Characterising in vivo cardiac function in the rat following supplementation of human dietary achievable fish oil doses

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CHARACTERISING IN VIVO CARDIAC FUNCTION IN THE RAT FOLLOWING SUPPLEMENTATION OF HUMAN DIETARY ACHIEVABLE FISH OIL DOSES

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Abbreviations commonly used

14:0  Myristic acid
16:0  Palmitic acid
16:1  Palmitoleic acid
18:0  Stearic acid
18:1n-9 Oleic acid
18:1n-7 Vaccenic acid
18:2n-6 Linoleic acid (LA)
18:3n-3 α-Linolenic acid (ALA)
20:4n-6 Arachidonic acid (AA)
20:5n-3 Eicosapentaenoic acid (EPA)
22:5n-3 Docosapentaenoic acid (DPA)
22:6n-3 Docosahexaenoic acid (DHA)
ATP  Adenosine triphosphate
CHD  Coronary heart disease
dP/dt\text{max} Maximum rate of force development
dP/dt\text{min} Maximum rate of relaxation
ECG  Electrocardiogram
ex \text{vivo} Experiment conducted on tissue outside of a living organism
FG   Fast glycolytic
FOG  Fast oxidative glycolytic
HRV  Heart rate variability
in \text{vivo} Experiment conducting within a living organism
LAD  Left anterior descending coronary artery
LC \text{n-3 PUFA} Long chain omega-3 fatty acids
MHC  Myosin heavy chain
MUFA Monounsaturated fatty acid
MVO\text{2} Myocardial oxygen consumption
n3:n6 ratio The ratio of phospholipid LC n-3 PUFA to n-6 PUFA
n-6 PUFA Omega-6 fatty acids
\text{Omega-3 index} percentage of total phospholipid fatty acids
PUFA Polyunsaturated fatty acid
PV   Pressure and volume
PVB  Premature ventricular beats
RCT  Random control trial
SCD  Sudden cardiac death
SERCA Sarcoplasmic reticulum Ca\text{2+}-ATPase
SFA  Saturated fatty acid
SO   Slow oxidative
SR   Sarcoplasmic reticulum
VF   Ventricular fibrillation
VT   Ventricular tachycardia
wt/wt Percentage of diet dry weight
ABSTRACT

**Background:** Regular fish consumption is consistently associated with a range of health benefits, particularly cardiovascular related. In excitable tissue such as skeletal and cardiac muscle, fish or fish oil (FO) intake increases membrane phospholipid concentration of long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA), especially docosahexaenoic acid (DHA). Of physiological consequence, skeletal muscle and cardiac function has been demonstrated to be modified, even during episodes of hypoxia/ischaemia. There is evidence that shows variation in the patterns by which DHA phospholipid incorporation occurs according to tissue type. However, little is known about whether incorporation of DHA is associated with specific physiological properties unique to the tissue. In addition, it is not known if physiological effects can be attributed to the supplementation of FO doses achievable within a typical Western-style human diet (high SFA and n-6 PUFA). Some physiological effects have been reported in skeletal muscle using human dietary achievable FO doses. The physiological effects demonstrated within the myocardium and pacemaker regions of the heart are postulated to underpin the cardiovascular health benefits of regular fish intake observed at a population level. However, previous studies have relied upon FO doses well above what could be achieved in the habitual human diet. This research therefore aimed, firstly, to characterise patterns of phospholipid DHA incorporation throughout muscle types with unique physiological properties, including ventricular myocardium, following supplementation of FO doses achievable within a typical Western-style human diet. Secondly, it aimed to relate ventricular myocardial phospholipid DHA concentrations to cardiac physiology at rest; and during ischaemia and reperfusion; that may explain the cardiovascular health benefits associated with regular fish consumption in humans.

**Methods:** The research commenced with a study which evaluated the optimal anaesthetic to use while characterising *in vivo* cardiac function in the rat using a Millar conductance catheter. The remainder of studies investigated the effect of supplementing FO to a control diet (10% fat wt/wt) devoid of LC n-3 PUFA and containing high amounts of SFA (5%), MUFA (2.5%) and n-6 PUFA (2.5%), typical of a Western-style diet in rats for 4 – 6 weeks. Dietary achievable FO doses (LowFO: 0.32% and ModFO: 1.25% wt/wt) were exchanged for MUFA in the control diet to ensure SFA and n-6
PUFA content remained high. Following dietary supplementation, cardiac function was assessed in vivo under pentobarbital anaesthetic (60mg/kg i.p), instrumented with lead-I ECG and a 6mm miniaturised 2-French pressure-volume conductance catheter. The heart rate, heart rate variability and ventricular haemodynamic function was evaluated: under resting conditions; during prolonged regional ischaemia; and the post-ischaemic recovery. Cardiac arrhythmias were evaluated from continuous ECG and aortic pressure recordings during: 15 min regional ischaemia; and 15 min reperfusion. Regional ischaemia was achieved by surgical occlusion of the left anterior descending coronary artery with reperfusion induced by release of the occlusive snare. Tissue was collected at completion of cardiac haemodynamic studies for morphological measurements and the analysis of phospholipid fatty acid composition via gas chromatography.

**Results:** Relative fatty acid phospholipid concentrations of LC n-3 PUFA, particularly DHA was higher in myocardial (Control: 5.0 ±0.2%; LowFO: 13.1 ±0.9%; ModFO: 18.3 ±0.4%, \(p <0.001\)) and skeletal muscles following supplementation with both doses of FO. Across five different hind-limb muscles within the rat, selected for their unique fibre type proportions, DHA preferentially incorporated in relation to the proportion of fast oxidative glycolytic (FOG) fibre type (Control soleus DHA: 8.3 ±0.1%, proportion of soleus FOG fibre type: 13%; Control gastrocnemius(red) DHA: 12.6 ±0.2%, proportion of FOG fibre type: 62%; \(\rho = 0.94, r^2 = 0.89\)). Phospholipid concentrations of DHA were further increased with FO feeding in a dose-dependent manner across all muscle types (ModFO soleus DHA: 18.9 ±0.7%; ModFO gastrocnemius(red) DHA: 23.0 ±0.5%). Supplementation of FO was associated with slower resting heart rate in a dose-related manner (Control: 453 ±6, LowFO: 433 ±4, ModFO: 402 ±5 beats.min\(^{-1}\), \(p <0.01\)) with no compromise to cardiac output and without change in time or frequency domains of heart rate variability. During 15 min regional ischaemia, the ModFO dose reduced the incidence and severity of arrhythmias, including reduced cases of fatal ventricular fibrillation (Fatal VF: Control: 61%, LowFO: 40%, ModFO: 25%, \(p <0.05\)). During 15 min reperfusion, the incidence and severity of arrhythmias was reduced by both FO doses, with few statistical differences between FO doses. Prolonged ischaemia-induced reductions in rate pressure product (indirect index of myocardial oxygen consumption) recovered faster in the ModFO group and post-ischaemic left ventricular pressure-volume loop integrity (shifted downwards and right with reduced ejection fraction in Control) was maintained in both FO groups (\(\Delta\) in post-ischaemic ejection
fraction: Control: -18% LowFO: -11% ModFO: -10%, p <0.05). Ischaemic zone at risk was not statistically different between groups, indicating an equivalent ischaemic insult.

**Discussion and conclusions:** This research establishes that supplementing FO doses achievable within a typical Western-style human diet produces robust and preferential fibre type dependent incorporation of DHA into excitable membranes of skeletal muscles and the heart. Of physiological consequence, resting heart rate was slowed, hearts remained in normal sinus rhythm for longer during an arrhythmogenic stimulus and heart function was controlled during ischaemia to ensure maintenance of mean arterial pressure. These effects occurred in the absence of any well-defined changes in heart rate variability, indicative that the autonomic nervous system was not involved. Rather, it appears that FO facilitates adaptations in the intrinsic control of the heart to provide cardiac nutritional pre-conditioning, similar to ischaemic pre-conditioning and exercise training. The physiological changes described in the heart were observed, for the first time, after supplementing FO doses achievable within a typical Western-style human diet (Human equivalence: LowFO ≈ EPA+DHA 570mg/d or ≈ 2 salmon [100g] serve/week; ModFO ≈ EPA+DHA 2.3g/d or ≈ 6 capsules per/day). The LowFO dose appears to be close to the threshold required for improving ischaemic heart function. Whereas, during reperfusion both FO doses were almost equally effective, particularly in relation to arrhythmia based outcomes, suggesting that the threshold for effectiveness may be even lower. This research provides evidence to rationalise how increasing dietary FO intake in a typical Western-style diet high in SFA and n-6 PUFA offers the potential to slow resting heart rate, prevent sudden (arrhythmic) cardiac death and attenuate the decline of post-ischaemic myocardial function leading to heart failure. Consequently, increasing fish or FO intake in the Western diet offers a safe and effective tactic for improving heart health and reducing the risk of cardiovascular disease.
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Michael
Abstracts arising from this thesis

Michael J. Macartney, Gregory E. Peoples, Peter L. McLennan, (2017) “Cardiac in vivo haemodynamic function is modified by myocardial membrane DHA incorporation attributable to fish oil doses achievable in the human diet.” Presented at AuPS: (Australian Physiological Society), Melbourne, Australia.

Michael J. Macartney, Gregory E. Peoples, Peter L. McLennan, (2017) “Myocardial membrane is modified and ischaemic arrhythmias are prevented by low fish oil dosing in the rat, achievable in a usual human diet.” Presented at Euro-Prevent: (European Association for Cardiovascular Prevention and Rehabilitation (EACPR)), Malaga, Spain.


CHAPTER 1

Introduction
1.1 INTRODUCTION

1.1.1 Background

According to the World Health Organisation approximately 38 million deaths worldwide each year are caused by non-communicable or ‘chronic disease’ and this number is disproportionately increased in populations with low to middle socioeconomic status. Lifestyle factors such as dietary patterns have been identified to place substantial influence on mortality and morbidity rates of chronic disease. Dietary fish oil (FO) is a rich source of poly-unsaturated fatty acids (PUFA), in particular, it contains high levels of long chain omega-3 PUFA (LC n-3 PUFA) which is a subclass of PUFA, with the other being omega-6 (n-6 PUFA). Recently, blood levels of LC n-3 PUFA, specifically eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) have been demonstrated to be in the very low or low range in populations investigated across the globe (Stark et al., 2016). This is alarming given that decades of evidence from numerous epidemiological, clinical, cohort, observational and animal studies demonstrate dietary intake of fish or FO to be closely associated with a significant number of health benefits and decreased risk of chronic disease.

Health benefits of FO include: improving brain development and functioning; mental health outcomes; attenuating cognitive decline; and beneficial effects on metabolic syndromes, dyslipidaemias and inflammatory problems (Gogus & Smith, 2010; Ruxton et al., 2004; Sidhu, 2003). The most well known and researched effect, is the association FO has with reduced cardiovascular disease morbidity and mortality outcomes (Gogus & Smith, 2010; Yashodhara et al., 2009). However, limited understanding of the underlying physiological mechanisms and FO doses required to improve cardiovascular health is leading to confusing and mixed conclusions being made about their benefits (Alexander et al., 2017; Nestel et al., 2015). The majority of previous research has relied upon ex vivo animal models and unrealistic doses of FO, which would not be achievable in the human diet to demonstrate physiological effects (Matthan et al., 2005; McLennan, 2014). As such, this thesis aimed to characterise cardiac function following supplementation of FO doses achievable in the typical Western-style human diet whilst using a physiologically appropriate in vivo animal model. This will help to establish the action by which increased fish or FO intake benefits heart health in humans.
1.1.2 What are fatty acids?

Dietary fats, along with carbohydrates and protein make up the three main macronutrients of the mammalian diet. Most dietary fats are found in the form of triglycerides which is a glycerol head group attached to three individual aliphatic fatty acid chains. Other less common forms of dietary fat include phospholipids, phytosterols and lipoproteins which are associated with cholesterol. Triglycerides are important dietary sources of fuel for mammals because they yield substantial quantities of energy (ATP) when they are metabolised into free fatty acids. Metabolism of triglycerides occurs in the duodenum following the secretion of pancreatic lipases and bile which hydrolyse the ester bond between the glycerol head group of the triglyceride and the individual fatty acid chains. This process splits triglycerides into diglycerides, monoglycerides and free fatty acids. They are subsequently mixed with fat soluble contents of the diet such as vitamins, cholesterol and bile salts to form micelles. Micelles are absorbed into enterocyte cells and their contents are resynthesized into triglycerides before being repackaged with cholesterol and proteins to form lipoprotein lipids. Lipoproteins are excreted into the lymphatic system and are then able to circulate in the blood stream. Lipoprotein lipase enzymes located on endothelial surfaces of capillaries throughout the body partially digest lipoproteins to release free fatty acids and glycerol into the plasma (Kratky et al., 2005). Plasma free fatty acids can diffuse rapidly across phospholipid bilayers, but there are a wide range of membrane associated proteins which also act to facilitate the uptake of free fatty acids into the membrane, for a detailed review see (Su & Abumrad, 2009). Free fatty acids are then incorporated into the membrane phospholipid bilayer in variable proportions throughout different tissues and play an important role in the functioning of cellular processes (Spector & Yorek, 1985).

Fatty acids are made up of a hydrocarbon chain with a hydrophobic methyl group at one end and a hydrophilic carboxyl group at the other. Fatty acids chains contain different numbers of carbon atoms and can therefore be categorised into short chain (< 6 carbon atoms), medium chain (< 12 carbons atoms) and long chain (>18 carbon atoms). Furthermore, each fatty acid can contain saturated or varied amounts of unsaturated bonds, and each chain attached to the glycerol head group may be the same or different. The degree of saturation refers to the bonding structure (i.e. whether each carbon is
saturated with hydrogen bonds or one/more carbon atom is bonded to another carbon via a double bond) of the carbon atoms found in the fatty acid aliphatic chain. There are three major classes of fatty acids: saturated fatty acids (SFA) which contain no double bond, monounsaturated fatty acids (MUFA) which contain one double bond and poly-unsaturated fatty acids (PUFA) which contain more than one double bond (Figure 1.1). The most common SFA found in the mammalian diet are Palmitic acid (16:0) and Stearic acid (18:0) and are mainly derived from animal-based products including meat and dairy. The typical Western-style diet contains high amounts of SFA (USDA, 2016). The most relevant MUFA to the mammalian diet is Oleic acid (18:1n-9) which is also found in animal fats but commonly sourced from plant-based foods such as processed oils including olive, canola and sunola oil. There are several types of important PUFA which will be discussed in the next section.

Several methods can be used for the nomenclature of fatty acid chains, most commonly in nutritional literature, they are referred to using their trivial name e.g. Linoleic acid. However, this method is often not concise enough to describe the chemical structure of the fatty acid chain. Therefore ‘lipid number’ nomenclature which identifies the number of carbon atoms and number/location of double bonds is also used. This method takes the form of C:Dn-x, where C is the number of carbon atoms, D refers to the number of double bonds and n-x gives information about the location of the first double bond. The methyl end of the aliphatic chain is referred to as the ‘omega’ carbon which is synonymous with ‘n’ and ‘ω’. Therefore n-x identifies precisely which carbon atom the first double bond is found. For example, the omega-6 fatty acid Linoleic acid is named 18:2n-6, informing the reader that Linoleic acid is made up of eighteen individual carbon atoms of which two have double bonds and the first double bond is found at the sixth carbon from the omega end of the aliphatic chain (Figure 1.2). Throughout this thesis, fatty acids will be referred to using their trivial name followed by the lipid number description at the first instance and then abbreviated.
Figure 1.1: Overview of fatty acid classification/nomenclature and biosynthesis of n-3 & n-6 PUFA

Example SFA (No double bonds), MUFA (1 double bond), n-6 PUFA (The first double bond appears at the 6th carbon atom from the omega carbon) and n-3 PUFA (The first double bond occurs at the 3rd carbon atom away from the omega carbon) are shown.
There are several important PUFA present in the mammalian diet which can be further classified into n-6 or n-3 PUFA depending on the location of the first double bond from the omega carbon (Figure 1.1). Mammals have the ability to produce a range of de novo fatty acids from palmitic acid via elongation, desaturation and hydroxylation. However, mammals lack the enzymes necessary to introduce double bonds beyond carbon 9, meaning both n-6 and n-3 PUFA cannot be synthesised de novo. These fatty acids must be obtained in the diet or formed from metabolism of other PUFA present in the diet. PUFA that can only be obtained from the diet are referred to as ‘essential’ fatty acids. The n-6 and n-3 biosynthetic pathways which are available in mammals predominantly take place in the endoplasmic reticulum except for the final conversion which occurs in the peroxisome (Figure 1.2). Linoleic acid (LA: 18:2n-6) is an essential n-6 PUFA in the mammalian diet and acts as the precursor for n-6 PUFA de novo enzymatic elongation and desaturation to form a range of longer chain n-6 fatty acids including Arachidonic acid (AA: 20:4n-6), which is particularly important for platelet and endothelial wall physiology.

The typical Western-style diet is rich in LA due to its presence in many common cooking oils such as corn oil, sunflower oil, soybean oil and safflower oil. Short-chain Alpha-linolenic acid (ALA: 18:3n-3) which is found in seeds and nuts such as flaxseeds, soybeans and walnuts is an essential n-3 PUFA in the mammalian diet. 18C ALA acts as a pre-cursor for n-3 PUFA de novo enzymatic synthesis of eicosapentaenoic (EPA: 20:5n-3) acid and docosahexaenoic acid (DHA: 22:6n-3). For this reason, it has been argued that EPA and DHA should not be considered essential fatty acids (Sinclair et al., 2002). However, there is considerable debate regarding the efficiency rate in the conversion of ALA to DHA in mammals, with studies showing oral doses of ALA to produce blood levels of DHA ranging from non-detectable to 9.8% (Domenichiello et al., 2014; Gibson et al., 2013; Hussein et al., 2005; Metherel et al., 2016). This is a result of delta (Δ) six desaturase enzymes which are used in two steps in the pathway for the conversion of ALA to EPA and DHA (Figure 1.2). Firstly, ALA competes with the n-6 PUFA LA to produce EPA, secondly the EPA metabolite docosapentaenoic acid (DPA: 22:5n-3), competes with both LA and ALA for the Δ six desaturase before the formation of DHA (Gibson et al., 2013; Leonard et al., 2004). A recent systematic review suggested that increasing dietary intakes of ALA results in
some increased formation of EPA, but in the presence of high LA concentrations, saturation of the Δ six desaturase enzyme reduces the conversion of DPA to DHA (Figure 1.2) (Wood et al., 2015). Further evidence for this suggestion is found in a study that demonstrated synthesis rates of DHA to be higher when using precursors beyond ALA (Metherel et al., 2018). Therefore, dietary intakes of foods high in pre-formed DHA & EPA, such as marine based foods, are essential to increase the concentration of LC n-3 PUFA in the mammalian phospholipid membrane.

![Figure 1.2: Overview of n-3 & n-6 PUFA biosynthesis in the mammalian body.](image)

The pathway of enzymatic desaturation and elongation is shown for both n-3 & n-6 PUFA. Steps labelled with an * occur in the endoplasmic reticulum, the final step labelled with # occurs in the peroxisome. It can be observed how increased dietary intakes of ALA can saturate the Δ6 desaturase enzyme and reduce the conversion of DPA – DHA. The figure was adapted from (Gibson et al., 2013).

**Abbreviations:** LA = Linoleic acid; ALA = Alpha-linolenic acid; AA = Arachidonic acid; EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; DPA = Docosapentaenoic acid.
1.1.3 Membrane phospholipid fatty acid incorporation

Phospholipids and cholesterol are the main components, which make up the membrane lipid bilayer found in mammals. The phospholipid composition of the membrane lipid bilayer, however, is not a static environment. Alterations of the fatty acid composition of membrane phospholipids can occur through a range of factors including exercise (Andersson et al., 2000; Andersson et al., 1998; Helge et al., 2001), oxidative stress (Hulbert et al., 2007), ageing (Paradies et al., 1992) and pathological states (Gudbjarnason, 1980). Perhaps of most importance to this thesis is the potent influence that dietary interventions can exert on the membrane phospholipid composition (Abeywardena et al., 2016; Charnock et al., 1986; Henry et al., 2015; Slee et al., 2010).

The proposed consequences of altering membrane phospholipid composition include direct effects on: membrane fluidity and the environment surrounding membrane receptors, proteins and channels; phospholipase release of fatty acids and indirect effects of free fatty acids including: enzymatic and non-enzymatic eicosanoid production; altered intracellular signalling processes; fatty acid oxidation and free radical production and free radical induced upregulation of antioxidants (Mozaffarian & Wu, 2011).

Tissue membrane phospholipid composition in the rat is made up predominantly of Stearic acid (18:0), LA and AA, together these fatty acids represent approximately 45 – 60% of the relative phospholipid composition. The next greatest contributors are Oleic acid (18:1n-9) which makes up approximately 10% and DHA which adds approximately 10%; the remaining fatty acids contribute < 5%. The composition of liver, cardiac, skeletal muscle and neural phospholipid has been demonstrated to maintain relatively constant SFA and MUFA concentrations when changes to the diet are made (Ayre & Hulbert, 1996a; Hulbert et al., 2005). However, when small changes to n-3 and n-6 PUFA content are made in the diet, phospholipids are very responsive, and the PUFA relative phospholipid composition can change dramatically (Hulbert et al., 2005). Interestingly, AA is preferentially incorporated into phospholipids over EPA when diets which are low in LC n-3 PUFA are supplied to rats (Slee et al., 2010). It is thought this occurs as a result of any available EPA being metabolised to DHA to maintain abundant phospholipid DHA concentrations (Tran et al., 2001). However, when LC n-3 PUFA is made available in the diet via dietary FO, EPA and DHA are
preferentially incorporated into phospholipids at the expense of n-6 PUFA such as AA and LA (Owen et al., 2004; Slee et al., 2010). The level of incorporation of individual fatty acids into phospholipids strongly reflects the type of fatty acids included in the diet and varies in tissue with different functions including cardiac, kidney, liver, lungs, plasma, neural, skeletal muscle and erythrocytes (Charnock et al., 1989; Spector & Yorek, 1985).

1.1.4 The role of fish oil in excitable tissue

Excitable tissues are made up of cells which use the movement of ions between cellular compartments and the extracellular environments to create a resting membrane potential that is capable of depolarisation. In myocardial and skeletal muscle cells, depolarisation culminates into a contraction via excitation-contraction coupling. Excitation-contraction coupling is the process which allows an electrical stimulus to be converted into mechanical energy at the actin and myosin filaments in skeletal and myocardial cells. This process is dependent upon the movement of a range of ions between cellular compartments. However, cellular membranes are impermeable to the movement of ions; therefore this passage of ions relies upon a vast array of receptors, ligands, gates and channels embedded within the membrane (Spector & Yorek, 1985). Interactions between the hydrophilic fatty acid tails and membrane bound proteins, receptors and channels are closely linked to cellular function (Stubbs & Smith, 1984). As such, alterations in membrane phospholipid composition can lead to altered cellular functioning.

The incorporation of DHA into skeletal and myocardial membrane phospholipids is an important example of linking cellular function to membrane composition. It has been well documented that excitable tissues including myocardial and skeletal muscle in humans (Andersson et al., 2002; Harris et al., 2004; Innis, 2007) and animals (Ayre & Hulbert, 1996a; McLennan & Abeywardena, 2005; Peoples & McLennan, 2010; Stark et al., 2007b) exhibit similar phospholipid fatty acid composition, much higher in DHA compared to tissues such as kidney, liver and plasma (Charnock et al., 1989; Nikolaidis et al., 2006). Of particular interest to this thesis, it has now been established that very small intakes of FO which are in the human dietary achievable range significantly
influence phospholipid concentrations of LC n-3 PUFA, particularly DHA in excitable membranes of the myocardium and skeletal muscles (Henry et al., 2015).

1.1.5 Skeletal muscle structure and function

Skeletal muscle can be classified into two overarching categories based on the abundance of fibres making up the myofibril. The first class is known as slow-twitch oxidative fibres otherwise referred to as Type I muscle fibres. The second are known as fast twitch fibres. Fast twitch fibres can be further categorised to include two subcategories: 1) fast oxidative glycolytic and 2) fast glycolytic fibres otherwise known as Type IIa and Type IIb respectively. Type I, IIa & IIb categorisation of skeletal muscles is based off contractile properties of the muscle, whereas oxidative or glycolytic categorisation is based on enzymatic processes. Both methods aim to describe the unique rate of contraction, metabolism and fatigue profiles displayed between fibre types. For the simplicity of reading, discussion of muscle fibres will be referred to using the slow oxidative (SO), fast glycolytic (FG) and fast oxidative glycolytic (FOG) terminology throughout this thesis.

The specific muscle function characteristics observed throughout different muscle fibres can be attributed to various isoforms of the myosin heavy chain (MHC) proteins located in the thick filaments of the sarcomere (Wells et al., 1996). The MHC proteins found within muscle fibres mediate metabolic and contractile responses of skeletal muscle (Hashimoto et al., 2016). Skeletal muscles made up with predominately SO fibres are characterised by a slow rate of contraction, primarily use oxidative metabolism and display resistance to fatigue. Slow oxidative muscle fibres are primarily made up of MHC<sub>slow</sub> isoform. This MHC isoform is associated with low basal rates of ATP consumption and has a high ATP reserve capacity; this leads to the fatigue resistant characteristics associated in muscles with high proportions of MHC<sub>slow</sub> isoform (Han et al., 2001). In contrast, FG fibres are characterised by a fast rate of contraction, primarily rely upon glycolytic metabolic pathways and display a rapid fatigue profile. Muscles containing high proportions of FOG fibres display characteristics of both SO and FG muscle fibres. For example, the FG and FOG have a high proportion of the MHC<sub>2</sub> isoform (Sieck & Regnier, 2001). Muscles displaying a high proportion of the MHC<sub>2</sub>
isoform rapidly fatigue because they rely upon high rates of ATP consumption and do not have large ATP reserves (Han et al., 2001).

1.1.6 The influence of fish oil on skeletal muscle function

Human studies have demonstrated several unique physiological roles for FO which may contribute to improved muscular performance (Mickleborough, 2013). These changes in function include increased muscle blood flow (Walser et al., 2006), attenuated post-exercise inflammation after eccentric exercise (Tartibian et al., 2011) and attenuated loss of twitch potentiation following downhill running (Mickleborough et al., 2015). Animal research investigating the health effects of adequate FO intake on skeletal muscle function has produced contradicting results. It was suggested that both n-6 PUFA and n-3 PUFA are essential to maintaining muscle function in rats, as deficiencies in either class of fatty acid were demonstrated to result in poor contractile performance compared to rats receiving diets high in n-6 and n-3 PUFA (Ayre & Hulbert, 1996b). Whereas, more recent studies have demonstrated that incorporation of LC n-3 PUFA into skeletal muscle phospholipids contributes to improved fatigue resistance (Henry et al., 2015; Peoples & McLennan, 2010, 2014) and enhanced oxygen efficiency (Peoples & McLennan, 2010).

Perhaps most important to this research is evidence which demonstrates dietary achievable FO doses to maintain skeletal muscle contractile performance during normoxic conditions (Henry et al., 2015). Diving mammals such as the seal are required to maintain contractile function during periods of hypoxia when they dive for >12 minutes. It has now been demonstrated that the seal concentrates large amounts of DHA in swimming skeletal muscle phospholipids and it has been hypothesised that this helps conserve oxygen (Trumble & Kanatous, 2012). Additionally, it has been demonstrated that therapeutic doses of FO in the auto-perfused in vivo rat hind-limb delays hypoxic skeletal muscle fatigue, suggesting a physiological role of DHA in skeletal muscle during low-oxygen stress (Peoples & McLennan, 2017). The outcomes of both animal and human studies combined suggest DHA play an important role in skeletal muscle function and health. However, the recent identification of a specific physiological role of DHA in skeletal muscle function, particularly during hypoxia/ischaemia has never been investigated using human nutritionally relevant FO doses in the rat heart.
1.1.7 Myocardial membrane structure and function

Striated myocardium is an involuntary excitable tissue that is found in the walls of the heart. Myocardial cells (myocytes) form a thick muscular layer between the epicardium and endocardium of the heart and coordinate contractions to ensure blood is rhythmically pumped away from the heart into the circulation. Like skeletal muscle, calcium ions (Ca\(^{2+}\)) are the principal intracellular signalling ions which play critical roles in both the action potential and contractile phase of the myocyte through excitation-contraction coupling. The handling, storage and re-uptake of Ca\(^{2+}\) in the myocardium are tightly regulated throughout the entire cardiac cycle and has been described in depth elsewhere (Berridge, 2006; Bers, 2008; Fearnley et al., 2011).

Ventricular myocytes contain a slightly different MHC isoform to what is observed for skeletal muscle. Ventricular myocytes predominately contain the MHC\(_{\beta}\) isoform, which is associated with low basal rates of ATP consumption and high ATP reserves (Sieck & Regnier, 2001). Interestingly, MHC\(_{\beta}\) isoform found in myocytes is somewhat similar to the the MHC\(_{\text{slow}}\) isoform found predominantly in SO skeletal muscle fibres. This similarity may be reflective of both types of muscle requiring a fatigue resistant physiological role. Both SO skeletal and cardiac muscles are required to sustain repeated contractile force production and relaxation over prolonged periods of time. Given that SO skeletal muscle and cardiac muscle share a somewhat similar MHC isoform and fatigue resistant profile, it is likely that the fatty acid incorporation patterns between the two types of muscle are also similar.

1.1.8 The influence of fish oil on the cardiovascular system

Dietary fish intake has been consistently associated with a cardio-protective effect in decades of human epidemiological and observational studies (Bang & Dyerberg, 1972; Burr et al., 1989; Hirai et al., 1980; Kagawa et al., 1982; Kromhout et al., 1985). Initially, research suggested that reduced CVD outcomes associated with FO intake was attributable to an anti-atherogenic and anti-thrombotic mechanism (Fischer & Weber, 1984; Siess et al., 1980). More recently, research has identified other vascular effects including favourable changes to blood pressure and endothelial functioning, all of which likely contribute to the cardiovascular protective effect observed (Adkins & Kelley, 2010; De Caterina et al., 2006; Robinson & Stone, 2006). However, the
majority of experiments which have demonstrated anti-atherogenic, anti-hypertensive, anti-thrombotic actions from FO have relied on very high doses to produce a typically modest effect, and this effect is usually only demonstrable in previously diseased populations (Geleijnse et al., 2002; Sekikawa et al., 2008). Despite the focus of initial research on vascular related outcomes, there is now growing evidence which demonstrates that regular fish consumption has a direct cardio-protective effect on the myocardium rather than vascular outcomes (Flock et al., 2013; McLennan, 2014). It is hypothesised that the direct cardio-protective action of FO upon the myocardium is closely linked to the incorporation of LC n-3 PUFA into myocardial membrane phospholipids (Abeywardena et al., 2016; McLennan, 2014). The effects of FO supplementation attributable to the heart directly include modification of heart rate (Dallongeville et al., 2003; Geelen et al., 2005; Grimsgaard et al., 1998; Mozaffarian et al., 2005), heart rate variability (Christensen et al., 2001; Xin et al., 2013) and improved cardiac contractile function in healthy (McLennan et al., 1992a) and ischaemic hearts (Pepe & McLennan, 2002). However, the most extensively studied and important effect of FO is its anti-arrhythmic action (Matthan et al., 2005; McLennan, 2014).

The current thesis builds on several decades of original animal research that points to the essential role that DHA, derived from FO, plays in optimal heart function in both health and disease (Abdukeyum et al., 2008; Abeywardena et al., 2016; Charnock et al., 1992; Hartog et al., 1987; McLennan, 2014; McLennan & Abeywardena, 2005; McLennan et al., 1992a; McLennan et al., 2007; Peoples et al., 2008; Pepe & McLennan, 1996, 2002). Such studies have investigated the physiological effects of LC n-3 PUFA on cardiac function in animals using doses of FO ranging from 1.25% to 12% wt/wt. Despite the consistent demonstration of FO providing a cardio-protective action in epidemiological and animal studies, there is still debate as to the potential cardiovascular health benefits of FO in humans. This is because of the consistent mixed conclusions published regarding FO by large-scale random control trials (RCT) using supplements in order to replicate dietary fish intake and the meta-analyses of these trials (Alexander et al., 2017; Bosch et al., 2012; Galan et al., 2010; Kromhout et al., 2010; O’Keefe et al., 2017; Rauch et al., 2010; Roncaglioni et al., 2013; Svensson et al., 2006; Tavazzi et al., 2008; Valagussa et al., 1999; Yokoyama et al., 2007). An example of this can be found in the recently released Heart Foundation of Australia updated
recommendations on clinical evidence for LC n-3 PUFA in the prevention and treatment of cardiovascular disease. The review concludes that while there are clear benefits of eating fish, there is no support from RCT or their meta-analyses to recommend the use of refined FO supplements (Nestel et al., 2015). Given that the observational and animal evidence remains consistent, yet there are regularly differences reported between RCT, perhaps the design and interpretation of RCT outcomes requires a better understanding of the doses of FO required and the underlying physiological mechanisms of action of FO. The challenge in explaining physiologically plausible mechanisms of action of FO requires animal and cellular work to be completed.

1.1.9 Linking animal fish oil studies to human health

Historically, animal work has relied upon high doses of FO which would not be easily achieved in the human diet. Consequently, the relevance of findings from such studies are ambiguous when extrapolated to human health. However, diets supplemented with 1.25% wt/wt FO consistently exhibit the same or very similar physiological effects as diets supplemented 12%. For example, the anti-arrhythmic, heart rate slowing and enhanced oxygen efficiency actions attributable to FO have been demonstrated at doses of 1.25, 3, 6 and 12% FO (Abeywardena et al., 2016; McLennan, 1993; McLennan et al., 1992a; McLennan et al., 1992b; Pepe & McLennan, 2002; Pepe & McLennan, 2007). Despite this observation, no studies to date have characterised the effect of FO on cardiac function using dietary achievable equivalent doses. Currently, 250mg/day EPA+DHA has been identified in epidemiology and randomized trials for cardiovascular protection (Kris-Etherton et al., 2009; Mozaffarian et al., 2005a) and 500mg/day of EPA+DHA is recommended for cardiovascular health in males (Colquhoun et al., 2008). Furthermore, it has been demonstrated that dietary intakes of FO ranging from as low as 180mg/day up to 1g/day in men and women are associated with reduced risk of primary cardiac arrest and sudden cardiac death, (Patterson et al., 2015; Siscovick et al., 1995). Therefore, the minimum dose of FO this series of projects will supplement animals will be equivalent to ~570mg/day EPA+DHA in humans (Chapter 2). In addition, to ensure the doses of FO are nutritionally relevant to human
health, they will be supplemented to a background diet high in SFA and high in n-6 PUFA, typical of the current Western-style dietary fat proportions (USDA, 2016).

Characterising the effectiveness of FO in the heart also requires the most physiologically stable experimental model. The importance of using a physiologically stable model is highlighted in skeletal muscle studies investigating muscle function. It has been demonstrated that the auto-perfused in vivo hind-limb model provides improved oxygen delivery, long term viability, physiologically relevant blood flow and vascular tone when compared to in situ hind-limb perfusion methods (Peoples et al., 2013). This explains the different outcomes observed in original animal investigations of skeletal muscle metabolism using an in situ rat hind-limb model (Ayre & Hulbert, 1996b) compared to research using the auto-perfused in vivo hind-limb model (Henry et al., 2015; Peoples & McLennan, 2010, 2014, 2017). Both skeletal (Bonen et al., 1994) and cardiac (Kuzmiak-Glancy et al., 2015) muscle performance are intimately linked to blood flow and oxygen delivery. This makes the choice of experimental model critical when trying to evaluate physiologically relevant muscle function. In fact, recently it has been suggested that FO does not enhance myocardial efficiency (Goo et al., 2014), yet the experiment was conducted using perfusate in an in situ model. There is extensive evidence demonstrating the importance of whole blood perfusion when using this type of model (Pepe & McLennan, 1993; Topping & Trimble, 1985). In effect, the physiologically relevant aspect of oxygen conductance is altered and renders the heart to conditions never experienced in vivo when perfusate is used ex vivo instead of whole blood. Although ex vivo studies using perfusate have provided many insights, a whole blood perfusion method offers specific advantages for the physiological questions relating to FO and cardio-protection being explored in this thesis. As such, this research will use a physiologically stable in vivo model of cardiac function to assess the effects of FO on healthy and ischaemic cardiac function. Not only does an in vivo model optimise oxygen diffusion and conductance to the heart but it also gives a more accurate representation of normal physiological function because metabolic waste clearance, vascular responses, humoral changes and neural inputs to the heart are maintained.

