 3.17 Representative traces of the responses of isolated soleus muscles (Fig. 3.17 A) and EDL muscles (Fig. 3.17 B) to low frequency stimulation. Muscles were stimulated at 60% of their optimal frequency (i.e. the frequency at which maximum tetanic tension occurred after 500 ms stimulation) with 330 ms square-wave pulses at 1 Hz for 4 minutes. Soleus muscles develop a progressive increase in baseline tension during stimulation which is evident in Fig. 3.17 A. This does not happen in EDL muscles (Fig. 3.17 B).


A
Soleus
Tension
(g)
5
4
3
2
1
0
-1
0
1
2
3
4
Time (mins)


B
EDL
Tension
(g)
5
4
3
2
1
0
-1
0
1
2
3
4
Time (mins)

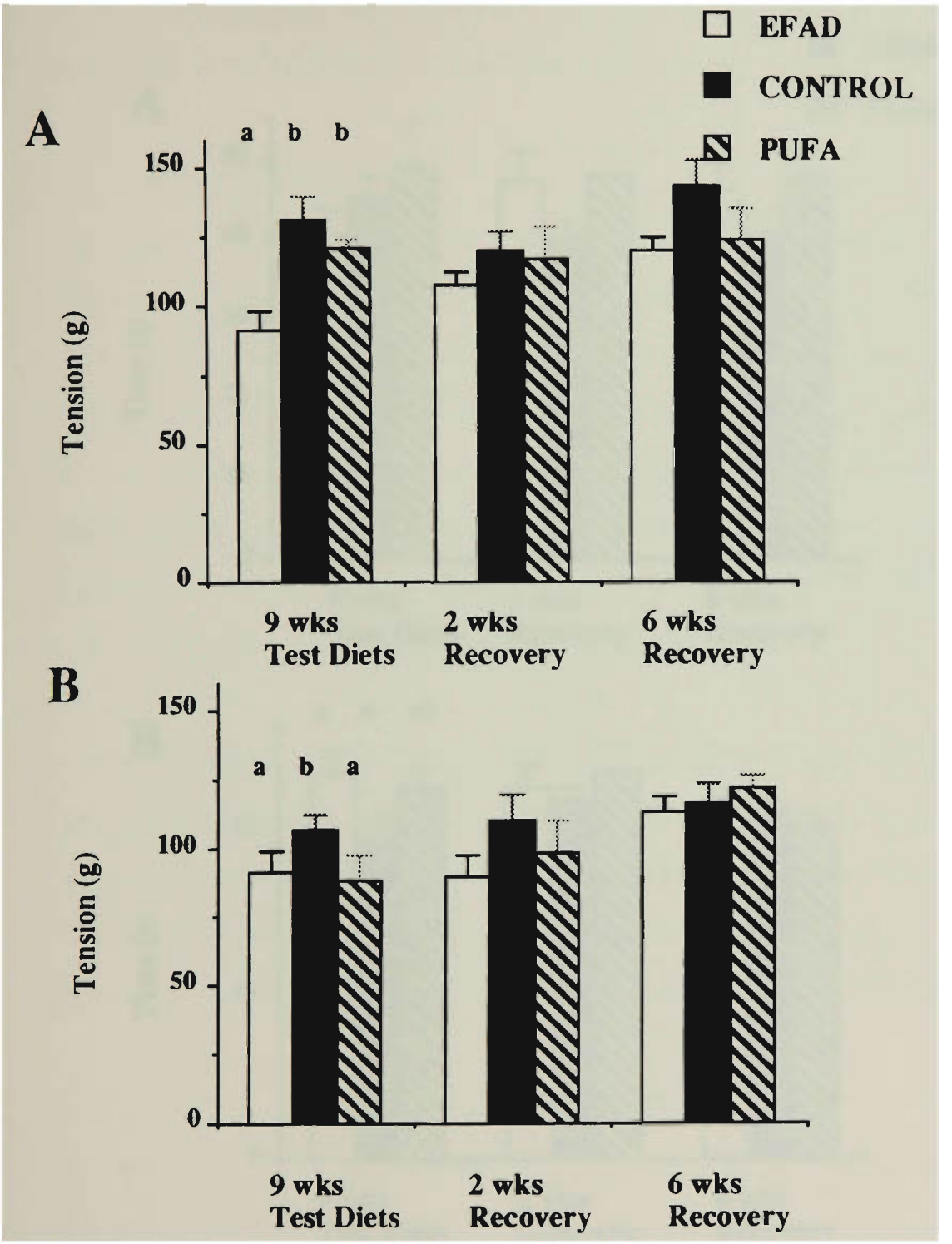


Figure 3.18

Peak tension (g) generated during low frequency stimulation of isolated soleus muscles (Fig. 3.18 A) and EDL muscles (Fig. 3.18 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 60% of their optimal frequency (i.e. the frequency required for maximal force generation) for 330 ms each second for 4 minutes. Diet had a significant effect on both soleus ($P < 0.005$) and EDL ($P < 0.05$) after nine weeks. Significantly different treatment means are denoted by different superscripts.

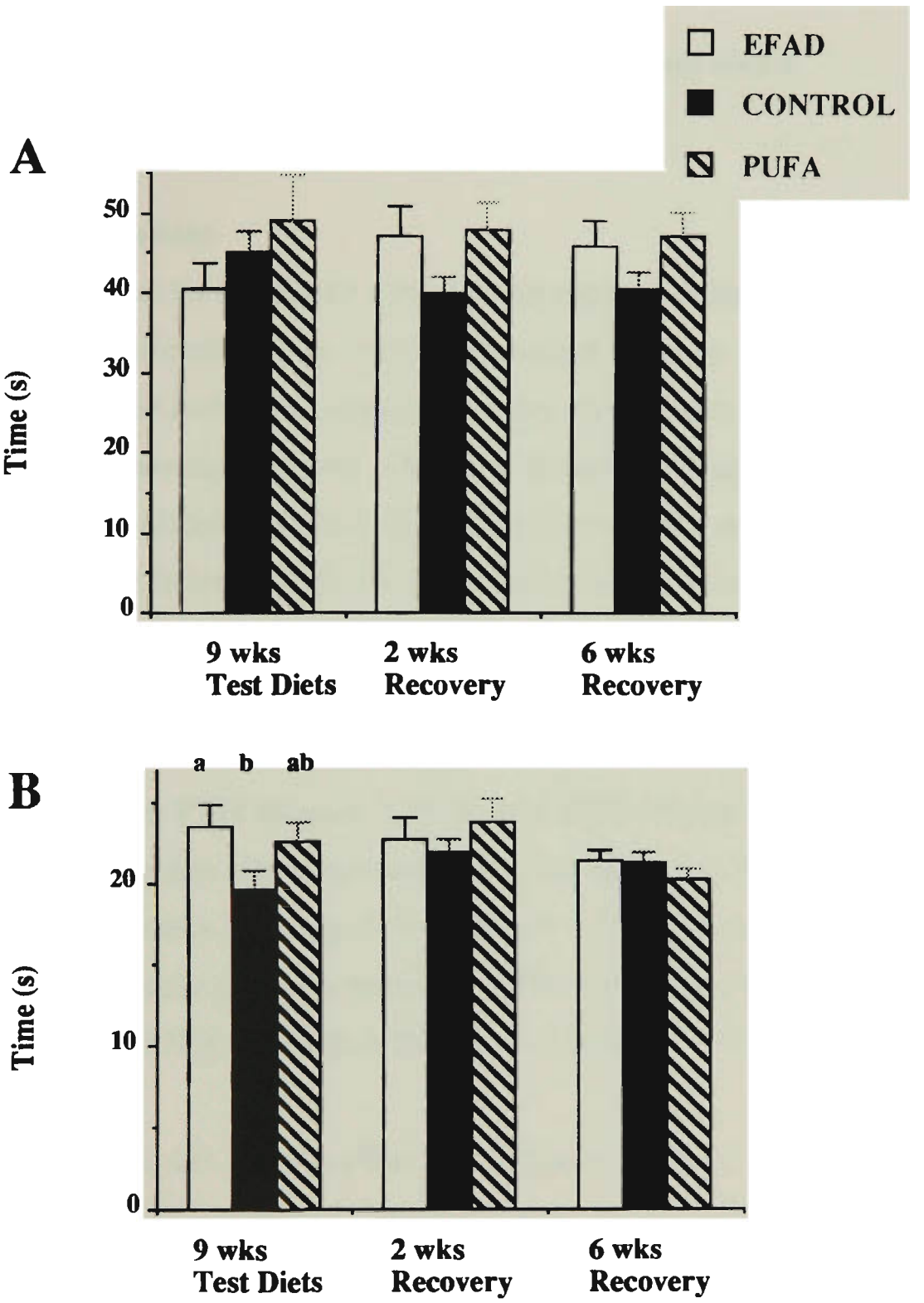


Figure 3.19
Fatigue time (ms) to half peak tension ("endurance") during low frequency stimulation of isolated soleus muscles (Fig. 3.21 A) and EDL muscles (Fig. 3.21 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 60% of their optimal frequency (i.e. the frequency required formaximal force generation) for 330 ms each second for 4 minutes. Diet did not significantly affect fatigue time in soleus after nine weeks ($P > 0.5$) but did have a significant effect on EDL ($P < 0.02$). Significantly different treatment means are denoted by different superscripts.

for the EFAD group than the Control group. There was also a significant litter effect after nine weeks ($P < 0.05$).

(iii) Fatigue Indices

Since soleus muscles developed a progressive increase in baseline tension during stimulation, as reported elsewhere (Carlsen & Walsh, 1987), the fatigue index was calculated in two ways to take the different baselines into account. The Total Tension Fatigue Index (TTFI) was calculated using the original baseline and the Active Tension Fatigue Index (ATFI) was calculated using the new baseline (Carlsen & Walsh, 1987). These indices were equivalent for EDL, as there was no change in the position of the baseline

In soleus, neither TTFI (Figure 3.20 A) nor ATFI (Figure 3.21 A) were affected by diet after nine weeks ($P < 0.2$ and $P > 0.5$), or following two weeks recovery ($P < 0.2$ and $P < 0.5$, respectively). Following six weeks recovery however, ATFI was significantly greater for the PUFA group than the Control group ($P < 0.001$).

In EDL muscles, diet did not affect TTFI (Figure 3.20 B) ($P > 0.05$), after the nine week test period. Similarly, there was no effect of diet after two weeks recovery ($P > 0.5$) or six weeks recovery ($P > 0.5$).

3.3.3 Summary

Two main generalizations can be made from this part of the study. Firstly, muscles from rats on the EFAD diet were affected much more than muscles from rats on the PUFA diet. In fact, there were no significant differences between PUFA and Control groups after

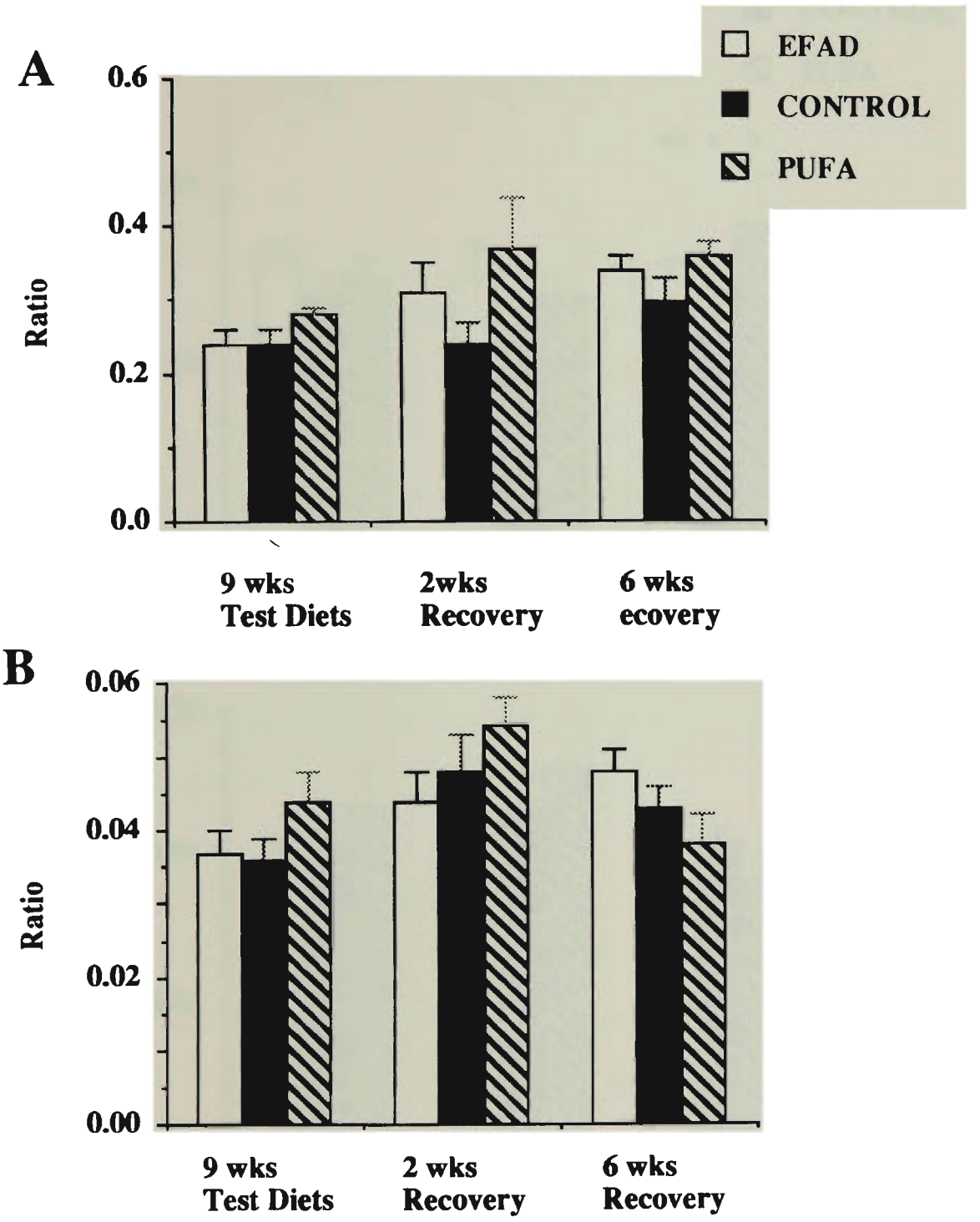


Figure 3.20
Total tension fatigue index (TTFI) during low frequency stimulation of isolated soleus muscles (Fig. 3.20 A) and EDL muscles (Fig. 3.20 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 60% of their optimal frequency (i.e. the frequency required for maximal force generation) for 330 ms each second for 4 minutes. TTFI is the ratio of final train tension, above the baseline, to initial train tension. Diet had no effect on TTFI in soleus at any stage.

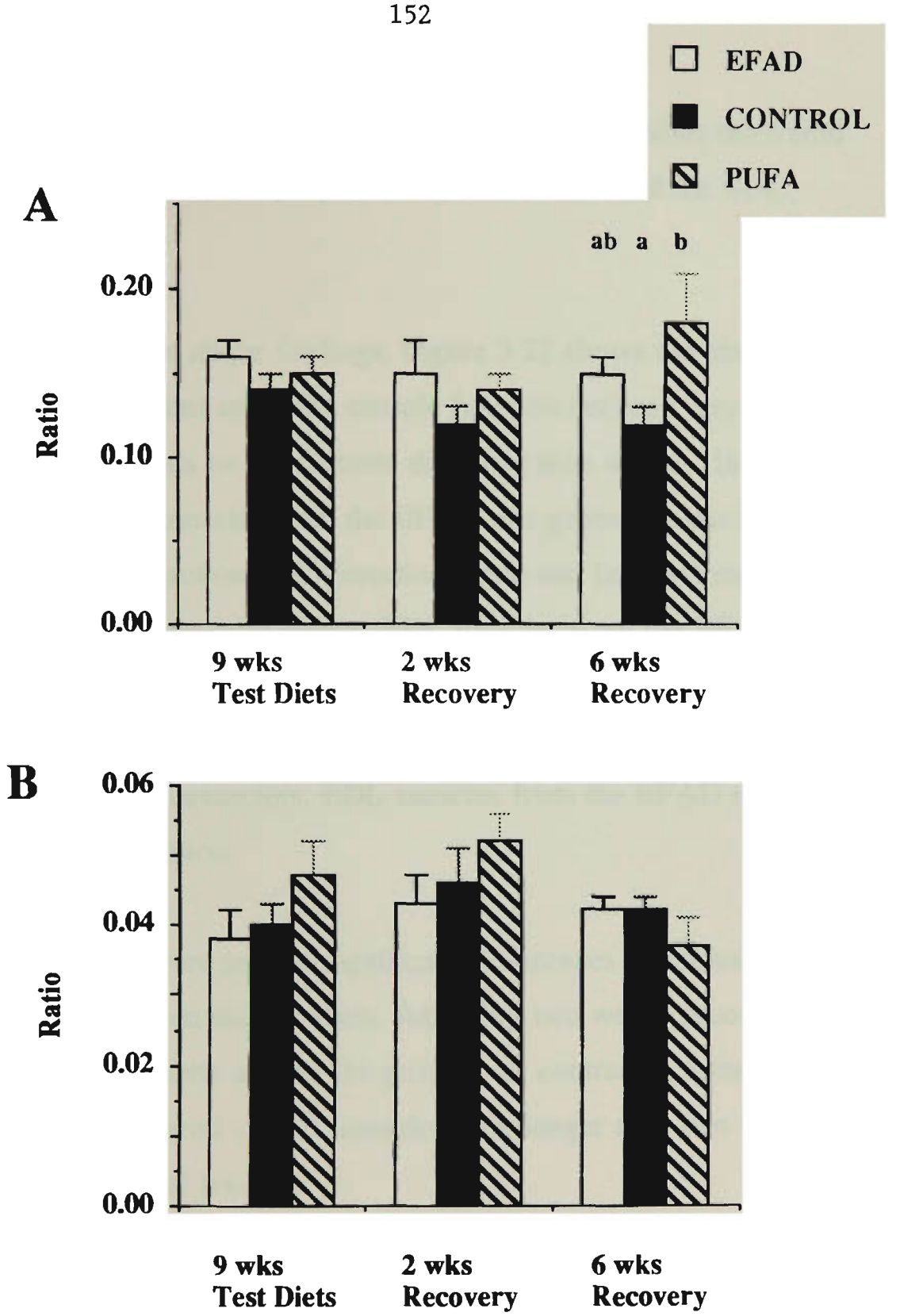


Figure 3.21
Active tension fatigue index (ATFI) during low frequency stimulation of isolated soleus muscles (Fig. 3.21 A) and EDL muscles (Fig. 3.21 B) from groups of five to seven male Wistar rats. Muscles were stimulated at 60% of their optimal frequency (i.e. the frequency required for maximal force generation) for 330 ms each second for 4 minutes. ATFI is the ratio of final train tension in excess of the elevated baseline to initial train tension. Diet had a significant effect on ATFI after six weeks recovery ($P < 0.001$).

nine weeks on the test diets. Secondly, the soleus muscles recovered more quickly from dietary lipid manipulation than did the EDL muscles.

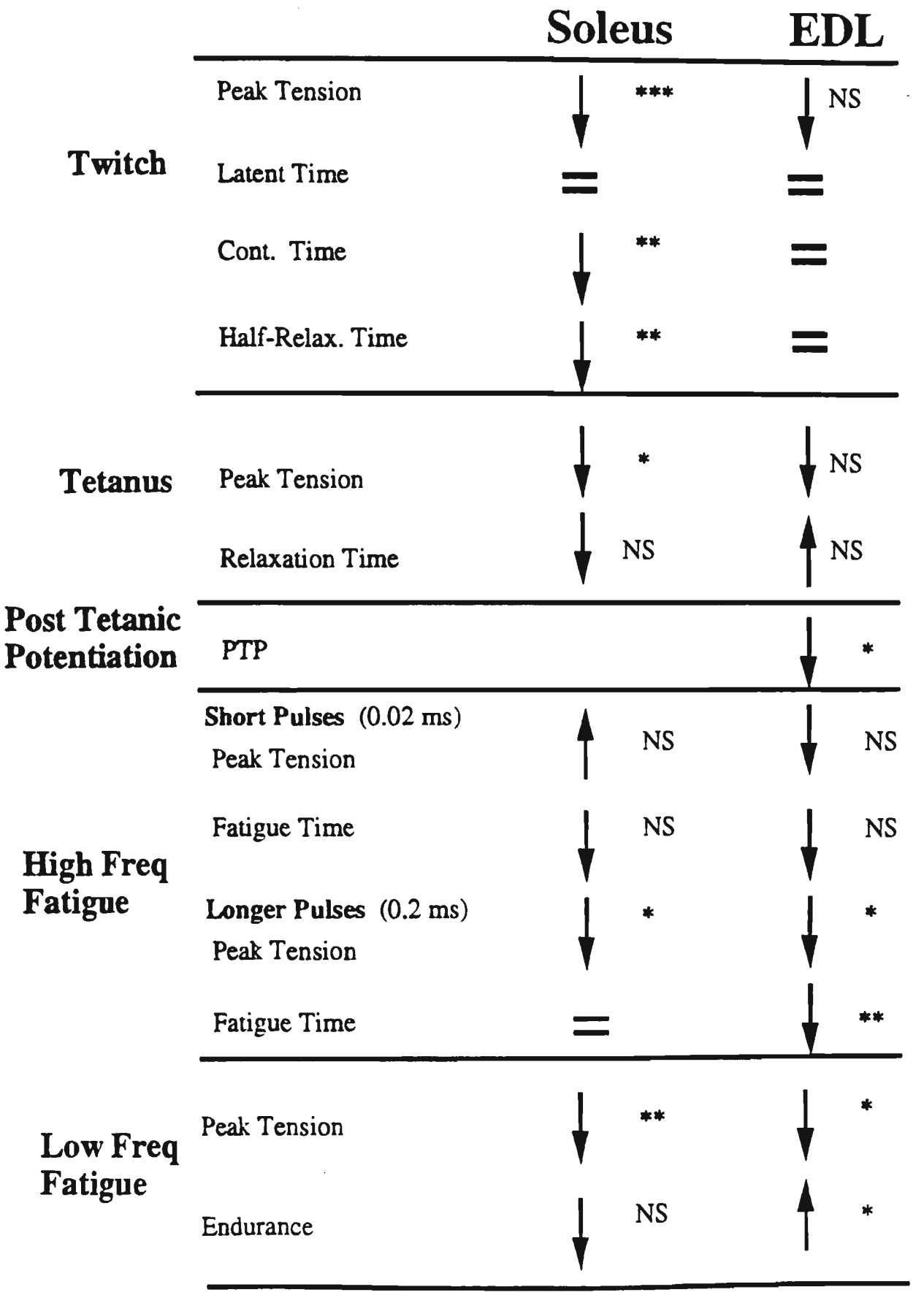
To summarize the major findings, Figure 3.22 shows the main differences in soleus and EDL muscle function between rats on the EFAD diet and rats on the Control diet after nine weeks. In almost all cases, soleus muscles from the EFAD rats generated less tension, had shorter contraction and relaxation times and fatigued more rapidly. In contrast with soleus, EDL muscles from the EFAD rats showed increased relaxation time after maximum tetanus and increased endurance after low frequency stimulation. However, in strength-related parameters, EDL muscles from the EFAD rats also generated less tension.

Although there were several significant differences in soleus muscles after nine weeks on the test diets, following two weeks recovery there was no heterogeneity among the groups. In contrast, a number of parameters measured in EDL muscles took longer than two weeks to recover to Control levels.

3.4 Discussion

This study provides clear evidence that the fatty acid composition of the diet can affect a range of skeletal muscle functions in rats. It also shows that fast and slow muscles respond differently to dietary changes.

Figure 3.22 Summary of the major differences in soleus and EDL muscle function between rats on the EFAD diet and rats on the Control diet after nine weeks. The direction of the arrows indicates whether the response was increased or decreased. For example, mean twitch tension in soleus muscles from the EFAD group of rats was significantly decreased ($P < 0.001$). No difference between the groups is denoted by "="; *** indicates $P < 0.001$; ** indicates $P < 0.01$ and * indicates $P < 0.05$.


Soleus
EDL
Twitch
Peak Tension
***
NS
Latent Time
Cont. Time
**
Half-Relax. Time
**
Tetanus
Peak Tension
*
NS
Relaxation Time
NS
NS
Post Tetanic Potentiation
PTP
*
Short Pulses (0.02 ms)
Peak Tension
NS
NS
Fatigue Time
NS
NS
High Freq Fatigue
Longer Pulses (0.2 ms)
Peak Tension
*
*
Fatigue Time
**
Low Freq Fatigue
Peak Tension
**
*
Endurance
NS
*

The effects of EFA deficiency were typically more pronounced than those of the PUFA diet and resulted in impaired performance. In isometric twitch and tetanic contractions of soleus muscles, the EFAD diet produced lower tensions but faster response times. There were similar, non-significant effects on tension in EDL muscles, but there were no effects on response times. Effects of the PUFA diet were less apparent and performance was sometimes, but not always, enhanced.

During prolonged stimulation at both maximal and submaximal frequencies, the EFAD group again generated lower tensions in soleus and EDL, but the two muscles differed in endurance. Diet did not affect endurance in soleus muscles, but it did have significant effects on endurance in EDL muscles. Again, there were fewer effects of the PUFA diet, but some non-significant differences in tension after nine weeks were statistically significant following recovery in EDL muscles.

Recovery from the dietary changes occurred rapidly, although the two muscles differed in their rates of recovery. In soleus muscles, all parameters which had shown significant dietary effects after the nine week test period were statistically homogeneous within two weeks on rat pellets. However in EDL, although several parameters took longer to recover, most results were statistically homogeneous within six weeks. It is important to note that, in this study, there was no effect of diet on either body weight or muscle dimensions. Thus, when the tension values were corrected for muscle weight and cross-sectional surface area, the differences seen between "whole muscle" values were mostly still evident. This indicates that the cause of the

reduced tension is unrelated to differences in either rat weight, muscle weight or cross-sectional surface area.

There were several strong litter effects throughout this study which were taken into account in the analysis. Nevertheless, their presence highlights the importance of random allocation of litter-mates to treatment groups, and the implementation of a design which can factor out the effects of variation among litters. Most other studies in this area do not take litter into account, instead they randomly allocate animals to treatments.

There appears to have been very little work done on the role of specific types of lipids on muscle function and published results on the effects of malnutrition on muscle function are contradictory. As discussed in Chapter 1, some studies on the effects of malnutrition have shown that skeletal muscles generate less power, fatigue more easily and relax more slowly than control muscles (Lopes *et al.*, 1982; Choong *et al.*, 1984; Russell *et al.*, 1983a; b; Russell *et al.*, 1984a; Chan *et al.*, 1986; Nishio & Jeejeebhoy, 1991) whereas others have reported no effect of malnutrition on muscle function (McCarter *et al.*,1978; Gardiner *et al.*, 1980; Lennmarken & Larsson, 1986).

3.4.1 Specific Effects

An obstacle to interpretation of the importance of these data is the problem of deciding whether a difference observed between the dietary groups can be interpreted as impaired or enhanced performance. Although the results have been interpreted as single factor effects, as in most other studies, this is clearly an over-

simplification. Contraction time (time to contract to peak tension) must be dependent on the size of peak tension, as must be the half-relaxation time (time to relax to half peak tension). Since twitch tension was reduced by 20% in soleus ($P < 0.001$) and by 16% in EDL ($P < 0.10$), it seems logical that it would take less time to contract to a smaller peak tension (T_C). This was the case in soleus muscle, where T_C decreased by 5% ($P < 0.01$), but T_C was not altered in EDL ($P > 0.5$). Hence parameters such as tension and rates of response are probably not independent. For example, in isometric twitches and tetanic contractions, an increased speed of contraction, might be considered to indicate improved performance, but if it is because peak tension is reduced, then is this "fast, poor response" indicative of impaired or enhanced performance? Schematic diagrams of the relative effects of EFA deficiency on muscle twitches and tetanus (Figures 3.23 and 3.24, respectively) show that tensions and response times are interdependent and that fast and slow muscles are affected differently. Isometric tensions and response times were significantly reduced in soleus whereas in EDL muscles, only tensions were reduced. Although effects of EFA deficiency on tetanic relaxation times were not significant, it was interesting to note that in the EFAD rats, relaxation following tetanus was 16% faster in soleus but 19% longer in EDL, than in Control rats.