Cardiovascular disease persists as a principal contributing factor to worldwide mortality rates and the impact of cardiovascular disease is expected to increase substantially as the proportion of the aged population increases (Heidenreich et al., 2011). Despite vast
improvements in the treatment and prevention of cardiovascular disease over recent
decades, it has been demonstrated that traditional risk factors are evolving and there are
increasing cases of cardiovascular disease in the absence of Standard Modifiable
cardiovascular Risk Factors ‘SMuRFs’ (Vernon et al., 2017). Therefore, elucidating the
physiological actions of FO doses achievable within a typical Western-style human diet
containing high amounts of SFA and n-6 PUFA, whilst using a physiological relevant in
vivo model, is important for informing further human research. A better understanding
of the benefits fish or FO intake provides the heart will contribute to informed
preventative approaches targeting cardiovascular disease in the 21st century.
AIMS AND HYPOTHESES

This thesis took a translational approach, using human research to inform *in vivo* animal studies, which in turn aims to inform further human research. The overarching aim relevant to all studies completed was to characterise cardiac function following supplementation of human dietary achievable FO doses, within a nutritionally relevant Western-style diet (high SFA and n-6 PUFA) and a physiologically appropriate *in vivo* animal model.

More specifically, this thesis aimed to determine the effects of supplementing dietary achievable FO doses on:

**Aim 1: (Chapter 2)**

incorporation patterns of LC n-3 PUFA throughout excitable tissue with unique physiological roles from within the same animal.

**Aim 2: (Chapter 4)**

resting cardiac function, including heart rate, heart rate variability and haemodynamic performance of the heart.

**Aim 3: (Chapter 5)**

arrhythmia generation during ischaemia and reperfusion injury.

**Aim 4: (Chapter 6)**

cardiac haemodynamic function during ischaemia and in the proceeding post-ischaemic recovery.

Hypotheses directly related to each of these aims are given in the corresponding chapters.
Membrane fatty acid incorporation, tissue mass and body weight following supplementation of human dietary achievable fish oil doses
2.1 INTRODUCTION

Investigations of FO supplementation in animals have historically used high intake ranges to achieve membrane phospholipid incorporation and elucidate physiological mechanisms of action of LC n-3 PUFA (Charnock et al., 1989; Charnock et al., 1986; Matthan et al., 2005; McLennan, 2014; McLennan et al., 1992a; Slee et al., 2010). However, phospholipid incorporation does not need the high intakes previously supposed. It has now been demonstrated that myocardial and skeletal muscle composition is particularly sensitive to DHA incorporation and changes occur in the rat, when using low doses of DHA-rich FO (Henry et al., 2015; McLennan et al., 2007; Slee et al., 2010).

2.1.1 Skeletal muscle phospholipid fatty acid composition

Comparative physiology studies have demonstrated that incorporation of PUFA into skeletal muscle phospholipids across mammals is closely linked to the contractile characteristics of the muscle. For example, phospholipids from the rapidly contracting skeletal muscle in the European brown hare which can reach peak running velocities of 80kmh\(^{-1}\) have been reported to contain a very high concentration of PUFA (Valencak et al., 2003). Furthermore, n-6 PUFA has been demonstrated to be associated with increased maximum running speed in a range of land based mammals (Ruf et al., 2006) and in salmon (McKenzie et al., 1998). Likewise, pectoral muscles in the hummingbird or shaker muscles in the rattlesnake which both have very high contractile frequencies have been shown to incorporate DHA preferentially compared to slow frequency contracting leg and ventral muscles within each animal respectively (Infante et al., 2001). Interestingly, despite having similar contractile profiles, the pectoral muscle of the hummingbird contained almost two-fold more DHA than the shaker muscle of the rattle snake. The authors attributed this difference to double packing of mitochondrial inner phospholipids (where the oxidative phosphorylation machinery is located) in the hummingbird pectoral muscles to optimise the rapid and sustained contraction (Infante et al., 2001).

It has also been hypothesised that enhanced muscle performance associated with increased PUFA content is due to changes in the membrane fluidity and permeability
(Daveloose et al., 1993; Stillwell & Wassall, 2003). However, the aforementioned studies are linked by the type of skeletal muscle fibre required to achieve these types of contraction. Muscle comprised of predominately FG and FOG fibres are characterised by a powerful and fast rate of contraction. To produce powerful and rapid contractions an extensive network of sarcoplasmic reticulum (SR) is needed for the distribution and reuptake of Ca$^{2+}$ by the SR Ca$^{2+}$-ATPase (SERCA) (Berchtold et al., 2000). Therefore, preferential DHA incorporation into muscles with greater SERCA content suggests that the role of PUFA in skeletal muscle is not only related to increased membrane fluidity and improved oxidative phosphorylation, but also intimately linked to Ca$^{2+}$ handling.

Exercise training also exerts an influence on membrane phospholipid fatty acid profiles observed in skeletal muscle. Human studies have demonstrated that low-intensity exercise increases oleic acid concentrations and reduces the concentration of total n-6 PUFA, particularly AA in skeletal muscle membrane phospholipids (Andersson et al., 1998). Similarly, oleic acid and DHA have been demonstrated to increase in the phospholipids of knee extensor muscles (Helge et al., 2001) and the vastus lateralis muscle following endurance training in healthy males when compared to untrained males consuming the same diet (Andersson et al., 2000). Therefore, it appears as though intrinsic contractile properties of the muscle combined with exercise training could partly explain the differences observed in relative phospholipid composition between muscle fibre types.

Importantly for this thesis, manipulation of dietary fatty acid profiles strongly influences skeletal muscle phospholipid fatty acid composition. Altering the diet has been demonstrated to produce rapid changes in fatty acid phospholipid profiles of skeletal and cardiac muscle in animals (Ayre & Hulbert, 1996a; Pan & Storlien, 1993) and in humans (Andersson et al., 2002; McGlory et al., 2014). In fact, it has been demonstrated that altering the diet produces a much stronger effect on the fatty acid profile of skeletal muscle than exercise training does in the rat (Turner et al., 2004). Furthermore, there is evidence to show that when FO is supplemented to the diet of rats, LC n-3 PUFA, in particular, DHA is preferentially incorporated into cardiac and skeletal muscle phospholipids (Henry et al., 2015; Peoples & McLennan, 2014; Stark et al., 2007b). Preferential incorporation of DHA into skeletal muscle phospholipids is
observed regardless of whether more EPA or DHA is present in the original oil preparation supplied (Ayre & Hulbert, 1996a).

2.1.2 Myocardial phospholipid fatty acid composition

Myocardial membrane phospholipid LC n-3 PUFA concentrations reflect the type of fatty acids supplied in the diet in a similar manner to what is observed in skeletal muscles. Increased incorporation of LC n-3 PUFA into myocardial phospholipids occurs at the cost of reduced total n-6 PUFA as also observed in skeletal muscle (Charnock et al., 1986; Metcalf et al., 2007; Slee et al., 2010). It has also been demonstrated that myocardial phospholipids preferentially accumulate DHA, well above circulating fatty acid levels (Christensen & Hoy, 1992; Owen et al., 2004; Slee et al., 2010), regardless of whether a DHA (Slee et al., 2010) or EPA (Pepe & McLennan, 1996) predominant oil is used in the diet, similar to skeletal muscle. Dose-response investigations have shown a linear relationship between FO supplementation and LC n-3 PUFA phospholipid concentrations in myocardial cells (Matthan et al., 2005; Owen et al., 2004). However, these findings have been reconsidered after Slee, E.L. et al., 2010 demonstrated dosages above 1.25% wt/wt lay in the asymptotic range of a hyperbolic curve when doses below 1.25% are considered, particularly for DHA (Figure 2.1). This study also demonstrated that the lowest concentration of FO in the rat to alter phospholipid incorporation is 0.16%, and the authors predicted that the threshold for DHA incorporation is even lower at 0.0027%. This observation, together with the demonstration that similar cardiac physiological effects occur when using doses of 1.25% or 12%, suggests that doses above 1.25% are beyond a critical saturation point to produce phospholipid and physiological changes. Time-course investigation of fatty acid incorporation demonstrates that DHA is rapidly up-taken by the myocardium before reaching a plateau point, whereas EPA increases linearly (Owen et al., 2004). However, the optimal supplementation time for dietary achievable doses to achieve maximal myocardial phospholipid DHA incorporation in the rat is four weeks (Slee et al., 2010).

Throughout this thesis, ‘low doses’ of FO will be referred to as ‘dietary achievable FO doses’. This terminology will be used because ‘low intake’ is commonly used to describe the problem (deficiencies of LC n-3 PUFA intake (de Goede et al., 2010)),
whereas ‘low doses’ describes the treatment (supplementing FO using doses lower than previous studies (Owen et al., 2004)). Human ‘dietary achievable’ equivalent doses of FO in the rat, range from 0.16 – 1.25% wt/wt with 0.16% representing an easily achievable dose from direct fish consumption (≈1 salmon [100g] serve/week) and 1.25% representing a dose achieved via capsule supplements (≈6 capsules per/day) (Slee et al., 2010). Doses higher than 1.25% and below 3% (≈15 capsules per/day) would not be easily achievable in the human diet except with concentrated products; they will be referred to as ‘therapeutic’. Doses above 3% will be referred to as ‘supra-therapeutical’ because they would be hard to achieve in the human diet even with concentrated capsule supplements, making findings from previous studies using such high doses inappropriate to extrapolate to human health (Table 2.1).
Figure 2.1: Dose relationships of dietary fish oil feeding & rat myocardial DHA & EPA incorporation. Colours bands represent doses described in Table 2.1. Figure adapted from Slee, E.L et al., 2010.

**Table 2.1: Equivalence of rat fish oil doses to the human diet.**

<table>
<thead>
<tr>
<th>Rat Diet Fish oil % weight diet</th>
<th>Human* EPA+DHA per/day (g)</th>
<th>Human† Serve (100g) salmon/week</th>
<th>Human‡ Fish Oil capsule/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>9.1</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>1.25</td>
<td>2.3</td>
<td>8</td>
<td>6.8</td>
</tr>
<tr>
<td>0.31</td>
<td>0.57</td>
<td>2</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Based on human energy intake of 8700 kJ per day, †Based on salmon n-3 content of 1.9g/100g, ‡Based on typical FO capsule content of 330mg EPA+DHA. (Slee et al., 2010)

- Fill represents historically common doses which will be referred to as supratherapeutical in this thesis.
- Fill represents the ModFO dose of this study which would require ≈6 FO capsules per/day in humans.
- Fill represents LowFO dose of this study which would require ≈2 salmon [100g] serve/week in humans.

**Abbreviations:** DHA = Docosahexaenoic acid; EPA = Eicosapentaenoic acid.
2.1.3 The effect of fish oil on excitable membrane composition

Skeletal muscle membrane phospholipids incorporate DHA in a very similar fashion to myocardial phospholipids with dietary intervention. However, very few studies have investigated the specific incorporation patterns of DHA throughout skeletal muscle fibre types, and the conclusions have been mixed. Originally, studies demonstrated that DHA concentrations are highest in SO muscles such as the soleus (Blackard et al., 1997; Kriketos et al., 1995), but more recently it has been demonstrated that DHA levels are higher in mixed FOG and FG fibres of the gastrocnemius (Henry et al., 2015). To the knowledge of the author, no studies to date have investigated the specific phospholipid incorporation patterns of DHA throughout an entire array of skeletal muscle fibre types within the one animal. Therefore, systematic investigations to be completed in this thesis, within the rat, will allow observations to be made for the first time. Phospholipid DHA incorporation patterns will be determined, with knowledge of the type of muscle fibre predominantly making up each individual skeletal muscle. Using the rat hind-limb is the ideal model to investigate a cross section of muscles due to the well-known and consistent fibre type composition profile available (Armstrong & Phelps, 1984). Additionally, the use of the rat allows for sampling of heart and skeletal muscle tissue from within the same animal. Most importantly, the use of an animal model allows for precision dosing of LC n-3 PUFA and removes the influence of exercise training which is difficult to control in human trials, allowing definitive separation of dietary groups and confident conclusions to be made as a direct result of altering the diet only. Furthermore, all animals in the dietary investigations of this thesis will be fed a Control diet which contains 5% SFA, 2.5% SSO and the remaining 2.5% will be OO which will be exchanged for FO to provide a LowFO (0.31%) and ModFO (1.25%) diet. This replicates a typical Western style diet more closely with a high SFA and high ratio of n-6 to n-3 PUFA content (USDA, 2016) than previous low dose studies (Henry et al., 2015; Slee et al., 2010). Therefore, the primary objective of the this study was to characterise the influence of providing human dietary achievable FO doses, for four weeks, on phospholipid DHA incorporation across a range of skeletal muscles and myocardium within the rat.
2.1.4 The effect of fish oil on skeletal muscle mass

Incorporation of LC n-3 PUFA into skeletal muscle phospholipids is associated with anti-catabolic and/or anabolic properties leading to maintenance of and increased lean muscle mass. Skeletal muscle hypertrophy occurs when the ratio of muscle protein synthesis exceeds that of muscle protein breakdown (Phillips et al., 1997). Supplementing therapeutic doses of FO has been demonstrated to improve muscle protein synthesis rates independent of training in elderly, middle-aged and young individuals (Smith et al., 2011; Smith et al., 2015). Muscle protein synthesis rates in mammals are closely linked to the rapamycin complex and key regulatory kinases, including mTOR, p70S6K1 and focal adhesion kinase (Dickinson et al., 2011). Supplementation of FO in humans has been demonstrated to increase phosphorylation of mTOR and p70S6K1 (Smith et al., 2011), increase the total protein content of focal adhesion kinase (McGlory et al., 2014) and altered mitochondrial function along with extracellular matrix organisation (Yoshino et al., 2016). Animal studies have demonstrated that supra-therapeutical doses of FO stimulates endogenous glucose production and increases phosphorylation of anabolic signaling kinases in ageing rats (Kamolrat et al., 2013), enhances insulin-sensitive protein metabolism in steers (Gingras et al., 2007), reduce myosin heavy chain loss during hindlimb immobilisation in the rat (You et al., 2010) and enhance gene expression of growth regulatory factors involved in myogenesis (Castillero et al., 2009). Given the evidence, it is likely that LC n-3 PUFA is acting through a combination of the mechanisms discussed to increase muscle protein synthesis rates resulting in increased skeletal muscle mass. As such, the second objective of this study was to determine the influence of human dietary achievable FO doses on skeletal muscle mass.

2.1.5 The effect of fish oil on body weight

In contrast to the lean tissue anabolic effects of LC n-3 PUFA discussed, it has also been suggested that increasing FO intakes may help reduce body weight in humans via an anti-obesity effect (Buckley & Howe, 2009, 2010; Couet et al., 1997; Kabir et al., 2007). The changes in body weight observed with FO supplementation in humans have been attributed to changes in fat oxidation pathways, gene expression, altered energy expenditure (Baillie et al., 1999; Couet et al., 1997), suppression of appetite
(Thorsdottir et al., 2007) and reduced fat deposition (Baillie et al., 1999). Animal studies have provided some evidence to support this by demonstrating that FO supplementation can attenuate body fat gain in obesogenic dietary models (Belzung et al., 1993), particularly visceral fat accumulation (Ruzickova et al., 2004) and reduce body weight in mice which were obese before supplementation began (Huang et al., 2004). However, the animal research that supports effects in obesity to date has used FO doses which are extremely high even in reference to supra-therapeutical doses (5 to 60% wt/wt, equivalent in man to FO: 28g/d and more) and could not be achieved in the human diet. Therefore, the third objective of this study was to determine the influence of human dietary achievable FO doses, provided in the presence of a typical Western-style background diet, on body weight in rats.
2.2 AIMS AND HYPOTHESES

The primary aim of this study was to establish fatty acid composition of a range of skeletal muscles and myocardial phospholipids in the rat following supplementation of FO doses achievable within a typical Western-style human diet. The secondary aim was to describe the effect of FO on skeletal muscle mass and identify any changes to whole body weight.

Specifically, these experiments had the following aims and hypotheses:

**Aim 1:** Determine the influence of supplementing human dietary achievable FO doses to a typical Western-style diet on phospholipid DHA concentrations in excitable tissues including cardiac and skeletal muscle from within the same animal.

- **Hypothesis 1:** Myocardial and skeletal muscle phospholipids will incorporate DHA at the expense of total n-6 PUFA concentrations, in particular AA.
- **Hypothesis 2:** Incorporation of DHA into skeletal muscle phospholipids will be muscle fibre type specific.

**Aim 2:** Quantify if the supplementation of human dietary achievable FO doses to a typical Western-style diet high in SFA and n-6 PUFA influences muscle mass.

- **Hypothesis 3:** Incorporation of LC n-3 PUFA into skeletal muscle phospholipids will result in increased muscle mass.

**Aim 3:** Describe the influence of supplementing human dietary achievable FO doses to a typical Western style diet high in SFA and n-6 PUFA on body weight.

- **Hypothesis 4:** The addition of human dietary achievable FO doses to a typical Western-style diet will attenuate weight gain.
2.3 METHODS

2.3.1 Animals & ethical considerations

A primary cohort (cohort 1) of twenty three (23) male rats (*Sprague Dawley*) was used for the general methods of this thesis (Chapter 3) and a second cohort (cohort 2) of sixty two (62) male rats (*Sprague Dawley*) were used for the investigations of the effects of LC n-3 PUFA on biochemical and physiological outcomes (Chapters 2, 4, 5 & 7). All animals were obtained from the Animal Resource Centre (Perth, Western Australia) and housed at the University of Wollongong’s animal housing facility. All experiments contained within this thesis were approved by the University of Wollongong animal ethics committee and were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004).

2.3.2 Standardisation

The animals were housed with a 12-hour light-dark cycle, and the temperature maintained between 20°C-22°C. Animals were able to access food and water *ad libitum* prior to the feeding protocol beginning. All animals were weighed upon arrival at the animal housing facility and before any experiments taking place.

2.3.3 Diet composition

Animals were randomly assigned to one of three isoenergetic prefabricated diets after consuming regular lab chow for a minimum of one week. The pre-fabricated diets have been previously developed for use in similar feeding studies (Slee *et al.*, 2010). All diets contained 10% fat, 50% carbohydrate and 20% protein by weight plus minerals and vitamins based on the American Institute of Nutrition AIN-93M diet (Reeves, 1997), differing only in the fatty acid composition making up the total fat. All components used in the diets were carefully weighed and combined in the laboratory by the primary investigator and then frozen at -20°C to ensure quality control. A small amount of food dye was added to the mixture to allow identification of the different dietary groups. The diets contained a balanced mix of macro and micro nutrients consisting of (as a percentage of dry weight) 56.5% corn-starch, 10% sucrose. 9% casein, 5% fibre, 10% oil, 1% vitamin mix and 3.5% mineral mix (*Table 2.2*). Four sources of fats and oils
were used to make up the total 10% of fat in the diet. All diets contained 5.5% beef tallow, 2.5% n-6 PUFA rich sunflower seed oil and the remaining 2.5% was made up of olive oil. This specific mix of oils was used to replicate the high amounts of SFA and n-6 PUFA found in a typical western diet (Simopoulos, 2008; USDA, 2016). Extra light olive oil was used as the refining process to produce extra light olive oil removes the majority of the antioxidant polyphenols commonly present in less refined oils. FO (0.31% - LowFO, 1.25% - ModFO) was substituted for olive oil using DHA-rich tuna FO (Nu-Mega Ingredients Pty Ltd, Australia) to create experimental groups. The DHA-rich tuna FO provided LC n-3 PUFA concentrations of 26% DHA and 7% EPA, with the remaining major fatty acids being made up of 20% 16:0 Palmitic acid and 15% 18:1 Oleic acid (Table 2.3). The experimental doses of FO used in this thesis were selected as they were calculated to be equivalent to doses that are achievable in the human diet and they have previously been demonstrated to increase incorporation of LC n-3 PUFA into myocardial and skeletal muscle phospholipids (Henry et al., 2015; Slee et al., 2010). Conversion of doses from rat to human equivalent is completed based on an average human energy intake of 8700kJ per/day, average salmon n-3 PUFA content of 1.9g/100g and the typical capsule content of 330mg EPA+DHA (Slee et al., 2010). The equivalent rat energy intake is approximately 325kJ/day, based on a 300g rat eating approximately 20g/day prefabricated food.
Table 2.2: Nutrient composition of rat diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>565g</td>
<td>Frutex (Aus)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100g</td>
<td>Commercially available</td>
</tr>
<tr>
<td>Casein</td>
<td>90g</td>
<td>Rogers &amp; Company (Aus)</td>
</tr>
<tr>
<td>Fibre</td>
<td>50g</td>
<td>MP BioMedicals (Aus)</td>
</tr>
<tr>
<td>Vitamin</td>
<td>10g</td>
<td>MP BioMedicals (Aus)</td>
</tr>
<tr>
<td>Mineral</td>
<td>35g</td>
<td>MP BioMedicals (Aus)</td>
</tr>
<tr>
<td>Oil*</td>
<td>100g</td>
<td>Variable sources</td>
</tr>
<tr>
<td>Gelatine</td>
<td>50g</td>
<td>Commercially available</td>
</tr>
<tr>
<td>Total</td>
<td>1000g</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>400mL</td>
<td></td>
</tr>
</tbody>
</table>

*Oil composition was different in each diet, see Table 2.3.
Table 2.3: Proportion of oils and fatty acids of all three diets.

<table>
<thead>
<tr>
<th>Fat Source (% of diet)</th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Tallow</td>
<td>5.5%</td>
<td>5.5%</td>
<td>5.5%</td>
</tr>
<tr>
<td>Sunflower Oil</td>
<td>2.5%</td>
<td>2.5%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>2.0%</td>
<td>1.69%</td>
<td>0.75%</td>
</tr>
<tr>
<td>DHA-rich Tuna FO</td>
<td>0%</td>
<td>0.31%</td>
<td>1.25%</td>
</tr>
</tbody>
</table>

Fatty Acid Profile (% fat in diet)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>2.04</td>
<td>2.13</td>
<td>2.41</td>
</tr>
<tr>
<td>16:0</td>
<td>17.21</td>
<td>17.51</td>
<td>18.40</td>
</tr>
<tr>
<td>18:0</td>
<td>12.07</td>
<td>12.14</td>
<td>12.37</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>41</td>
<td>39.12</td>
<td>33.45</td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>19.29</td>
<td>19.07</td>
<td>18.43</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.55</td>
<td>0.55</td>
<td>0.56</td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>0.02</td>
<td>0.08</td>
<td>0.24</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>0</td>
<td>0.22</td>
<td>0.87</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>0</td>
<td>0.03</td>
<td>0.14</td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>0</td>
<td>0.89</td>
<td>3.61</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>32.22</td>
<td>32.73</td>
<td>34.28</td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>43.73</td>
<td>42.06</td>
<td>37.01</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>19.9</td>
<td>20.89</td>
<td>23.87</td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
<td>19.35</td>
<td>19.19</td>
<td>18.7</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>0.55</td>
<td>1.69</td>
<td>5.17</td>
</tr>
<tr>
<td>Σ EPA + DHA</td>
<td>0</td>
<td>1.11</td>
<td>4.48</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>35.35</td>
<td>11.32</td>
<td>3.62</td>
</tr>
</tbody>
</table>

Abbreviations: 14:0 = myristic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA); SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
2.3.4 Dietary feeding protocol

Animals were randomly placed on the pre-fabricated diet between 8-10 weeks of age and allowed *ad libitum* access to the diet and water for a minimum of four (4) weeks and a maximum of six (6) weeks. Animals and their food were checked every two days to ensure the diet was being consumed and there were no signs of animal distress. All animals were weighed before beginning the diet and then weighed once again before experimental procedures.

2.3.4a Body weight and food intake measurements

A subgroup (N=32) of animals were investigated throughout the feeding protocol to determine if there were any differences in feeding patterns or weight gain with the different oils. Food intake was measured twice weekly to the nearest gram and then normalised to grams consumed/100 g body weight/day. Body weight change was measured weekly to the nearest gram, and tissue was collected at the end of the feeding protocol. A correction factor was calculated for the daily evaporation of water weight in the different diets while stored in the same environmental conditions as the animals to make an accurate measurement of the amount of food consumed by the animals.

2.3.5 Tissue collection

Note: The methods and results for tissue collection and biochemical analyses are reported in this chapter. However, the animals used for tissue collection were anaesthetised and physiological protocols (Chapter 4, 5 and 7) were undertaken beforehand. Upon completion of the protocols animals were euthanased via rapidly removing the heart from the animal and exsanguination and tissue collection began.

2.3.5a Cardiac tissue collection

The major vessels were cut from the heart, and the heart was submerged into an ice cold perfusate to arrest beating. Excised hearts were then flushed with an ice cold 0.9% NaCl solution to remove any remaining blood. The great vessels were then dissected off the heart, and the heart was weighed to obtain a heart mass. The atria were dissected off to obtain a ventricle mass, and the right ventricle was dissected off to obtain a left ventricle mass. The left ventricle was then sliced in 2mm diameter pieces and immediately frozen.
in liquid nitrogen, then stored at -80°C until fatty acid or tissue morphological and biochemical analyses.

2.3.5b Skeletal muscle tissue collection

Following exsanguination and removal of the heart, muscles were dissected from the left lower limb. An incision was made from the ileum toward the foot, and the skin was removed via blunt force. A selection of muscles from the upper thigh and lower limb were then carefully dissected. Physical force was used to separate larger muscle groups into red, white and red/white sections (Armstrong & Phelps, 1984). Surgical scissors and curved forceps were used to separate the fascia between smaller muscle groups being dissected; scissors were used to remove any remaining connective tissue/tendon. Following muscle dissection, the tibia was removed via cutting the ligaments at the knee and ankle using a scalpel and then measured to the nearest millimetre.

2.3.5c Skeletal muscle mass measurement

Following dissection, a pre-defined selection of muscles representing a cross section of SO, FG and FOG fibre types from the left thigh and lower limb were weighed to the nearest milligram. The muscles of the upper limb included the: quadriceps\textsubscript{red}, quadriceps\textsubscript{white}, quadriceps\textsubscript{mixed}, pectineus and adductor longus. Muscles from the lower limb included the: soleus, gastrocnemius\textsubscript{red}, gastrocnemius\textsubscript{white}, gastrocnemius\textsubscript{mixed} plantaris, extensor digitorum longus (EDL) (Table 2.4). Muscles which were predominantly made up of SO fibres included: soleus and Adductor longus. Muscles which were predominantly made up of FG fibres included: quadriceps\textsubscript{white}, gastrocnemius\textsubscript{white}, EDL and pectineus. Muscles which were predominantly made up of FOG fibres included: quadriceps\textsubscript{red}, gastrocnemius\textsubscript{red} and plantaris (Armstrong & Phelps, 1984). Following measurements, each muscle was immediately frozen in liquid nitrogen using liquid nitrogen cooled tongs. Muscles were then stored at -80°C until fatty acid or tissue morphology analysis.
Table 2.4: Known fibre-type composition of the selected rat hind-limb muscles used to analyse LC n-3 PUFA phospholipid concentrations.

<table>
<thead>
<tr>
<th>Skeletal muscle</th>
<th>Mass (g)</th>
<th>FOG</th>
<th>FG</th>
<th>SO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upper-limb</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>quadriceps&lt;sub&gt;(red)&lt;/sub&gt;</td>
<td>0.75 ± 0.02</td>
<td>53 ± 9</td>
<td>40 ± 8</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>quadriceps&lt;sub&gt;(white)&lt;/sub&gt;</td>
<td>0.78 ± 0.06</td>
<td>25 ± 6</td>
<td>74 ± 6</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>pectineus</td>
<td>0.28 ± 0.01</td>
<td>35 ± 4</td>
<td>55 ± 4</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>adductor longus</td>
<td>0.10 ± 0.01</td>
<td>18 ± 4</td>
<td>1 ± 1</td>
<td>81 ± 4</td>
</tr>
<tr>
<td><strong>Lower-limb</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>soleus</td>
<td>0.19 ± 0.02</td>
<td>13 ± 4</td>
<td>0</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>gastrocnemius&lt;sub&gt;(red)&lt;/sub&gt;</td>
<td>0.15 ± 0.03</td>
<td>62 ± 3</td>
<td>8 ± 4</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>gastrocnemius&lt;sub&gt;(white)&lt;/sub&gt;</td>
<td>0.13 ± 0.01</td>
<td>16 ± 3</td>
<td>84 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>plantaris</td>
<td>0.45 ± 0.03</td>
<td>50 ± 3</td>
<td>41 ± 3</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>extensor digitorum longus (EDL)</td>
<td>0.23 ± 0.08</td>
<td>42 ± 7</td>
<td>56 ± 8</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

Typical muscle mass and fibre type population (%) according to FOG, FG & SO classifications of the rat hind-limb skeletal muscles measured in this study. Ordered by muscle mass (heaviest to lightest). Table adapted from Armstrong et al. 1984.

**Abbreviations**: FOG = fast oxidative glycolytic; FG = fast glycolytic; SO = slow oxidative.
2.3.6 Membrane phospholipid fatty acid analysis

The fatty acid composition of heart and skeletal muscle tissue collected at the end of experimental procedures was analysed using gas chromatography to determine phospholipid LC n-3 PUFA incorporation of DHA and EPA. The following outlines in detail the acidified total lipid extraction (Folch et al., 1957), solid phase separation into phospholipid fractions (Kim & Salem, 1990) and transesterification (Lepage & Roy, 1986) techniques used in this study. All solvents used throughout the process contained 0.01% butylated hydroxytoluene as an antioxidant.

2.3.6a Total lipid extraction

Heart and skeletal muscle tissue samples were thawed on ice before preparation occurred. Tissues were cut into sample sizes of 100-150mg and washed with a phosphate buffered saline solution to remove any external contaminants (blood etc.) and placed into labelled 2mL Eppendorf tubes. An equal volume of stainless steel beads to tissue mass were then added to the tube along with 1mL of chloroform: methanol (2:1). The tubes were capped and then placed in a cardiac tissue homogenization blender (Bullet Blender®, Next Advance Inc, New York, USA) and homogenised following the manufacturer's instruction’s for 4 minutes at speed 8. The homogenates were then transferred to labelled Pyrex PTFE screw cap tubes and another 2mL of chloroform: methanol was used to wash out Eppendorf tubes. The samples were then rotated for 3-4 hours at 4ºC to separate lipid from protein. Following rotation, 2mL of 1M H₂SO₄ was added to the tubes, and they were vortexed for 10seconds before being centrifuged at 1000rom (210 x g) and 4ºC for 10min (Hettich Zentrifugen Rotina 46 R). The top (acid) layer was removed to a waste beaker, and a Pasteur pipette was used to go through the middle protein plug and transfer the bottom solvent layer into new tubes. A further 2mL of 1M H₂SO₄ was added, and the procedures described above were repeated. The bottom layer was then again collected, and a small amount of sodium hydrosulphite was added, and the tube was shaken to remove any remaining aqueous phase. The solution was then filtered through a Pasteur pipette with a small amount of silane treated glass wool pushed down into the stem of the pipette and stored at -20ºC overnight.
2.3.6b Phospholipid separation

The samples were dried down under nitrogen while sitting in a heating block set at 37°C. The phospholipid filters (Sep-Pak Silica Classic Filters, Waters Corporation, NSW, AUS) were numbered and placed into holding racks with 10mL glass syringes inserted into the filters as the reservoir for solvents. The filters were washed with 5mL hexane before running the samples through the filter. Once samples were dry, 5mL of hexane was added to bind the lipids in the tube and given a vortex (10sec) before being passed through the filters. A further 3mL hexane was used to wash out test tubes, and this was run through the filter also. Triglycerides were then eluted with 3 x 5mL ethyl acetate and discarded. Phospholipids were eluted with 3 x 5mL methanol.

2.3.6c Transesterification

Extracted phospholipids were dried under nitrogen in a heating block and then redissolved in 2mL methanol: toluene (4:1 v/v). A Gilson positive displacement pipette was used to add 200µL of the volatile acetyl chloride to the culture tubes very slowly while vortexing to methylate the samples. Teflon tape was then wrapped clockwise around the thread of the tubes, and they were capped tightly. The tubes were placed into a heating block which had been pre-heated to 100°C for sixty minutes. This enhances the methylation process of the fatty acids. Following methylation, the tubes were transferred to a metal rack sitting in icy water and allowed to cool for ten minutes. Very slowly, 5mL of a 6% solution of potassium carbonate (K2CO3) was added to neutralise the volatile acetyl chloride. The tubes were transferred to a pre-cooled centrifuge (4°C) and spun at 3000rpm (1895 x g) for ten minutes resulting in an upper toluene phase of fatty acid methyl esters. This upper toluene phase was carefully drawn off and transferred into labelled gas chromatography vials.
2.3.6d Analysis using gas chromatography

Samples were analysed using a Shimadzu GC-17A gas chromatograph with flame ionisation detection. The gas chromatograph used a 30m x 0.25mm internal diameter capillary column under the following conditions:

Running Conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injector Temperature</td>
<td>260 °C</td>
</tr>
<tr>
<td>Detector Temperature</td>
<td>260°C</td>
</tr>
<tr>
<td>Carrier Gas</td>
<td>Ultra-high purity hydrogen</td>
</tr>
<tr>
<td>Column Flow</td>
<td>1.54ml/min</td>
</tr>
</tbody>
</table>

Temperature Program:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>185 °C</td>
</tr>
<tr>
<td>Ramp</td>
<td>5°C / minute for 15 minutes</td>
</tr>
<tr>
<td>Maintain</td>
<td>260 °C for 5 minutes</td>
</tr>
<tr>
<td>Total Run time</td>
<td>20 minutes</td>
</tr>
</tbody>
</table>

Individual fatty acid peaks on the chromatograph were identified by comparison to authentic fatty acid methyl ester standards (F.A.M.E MIX C4-C24, Sigma-Aldrich, Castle Hill, Aus). Peak quantitation was calculated by area for corrected normalisation. The relative proportions of EPA+DHA and other fatty acids in the tissue samples were divided by the total area of fatty acids to give the relative amount of each fatty acid (% total fatty acids).

2.3.7 Chemicals

All chemicals used in this study were sourced from Sigma-Aldrich (Castle Hill, Aus). Solvents used in this study including chloroform, hexane, methanol, ethyl acetate and toluene were analytical grade. Butylated hydroxyl toluene was made up to 0.01% w/v in saline and added to all solvents as an antioxidant. Sulphuric acid H$_2$SO$_4$ was purchased
in concentrate and diluted to make a stock 1M solution. Sodium hydrosulphite and Potassium carbonate (K$_2$CO$_3$) were purchased as powders and stored according to manufacturer’s instructions before use. Fatty acid standards (F.A.M.E MIX C4-C24, Sigma-Aldrich, Castle Hill, Aus) were purchased neat and were stored at -20ºC until use; they were then dissolved in toluene for analysis.

2.3.8 Statistics

All results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The effects of diet treatment on experimental outcomes were tested via ANOVA. Individual group means were compared using Bonferroni’s post-hoc analysis. All statistical analyses were performed in a blinded manner and completed using SPSS 21 for Windows (SPSS Inc, Chicago, IL, USA). Alpha was set at $p < 0.05$. 
2.4 RESULTS

Fatty acids are reported as relative percent composition of phospholipid membranes; minor fatty acids were analysed but omitted from the reported results. Fatty acid analysis was started from Stearic acid (16:0) in cardiac phospholipids and Myristic acid (14:0) in skeletal muscle phospholipids and continued until docosahexaenoic acid (DHA - 22:6n-3).

2.4.1 The effect of fish oil supplementation on cardiac phospholipid fatty acids

Total LC n-3 PUFA content was significantly higher in the LowFO and ModFO group compared to the Control group. Elevated LC n-3 PUFA concentrations were predominantly associated with a significantly greater DHA incorporation in both FO supplemented groups. The composition of DHA in myocardial phospholipids was 5.02% in the Control group, 13.10% in the LowFO group and 18.35% in the ModFO group (Table 2.5). EPA was not detected in the Control group but was present in both FO groups (LowFO: 0.11%, ModFO: 0.39%) in very small amounts. Elevated LC n-3 PUFA concentrations were associated with lower total n-6 PUFA content. More specifically, the LowFO and ModFO groups displayed significantly less AA and LA in their phospholipid composition compared to the Control group (Table 2.5). As a result of these differences the n-6:n-3 ratio for both the FO supplemented groups was lower than the Control group. Additionally, Oleic acid (18:1n-9) and total MUFA was significantly lower in the ModFO group compared to both the Control and LowFO group.
Table 2.5: The effect of fish oil on phospholipid fatty acid composition in the left ventricle.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>12.0 (±0.5)</td>
<td>13.1 (±0.4)</td>
<td>13.1 (±0.3)</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.79 (±0.1)</td>
<td>0.79 (±0.1)</td>
<td>0.55 (±0.06)*†</td>
<td>0.031</td>
</tr>
<tr>
<td>18:0</td>
<td>22.2 (±0.7)</td>
<td>21.4 (±0.3)</td>
<td>23.0 (±0.5)</td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>9.3 (±0.5)</td>
<td>8.5 (±0.3)</td>
<td>6.0 (±0.3)*</td>
<td>0.002</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>3.4 (±0.1)</td>
<td>3.3 (±0.2)</td>
<td>3.2 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>26.1 (±1.4)</td>
<td>20.8 (±0.6)*</td>
<td>18.5 (±0.6)*</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.39 (±0.02)</td>
<td>0.43 (±0.01)</td>
<td>0.39 (±0.02)</td>
<td></td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>19.7 (±0.6)</td>
<td>16.7 (±0.4)*</td>
<td>14.2 (±0.7)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>ND</td>
<td>0.11 (±0.01)</td>
<td>0.39 (±0.02)†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>0.98 (±0.08)</td>
<td>1.3 (±0.1)*</td>
<td>1.3 (±0.02)*</td>
<td>0.002</td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>5.0 (±0.2)</td>
<td>13.1 (±0.9)*</td>
<td>18.3 (±0.4)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>34.2 (±0.4)</td>
<td>34.5 (±0.3)</td>
<td>36.1 (±0.8)</td>
<td></td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>13.5 (±0.7)</td>
<td>12.7 (±0.5)</td>
<td>9.8 (±0.3)*</td>
<td>0.007</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>52.2 (±0.9)</td>
<td>52.7 (±0.3)</td>
<td>53.9 (±0.8)</td>
<td></td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
<td>45.7 (±1.0)</td>
<td>37.7 (±0.7)*</td>
<td>33.3 (±1.1)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>6.4 (±0.3)</td>
<td>14.9 (±0.8)*</td>
<td>20.5 (±0.4)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ EPA + DHA</td>
<td>5.0 (±0.2)</td>
<td>13.1 (±0.9)*</td>
<td>18.7 (±0.4)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>7.1 (±0.4)</td>
<td>2.5 (±0.2)*</td>
<td>1.6 (±0.07)*</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 4 – 5 per group). ND = Not Detected. *p < 0.05 vs Control diet. †p < 0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

Abbreviations: 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:1n-7 = vaccenic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
2.4.2 The effect of fish oil supplementation on skeletal muscle phospholipid fatty acids

soleus phospholipid DHA composition was significantly greater in the LowFO (13.79%) and ModFO (18.98%) groups compared to the Control (8.35%) group (Table 2.6). EPA was not detected in the soleus of the Control or LowFO group but was present in the ModFO group in a very small amount. The Control group displayed the highest AA phospholipid concentration (16.61%) and reduced in a dose-dependent manner in the LowFO (13.46%) and ModFO (10.89%) groups. Other differences unique to the soleus which occurred in the phospholipid composition included a significantly lower concentration of LA and Stearic acid in the ModFO group compared to the Control group (Table 2.6).

quadriceps (red) phospholipid DHA composition was significantly greater in the LowFO (15.94%) and ModFO (21.72%) groups compared to the Control (11.24%) group (Table 2.7). EPA was not detected in the quadriceps (red) of the Control but was present in the LowFO and ModFO groups. The Control group displayed the highest AA phospholipid concentration (16.19%) and reduced in a dose-dependent manner in the LowFO (12.23%) and ModFO (9.37%) groups. Other differences unique to the quadriceps (red) which occurred in the phospholipid composition included a significantly lower concentration of Vaccenic acid (18:1n-7), LA and ALA in the ModFO group compared to the Control group (Table 2.7).
Table 2.6: The effect of fish oil on phospholipid fatty acid composition of the soleus.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>4.3 (±0.3)</td>
<td>5.6 (±0.3)</td>
<td>5.9 (±0.4)</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>12.9 (±0.2)</td>
<td>13.6 (±0.6)</td>
<td>13.9 (±0.5)</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.94 (±0.1)</td>
<td>0.90 (±0.1)</td>
<td>0.7 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>19.9 (±0.1)</td>
<td>18.9 (±0.3)</td>
<td>18.4 (±0.2)*</td>
<td>0.03</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>9.7 (±0.2)</td>
<td>9.9 (±0.9)</td>
<td>7.9 (±0.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>2.7 (±0.1)</td>
<td>2.2 (±0.07)</td>
<td>2.3 (±0.0)</td>
<td></td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>21.7 (±0.5)</td>
<td>18.9 (±0.3)</td>
<td>17.8 (±0.7)*</td>
<td>0.014</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.70 (±0.02)</td>
<td>0.61 (±0.01)*</td>
<td>0.64 (±0.02)</td>
<td>0.013</td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>16.6 (±0.2)</td>
<td>13.4 (±0.4)*</td>
<td>10.8 (±0.6)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>ND</td>
<td>ND</td>
<td>0.55 (±0.09)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>1.7 (±0.1)</td>
<td>1.6 (±0.1)</td>
<td>1.7 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>8.3 (±0.1)</td>
<td>13.7 (±0.2)*</td>
<td>18.9 (±0.7)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>37.3 (±0.2)</td>
<td>38.3 (±0.4)</td>
<td>38.2 (±0.3)</td>
<td></td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>13.4 (±0.3)</td>
<td>13.1 (±1.2)</td>
<td>11.0 (±0.3)</td>
<td></td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>49.1 (±0.4)</td>
<td>48.5 (±1.4)</td>
<td>50.7 (±0.6)</td>
<td></td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
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<td>32.4 (±1.3)*</td>
<td>28.7 (±1.1)*</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>10.7 (±0.1)</td>
<td>16.1 (±0.4)*</td>
<td>21.9 (±0.7)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ EPA + DHA</td>
<td>8.3 (±0.1)</td>
<td>13.7 (±0.2)*</td>
<td>19.5 (±0.7)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>3.5 (±0.07)</td>
<td>2.0 (±0.07)*</td>
<td>1.3 (±0.09)*†</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 5 per group). ND = Not Detected. *p <0.05 vs Control diet, †p <0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

**Abbreviations:** 14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:1n-7 = vaccenic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
Table 2.7: The effect of fish oil on phospholipid fatty acid composition in the quadriceps (red).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>4.6 (±0.8)</td>
<td>4.9 (±0.3)</td>
<td>5.3 (±0.4)</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>15.5 (±0.3)</td>
<td>16.8 (±0.5)</td>
<td>15.8 (±0.5)</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.82 (±0.09)</td>
<td>1.0 (±0.2)</td>
<td>0.79 (±0.05)</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>18.1 (±0.4)</td>
<td>17.6 (±0.9)</td>
<td>16.9 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>7.4 (±0.2)</td>
<td>8.0 (±0.2)</td>
<td>7.1 (0.3)</td>
<td></td>
</tr>
<tr>
<td>18:1n-7</td>
<td>3.0 (±0.1)</td>
<td>2.7 (±0.08)</td>
<td>2.4 (±0.09)*</td>
<td>0.008</td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>20.5 (±0.7)</td>
<td>17.9 (±0.1)</td>
<td>17.8 (±0.8)*</td>
<td>0.029</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.62 (±0.04)</td>
<td>0.68 (±0.03)</td>
<td>0.5 (±0.02)*</td>
<td>0.022</td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>16.1 (±0.2)</td>
<td>12.2 (±0.3)*</td>
<td>9.3 (±0.5)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>ND</td>
<td>0.31 (±0.02)</td>
<td>0.5 (±0.06)*</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>1.7 (±0.1)</td>
<td>1.7 (±0.1)</td>
<td>1.4 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>11.2 (±0.3)</td>
<td>15.9 (±0.2)*</td>
<td>21.7 (±0.6)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>38.3 (±0.3)</td>
<td>39.4 (±0.6)</td>
<td>38.1 (±0.5)</td>
<td></td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>11.3 (±0.1)</td>
<td>11.8 (±0.4)</td>
<td>10.4 (±0.4)</td>
<td></td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>50.3 (±0.2)</td>
<td>48.6 (±0.5)</td>
<td>51.4 (±0.8)†</td>
<td>0.021</td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
<td>36.7 (±0.5)</td>
<td>30.1 (±0.3)*</td>
<td>27.2 (±0.8)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>13.6 (±0.4)</td>
<td>18.4 (±0.3)*</td>
<td>24.2 (±0.5)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ EPA + DHA</td>
<td>11.2 (±0.3)</td>
<td>16.0 (±0.3)*</td>
<td>22.2 (±0.6)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>2.6 (±0.1)</td>
<td>1.6 (±0.03)*</td>
<td>1.1 (±0.05)*†</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 4 – 5 per group). ND = Not Detected. * Only detected in 3 samples. **p < 0.05 vs Control diet, †p < 0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

Abbreviations: 14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:1n-7 = vaccenic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
quadriceps\textsubscript{(white)} phospholipid DHA composition was significantly greater in the LowFO (14.05\%) and ModFO (20.50\%) groups compared to the Control (8.56\%) group (Table 2.8). EPA was not detected in the quadriceps\textsubscript{(white)} of the Control group but was present in the LowFO and ModFO groups. The Control group displayed the highest AA phospholipid concentration (17.45\%) and reduced in a dose-dependent manner in the LowFO (15.29\%) and ModFO (12.83\%) groups. Other differences unique to the quadriceps\textsubscript{(white)} included a significantly lower concentration of Oleic acid (18:1n-9) in the LowFO and ModFO group compared to the Control group and a significantly lower concentration of LA and Palmitoleic acid in the LowFO group compared to the Control group (Table 2.8).

extensor digitorum longus (EDL) phospholipid DHA composition was significantly greater in the LowFO (15.82\%) and ModFO (20.71\%) groups compared to the Control (10.82\%) group (Table 2.9). EPA was detected in small amounts in the EDL of all groups; however it was significantly greater in both FO supplemented groups. The Control group displayed the highest AA phospholipid concentration (15.85\%) and reduced in a dose-dependent manner in the LowFO (13.49\%) and ModFO (9.96\%) groups. Other differences unique to the EDL included a significantly higher total PUFA concentration in the ModFO group compared to the Control group (Table 2.9).
Table 2.8: The effect of fish oil on phospholipid fatty acid composition in the quadriceps (white).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>5.9 (±0.3)</td>
<td>6.3 (±0.1)</td>
<td>5.1 (±0.1)†</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>21.7 (±0.4)</td>
<td>22.3 (±0.2)</td>
<td>21.9 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.92 (±0.1)</td>
<td>0.82 (±0.03)</td>
<td>0.93 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>14.5 (±0.3)</td>
<td>14.2 (±0.5)</td>
<td>13.7 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>8.7 (±0.1)</td>
<td>7.1 (±0.09)*</td>
<td>7.6 (±0.1)*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>2.9 (±0.1)</td>
<td>2.7 (±0.1)</td>
<td>2.5 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>15.3 (±0.3)</td>
<td>12.7 (±0.6)*</td>
<td>11.6 (±0.07)*</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.96 (±0.05)</td>
<td>0.8 (±0.03)</td>
<td>0.79 (±0.03)</td>
<td></td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>17.4 (±0.6)</td>
<td>15.2 (±0.1)*</td>
<td>12.8 (±0.3)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>ND</td>
<td>0.07 (±0.01)*</td>
<td>0.37 (±0.09)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>2.5 (±0.3)</td>
<td>2.3 (±0.1)</td>
<td>1.7 (±0.3)*</td>
<td>0.045</td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>8.5 (±0.2)</td>
<td>14.0 (±0.4)*</td>
<td>20.5 (±0.3)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>42.5 (±0.9)</td>
<td>43.8 (±0.6)</td>
<td>40.9 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>12.6 (±0.2)</td>
<td>10.7 (±0.2)*</td>
<td>11.1 (±0.09)*</td>
<td>0.021</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>44.8 (±0.9)</td>
<td>45.4 (±0.7)</td>
<td>47.9 (±0.2)*</td>
<td>0.035</td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
<td>32.7 (±0.6)</td>
<td>28.0 (±0.5)*</td>
<td>24.4 (±0.2)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>12.0 (±0.4)</td>
<td>17.3 (±0.4)*</td>
<td>23.4 (±0.2)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ EPA + DHA</td>
<td>8.5 (±0.2)</td>
<td>14.1 (±0.4)*</td>
<td>20.8 (±0.2)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>2.7 (±0.06)</td>
<td>1.6 (±0.04)*</td>
<td>1.0 (±0.02)*†</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 5 per group). ND = Not Detected. *p <0.05 vs Control diet, †p <0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

Abbreviations: 14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:1n-7 = vaccenic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
Table 2.9: The effect of fish oil on phospholipid fatty acid composition in the extensor digitorum longus (EDL).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>3.2 (±0.1)</td>
<td>2.8 (±0.2)</td>
<td>2.8 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>19.8 (±0.3)</td>
<td>20.7 (±0.5)</td>
<td>20.2 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.85 (±0.09)</td>
<td>0.77 (±0.07)</td>
<td>0.82 (±0.06)</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>18.1 (±0.5)</td>
<td>17.2 (±0.2)</td>
<td>16.7 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>8.2 (±0.2)</td>
<td>7.0 (±0.08)</td>
<td>7.9 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>18:1n-7</td>
<td>2.8 (±0.1)</td>
<td>2.5 (±0.1)</td>
<td>2.4 (±0.05)</td>
<td></td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>17.7 (±0.5)</td>
<td>16.7 (±0.7)</td>
<td>15.4 (±0.3)</td>
<td></td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.73 (±0.05)</td>
<td>0.74 (±0.04)</td>
<td>0.71 (±0.02)</td>
<td></td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>15.8 (±0.2)</td>
<td>13.4 (±0.4)*</td>
<td>9.9 (±0.2)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>0.03 (±0.02)</td>
<td>0.15 (±0.04)*</td>
<td>0.48 (±0.04)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>1.6 (±0.09)</td>
<td>1.7 (±0.05)</td>
<td>1.6 (±0.04)</td>
<td></td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>10.8 (±0.2)</td>
<td>15.8 (±1.1)*</td>
<td>20.7 (±0.4)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>41.2 (±0.3)</td>
<td>40.8 (±0.4)</td>
<td>39.8 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>11.9 (±0.2)</td>
<td>10.3 (±0.2)</td>
<td>11.2 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>46.7 (±0.5)</td>
<td>48.7 (±0.5)</td>
<td>48.9 (±0.4)*</td>
<td>0.047</td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
<td>33.5 (±0.3)</td>
<td>30.2 (±0.8)*</td>
<td>25.3 (±0.2)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>13.2 (±0.1)</td>
<td>18.4 (±1.0)*</td>
<td>23.5 (±0.5)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Σ EPA + DHA</td>
<td>10.8 (±0.2)</td>
<td>15.9 (±1.1)*</td>
<td>21.1 (±0.4)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>2.5 (±0.02)</td>
<td>1.6 (±0.1)*</td>
<td>1.1 (±0.03)*†</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 4 – 5 per group). *Only detected in 2 samples. *p < 0.05 vs Control diet, †p < 0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

Abbreviations: 14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:1n-7 = vaccenic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
gastrocnemius\(_{\text{red}}\) phospholipid DHA composition was significantly greater in the LowFO (19.32%) and ModFO (23.07%) groups compared to the Control (12.61%) group (Table 2.10). EPA was detected in small amounts in the gastrocnemius\(_{\text{red}}\) of all groups; however it was significantly greater in both FO supplemented groups. The Control group displayed the highest AA phospholipid concentration (15.83%) and reduced in a dose-dependent manner in the LowFO (12.29%) and ModFO (9.44%) groups. Other differences unique to the gastrocnemius\(_{\text{red}}\) included a significantly lower concentration of Vaccenic acid, ALA and total MUFA in the LowFO and ModFO groups compared to the Control group. The ModFO group also showed a significantly lower amount of ALA compared to the LowFO group (Table 2.10).
### Table 2.10: The effect of fish oil on phospholipid fatty acid composition in the gastrocnemius (red).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>3.9 (±0.1)</td>
<td>4.9 (±0.4)</td>
<td>4.1 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>16.5 (±0.4)</td>
<td>16.0 (±0.2)</td>
<td>17.3 (±0.5)</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.23 (±0.08)</td>
<td>0.20 (±0.01)</td>
<td>0.23 (±0.07)</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>17.0 (±0.4)</td>
<td>16.4 (±0.3)</td>
<td>17.4 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>8.3 (±0.2)</td>
<td>7.6 (±0.04)</td>
<td>7.6 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>18:1n-7</td>
<td>2.8 (±0.1)</td>
<td>2.3 (±0.08)*</td>
<td>2.2 (±0.06)*</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>19.7 (±0.8)</td>
<td>18.1 (±0.7)</td>
<td>16.0 (±0.4) *</td>
<td>0.01</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.70 (±0.03)</td>
<td>0.59 (±0.07)*</td>
<td>0.49 (±0.07)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:4n-6 (AA)</td>
<td>15.8 (±0.4)</td>
<td>12.2 (±0.1)*</td>
<td>9.4 (±0.3)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>0.11 (±0.01)</td>
<td>0.28 (±0.01)*</td>
<td>0.54 (±0.06)*†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>1.9 (±0.1)</td>
<td>1.6 (±0.1)</td>
<td>1.3 (±0.1) *</td>
<td>0.021</td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>12.6 (±0.2)</td>
<td>19.3 (±0.2)*</td>
<td>23.0 (±0.5)*†</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

|         |         |         |         |         |
| Σ SFA   | 37.6 (±0.2) | 37.4 (±0.3) | 38.9 (±0.7) |           |
| Σ MUFA  | 11.4 (±0.2) | 10.2 (±0.1)* | 10.1 (±0.3) * | < 0.01    |
| Σ PUFA  | 50.9 (±0.3) | 52.3 (±0.4) | 50.9 (±0.7) |           |
| Σ n-6 PUFA | 35.6 (±0.5) | 30.4 (±0.6)* | 25.5 (±0.7)*† | < 0.001 |
| Σ n-3 PUFA | 15.3 (±0.2) | 21.8 (±0.1)* | 25.4 (±0.5)*† | < 0.001 |
| Σ EPA + DHA | 12.7 (±0.2) | 19.6 (±0.2)* | 23.6 (±0.5)*† | < 0.001 |
| n-6:n-3 | 2.3 (±0.06) | 1.3 (±0.04)* | 1.0 (±0.04)*† | < 0.001 |

Values are mean ± SEM ($n = 5$ per group). *$p < 0.05$ vs Control diet, †$p < 0.05$ vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

**Abbreviations:** 14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1n-9 = oleic acid; 18:1n-7 = vaccenic acid; 18:2n-6 = linoleic acid (LA); 18:3n-3 = α-linolenic acid (ALA); 20:4n-6 = arachidonic acid (AA); 20:5n-3 = eicosapentaenoic acid (EPA); 22:5n-3 = docosapentaenoic acid (DPA); 22:6n-3 = docosahexaenoic acid (DHA). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
2.4.2a Differences in phospholipid composition between muscles following fish oil supplementation

Total phospholipid LC n-3 PUFA, particularly DHA following dietary FO supplementation was significantly greater in cardiac muscle and across all skeletal muscles sampled in the hind-limb for the LowFO and ModFO groups compared to the Control group. The lowest concentration of phospholipid DHA were observed in the Control group left ventricle (5.02%) phospholipids and the greatest DHA concentrations were observed in the gastrocnemius(red) (23.07%) skeletal muscle phospholipids of the ModFO group (Figure 2.2). When DHA was plotted against the percentage of muscle fibre type typical for each muscle there was a strong association between % fast oxidative glycolytic and % phospholipid DHA (Spearman correlation \( \rho = 0.91, r^2 = 0.87 \)). However, no relationship was apparent between % fast glycolytic or % slow oxidative fibres (Figure 2.3). The association was even stronger when the left ventricle (not containing FOG) was included in the analysis (\( \rho = 0.94, r^2 = 0.89 \)). Interestingly, different muscle’s with unique fibre type profiles such as the Control gastrocnemius(red) (%FOG: 62) and LowFO soleus (%FOG: 13) showed ‘overlap’ of DHA concentrations (Control gastrocnemius DHA: 12.61%, LowFO soleus DHA: 13.79%) when comparing between dietary treatments.

Total phospholipid n-6 PUFA following dietary supplementation was significantly lower in cardiac muscle and across all skeletal muscles sampled in the hind-limb for the LowFO and ModFO groups compared to the Control group. The lowest concentration of phospholipid AA were observed in the ModFO gastrocnemius(red) (9.44%) skeletal muscle phospholipids and the greatest phospholipid AA concentrations were observed in the left ventricle (19.74%) phospholipids of the Control group. Additionally, LA was significantly lower in all muscles of the ModFO group except the EDL when compared to the Control group. These changes in muscle phospholipid composition were reflected by a significantly lower n6:n3 ratio across all muscle in the LowFO and ModFO groups following supplementation compared to the Control group.
Figure 2.2: The effect of supplementing dietary achievable fish oil doses on phospholipid concentrations of DHA and AA across different muscles.

Values are mean ± SEM (n = 4 – 5 per group). *p <0.05 vs Control diet, †p <0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).

Abbreviations: EDL = extensor digitorum longus; DHA = docosahexaenoic acid; AA = arachidonic acid.
Figure 2.3: Spearman correlations of DHA incorporation across cardiac and skeletal muscles with different proportions of muscle fibre types.

Overlap Zone: A potential area where similar metabolic properties may be displayed in muscles with different fibre type composition but similar DHA concentration following supplementation. For example, the LowFO muscle with 0% FOG fibres shares similar DHA levels to the Control muscle with over 60% FOG fibres.
2.4.3 The effect of fish oil supplementation on tissue mass

The mass of the muscles collected in this study was comparable to previously published data in the rat (Armstrong & Phelps, 1984). Total heart mass and tibia length were in the healthy range (Yin et al., 1982). Total heart mass was not significantly different between groups and this did not change when normalised to tibia length (Table 2.11). Individual hind-limb skeletal muscles were anatomically referenced to the upper hind-limb or lower hind-limb to calculate a total mass. There were no significant differences demonstrated between any of the groups for total upper hind-limb or lower hind-limb muscle mass. Comparison of individual muscle mass was also completed. There were no significant differences between dietary groups for the mass of any of the individual muscles measured (Table 2.11). Muscles were categorised into fibre type groups (SO, FG, FOG) based on the proportion of muscle fibres present within the muscle (Armstrong & Phelps, 1984). When analysing muscles categorised as SO, FG or FOG the gastrocnemius\textsubscript{(mixed)} and quadriceps\textsubscript{(mixed)} were not included as it is unclear what the fibre type proportions would have been in these muscles. There were no significant differences between any of the groups when muscle were analysed in this way (Table 2.11).
Table 2.11: The effect of fish oil on tissue mass in the rat.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tibial length (cm)</strong></td>
<td>4.6 (±0.04)</td>
<td>4.68 (±0.09)</td>
<td>4.63 (±0.04)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Cardiac muscle mass (g)</strong></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total heart</td>
<td>1.51 (±0.05)</td>
<td>1.55 (±0.05)</td>
<td>1.62 (±0.04)</td>
</tr>
<tr>
<td>Ventricular</td>
<td>1.27 (±0.03)</td>
<td>1.28 (±0.03)</td>
<td>1.32 (±0.03)</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>0.98 (±0.02)</td>
<td>0.99 (±0.03)</td>
<td>1.04 (±0.03)</td>
</tr>
<tr>
<td>Total heart/Tibial length</td>
<td>0.32 (±0.01)</td>
<td>0.34 (±0.01)</td>
<td>0.35 (±0.01)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Skeletal muscle mass (g)</strong></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower hind-limb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>soleus</td>
<td>0.19 (±0.03)</td>
<td>0.18 (±0.02)</td>
<td>0.24 (±0.03)</td>
</tr>
<tr>
<td>gastrocnemius&lt;sub&gt;(red)&lt;/sub&gt;</td>
<td>0.23 (±0.03)</td>
<td>0.24 (±0.01)</td>
<td>0.29 (±0.04)</td>
</tr>
<tr>
<td>gastrocnemius&lt;sub&gt;(white)&lt;/sub&gt;</td>
<td>0.22 (± 0.02)</td>
<td>0.20 (±0.01)</td>
<td>0.17 (±0.02)</td>
</tr>
<tr>
<td>gastrocnemius&lt;sub&gt;(mixed)&lt;/sub&gt;</td>
<td>2.03 (±0.09)</td>
<td>2.02 (±0.06)</td>
<td>1.77 (±0.07)</td>
</tr>
<tr>
<td>plantaris</td>
<td>0.49 (±0.02)</td>
<td>0.52 (±0.04)</td>
<td>0.47 (±0.03)</td>
</tr>
<tr>
<td>extensor digitorum longus</td>
<td>0.19 (±0.01)</td>
<td>0.17 (±0.02)</td>
<td>0.20 (±0.01)</td>
</tr>
<tr>
<td>Total – Lower hind-limb</td>
<td>3.39 (±0.12)</td>
<td>3.31 (±0.07)</td>
<td>3.33 (±0.27)</td>
</tr>
</tbody>
</table>

| Upper hind-limb               |          |          |          |
| quadriceps<sub>(red)</sub>    | 0.26 (±0.02) | 0.25 (±0.01) | 0.31 (±0.03) |
| quadriceps<sub>(white)</sub>  | 0.30 (±0.02) | 0.29 (±0.01) | 0.29 (±0.03) |
| quadriceps<sub>(mixed)</sub>  | 2.23 (±0.11) | 2.28 (±0.20) | 2.38 (±0.14) |
| adductor longus               | 0.14 (±0.03) | 0.11 (±0.01) | 0.10 (±0.02) |
| pectineus                     | 0.14 (±0.04) | 0.12 (±0.02) | 0.13 (±0.02) |
| Total – Upper hind-limb       | 2.97 (±0.15) | 3.08 (±0.19) | 3.20 (±0.17) |

| **Fibre type**                |          |          |          |
| Slow oxidative               | 0.34 (±0.03) | 0.29 (±0.02) | 0.42 (±0.27) |
| Fast glycolytic              | 0.88 (±0.06) | 0.80 (±0.03) | 0.81 (±0.04) |
| Fast oxidative glycolytic    | 1.03 (±0.05) | 0.99 (±0.05) | 1.07 (±0.08) |

Values are mean ± SEM (n = 12 – 16 per group). ^ Total heart mass included the atria but not the great vessels. # Ventricular mass included the left and right ventricle. No statistical significant differences were present between dietary groups.
2.4.4 The effect of fish oil supplementation on body weight and food consumption

Body weight and food consumption were monitored in a subgroup from the Control, LowFO and ModFO diets throughout the feeding protocol. There were no significant differences between the baseline body weights of all groups prior to dietary supplementation (Table 2.12). Body weight significantly increased at each week of measurement for all groups (Figure 2.4A). However, there were no significant differences between any of the groups for total weight gain. The ModFO group displayed a significantly greater weight gain to week three and four compared to the Control group (Figure 2.4B). At week four the LowFO group had significantly greater weight gain compared to the Control group. At the end of the feeding protocol, there were no significant differences between the groups in post supplementation body weight or body weight normalised to tibia length (Table 2.10).

Table 2.12: The effect of fish oil on body weight measurements.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (pre) (g)</strong></td>
<td>426 (±9)</td>
<td>434 (±11)</td>
<td>425 (±9)</td>
</tr>
<tr>
<td><strong>Body weight (post) (g)</strong></td>
<td>512 (±16)</td>
<td>540 (±18)</td>
<td>542 (±14)</td>
</tr>
<tr>
<td><strong>Body weight (post)/Tibial length</strong></td>
<td>109 (±3.5)</td>
<td>116 (±4.2)</td>
<td>116 (±2.9)</td>
</tr>
</tbody>
</table>

**Key:** Pre = Prior to fatty acid supplementation, Post = Following 4 weeks of dietary fatty acid supplementation.
Figure 2.4: The effect of fish oil on A: Change in body weight from baseline B: Body weight at weekly intervals.

Values are mean ± SEM (n = 20 – 22 per group). *p < 0.05 vs Control diet, †p < 0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test).
Food intake is represented as grams consumed/100grams body weight/day. Food intake was reduced at each week of the feeding period by all groups (Figure 2.5). There was no statistically significant interaction found between dietary treatment and food intake over the four-week feeding period ($p = 0.220$).

**Figure 2.5:** The effect of fish oil on food intake at weekly intervals.

Values are mean ± SEM ($n = 20 – 22$ per group). All values are in grams / 100grams body weight / day.
2.5 DISCUSSION

Excitable tissues incorporated LC n-3 PUFA, particularly DHA following supplementation of FO doses achievable within a typical Western-style human diet (high SFA and n-6 PUFA). Compared to the Control group, concentrations of DHA in myocardial phospholipids were more than twice as high in the LowFO diet and approximately three-fold higher in the ModFO group; some skeletal muscle phospholipids displayed even greater changes. Higher phospholipid concentration of DHA predominantly occurred at the expense of n-6 PUFA, particularly AA, although it was not in direct exchange for only one type of fatty acid or even for n-6 PUFA entirely. For the first time, this study demonstrated that DHA is incorporated preferentially into predominantly fast oxidative glycolytic (FOG) fibre type skeletal muscle membranes compared to slow oxidative (SO) and fast glycolytic (FG) fibre type membranes. Nonetheless, FO supplementation did not influence individual skeletal muscle mass or total hind-limb skeletal muscle mass. However, and in contrast to the fourth hypothesis, relative change of body weight at the completion of supplementation was greater in the FO diets. The robust and preferential incorporation of DHA into excitable membrane phospholipids when made available via the diet emphasises the important role it plays in the healthy physiological functioning of excitable tissue.

2.5.1 Excitable tissue phospholipid LC n-3 PUFA concentrations

Following supplementation of dietary achievable FO doses to animals, substantial changes were observed in the membrane phospholipid fatty acid composition of all excitable tissues measured. The current study is the first to report changes in cardiac and skeletal muscles encompassing a full range of contractile properties. The doses used in this study were chosen as they are equivalent to what can be achieved in the human diet either by fish consumption or via supplementation (Table 2.1). A 100g serve of salmon has been demonstrated to contain 2000mg of LC n-3 PUFA, in the form of EPA ≈550mg and DHA ≈1450mg (USDA, 2009). Considering an average salmon portion is ≈250g, this would require only a single salmon meal per week to achieve equivalent levels of incorporation as the LowFO dose. The ModFO dose would represent approximately 7-8 fish meals per week which would be towards the upper boundary of human dietary achievable doses but is also achievable by taking six FO capsules per day.
(Slee et al., 2010). Therefore, the doses used in this study indicate that a modest increase in fish intake in humans would alter phospholipid DHA incorporation and could contribute to some of the physiological benefits displayed in populations with high habitual intakes of fish.

Higher total phospholipid LC n-3 PUFA concentrations were driven via DHA that appeared to be preferentially incorporated into excitable phospholipids rather than EPA. Incorporation of DHA into excitable membrane phospholipids was largely due to the DHA-rich tuna oil used in the FO diets which contains 30% DHA and only 7% EPA. Interestingly, myocardial phospholipid DHA composition has been demonstrated to increase (15.4 > 27.2, ↑11.8%) more than phospholipid EPA (0.3 > 3.1, ↑2.8%) composition even when a high EPA oil is used (Pepe & McLennan, 1996). Notwithstanding, DHA-rich tuna oil was specifically used to better replicate the oils available in typical oily table fish. Table fish such as tuna, salmon, sardine, mackerel and anchovy contain naturally higher concentrations of DHA than EPA (Strobel et al., 2012). Whereas, the supplements used in the majority of RCT predominantly have much higher doses of EPA than DHA (Bays, 2006). Therefore, the use of DHA-rich tuna oil in future RCT would allow stronger conclusions to be drawn between the outcomes described and directly relating them to an increase in fish intake in the human diet.

The current study demonstrated that when n-6 PUFA and SFA were held constant, in-line with levels consumed in the typical Western diet, the supplementation of dietary achievable FO doses resulted in higher phospholipid DHA concentrations in excitable tissues of the animals. It has been well established that incorporation of fatty acids into excitable membrane phospholipids of the rat are reflective of the fatty acids being supplied in the diet, particularly at therapeutic and supra-therapeutical doses, not easily achievable in the human diet (Charnock et al., 1986; Peoples & McLennan, 2010; Pepe & McLennan, 2002). However, in recent centuries the typical n-6 to n-3 ratio in humans has increased to ≈10:1 from an estimated 1:1 prior to the agricultural revolution (DeFilippis & Sperling, 2006). It has been suggested that it is essential to reduce the n-6:n-3 ratio, by altering intakes of both n-3 and n-6 PUFA, to reduce competition for phospholipid space and enable phospholipid incorporation of LC n-3 PUFA (Hulbert et al., 2005; Simopoulos, 2008). However, the recommendation to balance the n-6:n-3
ratio is based on trials which have altered the amount of LC n-3 PUFA supplied while maintaining n-6 PUFA levels (Ayre & Hulbert, 1996a; Hulbert et al., 2005). Studies which have altered both sub-classes of PUFA, demonstrate that incorporation of DHA into myocardial phospholipids occurs relative to the absolute LC n-3 PUFA supplied in the diet, regardless of background concentrations of n-6 PUFA (Slee et al., 2010; Stark et al., 2007a; Stark et al., 2007b). Comparison of myocardial phospholipid incorporation patterns between studies using dietary achievable FO doses demonstrates that DHA is preferentially incorporated into myocardial phospholipids, regardless of the background fat profile (Table 2.13). Results from this study support the latter observations and extend them to demonstrate that high concentrations of SFA together with high concentrations n-6 PUFA does not prevent the incorporation of DHA into excitable phospholipids of the rat. In fact, the incorporation of DHA into myocardial and skeletal phospholipids resulted in a concomitant reduction of AA in all tissues measured, with some equal and smaller decreases in LA in some tissues, causing a reduction in total n-6 PUFA content. These changes resulted in a reduction of the n3:n6 ratio of the phospholipid without changing the total n-6 PUFA being supplied in the diet.

Table 2.13: Comparison of myocardial phospholipid DHA concentrations from studies using diets with different background oil blends.

<table>
<thead>
<tr>
<th>Study</th>
<th>Background oil blend</th>
<th>Membrane phospholipid DHA composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0.31%FO</td>
</tr>
<tr>
<td>Henry et al. (2015)</td>
<td>OO</td>
<td>6.2</td>
</tr>
<tr>
<td>Slee et al. (2010)</td>
<td>OO</td>
<td>7.7</td>
</tr>
<tr>
<td>Slee et al. (2010)</td>
<td>OO/SSO (High n-6 PUFA)</td>
<td>6.7</td>
</tr>
<tr>
<td>Current study</td>
<td>SFA/SSO (High SFA &amp; n-6 PUFA)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Abbreviations: SFA = saturated fatty acid; OO = olive oil; SSO = sunflower seed oil; ∆ = change from control.

Interestingly, phospholipid DHA composition was not absent in Control animals despite LC n-3 PUFA being removed from the diet. Animals in the Control diet likely obtained their phospholipid DHA from several sources. Long chain essential fatty acids,
particularly DHA and AA are crucial in the healthy development of the foetus neural and vascular systems (Crawford, 1993; Crawford et al., 1997). Because of this, these fatty acids are incorporated very efficiently from maternal into foetal phospholipids via the placenta throughout the third trimester of pregnancy in a process known as biomagnification (Ruyle et al., 1990). Additionally, it is well established that the newborn continues to receive DHA and AA postnatally through maternal breast milk (Makrides et al., 1996). This means the newborn tissue is supplied with abundant DHA and is one of the reasons why deficiency studies need to use two generations of animals to produce a truly DHA-deficient model (Moriguchi et al., 2001).

Another possible source of DHA in the Control animals is via conversion from ALA that was present in small amounts from OO using Δ six desaturase enzymes (Gibson et al., 2013). The conversion of EPA to DHA using this pathway also explains why EPA was either not detected or only detected in very small amounts, yet DHA was present in Control animal phospholipids. Incorporation of DHA may have also occurred during the lab chow feeding before the beginning of the animals being placed on the experimental diets. Lab chow contains a mixture of protein sources, one of which is fishmeal containing LC n-3 PUFA (Pepe & McLennan, 1996). Trace amounts of ALA and LC n-3 PUFA may also have been present in the Control diet due to their presence in the casein and starch being used in the diet mixture and contamination of equipment used to mix the different diets (Greiner et al., 2003). Importantly, this demonstrates the strong response of excitable tissue including cardiac and skeletal muscle to preferentially incorporate and maintain even very small amounts of DHA made available.