While some properties of skeletal muscle function cannot be interpreted independently, others such as post-tetanic potentiation, appear to be relatively poorly understood. Repetitive stimulation of fast-twitch skeletal muscle has been shown to cause potentiation of twitch tension (Close & Hoh, 1968a; Krarup, 1981) but the underlying cause is not clear. Carlsen & Walsh (1987) observed a

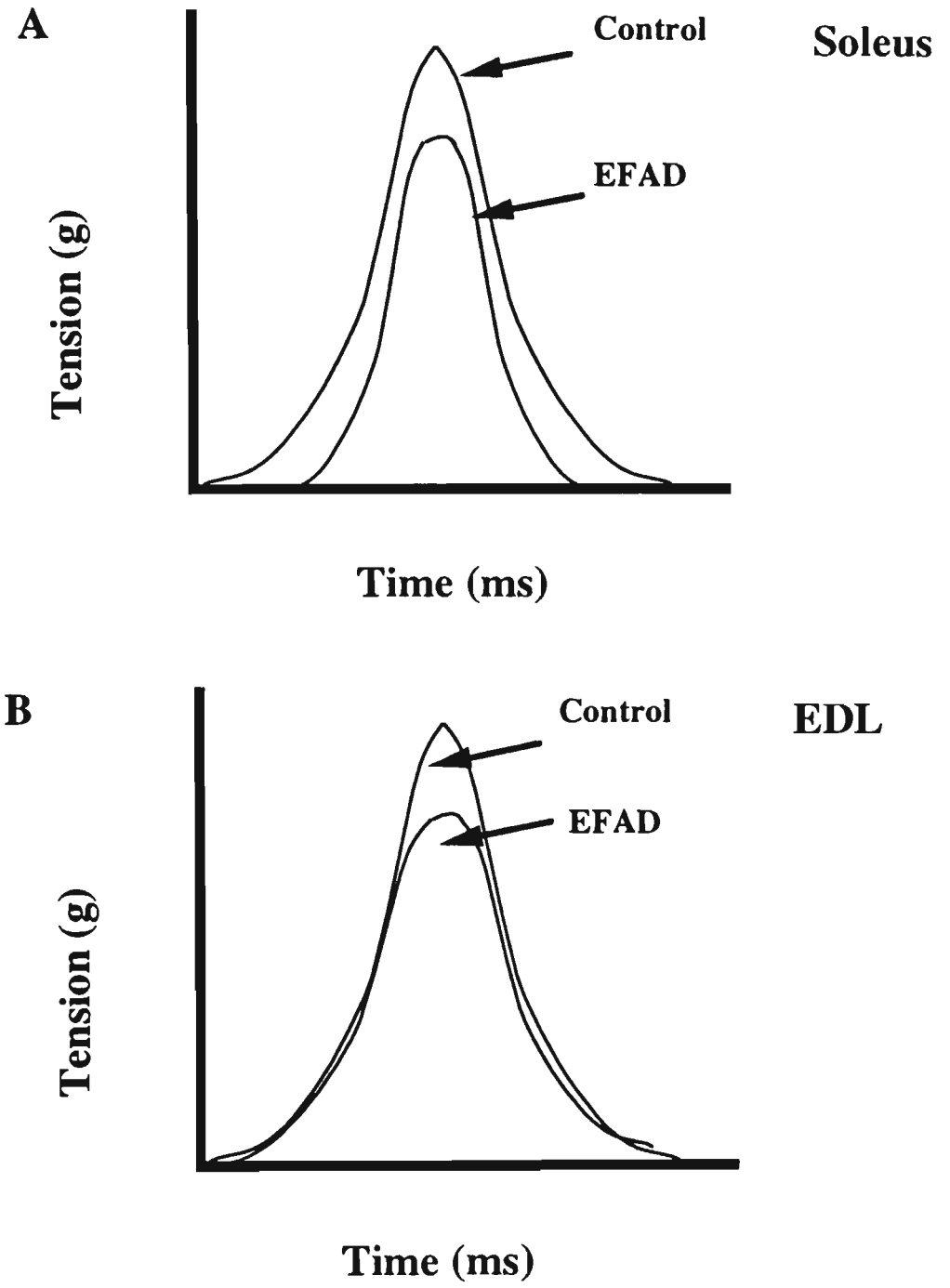


Figure 3.23
Schematic diagram of the relative effects of nine weeks of Control and EFAD diets on single twitches in isolated soleus (Fig. 3.23 A) and EDL (Fig. 3.23 B) muscles from male Wistar rats.

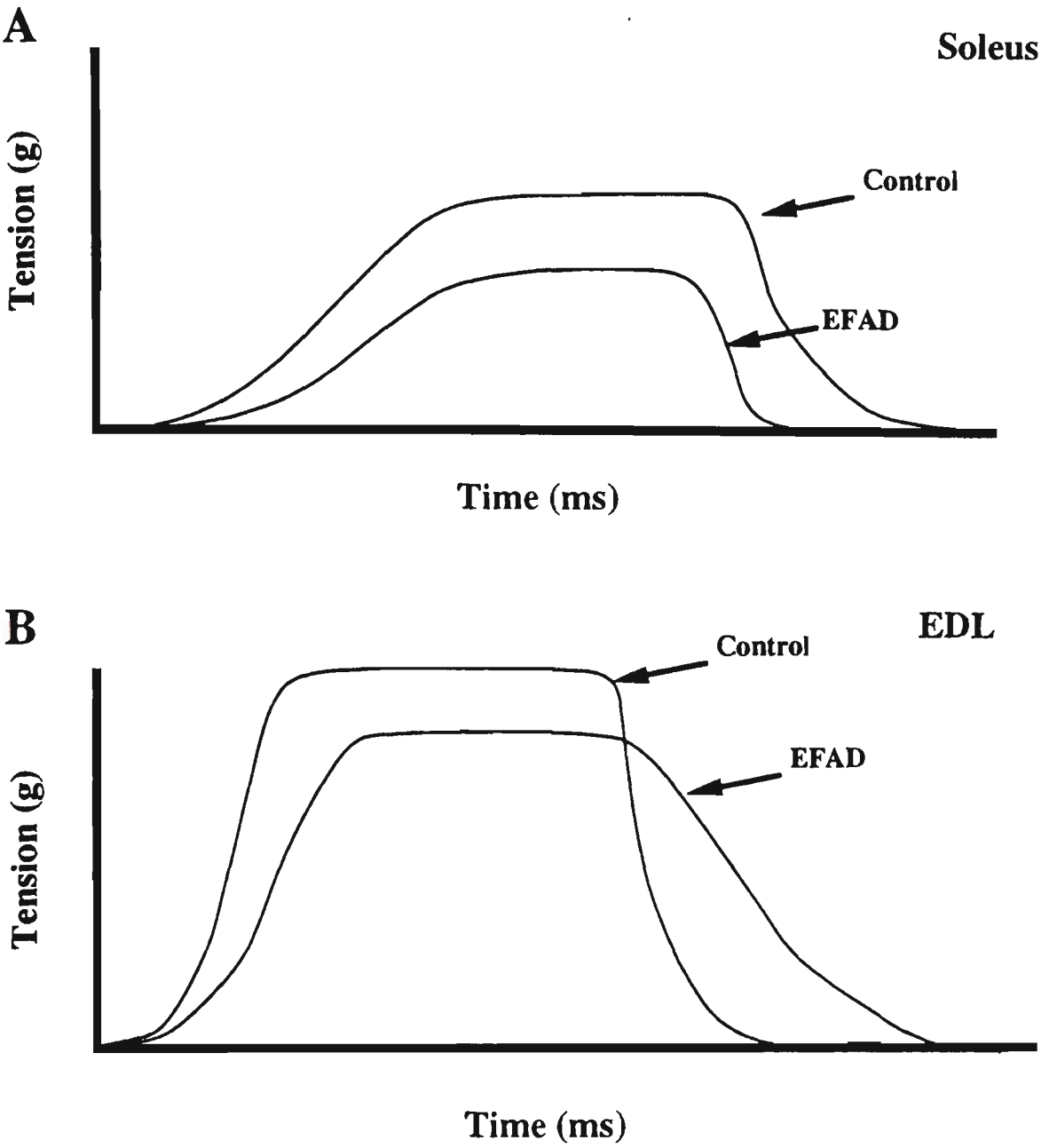


Figure 3.24
Schematic diagram of the relative effects of nine weeks of Control and EFADdiets on maximum tetanic tension and tetanic relaxation time in isolated soleus (Fig. 3.24 A) and EDL (Fig. 3.24 B) muscles from male Wistar rats.

decline in PTP in aging fast twitch muscle which they suggested was due to changes in excitation-contraction coupling and the release of Ca^{2+} from the sarcoplasmic reticulum. They linked increased half-relaxation time to prolonged calcium re-uptake. This could mean that the activity of the enzyme responsible for re-uptake, Ca^{2+}-ATPase, was affected. In this study, there was no significant effect of diet on PTP but there was also no effect of changes in dietary lipids on Ca^{2+}-ATPase activity (see Chapter 5).

Similarly, the effects of high frequency and low frequency stimulation on performance were difficult to interpret. Schematic diagrams of the relative effects of EFAD on high and low frequency fatigue are shown in Figures 3.25 and 3.26, respectively. Again, the effect of EFA deficiency was to decrease peak tensions for both muscles during high and low frequency fatigue, but to increase fatigue time at high frequency and increase endurance at low frequency in EDL muscles.

In this study, peak tension was decreased during single twitches and tetanic contractions in soleus muscles and during high and low frequency stimulations in both soleus and EDL muscles from rats on the EFAD diet. The observed effects of essential fatty acid deficiency on muscle function could be related to changes in sarcolemmal properties, such as changes in propagation of action potentials or maintenance of ionic concentration gradients, or sarcoplasmic reticulum membrane properties, such as release and re-uptake of calcium.

The restoration of ionic gradients following contraction is carried out by the sodium pump. Maximum pump rates in skeletal muscle have

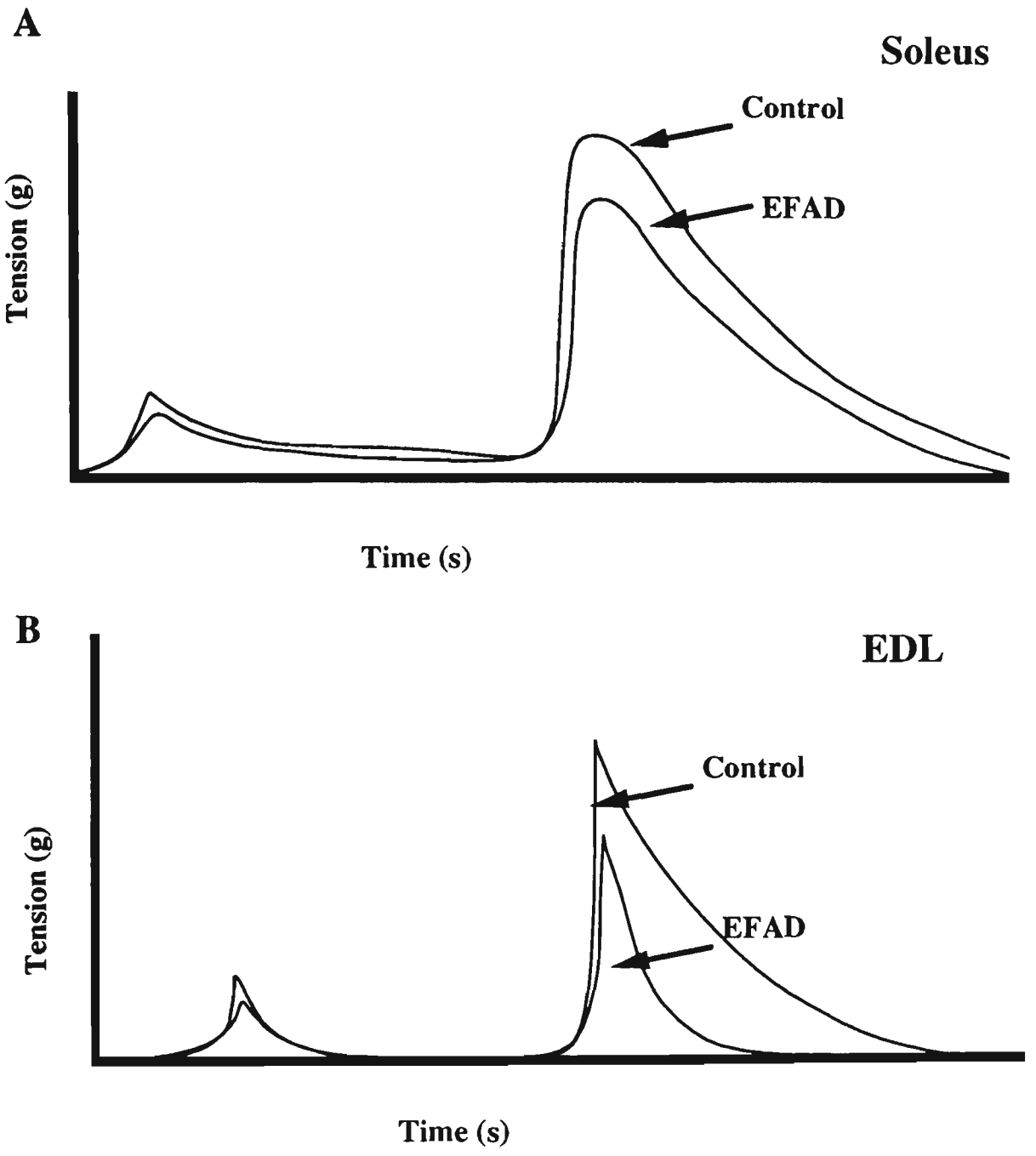


Figure 3.25
Schematic diagram of the relative effects of nine weeks of Control and EFAD diets on high frequency fatigue in isolated soleus (Fig. 3.25A) and EDL (Fig. 3.25 B) muscles from male Wistar rats.

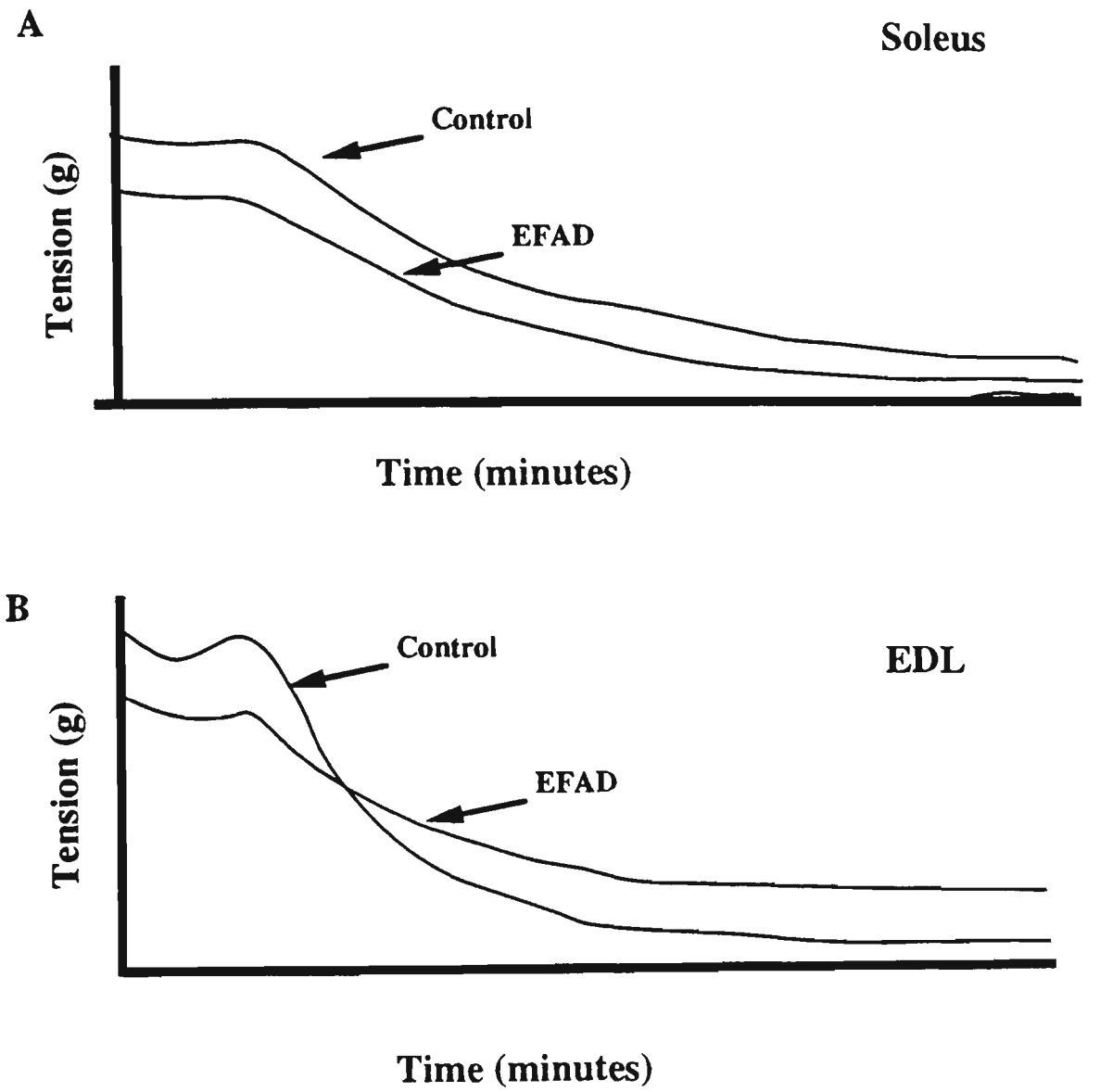


Figure 3.26
Schematic diagram of the relative effects of nine weeks of Control and EFADdiets on low frequency fatigue in isolated soleus (Fig. 3.26 A) and EDL (Fig. 3.26 B) muscles from male Wistar rats.

been used to predict tolerance limits for stimulation frequency. In normal human muscle, the reserve capacity of the pump corresponds to a stimulation frequency of about 40 Hz (Sejersted & Hallén, 1987). Higher stimulation frequencies would result in decreased intracellular K^+ and increased intracellular Na^+ until the intensity of exercise decreased and the sodium pump could restore the normal concentrations.

If the sarcolemma became more "leaky" as a result of changes in dietary fatty acids, or the number or activity of sodium pumps was decreased, then there would be an increased K^+ efflux and the effect would be to lower resting membrane potential. This would lead to greater depolarization, a reduction in the amplitude of action potentials (since ionic gradients would not be maintained), a reduction in calcium release from the sarcoplasmic reticulum and therefore a reduced response during contraction.

Alternatively, if the number or activity of calcium pumps is reduced by changes in dietary fatty acids, this could lead to a reduced re-uptake of calcium following contraction. The result would be longer relaxation times and increased time between contractions, leading to fewer contractions and reduced muscular tension.

Jones (1981) reported that an effect of high frequency stimulation in isolated muscles is an alteration of the excitation threshold. He showed that for pulses between 0.02 ms and 0.2 ms duration, twitch tension was similar and for tetanic stimulations at 200 Hz, pulses between 0.02 ms and 0.2 ms duration also produced similar tensions in fresh muscle. However, following prolonged stimulation at high

frequency, force is rapidly reduced but it can be rapidly restored by decreasing the frequency or increasing the duration of stimulation.

The effect of increased duration during high frequency stimulation was demonstrated in the current study by a sudden increase in force in both soleus and EDL muscles. Increasing the duration of stimulation is likely to allow a greater efflux of K^+ and influx of Na^+ than during stimulation with shorter pulses and lead to a restoration of force, although only temporarily. In this study, there was no significant effect of diet on tension generated during the first test of HFF when the stimulus pulses were 0.02 ms duration, but the results were extremely variable. However, when pulse duration was increased 10 fold, although force immediately increased in all dietary groups, there was a significant reduction in tension in both soleus and EDL muscles from rats on the EFAD diet. If the membrane was changed in such a way that there was greater accumulation of K^+ in the extracellular fluid during stimulation, this could be responsible for the reduced tension observed during high frequency fatigue in the EFAD rats.

Under normal conditions *in vivo*, or in isolated preparations with intact blood flow, there should not be a build-up of either metabolites or ions extracellularly during low frequency stimulation, since these will be rapidly removed in blood. However, isolated muscles studied *in vitro* are unable to remove unwanted metabolites and this build-up of potassium and metabolites may explain fatigue seen in these conditions. In this study, maximum tension generated during low frequency stimulation was reduced in both soleus and EDL muscles from the EFAD rats. Again, if the effect of EFA deficiency is to increase the accumulation of extracellular potassium, this may also

explain the decreased tensions observed during low frequency stimulation.

While the 20% longer endurance times seen in EDL muscles from the EFAD rats may appear to offer some protection against the induction of muscle fatigue, this effect should be viewed in the context of the marked loss in muscle strength (14%, $P < 0.02$) which also occurred.

In summary then, if fatigue is due to the loss of intracellular potassium which builds up in the extracellular fluid and impairs the propagation of action potentials, then at high frequency stimulation, isolated muscles would quickly fatigue since there is no blood flow. If the loss of K^+ was increased by changes in membrane composition as a result of changes in dietary fatty acids, the effect would be greater, i.e. tensions would be reduced. Similarly, during stimulation of isolated muscles at low frequency, since there is no flow of blood to remove the increased concentration of potassium, the high level of extracellular potassium in the extracellular fluid could alter the transmission of action potentials in a similar way to high frequency fatigue, but at a lower rate.

High frequency stimulation was used in this study to demonstrate fatigue and it resulted in a reduction in force but rapid recovery when pulse duration was increased. This type of protocol has been used to approximate maximal voluntary contractions in humans, but there are some important differences. In a maximal voluntary contraction, the firing frequency of motorneurons declines in order to optimise force production (Bigland-Ritchie *et al.*, 1979; Jones *et al.*, 1979) and the muscle fibres are ischaemic (Bigland-Ritchie & Woods, 1984;

Sjøgaard *et al.*, 1988). Since it is unlikely that high-tetanic rates of stimulation ever actually occur, it is unclear as to how relevant high frequency fatigue studies are to voluntary muscular contraction. During high intensity exercise in humans, a high extracellular [K^+] may be connected to exhaustion, but with lower intensity exercise there is no continuous build-up of extracellular [K^+], and a steady state seems to be reached within five to ten minutes (Vøllestad & Sejersted, 1988). Therefore, more confidence could be placed on results from studies of low frequency fatigue, since these stimulation rates more closely approximate what may occur during natural activity. According to Jones *et al.* (1979), the natural firing rate at the start of a maximal voluntary contraction in human subjects is about 80 Hz for at least some of the motor units. Since the mean optimal frequency for the muscles in this study ranged from 99 Hz to 111 Hz, the frequency used during the low frequency fatigue test ranged from 60 Hz to 67 Hz (i.e. 60% of the optimal frequency), which is similar to the frequency found during natural activity. During the low frequency repetitive stimulation tests, similar patterns of effects occurred as in the previous tests. Tensions were significantly reduced in soleus muscles and EDL muscles from rats on the EFAD diet, but endurance was increased in EDL muscles from these rats when compared with the Control group. These differences between fast and slow muscles were very similar to those observed during maximum tetanic contractions. As with high frequency stimulation, the soleus muscles were more resistant to low frequency fatigue than EDL muscles since total tension fatigue indices were about seven times greater in soleus.

It can be seen that the overall effect for each major test of muscle function was a general change in the nature of the response. In EFA deficient rats, isometric tensions and response times and fatigue tensions decreased in soleus; fatigue tensions decreased and endurance increased in EDL. In the context of this study, it is important that many measures of muscle function were clearly altered by the test diets and returned to normal during the recovery period.

3.4.2 Interpretations

Since several tests were performed on each muscle, the muscles could be expected to fatigue cumulatively although there were recovery periods between successive tests. The standard tests for fatigue, as used previously by others, may not be equally relevant to performance of whole animals or to different types of muscles. For example, the stimulus rate required for the production of maximum force of a motor unit depends on its contractile speed; stimulation rates that cause tetanic fusion in fast twitch units are supratetanic in slow twitch units (Bigland-Ritchie *et al.*, 1986).

The physiological significance of high frequency fatigue during maximal voluntary contractions is unclear since it is unlikely that such high motorneuron firing rates would ever be achieved (MacLaren *et al.*, 1989). During sustained voluntary contractions in humans, muscle fatigue is minimized by a reduction in motor neuron firing frequency (Jones *et al.*, 1979; Bigland-Ritchie *et al.*, 1979). Other studies have reported that slow-twitch fibres are more resistant to HFF than fast-twitch fibres (Jones *et al.*, 1981; Clamann & Robinson, 1985; Sandercock *et al.*, 1985). This difference was marked in this

study since during high frequency fatigue, soleus muscles took 10 - 20 times longer to fatigue to half peak tension than EDL with short pulses and three to four times longer than EDL with long pulses, even though EDL muscles generated greater tensions.

In estimating CSSA by dividing muscle mass by muscle length, the assumption is made that specific gravity of the muscle is equal to 1 and that all the muscle fibres were the same length and equal to the length of the muscle (Close, 1972). When tension results were adjusted for CSSA, this second assumption results in an overestimation of muscle strength since it is unlikely that muscle fibre length is equal to muscle length. In a comparison of six methods of force normalization, including CSSA, Nishio *et al.* (1992) concluded that results for measures of force may depend on the method used for normalization. In this study, changes in dietary fatty acids had no effects on muscle mass, length or the calculated CSSA and results for the different dietary groups for all measures of tension were similar, regardless of whether they were expressed as "whole animal" values, or adjusted for weight or CSSA.

In this study, the frequency used for high frequency stimulation (300 Hz) was extremely high and resulted in very rapid fatigue. This frequency was chosen to match that used for maximum tetanic contraction. A more relevant frequency may have been 200 Hz, as used by Jones (1981). However, since the aim of the study was to determine whether dietary changes affected contractile responses, the protocol used was relatively unimportant since all dietary groups were treated identically.

The response times (T_c and $T_{1/2\ R}$) in both soleus and EDL were greater than in some other published studies, but comparable with others, (see section 3.2, Methods). However, as discussed, results were very repeatable and rats and muscles from all dietary groups were treated identically. Also, the large number of tests performed should be sufficient to detect any effects of the diets on muscle function. One possible explanation for the increased response times was that temperature was less than it seemed. Close & Hoh (1968b) reported that a decrease in temperature from 35 °C to 20 °C increased isometric twitch contraction time from 10.8 ms to 37.5 ms for EDL and from 28 ms to 122 ms for soleus, increased half-relaxation time from 9.2 ms to 42 ms for EDL and from 36 ms to 170 ms for soleus and almost doubled peak tension in EDL, but had little effect in soleus. However, temperature of the medium surrounding the muscles was carefully monitored and averaged 33 - 34 °C and peak tension in this study were similar to those of other studies. The most likely explanation is that none of these other studies used male Wistar rats. The studies by Close (1964; 1967) and Close & Hoh (1968a; b) used female Wistar rats and all others used different strains (Gardiner *et al.*, 1980; Krarup, 1981, Sahlin *et al.*, 1981; Carlsen & Gray, 1987). Nevertheless, the results were highly repeatable (coefficients of variation < 15%) and so provide a valid comparison of the effects of the three diets.

Of interest throughout this study, was the seemingly delayed response of EDL muscles to dietary changes in several patterns of stimulation in that differences between the dietary groups were not statistically significant until after two weeks recovery. This occurred for peak twitch tension (Figure 3.3), maximal tetanic tension (Figure 3.5) and

peak tension during high frequency fatigue (with short duration pulses) (Figure 3.13). The question is: do these differences reflect delayed onset of the effect, or just a statistical artefact that was present, but not significant, after nine weeks on the test diets? Since the size differences between the dietary groups for twitch and tetanic tensions are similar after both the nine week test period and following recovery, it is likely that the variation at nine weeks was too large to detect significant statistical heterogeneity. Similarly, very large differences in high frequency fatigue were present between the dietary groups at each stage, but again, the errors were large at nine weeks.

3.4.3 Implications for Whole Animals

Some predictions can now be made for the effects of changes in dietary lipids on whole animal performance. The results of this isolated muscle study suggest that rats maintained on an EFA deficient diet may show an overall impairment of physical performance. Since the composition of the rat hindlimb is proportionately similar to an EDL muscle (i.e. about 95% fast-twitch fibres and 5% slow-twitch fibres), rats might be expected to show reduced strength but possibly, increased endurance during low frequency stimulation e.g. running. The PUFA diet now appears likely to have little effect on physical performance. The only other studies to investigate the effects of dietary changes on skeletal muscle function in both individual muscles and whole organisms appear to be those of Lopes *et al.* (1982) and Russell *et al.* (1983a; b; 1984a; b) who found similar effects of fasting and hypocaloric dieting on isolated muscle function and malnourished patients. Nevertheless, predictions are confounded

by the fact that physical performance at the level of the whole animal will reflect other effects of changes in dietary lipids on factors such as the composition of muscle membranes, adipose fat stores, the nervous system and activity of membrane-bound enzymes, metabolism of fats, the pulmonary and cardiovascular systems and integration of the entire skeletal muscle system.