2.5.2 Excitable tissue membrane phospholipid fatty acid differences

Very few studies have investigated the specific incorporation patterns of DHA throughout muscle fibre types, and the conclusions have been mixed. Some studies have demonstrated that DHA levels are highest in SO muscles such as the soleus (Blackard et al., 1997; Kriketos et al., 1995) but more recently it has been demonstrated that DHA levels are higher in mixed FOG and FG fibres of the gastrocnemius (Henry et al., 2015). In this study, muscles with an abundance of SO fibres such as the soleus and quadriceps\textsubscript{(white)} had the lowest concentration of DHA, whereas muscles with high proportions of FOG fibres such as the gastrocnemius\textsubscript{(red)} and quadriceps\textsubscript{(red)} had the
highest DHA concentrations. Therefore, this study supports the findings demonstrated by Henry et al. 2015, and extends them to demonstrate that DHA incorporation into skeletal muscles is strongly correlated with the population of FOG muscle fibres in the rat hind-limb. The contrasting findings demonstrated by Kriketos et al. 1995 and Blackard et al. 1997 can be accounted for by differences in dietary feeding protocols. The current study used a diet which contained 10% fat wt/wt, whereas the diet used by Kriketos et al. 1995 contained 59% fat wt/wt, which does not represent a diet relevant to human nutrition and likely influenced muscle phospholipid fatty acid incorporation patterns. Animals in the study conducted by Blackard et al. 1997 were fed lab chow ad libitum before measurements were made. Lab chow contains a mixture of protein sources, one of which is fishmeal containing DHA (Pepe & McLennan, 1996). Incorporation of DHA may have occurred as a result of lab chow feeding artificially increasing phospholipid DHA concentrations of the soleus. Importantly, Control animals in this study were fed a pre-fabricated diet which did not contain any DHA and all muscles compared were sampled from within the same animal, allowing a precise representation of incorporation patterns. Therefore, this study investigated tissue DHA incorporation in a systematic way, confirming differences and establishing a pattern associated with muscle fibre type. For the first time, muscle fibre type dependent incorporation of DHA was demonstrated in the rat, with FOG muscles able to preferentially incorporate DHA and maintain it in the phospholipid at higher concentrations than FG and SO muscles, even in the absence of FO supplementation.

Previous research has demonstrated that incorporation of DHA into skeletal muscle membrane phospholipids results in improved fatigue resistance (Peoples & McLennan, 2010), maintained force development over a range of contraction patterns (Henry et al., 2015) and enhanced oxygen efficiency (Peoples & McLennan, 2010). Human studies have also demonstrated several unique physiological roles for FO during exercise which may contribute to improved muscular performance (Mickleborough, 2013) and improved whole body oxygen efficiency (Peoples et al., 2008). Therefore, although not investigated directly in this study, the correlation between greater DHA concentrations and high proportions of FOG muscle fibres is likely linked to the physiological properties of the muscle.
Muscle fibres of the quadriceps\textsubscript{(red)} and gastrocnemius\textsubscript{(red)} are predominantly used for powerful and rapid contractions such as jumping and sprinting. This type of contraction is suited to FOG muscle fibres as it requires a high turnover of rapidly produced ATP via non-oxidative pathways. These types of muscles display a high proportion of the MHC\textsubscript{2} isoform that rapidly fatigues due to high rates of ATP consumption and small ATP reserves (Han \textit{et al.}, 2001). Comparative physiology studies have demonstrated that mammals which rely upon FOG muscle fibres for increased running speeds are closely associated with high concentrations of PUFA in skeletal muscle membrane phospholipids irrespective of the animals diet (Ruf \textit{et al.}, 2006; Valencak \textit{et al.}, 2003). Whereas, cardiac and soleus membranes which, respectively, display high amounts of the MHC\textsubscript{β} and MHC\textsubscript{slow} isoform, had the least amount of DHA. These MHC isoforms are associated with low basal rates of ATP consumption and high ATP reserve capacity to serve their similar physiological function of slow and regular contractions without fatigue (Han \textit{et al.}, 2001). Therefore, differences in ATP turnover and storage displayed within these isoforms of MHC may be one reason for the associated differences in DHA incorporation displayed between FOG and SO muscles in this study.

It is well-established that mitochondrial membrane phospholipids are intricately involved in the electron transfer and oxidative phosphorylation pathways of ATP production (Fleischer \textit{et al.}, 1962). Due to the reliance upon ATP, the ventricle and soleus contain very high concentrations of mitochondria relative to FOG muscles fibres of the quadriceps\textsubscript{(red)} and gastrocnemius\textsubscript{(red)}, which predominantly use non-oxidative metabolism to generate ATP. Mitochondrial phospholipids naturally contain less PUFA than the whole tissue; this difference in phospholipid composition is proposed to be a result of selective pressure towards oxidative damage resistant membranes to counteract the increased concentrations of reactive oxygen species in the vicinity (Tsalouhidou \textit{et al.}, 2006). Membrane fractions were not separated in this study when measuring fatty acid composition. This is important because the mitochondrial membrane would contain lower relative concentrations of PUFA, which would contribute to reduced whole tissue phospholipid LC n-3 PUFA. Therefore, the lower phospholipid DHA concentrations measured in ventricular and soleus tissue could also be associated with the overall high proportion of mitochondrial membranes making up the whole tissue measurement.
Cardiac and skeletal muscles are intimately reliant upon the efficient movement and handling of Ca\(^{2+}\) in the sarcoplasmic reticulum (SR). In particular FOG muscle fibres have been demonstrated to have extensive networks of SR (Welch & Altshuler, 2009), and these muscles were associated with much higher levels of DHA. Phospholipids in the rabbit (Gould et al., 1987) and trout SR have been demonstrated to incorporate DHA at high levels, even when the diet is deficient of PUFA (Ushio et al., 1997). Higher phospholipid DHA concentrations in SR phospholipids helps explain why SR-rich predominantly FOG muscle fibres of the quadriceps\(_{\text{red}}\) and gastrocnemius\(_{\text{red}}\) were observed to have the highest phospholipid concentrations of DHA in this study. The SR is responsible for excitation-contraction coupling in both cardiac and skeletal muscle. This is an energy dependent pathway which relies on the sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) active pump.

It is well established that membrane protein interactions and function can be affected by changing the phospholipid fatty acid composition (Bretscher, 1973). The role of SERCA in the membrane is to transfer Ca\(^{2+}\) from the cytosol to the lumen of the SR via active ATP hydrolysis during muscle relaxation (Endo, 1977). Therefore, muscles made up of majority FOG fibres which produce rapid and powerful contractions such as the quadriceps\(_{\text{red}}\), and gastrocnemius\(_{\text{red}}\) rely upon SERCA to cycle Ca\(^{2+}\) efficiently to allow repeated contractions. Comparative biology studies have shown that pectoral muscles in the hummingbird or shaker muscles in the rattlesnake which both contract very rapidly and require efficient SERCA activity incorporate LC n-3 PUFA, particularly DHA preferentially into the SR compared to other muscles within the animal (Infante et al., 2001). Additionally, skeletal muscle in rat’s supplemented FO has been demonstrated to be more resistant to fatigue and able to maintain contractile force production (Henry et al., 2015; Peoples & McLennan, 2010, 2014). Therefore, it appears likely that when made available in the diet, DHA is preferentially incorporated into FOG membranes because of the high proportion of SR membrane, leading to improved SERCA activity.
2.5.3 Skeletal muscle mass

There was no evidence of catabolic or anabolic properties associated with FO supplementation from the skeletal muscle mass measured in healthy animals from this study. This finding contrasts the demonstration of FO producing anabolic effects in healthy elderly subjects with (Da Boit et al., 2016) or without resistance training (Smith et al., 2015). However, the Control group in the study conducted by Smith et al. 2015, on average had a reduction of 1% thigh muscle volume, and the FO group showed an increase of 2%. The catabolic changes observed in the Control groups muscle volume were much greater (~1% over six months) than what normally occurs with ageing (~0.5%-1%/y) (Goodpaster et al., 2006). This made the overall muscle volume change effect between groups larger than if FO had only been compared to the group’s relative baseline levels.

The evidence provided of an anabolic effect of FO supplementation in these studies is when the skeletal muscles are in a state of decay e.g., sarcopenia associated with ageing rather than a healthy state which is likely why they contrast to the current findings of this study. In fact, eight weeks of FO supplementation in healthy, resistance-trained males has been shown to have no effect on muscle protein synthesis (McGlory et al., 2016). Several recent reviews have also come to similar conclusions that FO supplementation results in an anti-catabolic rather than an anabolic effect in skeletal muscle (Jeromson et al., 2015), particularly in older individuals (Molfino et al., 2014). A large-scale three-year cross-sectional study of elderly patients supplemented FO demonstrated protection against physical performance decline (Abbatecola et al., 2009). The maintenance of physical performance was likely due to attenuation of muscle mass loss rather than increased muscle protein synthesis. Animal studies have demonstrated that muscle mass loss is attenuated in the tibialis anterior during a cachetic cancer model (van Norren et al., 2009) and also in a burn injury model using the guinea pig (Alexander et al., 1986). Similarly, soleus muscle atrophy measured via muscle mass and myosin heavy chain levels was alleviated in an immobilisation protocol of rats fed a diet rich in FO (You et al., 2010). Lean body mass is also preserved in patients following oesophageal cancer surgery when EPA enriched enteral nutrition is used following the surgery (Ryan et al., 2009). When these findings are combined with results observed in this study, it does not seem likely that dietary achievable FO doses
induce an anabolic or catabolic effect in skeletal muscle. Dietary achievable FO doses may play a role in attenuating muscle loss in diseased conditions or conditions of muscle decline such as sarcopenia associated with ageing. However, further research is needed to understand whether it has a role in promoting anabolic actions in healthy skeletal muscle.

2.5.4 Body weight management

Both FO groups had gained significantly more weight than the Control group by week four of the feeding protocol, despite no increases in skeletal muscle mass and no changes observed in food intake. This indicates that FO did not restrict body weight gain in healthy lean animals following four weeks of supplementation with dietary achievable doses. This finding aligns with non-obesogenic animal studies which consistently demonstrate that there is no significant effect of FO on weight management when using dietary achievable, therapeutic and supra-therapeutical doses of FO, and feeding durations ranging from four weeks to thirty months (Table 2.14). However, these findings along with the findings from the current study are in contrast to some studies in rodents that have demonstrated an attenuation of body fat accumulation in growing (Baillie et al., 1999; Hainault et al., 1993) and adult (Belzung et al., 1993; Huang et al., 2004; Ruzickova et al., 2004) animals exposed to obesogenic high-fat diets incorporating supra-therapeutic doses of LC n-3 PUFA. The conflicting conclusions between studies investigating a weight management effect of FO can be explained by the trial design and dietary supplementation methods used.

The anti-obesity effect is consistently attributed to reductions in visceral adipose tissue deposition (Baillie et al., 1999; Hainault et al., 1993; Huang et al., 2004; Ruzickova et al., 2004). Diets high in SFA promote fat deposition to visceral adipose tissue (Rosqvist et al., 2014). The diets used in this study contained 10% background fat made up by 5% animal fat (high in SFA), 2.5% SSO (high in n-6 PUFA) and 2.5% OO (high in MUFA, which was substituted to make FO diets) to replicate a typical Western-style dietary fat distribution. Importantly, the diets in this study did not exchange SFA out of the diet for increased amounts of FO as is commonly performed in obesogenic models (Baillie et al., 1999; Hainault et al., 1993; Huang et al., 2004; Ruzickova et al., 2004). It has also been hypothesized that appetite suppression may be a mechanism by which FO can lead
to weight reduction (Thorsdottir et al., 2007). However, there were no significant differences in food intake levels in FO supplemented animals in this study, in line with several other animal studies (Kamolrat et al., 2013; Peoples & McLennan, 2014).

Interestingly, some human studies have also suggested that supplementing LC n-3 PUFA to the diet has a weight loss effect (Couet et al., 1997; Kabir et al., 2007), particularly when supplementation occurs in combination with exercise training (Buckley & Howe, 2009, 2010). The results from the current study showing greater weight gain, aligns with evidence of increased oxygen efficiency following FO supplementation in animals (Pepe & McLennan, 2002; Pepe & McLennan, 2007) and humans (Hingley et al., 2017; Peoples et al., 2008). This suggests that there is no potential for FO alone to produce a weight reduction effect and perhaps its efficacy in conjunction with exercise is due in part to the ability of FO to increase exercise capacity through improved fatigue-resistance (Henry et al., 2015; Hingley et al., 2017; Peoples & McLennan, 2010). Therefore, it appears that in the presence of unchanged SFA intakes, dietary achievable and therapeutic FO doses alone do not produce a weight loss effect but may provide fatigue-resistance to enhance the weight loss effects attributable to exercise.

2.5.5 Conclusions

It is now well established that obesity may lead to the development of hypertension, type II diabetes and atherosclerosis which are all high-risk factors for cardiovascular disease (Yusuf et al., 2001). Collectively, previous epidemiology, intervention trials and animal research has demonstrated a cardio-protective effect of fish consumption (Burr et al., 1989; McLennan, 2014; Mozaffarian & Wu, 2011). It is well established that obesity is associated with CVD outcomes, as such it has been hypothesised that the ability of FO to aid weight loss in obesity may reduce the risk of obesity driven CVD outcomes (Buckley & Howe, 2010). However, this study showed results that FO doses achievable within a typical Western-style human diet did not have a weight reduction action. This suggests that FO at these doses has questionable relevance to weight gain management and is likely acting via another mechanism (anti-arrhythmic, heart rate lowering, improving heart rate variability) to provide protection from cardiovascular disease, which will be explored further in the proceeding chapters. Confirmation of
DHA phospholipid incorporation allows any altered cardiovascular function demonstrated in the proceeding chapters to be confidently related directly to higher myocardial phospholipid DHA concentrations.
Table 2.14: Comparison of the effect of fish oil on body weight in animals from different studies.

<table>
<thead>
<tr>
<th>FO Dose</th>
<th>Animal</th>
<th>Duration of feeding</th>
<th>Outcome</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1.25%</td>
<td>Human</td>
<td><strong>Human dietary achievable (≈2 serves salmon/wk – 6 capsules/day)</strong></td>
<td>↔ Body weight, muscle mass or muscle mass:tibia length</td>
<td>(Henry et al., 2015)</td>
</tr>
<tr>
<td>Rat (Sprague Dawley)</td>
<td>15 weeks</td>
<td>↔ Body weight, muscle mass or muscle mass:tibia length</td>
<td>(Henry et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>Rat (Sprague Dawley)</td>
<td>12 weeks</td>
<td>↔ Energy intake, body weight or body weight gain</td>
<td>(Slee et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>1.25 – 6%</td>
<td>Human therapeutic range (≈6 capsules/day – 28 capsules/day)</td>
<td>↔ Food intake, body weight or body composition</td>
<td>(Kamolrat et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Rat (Rowett)</td>
<td>8 weeks</td>
<td>↔ Tibia length, body weight, body size or heart mass</td>
<td>(McLennan et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Rat (Sprague Dawley)</td>
<td>12 weeks</td>
<td>↔ Energy intake, body weight or body weight gain</td>
<td>(Slee et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Rat (Sprague Dawley)</td>
<td>12 weeks</td>
<td>↔ Food intake, body weight or muscle mass.</td>
<td>(Peoples &amp; McLennan, 2014)</td>
<td></td>
</tr>
<tr>
<td>Rat (Wistar)</td>
<td>8 weeks</td>
<td>↔ Food intake, body weight or muscle mass.</td>
<td>(Abdukeyum et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>Rat (Wistar)</td>
<td>6 weeks</td>
<td>↔ Body weight, body size as indicated by tibia length or heart mass</td>
<td>(Pepe &amp; McLennan, 1996)</td>
<td></td>
</tr>
<tr>
<td>Rat (Wistar)</td>
<td>16 weeks</td>
<td>↔ Final body weight and body weight gain.</td>
<td>(Belzung et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>Rat (Wistar)</td>
<td>4 weeks</td>
<td>↔ Body weight or heart mass</td>
<td>(Abeywardena et al. 1991)</td>
<td></td>
</tr>
<tr>
<td>Marmoset Monkey</td>
<td>30 months</td>
<td>↔ Weight gain or food intakes</td>
<td>(Charnock et al., 1992)</td>
<td></td>
</tr>
<tr>
<td>Rat (Wistar)</td>
<td>10 months</td>
<td>↔ Weight gain or food intakes</td>
<td>(McIntosh et al., 1985)</td>
<td></td>
</tr>
<tr>
<td>Rat (Wistar)</td>
<td>8 months</td>
<td>↔ Weight gain or food intakes</td>
<td>(McIntosh et al., 1985)</td>
<td></td>
</tr>
</tbody>
</table>

↔ = No significant difference between FO and Control group.
CHAPTER 3

General Methods
3.1 INTRODUCTION

Cardiac function has been demonstrated to be positively influenced by FO supplementation in healthy populations (Macartney et al., 2014; Peoples et al., 2008) and is consistently associated with reduced CVD rates in epidemiological and cohort studies (Burr et al., 1989; Kagawa et al., 1982; Kromhout et al., 1985). Several meta-analyses have provided evidence to support these observations (Mozaffarian et al., 2008; Mozaffarian et al., 2005; Xin et al., 2013), however some RCT (Galan et al., 2010; Roncaglioni et al., 2013; Svensson et al., 2006) and meta-analyses (Alexander et al., 2017; Nestel et al., 2015) of studies using supplements have produced mixed results which do not support the notion that FO is acting in a cardio-protective manner. The discrepant findings produced in large scale clinical trials can be explained by poor design because of i) misunderstandings of the underlying mechanisms and/or ii) misunderstanding the doses of FO required to modify cardiac function and dysfunction and/or iii) failure to account for significant pre-existing DHA incorporation in control groups (Ip et al., 2017; McLennan & Pepe, 2015).

Chapter two of this thesis demonstrated that supplementing dietary achievable FO doses to a typical Western-style diet in the rat caused robust and preferential incorporation of DHA into phospholipids of excitable tissue, most notably skeletal muscle phospholipids. It is well-established that incorporation of DHA into skeletal muscle phospholipids improves fatigue resistance during both normoxic (Henry et al., 2015; Peoples & McLennan, 2010, 2014) and hypoxic contractions (Peoples & McLennan, 2017), even while using dietary achievable FO doses. However, there are no studies which have investigated the effects of myocardial phospholipid DHA incorporation attributable to dietary achievable FO doses on healthy and ischaemic cardiac physiological function. As such, the remaining studies in this thesis aim to characterise whether dietary achievable FO doses alter rested healthy cardiac function and confer protection to cardiac function during and directly following ischaemia and reperfusion injury of the heart. Such experiments will help establish the physiological relevance of any health benefits associated with supplementing dietary achievable FO doses to a typical Western-style human diet high in SFA and n-6 PUFA.
Prior research has established the importance of physiological conditions, principally oxygenation and temperature (Henry et al., 2015; Peoples et al., 2013; Pepe & McLennan, 1993), in establishing real effects of altered cardiac and skeletal muscle phospholipid composition. Functional evaluation of the rat heart in vivo meets that requirement and is coupled with scaled down clinically relevant techniques to describe cardiac function in clinical terms and thereby optimise interpretation and translation of the findings into practice. Therefore, the primary objective of this study was to investigate the use of left ventricular pressure volume loops (PV-loops) for the assessment of cardiac performance and the optimisation of surgical protocols used to measure in vivo PV-loops in the rat heart.

3.1.1 Left ventricular pressure volume loops

To date, multiple techniques have been used to assess left ventricular cardiac haemodynamic performance such as ultrasonography, flow probes, micromanometers, magnetic resonance imaging (MRI), and more recently miniaturised conductance catheters (Burkhoff et al., 2005). The use of conductance catheters allows for the generation of instantaneous pressure and volume measurements (Figure 3.1 & Figure 3.2). Plotting instantaneous pressure against volume creates a two-dimensional representation of the cardiac cycle and generates a PV-loop. The PV-loop has long been recognised as an indicator of the work done by a system (the heart in this case) and the efficiency at which it completes this work. Although instantaneous pressure and volume measurements alone can provide valuable insights into the performance of the left ventricle, PV-loops provide a more powerful tool to assess left ventricular function. For these reasons, PV-loops provide a unique framework to assess a range of physiological relevant haemodynamic parameters and describe left ventricular performance of the heart.
Figure 3.1: Presentation of the Millar 6mm micromanometer conductance catheter along a longitudinal section of the left ventricle.

**Figure 3.1 notes:** The catheter is placed along the longitudinal axis of a sliced rat left ventricle. The tip of the catheter sits at the apex of the ventricle and the proximal part runs through the aortic valve. Two conductance (volume) electrodes can be observed at the top (E1 & E2) and bottom (E3 & E4) of the catheter and the pressure transducer is found in between them.

![Diagram of catheter placement](image)

Figure 3.2: Representative instantaneous pressure and volume traces coupled with ECG.

**Figure 3.2 notes:** This recording is an un-calibrated pressure and volume trace which was recorded in the left ventricle of a rat heart using the Millar 6mm micromanometer conductance catheter. Red trace = Pressure (mmHg), Blue trace = Volume (μL) & Pink trace = ECG (mV).
The conductance catheter can be used to assess both in vivo and ex vivo cardiac function from large and small animals. The use of conductance catheters in animal models permits detailed characterisation of a range of invasive parameters which are not easily measured in human subjects for research purposes (Pacher et al., 2008). Using an animal model gives the researcher the ability to have precise nutritional control of the diet, ease of handling and housing, and control over a range of stimuli during experimentation which can influence cardiac function including temperature, anaesthesia and blood oxygenation concentrations (Pacher et al., 2008). As such, the rat in vivo and ex vivo heart model are commonly used in research laboratories, both of which offering their own set of advantages and disadvantages (Milani-Nejad & Janssen, 2014). The ex vivo model allows direct conclusions to be made in regard to the heart itself as measurements are collected in the absence of confounding homeostatic feedback responses. The ex vivo model is preferred in some research settings investigating the effects of FO because it provides a highly reproducible preparation which allows easy access for measurement of oxygen consumption, cardiac workload and other coronary biochemistry (McLennan et al., 2012; Pepe & McLennan, 2002). However, Pepe & McLennan have suggested that using whole-blood perfusate to ensure adequate oxygenation is critical when using this model to draw accurate observations, particularly when considering mechanisms related directly to oxygen efficiency (Pepe & McLennan, 2002). Advances in technology such as the micro-conductance pressure-volume catheters allow the in vivo heart model to provide a relatively easier and inexpensive method to measure clinically relevant indices of cardiac function. However, measurements can be confounded by factors such as preload, afterload and heart rate. The retention of autonomic control, neurohormonal influences on the heart and most importantly beat-to-beat whole blood perfusion of the heart via the coronary arteries, makes the measurements collected in vivo more physiologically relevant to this thesis. Therefore, using the conductance catheter in the rat in vivo heart was chosen to investigate the hypotheses in this thesis regarding identification of any cardiac physiological changes attributable to LC n-3 PUFA. This approach permits more confident extrapolation of observations made in the studies to human heart health. Consequently, the first focus of this study was to successfully reproduce relevant cardiac measurements from the rat in vivo heart to evaluate cardiac performance.
A PV-loop from a single cardiac cycle is illustrated in *Figure 3.3*, labelled according to key phases and events (Each point is labelled A-D). The following discussion will start from the bottom right corner of the loop (A) with the heart filled and about to contract. Point A of the loop represents completion of the diastolic filling phase. Closure of the mitral valve occurs as the left ventricle begins to contract and build pressure at this point but there is no movement of blood from the ventricle as the aortic valve is also closed at this point, this is known as the isovolumic contractile phase (A to B). Point B indicates the opening of the aortic valve, this occurs when the pressure in the left ventricle becomes higher than the pressure in the aorta. Increased left ventricular pressure forces the aortic valve open, allowing blood to travel down its pressure gradient from the left ventricle into the aorta; this is known as the ejection phase (B to C). Point C illustrates when ventricular pressure falls below aortic pressure as myocardial relaxation begins, causing aortic valve closure as blood begins to flow in a retrograde direction towards the left ventricle. Myocardial relaxation occurs before and following the closure of the aortic valve. Once both the mitral and aortic valves are closed, the isovolumetric relaxation phase occurs (C to D). At this point, no blood enters the ventricle and the ventricle relaxes causing a substantial decrease in pressure with no change in volume. Point D signifies completion of relaxation and the mitral valve opens again. When the mitral valve opens the left ventricle begins the next diastolic filling phase (D to A) which occurs passively as blood flows down its pressure gradient from the left atrium into the left ventricle.
Figure 3.3: Illustration of key phases and events in the left ventricle cardiac cycle as observed through a PV-loop.
Commonly reported indices of cardiac physiological function are illustrated on a P-V loop in Figure 3.4. Point 1 represents end-diastolic volume and end-diastolic pressure; this shows the volume of blood which has filled the left ventricle during the diastolic filling phase and the resultant ventricular pressure. Physiologically, this point is synonymous with what is known as the ‘preload’ of the heart. Preload represents the extent to which myocardial sarcomeres are stretched at the time contraction commences. Thus, a greater end-diastolic volume produces increased distention of the ventricle, resulting in increased preload of myocardial sarcomeres, the opposite occurs with a reduced end-diastolic volume. In a healthy beating heart this is usually the lowest pressure reported between heart beats. The pressure at point 2 represents what is known as the ‘afterload’ of the heart. Afterload is an indirect representation of the tension that the left ventricular sarcomeres need to generate to overcome the resistance provided by aortic blood pressure. Changes in afterload are closely linked to the force-velocity relationship for cardiac myocytes. For example if afterload increases the velocity of myocyte shortening decreases, reducing the rate of volume ejection during the ejection phase, leaving more blood in the ventricle at the end of systole, the opposite occurs for decreases in afterload. The pressure measured at point 3 represents end-systolic pressure and the volume at point 3 represents end-systolic volume; this shows the volume of blood remaining in the ventricle after the ejection phase. The difference between end-systolic pressure and end-diastolic volume is known as stroke volume. With analogy to a piston pump, this is the amount of blood pumped out of the ventricle in one heartbeat (Dashed line in Figure 3.4).
Figure 3.4: Illustration of common physiological indices measured in the left ventricular cardiac cycle as observed through a PV-loop.

**Abbreviations:**

SV = stroke volume; SW = stroke work; ESV = end-systolic volume; EDV = end-diastolic volume; ESP = end-systolic pressure; EDP = end-diastolic pressure.

‘Preload’ = The extent to which myocardial sarcomeres are stretched at the time contraction commences.

‘Afterload’ = An indirect representation of the tension that the left ventricle sarcomeres need to generate to overcome the resistance provided by aortic blood pressure.
In addition to the physiological indices that can be measured directly from the PV-loop, there are a range of measurements which can be calculated from the PV-loop. Cardiac output can be calculated by multiplying the stroke volume with heart rate. Ejection fraction can be calculated using stroke volume as a proportion of end-diastolic volume (stroke volume/end-diastolic volume*100), this is an important measure for tracking pumping function of the left ventricle and in a healthy heart ejection fraction is usually fifty five percent or higher. The area within the loop can be calculated (shaded blue in Figure 3.4) and represents the stroke work completed by the left ventricle to eject blood into the aorta each cardiac cycle. The maximal rate of pressure rise (dP/dt max) and maximum rate of relaxation (dP/dt min) can also be calculated from the ventricular pressure measurements. The dP/dt max calculated represents the maximal rate at which the heart generates pressure during the isovolumic contraction phase of each beat and is a good indicator of systolic function. The dP/dt min calculated represents the maximal rate at which the heart relaxes during the isovolumic relaxation phase and is a good indicator of diastolic function. It can be appreciated that all aspects of the cardiac cycle can be intimately characterised through direct and calculated measurements made from the PV-loop. Therefore, the PV-loop provides a unique framework to assess physiological relevant cardiac haemodynamic parameters and describe left ventricular performance. For these reasons, PV-loops are regarded as the gold standard for assessment of in vivo cardiac haemodynamic function in animals (Calligaris et al., 2013; Clark & Marber, 2013; Pacher et al., 2008).

3.1.2 The effect of anaesthetic on cardiac performance

The use of anaesthetic for surgical protocols in animals is essential when investigating invasive measurements. However, anaesthetics can significantly alter baseline inotropic and chronotropic function of the cardiovascular system (Stein et al., 2007). As such, careful consideration of the anaesthetic used is needed when planning a study such as this, which aimed to measure cardiovascular performance. Sodium pentobarbital (pentobarbital) or a ketamine + xylazine mixture are amongst the most common anaesthetic agents used when measuring cardiovascular function in rats (Hanusch et al., 2007).

Pentobarbital is an oxybarbiturate that has been used for veterinary anaesthesia since the 1930s. Evidence suggests that pentobarbital provides anaesthesia via acting as an
agonist at the GABA<sub>A</sub> receptor which is the principle inhibitory neurotransmitter in the central nervous system in mammals (Krasowski & Harrison, 1999). When given at higher doses in the anaesthetic relevant range, pentobarbital also acts to block excitatory glutamate receptors in the central nervous system and neuronal nAChR channels (Krasowski & Harrison, 1999). These mechanisms of action result in pentobarbital having a slightly depressive chronotropic and inotropic effect on the cardiovascular system (Saha et al., 2007), and it has been demonstrated that when used for prolonged periods of anaesthesia (+3hrs), cardiac output can be significantly reduced (Nash et al., 1956).

The pharmacology of ketamine is quite complex as it is known to interact with N-methyl-D-aspartate (NMDA) receptors directly, opioid receptors, monoaminergic receptors, muscarinic receptors and voltage sensitive Ca<sup>2+</sup> channels (Bergman, 1999). However, the main action of ketamine is through its non-competitive binding of NMDA receptors resulting in antagonism of the central nervous system. There is also some evidence that ketamine binds to α & β adrenergic receptors (Bevan et al., 1997). This results in ketamine having a sympathomimetic action on the cardiovascular system. As such, induction of anaesthesia with ketamine leads to increases in heart rate and increased systemic and vascular blood pressure. To diminish the effects of ketamine on the cardiovascular system, the concomitant administration of xylazine is commonly used. Xylazine is a α<sub>2</sub>-receptor agonist from the non-opioid group that blocks the release of catecholamine’s by sympathetic nerves, thereby reducing the sympathomimetic effect of ketamine alone (Sumitra et al., 2004).

Both pentobarbital and ketamine offer a surgical grade of anaesthesia suitable for invasive measurements without pain to the animal. However, as just described, they are different classes of anaesthetic and act via different mechanisms of action to achieve this. Therefore, the secondary objective of this study is to compare these two anaesthetic agents for the purpose of reliably quantifying in vivo cardiac haemodynamic function in the rat.
3.2 AIMS AND HYPOTHESES

This study aimed to optimise the reliability and reproducibility of the in vivo animal surgical model used to measure cardiac function and identify the most appropriate anaesthetic for cardiac measurements.

Specifically, this experiment had the following aims:

**Aim 1:** Optimise the surgical techniques and protocols required to measure cardiac haemodynamics with a conductance catheter in a physiologically stable in vivo model relevant to normal bodily cardiovascular function.

**Aim 2:** Directly compare the effect of pentobarbital and ketamine + xylazine on cardiovascular haemodynamic function to determine which anaesthetic produces the most reliable and reproducible measurements of cardiac function.
3.3 METHODS

3.3.1 Animals, ethical considerations & standardisation

A primary cohort (cohort 1) of male rats (Sprague Dawley) were used in this study and ethical approval was completed (Section 2.3.1). Animals were housed and weighed according to procedures outlined in Section 2.3.2. Animals for this study were supplied lab chow and water ad libitum, but were not provided with dietary supplementation of FO.

3.3.2 Groups

Twenty-three (N=23) animals were randomly assigned to receive i.p injection of either pentobarbital (Hospira, Lyppards, SA, Aus) (n=11) or a mixture of ketamine + xylazine (Ceva brand Ketamine & Ilium brand Xylazine, Lyppards, SA, Aus) (n=12). Intraperitoneal injections of pentobarbital or ketamine + xylazine were chosen because of their ease of use when compared to gaseous anaesthetics which require specialised ventilation and gas extraction equipment.

3.3.3 Experimental preparation

Haemodynamic variables were obtained using a Millar pressure-volume single segment foundation system for animals connected to PowerLab 8/35 software (ADInstruments, Bella Vista, NSW, Aus) following established methods from previous studies (Abraham & Mao, 2015; Pacher et al., 2008). A 6mm miniaturised 2-French (2F) pressure-volume conductance catheter (SPR-638 Millar Instruments, Houston, USA) was used to assess in vivo left ventricular function in the animals. The sampling rate of the conductance catheter was set at 1kHz in PowerLab. The conductance catheter has four electrodes (E1, E2, E3 and E4) with a spacing of 6mm between electrode E2 and E3 which contained a 2F pressure transducer (Figure 3.1). To measure volume, the catheter emits a constant current to the blood inside the left ventricle from the two most outer electrodes (E1 & E4) and the voltage changes in the electrical field are then detected by the inner two electrodes (E2 & E3). Therefore, by Ohm’s law, the conductance of the blood surrounding the catheter in the left ventricle is inversely proportional to the measured electrical potential. A constant sinusoidal alternating current (0.02 mA root means square at 20kHz) is applied to drive the conductance. The catheter was placed in
saline heated to 37°C for 30 minutes prior to experimentation to replicate the biological working environment and electronically calibrated before being used in the procedures.

The catheter was connected to the Millar pressure volume suite (MPVS) ultra-control interface and the pressure and volume signals were electronically calibrated following the manufacturer’s instructions. This consisted of a two point electronic pressure calibration. The software output a pressure trace which was recorded at 0mmHg followed by 100mmHg and then the corresponding voltages were assigned to the trace. The electronic pressure calibration was confirmed using a manual mercury column sphygmomanometer calibration. The sphygmomanometer cuff port was fit with a 3-way stopcock. The transducer was carefully placed into a fluid filled (0.9% NaCl solution) haemostasis valve and gently secured. The haemostasis valve was then connected to the sphygmomanometer via the stopcock, and the sphygmomanometer was then inflated to 100mmHg. The pressure measured via the MVPS software was then checked to determine if it matched the pressure in the inflated sphygmomanometer. Volume was calibrated using the relative volume unit (RVU) method which converts conductance to RVU (Pacher et al., 2008). A volume tracing was output from the software and recorded at 119 RVU and 289 RVU and voltages were assigned to the corresponding volume traces. Volume measurements were then recorded throughout the experimental procedures as raw RVU (conductance). Further calibration was completed at the end of procedures to convert conductance into true volumes (μL).

3.3.4 Surgical procedures

3.3.4a Animal anaesthesia

Animals were anaesthetised throughout all procedures to ensure physiological stability. Animals were weighed and then given an i.p injection of anaesthetic using a 1mL BD micro-fine insulin needle. Anaesthetic was dosed according to body weight (pentobarbital: 60mg/kg or ketamine 100mg/kg + xylazine 10mg/kg) using the formulary provided by the British small animal veterinary association (BSAVA) manual of practical animal care for small animal anaesthesia and analgesia. The depth of anaesthesia was confirmed prior to beginning procedures via monitoring of the foot withdrawal reflex to toe pinch and corneal reflex to touch (on average this was <15min). The depth of anaesthesia was continuously monitored throughout protocols via toe
pinch reflexes for signs of inadequate anaesthesia. Additional doses (ketamine 25mg/kg, pentobarbital 20mg/kg) of anaesthetic were provided according to the standard practices on checking the animal’s reflex reaction responses (Hanusch et al., 2007).

3.3.4b Temperature control

Once anaesthetised, all surgical procedures and experimental protocols were performed with the animals placed on a heating mat (T-CAT 2 temperature controller, Braintree Scientific, MA, USA) which maintained animal core body temperature at 37°C as measured via a rectal probe (T-CAT 2 rectal probe, Braintree Scientific, MA, USA) throughout the procedures. Because of the automated nature of the heating mat with instantaneous feedback which ensured body temperature fluctuated from 37 °C only minimally at any time point, body core temperature was not recorded and is not reported in any of the studies conducted in this thesis.

3.3.4c Electrocardiogram

Electrical activity within the heart was monitored throughout surgical procedures and experimental protocols using the single lead surface electrocardiogram (ECG – lead I) method. This involved the placement of a positive electrode on the left fore-limb, a negative electrode on the right fore-limb and an earth electrode on the left hind-limb of the animal shortly after the animal was placed on the heating mat. Butterfly clip electrodes were attached to needles (27gx1/2, Terumo, Elkton, MD, USA) which were then placed through the superficial skin of the animals at each site. The surface ECG recorded at a frequency of 1000Hz to a computer with PowerLab software (ADInstruments Dual Bioamp, Bella Vista, NSW, Aus) and then saved for offline analysis. The ECG measurements record electrical activity of the heart, thus, tracing ventricular depolarisation periods. The ventricular depolarisation of the heart generates the R wave found in the QRS phase of the ECG recording of the cardiac cycle. Each QRS phase represents a new heart beat therefore the time between consecutive R waves was used to calculate instantaneous heart rate.
3.3.4d Tracheal, venous and arterial cannulation

Following placement of electrodes and collection of baseline resting ECG measurements, a 3-4cm superior-inferior incision was made in the midline anterior neck of the animal and the trachea was bluntly dissected from the surrounding tissue. A small anterior incision was made between the c-shaped cartilage rings of the trachea to allow the placement of a 40mm polyethylene tube (Microtube Extrusions, North Rocks, NSW, Aus) into the trachea. Using blunt scissors and micro-curved forceps, the left internal jugular vein was then bluntly dissected away from the surrounding connective tissue just distal to where it merges with the left subclavian vein to form the left brachiocephalic vein. Two sterile siliconized (Dysilk, Dynek Pty Ltd, Australia) 6/0 silk sutures were loosely tied around the proximal and distal ends of the vein. The suture which was most distal (away from the chest) was then tightened and tied to stop blood flow returning from the head. A small transverse incision was then made in the vein using microsurgical scissors and a length of 0.96 x 0.58mm PE tubing was placed through the incision into the left internal jugular vein. The remaining suture at the proximal end (closer to the chest) was then pulled tight to secure the vein to the tubing. The distal suture was then also re-tied to the tubing to ensure it did not come loose during the procedures. The tubing was connected to a three-way valve to allow for the administration of physiological (0.9%) and hypertonic (30%) saline during experimental protocols.

Following jugular vein cannulation, using blunt scissors and micro-curved forceps, the right common carotid artery was bluntly dissected away from the vagus nerve and surrounding connective tissue. Three sterile siliconized (Dysilk, Dynek Pty Ltd, Australia) 6/0 silk sutures were loosely tied around the artery, one in the proximal location and two distally. One of the distal sutures was then tightened and secured to occlude blood flow from the head. The proximal suture was held in a pair of hemostat scissors and then gently pulled and secured by clamping to the skin to occlude blood flow from the aorta. A small transverse incision was then made in the right common carotid artery using microsurgical scissors just in front of the distal suture. The pre-calibrated miniaturised 2F pressure-volume conductance catheter was then advanced into the artery via the incision. The third suture was then secured to the catheter using a single loop knot and the proximal suture was released from the hemostat which removed the occlusion in the artery ahead of the catheter. The catheter was then able to
be advanced through the right common carotid artery and into the aortic arch retrograde towards the heart. At this point the rodent ventilator system was turned on and artificial ventilation began.

3.3.4e Artificial ventilation

The tube placed into the trachea was attached to a rodent ventilator (Rodent Ventilator, Basile 7025, Italy) for the animal’s breathing to be maintained by machine (artificial ventilation); the rate and volume for ventilations were calculated using the following formula: Tidal Volume \( V_t, \text{ ml} \) = \( 6.2M^{1.01} \) (\( M \) = animal mass, kg); Respiration Rate (RR, \( \text{min}^{-1} \)) = \( 53.5 \times M^{0.26} \) (Pacher et al., 2008). For example, a rat weighing 400g would have a \( V_t = 2.4 \text{mL} \) and an RR = 68. This ensured that the concentration of oxygen in the arterial blood remained high and constant, independently of the animal’s natural breathing cycle and depth throughout the experimental protocol. Additionally, controlling the respiratory rate maintains an adequate acid base balance during the experimental period. Changes in acid base balance can have a strong influence on the oxyhaemoglobin curve and unloading of oxygen (Stickland et al., 2011). Therefore, the primary role of the artificial ventilation was to deliver a standardised high level of arterial oxygen to all animals, and the secondary role was to maintain an adequate acid base balance which did not influence unloading of oxygen at the tissue.

3.3.5 Experimental protocol and measurements

Prior to surgery occurring heart rate was calculated from the ECG recording, using one (1) minute R-R interval averages. Following surgical procedures, the conductance catheter was placed in the aortic arch for baseline measurement of arterial pressures and heart rate was calculated from the ECG recording. Placement of the catheter in the aortic arch was confirmed in real time via the presence of the dicrotic notch in the pressure wave channel on PowerLab software (Figure 3.5). The catheter was then advanced across the aortic semilunar valve into the left ventricle of the heart via probing the valve with small to-and-fro movements of the catheter against the valve while rotating it, so the catheter tip moved across different parts of the valve. Once the catheter advanced past the aortic valve and entered the ventricle the pressure wave would drop from aortic pressure ranges (120mmHg – 80mmHg) to ventricular pressure ranges (120mmHg – 5-10mmHg). Thus, real-time placement of the catheter within the
left ventricle was confirmed via the pressure output channel in the Powerlab software (Figure 3.6).

Figure 3.5: Experimental pressure tracing showing the dicrotic notch which confirmed placement of the transducer in the aorta.

Figure 3.6: Experimental pressure tracing showing pressure drop as catheter moved from the aorta into the left ventricle.
Once catheter placement was confirmed in the left ventricle, animals were given fifteen minutes rest to allow stabilisation of left ventricular function, heart rate was calculated from the ECG at this stage. Resting cardiac haemodynamic function was then recorded for twenty minutes via the conductance catheter, the final five minutes of the recorded data was used for analysis of baseline resting cardiac haemodynamic function and PV-loops, and heart rate was calculated from the ECG recording at this time point. The catheter was then withdrawn from the left ventricle into the aortic arch to measure aortic blood pressure. The catheter was then returned to the ventricle for saline calibration procedures.

### 3.3.5a Saline calibration of the conductance catheter

The current applied to the excitation electrodes on the catheter is conducted through the blood in the left ventricle, but also it flows through the surrounding ventricular tissue. If the conductance of the surrounding tissue is not accounted for, it can lead to an overestimation of the blood volume within the left ventricle. This is known as the parallel conductance of the ventricle ($G_p$). Therefore, a value for ($G_p$) was obtained by providing a bolus dose of hypertonic saline (30% NaCl) to the left ventricle via the right internal jugular vein at the end of experimental measurements. The saline was injected into the right internal jugular vein (Section 3.3.4d). The respirator was turned off for five seconds, and then 20-40μL of a 30% NaCl solution was injected into the right internal jugular vein until the PV loop visibly shifted to the right without a significant decrease in the pressure signal. This was conducted three times in each animal with 3-5min rest periods between to allow the volume signal to recover between injections. Following completion of saline calibration procedures the conductance catheter was withdrawn into the aorta and remained there while the animals were euthanised via exsanguination and rapid removal of the heart. An overview of the experimental protocol and measures used can be seen in Figure 3.7.
Figure 3.7: Overview of the surgical preparation and experimental protocol.

1. Rodent ventilator
2. Cannulation of left internal jugular vein for administration of saline
3. Cannulation of right common carotid artery for entry of the conductance catheter
4. Rectal temperature probe with automatic feedback loop to heat pad set at 37°C

**Abbreviations:** HR = heart rate; BP = blood pressure; Xyl = xylazine.
3.3.6 Euthanasia & tissue collection

All animals were euthanised without recovery from the anaesthetic using methods that were approved by the University of Wollongong’s animal ethics committee. Euthanasia was completed via removing the artificial ventilator and then rapid exsanguination and removal of the heart.

3.3.6a Blood collection

Exsanguination was completed using a 21-gauge needle (Terumo Europe, Leuven, Belgium) inserted into the bifurcation of the aorta at the common iliac artery. The blood was collected into a 5mL syringe containing 0.2mL of a 5,000 U/mL Heparin Sodium solution (Hospira, Lyypards, SA, AUS). The blood was then separated into 1mL EDTA-treated tubes. One tube of blood was used to complete cuvette calibration in order to calculate blood viscosity. The blood sample was immediately placed into a rat insulator-type calibration cuvette with known well diameters provided by the manufacturer (SPR-638 Millar Instruments, Houston, USA). The first five wells were filled with blood and the cuvette was placed on a heating pad at 37°C to keep the blood at biological temperature. The catheter was then placed into each of the five wells and held in the centre of each well while submerging all four electrodes by blood for 10-15secs. The conductance changes were recorded in the volume channel in RVU via the PowerLab software (LabChart 8, PV Loop module, ADInstruments, Bella Vista, NSW, Aus). The remaining blood tubes were frozen at -80°C for further analysis.

3.3.6b Cardiac tissue collection

The heart was rapidly removed from the animal following exsanguination through a thoracic incision of the sternum. The major vessels were cut from the heart, and the heart was submerged into an ice cold perfusate to arrest beating. Excised hearts were then flushed with an ice cold 0.9% NaCl solution to remove any remaining blood. The great vessels were then dissected off the heart, and the heart was weighed. The atria were dissected off to obtain a ventricle weight, and the right ventricle was dissected off to obtain a left ventricle weight. The left ventricle was then sliced in 2mm diameter pieces and immediately frozen in liquid nitrogen, then stored at -80°C until fatty acid or tissue morphological and biochemical analyses.
3.3.7 Post-experimental conductance catheter calibration

The recorded raw conductance data was converted to true volumes (μL) to allow for a more physiologically relevant assessment of ventricular work. Previously established cuvette and saline calibration calculations were completed using LabChart 8 PV loop software (Pacher et al., 2008). The conductance output collected in RVU during cuvette calibration was correlated with known volumes to develop a calibration curve that converted all recorded data from RVU into units of estimated volume (μL). However, these estimated volume recordings are still larger than expected due to the parallel conductance of the tissue surrounding the left ventricular blood volume. Therefore, parallel conductance was calculated using a series of linear equations. The LabChart 8 PV-loop software calculated both of the linear equations; the first plotted the saline calibration data as the end-diastolic volume vs end-systolic volume. The second plotted end-diastolic volume = end-systolic volume, which represented the equivalent of a heart chamber devoid of blood. The value at the intersection between the two plotted lines was then used as a value to represent the parallel conductance of the left ventricular tissue. This value was then automatically subtracted from the estimated volumes calculated via the cuvette calibration curve. Following the cuvette and saline calibration calculations the recordings were in units of true volume (μL). This allowed for accurate and detailed assessment of the ventricular haemodynamics measured with the catheter in animals.

3.3.8 Post-experimental data analysis

3.3.8a Heart rate

Offline analysis of the saved ECG recordings was completed using LabChart 8 software. Files were visually inspected and filtered for the presence of ectopic beats, unusual electrical activity or interference. The ventricular depolarisation of the heart generates the R wave found in the QRS complex of the ECG recording of each cardiac cycle. Each QRS complex represents a new ventricular depolarisation therefore the time between consecutive R waves can be used to calculate instantaneous heart rate.
3.3.8b Left ventricular cardiac haemodynamics

Resting cardiac haemodynamic function was analysed from the final five minutes of the recorded data collected. A smoothing filter (Triangular Barlett, window with 25 points) was applied to the volume channel due to regular noise which was distorting the channel and most likely due to the electrical network that the system was plugged into, the pressure signal was never filtered in order to maintain integrity of maximal and minimal rate of pressure development measurements (Pacher et al., 2008). Systolic and diastolic function was assessed via investigation of volume and pressure related indices measured and collated directly by the PowerLab software analysis tools. Analysis of pressure and volume indices was conducted on a minimum of thirty (30) consecutive cardiac cycles to create an average value for each animal.

3.3.8c Statistics

All results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The effects of anaesthetic treatment on experimental outcomes were tested via ANOVA. Individual group means were compared using Bonferroni’s post-hoc analysis. All statistical analyses were performed in a blinded manner and completed using SPSS 21 for Windows (SPSS Inc, Chicago, IL, USA). Alpha was set at $p < 0.05$. 
3.4 RESULTS

3.4.1 Anthropometrics

Anthropometric measures were collected on all animals. There were no statistically significant differences between the groups for age, body weight, respiratory rate and respiratory volume (Table 3.1). The pentobarbital group displayed a slightly larger total heart weight than the ketamine + xylazine group which approached significance \((p = 0.06)\). However, once heart weight was normalised to body weight, there was no statistical significant difference between the groups \((p = 0.17)\).

<table>
<thead>
<tr>
<th></th>
<th>Ketamine + xylazine</th>
<th>Pentobarbital</th>
<th>Trend (p)</th>
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<tbody>
<tr>
<td>Animals (N)</td>
<td>11</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>13.8 (±0.2)</td>
<td>14.1 (±0.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>505 (±8)</td>
<td>517 (±10)</td>
<td>NS</td>
</tr>
<tr>
<td>Ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory Rate (b/min)</td>
<td>63 (±2)</td>
<td>64 (±2)</td>
<td>NS</td>
</tr>
<tr>
<td>Respiratory Volume (mL)</td>
<td>3.1 (±0.1)</td>
<td>3.2 (±0.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>1400 (±22)</td>
<td>1484 (±36)</td>
<td>0.06</td>
</tr>
<tr>
<td>Heart/Body weight Ratio (mg/g)</td>
<td>2.78 (±0.04)</td>
<td>2.86 (±0.03)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Values are mean ± SEM \((n = 11 – 12\) per group). NS = Not significant.
3.4.2 The effect of anaesthetic on in vivo cardiovascular function

The heart rate of animals anaesthetised with ketamine + xylazine was significantly lower at all time points measured from the induction of anaesthesia until the end of the protocol when compared to animals anaesthetised with pentobarbital (Figure 3.8). Baseline resting heart rate was significantly lower in the ketamine + xylazine group compared to the pentobarbital group. Following surgery, heart rate of the ketamine + xylazine group (240 ±7 beats.min⁻¹) had dropped further, whereas heart rate in the pentobarbital group remained stable (424 ±11 beats.min⁻¹). At the beginning and end of cardiac function measurements heart rate remained stable in both groups.

![Graph showing heart rate calculations during different time points of the experimental protocol.](image)

**Figure 3.8: Heart rate (beats.min⁻¹) calculations during different time points of the experimental protocol.**

Values are mean ± SEM (n = 11 – 12 per group). *p <0.05 vs Ketamine + xylazine (One-way ANOVA with Bonferroni post-hoc test).

**Figure 3.8 notes:** Time 0 = Anaesthetic injection, Time 5 = Pre-surgery, Time 45 = Post-surgery. Time 60 = Start of baseline measures following rest period, Time 80 = End of baseline measures. Error bars are omitted when smaller than the symbol.
The ketamine + xylazine group displayed a significantly lower systolic blood pressure, diastolic blood pressure and mean arterial pressure compared to the pentobarbital group at both measurement points (Table 3.2). Measurement of left ventricular function demonstrated cardiac output and heart rate to be significantly lower in the ketamine + xylazine group compared to the pentobarbital group. However, the stroke volume of the ketamine + xylazine group was significantly greater than the pentobarbital group. There were no significant differences between groups in any of the other haemodynamic measurements recorded including end-systolic volume, end-diastolic volume, end-systolic pressure and end-diastolic pressure. Ejection fraction was significantly lower in the pentobarbital group compared to the ketamine + xylazine group, however there were no differences in any of the other systolic contractile indices measured including stroke work and dP/dt\textsubscript{max} (Table 3.2). There was no difference in dP/dt\textsubscript{min} between groups but the isovolumic relaxation constant Tau was significantly higher in the ketamine + xylazine group compared to the pentobarbital group.
Table 3.2: Haemodynamic parameters measured by conductance catheter in rats while anaesthetised with Pentobarbital or Ketamine + xylazine and expected normal range.

<table>
<thead>
<tr>
<th></th>
<th>Ketamine + xylazine</th>
<th>Pentobarbital</th>
<th>Normal range&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood Pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic (Pre)</td>
<td>111(±2)</td>
<td>122(±3)*</td>
<td></td>
</tr>
<tr>
<td>Systolic (Post)</td>
<td>115(±3)</td>
<td>127(±2)*</td>
<td></td>
</tr>
<tr>
<td>Diastolic (Pre)</td>
<td>78(±3)</td>
<td>95(±3)*</td>
<td></td>
</tr>
<tr>
<td>Diastolic (Post)</td>
<td>79(±2)</td>
<td>95(±3)*</td>
<td></td>
</tr>
<tr>
<td>MAP (Pre)</td>
<td>92(±2)</td>
<td>107(±3)*</td>
<td>93 - 120</td>
</tr>
<tr>
<td>MAP (Post)</td>
<td>94(±2)</td>
<td>108(±2)*</td>
<td></td>
</tr>
<tr>
<td><strong>Ventricular function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats.min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>285 (±25)</td>
<td>457 (±8)*</td>
<td>300 – 450</td>
</tr>
<tr>
<td>SV (μL)</td>
<td>105 (±3)</td>
<td>95 (±4)*</td>
<td>101 – 154</td>
</tr>
<tr>
<td>CO (mL.min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>26 (±3)</td>
<td>37 (±2)*</td>
<td>42 – 62</td>
</tr>
<tr>
<td>&lt;sup&gt;b&lt;/sup&gt;Estimated TPR (mmHg.min.ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3.62 (±0.4)</td>
<td>2.92 (±0.3)*</td>
<td></td>
</tr>
<tr>
<td>ESV (μL)</td>
<td>29 (±5)</td>
<td>44 (±7)</td>
<td>36 – 160</td>
</tr>
<tr>
<td>EDV (μL)</td>
<td>117 (±10)</td>
<td>139 (±11)</td>
<td>170 – 266</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>108 (±13)</td>
<td>132 (±9)</td>
<td>113 – 142</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>13 (±1)</td>
<td>14 (±2)</td>
<td>1 – 7</td>
</tr>
<tr>
<td><strong>Systolic indices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke work (mmHg*μL)</td>
<td>8286 (±612)</td>
<td>8990 (±321)</td>
<td>12,800 – 21,000</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>89 (±3)</td>
<td>68 (±4)*</td>
<td>42 – 87</td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg.s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>9119 (±987)</td>
<td>8965 (±647)</td>
<td>7,600 – 11,500</td>
</tr>
<tr>
<td><strong>Diastolic indices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;min&lt;/sub&gt; (mmHg.s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-7794 (±561)</td>
<td>-7193 (±498)</td>
<td>-5,970 – -9,970</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>12.7 (±0.6)</td>
<td>10.4 (±0.4)*</td>
<td>10 – 13</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (<i>n</i> = 11 – 12 per group). *<i>p</i> < 0.05 vs Ketamine + xylazine (One-way ANOVA with Bonferroni post-hoc test).

<sup>a</sup> Values for normal range are obtained from experimental data provided by Pacher, P. et al., 2008. Data was collected from various rat strains anaesthetised with pentobarbital across years of experiments.  
<sup>b</sup> Estimated TPR calculated by dividing MAP (post) by CO.

**Abbreviations:** SV = stroke volume; CO = cardiac output; ESV = end-systolic volume; EDV = end-diastolic volume; ESP = end-systolic pressure; EDP = end-diastolic pressure.

**Systolic indices:** dP/dt<sub>max</sub> = peak rate of pressure rise

**Diastolic indices:** dP/dt<sub>min</sub> = peak rate of pressure decline; Tau = relaxation time constant calculated by Glantz method (regression of dP/dt versus pressure)
In this study, the surgical techniques and protocols for measuring *in vivo* left ventricular haemodynamics were optimised. The study demonstrated that using a conductance catheter to measure *in vivo* left ventricular haemodynamic performance, in animals anaesthetised with pentobarbital, produced sensitive and reproducible results which were comparable with previously described normative values (Pacher *et al.*, 2008). Comparison of anaesthetic agents demonstrated that pentobarbital had less depressive influence on cardiac function than a ketamine + xylazine mixture. In fact, cardiovascular depression was evident directly following anaesthesia onset when using ketamine + xylazine and did not recover throughout the entire experimental protocol. In addition, anecdotal evidence observed of animal depth of anaesthesia during surgical procedures indicated that pentobarbital provided a more adequate grade of anaesthesia than ketamine + xylazine. Therefore, using pentobarbital as the anaesthetic was concluded to be more appropriate than the ketamine + xylazine mixture for surgical procedures and measurement of *in vivo* haemodynamic indices in the following FO studies.

Compared to pentobarbital, the use of the ketamine + xylazine mixture slowed heart rate and increased systolic blood pressure. Heart rate, systolic blood pressure and the product of both, rate pressure product have been demonstrated to correlate strongly with myocardial oxygen consumption (Kitamura *et al.*, 1972). Human and animal experimental studies consistently demonstrate FO supplementation to cause reductions in working myocardial oxygen consumption and heart rate (Macartney *et al.*, 2014; Peoples *et al.*, 2008; Pepe & McLennan, 2002). Heart rate and blood pressures measured in the pentobarbital group of this study were within the normal range and directly comparable to other studies using rats anaesthetised with barbiturates (Bal *et al.*, 2005; Bátkai *et al.*, 2004; Cingolani *et al.*, 2004; Pacher *et al.*, 2008) making it the more appropriate anaesthetic for assessment of these indices.

Compared to the pentobarbital group, the use of ketamine + xylazine slightly resulted in a larger stroke volume, however cardiac output was smaller. Experimental studies have demonstrated FO supplementation to attenuate reductions in cardiac output in hypertrophic induced heart failure models (McLennan *et al.*, 2012), therefore the use of ketamine + xylazine may mask this effect. Pacher *et al.*, 2008 reported the normal range
for stroke volume and cardiac output to be 101-154 µL and 42-62mL.min⁻¹ respectively, in rats anaesthetised with barbiturates. Stroke volume and cardiac output in the pentobarbital group of this study was slightly lower in comparison to the normative data reported by Pacher et al., 2008. However, it has been demonstrated that underestimation of stroke volume occurs as a result of the single volume segment present on four-electrode conductance catheters (Bal et al., 2005). To correct this underestimation, flow probe measurements are commonly collected from the aorta to calculate a gain factor and calibrate volume readings from the conductance catheter (Pacher et al., 2008). Flow probe measurements were not collected in this study due to experimental constraints. It appears likely that this contributed towards a slight underestimation of stroke volume. In addition, the heart rate observed in this study was at the higher end of the normal range and therefore would not provide as much time for the diastolic filling phase, thereby contributing to a reduction in stroke volume.

End-diastolic volume was higher in pentobarbital anaesthetised animals compared to ketamine + xylazine anaesthetised animals, but was slightly lower than values reported in other studies using barbiturates (Bal et al., 2005; Pacher et al., 2008). As stroke volume was similar between groups in this study, the greater end-diastolic volume contributed to a lower ejection fraction in pentobarbital anaesthetised animals compared to the ketamine + xylazine group. Importantly however, ejection fraction of the pentobarbital group was within the normal range and comparable to other studies (Cingolani et al., 2004; Pacher et al., 2008), whereas ejection fraction of ketamine + xylazine anaesthetised animals was beyond the upper limit of the normal range. Pressure derived indices (dP/dt max and dP/dt min) were within the normal range for both anaesthetic groups confirming that systolic and diastolic contraction and relaxation was not influenced by anaesthetic, however stroke work was lower than expected in both groups owing to the lower volumes observed.

Overall, this study demonstrated that the Millar micromanometer conductance catheter is appropriate to collect accurate and detailed recordings of in vivo left ventricular haemodynamics, which were comparable with previously described normative physiological values. However, using the ketamine + xylazine mixture as the anaesthetic in the remaining studies of this thesis would make it considerably more difficult to definitively identify any further changes in cardiac functional indices and
attribute them to FO supplementation alone, rather than side-effects of anaesthesia. The minor differences observed between normative values and collected data for some indices measured may be dependent on differences in rat strain used between studies (Fisher vs Wistar vs Sprague-Dawley) and/or the effect of gender or anaesthetic dosing (Pacher et al., 2008).

Research has demonstrated that cardiovascular depression is a common side-effect of ketamine + xylazine induced anaesthesia if it is not dosed precisely (Hart et al., 2001). Although not quantified with data in this study, the experimenter made anecdotal observations that dosing is especially difficult using the ketamine + xylazine mixture compared to pentobarbital. Animals dosed with the ketamine + xylazine mixture according to the formerly provided for body weight by the British small animal veterinary association took longer to reach the surgical plane of anaesthesia and required consistent additional doses to maintain this level. Whereas, the use of pentobarbital provided a faster onset of anaesthesia, a more satisfactory surgical grade of anaesthesia which continued for the entire 90 min with minimal additional doses required throughout the experiment. It is particularly important that the anaesthesia used for the FO studies performs in this manner as the experiments will run for an extensive time. Given the need for a surgical plane of anaesthesia, it is possible that the additional dosing was causing the cardio-depressive effects observed with ketamine + xylazine in this study. Importantly however, this study demonstrated that pentobarbital provides adequate long-term surgical grade anaesthesia.

Previous research aiming to characterise the effect of FO on cardiac performance have predominately used isolated heart protocols with either a Langendorff or working heart perfusion system (Abdukeyum et al., 2008; McLennan et al., 2012; Pepe & McLennan, 1996). As such, isolated heart measurements have been useful in describing changes to myocardial metabolic efficiency, inotropy, lusitropy and chronotropy associated with myocardial phospholipid DHA incorporation. These changes demonstrate many direct effects of FO in the myocardium and provide substantial evidence to support a mechanism of action for the cardio-protective effects associated with fish or FO intake. However, extension of these findings to humans is limited due to the isolation of the heart from a physiological system including neural feedback mechanisms, humoral factors and perfusion of the coronary arteries with whole blood.
Neural and humoral influences along with adequate oxygenation of the heart are essential for optimal cardiac performance in a working physiological system. In particular, maintaining oxygenation of the myocardium is critical for obtaining physiologically relevant measurements of cardiac function (Kuzmiak-Glancy et al., 2015). Diminished cardiac function is attributed to the high oxygen demand required by the myocardium to complete physiological work as demonstrated by a linear relationship between mechanical energy and myocardial oxygen consumption (Suga, 1979). In fact, inadequate oxygenation of the myocardium through the use of perfusate compared to erythrocytes in ex vivo models has been demonstrated to diminish cardiac function in the absence of injury (Pepe & McLennan, 1993) and has now been argued to be one of the biggest limitations to findings made in ex vivo research (Gutterman & Cowley, 2006; Kuzmiak-Glancy et al., 2015). The current study demonstrated that measurement of cardiac haemodynamic function indices using the conductance catheter is achievable in the in vivo model. This is important because the in vivo model together with artificial ventilation ensures high arterial oxygen concentrations are maintained throughout the experiment to allow oxygenation of the heart through beat-to-beat whole blood perfusion of the coronary arteries. Furthermore, the results exhibited in the study showed that the methods used are sensitive enough to identify minor changes of cardiac haemodynamic function and robust enough to allow reproducibility between animals to allow statistical comparisons. This will be particularly important for the characterisation of the effects of FO on cardiac haemodynamic function as the changes that will occur within the heart are hypothesized to be subtle functional shifts, particularly at rest, compared to the Control group.

In conclusion, this study demonstrated the suitability of the surgical procedures and anaesthetic to use in order to attain physiologically stable measurements of in vivo left ventricular haemodynamic function in the FO studies. Chapter two of this thesis used precision dosing of FO and definitively separated dietary groups to successfully describe phospholipid LC n-3 PUFA incorporation patterns between groups. As such, the following studies of this thesis used the conductance catheter and pentobarbital induced anaesthesia in an in vivo model to characterise the effects of FO on cardiac function.
CHAPTER 4

Characterising rested \textit{in vivo} cardiac function following supplementation of human dietary achievable fish oil doses
4.1 INTRODUCTION

4.1.1 Cardiovascular function

The principal role of the cardiovascular system is to ensure all organs are constantly perfused with an adequate supply of oxygenated blood. To achieve this goal, the heart generates an arterial pressure wave via rhythmically contracting its muscular walls to propel blood into the vasculature and towards the organs. To ensure the metabolic needs of each tissue are met, cardiac function continually adjusts in response to a range of stimuli including stress, illness, trauma, hydration status, temperature, metabolic rate and physical activity levels (Degaute et al., 1991). Instantaneous cardiac function is controlled via a combination of mechanisms, which can be separated into two broad umbrella categories; 1) ‘Intrinsic control’ e.g. The Frank-Starling law of the heart (Starling & Visscher, 1927) and spontaneous depolarisation of pace-making cells (Mangoni & Nargeot, 2008) and 2) ‘Extrinsic control’ e.g. The autonomic nervous system and effect of circulating hormones (Abboud & Thames, 2011). Intrinsic control of the heart ensures that the volume of blood pumped into the arterial circulation (stroke volume) is equal to the volume of blood returning to the heart from venous circulation via changing myocardial contractility and beat rate. The autonomic nervous system including both vagal (parasympathetic) and sympathetic input also play an integral controlling role through feedback loops. Feedback integrates afferent inputs and transmits efferent signals to control beat rate and stimulate the release of hormones which can influence cardiac contractility. Although these mechanisms are categorised separately they work in synchrony throughout every heart beat in order to maintain optimal cardiac function. Investigation of acute and chronic cardiac function during rested conditions through measurement of parameters including and not limited to: heart rate, heart rate variability, cardiac contractility, systolic function indices, diastolic function indices, stroke volume and cardiac output are useful for identifying functional cardiac differences between intervention groups.

4.1.2 Resting heart rate

The relationship between heart rate and cardiovascular or all-cause mortality is of particular interest to researchers and clinicians, making investigation of such parameters invaluable (Cook et al., 2006; Palatini, 2009; Zhang & Zhang, 2009). In the presence of
prior cardiovascular disease, the role of heart rate may differ according to pathophysiological settings. Reducing a previously elevated resting heart rate in patients with CAD and left ventricular systolic dysfunction has been demonstrated to be associated with a small reduction in the risk of cardiovascular mortality and morbidity, the association strengthens in heart failure related outcomes (Fox et al., 2008). Furthermore, there is evidence that in patients with chronic heart failure reducing heart rate with ivabradine improves prognosis (Swedberg et al., 2010). Conversely, it has been shown that in patients with stable CAD without heart failure there is no improvement to prognosis when heart rate is reduced with ivabradine (Fox et al., 2014). The conflicting results suggest that elevated heart rate may be a risk factor in heart failure whereas elevated heart rate in the absence of heart failure is a risk indicator of other pathophysiological processes. This suggestion is supported by evidence from studies of the use of beta-blockers in heart failure that demonstrates the magnitude of resting heart rate reduction to be associated with the survival benefit, whereas the dose of beta-blocker is not (Cullington et al., 2012; McAlister, 2009).

However, human studies stratified by resting heart rate have demonstrated that, in healthy population groups free from prior CVD, increased resting heart rate is associated with greater risk of cardiovascular and all-cause mortality (Hartaigh et al., 2014; Zhang & Zhang, 2009). In particular, heart rate profiling during exercise and recovery has been demonstrated to be a powerful predictor of the risk of sudden cardiac death in healthy men (Jouven et al., 2005), and independent of fitness, levels resting heart rate is inversely associated with all cause-mortality in both men and women (Aladin et al., 2014). Increases in resting heart rate over five years has also been shown to be an independent predictor of mortality in a 20 year follow up period with healthy middle aged men free from clinically detectable CVD (Jouven et al., 2009). Furthermore, it has been demonstrated that higher resting heart rate is associated equally with both cardiovascular and all-cause mortality outcomes (Alhalabi et al., 2017). These findings are supported by a recent meta-analysis of eighty seven prospective studies that demonstrated a positive association between higher resting heart rate and the risk of coronary heart disease, sudden cardiac death, heart failure, stroke, CVD, total cancer and mortality (Aune et al., 2017). This highlights the important role of therapeutic nutritional investigations, alongside pharmacological and exercise interventions, which
could offer a favourable effect of slowing resting heart rate in the general population free from prior CVD.

4.1.2a The effect of fish oil on heart rate

The attributable advantage of DHA incorporation is a reduction in resting heart rate by up to 5 beats.min\(^{-1}\) in healthy and patient populations (Dallongeville \textit{et al.}, 2003; Geelen \textit{et al.}, 2005; Grimsgaard \textit{et al.}, 1998; Mozaffarian \textit{et al.}, 2005) and across many animal species, including; rat (Abdukeyum \textit{et al.}, 2008; Lortet & Verger, 1995) rabbit (Verkerk \textit{et al.}, 2009); dog (Billman & Harris, 2011; Billman \textit{et al.}, 1999); pig (Hartog \textit{et al.}, 1987) and horse (O’Connor \textit{et al.}, 2004). Heart rate has also been demonstrated to be reduced in athletes during exercising conditions without a compromise to peak heart rate (Buckley \textit{et al.}, 2009; Macartney \textit{et al.}, 2014; Peoples & McLennan, 2008) and heart rate recovery has been demonstrated to be faster in healthy males (Macartney \textit{et al.}, 2014) and in post myocardial infarction patients (O’Keefe \textit{et al.}, 2006). The combination of these observations suggests that DHA is acting to modify heart rate, however the doses of FO used in the vast majority of published research to date has been much higher than easily achievable from fish consumption in the diet. Therefore, the first objective of this study was to confirm whether dietary achievable FO doses cause slowing of resting heart rate in the rat.

4.1.3 Left ventricular haemodynamics

The left ventricle of the heart is responsible for the efficient pumping of oxygenated blood to the systemic circulation. To regulate mean arterial blood pressure, the myocardium must be able to relax and contract rapidly and adjust to acute and chronic influences to increase or reduce its pumping capacity (Abboud & Thames, 2011). Injury or degradation to the left ventricular myocardium can result in severe consequences including death. Therefore measurement of left ventricular cardiac performance is an important diagnostic and experimental tool in the assessment of cardiac performance.

4.1.3a The effect of fish oil on left ventricular haemodynamics

The intake of fish or FO is associated with reduced incidence of heart failure (Levitan \textit{et al.}, 2010; Mozaffarian \textit{et al.}, 2005b) and non-arrhythmic cardiac deaths (Valagussa \textit{et al.}, 1999) indicating that FO alters cardiac haemodynamic performance. Experimental studies have demonstrated FO to preserve contractility in a rat hypertrophic heart failure
model (McLennan et al., 2012), limit ischaemic cardiac damage, reduce oxygen consumption at any given work output and increase post-ischaemic contractile recovery (Abdukeyum et al., 2008; Pepe & McLennan, 2002). Ejection fraction of the left ventricle has been demonstrated to increase as a result of enhanced ventricular filling in the marmoset heart (McLennan et al., 1992a). Furthermore, isolated working heart studies in the rat using supra-therapeutical doses of FO have demonstrated improved haemodynamic function and the authors postulated that these effects should extend to lower doses (Pepe & McLennan, 2002; Pepe & McLennan, 2007). Cohort and experimental studies provide strong evidence that dietary FO supplementation can augment cardiac haemodynamics during diseased states, particularly when using therapeutic and supra-therapeutical doses. However, it is unknown if dietary achievable FO doses alters cardiac haemodynamics during rested conditions. Given the evidence of reductions in heart rate following FO supplementation, the second objective of this study was to determine whether cardiac haemodynamic performance changes are observed in parallel to ensure cardiac output is maintained.

4.1.4 Heart rate variability

Absolute heart rate cannot predict the balance of autonomic control pertaining to cardiac function. This is because, even during periods of rest when the heart rate is stable, the timing between consecutive heart beats fluctuates in order to maintain a stable cardiac output and mean arterial blood pressure. The speeding and slowing of the cardiac cycle between beats, as a result of vagal and sympathetic tone, is referred to as heart rate variability (HRV). Measurement of HRV can be categorised within time, frequency and non-linear domains in order to describe the oscillations between consecutive heart beats. Animal and human studies use HRV as a non-invasive surrogate assessment of autonomic control of the heart (Akselrod et al., 1981; Camm et al., 1996; Sztajzel, 2004). Decreased HRV is strongly associated with sudden cardiac death in post-myocardial infarction patients (Bigger Jr et al., 1992; Bilehick et al., 2002; Hull et al., 1990). Furthermore, HRV has been demonstrated to independently predict cardiovascular disease mortality in populations with prior cardiovascular disease history (Carpeggiani et al., 2004; Galinier et al., 2000) as well as in healthy populations (Dekker et al., 2000).
4.1.4a The effect of fish oil on heart rate variability

The effect of FO on HRV is unclear and inconsistent. Traditionally high cardiac risk subgroups or post-myocardial infarction patient’s display reduced HRV, however, research has demonstrated some evidence that FO is associated with modest improvements in HRV indices in such patients (Christensen et al., 1999; Christensen et al., 2001; Mozaffarian et al., 2008; O’Keefe et al., 2006; Xin et al., 2013). However, some studies have demonstrated conflicting and inconsistent results when assessing the effect of DHA on time-domain (Dyerberg et al., 2004; O’Keefe et al., 2006) frequency-domain (Billman & Harris, 2011; Carney et al., 2010; Ninio et al., 2008) and non-linear domain parameters (Macartney et al., 2014). However, a recent meta-analysis of human trials concluded that there is an association between short term consumption of FO with improved HRV, particularly frequency domain indices (Xin et al., 2013). Considering this evidence, the final objective of this study was to investigate any associations between HRV measures and resting heart rate following FO supplementation.

In summary, there is strong evidence that demonstrates FO to have a direct cardio-protective effect on the myocardium through slowing heart rate and altering cardiac haemodynamics. However, previous research has relied on therapeutical and supra-therapeutical doses of FO and predominately ex vivo animal models to demonstrate these effects. Therefore, investigation of the physiological effects of dietary achievable FO doses, using a physiologically stable in vivo model, on resting cardiac function is warranted. Evidence-based observations of dietary FO altering cardiac haemodynamic performance and electrophysiology at these doses will help provide plausible physiological mechanisms of action of DHA on rested cardiac function. This will provide direct evidence and allow more confident extrapolation of findings from previous therapeutical and supra-therapeutical dose studies to explain the cardio-protective action of FO demonstrated in human epidemiological and cohort studies.
4.2 AIMS AND HYPOTHESES

This study investigated electrophysiological and haemodynamic function in the rested *in vivo* rat heart following supplementation of human dietary achievable FO doses to a typical Western-style diet (high SFA and n-6 PUFA).