Chapter 4 Effects of Changes in Dietary Fatty Acids on Physical Performance in Rats

4.1 Introduction

In Chapters 2 and 3, I showed that manipulation of dietary fats in rats can alter both muscle membrane composition and muscle function; that these changes can be reversed and that fast EDL muscles take longer to recover than slow soleus muscles. The logical question arising from such findings was whether dietary lipid manipulation could also alter physical performance of whole animals. If so, then it might be possible to make dietary recommendations to improve performance or work productivity.

The vast majority of studies involving exercise tests in rats have focused on the effects of training (i.e. comparisons between fit and unfit rats) and only a few studies have determined the effects of exercise in untrained rats (Flaim *et al.*, 1979; Gleeson & Baldwin, 1981; Baldwin *et al.*, 1982; Musch *et al.*, 1988). In this study, I was not interested in training, but rather the effects of different diets on measures of physical performance.

Since dietary manipulation could affect physical performance in a variety of ways, a series of tests was used in order that overall patterns of response to dietary manipulation could be described. Unlike the studies on isolated muscles in Chapter 3, differences in whole animal performance tests could quite easily be categorized as being an improvement or impairment of performance. In this study, four tests were used. They were oxygen consumption at rest ($\dot{V}O_2$

basal) and during maximal exertion ($\dot{V}O_{2\ peak}$), submaximal endurance and strength. Both $\dot{V}O_{2\ basal}$ and $\dot{V}O_{2\ peak}$ were measured as an indication of dietary effects on resting oxygen consumption and exercise capacity, respectively, and time to exhaustion was measured during running at submaximal intensity to provide an estimate of endurance. Also, the many effects on isolated muscle tension during isometric contractions observed in Chapter 3 indicated that strength may be affected by changes in dietary fatty acids. Therefore, forelimb grip strength was also measured.

Both maximal oxygen consumption and endurance were measured during treadmill running. To measure oxygen consumption during exercise in rats, there must be some way of sampling both inspired and expired air. Two techniques used for sampling oxygen during tests of oxygen consumption in rats during exercise include the use of a running chamber (Brooks & White, 1978) and a mask (Gleeson & Baldwin, 1981). Both methods have advantages and disadvantages, as discussed by by Musch *et al.* (1988) and in this study, a metabolic chamber was used (see Methods).

The aims of this part of the study were:

(1) to investigate whether changes in dietary fatty acid composition can affect physical performance parameters including oxygen consumption, submaximal endurance and strength and

(2) to determine whether any changes in physical performance can be reversed by a period on a normal diet.

4.2 Materials and Methods

4.2.1 Rats

Male albino Wistar rats, weighing 54.3 ± 1.0 g at weaning, were used. Maintenance and diets were as described in Chapter 2. Litter-mates were assigned at random, one per litter, to each of three dietary groups: Control, EFAD or PUFA. In this experiment, there was only one recovery period of six weeks. Rats were maintained on the test diets for nine weeks, followed by six weeks recovery on normal rat pellets.

4.2.2 Outline of Testing Programme

All physical performance tests were done as double-blind trials. Each rat was tested twice: once after nine weeks on the test diets and again following six weeks recovery). Four tests were performed on each rat at both times. The tests were grip strength (GS); basal oxygen consumption ($\dot{V}O_{2\ basal}$); peak oxygen consumption ($\dot{V}O_{2\ peak}$) and endurance.

Peak oxygen consumption and endurance were measured while rats ran on a motor-driven treadmill. In the week prior to each testing period (i.e. after eight weeks on the test diets and after five weeks on rat pellets), rats underwent an habituation programme to ensure that they would be familiar with running in a chamber and on a treadmill. When animals are taught to run on a treadmill, some form of motivation is essential to ensure that they keep running to exhaustion. Two devices commonly used are the electric shock system in which

electrified prods protrude into the rear of the chamber (Shepherd & Gollnick, 1976; Birrell & Roscoe, 1978; Brooks & White, 1978; Gleeson & Baldwin, 1981; Baldwin *et al.*, 1982; Musch *et al.*, 1988) and an air stimulation system (Flaim *et al.*, 1979; Russell *et al.*, 1980). When animals lag towards the rear of the chamber, they will feel a mild electric shock or jets of air respectively, which keep them running close to the front (Oscai & Molé, 1975). According to Oscai & Molé (1975), these devices persuade animals to run within a few sessions. In this study, an air stimulation system was used for rats running free on the treadmill (endurance test) and loud "taps" on the box were used for rats running in the metabolic chamber ($\dot{V}O_{2\ peak}$).

Maximal oxygen consumption can be measured either with the test animal running inside a metabolic chamber (Shepherd & Gollnick, 1976; Brooks & White, 1978 Bedford *et al.*, 1979; Baldwin *et al.*, 1982; Pica & Brooks, 1982; McDonald *et al.*, 1988; Hilty *et al.*, 1989; Cortez *et al.*, 1991), or with the test animal wearing a mask (Russell *et al.*, 1980; Gleeson *et al.*, 1981; 1983) and both methods have received wide acceptance. In a comparison of the two methods, Musch *et al.* (1988) found that the volumes of oxygen ($\dot{V}O_2$) consumed, at rest and during maximal exercise were similar, but that there were some significant differences during submaximal exercise, particularly at the lower workloads. They also observed gait differences between the two groups. Rats wearing masks had to contend with the added weight of the mask and tended to run with their heads hyperextended. These differences in gait appeared to dissipate with increasing work loads. Musch *et al.* (1988) also pointed out that more rats were willing to run in a chamber than when wearing a mask, and that it took less time to accustom rats to a

chamber. After extensive initial trials with both masks and a metabolic chamber, it was decided to conduct the $\dot{V}O_{2\ peak}$ tests in the metabolic chamber. Trial results supported the findings that rats ran more willingly in a chamber than when wearing a mask.

Following habituation, GS, $\dot{V}O_{2\ basal}$, $\dot{V}O_{2\ peak}$ and submaximal endurance were measured in each rat over a four day period. Rats were fasted overnight before $\dot{V}O_{2\ basal}$ and $\dot{V}O_{2\ peak}$ tests and all tests were performed between 06.00 and 12.00. Rats were given one day to recover between $\dot{V}O_{2\ peak}$ and endurance tests. Each of the $\dot{V}O_{2\ peak}$ and endurance tests was conducted only once during each testing period (i.e. once after nine weeks on the test diets and again, following six weeks recovery). In preliminary trials, these tests were conducted three times on each animal. However, for several reasons - (a) the results were found to be very repeatable in these trial tests (Table 4.1); (b) due to time constraints of animals being ready for testing and needing a recovery day between running tests and (c) a number of rats seemed to be reluctant to run on consecutive days, the number of measurements of $\dot{V}O_{2\ peak}$ and endurance on each rat was reduced to one.

4.2.3 Test Details

Results for GS, $\dot{V}O_{2\ basal}$ and $\dot{V}O_{2\ peak}$ were recorded on an Apple Macintosh SE computer with the aid of a MacLab (Analog Digital Instruments, Sydney, Australia), as described in Chapter 3.

Table 4.1 Preliminary results (mean ± SE) for $\dot{V}O_{2\ basal}$, $\dot{V}O_{2\ peak}$ and endurance from 12-week old male Wistar rats which have been fed normal rat pellets. Numbers in brackets represent the number of trials for each rat.

Rat #	$\dot{V}O_{2\ basal}$ (ml $O_2.kg^{-1}.min^{-1}$)	$\dot{V}O_{2\ peak}$ (ml $O_2.kg^{-1}.min^{-1}$)	Endurance (mins)
1	10.9 ± 1.6 (2)		
2	12.1 ± 2.5 (3)		
3	12.8 ± 2.5 (3)		
4	13.5 ± 0.5 (3)		
5	14.2 ± 2.4 (3)		
6	10.6 ± 3.6 (3)		
7	14.0 (1)	35.5 ± 1.6 (3)	17.0 ± 0.9 (3)
8	12.0 (1)	32.8 ± 2.8 (3)	6.2 ± 0.4 (3)
9	13.0 (1)	30.1 ± 0.1 (2)	4.2 ± 1.6 (3)

4.2.3.1 Grip Strength

Grip strength was measured using a modified version of a grip strength testing apparatus described by Meyer *et al.* (1979) (Plate 4.1). It consisted of a suspended T-bar connected to a force-displacement transducer (Grass, type FT 03 C, Quincy, U.S.A.). The output from the transducer was recorded by a MacLab and Macintosh SE computer. Prior to testing, the transducer was calibrated with a set of known weights and confirmed that transducer output (mV) and tension (g) were linearly related to 2000 g ($r^2 = 0.998$).

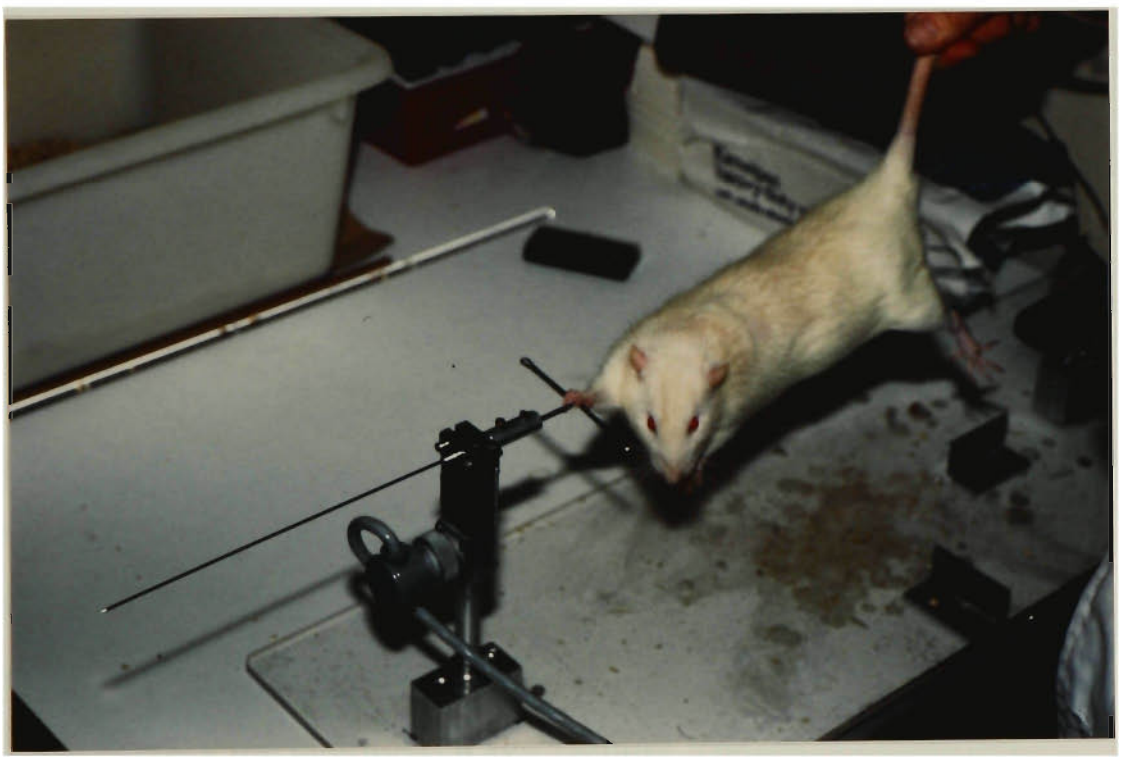
When rats were suspended by the tail, they tended to hold themselves at an angle of approximately 60° to the ground. When held towards the T-bar, they grasped hold and as they pulled on the bar, the tension was recorded by the force transducer and computer. This test was performed four times on each of four days. The highest tension for each day was recorded and the value for each rat represents a mean of these four highest results.

In the grip-strength testing apparatus described by Meyer *et al.* (1979), the rat was placed horizontally on a perspex surface with its paws inside a triangular ring. They were then pulled away from the ring until their grip was broken. However, although an identical apparatus was constructed and tested in this study, the rats would not pull on the T-bar when placed on a platform but did so when suspended approximately horizontally, as described.

4.2.3.2 Habituation to Treadmill Running

Rats were habituated to running in a chamber and on a treadmill by a five minute exercise period each day in the week prior to testing, with

Plate 4.1 Apparatus used for measuring forearm grip strength in rats (modified from Meyer *et al.*, 1979). The rat was suspended by the tail and held towards the T-bar. When it grasped the T-bar, the tension generated was recorded using a force transducer and Maclab.



speed and incline being increased each day (Table 4.2). The habituation programme used was a modified version of those used in other studies (Patch & Brooks, 1980; Baldwin *et al.*, 1982; Pica & Brooks, 1982) and rats quickly became accustomed to running on the treadmill.

As stated above, some rats appeared reluctant to run on consecutive days. A few other studies have reported rat refusal rates of 10 - 15% (Bedford *et al.*, 1979; Russell *et al.*, 1980; Gleeson *et al.*, 1983). In this study, after nine weeks on the test diets, only one rat refused to run a second time, having run well on the previous day. Along with his litter-mates, he also refused to run after six weeks recovery. In the majority of cases, however, a shot of compressed air or a loud noise kept the rats running until they appeared to be exhausted.

4.2.3.3 Oxygen Consumption

Oxygen consumption was measured using a rapid-flow open-circuit system. A constant flow of ambient air was drawn through a metabolic chamber by a vacuum pump at an average rate of 4698 ± 42 ml . min^{-1} (at standard temperature and pressure; 0°C, 760 Torr). Of the gas leaving the chamber, a sub-sample was passed through Drierite (anhydrous $CaSO_4$) (BDH, Melbourne, Australia) and Vivalyme (indicating soda lime) (CIG Medishield, Sydney, Australia) to remove water and carbon dioxide, respectively. The oxygen content of outlet air ("animal air") was continually monitored and analysed by a paramagnetic oxygen analyzer (Taylor Servomex, type OA 272, Sussex, England). With the use of three-way taps, inlet air ("atmospheric air") was intermittently analysed. The volume of air passed through the chamber was constantly monitored with a gas

Table 4.2 Habituation programme used to accustom rats to running on a treadmill (this programme was modified from Patch & Brooks, 1980; Baldwin *et al*, 1982; Pica & Brooks, 1982). Rats were habituated to running in a chamber and on a treadmill by a five minute exposure period each day for a week prior to testing.

	Time (mins)	**Speed** ($m.min^{-1}$)	**Incline** (°)
Day 1	2	0.0	0
	3	14.6	0
Day 2	5	14.6	0
Day 3	5	20.0	0
Day 4	5	20.0	5
Day 5	5	25.2	5
Day 6	5	25.2	10

meter (Singer # DTM-115) and its output was recorded by the MacLab. Digital outputs of the oxygen analyser and gas meter were recorded by a MacLab and displayed visually on a Macintosh computer. The response time of the system for gas analysis was established by the admission of known gases into the chamber and was found to be 70 s.

The oxygen analyser was calibrated before and after each experiment by flushing with dried inlet air, closing the system to the atmosphere and varying the pressure (measured with a water manometer) within the system. The composition of inlet air was assumed to be 20.95% O_2 and the calculated change in the pressure of O_2 in an air sample (measured by the oxygen analyzer) was equated with the analyzer output (in mV).

The rate of oxygen consumption was calculated using the measured flow of air through the chamber (corrected to standard temperature and pressure) and the fraction of oxygen in both inlet and outlet air, according to equation 4a of Withers (1977).

$$\dot{V}O_2 = \text{flow rate} \bullet \Delta\, O_2 \text{ (between inspired and expired air)}$$

Oxygen consumption is expressed in ml O_2.kg body wt^{-1} . min^{-1}.

4.2.3.4 Basal Oxygen Consumption ($\dot{V}O_{2\ basal}$)

Rats were in an airtight metabolic chamber consisting of a cylindrical perspex chamber surrounded by a circulating water bath. (350 ml volume) at 30°C. After approximately 30 minutes acclimation,

oxygen consumption was measured continuously for three hours. For each animal, $\dot{V}O_{2\ basal}$ was recorded as the mean difference between inlet oxygen concentration ("atmospheric air") and outlet oxygen concentration ("animal air"). The average of the four lowest 10-minute periods of oxygen consumption during this time was used as the rat's basal oxygen consumption rate.

4.2.3.5 Peak Oxygen Consumption ($\dot{V}O_{2\ peak}$)

Maximal oxygen consumption was estimated while rats performed a maximal exercise test. Rats were weighed and placed in a perspex running chamber (9.5 x 32.5 x 11.5 cm), as described by Brooks & White (1978), which fitted into a rodent treadmill with 0.5 HP motor (Tadco, Australia). Speed was regulated by a variable transformer (Yokoyama Electric Works, Ltd, type SB-10, Japan). Air was drawn through the chamber by the vacuum pump. The front end of the chamber contained six small holes (5 mm diameter) which allowed for entrance of ambient air and a unidirectional flow past the rat. The bottom edges of the chamber were covered with carpet and in contact with the treadmill belt around the entire perimeter.

Oxygen consumption during maximal exertion was measured with rats running continuously at increasing speeds and treadmill inclination following an initial warm-up period. The exercise programme of Musch and co-workers (Hilty *et al.*, 1989; Musch *et al.*, 1988; 1991) was used to measure $\dot{V}O_{2\ peak}$ (Table 4.3). Commonly used criteria for maximal oxygen consumption ($\dot{V}O_{2\ max}$) include no further increase in $\dot{V}O_2$ with further increases in workload, or when the rat refuses (or is unable) to continue running (Baldwin *et al.*, 1982; Musch *et al.*, 1988). It has been recognised in

Table 4.3 Exercise programme used to test oxygen consumption during maximal exertion ($\dot{V}O_{2\ peak}$) in rats. This programme was used by Musch *et al.* (1988) and Hilty *et al.* (1989). $\dot{V}O_{2\ peak}$ was measured while rats performed a maximal exercise test running inside a metabolic chamber on a motor-driven treadmill. A one-minute period of peak oxygen consumption ($\dot{V}O_{2\ peak}$) was used to estimate oxygen consumption during maximal exertion.

Time (mins)	**Speed** ($m.min^{-1}$)	**Incline** (^{o})
0	16	0
2	19	5
4	24	10
6	31	15
8	37	20
9	40	20
10	43	20
11	46	20

the literature that not all animals are able to demonstrate a plateau in oxygen consumption in response to further increases in workload (Bedford *et al.*, 1979; Baldwin *et al.*, 1982; Musch *et al.*, 1988; Sonne, 1989). Such a plateau is an accepted criterion of $VO_{2\ max}$ in humans (McConnell, 1988). However, Bedford *et al.* (1979) examined plateau and non-plateau results in the same rats and found no statistically significant differences between the two groups. Use of this criterion in humans has also been criticised (Noakes, 1988). In this study, the test was stopped when rats were unwilling or unable to continue running and $VO_{2\ peak}$ was defined as the highest value sustained for a one minute period by each rat (Patch & Brooks, 1980; Gleeson & Baldwin, 1981). The greater volume of a metabolic chamber, and the related increase in sampling time, in relation to a mask, means that the change in the pressure of oxygen between inlet and outlet air will be reduced. To minimise this effect, the one-minute period of peak oxygen consumption ($VO_{2\ peak}$) was used as an estimate of maximal oxygen consumption ($VO_{2\ max}$).

4.2.3.6 Endurance

The protocol of Baldwin *et al.* (1982) was used to measure submaximal endurance to exhaustion (Table 4.4). Exhaustion (or the limit of endurance) has been defined as "an in ability to sustain contractions / exercise at the target force / intensity" (Vøllestad *et al.*, 1988). According to Baldwin *et al.* (1982), the final intensity used in this test represents a workload corresponding to about 70 - 80% $VO_{2\ max}$ (thus "submaximal" endurance). A rat was considered to have reached exhaustion when it positioned itself at the back of the chamber for 10s or more and no longer responded to repeated jets of air.

Table 4.4 Exercise programme used to measure endurance in rats. This programme has been used by Baldwin *et al.* (1982). Endurance was measured while rats ran on a motor-driven treadmill until they were unable or unwilling to run any further and did not respond to repeated jets of air.

Time (mins)	**Speed** ($m.min^{-1}$)	**Incline** (°)
0	12	0
3	27	2
13	27	4
23	27	6

4.2.4 Statistical Methods

All measurements are expressed as mean ± SE. The level of statistical significance chosen for this experiment was $P < 0.05$.

To test for differences in physical performance between the dietary groups, a general linear models procedure was used to perform multiple analysis of variance (MANOVA) among the dietary groups at each of two times - after nine weeks on the test diets, and following six weeks recovery. The statistical package, SAS (SAS Institute, 1979), was used to perform Model lll two-factor ANOVAs without replication; the fixed factor was diet and the random factor was litter. Wherever ANOVA revealed significant effects of diet, Tukey's studentized range test was used to determine which dietary groups produced significantly different responses.

4.3 Results

All figures show mean ± SE from groups of eleven rats ($\dot{V}O_{2\ basal}$) and nine rats (grip strength, $\dot{V}O_{2\ peak}$ and endurance) which were fed one of the three diets: Control, EFAD or PUFA for nine weeks, followed by six weeks recovery on normal rat pellets. Significantly different dietary treatment means are denoted by different superscripts. As in Chapter 3, variation between groups is exaggerated in these plots since the effect of variation among litters is not factored out.

4.3.1 Rat Growth

Both diet ($P < 0.001$) and litter ($P < 0.001$) had a significant effect on rat weights after nine weeks on the test diets. However, mean weights of rats in all groups were very similar to those obtained in Chapter 3 and relative differences between the dietary groups were not only almost identical, but very small. Mean weight for the EFAD group was only 6% and 7% lighter than for the Control and PUFA groups, respectively. Since diet did have a significant effect, both absolute and weight-adjusted values are presented for grip strength, as well as weight-adjusted values for basal oxygen consumption and maximal oxygen consumption. All data and matching statistical analyses are presented in Appendix 3. Mean rat weights after nine weeks on the test diets were 408 ± 9g for the Control group, 383 ± 8g for the EFAD group and 414 ± 6g for the PUFA group. Following the six week recovery period on normal rat pellets, diet ($P > 0.2$) no longer affected rat weights but there was a significant litter effect ($P < 0.0002$). Final weights were 472 ± 14g for the Control group, 464 ± 21g for the EFAD group and 492 ± 10g for the PUFA group.

4.3.2 Grip Strength

Forearm grip strength was extremely consistent both for individual animals and among the dietary groups. Coefficients of variation were typically less than 10% for the four trials done on each rat. Absolute grip strength was unaffected by diet after both nine weeks on the test diets ($P > 0.5$) and following six weeks recovery ($P > 0.5$) (Figure 4.1 A). Interestingly, there was almost no change in absolute grip strength over the recovery period. Although there were no

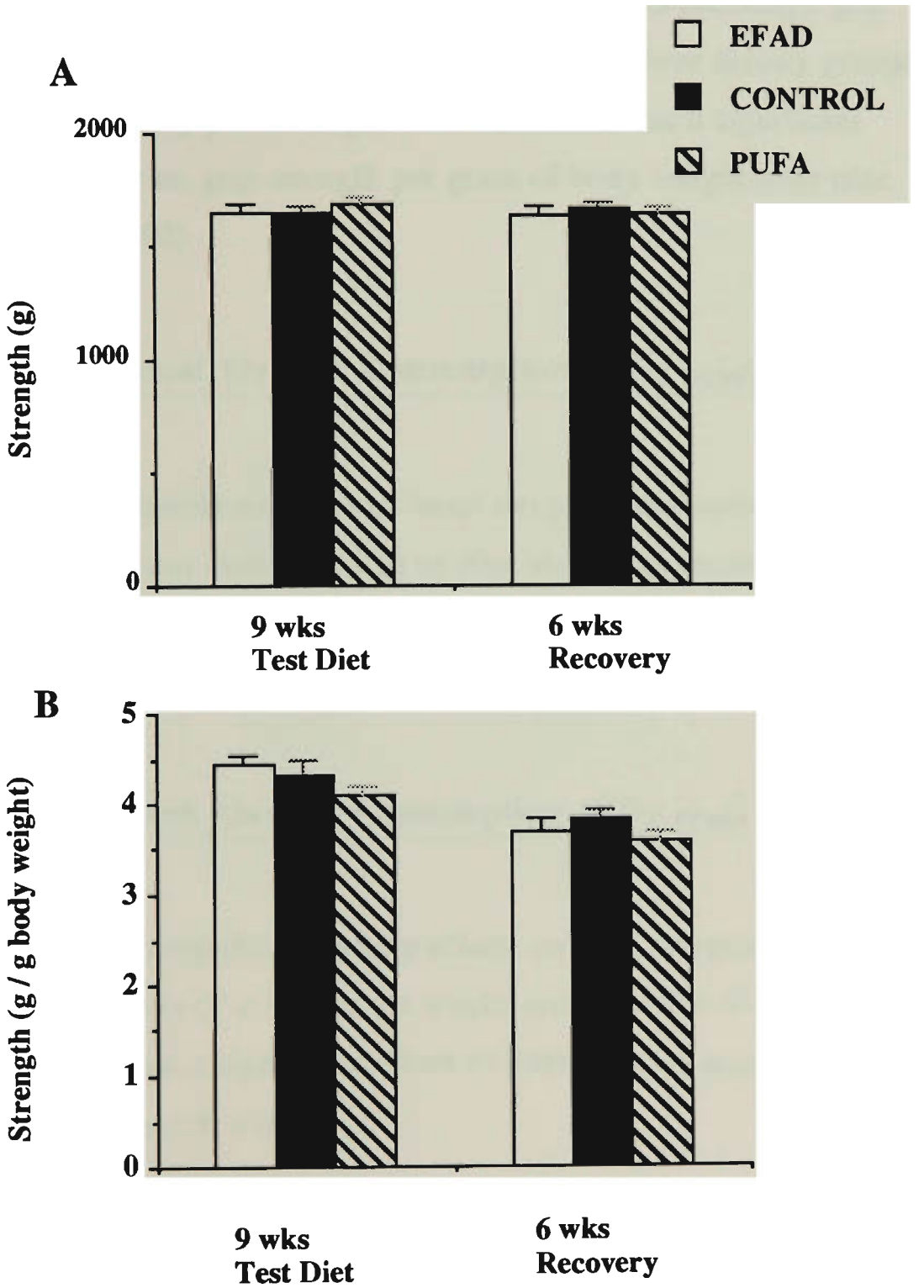


Figure 4.1
Forearm grip strength for groups of nine male Wistar rats. Grip strength was measured when rats pulled on a T-bar attached to a force transducer. Absolute values (Fig. 4.1 A) and weight-adjusted values (Fig. 4.1 B) are presented. Diet did not affect either absolute or weight-adjusted grip strength after nine weeks on the test diets ($P > 0.5$ for both) or following six weeks recovery ($P > 0.5$ (absolute); $P < 0.5$ (weight-adjusted)).

significant differences when adjusted for body weight ($P > 0.5$ after nine weeks on the test diets; $P < 0.5$ after six weeks recovery), grip strength decreased by 11 - 17% for each of the three dietary groups over the recovery period (Figure 4.1 B). There was a significant effect of litter on grip strength per gram of body weight after nine weeks ($P = 0.02$).

4.3.3 Basal Oxygen Consumption ($\dot{V}O_{2\ basal}$)

Diet had no significant effect on basal oxygen consumption after nine weeks on the test diets ($P > 0.5$) or after six weeks recovery ($P > 0.5$) (Figure 4.2) and there was no change in the rate of basal oxygen consumption during the recovery period.

4.3.4 Peak Oxygen Consumption ($\dot{V}O_{2\ peak}$)

There were no significant dietary effects on peak oxygen consumption after nine weeks ($P < 0.5$) or six weeks recovery ($P > 0.5$) (Figure 4.3). There was a significant effect of litter on $VO_{2\ peak}$ after 6 weeks recovery ($P < 0.01$).

Peak oxygen consumption was approximately three to four times greater than basal oxygen consumption after both nine weeks on the test diets and following six weeks recovery, but there was no difference between the dietary groups in their ability to increase oxygen consumption during maximal exertion ($P > 0.5$ after nine weeks; $P > 0.5$ following recovery).