Specifically, these experiments had the following aims and hypotheses:

**Aim 1:** Determine the influence of supplementing dietary achievable FO doses on ‘intrinsic’ cardiac function.

*Hypothesis 1:* Animals supplemented with FO will have a slower resting heart rate than animals from the Control group.

*Hypothesis 2:* Slower resting heart rate will be observed in direct relationship with modified cardiac haemodynamic performance in order to maintain cardiac output at rest.

**Aim 2:** Determine the influence of supplementing dietary achievable FO doses on ‘extrinsic’ autonomic control of cardiac function.

*Hypothesis 3:* Slower resting heart rate will be associated with higher HRV. Furthermore, the modification will be most evident in the frequency domain.
4.3 METHODS

4.3.1 Animals, ethical considerations and standardisation

Male rats (Sprague Dawley) were obtained and used under University of Wollongong ethical approval for this study (Section 2.3.1). Animals were housed and weighed according to procedures outlined in Section 2.3.2.

4.3.2 Groups

Sixty two (N = 62, cohort 2) animals were used supplied lab chow and water *ad libitum* for one week prior to dietary supplementation of FO beginning. Animals were then randomly split into three groups and supplied water and one of three pre-fabricated diets *ad libitum* for four weeks before experimentation. The diets contained different amounts of FO (Control – 0%: n = 22, LowFO – 0.31%: n = 20, ModFO – 1.25%: n = 20).

4.3.3 Experimental preparation

Left ventricular *in vivo* haemodynamic variables were obtained using a 6mm miniaturised 2-French (2F) Millar pressure-volume conductance catheter (SPR-638 Millar Instruments, Houston, USA) for rats. ECG was also used to collect cardiac electrical activity, both the conductance catheter and ECG were connected to PowerLab 8/35 software (ADInstruments, Bella Vista, NSW, Aus). Experimental preparation of the conductance catheter was completed according to the methods outlined in Section 3.3.3.

4.3.4 Surgical procedures

Animals were prepared for experimental measurements according to the surgical procedures outlined in Section 3.3.4. Briefly, animals were removed from their cages, weighed and given an i.p injection of anaesthetic (Pentobarbital: 60mg/kg) before being returned to their cage and covered with a towel until they became non responsive to the toe pinch test. Animals were then placed on a heating pad and animal core temperature was maintained at 37°C via automated feedback loop from a rectal probe measuring core temperature. ECG was connected to the animal via the traditional three-lead method. An incision was made in the midline neck to allow for artificial ventilation via
the trachea, and cannulation of the right common carotid artery and the left internal jugular vein was completed to allow entrance of the conductance catheter and fluid lines respectively. The conductance catheter was prepared prior to each experiment and calibrated using the cuvette and saline calibration methods following the end of each experiment.

4.3.4a Left ventricular load-independent haemodynamics

To collect load-independent measurements using the pressure-volume conductance catheter the inferior vena cava was transiently occluded after resting haemodynamics had been collected. The abdominal wall was opened via a transverse incision just inferior to the xiphoid process and diaphragm. The coronary ligament of the liver was cut and removed using micro surgical scissors and the liver was held inferiorly to allow access to the inferior vena cava. A cotton tip applicator was then used to apply pressure on the inferior vena cava against the diaphragm and occlude it for four to six seconds, reducing venous return to the heart and allowing load-independent indices to be measured. Occluding the vena cava for short time frames leads to a reduction in venous return and thereby reduces end-diastolic volume and preload. The typical response observed in the PV-loop during vena cava occlusion is a shift of the loop towards the left and drop in the left ventricular pressure developed (Figure 4.1). Measuring end-systolic and end-diastolic pressure-volume relationships allows assessment of cardiac contractility and diastolic relaxation. The passive tension filling curve for the ventricle is known as the end-diastolic pressure-volume relationship (EDPVR), the slope of this curve is the reciprocal of ventricular compliance. Increases in EDPVR indicate impaired diastolic relaxation or ‘stiffness’ in the left ventricle myocytes. The end-systolic pressure-volume relationship (ESPVR) describes the maximal pressure that the ventricle can generate at any given left ventricular volume. The ESPVR is analogous to the total tension that the ventricle can generate as the PV-loop cannot cross the ESPVR under a given inotropic state. Therefore, ESPVR is a useful indicator of left ventricular systolic contractile function. The ability to measure left ventricular cardiac parameters independently from loading conditions is particularly important for the assessment of acute changes in left ventricular performance (Pacher et al., 2008) and emphasises the importance of the current model.
Figure 4.1: Representation of the effect of inferior vena cava occlusion on PV-loops to calculate the ESPVR and EDPVR.

ESPVR = End systolic pressure-volume relationship
EDPVR = End diastolic pressure-volume relationship
4.3.5 Experimental protocol and measurements

Once animals were sufficiently anaesthetised and ECG was connected, animals were left to rest for five minutes before baseline resting ECG recording was collected for fifteen minutes. Baseline resting heart rate and HRV analysis of data collected via the ECG was completed in the last five minutes of recording. Upon completion of ECG measurements surgical procedures began. Following surgical procedures animals were given ten minutes rest while anaesthetised with the conductance catheter in the left ventricle of the heart. Resting cardiac function and PV-loops were then recorded via the conductance catheter for ten minutes, the last five minutes of the recorded data was used for analyses. The abdominal cavity was then opened and load-independent indices were measured via occlusion of the inferior vena cava on three separate occasions for each animal with five minutes recovery between each occlusion. An overview of the experimental protocol is given in Figure 4.2.
**Figure 4.2:** Overview of the resting in vivo cardiac function experimental protocol carried out.

**Groups:** Control, LowFO – 0.31% fish oil & ModFO – 1.25% fish oil

**Abbreviations:** ECG = electrocardiogram; PV = pressure-volume; LV = left ventricle; IVC = inferior vena cava.
4.3.6 Post-experimental data analysis

4.3.6a Heart rate and heart rate variability

Offline analysis of the saved ECG recordings was completed on the final five minutes of data using LabChart 8 software to investigate heart rate (Section 3.3.8a) and HRV. LabChart 8 was used to detect R waves and calculated 512 RR interval time periods from the cleanest segment of the five minute recording for HRV analysis. Data sets of 512 RR intervals were chosen as this length has been recommended to be the best compromise between the need for a large time series in order to achieve frequency domain accuracy and the practicality of shorter time periods (Kuwahara et al., 1994). The data sets were converted into a text file and exported into Kubios HRV analysis program (Version 2.2, Department of Physics of the University of Kuopio, Finland). The recordings were placed through an automatic filter set to low which is part of the Kubios program. The filter was used to remove any artefacts and/or ectopic beats and leave only normal beats. This allowed the remaining data to be referred to as NN intervals rather than R-R intervals as they are all normal beats. HRV measures analysed included time, frequency and nonlinear indices.

Time domain heart rate variability

Time domain parameters measured included the mean NN interval (R-R time between successive beats), the standard deviation of NN intervals and the root mean square of differences of successive NN intervals (Figure 4.3). The standard deviation of NN reflects cyclic elements responsible for the variability in the corresponding period of recording; therefore standard deviation of NN represents long term HRV (Camm et al., 1996). The standard deviation of NN captures short term changes in variability of the heart and has been shown to be an acceptable index of vagal (parasympathetic) mediated change to the heart (Camm et al., 1996).
Frequency domain heart rate variability

Frequency domain analysis was used to calculate power spectrum density estimates present in the recordings. The RR interval series recording was detrended prior to analysis using the smoothness priors method with regularization parameter ($\lambda$) set to 500 and a cubic spline interpolation technique described previously (Tarvainen et al., 2002). Power spectrum density estimates were calculated using the fast fourier transformation. RR interval series were resampled at 4Hz and fast fourier transformation window length of 256 samples was used. The frequency-domain measures calculated were low frequency and high frequency power in normalised units and as a percentage of overall power. The frequency bands identified for analysis were: low frequency (0.04 - 0.8Hz) which represented both sympathetic and parasympathetic activity and high frequency (0.8 – 1.5Hz) which is considered an index of cardiac vagal control.

Non-linear domain heart rate variability

Sample entropy and approximate entropy were assessed via non-linear domain analyses. In terms of HRV analysis, accurate long period data sets can be hard or impossible to obtain in animals, therefore sample entropy and approximate entropy calculations are
acceptable to allow the measure of regularity from shorter recordings. Additionally, Poincaré plots were used for quantitative and qualitative beat-to-beat analysis of HRV. The Poincaré plot is a scattergram of all NN intervals in which each NN interval is plotted as a function of the previous one (Figure 4.4). As the RR interval lengthens progressively to its maximum then shortens progressively to minimum repeatedly, the shape of the plot allows the fit of an ellipse, which can be used to quantify SD1 and SD2. SD1 represents dispersion of the points perpendicular to the line of identity and is thought to be an index of instantaneous (parasympathetic) beat-to-beat variation (Camm et al., 1996). SD2 represents dispersion of the points along the line of identity, and represents the slow variability (sympathetic) of the heart (Camm et al., 1996). Additionally, the ratio of SD1:SD2 was calculated which represents the randomness in the HRV time series recording (Camm et al., 1996).

![Figure 4.4: Representation of a Poincaré plot measured in a rat during rested cardiac function.](image)

**Figure 4.4 notes:** The identity line divides the Poincaré plot into two areas, decelerations are located above and accelerations below this line. As described the variance of the projection of the Poincaré plot points along the identity line is the short-term variability SD1 and perpendicular projection leads to long-term variance SD2.
4.3.6b Left ventricular cardiac haemodynamics

Resting left ventricular cardiac haemodynamic function was analysed from the post-surgery measurements in the final five minutes of the recording period. This gave each animal time to stabilise following surgery and reduced the chance of surgical procedures influencing the haemodynamic measurements collected. Analysis was completed following the methods outlined in section 3.3.8b. Load-independent indices were analysed at each inferior vena cava occlusion and the average of all three measurements were used to assess the ventricular systolic and diastolic function of dietary groups.

4.3.6c Statistics

All results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The effects of dietary treatment were tested via ANOVA. A Bonferroni post-hoc test for comparison between group means was then used to identify differences. A Dunnett’s T-test was used for comparison of individual diets against the Control group. A repeated measures ANOVA (Dietary treatment*time) was completed to investigate changes in heart rate between the groups during three time points across the experiment. All statistical analyses were performed in a blinded manner and completed using SPSS 21 for Windows (SPSS Inc, Chicago, IL, USA). Alpha was set as $p < 0.05$. 
4.4 RESULTS

Sixty two (N=62) animals were used for the experimentation following dietary supplementation (*Figure 4.5*). Two (2) animals in the Control and one (1) animal in the LowFO groups died directly following injection of the anaesthetic. During surgery a further three (3) animals in the LowFO and two (2) animals in the ModFO groups died, likely caused by surgical damage to the conduction pathways, surgical complication resulting in cardiogenic shock or anaesthetic overdose. Separate to these, the conductance catheter was unable to be guided into the left ventricle of two (2) animals in Control group and one (1) animal in the LowFO group; this meant only ECG measurements were collected from these animals at the post-surgical stage of the experiment. Final group numbers for resting ECG and HRV measurements were $n = 19 - 20$ respectively and resting left ventricle cardiac haemodynamic measurements were collected from $n = 15 - 18$ respectively.

*Figure 4.5: Overview of animal group numbers throughout experimentation.*

*Abbreviations: ECG = electrocardiogram; PV = pressure-volume; LV = left ventricle.*
4.4.1 The effect of fish oil supplementation on resting heart rate

Resting heart rate was analysed \((n = 16 – 20)\) at three different time points during the experiment including baseline, pre-surgery and post-surgery (Figure 4.6). Baseline, pre-surgical and post-surgical resting heart rate of the Control group was between 447 – 454 beats.min\(^{-1}\). The FO groups displayed a dose-related slowing in baseline resting heart rate compared to the Control group. The LowFO group (429 – 445 beats.min\(^{-1}\)) trended slower at all time points but was only significantly slower during baseline measures compared to the Control group \((p < 0.01)\). Whereas, resting heart rate of the ModFO group (420 – 426 beats.min\(^{-1}\)) was significantly slower at all three time points compared to the Control group \((p < 0.05)\).

Figure 4.6: The effect of fish oil on resting heart rate (beats.min\(^{-1}\)) calculated at different times throughout experimentation.

Values are mean ± SEM. \((n = 16 – 20)\) *p <0.05 vs Control **p <0.01 vs Control †p <0.001 vs Control (One-way ANOVA with Dunnett’s T-test vs Control group).
4.4.2 The effect of fish oil supplementation on resting heart rate variability

Time domain, frequency domain and non-linear domain HRV parameters were analysed during the pre-surgery measurements after fifteen minutes of rest. Both FO groups displayed a dose-related higher mean NN interval time than the Control group (Control: 134 ±2, LowFO: 140 ±2, ModFO: 145 ±3ms) compared to the Control group \( (p =0.015) \). This mirrored the slowed heart rate previously discussed. There were no significant differences displayed in either the SDNN or RMSSD between any of the groups (Table 4.1). Fast fourier transformation (FFT) analysis displayed high frequency band dominance (77 - 83% of total power) compared to low frequency (16 - 22% of total power) during the recording period regardless of dietary group. However, there were no statistical differences observed between dietary groups for any frequency domain measures calculated.

Non-linear domain parameters SD1, SD2, the ratio of SD1:SD2, approximate entropy and sample entropy were calculated from the Poincaré plots produced (Figure 4.7). The Control group displayed a SD1/SD2 ratio of 0.520 ±0.01, sample entropy of 1.154 ±0.04 and approximate entropy of 0.940 ±0.02. The SD1/SD2 ratio was demonstrated to be significantly higher in the LowFO (0.566 ±0.01) and ModFO (0.580 ±0.01) groups compared to the Control group (Table 4.1). Sample entropy was significantly higher in the ModFO group compared to the Control group \( (p <0.05) \). Approximate entropy trended higher in the LowFO and ModFO groups but did not reach significance \( (p =0.103) \). However, pooled analysis of both FO groups showed a significantly higher approximate entropy compared to the Control group \( (p =0.035) \).
### Table 4.1: The effect of fish oil on heart rate and heart rate variability indices during pre-surgical measurements.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time domain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean HR (b.min(^{-1}))</td>
<td>447 (±7)</td>
<td>430 (±6)</td>
<td>425 (±6)*</td>
<td>0.017</td>
</tr>
<tr>
<td>STD (b.min(^{-1}))</td>
<td>5 (±1)</td>
<td>5 (±1)</td>
<td>4 (±1)</td>
<td></td>
</tr>
<tr>
<td>Mean NN (ms)</td>
<td>134 (±2.2)</td>
<td>140 (±2.1)</td>
<td>145 (±3.6)*</td>
<td>0.015</td>
</tr>
<tr>
<td>SDNN (ms)</td>
<td>1.3 (±0.4)</td>
<td>1.4 (±0.4)</td>
<td>1.2 (±0.3)</td>
<td></td>
</tr>
<tr>
<td>RMSSD (ms)</td>
<td>1.3 (±0.4)</td>
<td>1.4 (±0.4)</td>
<td>1.3 (±0.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Frequency domain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFT LF Power (%)</td>
<td>16.4 (±2.4)</td>
<td>21.5 (±3.6)</td>
<td>21.1 (±3.7)</td>
<td></td>
</tr>
<tr>
<td>LF Power (nu)</td>
<td>16.4 (±2.4)</td>
<td>21.5 (±3.6)</td>
<td>21.2 (±3.7)</td>
<td></td>
</tr>
<tr>
<td>HF Power (%)</td>
<td>83.2 (±2.4)</td>
<td>78.2 (±3.7)</td>
<td>78.4 (±3.8)</td>
<td></td>
</tr>
<tr>
<td>HF Power (nu)</td>
<td>83.4 (±2.4)</td>
<td>78.3 (±3.6)</td>
<td>78.6 (±3.7)</td>
<td></td>
</tr>
<tr>
<td>LF/HF</td>
<td>0.22 (±0.1)</td>
<td>0.35 (±0.09)</td>
<td>0.36 (±0.09)</td>
<td>0.32</td>
</tr>
<tr>
<td>Pooled LF/HF</td>
<td>0.22 (±0.04)</td>
<td>0.35 (±0.06)</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Non-linear domain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD1 (ms)</td>
<td>0.94 (±0.3)</td>
<td>1.02 (±0.3)</td>
<td>0.93 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>SD2 (ms)</td>
<td>1.68 (±0.5)</td>
<td>1.76 (±0.6)</td>
<td>1.48 (±0.3)</td>
<td></td>
</tr>
<tr>
<td>SD1/SD2</td>
<td>0.52 (±0.1)</td>
<td>0.56 (±0.01)</td>
<td>0.58 (±0.01)*</td>
<td>0.031</td>
</tr>
<tr>
<td>SampEn</td>
<td>1.15 (±0.1)</td>
<td>1.30 (±0.1)</td>
<td>1.42 (±0.1)*</td>
<td>0.033</td>
</tr>
<tr>
<td>ApEn</td>
<td>0.94 (±0.02)</td>
<td>0.99 (±0.1)</td>
<td>1.03 (±0.1)</td>
<td>0.10</td>
</tr>
<tr>
<td>Pooled ApEn</td>
<td>0.94</td>
<td>1.02 (±0.02)*</td>
<td></td>
<td>0.035</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 19 – 20). *p <0.05 vs Control diet (One-way ANOVA with Dunnett’s T-test vs Control group). Pooled analysis compared both FO diets combined vs Control.

**Abbreviations:** SDNN = standard deviation of NN intervals; RMSSD = root mean square standard deviation of NN intervals; FFT = fast fourier transformation; LF = low-frequency power; HF = high-frequency power; SD1 = standard deviation of points perpendicular to the axis; SD2 = standard deviation of points along the axis of line-of-identity; ApEn = approximate entropy; SampEn = sample entropy.
Figure 4.7: Representative Poincaré plots from a Control and ModFO animal during rested cardiac function measurements.
4.4.3 The effect of fish oil supplementation on resting cardiac haemodynamics

Resting aortic blood pressure was analysed from data following the surgery but before the catheter had been advanced into the left ventricle. No significant differences were observed in diastolic pressure, systolic pressure or MAP between any of the groups ($p >0.05$). Resting left ventricular cardiac haemodynamics and preload-independent indices were analysed from the post-surgery measurements in the final five minutes of the recording. The average heart rate, stroke volume and cardiac output were $451 \pm 8$beats.min$^{-1}$, $100 \pm 7$µL and $44 \pm 3$mL.min$^{-1}$ respectively in the Control group. Average heart rate in the ModFO group was significantly slower than the Control group (Table 4.2). The slower heart rate observed in the ModFO group was accompanied with a non-significant elevation of stroke volume compared to both the other groups. No significant differences were observed in cardiac output between any of the groups ($p >0.05$).

Furthermore, there were no significant differences in systolic (ejection fraction – EF, maximal rate of pressure change – $dP/dt_{max}$) or diastolic function (Isovolumic relaxation constant – Tau, minimal rate of pressure change – $dP/dt_{min}$) between any of the dietary groups.
Table 4.2: The effect of fish oil on resting cardiac haemodynamics measured post-surgery with a pressure volume conductance catheter.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>94 (±4)</td>
<td>90 (±3)</td>
<td>93 (±3)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>122 (±6)</td>
<td>125 (±6)</td>
<td>127 (±4)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>103 (±5)</td>
<td>101 (±4)</td>
<td>104 (±4)</td>
</tr>
<tr>
<td>HR (beats.min(^{-1}))</td>
<td>451 (±8)</td>
<td>446 (±6)</td>
<td>426 (±8)*</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>88 (±7)</td>
<td>90 (±8)</td>
<td>108 (±9)</td>
</tr>
<tr>
<td>CO (mL.min(^{-1}))</td>
<td>39 (±3)</td>
<td>40 (±3)</td>
<td>46 (±4)</td>
</tr>
<tr>
<td>CI (mL.min(^{-1}) x kg)</td>
<td>23 (±2)</td>
<td>23 (±2)</td>
<td>25 (±2)</td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>65 (±10)</td>
<td>56 (±9)</td>
<td>71 (±10)</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>153 (±9)</td>
<td>145 (±12)</td>
<td>179 (±16)</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>139 (±7)</td>
<td>132 (±4)</td>
<td>140 (±5)</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>13 (±1)</td>
<td>12 (±1)</td>
<td>10 (±1)</td>
</tr>
<tr>
<td>DFP (sec)</td>
<td>0.068 (±0.001)</td>
<td>0.070 (±0.002)</td>
<td>0.078 (±0.002)*</td>
</tr>
<tr>
<td>Ea (mmHg/µL)</td>
<td>1.5 (±0.2)</td>
<td>1.5 (±0.2)</td>
<td>1.2 (±0.1)</td>
</tr>
</tbody>
</table>

**Systolic indices**
- EF (%): 67 (±5) 70 (±4) 63 (±4)
- \(dP/dt_{\text{max}}\) (mmHg.sec\(^{-1}\)): 8688 (±441) 8796 (±288) 8537 (±401)

**Diastolic indices**
- \(dP/dt_{\text{min}}\) (mmHg.sec\(^{-1}\)): -7735 (±483) -7773 (±364) -7440 (±313)
- Tau (ms): 10.0 (±0.3) 9.6 (±0.4) 10.0 (±0.4)

Values are mean ± SEM (n = 15 – 18). *p < 0.05 vs Control diet (One-way ANOVA with Bonferroni post-hoc test).

**Abbreviations:**
- HR = heart rate; SV = stroke volume; CO = cardiac output; CI = cardiac index, CO/Body weight; ESV = end-systolic volume; EDV = end-diastolic volume; ESP = end-systolic pressure; EDP = end-diastolic pressure; Ea = arterial elastance (measure of ventricular afterload).

**Systolic indices:** EF = ejection fraction; \(dP/dt_{\text{max}}\) = peak rate of pressure rise.

**Diastolic indices:** \(dP/dt_{\text{min}}\) = peak rate of pressure decline; Tau = relaxation time constant calculated by Glantz method (regression of \(dP/dt\) versus pressure).

**Table 4.2 notes:** Two (2) animals in the ModFO group were removed from the analysis due to bradycardia associated with atrioventricular block, without preceding arrhythmia. This was most likely caused by surgical damage to the conduction pathways, surgical complication resulting in cardiogenic shock or anaesthetic overdose.
Left ventricular cardiac haemodynamics during inferior vena cava occlusions were analysed to assess preload-independent changes of systolic and diastolic function. There were no statistical differences present between dietary groups for the slope calculated of the ESPVR, PRSW and $dP/dt_{\text{max}}$ – EDV relationships (Figure 4.8). Pooled analysis of FO vs Control animals did not show any statistical differences either but the trend became stronger (Table 4.3).

**Table 4.3: The effect of fish oil on preload-independent indices of left ventricular function during inferior vena cava occlusion.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th>Trend p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systolic indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW (mmHg*μL)</td>
<td>10933 (±963)</td>
<td>9693 (±744)</td>
<td>10226 (±917)</td>
<td></td>
</tr>
<tr>
<td>ESPVR</td>
<td>2.11 (±0.21)</td>
<td>2.14 (±0.18)</td>
<td>2.28 (±0.14)</td>
<td></td>
</tr>
<tr>
<td>- Pooled</td>
<td></td>
<td>2.22 (±0.11)</td>
<td></td>
<td>0.637</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>115 (±8)</td>
<td>120 (±8)</td>
<td>124 (±7)</td>
<td></td>
</tr>
<tr>
<td>- Pooled</td>
<td></td>
<td>122 (±5)</td>
<td></td>
<td>0.498</td>
</tr>
<tr>
<td>$dP/dt_{\text{max}}$–EDV (mmHg.s$^{-1}$ ml$^{-1}$)</td>
<td>46 (±8)</td>
<td>49 (±8)</td>
<td>55 (±6)</td>
<td></td>
</tr>
<tr>
<td>- Pooled</td>
<td></td>
<td>53 (±5)</td>
<td></td>
<td>0.517</td>
</tr>
<tr>
<td><strong>Diastolic indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDPVR</td>
<td>0.038 (±0.009)</td>
<td>0.041 (±0.008)</td>
<td>0.036 (±0.006)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM ($n = 9 – 12$). Pooled analysis completed with both FO diets combined vs Control.

**Abbreviations:**

**Systolic indices:** SW = stroke work; ESPVR = end-systolic pressure volume relation; PRSW = preload recruited stroke work (slope of stroke work–EDV relationship); $dP/dt_{\text{max}}$–EDV = $dP/dt_{\text{max}}$–end diastolic volume relation.

**Diastolic indices:** EDPVR = end-diastolic PV relation slope.
Figure 4.8: Typical PRSW and $dP/dt_{max}$ – EDV relationship from one animal in each dietary group.

*Regression lines shown are from one (1) animal per group, mean slope is calculated from entire group.
4.5 DISCUSSION

This study demonstrated that supplementing FO doses achievable within a typical Western-style human diet (high SFA and n-6 PUFA) modified in vivo resting cardiac function. Physiologically, resting heart rate was slowed in a dose-related manner, cardiac output was maintained despite the slower heart rate and non-linear domains of vagal tone were higher in FO supplemented animals. The dietary achievable FO doses used in this study, for the first time, provide a compelling physiological link to explain the consistent observation of fish or FO intake being associated with lower heart rates in humans (Dallongeville et al., 2003; Geelen et al., 2005; Grimsgaard et al., 1998; Mozaffarian. et al., 2005). The ability of FO to reduce resting heart rate in healthy populations is likely associated with its consistently demonstrated cardio-protective effect in previous epidemiology, intervention trials and animal research (Burr et al., 1989; McLennan, 2014; Mozaffarian & Wu, 2011). Importantly, the physiological measurements collected in all dietary groups using the conductance catheter in this study were consistent with previous values reported in Chapter 3, and within the normative physiological range outlined in a separate study (Pacher et al., 2008). Thus, the in vivo experimental model used in this study provides a strong platform to examine the differences observed in rested cardiac function between dietary groups.

4.5.1 Resting blood pressure, heart rate and cardiac haemodynamics

No differences were found for resting blood pressures following supplementation. Blood pressure acts to maintain perfusion of oxygen and essential nutrients to the organs, tissues and cells of the body. Thus, in a healthy population, the cardiovascular system acts to control contributing cardiac factors such as heart rate and stroke volume (cardiac output) and vascular factors such as total peripheral resistance in order to maintain mean arterial blood pressure. Dietary supplementation of fish oil has only been shown to reduce blood pressure in hypertensive subjects in a dose-dependent relationship, but/and only in supra-therapeutic doses (Appel et al., 1993; Kris-Etherton et al., 2009; Morris et al., 1993). Considering the normotensive blood pressures of the animals in the current study, it would be counterproductive if fish oil acted to reduce them further. Importantly however, this study is the first to demonstrate that resting
heart rate is slowed by $\sim 20 - 40$ beats min$^{-1}$ in a dose-related manner in the rat heart which has been supplemented with dietary achievable FO doses.

Previously, animal studies have demonstrated that DHA confers the ability to reduce heart rate in rats (Abdukeyum et al., 2008; Lortet & Verger, 1995; Pepe & McLennan, 1996), exercising conditioned horses (O'Connor et al., 2004) pigs (Hartog et al., 1987), and dogs (Billman & Harris, 2011). Additionally, FO supplementation has been reported to slow resting heart rate in generally healthy but untrained humans (Mozaffarian. et al., 2005), similar to the slowed resting heart rate associated with regular physical activity (Scheuer & Tipton, 1977). Heart rate has also been shown to be reduced by FO supplementation during cardiac induced stress from exercise protocols (Buckley et al., 2009; Macartney et al., 2014; Ninio et al., 2008; Peoples & McLennan, 2008). However, slowed heart rates in animal models have only been associated with the delivery of therapeutical and supra-therapeutical FO doses. Likewise, human studies demonstrating slowed resting or exercise heart rate have provided supplementation of FO doses in the therapeutic range (Mozaffarian. et al., 2005).

Physiologically, slowing of the heart rate in the ModFO group resulted in $\sim 40$ beats min$^{-1}$ less than Control animals, this equates to approximately 2400 less heart beats per hour. This slowing of heart rate is important when related to myocardial oxygen demand (MVO$_2$) of the heart. Cardiac MVO$_2$ is an index of the oxygen consumption and energy used by the heart for a given workload. Basal myocardial energy metabolism accounts for approximately 25% of myocardial MVO$_2$, myocardial wall tension, rate of contraction and contractility are the other primary factors determining MVO$_2$. Factors that enhance tension development by myocytes, such as the rate of tension development (systolic blood pressure), or the number of tension generating cycles (heart rate) will increase MVO$_2$. The importance of MVO$_2$ was highlighted almost a century ago in Ernest Starling’s laboratory which demonstrated that the heart is an aerobic organ which cannot develop an oxygen debt except for very brief periods (Evans & Ogawa, 1914). Dietary achievable FO doses slowed heart rate thereby leading to favourable reductions in MVO$_2$.

Slowed resting heart rate leads to increased myocardial perfusion time due to an increased time period of the diastolic phase in the cardiac cycle. Blood flow to the myocardium is greatest during the diastolic phase as the coronary vessels are not
compressed due to the higher pressures associated with systole (Spaan et al., 2008). Diastolic filling period was demonstrated to be approximately ten milliseconds longer in the ModFO group compared to the Control group and the LowFO group also showed a slightly longer diastolic filling period. Given that the resting heart rate observed in the ModFO group was 425 beats.min\(^{-1}\) and diastolic filling period was approximately 10 milliseconds longer for each heartbeat, this would result in cardiac perfusion time to myocardial membranes increasing by almost four and a half minutes per hour (425beats.min\(^{-1}\) * 0.01sec [diastolic filling period] * 60sec).

Cardiac efficiency is improved when diastolic filling period is longer because more volume enters the ventricle leading to a greater end-diastolic volume. The Frank-Starling law of the heart demonstrates that a greater end-diastolic volume otherwise referred to as ‘preload’ allows more myocardial stretch which results in a stronger contraction at a lower energy cost (Starling & Visscher, 1927). This phenomenon helps demonstrate how stroke volume is acutely adjusted when preload is changed in order to maintain forward movement of blood volume throughout the cardiac cycle. During periods of increased preload cardiac myocytes are stretched, this causes two processes to occur: (i) troponin C Ca\(^{2+}\) binding sites to become more sensitive to Ca\(^{2+}\) allowing increased rates of actin and myosin cross-bridge attachment and detachment (ii) the amount of Ca\(^{2+}\) supplied to the cardiac myocytes is increased (Allen & Kentish, 1985). Together this causes the myocyte to generate a stronger contraction when stretched. Stroke volume was not statistically different between any of the groups in this study. However, the ModFO group displayed the greatest stroke volume which is likely closely linked to the greater end-diastolic volume observed and the Frank-Starling law of the heart.

Previously, FO supplementation in animal models has shown associations with increased left ventricular ejection fraction in healthy hearts (McLennan et al., 1992a) and increased myocardial contractility in models of hypertrophic heart failure (McLennan et al., 2012) and ischaemia and reperfusion (Pepe & McLennan, 2002). Taken in combination, these findings suggest improved contractile properties of the myocardium following incorporation of DHA. Although this study did not identify any changes in left ventricular contractile properties at rest, the observation of maintained
cardiac output despite slowed resting heart rate suggests that contractile function was modified.

### 4.5.2 Heart rate variability

The intrinsic control of heart rate is modulated by the spontaneous depolarization of pacemaker cells in the sino-atrial node. Moment by moment changes in heart rate are then further mediated through the vagal nerve as part of the parasympathetic nervous system (PNS). Acetylcholine acts on muscarinic receptors to slow heart rate with high vagal tone, whereas vagal withdrawal causes heart rate to increase. Sympathetic nervous system (SNS) stimulation acts via noradrenaline on $\beta_1$-adrenergic receptors to increase heart rate and force, whereas sympathetic withdrawal acts to decrease heart rate and force. However, the SNS acts much slower than the PNS to modify heart function (Nicolini et al., 2012). The influence of the PNS or SNS over heart rate is generally evaluated through heart rate variability (HRV) measurements and analyses (Camm et al., 1996).

This study demonstrated higher sample entropy and a high SD1:SD2 ratio in both FO groups compared to the Control group. Entropy is defined as the rate of generation of new information over a large data set, or the regularity of the data, e.g. it is a measure of how likely an RR interval will repeat itself (Richman & Moorman, 2000). Thus, higher entropy suggests a more irregular heart rate (increased HRV) during the recording period. Higher SD1:SD2 has been proposed to indicate stronger vagal tone (Camm et al., 1996), suggesting that vagal tone in this study was higher in FO supplemented animals and may have facilitated the slowed resting heart rate. In contrast, high frequency power which is thought to represent vagal tone (Pomeranz et al., 1985), trended lower in both FO groups, implying decreased vagal tone. The conflicting nature of the non-linear and frequency domain measures analysed in this study makes it difficult to draw direct conclusions of autonomic tone slowing resting heart rate in this study. Nevertheless, the demonstration of higher sample entropy and a higher SD1:SD2 ratio aligns with conclusions drawn from previous studies that describe therapeutical and supra-therapeutical doses of FO to increase HRV, particularly vagal tone (Billman & Harris, 2011; Xin et al., 2013)
Despite the great awareness of HRV and its association with autonomic control of heart rate, the analysis and relation of HRV to autonomic control is still controversial (Karemaker, 2006). Animal and human studies use HRV as a non-invasive surrogate assessment of autonomic control of the heart (Akselrod et al., 1981; Camm et al., 1996; Sztajzel, 2004). However, it has also been demonstrated that HRV is primarily a non-linear surrogate of heart rate itself, making it inappropriate to draw conclusions on the autonomic nerve activity controlling the heart (Bolea et al., 2016; Monfredi et al., 2014; Rocchetti et al., 2000; Zaza & Lombardi, 2001). Furthermore, it has been demonstrated that variability in the beat rate remains in the human transplanted heart that cannot be attributed to sympathetic or parasympathetic nervous influences (Camm et al., 1996; Fallen et al., 1988; Sands et al., 1989). This variability in beat rate exists even in isolated rabbit hearts (Janoušek et al., 2010), isolated myocytes (Zaniboni et al., 2014) and isolated cardiomyocytes derived from human stem cells in culture (Mandel et al., 2012). As such, variability in beat rate is likely influenced by both autonomic tone and intrinsic mechanisms within the sino-atrial node of the heart.

The evidence for modified autonomic tone in this study was conflicting and did not allow conclusions to be drawn in relation to what caused the slowing of resting heart rate. Nevertheless, several studies have demonstrated slowed heart rate in cardiac transplant patients (Harris et al., 2006b) and in rat isolated hearts following FO supplementation (Abdukeyum et al., 2008; Pepe & McLennan, 1996, 2002), both of which would be void of neural inputs. Interestingly, resting heart rate has been demonstrated to remain unchanged despite a trending reduction in vagal tone which should increase heart rate, following supplementation of dietary achievable FO doses to healthy young males (Macartney et al., 2014). The same study also described a faster recovery of heart rate towards resting rates following intense exercise with an associated decrease in vagal tone. Given these observations in addition to the evidence from transplant and isolated heart studies, it is likely that slowing and variation of beat by beat rate is related to an intrinsic mechanism of action of DHA at the pacemaker region of the heart, rather than altered autonomic tone.

4.5.3 Conclusions

In a dose-related manner, in vivo resting heart rate was slowed in animal’s supplemented dietary achievable FO doses with no detriment to cardiac output. Whether
autonomic tone or intrinsic regulation of HRV is responsible for the slower heart rate described in this study requires further investigation. Slower resting heart rate has been associated with reduced risk of cardiovascular related mortality and morbidity (Ivanović et al., 2012; Palatini & Julius, 2004). In fact, clinical evidence has demonstrated that a reduction of resting heart rate by approximately 1.6 beats.min⁻¹ is estimated to represent a 5% decrease in the risk of developing cardiovascular disease (Mozaffarian et al., 2005). Therefore, the evidence of slowed resting heart rate provided in this study supports the notion that increased fish or FO intake reduces the risk of cardiovascular disease. Given these findings the next chapter will explore whether supplementing dietary achievable FO doses provides protection from arrhythmias during brief periods of regional in vivo ischaemia and reperfusion (acute coronary syndrome).
CHAPTER 5

Investigating arrhythmias during ischaemia and reperfusion following supplementation of human dietary achievable fish oil doses
5.1 INTRODUCTION

5.1.1 Background

The coronary arteries carry oxygenated blood and nutrients to the myocardium and are essential for healthy functioning of the heart. A common cause of cellular dysfunction, damage or death within the myocardium is via the temporary blockage (ischaemia) and return (reperfusion) of blood flow in one or more of the vessels supplying the myocardium. In cardiac patients, this is known as coronary or ischaemic heart disease. Coronary heart disease can present clinically as either an acute coronary syndrome or as angina, which is persistent chest pain. The heart can function during this disease process, and it is not uncommon for people to live for prolonged periods of time without a diagnosis. However, if left untreated the myocardial cells in the ischaemic area will undergo necrosis and a myocardial infarction will develop. Therefore, reperfusion of blood flow to the ischaemic area is necessary to preserve the viability of myocardial cells. Unfortunately, it has been demonstrated that reperfusion can lead to accelerated and additional damage to the myocardium beyond what was generated by the initial ischaemia (Moens et al., 2005). For this reason, reperfusion has been referred to as a ‘double edged sword’ because it causes further damage to previously ischaemic tissue, when it should attenuate damage (Braunwald & Kloner, 1985).