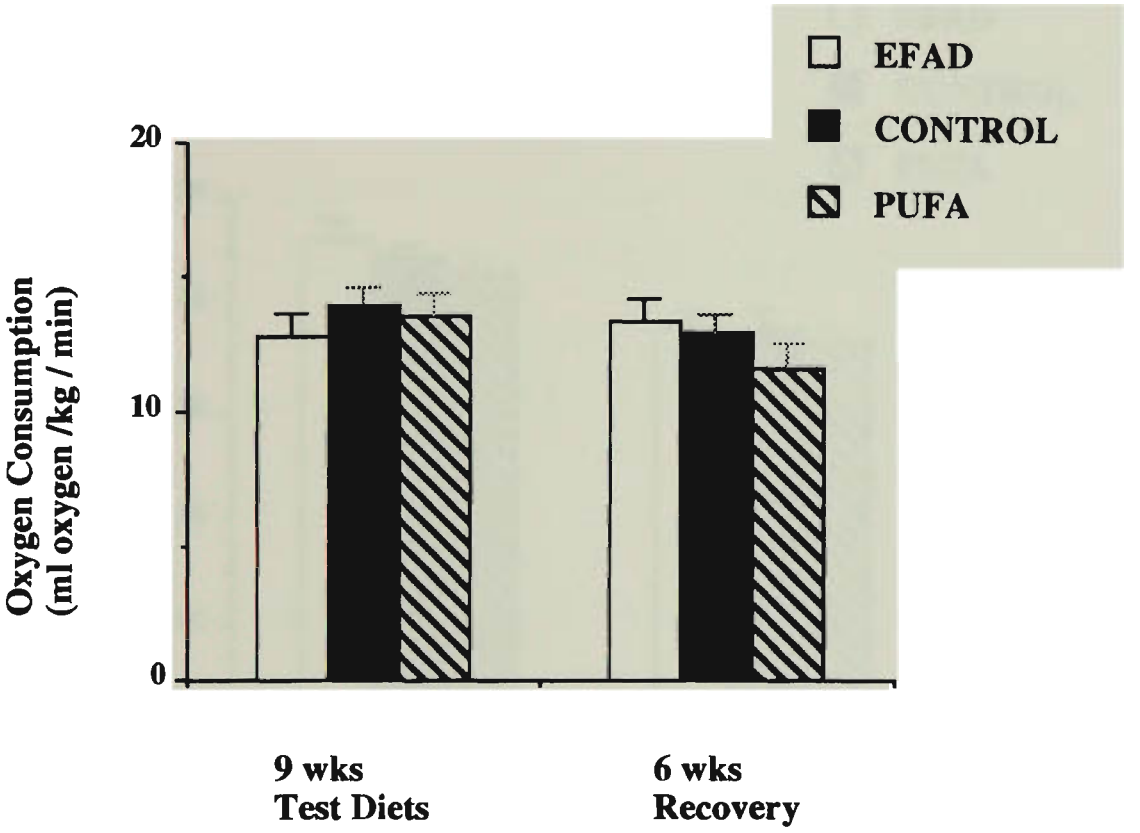


Figure 4.2
Basal oxygen consumption for groups of 11 male Wistar rats. Basal oxygen consumption was measured over a three hour period while rats were at rest in a metabolic chamber. Diet had no effect on basal oxygen consumption after nine weeks on the test diets or following recovery ($P > 0.5$).

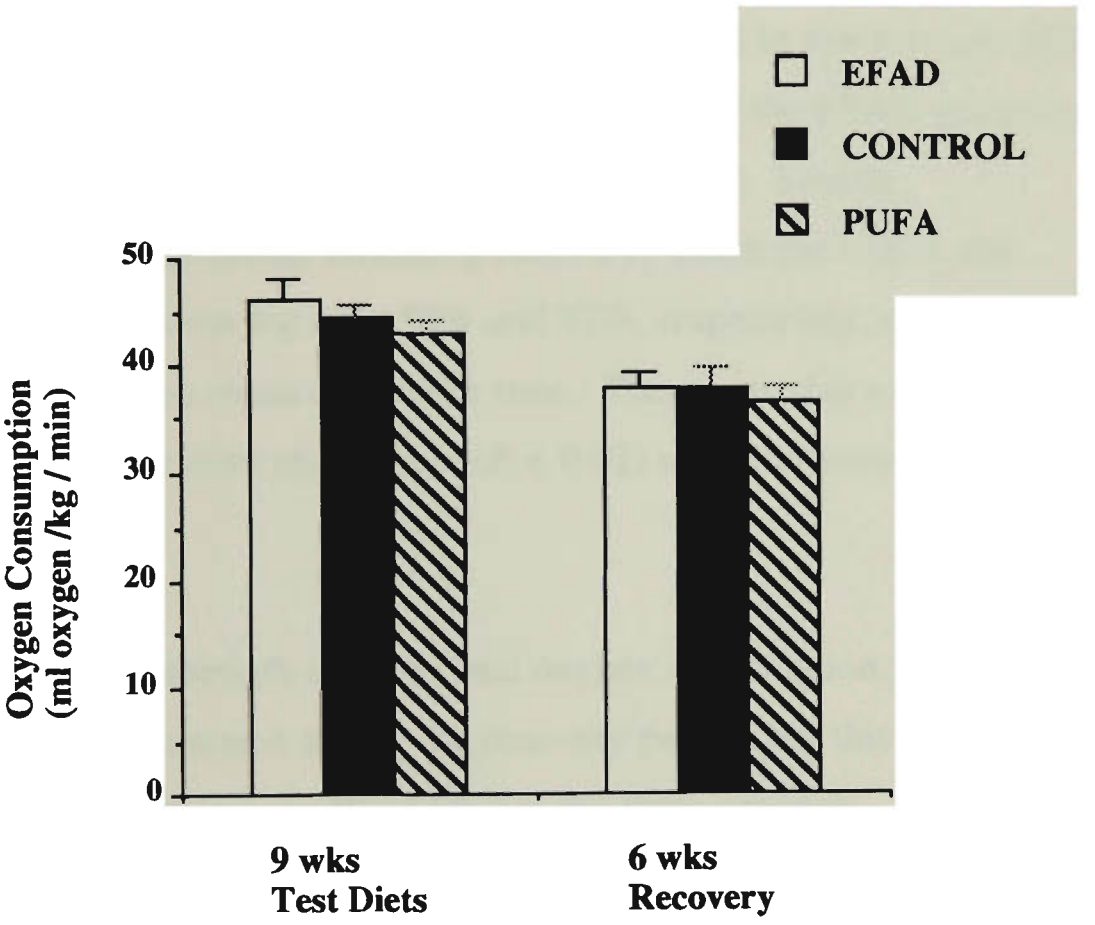


Figure 4.3
Peak oxygen consumption for groups of nine male Wistar rats. Peak oxygen consumption was measured while rats ran on a treadmill inside a metabolic chamber. Diet did not affect oxygen consumption during maximal exertion after nine weeks ($P < 0.5$) or following six weeks recovery ($P > 0.5$).

4.3.5. Endurance

There was a highly significant effect of diet on endurance after nine weeks on the test diets ($P < 0.02$) and again following recovery ($P < 0.005$) (Figure 4.4). The PUFA group was able to run for only 56% as long as the Control group. The endurance of the EFAD group was also (non-significantly) lower (73% of Control). Similar relationships persisted following recovery, where the PUFA and EFAD groups ran for only 50% and 72%, respectively, of the Control group's mean endurance time. There was also a significant effect of litter after nine weeks ($P < 0.02$) and after recovery ($P < 0.05$).

As with grip strength and maximal oxygen consumption, mean endurance decreased during the recovery period, but this effect was largest for endurance (49 - 55% for the three groups).

4.4 Discussion

This study demonstrated that manipulation of dietary fatty acids can affect physical performance in whole animals, although the detected patterns of change were not as predicted from the previous study on isolated muscle (Chapter 3). Endurance was affected after nine weeks on the PUFA diet and there was no sign of recovery after the six weeks on rat pellets, which indicates that recovery of whole animals from dietary manipulation may be slow.

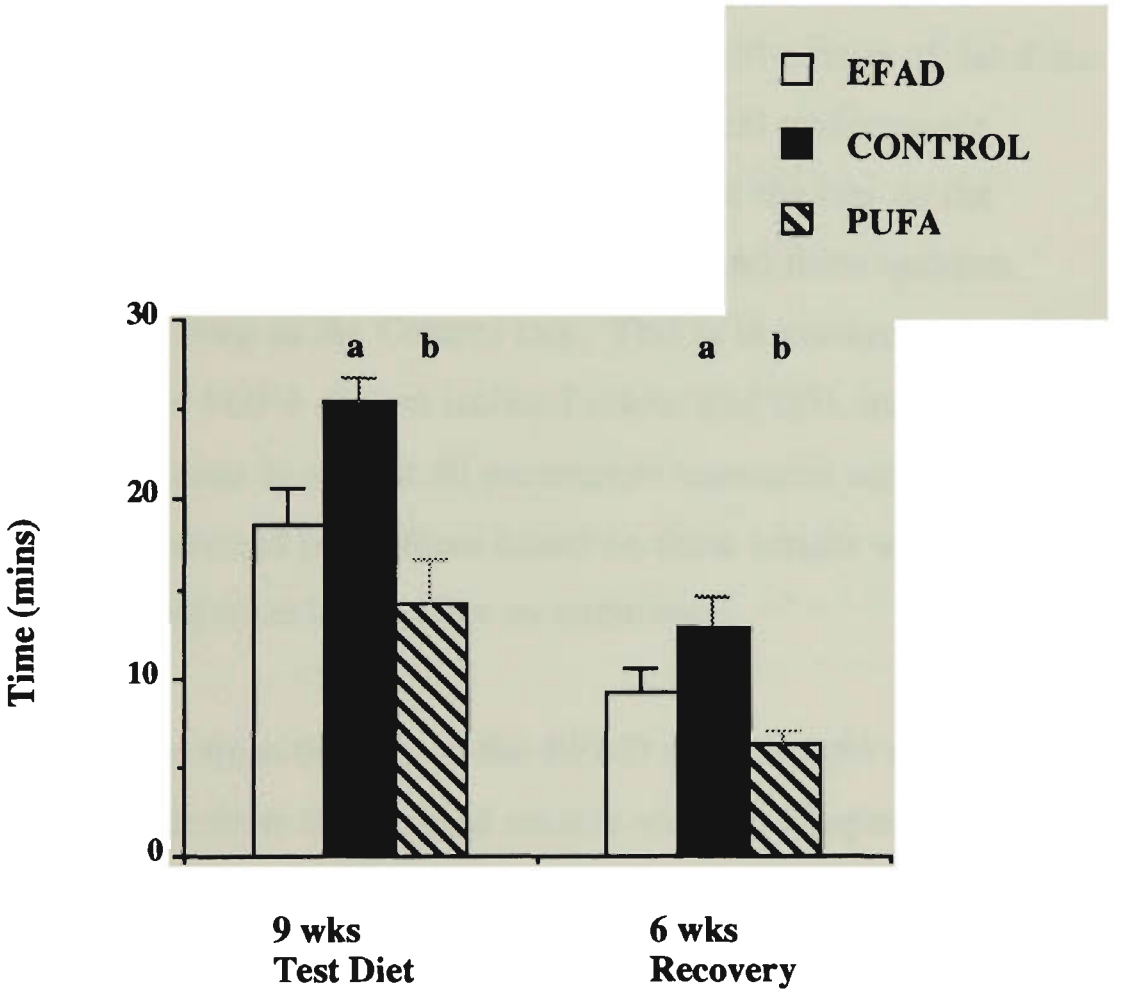


Figure 4.4
Endurance for groups of nine male Wistar rats. Endurance was measured while rats ran on a treadmill inside until exhaustion. Diet had a significant effect after nine weeks ($P < 0.02$) and again following six weeks recovery ($P < 0.005$). Significantly different treatments are denoted by different superscripts.

For the first time in this study, the effects of the PUFA diet were dramatic, but quite similar to those of the EFAD diet (although the change resulting from the EFAD diet was not statistically significant). None of the diets had significant effects on oxygen consumption, either at rest or during maximal exertion. Similarly, none of the diets affected forelimb grip strength. The only physical performance parameter that was affected was endurance where the rats on the PUFA and EFAD diets could only run for half and three quarters, respectively, as long as the Control rats. This is in marked contrast to the effects of the PUFA diet on isolated soleus and EDL muscles, where the responses to almost all parameters measured were similar to the Control rats and predictions based on these results were that the PUFA diet would have little effect on endurance.

Results obtained from the rats on the EFAD diet al.so did not fit the predictions made from the isolated muscle study in Chapter 3. Rather than the predicted decrease in grip strength and possible increased endurance, there was no effect of diet on grip strength, and rats on the EFAD diet al.so showed decreased endurance (although this was non-significant).

The pattern of recovery, too, was unexpected, but may be related to recovery of muscle membrane fatty acid composition. Skeletal muscle membrane composition (see Chapter 2) and isolated muscle function (see Chapter 3) of rats on the EFAD diet almost completely recovered from the effects of EFA deficiency, although in both cases, the fast EDL muscles took longer to recover than the slow soleus muscles. However, the fatty acid composition of muscle membranes from rats on the PUFA diet did not recover to Control levels, even

after six weeks. In the endurance test, which was the only parameter affected by dietary manipulation, there was also no indication of recovery, even after six weeks.

Only three other (very recent) studies have investigated the effects of dietary lipid manipulation on physical performance. These studies have all been in humans and there are no comparable data for rats. None of the studies investigated the effects of EFA deficiency but in each, the level of dietary n-3 fatty acids was altered and $\dot{V}O_{2\ max}$ was measured. Warner *et al.* (1989) showed in hyperlipidemic, sedentary human subjects that $\dot{V}O_{2\ max}$ was unaffected by daily consumption of fish oil for 12 weeks. In Brilla & Landerholms's (1990) study, there was no difference in $\dot{V}O_{2\ max}$ between Control subjects and subjects whose diets were boosted with n-3 fatty acids in the form of fish-containing meals for 10 weeks. In a similarly designed study, Ågren *et al.* (1991) also found no effect of fish meals on $\dot{V}O_{2\ max}$ in humans after 14 weeks. These results are all in agreement with those for $\dot{V}O_{2\ peak}$ in this study, but none of them investigated possible changes in endurance or strength.

Ivy *et al.* (1980) compared the metabolism of medium-chain and long-chain triglycerides (MCT and LCT, respectively) since MCT can enter mitochondria directly, are metabolised as quickly as glucose and could be an alternative fuel source during prolonged exercise. However, they found no difference between MCTand LCT in their effects on carbohydrate metabolism. In the light of these and similar results obtained by Decombaz *et al.* (1983), Coggan and Swanson (1992), in a review of nutritional manipulations before and during endurance exercise, suggested that there is little reason to expect a

beneficial effect from increased ingestion of medium-chain triglycerides.

Grip Strength

The results obtained in this study for grip strength, were three times higher those of Meyer *et al.* (1979) with 14 week old male rats. However, since Meyer *et al.* (1979) showed large differences (25%) between strains, and all rats in this study demonstrated extremely similar strength (coefficients of variation were typically less than 10%), this discrepancy may be due to strain differences, or differences in apparatus. In the grip-strength testing apparatus described by Meyer *et al.*(1979), the rats sat horizontally on a perspex surface and were pulled backwards until they released the bar. However, in this study, the rats showed no "interest" in pulling on the T-bar in such a position, and the only way to get a response was to suspend them from the tail so that they held themselves at about 60^{o} to the ground. This technique may have increased the strength with which they pulled at the bar since it was their only means of support.

Although the grip strength test gave a measure of strength in the forelimb, it is difficult to relate these results to those obtained in the hindlimb for isolated soleus and EDL muscles. There appears to have been no documentation of the proportionate composition of fibre types in rat forelimb muscles, as there has been in hindlimb muscles (Ariano *et al.*, 1973; Armstrong & Phelps, 1984), so it was not possible to make predictions about forelimb grip strength based on the Chapter 3 results of isolated muscles. Although it should be possible to measure hindlimb grip strength with the apparatus

described by Meyer *et al.* (1979), such a set-up did not work for the rats in this study.

Basal Oxygen Consumption

As discussed in Chapter 3, previous studies have found that increased basal metabolism is symptomatic of EFA deficiency (Holman, 1968). As with the other previously reported symptoms, there was no difference in basal oxygen consumption between any of the dietary groups in this study. Since relative humidity was kept quite high (averaged 57 ± 2%), evaporative water loss would not be expected to increase significantly, even if skin permeability increased as a result of EFA deficiency and there would be no need for an increase in BMR to maintain a constant body temperature. In other studies, values reported for $\dot{V}O_{2\ max}$ in rats vary from 19 - 28 ml O_2.kg body wt^{-1} . min^{-1} (Shepherd & Gollnick, 1976; Bedford *et al.*, 1979; Patch & Brooks, 1980; Russell *et al.*, 1980; Gleeson & Baldwin, 1981; Gleeson *et al.*, 1983; Musch *et al.*, 1988; Hilty *et al.*, 1989; Sonne *et al.*, 1989) but other factors such as strain, age, sex and level of training also vary. The mean values determined in this study ranged from 12 to 14 ml O_2.kg body wt^{-1} . min^{-1} for the three dietary groups and were very consistent. Most other studies of BMR have been done on female rats and their reported weights are around 300g. In this study, male rat weights were about 410g after nine weeks on the test diets and 470g after six weeks recovery. Since Sonne (1989) suggested that there is an inverse relationship between oxygen uptake and body weight, it is probably not surprising that $\dot{V}O_{2\ basal}$ was lower in this study and the sex and strain differed from other studies.

In this study, as in all others, basal metabolic rate was corrected for body weight. However, this should be done with caution in dietary studies because differences in the amount and type of body fat (and hence, body weight) could affect the results. For example, brown adipose tissue (BAT) in the body has the specific function of thermogenesis (Himms-Hagen, 1989) and studies have shown that changes in dietary lipids can alter the activity of BAT and diet-induced thermogenesis (for review, see Himms-Hagen, 1989). There is some evidence that dietary unsaturated fatty acids may be more effective than saturated fatty acids in increasing BAT thermogenesis (Nedergaard *et al.*, 1983) but Rafael *et al.* (1988) found no effect of increased linoleic acid. If the effect of dietary lipid manipulation is to alter the activity of BAT, then this may also affect BMR. In this study, although changes in dietary fatty acids did not affect basal oxygen consumption, this does not mean that there were no effects on BAT in these rats.

Peak Oxygen Consumption

While the results obtained for $\dot{V}O_{2\ peak}$ showed no significant differences between the dietary groups and were very consistent within groups, they also were lower than other reported values for $\dot{V}O_{2\ max}$. However, Gleeson & Baldwin (1981) reported that $\dot{V}O_{2\ max}$ generally occurred while rats ran at speeds of 27 - 38 m . min^{-1}, which was the case in this study where rats were usually running at 37 - 40 m . min^{-1} before they refused or were unable to run any further. In this study, values ranged from 43 to 46 ml O_2 . kg body wt^{-1} . min^{-1} after nine weeks on the test diets and from 36 - 38 ml O_2 . kg body wt^{-1} . min^{-1} after six weeks recovery. In each case, these were three to four times greater than the corresponding values for $\dot{V}O_{2\ basal}$. In

other studies, values of $\dot{V}O_{2\ max}$ for rats range from 67 - 96 ml O_2 . kg body wt^{-1} . min^{-1} (Shepherd & Gollnick, 1976; Bedford *et al.*, 1979; Patch & Brooks, 1980; Gleeson & Baldwin, 1981; Baldwin *et al.*, 1982; Pica & Brooks, 1982; Gleeson *et al.*, 1983; Musch *et al.*, 1988; 1991; Hilty *et al.*, 1989), but as for BMR, other studies have mostly used female rats from a variety of strains. Since Bedford *et al.* (1979) showed that $\dot{V}O_{2\ max}$ is different in different strains (and in fact was lowest in Wistar-Kyoto rats) and is higher in females than males, it is not surprising that values in this study are lower than previously reported. In fact, none of the reported studies used male Wistar rats. However, in other studies, the increase in $\dot{V}O_{2\ max}$ over BMR ranged from 2.9 to 4.4 times (Shepherd & Gollnick, 1976; Patch & Brooks, 1980; Gleeson & Baldwin, 1981; Gleeson *et al.*, 1983; Musch *et al.*, 1988; Hilty *et al.*, 1989; Sonne *et al.*, 1989), which is very similar to the range found between dietary groups in this study.

Endurance

The dramatic significant effect of nine weeks on the PUFA diet may not be due to direct effects of this diet on the muscles, since there were no observed effects of the PUFA diet on isolated muscles (Chapter 3). Although both the EFAD and PUFA diets resulted in decreased endurance in whole rats, these diets had different effects in isolated muscles. The PUFA diet had no effect on isolated muscle fatigue or endurance (either during high frequency or low frequency stimulation), where the EFAD diet decreased fatigue time during high frequency stimulation in EDL muscles, but increased endurance during low frequency stimulation in the same muscles (Figures 3.14,

3.16 and 3.19). The effects of the two diets may be on more than one part, or on different parts, of the system.

The PUFA diet may have wide-ranging effects on metabolism and physiology of these rats. A possible explanation for the observation of reduced endurance in the PUFA rats could be differences in the "natural" activity level between the dietary groups. For example, if an effect of the PUFA diet was to decrease the amount of time the rats spent moving around their box (perhaps by making them feel unwell), then they may be less accustomed to physical exertion and less able to cope with endurance exercise than other rats that have been more active. Logistical reasons prevented any more than habituation to running on a treadmill so these rats were certainly not trained to run. Further studies beg to be done on the effects of these diets on trained rats.

The large decrease (approximately 50%) observed in endurance (and obvious lack of recovery) during the six week recovery period could be due to the fact that the rats were six weeks older and 60 - 80g heavier, and were therefore unable to perform strenuous exercise for as long a period of time. Since these rats were kept in a cage, and were highly unlikely to exercise much, their muscles were probably atrophied in comparison with a wild rat of the same age. After a further six weeks of inactivity, their muscles would have atrophied even more, making them less able to perform physical work.

The few studies which have reported results for submaximal endurance tests in rats found that exhaustion occurred at about the same treadmill speed and elevation as in this study. Askew *et al.*

(1975) found that exhaustion occurred at 18 m . min^{-1} at an 8% grade in six - seven week old male rats, but did not report how long it took, whereas Baldwin *et al.* (1982), using the same experimental protocol as in this study, reported that time to exhaustion in ~12 week old female rats was 47 minutes (27 m . min^{-1}, 6% grade). In this study, time to exhaustion for the Control rats was 25 minutes (at 27 m . min^{-1}, 6% grade) after nine weeks on the test diets and 13 minutes (27m . min^{-1}, 4% grade) after six weeks recovery. The difference in endurance between the results of this study and those found of Baldwin *et al.* (1982) could be due to the fact that the rats used by Baldwin *et al.* were much smaller than the rats in this study, or to the fact that they were female, or they may be due to differences in the effectiveness of methods used to encourage rats to continue running.

Difficulties Associated with Whole Animal Performance

An important result from this study was that, of the four physical performance parameters measured, those that required physical exertion by the rats (i.e. GS, $\dot{V}O_{2\ peak}$ and endurance) decreased substantially over the recovery period. An analysis of changes in endurance and maximal oxygen consumption during recovery showed that no more than two rats out of nine in each dietary group actually increased their performances over time. Since there were no significant effects of diet and from observations of individual rats during the testing periods, it is possible that these decreases in $\dot{V}O_{2\ peak}$ and endurance may have been due to behavioural changes in the rats. Several rats did not appear to run "willingly" and required constant "encouragement" in the form of a jet of air or tapping on the running chamber. In the original experimental plan, each rat was expected to undergo all tests in triplicate. However, during trial tests,

it was obvious that some rats were reluctant to run and that it would not be possible to perform all tests on different days so the number of replicates per rat was reduced to one. Therefore, each rat performed only two tests each of endurance and maximal oxygen consumption, once after the test diets and again following recovery. Although several rats needed encouragement to run, there were few that refused. One rat that did refuse to run at nine weeks, also refused following recovery. Also, his two litter-mates which ran well after the nine week test period, also refused to run after recovery. These litter effects on endurance were statistically significant (see Appendix 3).

As for the observed decrease in grip strength adjusted for body weight during recovery, there was no obvious sign of reluctance after recovery, so it may be that by 12 weeks of age, the rats had attained maximum strength, which was unaffected by the change in diet, even though body weight continued to increase.

A few other studies have reported that approximately 10 - 15% of rats refuse to run on a treadmill (Bedford *et al.*, 1979; Russell *et al.*, 1980; Gleeson *et al.*, 1983). In their comparison of masks and metabolic chambers, Musch *et al.* (1988) reported a 27% refusal rate for the mask and a 6% refusal rate for the metabolic chamber. Other studies have not reported on the number of rats eliminated due to refusal, but in the light of these figures, the reluctance of rats in this study to take part in repeat runs and personal communication with others, it is felt that the normal refusal rate may actually be higher than reported.

Future Directions

Dietary fatty acids clearly exert significant effects on isolated muscle function and whole animal performance. Effects on whole animals may reflect changes in systems such as the hormones (some of which are derived from lipids) or the circulatory system (which is particularly affected by lipids), or they may reflect changes in energy stores (such as glycogen). However, since skeletal muscle membranes contain a substantial amount of of lipid, it is quite possible that the observed changes reflect differences in skeletal muscle membranes. With respect to biochemical function of muscle membranes, it is possible that changes in dietary lipids may alter the activity of membrane-bound enzymes involved in muscle function. The "sodium pump" (Na^+,K^+ -ATPase) in the cell membrane is particularly important in restoring normal ion concentration gradients following the passage of action potentials, and is likely to be very important in returning vast amounts of potassium to muscle cells following high frequency fatigue. The "calcium pump" (Ca^{2+}-ATPase) in the sarcoplasmic reticulum membrane is responsible for sequestering calcium following contraction. Alterations in its activity can affect relaxation of muscle and further contractions.

Else & Hulbert (1987) hypothesized that since mammals have a higher proportion of polyunsaturated fatty acids in cell membranes than reptiles, this could be responsible for making their membranes more "leaky" to sodium and potassium ions and suggested that the greater metabolic rate and homeothermic body temperature of mammals is, in part, the result of greater activity of ion pumps responsible for maintaining cellular ion gradients. Such a change in membrane fatty acid composition may also lead to a change in either activity or

concentration of enzymes such as Na^+,K^+ -ATPase and Ca^{2+}-ATPase If this is so, then biochemical analysis of muscles from the EFAD and PUFA groups may reveal changes in these enzymes. If this hypothesis is true, it is predicted that the concentration and / or activity of sodium pumps and / or calcium pumps would be greater in muscles from rats on a PUFA diet and reduced in muscles from rats on an EFAD diet.

Chapter 5 Effects of Changes in Dietary Fatty Acids on Membrane-Bound Enzymes

5.1 Introduction

This study has demonstrated that a dietary manipulation that can alter the composition of skeletal muscle membranes can also modify the function of isolated muscles and the physical performance of whole animals. This chapter aims to investigate the effects of the dietary fatty acid manipulation on the two major systems of facilitated ion exchange which are vital to muscle function. These systems are the "sodium pump" (Na^+,K^+-ATPase) of the sarcolemma and the "calcium pump" (Ca^{2+}-ATPase) of the sarcoplasmic reticulum. Both involve complex membrane-bound proteins which are intimately associated with the fats of the muscle membrane. The influence of dietary fats on membrane integrity has been implicated in severe whole body disorders (for discussions, see Holman, 1986 and McMurchie, 1988) and cardiac contractility and arrhythmias (Charnock *et al.*, 1982; 1985c; 1987; McLennan *et al.*, 1985; 1987 a; b; 1989, 1990). Changes in membrane lipid composition may be responsible for changes in the maintenance of ion homeostasis via effects on membrane-bound enzymes.

As discussed in Chapter 1, Na^+,K^+-ATPase is responsible for restoring the resting membrane potential following contractions by maintaining a low intracellular [Na^+] and a high intracellular [K^+] and Ca^{2+}-ATPase is responsible for the re-uptake of calcium ions from the sarcoplasm following muscle contraction.

The aims of this part of the study were to:

(a) determine whether dietary lipid manipulation affects concentration and activity of two membrane-bound enzymes which are vital for muscle contraction (Na^+,K^+-ATPase of the sarcolemma and Ca^{2+}-ATPase of the sarcoplasmic reticulum membrane) and

(b) compare the effects of dietary lipid manipulation on enzyme activity and concentration in fast and slow muscles.

5.2 Materials and Methods

5.2.1 Rats

Male albino Wistar rats were used and maintained as described in Chapter 2. Following weaning, litter-mates were fed one of three diets: Control, EFAD or PUFA for a period of nine weeks. Muscles from rats used for isolated muscle tests in Chapter 3, were used for assessment of the effects of dietary lipid manipulation on Na^+,K^+-ATPase concentration. Muscles from rats used for whole animal performance tests in Chapter 4 were used for assessment of the effects of dietary lipid manipulation on Na^+,K^+-ATPase activity and Ca^{2+}-ATPase activity. In this experiment, there were no recovery periods: enzyme assays were only conducted after rats had been on the test diets for nine weeks. Once soleus and EDL muscles from the left side of the rats had been removed and stimulated electrically, as described in Chapter 3, the contralateral muscles were removed, placed in pre-weighed vials, weighed, frozen in liquid nitrogen and stored at -80°C until analysis. Once rats had undergone all performance tests in

Chapter 4, soleus and EDL muscles from both sides were removed and frozen and stored. Dissection details are given in Chapter 2. All muscles were assayed in triplicate using double-blind trials.

5.2.2 Chemicals

All chemicals were of analytical grade and were obtained from BDH Chemicals (Melbourne, Australia) or Sigma Chemical Company (St. Louis, USA) except for [^{3}H]ouabain, ammonium molybdate, Hionic Fluor, Soluene 350, pyruvate kinase (PK) and lactate dehydrogenase (LDH). [^{3}H]ouabain (31.5 Ci/mmol and 99.3% purity) in ethanol was from New England Nuclear Du Pont (Wilmington, U.S.A.). (Ethanol was removed under a stream of nitrogen gas and the [^{3}H]ouabain re-suspended in distilled water). Tissue solubilizer (Soluene 350) and liquid scintillation cocktail (Hionic Fluor) were from Packard Instrument Pty Ltd (Melbourne, Australia). Ammonium molybdate was from Eastman Kodak (Melbourne, Australia). PK and LDH were from Boehringer (Mannheim, Germany).

5.2.3 Concentration of Na^+,K^+ -ATPase

The concentration of Na^+,K^+ -ATPase in intact muscle was quantified by measuring the uptake of a labelled cardiac glycoside (specifically [^{3}H]ouabain) using an equilibrium binding assay developed for small muscle biopsies by Nørgaard *et al.*(1983). The assay is based on the knowledge that Na^+,K^+-ATPase contains the only known receptor for cardiac glycosides (a class of drugs) and has only one such binding site (Clausen & Hansen, 1974; Hansen & Clausen, 1988) which is on the outer surface of the enzyme. Ouabain acts to inhibit Na^+,K^+-

ATPase and vanadate (in low concentrations in the buffer) facilitates [^{3}H]ouabain binding (Hansen, 1979).

In the assay, slices of skeletal muscle were incubated in a medium containing a range of ouabain concentrations. Each incubation mix contained 5 nM [^{3}H]ouabain titrated to the appropriate concentration with unlabelled ouabain giving a range of total ouabain concentrations of 5 - 1000 nM ouabain. When ouabain binds to the Na^+,K^+ -ATPase, the [^{3}H]ouabain and the unlabelled ouabain "compete" with each other for the ouabain-binding sites. At lower total concentrations of ouabain, there is a higher relative concentration of [^{3}H]ouabain, so a greater amount of [^{3}H]ouabain is bound (i.e. there is a greater relative uptake of [^{3}H]ouabain at low total ouabain concentrations) (measured as disintegrations per minute (DPM) in a liquid scintillation counter). At the higher total concentrations of ouabain, there is very little [^{3}H]ouabain relative to unlabelled ouabain (in fact, at 1000 nM ouabain, only 0.5% is [^{3}H]ouabain) and much less [^{3}H]ouabain is bound relative to unlabelled ouabain. The assay becomes much less accurate at these higher total concentrations since there is very little relative uptake of [^{3}H]ouabain due to the "swamping" effect of the large amount of unlabelled ouabain.

Studies relating [^{3}H]ouabain binding and ^{42}K uptake indicate that measurements of [^{3}H]ouabain binding quantify functional sodium pumps (Clausen, 1986). Clausen *et al.* (1987) showed that changes in [^{3}H]ouabain binding site concentration are associated with proportional changes of the measured maximum transport rate of the sodium pump. Therefore measurement of [^{3}H]ouabain binding provides a useful approach for the quantification of sodium pumps.

Calculation of Na^+,K^+-ATPase concentration is outlined below.

Relative ouabain uptake is equal to:
Total ouabain uptake - Non-specific ouabain uptake
(see section 5.2.3.2 for details)
and is measured in ml incubation medium [^{3}H]ouabain activity taken up per mg tissue wet wt.

It is calculated as:

$$\frac{\text{DPM . mg muscle}^{-1}}{\text{DPM . ml incubation medium}^{-1}}$$

Number of [^{3}H]ouabain binding sites is measured in pmoles ouabain per gram of muscle wet weight (pmoles . g wet wt^{-1}).
It as calculated as:
Relative uptake (ml . mg^{-1}) • ouabain concentration (μM) • 1000

Traditionally, results from equilibrium binding assays have been analysed graphically using linear, semilogarithmic, Scatchard, double reciprocal and Hill plots (Pratt & Taylor, 1990). In studies measuring the concentration of Na^+, K^+-ATPase, the most common method of analysis is the Scatchard (Scatchard, 1949) plot (Narayaneddy & Kaplay, 1982; Nørgaard *et al.*, 1983; Kjeldsen, 1986a; b; 1988; Kjeldsen & Nørgaard, 1987; Hanson *et al.*, 1993) in which the ratio of the concentrations of bound : free ouabain is plotted as a function of the concentration of bound ouabain. Linear regression analysis then enables an estimate to be made of the maximum number of binding sites (B_{max}) (y-intercept) by

extrapolation, as well as the affinity of ouabain for Na^+, K^+-ATPase (from the slope of the line).

Several assumptions are made in equilibrium binding assays (Pratt & Taylor, 1990):

1. The binding reaction is totally reversible;
2. The two reactants exist as either free or bound species;
3. All receptor sites are considered to have equal affinity for the ligand (in this case, ouabain) and to be independent, so that occupancy of some receptor sites does not alter the binding to unoccupied sites.

Also, underlying assumptions of linear regression analysis, such as a normal distribution and equal variances (Zar, 1984) must be met.

Although these assumptions are implicit in the use of the above equations, rigorous tests to validate them often have not been adequately performed in published studies of ligand-receptor interactions (see Discussion).

If linear regression is used to plot a straight line through a graph of bound : free ligand against free ligand, as in Scatchard plots, then the points of the straight line should form an S-shaped curve when plotted on a semi-logarithmic graph and the inflexion point should be at a concentration which is halfway to the saturation plateau. According to Klotz (1989), if there is no indication of a plateau when the data are plotted on a semi-logarithmic graph, then the points cannot be represented by a straight line in a Scatchard plot.

In this study, the number of binding sites in soleus muscles from rats on the Control diet did reach a clear plateau, but others did not. Therefore, the effects of changes in dietary lipids on the concentration of Na^+,K^+ -ATPase were analysed in two ways. Comparisons were made of:

(i) the number of binding sites at 1 μM (measured as the density of ouabain receptor sites) (This is the highest concentration at which ouabain binding can be measured before variation becomes too large due to the low concentration of [^{3}H]ouabain relative to unlabelled ouabain) and

(ii) the maximum number of binding sites (B_{max}) was inferred from extrapolation using Scatchard plots for each individual, as well as

(iii) the slopes, as a measure of binding affinity (K_D) (i.e. the strength of interaction between Na^+,K^+ -ATPase and ouabain).

The assay consisted of five steps:

(i) pre-incubation wash in unlabelled buffer

(ii) incubation with [^{3}H]ouabain

(iii) post-incubation wash in unlabelled buffer to remove unbound ouabain

(iv) weighing of muscle and solubilization

(v) counting in liquid scintillation counter.

5.2.3.1 Preparation of Samples

Frozen muscle was rapidly partially thawed (under running water), blotted dry on filter paper and cut into 1 mm thick slices (at least 24 slices / muscle) while semi-frozen using a home-built tissue chopper. Nørgaard *et al.* (1983) showed that for tissues within the size range 2

- 14 mg in mass, tissue size does not affect ouabain binding. In this study, muscles slices ranged from 2 - 12 mg. All muscle slices were immediately transferred to individual wells of a 24-well tissue culture plate (24 wells / plate); each well contained 2 ml of a potassium-free vanadate-Tris buffer (500 mM sucrose, 770 mM $MgSO_4$, 400 mM Tris base, 400 mM Tris HCl and 92.8 mM vanadate solution) which was adjusted to pH 7.3 with Tris. This vanadate-Tris buffer was used throughout since vanadate facilitates the binding of ^{3}H-ouabain to Na^+,K^+ -ATPase.

5.2.3.2 Measurement of Na^+,K^+ -ATPase Concentration

(i) Pre-incubation wash

Muscle samples were individually pre-washed for 20 minutes at 0°C by continuous agitation in a shaking water bath in order to reduce extracellular potassium concentration.

(ii) Incubation

Muscles slices were transferred to another 24-well tissue culture plate and incubated in 2 ml of buffer, containing both unlabelled ouabain and [^{3}H]ouabain. Total binding was determined over a concentration range of 5 - 1000 nM ouabain. Specific activity of [^{3}H]ouabain at each concentration was 154 nCi ml^{-1}. In each experiment a set of samples was incubated with an excess of unlabelled ouabain (10 mM) to allow correction for non-specific uptake of [^{3}H]ouabain (i.e. interaction of [^{3}H]ouabain activity with sites other than the specific receptor site on Na^+,K^+ -ATPase). All wells contained 5 nM [^{3}H]ouabain titrated to the appropriate concentration with unlabelled ouabain. Samples (20 µl) of each incubation medium were added to 180 µl of buffer and 2 ml Hionic Fluor scintillant for liquid

scintillation counting. (The volume of all samples of incubation medium and muscles prepared for beta counting was 200 µl). During incubation at 37°C, muscle slices were continuously agitated for three hours in a shaking water bath. The concentration of free [^{3}H]ouabain, used for Scatchard analysis, was determined prior to incubation by removing 20 µl of incubation medium from six wells. Incubation time was based on preliminary results obtained by varying time from 30 minutes to three hours which showed that maximum binding had occurred within three hours.

(iii) Post-incubation wash

Tissue wells were evacuated using a pump and re-filled with two ml of fresh unlabelled buffer. All samples were washed continuously for two x 15 minutes at 0°C to reduce the fraction of non-specifically bound [^{3}H]ouabain. Slices from the total binding wells were washed in standard buffer and slices from non-specific binding wells were washed in an excess (10^{-2}M) of unlabelled ouabain.

(iv) Weighing of tissue and solubilization

Individual muscles slices were blotted dry on filter paper, placed into pre-weighed mini scintillation vials (type Pico 'Hang-In' Vials, Packard Instrument Pty Ltd, Melbourne, Australia), weighed and solubilized overnight (at least 16 hours) in 200 µl of Soluene 350.

(v) Liquid Scintillation Counting.

Hionic Fluor (2 ml) was added to the scintillation vials and liquid scintillation counting was performed in a scintillation counter (type RackBeta 'Spectral', LKB, Turku Finland). Quench curves were

established to accurately calculate the number of ^{3}H-ouabain-binding sites.

The amount of [^{3}H] activity retained after the cold wash was calculated and expressed as the relative uptake of the incubation-medium [^{3}H]ouabain activity into the muscle samples (ml incubation-medium [^{3}H]ouabain activity taken up per gram of muscle wet weight, expressed as a percentage). Specific binding (i.e. number of binding sites) was determined from the difference between total and non-specific binding and expressed as pmol . $gram^{-1}$ wet weight of muscle tissue(Kjeldsen *et al.*, 1988).

Changes in [^{3}H]ouabain binding site concentration between dietary groups were assumed to reflect changes in Na^{+},K^{+}-ATPase concentration.

5.2.4 Activity of Na^{+},K^{+} -ATPase

The rate at which Na^{+},K^{+}-ATPase transfers ions across across membranes is believed to be directly proportional to its energy consumption and hence hydrolysis of ATP. The maximal activity of Na^{+},K^{+}-ATPase can therefore be inferred by measuring the amount of inorganic phosphate (P_i) released from ATP during incubation of normal tissue and tissue in which the activity of Na^{+},K^{+}-ATPase has been inhibited by ouabain, as described by Akera (1984). This important characteristic of Na^{+},K^{+}-ATPase is frequently used to distinguish this enzyme from other membrane-bound ATPases with which it is closely associated.

The conditions which result in hydrolysis of ATP by Na^+,K^+-ATPase also activate other Mg^{2+} dependent ATPase (Akera, 1984). Therefore, it is necessary to subtract this background activity by the addition of ouabain to identical incubation mixes which inhibits Na^+,K^+-ATPase but is assumed to have no effect on Mg^{2+}-ATPase.

Calculation of Na^+,K^+-ATPase activity is outlined below.

Na^+,K^+-ATPase activity is equal to:

Total ATPase activity - (Total ATPase activity - Na^+,K^+-ATPase activity)

and is measured in μmoles P_i . mg protein^{-1} . hour^{-1} and

μmoles P_i . mg tissue^{-1} . hour^{-1}.

Activity is calculated as:(μ moles Pi . mg protein wet weight^{-1} . hr^{-1})

$$\frac{\text{Activity (} \mu M\ P_i) \text{ in 1 hour}}{\text{Amount of protein in homogenate (mg . ml}^{-1}) \bullet \text{total amount of protein used (ml)}}$$

For details, see section 5.2.4.2.

Activity of Na^+,K^+-ATPase in skeletal muscle was determined in both homogenates and microsomes of skeletal muscles. Initial measurements were done in muscle homogenates, but since the level of Na^+,K^+-ATPase obtained was so low, it was decided to partially purify the muscle homogenates and analyse muscle microsomes as well.

Reactions were linear with respect to time of incubation and concentration of protein.

The assay consisted of four steps:

(i) incubation with and without ouabain

(ii) removal of precipitated protein

(iii) determination of inorganic phosphate

(iv) protein determination

5.2.4.1 Preparation of Samples

(i) Homogenates

Frozen muscle was thawed rapidly and homogenised in 250 mM sucrose , 5 mM EDTA, 20 mM imidazole and 2.4 mM sodium deoxycholate at a concentration of 20 mg tissue wet weight . ml^{-1} using a motor-driven Teflon-glass Elvehjem-Potter homogenizer. As mentioned above, in preliminary tests, Na^+,K^+ -ATPase activity in muscle homogenates showed such low levels of activity that it was evident that the muscle needed to be purified. Preliminary tests were performed in kidney, which has a very high Na^+,K^+ -ATPase activity (Jørgensen, 1969). Results were compared with published values to ensure that the assay was measuring Na^+,K^+ -ATPase activity accurately.

(ii) Microsomes

The methods of Asano *et al.* (1976) and Charamlambous *et al.* (1984) for partially purifying Na^+,K^+-ATPase in skeletal muscle were modified slightly to form microsomes. Muscle homogenate was transferred to a microfuge tube and spun at 12,300 g for 10 minutes at 4°C in an ultracentrifuge (type TLA 100 , rotor type TLA 100.3,

Beckman, Palo Alto, U.S.A.) to remove all debris, nuclei and mitochondria. The supernatant was further centrifuged at 100,000 g for 60 minutes at 4°C. The pellet was re-suspended in 1 ml of 25 mM Tris HCL / 2 mM EDTA at pH 7.6. This last procedure allows the formation of microsomes.

5.2.4.2 Measurement of Na^+,K^+ -ATPase Activity

(i) Incubation

The assay mix, with or without ouabain, contained 10-20 µg protein. In each assay, 100 µl of sample was suspended in 800 µl buffer. The buffer contained (final concentrations) 83.3 mM Tris/HCL, 5 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl and 5 mM NaN_3, pH 7.3 at room temperature. Identical assays contained 1 mM ouabain instead of 15 mM KCl. After an initial 10 minute pre-incubation period for temperature equilibrium, assays were started by the addition of 100 µl of 25 mM Na_2ATP (giving a final concentration of 5 mM) and samples were incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 1 ml of ice cold 0.8N perchloric acid with rapid mixing.

(ii) Removal of precipitated protein

Protein precipitates were removed by centrifugation in a Sorvall bench-top microfuge at 2500 g for 20 minutes at 0 - 2 °C. The supernatant was used for measuring the concentration of P_i by colorimetric assay.

(iii) Measurement of inorganic phosphate by colorimetry

The amount of inorganic phosphate was measured using the method of Bonting *et al.* (1961). A "colour reagent" was prepared by dissolving

1 g ammonium molybdate in 90 ml distilled water and adding 3.3 ml concentrated sulphuric acid and 4 g ferrous sulphate. The final volume was adjusted to 100 ml with distilled water. Assay supernatant and water (250 µl of each) were added to 500 µl of colour reagent at room temperature and mixed thoroughly in a 1 ml cuvette. Phosphate standards (0 - 250 nM) were made using KH_2PO_4. After five to seven minutes, absorption was measured at 750 nm in a spectrophotometer (type Ultrospec ll, LKB Wallac, Turku, Finland).

(iv) Protein Determination

The amount of protein was determined by the method of Lowry *et al.* (1951). Protein standards (0 - 200 µg) were made using bovine serum albumin (BSA).

5.2.5 Activity of Ca^{2+}-ATPase

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity was measured in skeletal muscle homogenates using a coupled enzyme assay, as described by Simonides & van Hardeveld (1990). The rate at which Ca^{2+}-ATPase sequesters calcium following muscle contraction is believed to be directly proportional to its energy consumption and hence hydrolysis of ATP which is calculated from spectrophotometric recordings of the oxidation of NADH (Simonides & van Hardeveld, 1990).

Conditions used in this assay maximized Ca^{2+}-ATPase activity while eliminating Ca^{2+}-dependent myofibrillar ATPase activity and minimizing background activity (Simonides & van Hardeveld, 1990). The rate of total ATP hydrolysis in the presence of a low level of

calcium was followed spectrophotometrically from recordings of the oxidation of NADH (340 nm). The molar absorption coefficient of NADH of 6.22 x 10^3 M^{-1} was used. The rate of hydrolysis by Ca^{2+}-ATPase was then inhibited by the addition of a high level of calcium. The difference in activity between the low and high levels of calcium is defined as the SR Ca^{2+}-ATPase activity.

Calculation of Ca^{+2}-ATPase Activity is outlined below.

SR Ca^{+2}-ATPase activity is equal to:

Total ATPase activity - (Total ATPase activity - Ca^{2+}-ATPase activity)

and is measured in µmoles ATP . mg.protein^{-1} . min^{-1} and

µmoles ATP . g tissue^{-1} . min^{-1}.

Activity is calculated as: (µmoles ATP . mg protein^{-1} . min^{-1})

$$\frac{\text{(Slope of line for 1 min / 6.22)} \bullet 1000}{\text{Amount of protein in homogenate (mg/µl)} \bullet \text{total amount of protein used (µl)}}$$

For details, see section 5.2.5.2.

5.2.5.1 Preparation of Samples

Frozen muscles were thawed rapidly and then homogenised in 100 mM Tris/HCl, and 0.3 M sucrose at pH 8.4 at a concentration of 100 mg tissue wet weight.ml^{-1}. Preliminary tests were conducted to ensure that optimal results for activity were obtained.

5.2.5.2 Measurement of Ca^{2+}ATPase Activity

Assays were conducted at room temperature. In each assay, 5 µl of homogenate was suspended in 1 ml buffer (i.e. 0.5 mg tissue). The buffer contained 1 mM EGTA, 10 mM phosphoenolpyruvate, purified pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) from rabbit (both 18 $U.ml^{-1}$), 0.2 mM NADH, 20 mM Hepes, 200 mM KCl, 15 mM $MgCl_2$ and 10 mM NaN_3, pH 7.5. Triton X-100 (0.005%) was added prior to the assay. Assays were started by the addition of 4 mM MgATP and the spectrophotometric absorbance signal (type Cary 210 Spectrophotometer, Varian Instrument Division, California) was recorded until the slope was constant. A low concentration of $CaCl_2$ (10 µl of 64 mM - giving a final concentration of 0.64 mM, final range should be 0.5 - 0.8 mM) was added and the slope was recorded. 10 µl of 2 M $CaCl_2$ was then added and the final slope recorded. The final high level of Ca^{+2} (i.e. 20.64 mM) immediately inhibits the SR Ca^{+2}-ATPase activity and any remaining activity is defined as background activity.

Protein content was determined as previously described.

5.2.6 Statistical Methods

All measurements are expressed as mean ± SE. The level of statistical significance chosen for this experiment was $P < 0.05$.

In measuring the concentration of Na^+,K^+-ATPase, not all muscles were saturated with ouabain. Accordingly, the results were analysed in two ways. Firstly, the number of binding sites for each dietary group at 1 µM ouabain was analysed by Kruskal-Wallis tests, since the

variances were heterogeneous. Secondly, results for [^{3}H]ouabain binding sites in muscle were then individually transformed by the method of Scatchard (1949) to enable an estimate of the maximum number of [^{3}H]ouabain binding sites (B_{max}) to be made, as well as the dissociation constant (K_D). (K_D represents the concentration of free ouabain at which the concentration of bound ouabain is 50% of B_{max} (Pratt & Taylor, 1990). Lines were fitted by linear regression analysis. Correlation coefficients for Scatchard plots ranged from 0.86 to 0.94. Values for B_{max} and K_D were then analysed by Kruskal-Wallis tests and wherever the test revealed significant effects of diet, Tukey's non-parametric multiple comparisons test was used to determine which dietary groups produced significantly different responses.

Although Scatchard plots were calculated separately for each muscle and analyses were done on individual B_{max} and K_D, Figures 5.1 and 5.2 represent the mean values for each group.

To test for differences in Na^+,K^+-ATPase activity and Ca^{2+}-ATPase activity between the dietary groups, Model lll 2-factor ANOVAs without replication; the fixed factor was diet and the random factor was litter.

All data and ANOVA details are presented in Appendix 4.

5.3 Results

5.3.1 Na^+,K^+ -ATPase

5.3.1.1 Concentration of Na^+,K^+ -ATPase

Diet did not significantly affect the concentration of [^{3}H]ouabain binding sites estimated from binding at 1 μM ouabain ($P > 0.1$) (Table 5.1; Figures 5.1 and 5.2). Mean estimates for each group showed little variation ranging from 301 ± 12 pmoles . g wet wt^{-1} to 345 ± 21 pmoles . g wet wt^{-1} for soleus and from 412 ± 42 pmoles . g wet wt^{-1} to 555 ± 77 pmoles . g wet wt^{-1} for EDL.

The relationship between the ratio of bound ouabain : free ouabain and bound ouabain (for the Scatchard plot) was approximately linear ($r^2 \geq 0.86$ for all groups) although only the soleus muscle from the Control group appeared to have reached saturation (Table 5.1; Figure 5.1). Extrapolated estimates of the maximum number of binding sites per gram of muscle by Scatchard analysis similarly indicated no significant difference in soleus muscles ($P > 0.1$) or EDL ($P > 0.1$) (Table 5.1). Mean B_{max} ranged from 343 ± 21 pmoles . g wet wt^{-1} to 379 ± 39 pmoles . g wet wt^{-1} for soleus and from 438 ± 41 pmoles . g wet wt^{-1} to 583 ± 76 pmoles . g wet wt^{-1} for EDL.

There was a significant difference in affinity for ouabain in soleus muscles ($P < 0.02$) (Table 5.1). Although Tukey's non-paramatric multiple comparisons tests was not sufficiently powerful to be able to distinguish between the means, it should be noted that K_D for the PUFA group (52 ± 10 nM) appeared to be lower than for the Control

Table 5.1 Concentration of Na^+, K^+-ATPase (pmoles g^{-1} tissue wet weight), measured as the number of [^{3}H]ouabain binding sites, in soleus and EDL muscles male Wistar rats which have been on one three diets; Control, EFAD or PUFA for nine weeks (mean ± SE). Diet did not affect the maximum number of binding sites (B_{max}) in soleus or EDL muscles or the number of binding sites at 1 μM ouabain. There was a significant effect of diet on affinity for ouabain in soleus (K_D - dissociation constant) but diet had no effect on affinity in EDL. B_{max} and K_D. were estimated from Scatchard analysis of individual muscle saturation binding isotherms. "r" is the mean correlation coefficient calculated for the linear regression of individual Scatchard plots. "H" is the Kruskal-Wallis test statistic.

	Dietary Group				
	Control	**EFAD**	**PUFA**	**H**	**P**
Soleus	(n = 9)	(n = 11)	(n = 9)		
Binding at 1 μM ouabain (pmoles . g wet wt $^{-1}$)	301 ± 12	345 ± 2	303 ± 25	3.1	N.S.
Mean correlation coefficient (r)	0.94 ± 0.02	0.93 ± 0.03	0.93 ± 0.02		
Total binding (B_{max}) (pmoles . g wet wt $^{-1}$)	343 ± 21	359 ± 18	379 ± 39	1.8	N.S.
K_D (nM)	85 ± 12	128 ± 48	52 ± 10	6.7	< 0.02
EDL	(n = 10)	(n = 15)	(n = 12)		
Binding at 1 μM ouabain (pmoles . g wet wt $^{-1}$)	412 ± 42	555 ± 77	497 ± 80	1.0	N.S.
Mean correlation coefficient (r)	0.92 ± 0.02	0.90 ± 0.03	0.86 ± 0.03		
Total binding (B_{max}) (pmoles . g wet wt $^{-1}$)	438 ± 41	583 ± 76	507 ± 62	1.6	N.S.
K_D (nM)	137 ± 31	148 ± 61	113 ± 22	0.7	N.S.

Figure 5.1 Saturation binding isotherm (Fig 5.1 A) for [^{3}H]ouabain binding to soleus muscle slices from groups of male Wistar rats and mean Scatchard plots derived from individual muscles (Fig. 5.1 B). Each point represents the mean ± SE for 9 (Control), 11 (EFAD) and 9 (PUFA) rats. In the Scatchard plot, the ratio of the concentrations of bound : free ouabain is plotted against the concentration of bound ouabain. (Diet did not affect total binding (B_{max}) ($P > 0.1$) or the number of binding sites at 1 μM ouabain ($P > 0.1$). There was a significant dietary effect on affinity (K_D) for ouabain ($P < 0.02$) but Tukey's multiple comparisons test was unable to distinguish between the dietary groups.