Of particular relevance to this study is the evidence which demonstrates a close link between ischaemia and reperfusion of the myocardium and the manifestation of electrical instability (arrhythmia) in the heart (Gaudron et al., 2001; Gorenek et al., 2015). Arrhythmias can originate from the atria or ventricle of the heart and have significant consequences on the hemodynamic functioning of the cardiovascular system. If the arrhythmia is of atrial origin, cardiac output can be significantly reduced, particularly when the heart is working at higher rates (>140beats.min⁻¹) resulting in exercise intolerance (Lee et al., 2005). However, arrhythmia of ventricular origin is much more serious as it can result in sudden cardiac death (SCD) in the absence of rapid medical intervention (Gorenek et al., 2015). In fact, in 20-25% of patients with myocardial ischaemia, the first clinical manifestation may be SCD (Luqman et al., 2007) and it has been reported that in most cases, the mechanism of onset is ventricular tachycardia that rapidly progresses to ventricular fibrillation (Zipes & Wellens, 1998).
5.1.2 Myocardial damage attributable to brief episodes of ischaemia and reperfusion

During ischaemia there is insufficient blood flow to maintain adequate oxygenation of the tissue and sustain healthy metabolism within myocardial cells. Myocardial ischaemic episodes as short as five minutes lead to depletion of energy stores and the production of reactive oxygen species and their oxidation products within the myocardium. The heart has very little energy stored in the form of ATP, yet very high energy demands (Lopaschuk et al., 2010). In fact, in a contracting human heart the entire pool of ATP is turned over six to eight times in a minute (Lopaschuk et al., 2010). Therefore, synthesis of ATP required by the healthy heart is primarily generated by aerobic mitochondrial oxidative metabolism of substrates including fatty acids, glucose, ketones, lactate, amino acids and pyruvate (Lopaschuk et al., 2010). During ischaemia the coronary vessels dilate up to three to five times in order to maintain oxygen delivery for aerobic metabolism (Spaan et al., 2008). However, if oxygen supply to the myocardium is not adequate even with this compensation, ATP concentrations cannot be maintained using anaerobic pathways and will rapidly deplete (Stanley et al., 2005). In addition, the alternative use of anaerobic metabolism to maintain ATP production creates an excess of intracellular hydrogen (H+) ions. Increased H+ ions and decreased washout of lactate and other metabolites associated with anaerobic metabolism leads to a reduction in the tissue pH (Moens et al., 2005).

The combination of oxidative stress due to increased reactive oxygen species, ATP depletion and changes in tissue pH lead to altered membrane integrity, lipid peroxidation, impaired functioning of membrane-bound proteins, ion pumps, channels, receptors, altered electrolyte balances and damage to DNA (Bolli, 1988; Bolli, 1990; Ferrari et al., 1998). These cellular changes lead to mechanical dysfunction of cardiac myocytes and functional abnormalities, particularly electrical instability even in the absence of cell necrosis. Intracellular Ca²⁺ concentrations are closely linked to these changes, as Ca²⁺ movement out of the cell is inhibited by the failure of ATP-reliant ion transporters. Therefore intracellular Ca²⁺ concentrations overload the cells. Calcium overload within the cell can cause the generation of further reactive oxygen species and the activation of a range of Ca²⁺ dependant enzymes including ATPase’s and phospholipases (Moens et al., 2005). The impaired ability to sequester Ca²⁺ can culminate in the development of haemodynamic dysfunction and also electrical
instability resulting in premature ventricular beats (PVB), ventricular tachycardia (VT) and ventricular fibrillation (VF) (Luqman et al., 2007; Pogwizd & Corr, 1986). However, the abnormalities of myocardial functioning in the absence of tissue necrosis usually recover in the proceeding 24 - 48 hours, hence the term “stunned myocardium” has been coined to describe the acute abnormalities observed (Bolli, 1992). The return of blood flow allows restoration of glucose and free fatty acids concentrations, which are essential for generating ATP via aerobic metabolism, and allows the recruitment of inflammatory cells to the damaged region.

The rapid reactivation of aerobic pathways is associated with an overload of reactive oxygen species due to Ca^{2+} sensitive proteases in the mitochondrial respiratory chain (Kitakaze, 2010). Furthermore, the return of blood flow causes metabolites and ions which have accumulated in the ischaemia area to be redistributed throughout the extracellular space (Moens et al., 2005). As such, when blood flow is restored to the ischaemic region there is rapid normalisation of the tissue pH but also overload of intracellular Ca^{2+} concentration gradients (Sanada et al., 2011). The rapid normalisation of extracellular H^{+} concentrations which occurs upon reperfusion leads to the activation of the Na^{+} - H^{+} exchanger to extrude H^{+} to the interstitium in exchange for sodium (Na^{+}) influx to the cytosol. It has been postulated that this generates an excess of Na^{+} within the cell upon reperfusion (Tani & Neely, 1989), however, some studies have been unable to demonstrate increased Na^{+} concentrations (Pike et al., 1990) or have reported decreased Na^{+} concentrations (Emous et al., 2001). Increased intracellular Na^{+} activates the Na^{+} - Ca^{2+} exchange to remove Na^{+} in exchange for Ca^{2+} into the cell. However, Ca^{2+} concentrations within the cell are already high as Ca^{2+} movement out of the cell during ischaemia was inhibited. The combination of accumulated intracellular Ca^{2+} and increased Ca^{2+} flux from activation of the Na^{+} - Ca^{2+} exchange leads to much higher than normal intracellular Ca^{2+} concentrations.

Ischaemia also induces an inflammatory response that leads to the production of a range of endogenous eicosanoids and other fatty-acid membrane derived bioactive compounds. These compounds are formed from membrane bound AA, EPA and DHA cleaved by phospholipases and then by cyclooxygenase enzymes (Serhan & Petasis, 2011). The eicosanoid end products derived mainly from AA include prostaglandins, prostacylcins, thromboxanes and leukotrienes. These compounds are generally
associated with a pro-inflammatory response and have been the target of many pharmacological interventions including aspirin (inhibits COX-2 mediated production of prostaglandins), which inhibit their production. They cause the recruitment and amplification of cytokines (TNF-α), interleukins (IL-1β, IL-6) and chemokines to the myocardium (Nah & Rhee, 2009). This inflammatory response is amplified and accelerated when the myocardium is reperfused allowing better access for inflammatory cells (Frangogiannis et al., 2002). The localized inflammation process is an integral part of the recovery for the myocardium as it mediates the removal of necrotic cells and debris and promotes tissue repair and scar formation (Nathan, 2002). However, a sustained, exacerbated or uncontrolled inflammatory response can impede the recovery process and lead to further damage of the ischaemic and surrounding areas. In addition, the presence of inflammation has been demonstrated to be associated post-operative arrhythmias (Bruins et al., 1997) and atrial fibrillation (Aviles et al., 2003). As such, the combination of rapid reactivation of aerobic metabolism, inflammatory cascades and the presence of catecholamines during reperfusion often facilitates the rapid generation (~15 seconds) of VT which frequently degenerates into fatal VF upon reperfusion of the myocardium (Brooks et al., 1995; Pogwizd & Corr, 1986; Sanada et al., 2011).

5.1.3 The anti-arrhythmic action of fish oil during ischaemia and reperfusion

Research has established a range of actions of FO on the cardiovascular system, which may contribute to the observed cardio-protective effects. These include an anti-thrombotic (Connor, 2000), anti-atherogenic (Balk et al., 2006) and an anti-hypertensive effect (Geleijnse et al., 2002). However, the most significant effect of FO appears to be its anti-arrhythmic action (McLennan, 2014). This effect has been observed at lower doses of FO than most other established effects with the consequences potentially being of more relevance to the cardio-protective action observed in populations which regularly consume fish (Harris et al., 2009).

Several large human based trials have demonstrated that consumption of FO leads to a reduction in SCD. The Diet and Reinfarction observational Trial (DART) followed the post-myocardial infarction recovery (secondary prevention) of over 2000 men and revealed that men who consumed fish regularly (2-3 meals/week) had a 52% reduction in the risk of SCD (Burr et al., 1989). The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI) - Prevenzione random control trial
investigated more than 11,000 post-myocardial infarction patients (secondary prevention) and also demonstrated a 45% reduction in the risk of SCD with the consumption of supplements containing 850mg/day of EPA + DHA (Valagussa et al., 1999). Additionally, doses as low as 180mg/day have been demonstrated to reduce the risk of primary cardiac arrest in a population-based case-control study of adult females and males free from prior clinical heart disease (Siscovick et al., 1995). As such, it has been suggested that there is sufficient evidence to show FO reduces the incidence of SCD in humans, however it is still unclear what dose is required and whether the background diet influences the cardio-protective action (Reiffel & McDonald, 2006). Physiological evidence of the direct anti-arrhythmic action of FO has been provided by studies using both paced and unpaced in vivo and ex vivo rat, monkey and pig hearts (Table 5.1).

In summary, it is still unknown if the anti-arrhythmic effect of FO demonstrated in animal studies is relevant to the observational and cohort studies of diet and SCD, because of the use of FO doses not relevant to human nutrition. Studies which have used dietary achievable FO doses have not supplied a typical Western-style background diet high in SFA and n-6 PUFA. Therefore, extrapolation of the findings from such studies must be used with caution to explain reductions of SCD observed in epidemiological and cohort trials, where arrhythmia prevention is ascribed to fish intake. As such, the objective of this study was to establish whether supplementing human dietary achievable FO doses to a typical Western-style diet protects against ischaemic and reperfusion generated arrhythmias. The surgical model which will be used in the present study to induce regional ischaemia and reperfusion was a modification of a technique first described in 1960 (Selye et al., 1960). The clinical relevance of this model to acute coronary syndrome has allowed it to be extensively used in the literature to investigate arrhythmia generation in both pharmaceutical anti-arrhythmic therapies (Altug et al., 1999; Walsh et al., 2010) and the investigation of anti-arrhythmic actions of FO (Abdukeyum et al., 2008; Charnock et al., 1989; McLennan et al., 1988).
Table 5.1: Comparison of animal interventions investigating fish oil and arrhythmia generation.

<table>
<thead>
<tr>
<th>Fish oil dose (% wt/wt)</th>
<th>Type of oil</th>
<th>Animal</th>
<th>Model</th>
<th>Outcome</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>12%</td>
<td>DHA-rich tuna oil</td>
<td>Rat</td>
<td>In vivo</td>
<td>↓ Vulnerability to I &amp; R induced VT and VF</td>
<td>McLennan. P.L. et al. (1988)</td>
</tr>
<tr>
<td>12%</td>
<td>Purified EPA-rich Shaklee oil</td>
<td>Rat</td>
<td>Ex vivo</td>
<td>Prevented reperfusion-induce VF and ↓ ischaemia induced arrhythmias</td>
<td>Pepe. S. et al. (1996)</td>
</tr>
<tr>
<td>8%</td>
<td>DHA-rich tuna oil</td>
<td>Marmoset monkey</td>
<td>In vivo</td>
<td>↑ Threshold to stimulated VF and ↓ VF incidence</td>
<td>McLennan. P.L. (1992b)</td>
</tr>
<tr>
<td>7%</td>
<td>DHA-rich tuna oil</td>
<td>Rat</td>
<td>Ex vivo</td>
<td>↓ Arrhythmia during I &amp; R</td>
<td>Abdukeyum. G.G. et al. (2008)</td>
</tr>
<tr>
<td>5%</td>
<td>DHA-rich Menhaden oil</td>
<td>Rat</td>
<td>In vivo</td>
<td>↓ Incidence and severity of VF and VT during I &amp; R</td>
<td>Hock. C.E. et al. (1990)</td>
</tr>
<tr>
<td>3%</td>
<td>Purified FO</td>
<td>Marmoset monkey</td>
<td>In vivo</td>
<td>↑ Threshold to stimulated VF and ↓ VF incidence</td>
<td>McLennan. P.L. et al. (1993)</td>
</tr>
<tr>
<td>3%</td>
<td>Purified EPA-rich Shaklee oil</td>
<td>Rat</td>
<td>Ex vivo</td>
<td>↓ Incidence of arrhythmias during 15min ischaemia</td>
<td>Pepe. S. et al. (2007)</td>
</tr>
<tr>
<td>1%</td>
<td>DHA-rich tuna oil</td>
<td>Rat</td>
<td>In vivo</td>
<td>↓ Episodes and duration of VF during reperfusion</td>
<td>Abeywardena. M. et al. (2016)</td>
</tr>
<tr>
<td>0.4-1.1%</td>
<td>Purified EPA or DHA rich oil</td>
<td>Rat</td>
<td>In vivo</td>
<td>Purified DHA ↓ VF incidence but Purified EPA did not</td>
<td>McLennan. P.L. et al. (1996)</td>
</tr>
<tr>
<td>1.25 &amp; 0.31%</td>
<td>DHA-rich tuna oil</td>
<td>Rat</td>
<td>In vivo</td>
<td>??</td>
<td>Present study</td>
</tr>
</tbody>
</table>

**Abbreviations:** VT = ventricular tachycardia; VF = ventricular fibrillation; I = ischaemia; R = reperfusion.

- Fill represents historically common supra-therapeutical doses of FO.
- Fill represents dietary achievable doses of FO.
5.2 AIMS AND HYPOTHESES

This study aimed to investigate arrhythmia generation, during a clinically relevant (acute coronary syndrome) ischaemia and reperfusion protocol, following supplementation of dietary achievable FO doses to a typical Western-style diet (high SFA and n-6 PUFA).

Specifically, this experiment had the following aims and hypotheses:

Aim 1: Determine the influence of supplementing dietary achievable FO doses on ischaemic generated arrhythmias.

Hypothesis 1: The severity, duration and incidence of ischaemic arrhythmias will be reduced by FO supplementation.

Hypothesis 2: The risk of fatality from arrhythmia will be reduced by FO supplementation.

Aim 2: Determine the influence of supplementing dietary achievable FO doses on reperfusion generated arrhythmias.

Hypothesis 3: The severity, duration and incidence of reperfusion generated arrhythmias will be reduced by FO supplementation.

Hypothesis 4: The risk of fatality from arrhythmia will be reduced by FO supplementation.
5.3 METHODS

5.3.1 Animals, ethical considerations and standardisation

The male rats (Sprague Dawley) from cohort 2 were used in this study. Directly following the end of experimental procedures described in Chapter 4 the experimental procedures for this study began.

5.3.2 Groups

Animals were supplied water and randomly assigned to one of three pre-fabricated diets ad libitum for four (4) weeks before experimentation. The diets contained different amounts of FO (Control – 0%: n = 18, LowFO – 0.31%: n = 15, ModFO – 1.25%: n = 18 (%wt/wt)).

5.3.3 Experimental preparation

Preparation of the 6mm miniaturised 2-French (2F) Millar pressure-volume conductance catheter (SPR-638 Millar Instruments, Houston, USA) and electrocardiogram (ADInstruments, Bella Vista, NSW, Aus) was completed (Section 3.3.3).

5.3.4 Surgical procedures

Animals were anaesthetised, and surgery conducted to allow cardiac measurements including: ECG, ventricular pressure and volume and aortic blood pressure (Chapter 4). Following this an established surgical technique which has been used extensively for the production of arrhythmias in the in vivo heart was completed (McLennan et al., 1988; Selye et al., 1960).

5.3.4a Thoracotomy

A 3-4cm superior-inferior midline incision was made in the chest and at the inferior end of the incision a 2-3cm transverse incision was to made towards the left mid-axillary line to allow the skin to be bluntly dissected and reflected back to reveal the musculature and ribs of the left thoracic cavity. Blunt scissors were inserted between the pectoralis major and minor muscles in order to break the fascia and allow retraction of these muscles from the chest to expose the ribs underneath. Care was taken to not break
the superficial thoracic vein during this step as injury to this vessel causes significant bleeding. Once the ribs were visible the chest was then opened at the fourth intercostal space using blunt surgical scissors to tear the intercostal muscles and make a 1-2cm transverse incision. The incision was made 3-4mm laterally from the sternum to avoid damaging the internal thoracic artery which results in heavy bleeding that is very difficult to control. The fourth rib was then sectioned using surgical scissors approximately 2-3mm from the sternum and blunt scissors were then used to increase the space of the incision via opening the scissors at an oblique angle to the ribs. The heart then became visible encased in the pericardium. The pericardium was grasped with curved forceps and opened using blunt scissors to avoid damage to the myocardium, once opened it would slide behind the heart and remain there making the heart freely accessible.

5.3.4b Exteriorisation of the heart and suturing of the LAD coronary artery

The heart was exteriorised via placing a scoop spatula with a small amount of 0.9% NaCl on it underneath the heart inside the thoracic cavity and then applying a small amount of external pressure on the superior and right chest wall. The LAD runs obliquely over the anterior surface of the left ventricle from the left coronary artery branch with the left circumflex artery towards the apex of the heart along the interventricular sulcus. Once the heart was exteriorised it was held between the left thumb and forefinger with the apex facing towards the right of the animal and slightly superiorly. A size 6-0 braided silk suture attached to a 12mm atraumatic taper needle (Dynek Pty Ltd, Australia) was placed under the LAD using curved tip hemostat scissors in the right hand. The LAD sits within the myocardium which makes it difficult to directly locate visually. Therefore the left auricular appendage and pulmonary cone were used as guides for insertion and exit points respectively of the suture. The heart was then replaced in the chest and the animal was allowed to recover for ten minutes. This entire process takes roughly ten to fifteen seconds.

5.3.5 Experimental protocol and measurements

Animals were checked for depth of anaesthesia (Section 3.3.3) directly following surgical procedures being completed and prior to experimental protocols and measurements beginning. After insertion of the occluding suture and return of the heart
to the thoracic cavity, each animal was allowed ten minutes recovery for stabilisation of cardiac function before commencing the ischaemia and reperfusion protocol (Figure 5.3).

5.3.5a LAD coronary artery occlusion and reperfusion

The suture was drawn through a 3mm length of polyethylene tubing with a small slit made in the side at one end of the tubing. The tubing was allowed to slide down the suture to come into contact with the heart where the LAD sits. The LAD was occluded via applying tension to the suture and the tension was maintained by drawing the suture down the slit in the tubing (Figure 5.1). Occlusion of the LAD was confirmed by checking for visible blanching of the anterior wall of the left ventricle after a few seconds and via confirming depression of the ST segment and elevation of R wave on the ECG (Figure 5.2). The LAD was occluded for fifteen minutes of ischaemia while arrhythmia generation was measured via ECG. The fifteen minutes ischaemic duration was used because the occurrences of arrhythmias induced by regional ischaemia in the rat peak between five to ten minutes and usually finish before fifteen minutes (Curtis et al., 1987). The suture was released from the slit in the tubing and tension relieved after fifteen minutes of ischaemia, allowing reperfusion of the heart following occlusion. Reperfusion arrhythmias peak in the first five minutes but can also still be observed up to fifteen minutes after release of the occlusion (McLennan et al., 1988), therefore reperfusion arrhythmias were measured for fifteen minutes via ECG.
Figure 5.1: Post-experimental isolated heart showing surgical LAD coronary artery occlusion via placing tension on a piece of tubing over the artery & Indian dye stained ischaemic/non-ischaemic tissue.

**Figure 5.1 notes:** The tubing places pressure on the area of myocardium where the LAD is located whilst the suture places pressure from below in order to occlude it. The tubing is held in place with tension generated from a suture being held in a slit of the tubing. Ischaemic region (non-stained) and non-ischaemic region (stained black) can also be observed after perfusion of coronary arteries with Indian dye while LAD is occluded.
Figure 5.2: Example of typical ECG trace from an animal as the LAD coronary artery occlusion is begun.

**Figure 5.3 notes:** The occlusion of the LAD artery was confirmed via sighting of elevation of the R wave and ST-segment depression along with blanching of the ventricular wall.
Figure 5.3: Overview of the experimental protocol used to investigate the effect of fish oil on arrhythmia generation.

Abbreviations: ECG = electrocardiogram; LAD = left anterior descending coronary artery.
5.3.6 Post-experimental data analysis

5.3.6a Classification and scoring of arrhythmias

Arrhythmia classification was completed according to the Lambeth conventions (Walker et al., 1988). Measurement of PVB was completed through identifying a QRS complex which occurred earlier than would be expected in the ECG tracing of regular sinus rhythm. Because PVB originate from the ventricle they are identifiable on the ECG as a QRS complex without a preceding P wave (Figure 5.4A), which is why they are also commonly referred to as Ventricular Ectopic Beats (VEB). Classification of VT was defined as four or more consecutive PVB, and VF was defined as indistinguishable QRS deflections where no rate could be measured along with accompanied drop in blood pressure, torsade de pointes was also classified as VF (Figure 5.4B). Identification of the arrhythmias allowed a previously established scoring scale to be used to rank the severity of the arrhythmias and allow comparison between groups (Curtis et al., 1987). This is achieved by taking into account the number of PVB, the duration and type of arrhythmia, the timing of the arrhythmia in relation to the ischaemic and reperfusion period and whether and when the animal spontaneously reverted to sinus rhythm or the arrhythmia resulted in death. The scale is from zero to nine with scores between zero and five indicating that the animal survived the entire ischaemia or reperfusion protocol. However, an animal which scored five would have had more incidents and/or duration of arrhythmia and/or more serious arrhythmia e.g., more VF than VT compared to an animal which scored one. Scores between six and nine are given to animals which did not survive the ischaemia, the difference in scoring for these animals is dependent upon the time that the fatal VF started, with a higher score given for fatal VF starting earlier in the ischaemia or reperfusion (Table 5.2). The scale was slightly modified to quantify reperfusion induced arrhythmias following an established method in a previous study with similar design and measurement outcomes to this one (McLennan et al., 1988). The modified scale was used to take into account the shorter time course of reperfusion arrhythmias. Using a scoring index to represent the severity and incidence of arrhythmias occurring is of particular value in aiding statistical evaluation between groups where there is a low incidence of VT and/or VF.
### Table 5.2: Scoring index used to quantify arrhythmias.

<table>
<thead>
<tr>
<th>Ischaemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = 0-49 PVB</td>
<td>0-49 PVB</td>
</tr>
<tr>
<td>1 = 50-499 PVB</td>
<td>50-249 PVB</td>
</tr>
<tr>
<td>2 = &gt;499 PVB and/or 1 episode of spontaneously reverting VT or VF</td>
<td>&gt;250 PVB and/or 1 episode of spontaneously reverting VT or VF</td>
</tr>
<tr>
<td>3 = &gt;1 episode of VT or VF or both (&lt;60 s total combined duration)</td>
<td>&gt;1 episode of VT or VF or both (&lt;20 s total combined duration)</td>
</tr>
<tr>
<td>4 = VT or VF or both (60-119 s total combined duration)</td>
<td>VT or VF or both (20-59 s total combined duration)</td>
</tr>
<tr>
<td>5 = VT or VF or both (&gt;119 s total combined duration)</td>
<td>VT or VF or both (&gt;60 s total combined duration)</td>
</tr>
<tr>
<td>6 = fatal VF starting &gt;15 min after occlusion</td>
<td>fatal VF starting at &gt;5 min after reperfusion</td>
</tr>
<tr>
<td>7 = fatal VF starting between 4 and 14 min 59 s after occlusion</td>
<td>fatal VF starting between 4 and 5 min after reperfusion</td>
</tr>
<tr>
<td>8 = fatal VF starting between 1 and 3 min 59 s after occlusion</td>
<td>fatal VF starting between 20 s and 2 min after reperfusion</td>
</tr>
<tr>
<td>9 = fatal VF starting &lt;1 min after occlusion</td>
<td>fatal VF starting &lt;20 s after reperfusion</td>
</tr>
</tbody>
</table>

**Abbreviations:** PVB = premature ventricular beats; VT = ventricular tachycardia; VF = ventricular fibrillation.

Ischaemic scoring index was established by Curtis. M.J. *et al.*, 1987; reperfusion scoring is a modified version from McLennan. P.L. *et al.*, 1988.
Figure 5.4: Example experimental ECG (Pink) and aortic blood pressure (Red) traces showing PVB (A), VT and VF (B).

A: Premature ventricular beat (PVB). It can be observed that the QRS complex occurs prior to the P wave and then a compensatory pause before next QRS complex.

B: Highlights a run of premature ventricular beats (PVB) leading into an episode of ventricular tachycardia (VT: ) and an episode of torsade de pointes (ventricular fibrillation VF: ) with the associated drop in aortic blood pressure.
5.3.6b Statistics

All results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The effects of diet treatment were tested via ANOVA. A Bonferroni post-hoc test for comparison between group means was then used to identify differences. The incidence of VT or VF was expressed as the percentage (%) of animals in which at least one episode was observed. Percentage incidence data was then tested using chi-square with Fischer’s exact test. All statistical analyses were performed in a blinded manner and completed using SPSS 21 for Windows (SPSS Inc, Chicago, IL, USA). Alpha was set at $p < 0.05$. 

5.4 RESULTS

Forty-nine (N = 49) animals were used for the analysis of ischaemic arrhythmias. Two (2) animals in the ModFO group were removed from the analysis due to bradycardia associated with atrioventricular block, without preceding arrhythmia. This was most likely caused by surgical damage to the conduction pathways, surgical complication resulting in cardiogenic shock or anaesthetic overdose. The final group numbers included in the analysis of each group were as follows: Control (n = 18), LowFO (n = 15) and ModFO (n = 16). Of the forty nine (49) animals that underwent LAD coronary artery occlusion only twenty-eight (28) animals survived the complete occlusion. Ischaemic induced arrhythmias were most pronounced between five and eight minutes after the occlusion had been drawn tight. Animals which did not die from fatal VF during the ischaemia had predominantly reverted to normal sinus rhythm after ten minutes of ischaemia. Twenty-one (21) animals died (44%) due to VF prior to the occlusion being released for reperfusion (Figure 5.5).

Figure 5.5: The total survivors/deaths during fifteen minutes LAD coronary artery occlusion.

*Two (N=2) animals in the ModFO group were removed from the analysis due to bradycardia (Abnormally low heart rate prior to the occlusion occurring) without preceding arrhythmia.
5.4.1 The effect of fish oil supplementation on ischaemia generated arrhythmias

Nearly all the animals in the Control (94%) and LowFO (91%) group experienced at least one VT episode during the ischaemia (Figure 5.6A). Significantly less animals experienced at least one incidence of VT in the ModFO (31%) group compared to both of the other groups ($p < 0.001$). The mean total duration of VT in animals which experienced ≥ one episode was significantly shorter in both FO groups compared to the Control group (Figure 5.6B).

![Figure 5.6](image)

*Figure 5.6: The effect of fish oil on the incidence and duration of ventricular tachycardia during LAD coronary artery occlusion induced ischaemia of the left ventricle.*

Values are mean ± SEM ($n = 15 - 18$). * $p < 0.05$ vs Control diet, † $p < 0.05$ vs LowFO diet (% Incidence analysed via chi-square with Fischer’s exact test, Duration analysed by One-way ANOVA with Bonferroni post-hoc test).
Almost all animals in the Control group (89%) experienced at least one VF episode during ischaemia. Less animals in the LowFO (60%) group experienced at least one episode of VF which trended toward significance ($p = 0.052$). In the ModFO (50%) group, a significant difference in the number of animals which experienced at least one episode of VF was evident (Figure 5.7A). There were no significant differences between groups in the mean number of VF episodes per animal which did experience VF during the ischaemia (Table 5.3). A trending reduction in cases of fatal VF during ischaemia in animals from the LowFO group (6/15 animals [40%]) compared to the Control (11/18 animals [61%]) was evident. Whereas, cases of fatal VF in the ModFO group (4/16 animals [25%]) were significantly less than the Control group. The mean total duration of VF in animals which experienced $\geq$ one episode of VF was not different between any group (Figure 5.7B).

Figure 5.7: The effect of fish oil on the incidence and duration of ventricular fibrillation during ischaemia of the left ventricle induced by occlusion of the LAD coronary artery.

Values are mean ± SEM ($n = 15 - 18$). * $p < 0.05$ vs Control diet (% Incidence analysed via chi-square with Fischer’s exact test, Duration analysed by One-way ANOVA with Bonferroni post-hoc test).
The arrhythmia score was significantly lower in a dose-related manner, the Control group displaying the highest score followed by the LowFO and the ModFO groups (Figure 5.8A). The FO supplemented groups spent significantly more time in normal sinus rhythm compared to the Control group throughout the entire fifteen minutes of ischaemia (Figure 5.8B). Furthermore, during the fifteen minutes of ischaemia both FO groups had less PVB than the Control group (Table 5.3). The ModFO group had an almost three-fold reduction in PVB which was significantly less compared to the Control group ($p = 0.05$).

Figure 5.8: The effect of fish oil on arrhythmia score and time spent in normal sinus rhythm during ischaemia of the left ventricle induced by occlusion of the LAD coronary artery.

Values are mean ± SEM ($n = 15 - 18$). * $p < 0.05$ vs Control diet (One-way ANOVA with Bonferroni post-hoc test). Arrhythmia score was calculated according to Curtis. M.J. et al., 1987 (Table 5.2).
5.4.2 The effect of fish oil supplementation on reperfusion generated arrhythmias

Twenty-one (N = 21) animals died of arrhythmias during the ischaemic period of the ischaemia and reperfusion protocol. This resulted in twenty-eight (28) animals being used for the analysis of reperfusion-induced arrhythmias. The final group numbers for analysis were: Control (n = 7), LowFO (n = 9) and ModFO (n = 12). Reperfusion induced arrhythmias were most pronounced between fifteen seconds and one minute after the LAD coronary artery occlusion had been released. Animals which did not have an arrhythmia during this time period predominantly would not have any arrhythmias during the remaining reperfusion time. Animals that did have non-fatal arrhythmias during this time had usually spontaneously reverted to normal sinus rhythm before five minutes of reperfusion. Of the twenty-eight (28) animals that underwent reperfusion of the LAD coronary artery only one (1) animal died of arrhythmia (Figure 5.9).

Figure 5.9: The total survivors/deaths during fifteen minutes LAD coronary artery reperfusion.
All the animals in the Control and LowFO group experienced at least one VT episode during reperfusion (*Figure 5.10A*). Significantly less animals experienced at least one incidence of VT in the ModFO (33%) group compared to both of the other groups (*p* <0.001). The mean total duration of VT in animals which experienced ≥ one episode was significantly shorter in both FO groups compared to the Control group (*Figure 5.10B*).

*Figure 5.10: The effect of fish oil on the incidence and duration of ventricular tachycardia during reperfusion of the LAD coronary artery.*

Values are mean ± SEM (*n* = 7 - 12). * *p* <0.05 vs Control diet, † *p* <0.05 vs LowFO diet (% Incidence analysed via chi-square with Fischer’s exact test, Duration analysed by One-way ANOVA with Bonferroni post-hoc test).
Almost all animals in the Control group (86%) experienced at least one VF episode during reperfusion. Significantly less animals in the LowFO (22%) and ModFO (8%) group experienced at least one episode of VF compared to the Control group (Figure 5.11A). Total mean number and duration of VF episodes per animal which did experience VF during reperfusion were unable to be statistically analysed due to the small number of animals in the ModFO (n=1) and LowFO (n=2) which experienced VF (Table 5.3). However, anecdotal observation shows that the duration of VF in the animals that did experience at least one case was dramatically lower in the ModFO and LowFO groups compared to the Control (Figure 5.11B).

Figure 5.11: The effect of fish oil on the incidence and duration of ventricular fibrillation during reperfusion of the LAD coronary artery.

Values are mean ± SEM (n = 7 - 12). * p <0.05 vs Control diet (% Incidence analysed via chi-square with Fischer’s exact test, Duration analysed by One-way ANOVA with Bonferroni post-hoc test).
The arrhythmia score was significantly lower in a dose-related manner across the FO groups compared to Control during reperfusion (*Figure 5.12A*). Additionally, the FO supplemented groups spent significantly longer in normal sinus rhythm compared to the Control group throughout the entire fifteen minutes of reperfusion (*Figure 5.12B*). There was one fatal VF episode in the Control group but there were no fatal VF episodes in the FO groups. During the fifteen minutes of reperfusion both FO groups had an approximately three-fold reduction in PVB compared to the Control group (*p* =0.006) (*Table 5.3*).

*Figure 5.12: The effect of fish oil on arrhythmia score and time spent in normal sinus rhythm during reperfusion of the LAD coronary artery.*

Values are mean ± SEM (*n* = 7 – 12).* *p* <0.05 vs Control diet, † *p* <0.05 vs LowFO diet (One-way ANOVA with Bonferroni post-hoc test). Arrhythmia score was calculated according to methods described in McLennan. P.L. *et al.*, 1988 (*Table 5.2*).
Table 5.3: The effect of fish oil on arrhythmia indices in the left ventricle during fifteen minutes ischaemia induced by occlusion of the LAD coronary artery and fifteen minutes reperfusion.

|                     | Ischaemia |            | Trend p = | |                     |            | Trend p = |
|---------------------|-----------|------------|-----------|---------------------|------------|-----------|
| **Animals (n)**     | Control  | LowFO | ModFO | Control  | LowFO | ModFO |
| 18                  | 15       | 16       | 0.005    | 7                    | 9       | 12      | <0.001 |
| **Time in NSR (Max = 900s)** | 716 (±13) | 794 (±24)* | 798 (±25)* | 0.050   | 795 (±34) | 884 (±5)* | 895 (±2)* | 0.006 |
| **PVB**             | 114 (±27) | 78 (±17) | 43 (±9)* | 0.005   | 155 (±36) | 58 (±15)* | 59 (±14)* | 0.006 |

<table>
<thead>
<tr>
<th><strong>Animals experienced VT (n)</strong></th>
<th>17</th>
<th>14</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>4</th>
<th>&lt;0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VT incidence</strong></td>
<td>94%</td>
<td>93%</td>
<td>31%*†</td>
<td>&lt;0.001</td>
<td>100%</td>
<td>100%</td>
<td>37%*†</td>
</tr>
<tr>
<td><strong>VT episodes</strong></td>
<td>7 (±2)</td>
<td>5 (±2)</td>
<td>4 (±3)</td>
<td>0.01</td>
<td>6 (±1)</td>
<td>2 (±1)*</td>
<td>2 (±1)</td>
</tr>
<tr>
<td><strong>VT duration</strong></td>
<td>64 (±14)</td>
<td>26 (±15)*</td>
<td>25 (±16)*</td>
<td>0.043</td>
<td>32 (±8)</td>
<td>13 (±5)*</td>
<td>10 (±8)*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Animals experienced VF (n)</strong></th>
<th>16</th>
<th>9</th>
<th>8</th>
<th>6</th>
<th>2</th>
<th>1</th>
<th>&lt;0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VF incidence</strong></td>
<td>89%</td>
<td>60%</td>
<td>50%*</td>
<td>0.042</td>
<td>86%</td>
<td>22%*</td>
<td>9%*</td>
</tr>
<tr>
<td><strong>Fatal VF</strong></td>
<td>61%</td>
<td>40%</td>
<td>25%*</td>
<td>0.045</td>
<td>14%</td>
<td>a 0%</td>
<td>a 0%</td>
</tr>
<tr>
<td><strong>VF episodes</strong></td>
<td>1.4 (±0.3)</td>
<td>1.7 (±0.4)</td>
<td>1.1 (±0.5)</td>
<td>1.6 (±0.4)</td>
<td>1.7 (±0.8)</td>
<td>1.1 (±0.5)</td>
<td>1.6 (±0.4)</td>
</tr>
<tr>
<td><strong>VF duration</strong></td>
<td>124 (±16)</td>
<td>122 (±22)</td>
<td>110 (±23)</td>
<td>85 (±33)</td>
<td>85 (±33)</td>
<td>85 (±33)</td>
<td>85 (±33)</td>
</tr>
</tbody>
</table>

| **Arrhythmia score**          | 6.1 (±0.4) | 4.6 (±0.5) | 3.1 (±0.7)* | 0.0026 | 4.6 (±0.3) | 3.1 (±0.3)* | 1.3 (±0.3)*† | <0.001 |

Values are mean ± SEM. * p <0.05 vs Control diet † p <0.05 vs LowFO diet, a too few numbers to complete statistical comparisons (% Incidence analysed via chi-square with Fischer’s exact test, Duration analysed by One-way ANOVA with Bonferroni post-hoc test).