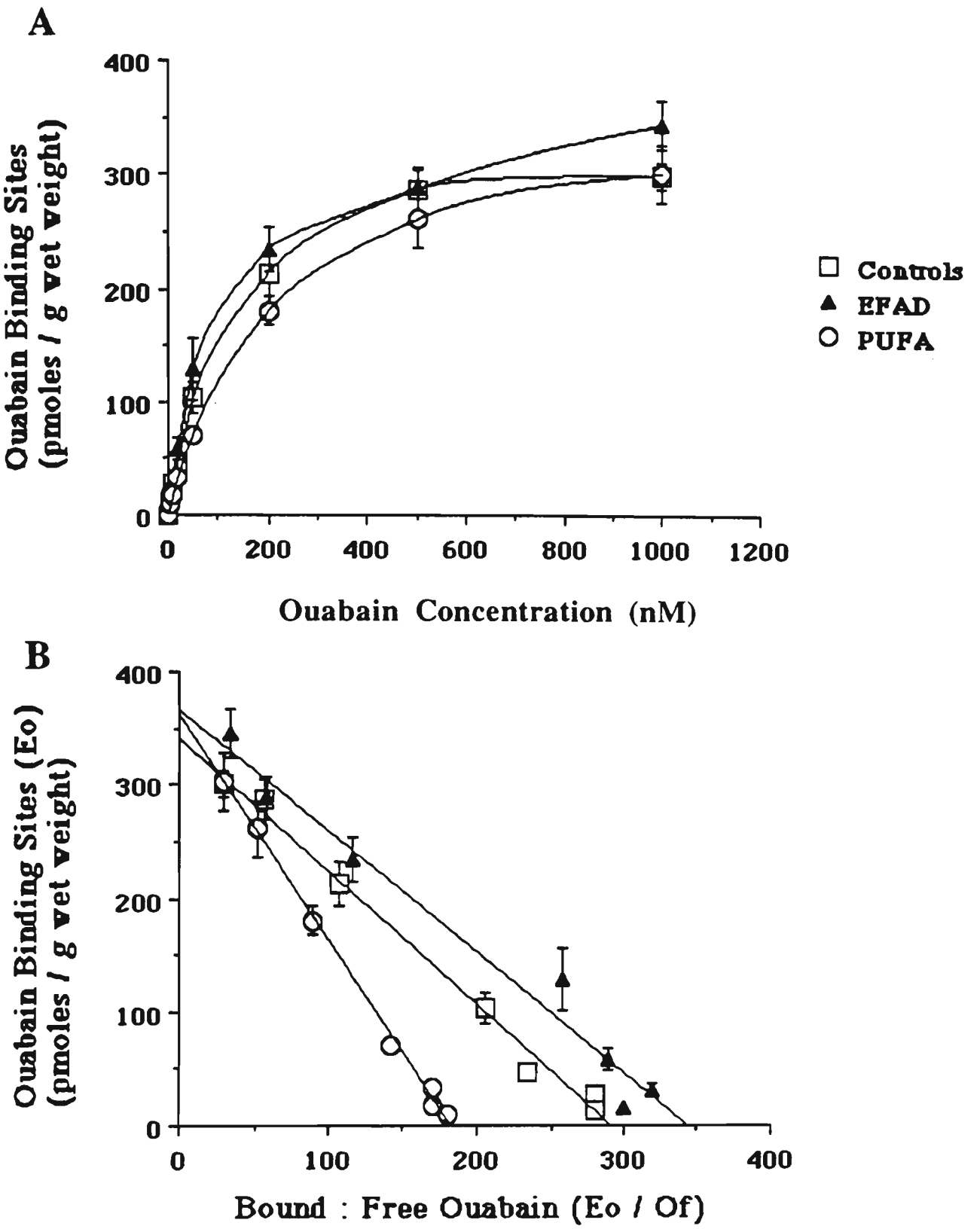
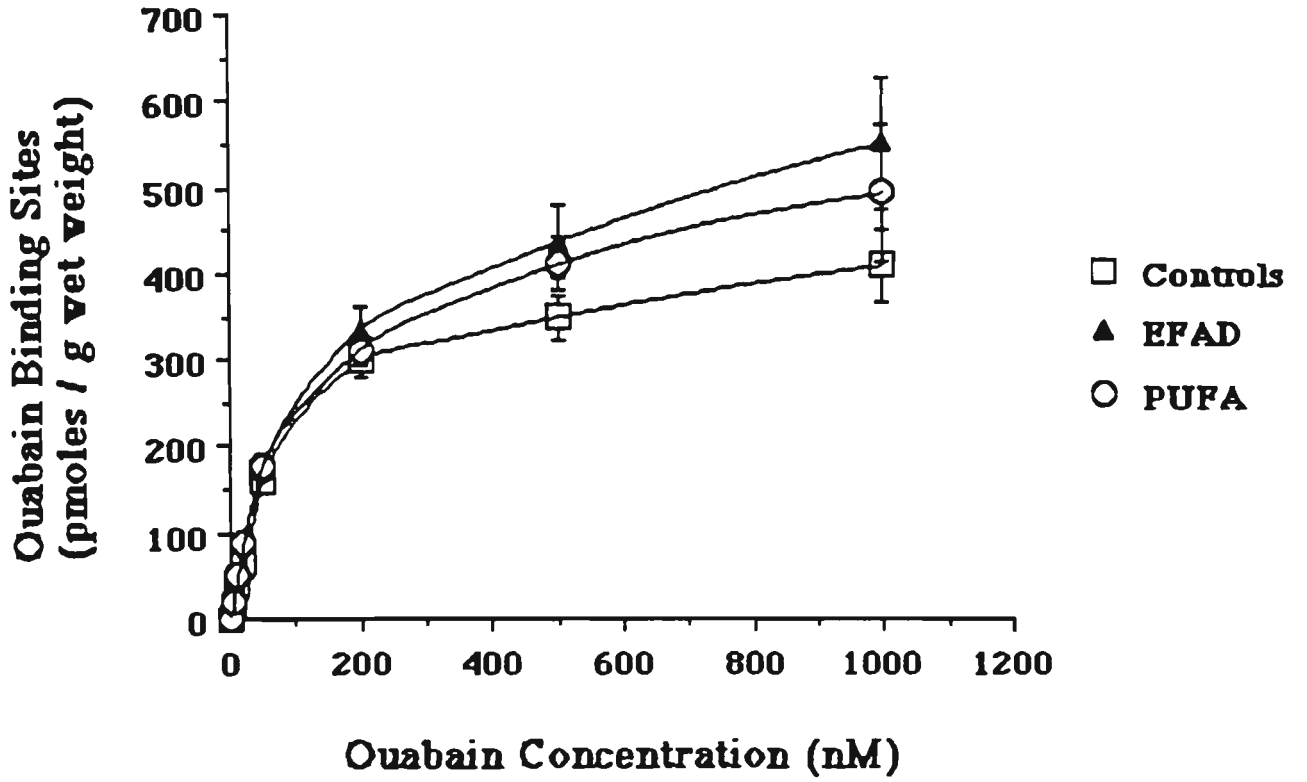
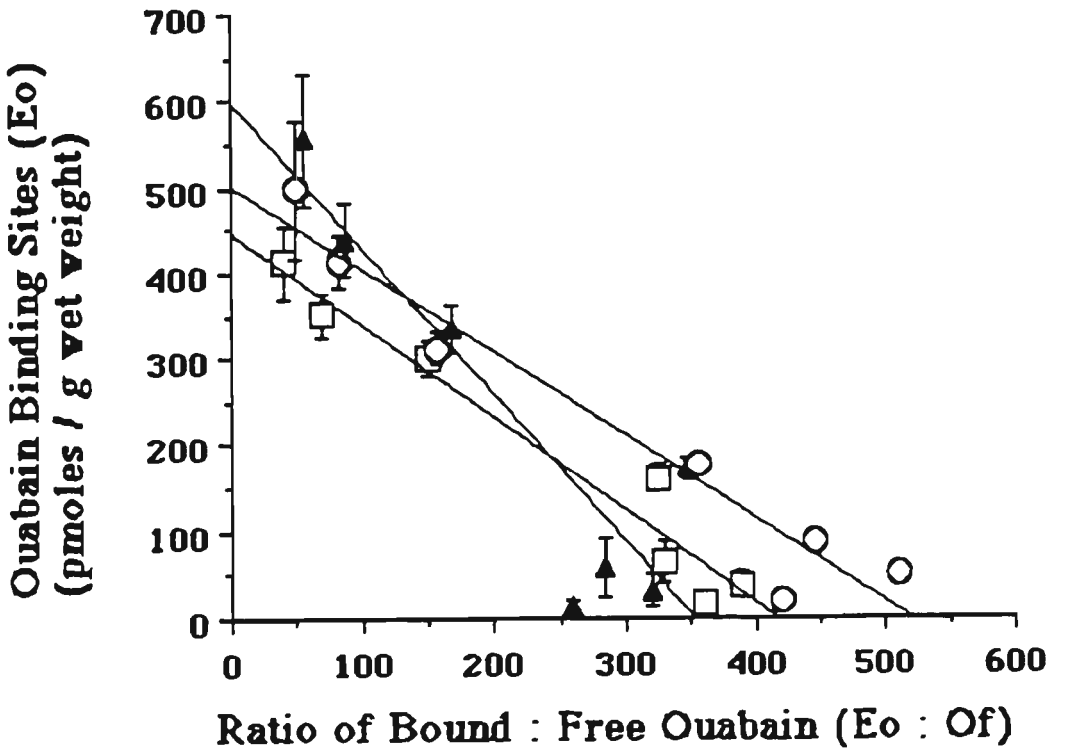

A
Ouabain Binding Sites
(pmoles / g wet weight)
400
300
200
100
0
0
200
400
600
800
1000
1200
Ouabain Concentration (nM)
Controls
EFAD
PUFA
B
Ouabain Binding Sites (Eo)
(pmoles / g wet weight)
400
300
200
100
0
0
100
200
300
400
Bound : Free Ouabain (Eo / Of)

Figure 5.2 Saturation binding isotherm (Fig 5.2 A) for [^{3}H]ouabain binding to EDL muscle slices from groups of male Wistar rats and mean Scatchard plots derived from individual muscles (Fig. 5.2 B). Each point represents the mean ± SE for 10 (Control), 15 (EFAD) and 12 (PUFA) rats. In the Scatchard plot, the ratio of the concentrations of bound : free ouabain is plotted against the concentration of bound ouabain. Diet did not affect total binding (B_{max}) ($P > 0.1$), the number of binding sites at 1 μM ouabain ($P > 0.1$) or affinity (K_D)for ouabain ($P > 01$).

A


Ouabain Binding Sites
(pmoles / g wet weight)
700
600
500
400
300
200
100
0
0
200
400
600
800
1000
1200
Ouabain Concentration (nM)
Controls
EFAD
PUFA

B


Ouabain Binding Sites (Eo)
(pmoles / g wet weight)
700
600
500
400
300
200
100
0
0
100
200
300
400
500
600
Ratio of Bound : Free Ouabain (Eo : Of)

(85 ± 12 nM) and EFAD (128 ± 48 nM) groups, respectively. Although diet had no statistically significant effect on ouabain affinity in EDL muscles ($P > 0.1$) the order was similar to that in soleus.

The amount of total binding in EDL was about 1.3 to 1.6 times greater than in soleus.

5.3.1.2 Activity of Na^+,K^+ -ATPase

Results are presented for activity per mg of muscle tissue and per mg of protein (Table 5.2).

Activity of Na^+,K^+ -ATPase was initially measured in muscle homogenates. Although there was no evidence of differences between the dietary groups, the levels of activity were very low, ranging in both soleus and EDL from 0.066 ± 0.011 to 0.082 ± 0.006 μmoles P_i . mg tissue^{-1} . hour^{-1} or from 0.33 ± 0.06 to 0.43 ± 0.05 μmoles P_i . mg protein^{-1} . hour^{-1} (Table 5.2).

When muscle homogenates were partially purified and the microsomal preparations were analysed, the level of activity increased by approximately three-fold. Values ranged in both soleus and EDL from 0.17 ± 0.04 to 0.24 ± 0.02 μmoles P_i.mg tissue^{-1} . hour^{-1} and from 0.95 ± 0.14 to 1.21 ± 0.13 μmoles P_i.mg protein^{-1} . hour^{-1}.

In soleus muscles, there was no significant effect of diet on Na^+,K^+ -ATPase activity in either muscle homogenates or muscle microsomes, whether expressed per mg tissue or per mg protein ($P > 0.5$ for each) (Table 5.2).

Table 5.2 Na^+, K^+-ATPase activity (µmoles Pi.mg tissue wet weight^{-1} . hr^{-1} and µmoles Pi.mg protein^{-1} . hr^{-1}) in soleus and EDL muscles from groups of nine male Wistar rats which have been on one three diets; Control, EFAD or PUFA for nine weeks (mean ± SE). Diet did not affect Na^+, K^+-ATPase activity in either muscle homogenate or purified microsomes in soleus or EDL. "H" is the Kruskal-Wallis test statistic.

	Dietary Group				
	Control	**EFAD**	**PUFA**	**H**	**P**
Soleus	(n = 9)	(n = 9)	(n = 9)		
Homogenate					
µmoles P_i. mg tissue^{-1}.hr^{-1}	0.082 ± 0.006	0.066 ± 0.011	0.069 ± 0.008	1.0	N.S.
µmoles P_i. mg protein^{-1}.hr^{-1}	0.40 ± 0.03	0.33 ± 0.06	0.41 ± 0.07	0.5	N.S.
Microsomes					
µmoles P_i. mg tissue^{-1}.hr^{-1}	0.24 ± 0.02	0.19 ± 0.02	0.20 ± 0.03	1.0	N.S.
µmoles P_i. mg protein^{-1}.hr^{-1}	1.21 ± 0.13	0.99 ± 0.11	1.19 ± 0.24	0.5	N.S.
EDL	(n = 9)	(n = 9)	(n = 9)		
Homogenate					
µmoles P_i. mg tissue^{-1}.hr^{-1}	0.066 ± 0.010	0.078 ± 0.017	0.078 ± 0.013	1.6	N.S.
µmoles P_i. mg protein^{-1}.hr^{-1}	0.39 ± 0.04	0.40 ± 0.07	0.43 ± 0.05	0.3	N.S.
Microsomes					
µmoles P_i. mg tissue^{-1}.hr^{-1}	0.17 ± 0.04	0.23 ± 0.05	0.22 ± 0.05	4.0	N.S.
µmoles P_i. mg protein^{-1}.hr^{-1}	0.95 ± 0.14	1.14 ± 0.22	1.15 ± 0.18	1.8	N.S.

Similarly, in EDL muscles, there was no significant effect of diet on Na^+,K^+ -ATPase activity in either muscle homogenates (per mg tissue; $P = 0.2$ and per mg protein; $P > 0.5$) or muscle microsomes (per mg tissue; $P < 0.1$; per mg protein; $P < 0.5$). There was a significant effect of litter on all measures of Na^+,K^+ -ATPase activity in EDL muscles ($P < 0.001$), but there were no effects of litter on soleus muscles.

5.3.2 Ca^{2+} -ATPase

Diet had no significant effect on the activity of Ca^{2+} -ATPase in either soleus or EDL regardless of whether results were expressed per mg protein or per g tissue (Table 5.3).

For soleus, values ranged from 5.7 ± 0.7 to 6.6 ± 0.7 μmoles . g tissue^{-1} . min^{-1} ($P > 0.5$) and from 48 ± 6 to 55 ± 5 μmoles . g protein^{-1} . min^{-1} ($P > 0.5$) (Table 5.3). For EDL, values ranged from 5.4 ± 0.5 to 5.9 ± 0.6 μmoles . g tissue^{-1} . min^{-1} ($P = 0.5$) or from 42 ± 4 to 48 ± 6 μmoles . g protein^{-1} . min^{-1} ($P > 0.5$) (Table 5.3).

5.4 Discussion

This study investigated the effects of dietary fatty acid manipulation on sarcolemmal Na^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase in fast (EDL) and slow (soleus) skeletal muscles. Diet had a significant effect on the affinity of Na^+,K^+-ATPase for ouabain in soleus muscles, but in both soleus and EDL muscles, there was no

Table 5.3 Ca^{2+}-ATPase activity (µmoles ATP.g tissue wet weight^{-1} . hr^{-1} and µmoles ATP.g protein^{-1} · hr^{-1}) in soleus and EDL muscles from groups of nine male Wistar rats which have been on one three diets; Control, EFAD or PUFA for nine weeks (mean ± SE). Diet did not affect Ca^{2+}-ATPase activity in either muscle homogenate or purified microsomes in soleus or EDL. "H" is the Kruskal-Wallis test statistic.

	Dietary Group				
	Control	**EFAD**	**PUFA**	**H**	**P**
Soleus	(n = 9)	(n = 9)	(n = 9)		
µmoles ATP. g tissue^{-1}min^{-1}	5.7 ± 0.7	6.5 ± 0.6	6.6 ± 0.7	1.3	N.S.
µmoles ATP. g protein^{-1}min^{-1}	48 ± 6	55 ± 5	54 ± 6	1.6	N.S.
EDL	(n = 9)	(n = 9)	(n = 9)		
µmoles ATP. g tissue^{-1}min^{-1}	5.6 ± 0.5	5.4 ± 0.5	5.9 ± 0.6	1.5	N.S.
µmoles ATP. g protein^{-1}min^{-1}	42 ± 4	47 ± 5	48 ± 6	1.3	N.S.

detectable effect of diet on either concentration or activity of Na^+,K^+-ATPase or the activity of Ca^{2+}-ATPase.

5.4.1 Na^+,K^+-ATPase

It has been argued that few studies have measured the true activity level of Na^+,K^+-ATPase in skeletal muscle because the recovery of Na^+,K^+-ATPase is usually very low (Kjeldsen, 1986; Hansen & Clausen, 1988). This is believed to be due to the high background level of nonspecific ATPases and as well as difficulties associated with separation of the plasma membrane from other cell structures. However, low recovery rates need not invalidate comparisons of the effects of diet on relative activity level. The critical issues are whether the assay can provide a sufficiently repeatable and representative assay of membrane Na^+,K^+-ATPase activity. In this study, Na^+,K^+-ATPase activity was measured in muscle homogenates, and as expected, activity levels were extremely low, ranging from 0.33 ± 0.06 to 0.43 ± 0.05 μ moles Pi . mg protein^{-1} . hr^{-1}, but showed no effect of diet (standard errors were 7% to 22% of the mean). In an attempt to overcome the problem of low activity, muscle membranes were partially purified and activity was measured in microsomes. This purification process increased the activity level by approximately three-fold, but did not reduce the already small level of variation detected for each dietary group (% SE ranged from 8% to 24%). Again, there was no effect of diet on Na+,K+-ATPase activity. Results obtained for activity of Na^+, K^+-ATPase (ranged from 0.99 - 1.2 μmoles Pi . mg protein^{-1} . hr^{-1}) are similar to those obtained in gastrocnemius (1.13 ± 0.34 μmoles Pi.mg protein^{-1} . hr^{-1}; Else, personal communication) and soleus (0.15 - 0.35 μmoles Pi . mg

protein^{-1} . hr^{-1}; Clausen & Hansen, 1974 and Kjeldsen *et al.*, 1984, respectively). Estimates of Na^+,K^+-ATPase activity should be made with caution since this assay measures maximum activity by using saturating substrate levels and the activity of Na^+,K^+-ATPase *in vivo* may be submaximal.

Precise estimates of the concentration of Na^+,K^+-ATPase within muscle membranes are considered to be achievable through ouabain binding assays (but see General Discussion). In this study, there was no significant effect of dietary fatty acid manipulation on the number of sodium pumps in soleus or EDL muscles, and the values obtained were similar to those reported in the literature. In soleus, mean values for total binding ranged from 301 to345 pmoles . g wet wt^{-1} for soleus and from 412 to 555 pmoles . g wet wt^{-1} for EDL. Kjeldsen (1988) reported that the total concentration of [^{3}H]ouabain binding sites in soleus muscles from 12-week old rats is 278 - 359 pmol . g wet wt^{-1} which is in good agreement with the results obtained here. Similarly, the values obtained for EDL muscles (30 - 60 % higher than for soleus muscles) were in agreement with previously reported results (Clausen *et al.*, 1982; Nørgaard *et al.*, 1983).

Minor differences in the concentration or activity of Na^+,K^+-ATPase may reflect differences in strain, sex or age of rats. It was not possible to detect litter effects on the concentration of Na^+,K^+-ATPase as these data were analysed by Kruskal-Wallis tests (due to their heterogeneous variances and values were not obtained for all members of litters). For activity of Na^+,K^+-ATPase however, it is interesting to note that although there were no effects of litter in

soleus muscles, there were significant litter effects on Na^+,K^+-ATPase activity in EDL muscles.

Although there was no effect of diet on total binding, in soleus there was a significant dietary effect on the affinity of Na^+,K^+-ATPase for ouabain which may be due to changes in the membrane fatty acid composition. Although Tukey's multiple comparisons test was unable to distinguish between the dietary groups, it appeared that affinity in the PUFA group was lower than for the Control and EFAD groups. Since affinity for ouabain was altered by changes in dietary fatty acids, it is possible that affinities for Na^+ and / or K^+ were also altered.

5.4.2 Ca^{2+}-ATPase

In this study, neither a deficiency of the essential polyunsaturated fatty acids nor high levels of n - 3 fatty acids affected the activity of Ca^{2+}-ATPase of the SR.

Simonides & van Hardeveld (1990) reported that maximum Ca^{2+}-ATPase activity in unspecified rat skeletal muscle homogenate was 50 µmoles . g wet wt^{-1} . min^{-1}.at 37°C. Using thin slices at 25 °C, they estimated that the activity of Ca^{2+}-ATPase is 45 ± 3 µmoles .g wet wt^{-1}. min^{-1} in EDL muscles and 8 ± 1 µmoles . g wet wt^{-1} . min^{-1} in soleus muscles. Using the same technique, Matsushita & Pette (1992), found that the activity of Ca^{2+}-ATPase in rabbit fast muscle homogenates was 113 ± 4. µmoles . mg wet wt^{-1} . min. In this study at 25 °C, values ranged from 5.7 ± 0.7 to 6.6 ± 0.7 µmoles . g wet

wt^{-1} . min^{-1} in soleus and from 5.4 ± 0.5 to 5.9 ± 0.6 μmoles . g wet wt^{-1} . min^{-1} in EDL. Although there is no difference in the values for soleus and EDL, as reported by Simonides & Van Hardeveld (1990), the standard errors are of similar relative magnitude, the results were highly repeatable and there were no differences between the dietary groups in the activity levels obtained.

The results of the PUFA diet, with a high proportion of n - 3 fatty acids (7%), support those of Stubbs & Kisielewski (1990), who found no effects of a 12% fish oil diet on Ca^{2+}-ATPase in rat skeletal muscle. In contrast, studies which have examined the effects of increased dietary polyunsaturated fatty acids in cardiac muscle have produced conflicting results. Swanson *et al.* (1989), found, in mice, that a 12% n - 3 enriched diet decreased both cardiac SR Ca^{2+}-ATPase and calcium pump activity whereas in their later study, with a 2% n - 3 enriched diet, calcium uptake was decreased but calcium pump activity was unaffected (Croset *et al.*, 1989). In rats, Abeywardena *et al.* (1984) found no effect of a 12 % (by weight) n - 6 fatty acid enriched diet on Ca^{2+}-ATPase.

5.4.3 Some General Considerations

Assays which measure the activity of membrane-bound enzymes or the concentration of binding sites *in vitro* are inevitably dependent on the assay conditions and there are a number of critical assumptions which are rarely tested or cannot be tested.

Firstly, there are three known isozymes of Na^+,K^+-ATPase (for review, see Sweadner, 1989). These are specific for species, tissue

and developmental stages (Orlowski & Lingrel, 1988) and their functional significance is not understood (Jewell *et al.*, 1992). In rat skeletal muscles, there appears to be a predominant high-affinity isozyme of Na^+,K^+-ATPase and lower amounts of a low-affinity binding site (Lytton *et al.*, 1985; Young & Lingrel, 1987; Kjeldsen *et al.*, 1985; 1988; Sweadner, 1989). Each of these isoforms may have a different affinity for ouabain. This is potentially a problem when using Scatchard plots to extrapolate binding data since there is an underlying assumption of linearity.

According to Everts & Clausen (1988) and Jørgensen (personal communication), the vanadate-facilitated [^{3}H]ouabain binding method probably only detects the high-affinity form of Na^+,K^+-ATPase in muscles and not the low affinity form. Recent studies on the distribution of the mRNAs encoding for the isoforms of Na^+,K^+-ATPase have shown that 90 - 95% of the mRNA in muscle encodes the high affinity isoform whereas the remaining 5 - 10% encodes the low affinity isoform (Young & Lingrel, 1987; Schneider *et al.*, 1988). However, results of studies relating [^{3}H]ouabain binding and ^{42}K uptake indicate that measurements of [^{3}H]ouabain binding quantify functional sodium pumps (Kjeldsen *et al.*, 1985) and Clausen *et al.* (1987) showed that induced changes in [^{3}H]ouabain binding site concentration are associated with proportional changes in the maximum transport rate of the sodium pump. Kjeldsen (1988), using the [^{3}H]ouabain binding technique, concluded that there is no evidence for a major pool of low-affinity [^{3}H]ouabain binding sites. Therefore, measurement of [^{3}H]ouabain binding provides a useful approach for the quantification of sodium pumps but it is important to bear in mind that more than one form of Na^+,K^+-ATPase exists in skeletal muscle.

Secondly, if the membrane is changed by dietary manipulation or some other treatment, then affinity for ouabain by the different isoforms of Na^+,K^+-ATPase may also change. The minor isoform of Na^+,K^+-ATPase in skeletal muscle membranes may exhibit low or high binding affinity under normal conditions, but this may change if dietary lipid manipulation alters the surrounding environment by changing membrane fatty acid composition. This may present problems when determining whether a Scatchard plot is linear, or represents more than one [^{3}H]ouabain binding site. In this study, there was in fact a significant effect of diet on affinity of Na^+,K^+-ATPase in soleus muscles for ouabain. Although Tukey's test was unable to distinguish between the dietary groups, it appeared that affinity in the PUFA group was lower than for the Control and EFAD groups. Since affinity for ouabain was altered by changes in dietary fatty acids, it is possible that affinities for Na^+ and / K^+ was also altered.

Thirdly, and perhaps most importantly, does the presence of substances such as ouabain (in assays measuring both concentration and activity of Na^+,K^+-ATPase) and calcium (used for measuring Ca^{2+}-ATPase) in very high concentrations change other cellular functions?. The methods of measuring both concentration and activity of Na^+,K^+-ATPase used here are the most commonly used. However, the assumption is made that the remaining functions of the cell are unaffected by direct or induced effects of ouabain (i.e. altered intracellular ionic concentrations since the effect is to inhibit Na^+,K^+-ATPase), or at least that the remaining cellular functions do not vary

among dietary groups. Similar criticisms must apply to the effect of high [Ca^{2+}] as a means of inhibiting calcium pumps.

Almost all studies determining the maximum number of binding sites in equilibrium binding assays appear to rely on the use of Scatchard plots. These are used even when saturation has clearly not been reached (Alam *et al.*, 1987) or in studies where the nature of the curves is unreported (Lin *et al.*, 1979; Alam *et al.*, 1989). Certainly, most studies do not report whether Scatchard analyses are performed on individual values or means for a group.

I have attempted to avoid the problems associated with [^{3}H]ouabain binding by analysing the data in two ways. However, despite comparing the number of binding sites at 1 μM ouabain and the use of Scatchard analysis to estimate the maximum number of binding sites, there was no evidence of a dietary effect on the concentration of Na^+, K^+-ATPase although soleus muscles from rats on the PUFA diet appeared to have a reduced affinity for ouabain.

Although no differences were detected in the concentration and activity of Na^+, K^+-ATPase and activity of Ca^{2+}-ATPase, it is possible that, given the limitations of these assays and the fact that they are only part of the metabolic machinery of the cell, effects at the level of the membrane may still exist. It must be remembered that these enzyme assays measure the maximum activity of the enzymes *in vitro*, which may be quite different to the rate of activity under normal working conditions in vivo. Also, just because these dietary manipulations did not affect concentration or activity of these enzymes does not mean that the dynamics of ion transport in

membranes is unaffected. It may be that membrane permeability may be altered which could in turn alter the efflux of K^+ and influx of Na^+ through the sarcolemma, or the rate of release of Ca^{2+} through the SR membrane. As stated above, since the affinity for ouabain was altered in soleus, the affinity for sodium and / or potassium might also be altered. The next step in this investigation would be to directly investigate the movement of these ions through membranes of rats which have been on EFAD and PUFA diets, by the use of ion-selective electrodes or by measuring their concentrations in blood entering and leaving muscles, particularly during exercise. It is clear that the story is not complete for EFA deficiency and enriched PUFA diets. It is important to approach the problem from different levels, and particularly, to determine the effects of dietary manipulation on the function of whole animals.

Chapter 6 General Discussion

It seems inevitable that a study which investigated such a broad area as diet and physical performance will produce at least as many questions as answers. This study has provided the first investigation of the effects of changed levels of polyunsaturated fatty acids on a range of aspects of muscle function and physical performance. It demonstrated that changes in the proportions of specific dietary fatty acids can significantly alter the composition of skeletal muscle membranes and at the same time, affect both muscle function and whole animal performance. Recovery from the effects of a deficiency in essential fatty acids (both n - 6 and n - 3) was shown to be rapid in the muscle membranes and in the functioning of isolated muscles; the turnover time for some membrane fatty acids must be a matter of just a few days. Recovery from the effects of an n - 3 enriched diet on membrane composition did not occur. In fact, the membranes from rats on this diet appeared to tenaciously retain the increased concentration of n - 3 fatty acids, even after six weeks recovery. This should not necessarily be interpreted as a failure to recover. It may be that some aspects of the function of these muscles are improved with the increased level of n - 3 fatty acids. There was no evidence of recovery from the detrimental effects of changed dietary lipids on endurance in whole animals. In addition, the fatty acid composition and function of fast and slow muscles were affected differently and they recovered at different rates. Both membrane fatty acid composition and some specific measures of muscle function took longer to recover from the effects of changed dietary fatty acids in EDL, an example of a fast-twitch muscle, than in soleus, a slow-twitch muscle.

This study showed preferential retention of n - 3 fatty acids in skeletal muscle. Abeywardena *et al.* (1987) showed that changes in myocardial fatty acids due to a long-term saturated fat diet and the accompanying arrhythmogenic effects on cardiac muscle (McLennan *et al.*, 1990) are reversed by crossing over to diets boosted with polyunsaturated fatty acids. However, they have not examined whether the reverse cross-over results in preferential retention of the n - 3 fatty acids. Anderson *et al.* (1992) showed that chicks fed diets enriched with a high level of n - 3 fatty acids (in fact, four times the level used in this study) retained a high level of these in brain and retina from three weeks of age. This is not surprising since the n - 3 fatty acids perform vital functions in these tissues (Neuringer *et al.*, 1988). They also showed that in other tissues (liver and serum) following dietary reversal, that levels of n - 3 fatty acids returned to control levels.

Whilst this study produced some surprising outcomes, partial support comes from earlier less specific studies. Although studied inadvertently in some cases, by the use of "high carbohydrate" (and therefore "low fat") diets, little effect of different total levels of dietary lipids on exercise has been found. Only a few studies have investigated the effects of different types of dietary fat (Ivy *et al.*, 1980; Decombaz *et al.*, 1983; Warner *et al.*, 1989; Brilla & Landerholm, 1990; Ågren *et al.*, 1991) on exercise and they have not reported any significant effects.

In the remainder of this chapter I will discuss again several aspects of the study and provide some directions for future work.

6.1 Membrane Fatty Acid Composition and Isolated Muscle Function

Recovery from the effects of the EFAD diet on both muscle membrane composition and isolated muscle function was rapid (less than two weeks) in soleus muscles (an example of a "slow-twitch" muscle), but took up to six weeks in EDL muscles (an example of a "fast-twitch" muscle).

Recovery from the effects of the PUFA diet on muscle membrane composition did not occur completely in either soleus or EDL muscles. After six weeks recovery, the level of n - 3 fatty acids was still significantly greater than in the Control and EFAD groups. It appeared that muscles from rats on the PUFA diet tenaciously retained the increased proportion of n - 3 fatty acids provided in the diet. This accumulation and aggressive retention of n - 3 fatty acids was also evident in the EFAD group. Although the EFAD diet contained no n - 3 fatty acids, the membranes from the muscles of rats on this diet contained 3 - 4% n - 3 fatty acids after nine weeks. This must have been retained from their period *in utero* and before weaning.

Very little is known about the rates of fatty acid turnover in muscle membrane and it is hard to generalise from these results. The differences, however, may be due to specific properties of the muscles which may have changed as a result of the diets or, since soleus is a postural muscle as well as being responsible for flexion of the foot, it may be used a great deal more than EDL, especially in a caged rat. If this is so, it may also account for the

observation that isometric contraction parameters were altered by dietary changes in soleus but not EDL. Both twitch and tetanic tensions were decreased in soleus muscles from rats on the EFAD diet, as well as contraction and relaxation times, but none of these parameters were altered in EDL muscles. To test this hypothesis, it would be necessary to compare the fatty acid composition and function of other postural and non-postural muscles.

6.2 Membrane Fatty Acid Composition and Whole Animal Performance

This is perhaps the first study to examine both isolated muscle function and whole animal performance and the results appear contradictory in terms of both inital effects and time to recovery. Overall, there was very little effect of changes in dietary fatty acids on physical performance, in comparison to the widespread effects on isolated muscle function. Neither basal oxygen consumption nor peak oxygen consumption were affected, and these are probably the most commonly used measures of metabolic function. Grip strength, also, was unaffected. However, the relationship between muscle function and whole animal performance is likely to be complex and the results of whole animal performance are harder to interpret since they may be confounded by psychological and other physiological effects.

Although measurements were made of basal oxygen consumption (as an estimate of basal metabolic rate), effects of changes in dietary fatty acids on this parameter may be quite different to effects on the normal activity level of rats. For example, if a group of rats on one particular diet feels well and healthy, it may normally be fairly active,

but another group which feels less well on its diet, may be quite inactive. This may be reflected in effects on metabolic rate during activity since inactive rats will be less accustomed to physical exertion. This difference in behaviour could be reflected in tests such as the endurance and aerobic power tests used in this study.

The most surprising aspect of whole animal performance was the reduced endurance of the PUFA rats both initially (when isolated muscle function appeared unaffected) and after the 6 week "recovery period". This result was important since diets enriched with n - 3 fatty acids are likely to be favoured for other (cardiovascular) reasons. Since this diet had been shown to exert only beneficial effects (in comparison to the effects of the EFAD diet) on both isolated soleus and EDL muscles, it was hypothesized that it would have similar effects on whole animal performance. However, the results were highly repeatable and showed clear differences between dietary groups.

There were three different aspects to endurance in this study: (i) decreased endurance of the PUFA rats; (ii) lack of recovery of the PUFA rats and (iii) reduced endurance of all groups of rats after the recovery period. (i) The observed decrease in endurance in rats on the PUFA diet is not likely to be due directly to changes in muscle function since all isolated muscle function parameters had recovered within two weeks in soleus, and within six weeks in EDL. This effect may be due to any of a number of factors that are affected by the change in the level of n - 3 fatty acids, and clearly, results obtained for whole animals must be interpreted with caution. (ii) One possible effect of these different diets is to change the "natural activity" level

of the rats, so that they differ in the amount of movement or exercise they get each day. Rats that are naturally more active would be likely to cope better with strenuous exercise and show increased endurance. The observation that the levels of n - 3 fatty acids in both soleus and EDL muscles were still significantly higher in rats from the PUFA group after six weeks recovery, may be related to the lack of recovery in endurance in these rats after six weeks. If the high level of n - 3 fatty acids was indeed the cause of reduced endurance during the nine weeks on the test diets, then since there is still a high level of n - 3 fatty acids in these rats following recovery, the recovery would still be expected to be reduced. (iii) Even though all groups showed reduced endurance after the recovery period, the important point is that the pattern of the results was identical to the results after nine weeks on the test diets. Since these rats were both heavier and six weeks older following recovery, they may have been more reluctant to exercise. However, it is also possible that their muscles, which were probably not particularly well developed anyway as a result of a sedentary life in a relatively small box, had atrophied even more during the six week recovery period. An important point to note is that this study used untrained rats and these diets may have very different effects on rats that have undergone a training programme. Training can be looked at in two ways. As discussed, some rats may have been effectively less trained than others due to reduced natural activity levels. It could be that the continued poor performance of the PUFA group reflected the lack of earlier exercise. The diets may have different effects in rats that have actually been trained with regular exercise. It is also possible that this effect may be restricted to male rats. For future studies, the effects of these diets on trained

subjects needs to be investigated, preferably with human volunteers. This may result in very different findings.

The lack of an effect of diet on grip strength was surprising since there were several dietary effects on isolated muscle tension recorded. Although it was possible to measure grip strength very consistently, it may be argued that rats are not ideal subjects for this type of study. Ideally, tension was to be measured in isolated muscles of the hindlimb but this proved impossible. In hindsight, a better comparison would have been to test isolated muscle function and grip strength in the same muscles. In future studies, it would be important to measure the effects of dietary changes on all whole animal parameters, including grip strength, on human subjects, since there could be more careful control of tests. However, it must be realised that with humans, there are likely to be more confounding effects of motivation since they are able to find more "excuses" for stopping a strenuous exercise test.

6.3 Membrane Fatty Acid Composition and Membrane-Bound Enzymes

Although there was no effect of changes in dietary lipids on either Na^+,K^+-ATPase or Ca^{2+}-ATPase, these enzymes warranted investigation as possible causes of the observed effects on exercise since they control such critical aspects of muscle function. Other studies have shown that both the Na^+,K^+-ATPase and Ca^{2+}-ATPase enzymes systems in skeletal muscles are dynamic and capable of being changed by malnutrition (Nørgaard et al., 1981; Kjeldsen *et al.*, 1986), dietary manipulation (Stubbs & Kisielewski, 1990) and

training (Kjeldsen *et al.*, 1986; McKenna *et al.*, in press). They are certainly prime candidates for modification by surrounding membrane fatty acids since changes in dietary fatty acids have been shown to alter both enzymes in other tissues and this approach has been used in studies of cardiac muscle. However, since Abeywardena *et al.* (1984) found no effect of dietary lipid manipulation on either enzyme in cardiac muscle, and the composition of cardiac and skeletal muscle membranes appear to be very similar (Charnock *et al.*, 1989; 1992), it is not surprising that there was no effect on skeletal muscle in this study. The observation that the concentration and / or activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase were unaltered by changes in membrane fatty acids may imply that there are no significant effects on either enzyme system. However, these assays measure maximum enzyme activity but enzymes are not always operating at a maximal rate. Changes in membrane fatty acids may have effects on enzyme activity in vivo, at reduced working rates, which are not apparent when enzymes are working maximally. Also, such enzyme systems are complex and it may be more important to study the dynamics of ion transport through membranes. It is apparent that a set of more technically sophisticated studies is required. It may be important to verify that Na^+,K^+-ATPase is equally able to maintain intracellular K^+ levels in rats on different diets and similarly, that Ca^{2+}-ATPase is equally capable of sequestering calcium following contraction. Other studies have suggested that membranes with increased levels of polyunsaturated fatty acids are more "leaky" to sodium and potassium (Hulbert & Else, 1989). The sodium and calcium pump systems may not be affected, but the rate of ion transport may be altered. To learn more about the dynamics of the system, it would be necessary to measure circulating levels of K^+ during exercise (e.g. for whole

exercising limbs in humans or by monitoring the concentration of venous and arterial K^+ in isolated muscles with intact blood flow), as well as measuring the rate of calcium uptake by the sarcoplasmic reticulum.

6.4 Dietary n - 3 Polyunsaturated Fatty Acids and Performance

Although a diet enriched with n - 3 fatty acids is likely be favoured for its beneficial effects on heart disease and thromobosis, the results suggest that they are of no benefit during prolonged exercise (and may in fact be detrimental). In other studies, fish oil and fish diets have been fed to subjects and have had no effect on aerobic power (VO_{2max}) (Warner *et al.*, 1989; Brilla & Landerholm; 1990; Ågren *et al.*,1991). Although these other studies have used very different levels of n - 3 fatty acids (0.25 ml.kg.day; 57 mg.kg.day and 12.8 mg.kg.day, respectively, for an average 70kg person), their findings support the findings in this study for oxygen consumption during peak exertion. Perhaps the level of fish oil used in this PUFA diet was too high. It may be that there was a toxic effect on other organ systems that counter-balanced any favourable effects on skeletal muscles. Although the n - 3 fatty acids have been used for the prevention of coronary heart disease, little research has focused on other general effects (for discussion, see Bourre *et al.*, 1993) but there have been no serious side-effects reported (Drevon, 1992). There are no comparable studies which have correlated membrane fatty acid analysis with physical performance in subjects whose diets are boosted with n - 3 fats. The recommended daily intake of about 2 g per day of very long chain n - 3 fatty acids (i.e. about 28 mg per kg body

weight per day) (Neutze & Starling, 1986; Gibson, 1988; Drevon, 1992) appears to be about 13 times less than the amount consumed by the rats in this study on the PUFA diet (about 375 mg per kg body weight per day). However, this difference is probably three times smaller when body size is taken into account (since the metabolic rate of rats is about four times greater than that of humans, Schmidt-Nielsen, 1975)). The results from dose-response studies are not clear but it appears that high doses of n - 3 fatty acids may cause unfavourable increases in low-density-lipoprotein cholesterol (e.g. Blonk *et al.*, 1990; Harris *et al.*, 1990).

An attempt was made to determine whether there were significant correlations between results from different parts of this study. However, due to various reasons, the only comparisons that could be made were between Na^+,K^+-ATPase concentration and isolated muscle function. Given the lack of variation seen in this study, the sample sizes needed for significant correlations would be enormous. However, parameters such as B_{max} & isolated muscle function parameters were extremely weakly correlated. For example, the correlation coefficient (r) between B_{max} and various isometric and fatigue tensions in soleus muscles ranged from 0.3 to 0.5.

Consistent findings throughout this study were significant litter effects. These may reflect important interactions between diet and parental genotype, but it should be emphasized that since it was normal practice to process only one litter each day for most assays, these effects may equally represent day to day variation in assay conditions. To really test for litter effects, members of litters should have been tested on different days. However, since the objective of

the study was to determine the effects of the diets, litters were tested together in order to minimise any variation between litter-mates.

6.5 Future Directions

In future studies of this kind, it would be important to examine the effects of changes in dietary fatty acids in rats whose mothers have also been subjected to the same dietary manipulation. This should provide more powerful tests of the onset and recovery of the effects of excesses or deficiencies of essential polyunsaturated fatty acids. A number of studies have investigated the changes that occur in essential fatty acids during development (for review, see Innis, 1991) and it appears that essential fatty acids are delivered preferentially in utero via the placenta and after birth via breast milk. Although Anderson *et al.* (1992) suggested that high levels of n - 3 fatty acids are retained in the brain and retina of chicks (where they perform vital functions (Neuringer *et al.*, 1988)), but not liver or serum, this study showed that they are retained in at least one peripheral tissue, skeletal muscle.

It is important for future research to involve dose-response studies of the effects of n - 3 fatty acids on, not only cardiovascular factors, but other systems of the body since they have such wide-ranging effects. Although they have been accepted as having beneficial ameliorative effects on the cardiovascular system, compared to the n - 6 fatty acids, it is clear that overdoses may causes harmful effects and it has now been shown by this study that large doses have huge detrimental effects on prolonged physical exercise in rats. Since reversal studies have shown that dietary n - 3 fatty acids may have lasting effects on the brain, even after dietary reversal (Anderson *et al.*, 1992), it will

also be important to find out how long-lasting the effects of n - 3 fatty acids are in humans as well as their long-term efficacy and toxicity. This study also has important implications in the area of maternal - fetal nutrition. Research has shown that n - 6 and n - 3 fatty acids are transferred to fetuses via the placenta (for review, see Innis, 1991) so the rats in this study were probably not as deficient in essential fatty acids as a subject suffering from severe malnutrition. To really examine the effects of essential fatty acid deficiency in young rats, it would be necessary to start rats on an EFAD diet at least one generation prior to the production of offspring.

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Appendix 1: Data and analysis for Chapter 2

PUFA Trials - Table 1

Mean rat weights after

(i) two weeks on diets containing 2%, 3% and 5% MaxEPA oil and 10% sesame oil (Control diet) and

(ii) six weeks on diets containing 2% and 3% MaxEPA oil and 10% sesame oil (Control diet).

The accompanying ANOVA details are for the PUFA diets only.

EFAD Trials - Table 2

(i) Food intake and

(ii) Rat weights

after six weeks on a Control diet (containing 10% sesame oil), an EFAD diet (containing 10% stearic acid) and a PUFA diet (containing 3% sesame oil and 7% MaxEPA oil).

Since the food intake of the EFAD rats was significantly greater and their body weight was significantly less than the other two groups after six weeks, the EFAD diet was changed to contain 10% coconut oil which had no effect on food intake or weight gain (see Figures 2.4 and 2.5).

Dietary Trials

Analysis of Variance Summary

(1-way)

PUFA Diets after 2 wks (2%, 3% & 5% Max EPA oil)

Weight	2 wks
2%	264 ± 8
3%	263 ± 7
5%	196 ± 15
Control	275 ± 6

Source of Variation	SS	DF	MS	F	P
Total	13154	16			
Diet	6798	2	3399	7.50	< 0.01
Litter	6356	14	454		

PUFA Diets after six weeks (2% & 3% MaxEPA oil)

Weight	6 wks
2%	418 ± 9
3%	416 ± 9
Control	416 ± 8

Source of Variation	SS	DF	MS	F	P
Total	5114	11			
Diet	18	1	18	0.03	> 0.5
Litter	5096	10	510		

Table 1

Dietary Trials

Analysis of Variance Summary
(2-way)

EFAD Diets (Food Intake with Stearic Acid)

Intake	9 wks	Source of Variation	SS	DF	MS	F	P
		Total	177170	21			
Control	863.4 ± 0.1	**Diet**	125892	2	62946	23.30	
EFAD	1000.7 ± 0.2	**Litter**	51278	19	2699	> 0.5	
PUFA	896.3 ± 0.1						

EFAD Diets (Rat Weight with Stearic Acid)

Weight	6 wks	Source of Variation	SS	DF	MS	F	p
		Total	28620	41			
Control	416 ± 8	**Diet**	17530	2	8765	12.5	< 0.001
EFAD	366 ± 7	**Litter**	11090	13	853	1.2	> 0.5
PUFA	429 ± 7	**Remainder**	18289	26	703		

Table 2

Appendix 2: Data and analysis for Chapter 3

Mean values for each parameter tested in isolated muscles after nine weeks on the test diets followed by zero, two or six weeks recovery and accompanying ANOVA details for the nine week data.

Details for soleus muscles are presented first (Tables 3a - 3i), followed by details for EDL muscles (Tables 4a - 4h).

ANOVA details for the two week and six week recovery periods are presented in Tables 5a - 5c.

ANOVA details for all data adjusted for muscle weight and muscle cross-sectional surface area are presented in Tables 6 and 7, respectively, for soleus and in Tables 8 and 9, respectively, for EDL.

Soleus Analysis of Variance Summary

Rat Weight (g)

Rat Wt	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	20216	20			
Control	413 ± 16	454 ± 13	425 ± 17	Diet	2988	2	1494	2.96	< 0.1
EFAD	390 ± 9	438 ± 14	438 ± 13	Litter	11180	6	1863	3.70	< 0.05
PUFA	417 ± 8	465 ± 8	433 ± 17	Remainder	6048	12	504		

Muscle Weight (mg)

Musc Wt	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.00849	20			
Control	190 ± 10	197 ± 7	196 ± 8	Diet	0.00049	2	0.0002	0.59	> 0.5
EFAD	179 ± 6	201 ± 7	202 ± 9	Litter	0.003	6	0.0005	1.20	> 0.5
PUFA	183± 7	203 ± 8	198 ± 10	Remainder	0.005	12	0.0004		

Length (cm)

Length	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.308	20			
Control	2.81±0.06	2.83±0.06	2.96±0.10	Diet	0.017	2	0.01	0.51	> 0.5
EFAD	2.75±0.03	2.94±0.07	2.89±0.07	Litter	0.09	6	0.02	0.90	> 0.5
PUFA	2.82±0.04	2.97±0.07	2.74±0.17	Remainder	0.201	12	0.02		

Table 3a

Soleus Analysis of Variance Summary

Cross-sectional Surface Area (mm-2)

CSSA	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.00148	20			
Control	68 ± 4	65 ± 2	65 ± 2	Diet	4E-05	2	0.0000	0.24	> 0.5
EFAD	70 ± 3	68 ± 3	68 ± 2	Litter	0.00044	6	0.0001	0.88	> 0.5
PUFA	66 ± 3	70 ± 3	76 ± 10	Remainder	0.001	12	0.0001		

Table 3 b

Soleus

Analysis of Variance Summary

Peak Tension (Pt) (g)

Pt	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	495.65	20			
Control	30.5 ± 1.7	31.8 ± 1.9	32.2 ± 2.0	Diet	151.65	2	75.83	17.02	< 0.001
EFAD	24.7 ± 1.6	31.7 ± 0.8	31.1 ± 1.6	Litter	290.53	6	48.42	10.87	< 0.001
PUFA	31.0 ± 1.6	30.2 ± 1.5	30.7 ± 1.3	Remainder	53.47	12	4.46		

Latent Time (T L) (ms)

T L	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	146.828	20			
Control	10.9 ± 0.8	12.2 ± 0.4	11.2 ± 1.0	Diet	7.639	2	3.82	2.15	< 0.5
EFAD	10.3 ± 1.2	12.3 ± 0.5	10.1 ± 0.8	Litter	117.845	6	19.64	11.04	< 0.001
PUFA	9.4 ± 1.1	11.5 ± 1.2	10.8 ± 1.2	Remainder	21.344	12	1.78		

Contraction Time (Tc) (ms)

Tc	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	195.244	20			
Control	63.6 ± 1.1	67.0 ± 1.2	67.5 ± 2.4	Diet	45.568	2	22.78	9.37	< 0.01
EFAD	60.2 ± 1.0	67.1 ± 0.8	67.3 ± 1.3	Litter	120.501	6	20.08	8.26	< 0.005
PUFA	63.1 ± 1.2	67.7 ± 1.2	66.8 ± 0.9	Remainder	29.175	12	2.43		

Table 3 c

Soleus

Analysis of Variance Summary

Half-Relaxation Time (T 1/2 R) (ms)

T 1/2 R	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	232.99	20			
Control	53.3 ± 0.9	56.4 ± 1.8	59.1 ± 2.0	Diet	55.4	2	27.70	10.20	< 0.01
EFAD	50.7 ± 1.5	57.1 ± 1.5	58.0 ± 1.0	Litter	145	6	24.17	8.90	< 0.002
PUFA	54.6 ± 1.2	53.4 ± 1.1	58.2 ± 1.5	Remainder	32.59	12	2.72		

Table 3 d

Soleus — Analysis of Variance Summary

Maximum Tetanic Tension (Po) (g)

Po	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	4133	19			
Control	183 ± 5	191 ± 7	192 ± 9	Diet	1534	2	767.00	5.70	< 0.05
EFAD	160 ± 6	190 ± 5	187 ± 6	Litter	983	6	163.83	1.22	> 0.5
PUFA	177 ± 3	191 ± 3	185 ± 8	Remainder	1616	12	134.67		

Twitch tension : Tetanic Tension (Pt : Po)

Pt : Po	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.0096	19			
Control	0.164±0.009	0.166±0.006	0.168±0.005	Diet	0.0017	2	0.00	5.10	0.05
EFAD	0.153±0.007	0.168±0.004	0.167±0.008	Litter	0.0059	6	0.00	5.90	< 0.01
PUFA	0.175±0.009	0.158±0.008	0.166±0.005	Remainder	0.002	12	0.00		

Tetanic RelaxationTime (TRT) (ms)

TRT	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	7887	19			
Control	166 ± 10	161 ± 9	166 ± 13	Diet	440	2	220.00	1.44	> 0.5
EFAD	140 ± 8	166 ± 15	164 ± 13	Litter	5619	6	936.50	6.15	< 0.01
PUFA	151 ± 8	165 ± 10	154 ± 13	Remainder	1828	12	152.33		

Table 3 e

Soleus Analysis of Variance Summary

Peak Tension during High Frequency Stimulation (short pulses) (HF PT 1) (g)

HF Pt 1	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	25283	20			
Control	58 ± 14	86 ± 17	67 ± 9	Diet	3176	2	1588.00	1.16	> 0.5
EFAD	89 ± 14	99 ± 17	82 ± 21	Litter	5651	6	941.83	0.69	> 0.5
PUFA	65 ± 12	96 ± 19	96 ± 21	Remainder	16456	12	1371.33		

Peak Tension during High Frequency Stimulation (long pulses) (HF PT 2) (g)

HF Pt 2	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	8924	20			
Control	193 ± 7	184 ± 9	202 ± 11	Diet	3859	2	1929.50	5.50	< 0.05
EFAD	156 ± 8	182 ± 6	186 ± 4	Litter	852	6	142.00	0.40	> 0.5
PUFA	172 ± 3	193 ± 6	189 ± 8	Remainder	4213	12	351.08		

Fatigue Time to Half-Peak Tension during High Frequency Stimulation (short pulses) (HF T 1) (s)

HF T 1	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	48501688	20			
Control	4.1 ± 1.4	3.0 ± 0.3	2.4 ± 0.3	Diet	2660744	2	1330372	0.42	> 0.5
EFAD	2.0 ± 0.4	2.6 ± 0.3	3.5 ± 0.9	Litter	7806662	6	1301110	0.41	> 0.5
PUFA	2.5 ± 0.3	3.5 ± 0.5	4.5 ± 1.4	Remainder	38034282	12	3169524		

Table 3 f

Soleus Analysis of Variance Summary

Fatigue Time to Half-Peak Tension during High Frequency Stimulation (long pulses) (HF T 2) (s)

	Test Diet	Recovery		Source of					
HF T 2	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	49596986	20			
Control	6.6 ± 0.5	6.4 ± 0.7	8.6 ± 0.8	Diet	979450	2	489725	0.24	> 0.5
EFAD	6.7 ± 0.8	8.0 ± 0.9	9.6 ± 1.7	Litter	24332602	6	4055434	2.00	< 0.5
PUFA	6.2 ± 0.5	10.1 ± 1.4	9.5 ± 1.4	Remainder	24284934	12	2023745		

Table 3 g

Soleus Analysis of Variance Summary

Peak Tension during Low Frequency Stimulation (LF Pt) (g)

	Test Diet	Recovery		Source of					
LF Pt	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	10155	20			
Control	131 ± 8	120 ± 6	144 ± 9	Diet	5118	2	2559.00	10.69	< 0.005
EFAD	91 ± 7	108 ± 5	120 ± 5	Litter	2522	6	420.33	1.76	< 0.5
PUFA	121 ± 3	117 ± 11	124 ± 11	Remainder	2872	12	239.33		

Fatigue Time to Half-Peak Tension during Low Frequency Stimulation (LF T) (s)

	Test Diet	Recovery		Source of					
LF T	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	2321	20			
Control	45.2 ± 2.6	40.1 ± 2.1	40.9 ± 2.1	Diet	256	2	128	1.37	< 0.5
EFAD	40.5 ± 3.3	47.1 ± 4.0	46.2 ± 3.1	Litter	946	6	158	1.69	< 0.5
PUFA	49.0 ± 5.6	48.1 ± 3.4	47.4 ± 3.0	Remainder	1119	12	93		

Total Tension Fatigue Index (TTFI)

	Test Diet	Recovery		Source of					
TTFI	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	0.037	20			
Control	0.24 ± 0.02	0.24 ± 0.03	0.30 ± 0.03	Diet	0.007	2	0.0035	3.23	< 0.2
EFAD	0.24 ± 0.02	0.31 ± 0.04	0.34 ± 0.02	Litter	0.017	6	0.0028	2.62	< 0.2
PUFA	0.28 ± 0.01	0.37 ± 0.07	0.36 ± 0.02	Remainder	0.013	12	0.0011		

Table 3 h

Soleus Analysis of Variance Summary

Active Tension Fatigue Index (ATFI)

ATFI	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.013	20			
Control	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	Diet	0.001	2	0.0005	1.20	> 0.5
EFAD	0.16 ± 0.01	0.15 ± 0.02	0.15 ± 0.01	Litter	0.007	6	0.0012	2.80	< 0.2
PUFA	0.15 ± 0.01	0.14 ± 0.01	0.18 ± 0.003	Remainder	0.005	12	0.0004		

Table 3 i

EDL Analysis of Variance Summary

Muscle Weight (mg)

Rat Wt	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.00306	20			
Control	188 ± 6	203 ± 7	187 ± 11	Diet	5.9E-05	2	0.0000	0.18	> 0.5
EFAD	185 ± 6	183 ± 7	192 ± 1	Litter	0.001	6	0.0002	1.00	> 0.5
PUFA	189 ± 1	203 ± 4	202 ± 7	Remainder	0.002	12	0.0002		

Length (cm)

Musc Wt	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.546	20			
Control	3.34±0.04	3.43±0.06	3.47±0.11	Diet	0.094	2	0.0470	1.77	< 0.5
EFAD	3.43±0.04	3.45±0.06	3.46±0.06	Litter	0.134	6	0.0223	0.84	> 0.5
PUFA	3.28±0.10	3.46±0.05	3.58±0.10	Remainder	0.318	12	0.0265		

Cross-sectional Surface Area (mm-2)

CSSA	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.00045	20			
Control	56 ± 2	53 ± 1	58 ± 2	Diet	5.3E-05	2	0.0000	1.06	> 0.5
EFAD	59 ± 3	54 ± 2	58 ± 1	Litter	0.0001	6	0.0000	0.67	> 0.5
PUFA	54 ± 2	55 ± 1	58 ± 2	Remainder	0.0003	12	0.0000		

Table 4 a

EDL Analysis of Variance Summary

Peak Tension (Pt) (g)

Pt	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	600.1	18			
Control	38.9 ± 2.4	39.9 ± 1.8	38.4 ± 1.6	Diet	147.5	2	73.75	4.05	< 0.1
EFAD	32.5 ± 1.8	35.0 ± 1.6	37.1 ± 1.2	Litter	234.2	6	39.03	2.14	< 0.5
PUFA	35.3 ± 1.7	36.9 ± 1.0	39.2 ± 0.6	Remainder	218.4	12	18.20		

Latent Time (T L) (ms)

T L	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	81.905	18			
Control	7.1 ± 0.9	8.8 ± 0.4	7.5 ± 0.5	Diet	1.783	2	0.89	0.33	> 0.5
EFAD	6.4 ± 0.9	8.4 ± 0.4	7.0 ± 0.8	Litter	47.952	6	7.99	2.98	< 0.2
PUFA	5.3 ± 1.2	8.1 ± 0.5	6.5± 0.7	Remainder	32.17	12	2.68		

Contraction Time (Tc) (ms)

Tc	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	112.697	18			
Control	37.4 ± 1.1	39.2 ± 0.6	37.9 ± 0.8	Diet	2.764	2	1.38	0.66	> 0.5
EFAD	36.0 ± 1.1	38.6 ± 0.4	36.0 ± 0.9	Litter	84.821	6	14.14	6.76	< 0.01
PUFA	34.2 ± 1.2	38.3 ± 0.7	36.7 ± 1.0	Remainder	25.112	12	2.09		

Table 4 b

EDL Analysis of Variance Summary

Half-Relaxation Time (T 1/2 R) (ms)

	Test Diet	Recovery		Source of					
T 1/2 R	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	10.42	18			
Control	27.3 ± 0.2	27.4 ± 0.3	27.1 ± 0.2	Diet	2.09	2	1.05	4.64	< 0.1
EFAD	27.0 ± 0.2	27.3 ± 0.2	26.8 ± 0.2	Litter	5.63	6	0.94	4.17	< 0.05
PUFA	26.3 ± 0.3	27.4 ± 0.1	26.8 ± 0.2	Remainder	2.7	12	0.23		

Post-Tetanic Potentiation

	Test Diet	Recovery		Source of					
PTP	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	0.089	18			
Control	1.23±0.02	1.26±0.04	1.28±0.02	Diet	0.008	2	0.0040	1.55	0.5
EFAD	1.27±0.02	1.27±0.01	1.03±0.02	Litter	0.05	6	0.0083	3.23	< 0.1
PUFA	1.30±0.05	1.28±0.02	1.29±0.02	Remainder	0.031	12	0.0026		

Table 4 c

EDL Analysis of Variance Summary

Maximum Tetanic Tension (Po) (g)

Po	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	7434	18			
Control	272 ± 4	286 ± 6	281 ± 15	Diet	2498	2	1249.00	4.46	< 0.1
EFAD	244 ± 6	253 ± 15	282 ± 7	Litter	1572	6	262.00	0.93	> 0.5
PUFA	257 ± 12	289 ± 3	310 ± 4	Remainder	3364	12	280.33		

Twitch tension : Tetanic Tension (Pt : Po)

Pt : Po	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.0087	18			
Control	0.143±0.008	0.143±0.004	0.137±0.004	Diet	0.0003	2	0.0002	0.36	> 0.5
EFAD	0.133±0.006	0.142±0.004	0.131±0.003	Litter	0.0034	6	0.0006	1.36	> 0.5
PUFA	0.138±0.006	0.128±0.004	0.126±0.008	Remainder	0.005	12	0.0004		

Tetanic Relaxation Time (TRT) (ms)

TRT	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	61165	18			
Control	265 ± 13	258 ± 15	278 ± 9	Diet	14754	2	7377	3.45	< 0.2
EFAD	316 ± 9	291 ± 14	285 ± 12	Litter	20747	6	3458	1.62	< 0.5
PUFA	326 ± 30	276 ± 26	266 ± 17	Remainder	25664	12	2139		

Table 4 d

EDL Analysis of Variance Summary

Peak Tension during High Frequency Stimulation (short pulses) (HF PT 1) (g)

	Test Diet	Recovery		Source of					
HF Pt 1	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	12898	18			
Control	44 ± 7	44 ± 9	30 ± 5	Diet	1472	2	736.00	1.91	< 0.5
EFAD	34 ± 5	32 ± 8	55 ± 8	Litter	6804	6	1134.00	2.94	< 0.2
PUFA	63 ± 21	85 ± 12	72 ± 13	Remainder	4622	12	385.17		

Peak Tension during High Frequency Stimulation (long pulses) (HF PT 2) (g)

	Test Diet	Recovery		Source of					
HF Pt 2	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	12271	18			
Control	229 ± 4	227 ± 6	231 ± 13	Diet	4194	2	2097.00	5.67	< 0.05
EFAD	195 ± 10	204 ± 14	233 ± 5	Litter	3639	6	606.50	1.64	< 0.5
PUFA	207 ± 12	241 ± 15	255 ± 5	Remainder	4438	12	369.83		

Fatigue Time to Half-Peak Tension during High Frequency Stimulation (short pulses) (HF T 1) (ms)

	Test Diet	Recovery		Source of					
HF T 1	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	384338	18			
Control	275 ± 68	280 ± 59	240 ± 66	Diet	78274	2	39137	3.35	< 0.2
EFAD	133 ± 16	323 ± 98	182 ± 23	Litter	165711	6	27619	2.36	< 0.2
PUFA	252 ± 88	214 ± 35	126 ± 22	Remainder	140353	12	11696		

Table 4 e

EDL Analysis of Variance Summary

Fatigue Time to Half-Peak Tension during High Frequency Stimulation (long pulses) (HF T 2) (s)

	Test Diet	Recovery		Source of					
HF T 2	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	4472053	18			
Control	2.4 ± 0.2	2.1 ± 0.2	2.4 ± 0.1	Diet	2294395	2	1147198	8.58	< 0.01
EFAD	1.6 ± 0.7	2.1 ± 0.2	2.30±0.09	Litter	573181	6	95530	0.71	> 0.5
PUFA	2.4 ± 0.7	2.3 ± 0.2	2.6 ± 0.2	Remainder	1604477	12	133706		

Table 4 f

EDL Analysis of Variance Summary

Peak Tension during Low Frequency Stimulation (LF Pt) (g)

	Test Diet	Recovery		Source of					
LF Pt	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	10155	18			
Control	107 ± 6	110 ± 10	116 ± 6	Diet	1998	2	999.00	5.52	< 0.05
EFAD	92 ± 7	90 ± 8	113 ± 6	Litter	2577	6	429.50	2.37	< 0.2
PUFA	88 ± 9	98 ± 12	122 ± 4	Remainder	2171	12	180.92		

Fatigue Time to Half-Peak Tension during Low Frequency Stimulation (LF T) (s)

	Test Diet	Recovery		Source of					
LF T	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	225.8	18			
Control	19.6 ± 1.3	21.9 ± 0.8	21.3 ± 0.6	Diet	69.6	2	34.80	7.68	< 0.02
EFAD	23.6 ± 1.3	22.7 ± 1.4	21.5 ± 0.6	Litter	101.8	6	16.97	3.74	< 0.05
PUFA	22.6 ± 1.2	23.9 ± 1.5	20.3 ± 0.7	Remainder	54.4	12	4.53		

Total Tension Fatigue Index (TTFI)

	Test Diet	Recovery		Source of					
TTFI	9 wks	2 wks	6 wks	Variation	SS	DF	MS	F	P
				Total	0.00074	18			
Control	0.036±0.003	0.048±0.005	0.043±0.003	Diet	1.2E-05	2	0.0000	0.22	> 0.5
EFAD	0.037±0.003	0.044±0.004	0.048±0.003	Litter	0.00041	6	0.0001	2.53	< 0.2
PUFA	0.044±0.004	0.054±0.004	0.038±0.004	Remainder	0.00032	12	0.0000		

Table 4 g

EDL Analysis of Variance Summary

Active Tension Fatigue Index (ATFI)

ATFI	Test Diet 9 wks	Recovery 2 wks	6 wks	Source of Variation	SS	DF	MS	F	P
				Total	0.00201	18			
Control	0.040±0.003	0.046±0.005	0.042±0.002	Diet	5.7E-06	2	0.0000	0.03	> 0.5
EFAD	0.038±0.004	0.043±.004	0.042±0.002	Litter	0.001	6	0.0002	2.00	< 0.5
PUFA	0.047±0.004	0.052±0.004	0.037±0.004	Remainder	0.001	12	0.0001		

Table 4 h

Table 5 a

	SOLEUS				EDL			
	Recovery 2 wks		6 wks		Recovery 2 wks		6 wks	
	F	P	F	P	F	P	F	P
Morphology								
Rat Weight								
Diet	2.13	< 0.1	0.12	> 0.5	1.20	> 0.5	0.17	> 0.5
Litter	3.09	< 0.1	1.43	> 0.5	2.83	< 0.2	0.99	> 0.5
Muscle Weight								
Diet	0.12	> 0.5	0.05	> 0.5	2.07	< 0.5	1.57	< 0.5
Litter	0.37	> 0.5	1.25	> 0.5	0.64	> 0.5	1.70	< 0.5
Muscle Length								
Diet	0.92	> 0.5	3.13	< 0.2	0.30	> 0.5	0.49	> 0.5
Litter	0.49	> 0.5	2.47	< 0.2	2.48	< 0.2	4.39	<0.05
Cross-sectional Surface Area								
Diet	0.12	> 0.5	0.43	> 0.5	1.93	< 0.5	1.47	> 0.5
Litter	0.83	> 0.5	0.43	> 0.5	0.95	> 0.5	0.71	> 0.5
Twitch								
Peak Tension								
Diet	0.32	> 0.5	0.35	> 0.5	10.69	<0.005	1.01	>0.5
Litter	0.33	> 0.5	1.44	> 0.5	4.21	> 0.05	1.4> 0.5	
Latent Time								
Diet	0.28	> 0.5	1.87	< 0.5	0.26	> 0.5	2.30	< 0.5
Litter	0.56	> 0.5	18.31	< 0.001	2.72	< 0.2	38.81	<0.0001
Contraction Time								
Diet	0.11	> 0.5	0.03	> 0.5	0.24	> 0.5	3.53	< 0.2
Litter	1.19	> 0.5	4.17	< 0.05	3.37	< 0.1	14.82	<0.0001
Half-Relaxation Time								
Diet	1.94	< 0.5	1.551	> 0.5	0.14	> 0.5	1.01	> 0.5
Litter	0.78	> 0.5	0.92	> 0.5	1.54	> 0.5	0.61	> 0.5

Table 5 b

	SOLEUS				EDL			
	Recovery				Recovery			
	2 wks		6 wks		2 wks		6 wks	
	F	P	F	P	F	P	F	P
Tetanus								
Peak Tension								
Diet	0.01	> 0.5	0.58	> 0.5	5.12	< 0.05	3.15	< 0.2
Litter	1.28	> 0.5	3.12	< 0.1	1.48	> 0.5	2.17	< 0.5
Ratio of Twitch Peak Tension to Tetanic Peak Tension								
Diet	0.45	> 0.5	0.40	> 0.5	4.05	< 0.1	0.77	> 0.5
Litter	0.40	> 0.5	0.47	> 0.5	1.56	< 0.5	1.88	< 0.5
Tetanic Relaxation Rate								
Diet	0.17	> 0.5	0.63	> 0.5	0.19	> 0.5	0.08	> 0.5
Litter	7.68	<0.005	6.89	< 0.01	1.19	> 0.5	0.56	> 0.5
Post-Tetanic Potentiation								
Diet					0.05	> 0.5	1.00	> 0.5
Litter								
High Frequency Stimulation								
Peak Tension (short pulses)								
Diet	1.06	> 0.5	0.33	> 0.5	7.73	< 0.02	7.2	< 0.02
Litter	17.73	<0.0001	1.72	< 0.5	0.69	> 0.5	1.49	> 0.5
Fatigue Time (short pulses)								
Diet	0.54	> 0.5	1.98	< 0.5	0.69	> 0.5	1.33	> 0.5
Litter	1.22	> 0.5	1.97	> 0.5	0.88	> 0.5	0.36	> 0.5
Peak Tension (long pulses)								
Diet	0.99	> 0.5	2.43	< 0.5	1.75	< 0.5	3.64	< 0.2
Litter	2.58	< 0.2	2.89	< 0.2	3.01	< 0.2	3.27	< 0.1
Fatigue Time (long pulses)								
Diet	4.80	< 0.1	3.49	< 0.2	0.60	> 0.5	1.64	< 0.5
Litter	1.13	> 0.5	7.40	< 0.005	1.18	> 0.5	1.76	< 0.5

Table 5 c

	SOLEUS				EDL			
	Recovery 2 wks		6 wks		Recovery 2 wks		6 wks	
	F	P	F	P	F	P	F	P
Low Frequency Stimulation								
Peak Tension								
Diet	0.66	> 0.5	0.46	> 0.5	2.13	< 0.5	0.45	> 0.5
Litter	0.80	> 0.5	0.87	> 0.5	2.00	< 0.5	1.22	> 0.5
Endurance								
Diet	2.14	< 0.5	3.30	< 0.2	0.79	> 0.5	0.97	> 0.5
Litter	0.61	> 0.5	2.43	< 0.2	0.80	> 0.5	0.41	> 0.5
Total Tension Fatigue Index								
Diet	2.82	< 0.2	2.0	< 0.5	1.28	> 0.5	0.73	> 0.5
Litter	2.91	< 0.2	1.67	< 0.5	1.00	> 0.5	1.93	< 0.5
Active Tension Fatigue Index								
Diet	2.00	< 0.5	4.92	< 0.001	3.08	< 0.2	0.00	> 0.5
Litter	1.00	> 0.5	2.49	< 0.2	3.34	< 0.1	4.23	< 0.05

Table 6

SOLEUS

	Test Diets 9 wks		Recovery 2 wks		6 wks	
	F	P	F	P	F	P
Normalized for Muscle Weight						
Peak Twitch Tension (P_t)						
Diet	9.49	< 0.01	0.31	> 0.5	0.73	> 0.5
Litter	7.33	< 0.005	0.54	> 0.5	0.39	> 0.5
Maximum Tetanic Tension (P_o)						
Diet	2.68	< 0.5	0.20	> 0.5	1.28	> 0.5
Litter	2.14	< 0.5	1.15	> 0.5	1.11	> 0.5
HFF Peak Tension during 0.02 ms pulses						
Diet	0.90	> 0.5	1.29	> 0.5	0.52	> 0.5
Litter	0.97	> 0.5	12.97	< 0.001	0.47	> 0.5
HFF Peak Tension during 0.2 ms pulses						
Diet	4.56	< 0.1	0.57	> 0.5	0.11	> 0.5
Litter	2.08	> 0.5	1.25	> 0.5	1.84	< 0.5
LFF Peak Tension						
Diet	8.41	< 0.02	0.27	> 0.5	1.86	< 0.5
Litter	3.35	< 0.1	0.67	> 0.5	0.16	> 0.5

Table 7

SOLEUS

	Test Diets 9 wks		Recovery 2 wks		6 wks	
	F	P	F	P	F	P
Normalized for Muscle CSSA						
Peak Twitch Tension (P_t)						
Diet	10.43	< 0.005	0.31	> 0.5	2.78	< 0.5
Litter	7.90	< 0.005	0.54	> 0.5	0.88	> 0.5
Maximum Tetanic Tension (P_o)						
Diet	1.86	< 0.5	0.20	> 0.5	1.45	> 0.5
Litter	1.75	< 0.5	1.15	> 0.5	1.96	< 0.5
HFF Peak Tension during 0.02 ms pulses						
Diet	1.26	> 0.5	1.28	> 0.5	0.32	> 0.5
Litter	1.00	> 0.5	12.98	< 0.001	1.11	> 0.5
HFF Peak Tension during 0.2 ms pulses						
Diet	4.00	< 0.1	0.57	> 0.5	2.07	< 0.5
Litter	1.88	> 0.5	1.25	> 0.5	0.88	> 0.5
LFF Peak Tension						
Diet	9.31	< 0.01	0.27	>0.5	5.38	< 0.05
Litter	3.86	0.05	0.67	> 0.5	2.87	< 0.2

Table 8

EDL

	Test Diets 9 wks		Recovery 2 wks		6 wks	
	F	P	F	P	F	P
Normalized for Muscle Weight						
Peak Twitch Tension (P_t)						
Diet	2.56	< 0.5	2.48	< 0.5	1.59	< 0.5
Litter	1.73	< 0.5	0.50	> 0.5	3.48	< 0.1
Maximum Tetanic Tension (P_o)						
Diet	1.26	> 0.5	0.31	> 0.5	1.59	< 0.5
Litter	0.92	> 0.5	0.48	> 0.5	6.58	< 0.01
HFF Peak Tension during 0.02 ms pulses						
Diet	1.53	> 0.5	3.16	< 0.2	8.44	< 0.02
Litter	3.00	0.1	1.36	> 0.5	2.17	< 0.5
HFF Peak Tension during 0.2 ms pulses						
Diet	2.74	< 0.5	0.38	> 0.5	1.61	< 0.5
Litter	1.79	< 0.5	0.94	> 0.5	5.34	< 0.02
LFF Peak Tension						
Diet	5.08	0.05	0.30	> 0.5	0.87	> 0.5
Litter	3.27	< 0.1	0.70	> 0.5	1.69	< 0.5

Table 9

EDL

	Test Diets 9 wks		Recovery 2 wks		6 wks	
	F	**P**	**F**	**P**	**F**	**P**
Normalized for Muscle CSSA						
Peak Twitch Tension (P_t)						
Diet	2.58	< 0.5	0.65	> 0.5	0.72	> 0.5
Litter	5.87	< 0.01	0.32	> 0.5	1.06	> 0.5
Maximum Tetanic Tension (P_o)						
Diet	0.23	> 0.5	0.23	> 0.5	10.79	< 0.05
Litter	0.41	> 0.5	0.41	> 0.5	1.57	> 0.5
HFF Peak Tension during 0.02 ms pulses						
Diet	0.78	> 0.5	4.32	< 0.1	4.39	< 0.2
Litter	1.05	> 0.5	0.50	> 0.5	0.74	> 0.5
HFF Peak Tension during 0.2 ms pulses						
Diet	2.44	< 0.5	0.67	> 0.5	3.44	0.2
Litter	1.52	> 0.5	1.43	> 0.5	1.20	> 0.5
LFF Peak Tension						
Diet	0.06	> 0.5	1.86	< 0.5	0.28	> 0.5
Litter	0.98	> 0.5	1.47	> 0.5	0.06	> 0.5

Appendix 3: Data and analysis for Chapter 4

Mean values for each test conducted in whole animals after nine weeks on the test diets followed by zero or six weeks recovery and accompanying ANOVA details.

Details for the nine week dietary test period are presented in Tables 10a and 10b and details for the six week recovery period are presented in Tables 11 a and 11b.

Test Diets

Analysis of Variance Summary

Grip Strength (g)

GS	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	413870	26			
Control	1646 ± 29	Diet	3292	2	1646	0.11	> 0.5
EFAD	1651 ± 40	Litter	174384	8	21798	1.48	< 0.5
PUFA	1691 ± 29	Remainder	236194	16	14762		

Grip Strength adjusted for Body Weight (g / g)

GS / g	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	3.79	26			
Control	4.3 ± 0.2	Diet	0.20	2	0.102	1.34	> 0.5
EFAD	4.4 ± 0.1	Litter	2.37	8	0.296	3.89	0.02
PUFA	4.1 ± 0.1	Remainder	1.22	16	0.076		

Basal Oxygen Consumption (ml oxygen / kg / min)

$\dot{V}O2$	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	216.3	32			
Control	14.0 ± 0.6	Diet	8.2	2	4.1000	0.54	> 0.5
EFAD	12.8 ± 0.9	Litter	56.3	10	5.6300	0.74	> 0.5
PUFA	13.6 ± 0.9	Remainder	151.8	20	7.5900		

Table 10 a

Test Diets — Analysis of Variance Summary

Peak Oxygen Consumption (ml oxygen / kg / min)

$\dot{V}O2$	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	542.8	26			
Control	44 ± 1	**Diet**	46.8	2	23.40	1.66	< 0.5
EFAD	46 ± 2	**Litter**	270.5	8	33.81	2.40	< 0.2
PUFA	43 ± 1	**Remainder**	225.5	16	14.09		

Ratio of Peak $\dot{V}O2$ to Basal $\dot{V}O2$

Ratio	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	15.352	26			
Control	3.4 ± 0.2	**Diet**	0.002	2	0.00	0.00	> 0.5
EFAD	3.6 ± 0.4	**Litter**	6.37	8	0.80	1.42	> 0.5
PUFA	3.4 ± 0.2	**Remainder**	8.98	16	0.56		

Endurance (mins)

End	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	1213	26			
Control	25 ± 1	**Diet**	241	2	120.50	6.45	< 0.02
EFAD	18 ± 2	**Litter**	673	8	84.13	4.50	< 0.02
PUFA	14 ± 2	**Remainder**	299	16	18.69		

Table 10 b

Recovery

Analysis of Variance Summary

Grip Strength (g)

GS	Recovery 6 wks	Source of Variation	SS	DF	MS	F	P
		Total	251529	26			
Control	1668 ± 34	Diet	4254	2	2127	0.26	> 0.5
EFAD	1639 ± 35	Litter	113951	8	14244	1.71	< 0.5
PUFA	1644 ± 32	Remainder	133324	16	8333		

Grip Strength adjusted for Body Weight (g / g)

GS / g	Recovery 6 wks	Source of Variation	SS	DF	MS	F	P
		Total	2.34	26			
Control	3.8 ± 0.1	Diet	0.24	2	0.120	1.54	< 0.5
EFAD	3.7 ± 0.1	Litter	0.85	8	0.106	1.36	> 0.5
PUFA	3.6 ± 0.1	Remainder	1.25	16	0.078		

Basal Oxygen Consumption (ml oxygen / kg / min)

$\dot{V}O_2$	Recovery 6 wks	Source of Variation	SS	DF	MS	F	P
		Total	252.5	32			
Control	13.0 ± 0.6	Diet	17.6	2	8.80	1.33	> 0.5
EFAD	13.3 ± 0.9	Litter	102.7	10	10.27	1.55	< 0.5
PUFA	11.6 ± 1.0	Remainder	132.2	20	6.61		

Table 11 a

Recovery — Analysis of Variance Summary

Peak Oxygen Consumption (ml oxygen / kg / min)

$\dot{V}O2$	Recovery 6 wks	Source of Variation	SS	DF	MS	F	P
		Total	589	26			
Control	38 ± 2	Diet	11	2	5.50	0.53	> 0.5
EFAD	38 ± 2	Litter	411	8	51.38	4.92	< 0.01
PUFA	36 ± 2	Remainder	167	16	10.44		

Ratio of Peak $\dot{V}O2$ to Basal $\dot{V}O2$

Ratio	Recovery 6 wks	Source of Variation	SS	DF	MS	F	P
		Total	11.36	26			
Control	3.2 ± 0.2	Diet	0.32	2	0.16	0.70	> 0.5
EFAD	3.2 ± 0.3	Litter	7.38	8	0.92	4.03	< 0.02
PUFA	3.4 ± 0.2	Remainder	3.66	16	0.23		

Endurance (mins)

End	Recovery 6 wks	Source of Variation	SS	DF	MS	F	P
		Total	479	23			
Control	13 ± 2	Diet	170	2	85.00	10.35	< 0.005
EFAD	9 ± 1	Litter	194	7	27.71	3.37	< 0.05
PUFA	6 ± 1	Remainder	115	14	8.21		

Table 11 b

Appendix 4: Data and analysis for Chapter 5

Mean values for biochemical assays conducted and accompanying statistical analysis details.

Details for Na^+,K^+-ATPase concentration in soleus muscles and EDL muscles are presented in Tables 12a and 12b, respectively. Results were analysed by the Kruskal-Wallis test.

Details for Na^+,K^+-ATPase activity in soleus muscles and EDL muscles are presented in Tables 13 and 14. Activity per mg tissue is presented in Table 13a (soleus) and 13b (EDL). Activity per mg protein is presented in Table 14a (soleus) and 14b (EDL). Results were analysed by 2-way ANOVA.

Details for Ca^{2+}-ATPase activity in soleus muscles and EDL muscles are presented in Tables 15 and 16, respectively. Results were analysed by 2-way ANOVA.

Soleus

Kruskal Wallis Summary

Sodium Pump Concentration (pmoles / g wet weight)
Maximum # of Binding Sites

Max BS	Test Diet 9 wks	# cases	# grps	DF	H	P
		29	3	2	1.8	> 0.1
Control	343 ± 21					
EFAD	259 ± 18					
PUFA	379 ± 39					

Sodium Pump Concentration (pmoles / g wet weight)
Binding Sites at 1 μM ouabain

1 μM BS	Test Diet 9 wks	# cases	# grps	DF	H	P
		29	3	2	3.11	> 0.1
Control	301 ± 12					
EFAD	345 ± 21					
PUFA	303 ± 25					

Affinity for Ouabain (K D)

K D	Test Diet 9 wks	# cases	# grps	DF	H	P
		29	3	2	6.74	< 0.02
Control	85 ± 12					
EFAD	128 ± 48					
PUFA	52 ± 10					

Table 12 a

EDL Kruskal Wallis Summary

Sodium Pump Concentration (pmoles / g wet weight)
Maximum # of Binding Sites

Max BS	Test Diet 9 wks	# cases	# grps	DF	H	P
		37	3.00	2	1.56	> 0.1
Control	438 ± 41					
EFAD	583 ± 76					
PUFA	507 ± 62					

Sodium Pump Concentration (pmoles / g wet weight)
Binding Sites at 1 μM ouabain

Activity	Test Diet 9 wks	# cases	# grps	DF	H	P
		37	3	2	1.02	> 0.1
Control	412 ± 42					
EFAD	555 ± 77					
PUFA	497 ± 80					

Affinity for Ouabain (K D)

Activity	Test Diet 9 wks	# cases	# grps	DF	H	P
		37	3	2	0.71	> 0.1
Control	137 ± 31					
EFAD	148 ± 61					
PUFA	113 ± 22					

Table 12 b

Soleus

Analysis of Variance Summary

Sodium Pump Activity (μmoles Pi/mg tissue) Homogenate

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.019	26			
Control	0.082 ± 0.006	Diet	0.001	2	0.0005	0.67	> 0.5
EFAD	0.066 ± 0.011	Litter	0.006	8	0.0008	1.00	> 0.5
PUFA	0.069 ± 0.008	Remainder	0.012	16	0.0008		

Sodium Pump Activity (μmoles Pi/mg tissue) Microsomes

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.152	26			
Control	0.24 ± 0.02	Diet	0.013	2	0.007	0.90	> 0.5
EFAD	0.19 ± 0.02	Litter	0.023	8	0.003	0.40	> 0.5
PUFA	0.20 ± 0.03	Remainder	0.116	16	0.007		

Table 13 a

EDL Analysis of Variance Summary

Sodium Pump Activity (µmoles Pi/mg tissue) Homogenate

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.032	26			
Control	0.066 ± 0.010	Diet	0.001	2	0.001	2.67	0.2
EFAD	0.078 ± 0.017	Litter	0.028	8	0.004	18.67	< 0.001
PUFA	0.078 ± 0.013	Remainder	0.003	16	0.000		

Sodium Pump Activity (µmoles Pi/mg tissue) Microsomes

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.372	26			
Control	0.17 ± 0.04	Diet	0.015	2	0.008	4.29	< 0.1
EFAD	0.23 ± 0.05	Litter	0.329	8	0.041	23.50	< 0.001
PUFA	0.22 ± 0.05	Remainder	0.028	16	0.002		

Table 13 b

Soleus

Analysis of Variance Summary

Sodium Pump Activity (µmoles Pi/mg protein) Homogenate

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.689	26			
Control	0.40 ± 0.03	**Diet**	0.03	2	0.015	0.51	> 0.5
EFAD	0.33 ± 0.06	**Litter**	0.189	8	0.024	0.80	> 0.5
PUFA	0.41 ± 0.07	**Remainder**	0.47	16	0.029		

Sodium Pump Activity (µmoles Pi/mg protein) Microsomes

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	6.636	26			
Control	1.21 ± 0.13	**Diet**	0.283	2	0.14	0.48	> 0.5
EFAD	0.99 ± 0.11	**Litter**	1.621	8	0.20	0.69	> 0.5
PUFA	1.19 ± 0.24	**Remainder**	4.732	16	0.30		

Table 14 a

EDL Analysis of Variance Summary

Sodium Pump Activity (μmoles Pi/mg protein) Homogenate

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.57	26			
Control	0.39 ± 0.04	Diet	0.006	2	0.0030	0.36	> 0.5
EFAD	0.40 ± 0.07	Litter	0.429	8	0.0536	6.36	< 0.002
PUFA	0.43 ± 0.05	Remainder	0.135	16	0.0084		

Sodium Pump Activity (μmoles Pi/mg protein) Microsomes

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	5.762	26			
Control	0.95 ± 0.14	Diet	0.204	2	0.102	2.11	< 0.5
EFAD	1.14 ± 0.22	Litter	4.786	8	0.598	12.40	< 0.001
PUFA	1.15 ± 0.18	Remainder	0.772	16	0.05		

Table 14 b

Soleus

Analysis of Variance Summary

Calcium Pump Activity (μmoles Pi/mg protein)

Activity	Test Diet 9 wks
Control	48 ± 6
EFAD	55 ± 5
PUFA	54 ± 6

Source of Variation	SS	DF	MS	F	P
Total	0.01	29			
Diet	0.001	2	0.0005	2.25	< 0.5
Litter	0.005	9	0.0006	2.50	0.1
Remainder	0.004	18	0.0002		

Calcium Pump Activity (μmoles Pi/mg tissue)

Activity	Test Diet 9 wks
Control	5.7 ± 0.7
EFAD	6.5 ± 0.6
PUFA	6.6 ± 0.7

Source of Variation	SS	DF	MS	F	P
Total	160.02	29			
Diet	9.237	2	4.62	1.34	> 0.5
Litter	88.963	9	9.88	2.88	< 0.1
Remainder	61.82	18	3.43		

Table 15

EDL Analysis of Variance Summary

Calcium Pump Activity (μmoles Pi/mg protein)

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	0.00645	29			
Control	42 ± 4	Diet	0.00045	2	0.0002	1.35	> 0.5
EFAD	47 ± 5	Litter	0.003	9	0.0003	2.00	< 0.5
PUFA	48 ± 6	Remainder	0.003	18	0.0002		

Calcium Pump Activity (μmoles Pi/mg tissue)

Activity	Test Diet 9 wks	Source of Variation	SS	DF	MS	F	P
		Total	72.153	29			
Control	5.6 ± 0.5	Diet	4.036	2	2.02	1.69	< 0.5
EFAD	5.4 ± 0.5	Litter	46.571	9	5.17	4.32	< 0.01
PUFA	5.9 ± 0.6	Remainder	21.546	18	1.20		

Table 16