Abbreviations: NSR = normal sinus rhythm; PVB = premature ventricular beats; VT = ventricular tachycardia; VF = ventricular fibrillation; Incidence = percentage of animals which experienced at least one episode; Fatal VF = percentage of animals which died from VF; Episodes = mean number of arrhythmic episodes per animal; Duration = mean total time in arrhythmia of animals having ≥ one episode.

Ischaemic arrhythmia score was calculated using previously outlined and established methods (Curtis et al., 1987). Reperfusion arrhythmia score was calculated using previously outlined and established methods (McLennan et al., 1988).

Fill represents data where the LowFO has produced a comparable effect to what was demonstrated with the ModFO dose.
5.5 DISCUSSION

This study, for the first time, demonstrated that supplementing FO doses achievable within a typical Western-style human diet (high SFA and n-6 PUFA) provides protection against ischaemia and reperfusion induced arrhythmias in the *in vivo* heart. Arrhythmia generation, including fatal arrhythmias, were reduced by both FO diets in a dose-related manner, suggesting that optimal cardiac electrophysiology is intimately linked with myocardial phospholipid DHA concentrations. However, during ischaemia anti-arrhythmic effects were not observed across all indicators in the LowFO diet, suggesting that it may be close to the threshold dose required for protection. Whereas, during reperfusion there were few statistically significant differences between the two FO doses, suggesting that the threshold for effectiveness may be even lower. The demonstration of reduced fatal ischaemia and reperfusion generated arrhythmias provides strong evidence, for the first time, to rationalise how FO can prevent sudden (arrhythmic) cardiac death (Burr *et al.*, 1989; Valagussa *et al.*, 1999). This finding underpins the importance of increasing fish or FO intake in a typical Western-style diet containing high amounts of SFA and n-6 PUFA.

5.5.1 Generation of arrhythmias via ischaemia and reperfusion model

Arrhythmias were successfully generated during the transient regional ischaemia and reperfusion periods induced by the surgical occlusion and release of the LAD coronary artery. During the ischaemic period approximately 60% of the Control group died due to fatal VF. The incidence of fatal VF in the Control group is very similar to the mortality rate displayed in Control groups of other studies using the same or very similar surgical methods for the induction of arrhythmias in the rat (Abeywardena *et al.*, 2016; McLennan, 1993). This confirms the reliability of the surgical model used to induce ischaemic arrhythmias in this study. Reperfusion of the myocardium is also a very strong stimulus for arrhythmia generation (Manning & Hearse, 1984). It has been suggested however that the preceding ischaemic period can lead to biased outcomes (Balke *et al.*, 1981). When LAD ligation induced ischaemia is limited to five minutes, the proceeding reperfusion produces VT in virtually all cases and VF in approximately 80%; less cases are generated with both shorter and longer periods of ischaemia (Manning & Hearse, 1984). The majority of arrhythmias in this study occurred between
five and eight minutes of ischaemia and animals usually spontaneously reverted to normal sinus rhythm for the remainder of the fifteen minute occlusion. During reperfusion the occurrence of VT was usually observed in all animals very rapidly after reperfusion began. The Control group incidence of VF was 86% and one case progressed into fatal VF. Therefore, the use of a fifteen minute ischaemic period did not seem to influence the occurrence of reperfusion VT or VF but did reduce the expected cases of fatal VF. A possible explanation for this observation is that the ischaemic period was sufficient enough to cause fatal VF in animals susceptible to it; therefore the animals which reached reperfusion were more likely to not die of fatal VF upon reperfusion. Importantly, compared to the Control group, the FO supplemented groups were protected from the arrhythmic stimulus throughout ischaemia and reperfusion.

5.5.2 Arrhythmia protection attributable to human dietary achievable fish oil doses

The current study has demonstrated, for the first time, that the anti-arrhythmic action of FO is present in animals when using human dietary achievable doses, equivalent to what could be achieved in the human diet, and in the presence of a background Western-style diet high in SFA and n-6 PUFA. This finding extends the demonstration of FO anti-arrhythmic action in previous studies (Abdukeyum et al., 2008; Abeywardena et al., 2016; Hartog et al., 1987; Hock et al., 1990; McLennan et al., 1988; McLennan et al., 1993; McLennan et al., 2007) using therapeutic and supra-therapeutical doses of FO. Importantly, for the first time this study has demonstrated that the anti-arrhythmic action of FO remains present at a dose (LowFO) equivalent to two 100g serves of fish per week or one average sized salmon fillet (≈250g) (Slee et al., 2010). Only one other study has described an anti-arrhythmic action of FO while using a comparable dose to the ModFO group (Abeywardena et al., 2016). The study completed by Abeywardena et al., 2016, supplemented 1% FO and induced in vivo arrhythmias via the same surgical protocol used in this study. The reduction in fatal VF episodes observed in the ModFO (1.25%) supplemented rats was comparable to the mortality rate demonstrated in rats supplemented 1% FO by Abeywardena et al., 2016. In combination, these findings provide strong evidence that infers the anti-arrhythmic action of FO is present using doses in the rat equivalent to what could be easily achieved in the human diet. Interestingly, even though the two studies supplemented FO
at similar doses, different background diets were used. This suggests that variations in background diet do not exert any influence on the anti-arrhythmic action of FO.

The anti-arrhythmic action of FO first described several decades ago using a whole animal model (McLennan et al., 1988) appears to be intimately linked to myocardial phospholipid DHA concentrations. In line with this observation, studies consistently demonstrate the anti-arrhythmic action of FO via only altering dietary LC n-3 PUFA intake. For example, initial research demonstrated that crossover to a supra-therapeutical FO diet reversed the arrhythmogenic effects of prolonged high SFA intake (McLennan et al., 1990). Further to this, addition of supra-therapeutical doses of FO to a diet high in SFA has been demonstrated to reduce the vulnerability of the rat heart to ischaemic arrhythmia, whereas the addition of n-6 PUFA to the high SFA diet did not show the same protection (Charnock et al., 1991). Similarly, ventricular fibrillation threshold has been demonstrated to be modulated by addition of supra-therapeutical doses of FO to a high SFA diet but it is not modified by the addition of sunflower seed oil (n-6 PUFA) in the marmoset heart (McLennan et al., 1993). More recently, ischaemic induced arrhythmias have been demonstrated to be inhibited by the addition of therapeutic and supra-therapeutical doses of FO to a high SFA-enriched diet in rats (Pepe & McLennan, 2007). It has been postulated that it is essential to change dietary intakes of both LC n-3 PUFA and n-6 PUFA (reduce the n-6:n-3 ratio) to observe cardiovascular benefits (Lands, 2003; Rupp et al., 2004; Simopoulos, 2008), based on the notion that phospholipid incorporation of LC n-3 PUFA only occurs when the n-6:n-3 ratio is reduced (Hulbert et al., 2005). However, there is significant evidence that indicates that dietary n-6 PUFA intake does not affect LC n-3 PUFA incorporation, particularly when preformed EPA+DHA are provided (Slee et al., 2010; Stark et al., 2007a; Stark et al., 2007b). The current research supports this by demonstrating that phospholipid concentrations of LC n-3 PUFA are closely associated with absolute amounts of LC n-3 PUFA provided, regardless of background SFA or n-6 PUFA levels. In addition, the current research has now demonstrated an anti-arrhythmic action without modifying SFA or n-6 PUFA concentrations in the diet, supporting clinical evidence which demonstrates that only LC n-3 PUFA levels need to be changed in the diet to produce cardioprotective actions (Harris, 2006; Harris et al., 2006a).
Previously studies have relied upon therapeutic and supra-therapeutical doses of FO to demonstrate an anti-arrhythmic action; however, background diets has varied considerably between studies (Charnock et al., 1991; McLennan, 1993; McLennan et al., 1988, 1990; Pepe & McLennan, 2007). The only other study to demonstrate anti-arrhythmic effects of FO with similar doses to this study used a diet which was rich in n-6 PUFA and monounsaturated fatty acids but did not contain large amounts of SFA (Abeywardena et al., 2016). In contrast, the monounsaturates portion (olive oil) of the diets used in this study was substituted for FO to ensure high levels of both SFA and n-6 PUFA were present in the FO diets. The variation in SFA between this study and the study completed by Abeywardena et al., 2016 is important as long term feeding with SFA has been demonstrated to be arrhythmogenic (McLennan et al., 1990). As such, this study provides novel evidence to demonstrate that the anti-arrhythmic action of FO is not inhibited by the normally arrhythmogenic stimulus associated with high amounts of dietary SFA, typical to the Western-style diet. In addition, it demonstrates that variations in the background diet do not appear to exert any influence on the anti-arrhythmic action of FO. Rather, the anti-arrhythmic action is closely linked to the absolute FO intake, regardless of background SFA and n-6 PUFA intake.

Several key changes in the arrhythmic indices measured from FO supplemented animals help explain the overall anti-arrhythmic action observed. During ischaemia, FO supplemented animals spent approximately one to one and a half minutes longer in normal sinus rhythm compared to Control animals, and during reperfusion this increased towards two minutes longer. The FO groups had a reduced total number of PVB compared to the Control group and in the case of the ModFO group the reduction observed was almost three-fold. Increased time in normal sinus rhythm and less PVB in FO supplemented animals is important as PVB have been demonstrated to be associated with the initiation of VT which can progress into fatal VF in patients with and without heart disease (Haïssaguerre et al., 2003; Haïssaguerre et al., 2002). As such, reduced PVB in FO animals provides plausible physiological evidence to describe the lower incidence and shorter mean duration of VT and VF observed in FO supplemented animals during ischaemia and reperfusion. Human clinical investigations have demonstrated that as the duration of VF increases, the defibrillation threshold required to revert out of VF becomes larger (Gradaus et al., 2002). Furthermore, supplementation of FO has been demonstrated to reduce the ventricular fibrillation.
threshold in the marmoset heart (McLennan et al., 1993). Unlike the human heart, the rat heart can spontaneously defibrillate (Manoach et al., 1980). As such, it is likely that the observation of reduced fatal VF cases in animals supplemented FO is due in part, to the smaller mean duration of time spent in VF, and modification of the ventricular fibrillation threshold improving the chances of the heart spontaneously defibrillating.

There has been evidence that suggests dietary FO does not protect against arrhythmias and may even act in a pro-arrhythmic manner (Billman et al., 2012; Billman et al., 2010; Mączewski et al., 2016). However, several reasons can be made for the conflicting evidence provided in these studies and the findings described in this study. The study conducted by Mączewski et al., (2016) investigated arrhythmias occurring between the sixth and ninth hour of ischaemia. Arrhythmias investigated in the present study and in the majority of previous work completed (Abdukeyum et al., 2008; Abeywardena et al., 2016; Hartog et al., 1987; Hock et al., 1990; McLennan, 1993; McLennan et al., 1988; McLennan et al., 1993) were generated during the first minutes of ischaemia. Arrhythmia which occur within minutes of the ischaemic onset are caused by different pathogenic mechanisms to arrhythmias which occur after hours (Opitz et al., 1995), making a comparison between the results of the Mączewski et al., 2016 study and others challenging. The studies completed by Billman et al., (2010 & 2012) demonstrated a pro-arrhythmic effect when supplementing supra-therapeutical FO doses to dogs. However, despite supplementing extraordinary doses of FO to dogs (1,2, 4g/d (840mg; 1680mg; 3360mg EPA+DHA/d to 20kg dogs), their erythrocyte EPA+DHA phospholipid concentrations were only 3.0% (1g); 3.7% (2g); and 6.7% (4g). This highlights that the dog is much less amenable to phospholipid LC n-3 PUFA incorporation following dietary FO supplementation than the rat and even humans (700mg/d EPA+DHA in 80kg males has been shown to produce phospholipid EPA+DHA concentrations of 6.3% (Macartney et al., 2014)). Furthermore, there is evidence to describe that the dog model used by Billman does not fit with other studies in the rat, mouse and human when investigating the effect of vagal tone on exercise induced bradycardia (Boyett et al., 2017b). Likewise, the evidence of pro-arrhythmic effects shown in dogs does not fit with the consistent anti-arrhythmic effect demonstrated in other animal models (Matthan et al., 2005) and humans (Reiffel & McDonald, 2006). Therefore, using dogs to investigate the effect of FO on arrhythmia
generation does not seem appropriate, whereas, this study used a more physiologically relevant in vivo rat model.

5.5.3 Linking reduced risk of sudden cardiac death and fish oil

The reduction in mortality associated with FO is usually attributed to reduced risk of SCD caused by fatal arrhythmia. Research shows that myocardial ischaemia and spontaneous reperfusion is linked to more than 80% of all fatal arrhythmias (Huikuri et al., 2001). During regional myocardial ischaemia caused by blockage of a coronary artery there is redistribution of a number of ions (H+, Na+, Ca2+, K+) (Rubart & Zipes, 2005), increased production of reactive oxygen species (Bolli, 1992), depletion of ATP stores (Stanley et al., 2005) and overload of intracellular Ca2+ concentrations throughout cells in the ischaemic area (Moens et al., 2005; Rubart & Zipes, 2005). The culmination of these cellular changes leads to slowed conduction, altered refractory periods and ultimately electric instability of the ischaemic zone which can trigger episodes of VT and/or VF (Rubart & Zipes, 2005; Zipes & Wellens, 1998). Paradoxically, reperfusion of the ischaemic area further propagates the rapid generation of VT and/or VF and is closely linked to SCD (Wit & Janse, 2001). Ischaemia and reperfusion was induced experimentally by occlusion of the LAD coronary artery in this study. In humans, pre-existing coronary artery disease is a common pathology which can produce episodes of ischaemia and spontaneous reperfusion of the myocardium, triggering cardiac arrhythmias and SCD. However, even in the absence of coronary artery disease, there are other pathologies including: acute coronary artery spasms, unstable angina and exercise induced ischaemia which can trigger spontaneous reperfusion induced fatal arrhythmias and SCD (Zipes & Wellens, 1998).

The unpredictable nature of these events makes it difficult to identify patients at risk of SCD and therefore preventative prophylactic drug therapy is not possible and usually only treatable with implantable defibrillation devices. For this reason, the evidence of FO reducing experimentally induced fatal ischaemic and reperfusion generated arrhythmias in this study, provides strong evidence to underpin the importance of including dietary FO as part of a balanced diet to help prevent SCD. The hypothesis of FO acting to prevent SCD rates through an anti-arrhythmic action is supported by evidence from human trials also. Two prominent large scale trials have demonstrated that increasing fish intake or supplementing doses of FO (equivalent to the ModFO diet)
cause a reduction in cardiovascular related mortality (Burr et al., 1989; Valagussa et al., 1999). Interestingly, these trials demonstrated that the incidence of SCD was significantly reduced but the incidence of new ischaemic events was not significantly reduced. Furthermore, it has been demonstrated that a 1% increase in the omega-3 index (red blood cell EPA + DHA concentrations expressed as weight percentage of total phospholipid fatty acids) in humans, reflects a 58% reduced risk of VF during the ischaemic phase of acute myocardial infarction caused by coronary artery disease (Aarsetoey et al., 2011; Aarsetoy et al., 2008). Taken in combination with the evidence provided in this study suggests that FO acts directly on the heart in an anti-arrhythmic manner rather than on vascular related outcomes influencing the initiation of acute coronary syndrome. Ex vivo research supports this notion via demonstrating that the anti-arrhythmic effect is independent of the circulating fatty acid concentrations (Pepe & McLennan, 1996). These findings provide a physiological plausible mechanism to assist in explaining the reduced cardiovascular mortality rates observed amongst populations that habitually consume fish in their diet (Kromhout et al., 1985).

5.5.4 Conclusions

For the first time, the anti-arrhythmic action of FO was demonstrated using a dose of FO equivalent to approximately two serves of fish per week in the human diet. The protection was evident despite animals consuming a typical Western-style background diet high in SFA and n-6 PUFA, suggesting that the anti-arrhythmic action of FO is intimately linked to absolute intake of FO. Reduced fatal ischaemic and reperfusion VF events strongly warrant the inference that FO is acting to reduce SCD outcomes, rather than on vascular outcomes influencing the occurrence of ischaemia (acute coronary syndrome). In view of these findings the next chapter explored whether supplementing dietary achievable FO doses protects the haemodynamic integrity of the heart during prolonged periods of regional ischaemia and post-ischaemic recovery.
CHAPTER 6

Investigating ischaemic and post-ischaemic \textit{in vivo} cardiac haemodynamics following supplementation of human dietary achievable fish oil doses
6.1 INTRODUCTION

6.1.1 Background

In contrast to brief periods of ischaemia, the primary outcome of prolonged ischaemia is myocardial tissue necrosis which can develop into a myocardial infarct. This leads to progressive left ventricle remodelling including dilation and hypertrophy of the myocardium and haemodynamic dysfunction (Sutton & Sharpe, 2000). There is strong evidence demonstrating that sudden cardiac death commonly occurs in patients with or without pre-existing infarct evident, showing that the significance of arrhythmic outcomes (Chapter 5) is not necessarily correlated to the extent of tissue necrosis (Huikuri et al., 2001). However, haemodynamic dysfunction is correlated with the extent of the tissue necrosis (Bolli, 1990) and evidence has highlighted that in the decade following their myocardial infarction, more than one third of patients will develop heart failure (Hellermann et al., 2003; Kenchaiah et al., 2004). The degree of tissue necrosis varies, depending on the duration of the ischaemia, the presence or absence of collateral circulation in the ischaemic zone, whether the occlusion is complete or incomplete and the individual demands of the myocardial cells for oxygen and nutrients (Thygesen et al., 2007).

6.1.2 The phenomenon of myocardial ischaemic pre-conditioning

Ischaemic pre-conditioning is a phenomenon in which submitting the myocardium to brief periods of ischaemia and then reperfusion prior to prolonged ischaemia protects and improves the recovery of the heart (Przyklenk & Kloner, 1998). Ischaemic pre-conditioning has been shown to significantly reduce arrhythmia generation and infarct size (Abdukeyum et al., 2008; Shiki & Hearse, 1987), attenuate post-ischaemic myocardial dysfunction (Efstathiou et al., 2001; Lasley et al., 1993; Przyklenk & Kloner, 1998) and improve the recovery of rate pressure product during reperfusion (Lasley et al., 1993). Additionally, hypoxic pre-conditioning before regional cardiac ischemia has been demonstrated to lead to preservation of ejection fraction and preload recruitable stroke work, indicating improved systolic function (Van der Mieren et al., 2008). The protection afforded by ischaemic pre-conditioning occurs in two stages. The first ‘early’ stage produces potent protection, however the protection only persists for a
few hours and repeated applications causes desensitisation (Qiu et al., 1997). The second ‘late’ stage occurs after twenty four hours and can be invoked repeatedly to provide prolonged cardio-protection (Qiu et al., 1997).

Identification of the cellular mechanisms contributing to the physiological outcomes observed with ischaemic pre-conditioning is of interest to researchers due to the potential clinical uses in the treatment of ischaemia and reperfusion injury. It has been demonstrated that the early stage protection offered by ischaemic pre-conditioning is caused by the production of free radicals and reactive oxygen species, creating oxidative stress (Yellon & Downey, 2003). However, because the stimulation is only for brief periods, the myocardial oxidative stress generated actually leads to the production and up-regulation of a range of antioxidant systems including: nitric oxide synthase, superoxide dismutase and heat-shock proteins (Lin et al., 2011) which in turn induce resistance to subsequent more prolonged ischaemic episodes (Das & Das, 2008). Whereas, late stage protection is thought to be mediated by changes in the expression of genes leading to altered inflammatory processes and mediation of excessive Ca\(^{2+}\) influx and opening of mitochondrial K\(^{+}\) ATP channels during subsequent ischaemic episodes (Das & Das, 2008; Yellon & Downey, 2003). Despite the extensive interest in research surrounding ischaemic pre-conditioning, the unpredictable onset of ischaemic/reperfusion injury and invasive methods required to implement remote or ischaemic pre-conditioning make it impractical in clinical practice (Iliodromitis et al., 2007).

6.1.3 Other potential methods to exert a pre-conditioning effect

There is evidence for other methods which are more clinically applicable to replicate the cardio-protection observed with ischaemic pre-conditioning. These approaches include exercise, remote and post ischaemic conditioning, heat stress, oxidative stress, stretch and some pharmacological interventions (Kharbanda et al., 2002; Kharbanda et al., 2009; Staat et al., 2005; Taylor & Starnes, 2003). Typically, the conditioning provided involves toxic stimuli acting directly at the heart that raise defences in anticipation of an upcoming event and the protection is persistent even after the initial agent has washed off. However, exercise and remote ischaemic conditioning are particular interesting therapeutic candidates because they do not require toxic stimuli to be applied directly to the heart and protection persists after the initial stimuli has finished (Kavazis, 2009;
Staat et al., 2005). This suggests the mechanism involves the release of protective agents into the circulation which can produce persistent protective effects. Specifically, studies reveal that chronic endurance exercise training protects the heart against arrhythmias, contractile dysfunction, oxidative injury, mitochondrial damage and cell death (Lennon et al., 2004; Liu et al., 2000). The mediators responsible for the cardioprotection offered by exercise and remote conditioning have not yet been fully explained, however studies have shown that upregulation of antioxidant enzymes plays a critical role in protecting the myocardium against I/R injury (Hamilton et al., 2003; Kavazis, 2009; Liu et al., 2000; Powers et al., 1993).

Supplementation of FO appears to offer similar cardio-protective features as to those observed when using other forms of conditioning. However, like remote and exercise conditioning, FO supplementation results in the upregulation of antioxidant enzymes within the myocardium without subjecting the heart to a toxic stimuli (Abdukeyum et al., 2016). The unsaturated nature of DHA makes it prone to oxidation; therefore increasing myocardial DHA concentrations elevates the peroxidation potential of membrane phospholipids in the myocardium (Serhan et al., 2004; Song et al., 2000), indirectly generating a toxic stimulus. In addition to this upregulation of antioxidant systems, it is well-established that FO acts in an anti-arrhythmic manner at therapeutic doses (Matthan et al., 2005; McLennan, 2014), and now at dietary achievable doses (Chapter 5). It has also been demonstrated that MVO\textsubscript{2} is reduced without diminishing work output, suggesting the heart is also using oxygen more efficiently following supplementation of therapeutic FO doses (Pepe & McLennan, 2002). Furthermore, early post-ischaemic contractile recovery of heart function is augmented (Pepe & McLennan, 2002), there is reduced production of ischaemic damage biomarkers and infarct size is attenuated (Abdukeyum et al., 2008; Pepe & McLennan, 2002; Pepe & McLennan, 2007).

As such, it appears that supplementing therapeutic FO doses promotes the upregulation of antioxidants and produces similar physiological outcomes to other well researched forms of conditioning. Importantly, the upregulation of antioxidant enzymes, anti-arrhythmic and improved ischaemic and post-ischaemic contractile actions of FO are on-going without the need for daily intake. It has been demonstrated that the most effective manner to increase tissue LC n-3 PUFA concentrations is from a single weekly
dose of FO (Ghasemifard et al., 2015). As such, FO supplementation, like remote and exercise conditioning offers a more lifestyle applicable, sustainable and effective form of cardio-protection referred to by Abdukeyum et al., 2008 as ‘nutritional pre-conditioning’. However, previous studies investigating conditioning of the myocardium by FO supplementation have relied upon therapeutic and supra-therapeutical doses and ex vivo experimental models to describe changes. Therefore, the objective of this study was to evaluate the conditioning efficacy of human dietary achievable FO doses in a physiologically stable in vivo ischaemia and reperfusion experimental model.
6.2 AIMS AND HYPOTHESES

This study investigated whether supplementing dietary achievable FO doses to a typical Western-style diet (high SFA and n-6 PUFA) provides ‘nutritional pre-conditioning’ of the heart during prolonged regional ischaemia and reperfusion, in a similar manner to ischaemic pre-conditioning, remote pre-conditioning and exercise training.

Specifically, this experiment aimed to test the following hypotheses:

**Aim 1:** Determine the influence of supplementing dietary achievable FO doses on ischaemic haemodynamic function.

*Hypothesis 1:* Cardiac haemodynamic dysfunction induced by ischaemia, particularly changes to heart rate, will be attenuated by supplementation of FO.

**Aim 2:** Determine the influence of supplementing dietary achievable FO doses on post-ischaemic haemodynamic recovery.

*Hypothesis 2:* Post-ischaemic haemodynamic recovery of the left ventricle as reflected by PV-loops will be modified in FO supplemented animals.
6.3 METHODS

6.3.1 Animals, ethical considerations and standardisation

The male rats (Sprague Dawley) from cohort 2 were used in this study. Directly following the end of experimental procedures described in Chapter 5 the experimental procedures for this study began.

6.3.2 Groups

Animals were supplied water and randomly assigned one of three pre-fabricated diets *ad libitum* for four weeks before experimentation. The diets contained different amounts of FO (Control – 0%; $n = 6$, LowFO – 0.31%; $n = 9$, ModFO – 1.25%; $n = 12$).

6.3.3 Experimental preparation

Preparation of the 6mm miniaturised 2-French (2F) Millar pressure-volume conductance catheter (SPR-638 Millar Instruments, Houston, USA) and electrocardiogram (ADInstruments, Bella Vista, NSW, Aus) was completed (Section 3.3.3).

6.3.4 Surgical procedures

Animals were anaesthetised, and surgery conducted to allow cardiac measurements including: ECG, ventricular pressure and volume and aortic blood pressure (Chapter 4). An established surgical technique was used to create an ischaemia and reperfusion model within the *in vivo* heart via occluding and reperfusing the LAD coronary artery (Chapter 5).

6.3.5 Experimental protocol and measurements

Following completion of the *in vivo* acute ischaemia and reperfusion experimental protocol and arrhythmia generation measurements (Chapter 5), animals were checked for depth of anaesthesia prior to experimental protocols and measurements being collected for this study. After depth of anaesthesia was confirmed, measurements of heart rate and aortic systolic blood pressure were collected as pre-ischaemic reference values. The LAD coronary artery was then occluded by placing tension on the ligature
for forty five minutes to generate a prolonged ischaemic episode, followed by the release of tension from the ligature, allowing reperfusion of the myocardium (Figure 6.1). During ischaemia the catheter was placed in the aorta to monitor arterial blood pressure, heart rate was also collected. Following the ischaemia, the myocardium was reperfused for forty five minutes before the conductance catheter was moved into the left ventricle and post-ischaemic cardiac function was measured for fifteen minutes. Upon completion of post-ischaemic cardiac function measurements, the inferior vena cava was occluded for four to six seconds using a cotton tip applicator. Three separate occlusions were completed for each animal with five minutes recovery between each occlusion, this allowed measurement of post-ischaemic load-independent cardiac function. Saline calibration procedures (Section 3.3.5a) were completed with the conductance catheter and then animals were euthanased.

6.3.5a Euthanasia and tissue collection

Following the completion of experimental procedures all animals were euthanased without recovery from the anaesthetic via removing the artificial ventilator and then rapid exsanguination and removal of the heart. During exsanguination, blood was collected into a 5mL syringe containing 0.2mL of a 5,000 U/mL Heparin Sodium solution (Hospira, Lyypards, SA, AUS). The blood was then used to fill the wells of calibration cuvette and then placed on a heating pad set to 37 °C for calibration of the pressure volume transducer (Section 3.3.7). Hearts were rapidly removed from animals following exsanguination, weighed and snap frozen in liquid nitrogen (Section 2.3.5a). Following exsanguination and removal of the heart, a pre-determined selection of skeletal muscles were dissected from the upper thigh and lower leg of the left hind-limb, weighed and snap frozen in liquid nitrogen (Section 2.3.5b).
Figure 6.1: Overview of the experimental protocol which occurred during the prolonged ischaemia and subsequent reperfusion.

Abbreviations: I = ischaemia; R = reperfusion; LAD = left anterior descending coronary artery; IVC = inferior vena cava; BP = blood pressure.
6.3.5b Zone at risk measurement

The heart was arrested and flushed with ice cold saline and the zone at risk was delineated according to previously established methods (McLennan et al., 1988). A 5mL plastic syringe filled with an Indian-dye solution was inserted into the aorta and the aorta was tightly bound to the syringe via suture. The coronary arteries were then re-occluded and pressure was placed on the syringe in order to cause retrograde perfusion of Indian dye into the aorta and coronary arteries. The ischaemic zone at risk was then delineated via staining of the perfused (non-ischaemic) and non-perfused (ischaemic) tissue. The heart was then weighed (total weight, total left + right ventricular weight and left ventricle weight only) and cut into five transverse sections (Figure 6.2). The slices were then separated into non-ischaemic and zone at risk and weighed again. The zone at risk was calculated as % of total wet left ventricle weight. Tissue was then snap frozen and stored at –80°C for future biochemical analysis following weight and zone at risk measurements.

Figure 6.2: An isolated heart slice showing ischaemic/non-ischaemic ventricular tissue following Indian dye tissue staining.

Figure 6.2 notes: Ischaemic region (non-stained) and non-ischaemic region (stained black) can be observed after perfusion of coronary arteries with Indian dye while LAD is occluded.
6.3.6 Post-experimental data analysis

Average heart rate was calculated (Section 3.3.8a) at five minute intervals during ischaemia and reperfusion from the saved electrocardiogram recordings in parallel with measurements of systolic blood pressure. Rate pressure product was calculated from these measurements at the corresponding time points. *In vivo* left ventricular cardiac haemodynamic function was analysed in the final five minutes of the recordings collected by the conductance catheter during post-ischaemic measurements. Analysis of haemodynamic indices was completed following the methods outlined in section 3.3.8b. Load-independent indices were analysed at each inferior vena cava occlusion and the average of all three measurements were used to assess the ventricular systolic and diastolic function of dietary groups.

6.3.6a Statistics

All results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The effects of dietary treatment during ischaemia and post-ischaemia were tested via ANOVA. A Bonferroni post-hoc test for comparison between group means was then used to identify differences. A Dunnett’s T-test was used for comparison of individual diets against the Control group. All statistical analyses were performed in a blinded manner and completed using SPSS 21 for Windows (SPSS Inc, Chicago, IL, USA). Alpha was set as $p <0.05$. 

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6.4 RESULTS

Six (6) animals died during the forty five minute ischaemia period due to cardiogenic shock and diminished mean arterial pressure. Therefore, final group numbers for comparisons of ischaemic cardiac function and recovery were \( n = 6 - 8 \). Zone at risk measurements showed that on average \( \sim 39 - 45\% \) of the left ventricle wall across all animals was ischaemic during the LAD occlusion and at risk of becoming infarcted (Figure 6.3). There were no statistical significant differences in the zone at risk between any groups.

![Diagram of heart with ischemic zone highlighted.](image)

**Figure 6.3:** The effect of fish oil on the percentage wet weight of left ventricle wall at risk of ischaemia following prolonged LAD coronary artery occlusion.

Values are mean ± SEM \((n = 6 - 8 \text{ per group})\). No statistical significant differences present. Figure obtained from Wikimedia commons and adapted.
6.4.1 The effect of fish oil supplementation on ischaemic cardiac function

There were no significant differences between any of the groups in pre-ischaemic heart rate, systolic blood pressure and rate pressure product. Five minutes after the onset of ischaemia heart rate and systolic blood pressure had dropped in all groups resulting in decreased rate pressure product (Cross hatch red, Figure 6.4). Ten minutes after the onset of ischaemia heart rate and systolic blood pressure returned toward pre-ischaemic levels in the ModFO group but remained depressed in the Control and LowFO groups. The recovery of heart rate and systolic blood pressure in animals from the ModFO group generated a significantly higher rate pressure product then the Control and LowFO group. The ModFO maintained the significantly higher rate pressure product for the remaining time in ischaemia. Five minutes after the onset of reperfusion, rate pressure product remained significantly higher in the ModFO group compared to the Control and LowFO group (Cross hatch green, Figure 6.4). There were no further significant differences between any of the groups for the remaining time in reperfusion. All groups displayed a recovery towards pre-ischaemic heart rate, systolic blood pressure and rate pressure product measurements.
Figure 6.4: The effect of fish oil on heart rate, aortic systolic blood pressure and rate pressure product throughout ischaemia and subsequent reperfusion of the left anterior descending coronary artery.

Values are mean ± SEM (n = 6 – 8 per group). *p < 0.05 vs Control diet (One-way ANOVA with Dunnett’s T-test vs Control)

Notes: ‘Pre’ measurements are taken directly prior to ischaemia occurring and are not equivalent to baseline measurements shown in Figure 6.5 which were collected prior to any surgery being completed.

Abbreviations: HR = heart rate; BP = blood pressure.
6.4.2 The effect of fish oil supplementation on post-ischaemic cardiac haemodynamic function

Heart rate was significantly slower during post-ischaemic measurement when compared to baseline measurements in the Control and LowFO groups, whereas the post-ischaemic heart rate of the ModFO group returned towards its resting baseline measurement (Figure 6.5).

![Figure 6.5: The effect of fish oil on resting heart rate (beats.min⁻¹) following ischaemia and reperfusion.](image)

Values are mean ± SEM ($n = 6 – 8$ per group). *$p < 0.05$ within group, vs baseline measurements (One-way ANOVA with Bonferroni post-hoc test). †$p < 0.05$ between group, vs Control diet (One-way ANOVA with Bonferroni post-hoc test).

Notes: ‘Baseline’ resting heart rates are the measurements reported in Chapter 4 (Table 4.2) which were collected using the conductance catheter prior to the chest cavity being surgical opened.
Stroke volume and cardiac output were lower in all groups, but the reductions did not reach statistical significance (*Table 6.1*). Compared to baseline, the Control group displayed a significantly lower end-systolic pressure and a significantly greater end-systolic and end-diastolic volume. Compared to baseline, both FO groups displayed a significantly lower end-systolic pressure, there were no other changes in the LowFO group but the ModFO group also showed a significantly greater end-diastolic pressure. As a result of these changes there was a clear downward shift of the PV-loop on the y-axis for all groups. However, rightward shifting along the x-axis was only reflected in the Control PV-loop (*Figure 6.6*). Comparison of calculated haemodynamic variables showed all groups to have a significantly lower $\dP/dt_{\text{max}}$ and significantly higher $\dP/dt_{\text{min}}$ and Tau (*Table 6.2*).
Figure 6.6: Representative in vivo baseline and post-ischaemic left ventricular PV-loops following FO supplementation.
Values are mean ± SEM (Baseline n = 15 – 18, Post n = 6 – 8) *p <0.05 vs baseline (One-way ANOVA comparing within diet conditions with Bonferroni post-hoc test).

Abbreviations: ESP = end-systolic pressure; ESV = end-systolic volume; EDP = end-diastolic pressure; EDV = end-diastolic volume.
Table 6.1: The effect of fish oil on baseline resting and post-ischaemic left ventricular measured haemodynamic variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post</td>
<td>∆</td>
</tr>
<tr>
<td>HR (beats.min⁻¹)</td>
<td>451 (±8)</td>
<td>382* (±17)</td>
<td>-69</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>100 (±7)</td>
<td>69 (±11)</td>
<td>-31</td>
</tr>
<tr>
<td>CO (mL.min⁻¹)</td>
<td>45 (±3)</td>
<td>26 (±4)</td>
<td>-19</td>
</tr>
<tr>
<td>CI (mL.min⁻¹ x kg)</td>
<td>23 (±2)</td>
<td>17 (±3)</td>
<td>-6</td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>65 (±10)</td>
<td>115* (±15)</td>
<td>50</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>154 (±9)</td>
<td>184* (±36)</td>
<td>30</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>139 (±7)</td>
<td>84* (±7)</td>
<td>-55</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>13 (±1)</td>
<td>13 (±2)</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (Baseline n = 15 – 18, Post n = 6 – 8) *p < 0.05 vs baseline (One-way ANOVA comparing baseline vs post-ischaemic measures within diet conditions using a Bonferroni post-hoc test).

**Abbreviations:** HR = heart rate; SV = stroke volume; CO = cardiac output; CI = cardiac index, CO/Body weight; ESV = end-systolic volume; EDV = end-diastolic volume; ESP = end-systolic pressure; EDP = end-diastolic pressure; Post = post ischaemia; ∆ = difference between baseline and post-ischaemia.
Table 6.2: The effect of fish oil on baseline resting and post-ischaemic left ventricular calculated haemodynamic variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
<th></th>
<th>LowFO</th>
<th>ModFO</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Post</td>
<td>Δ</td>
<td>Baseline</td>
<td>Post</td>
<td>Δ</td>
<td>Baseline</td>
<td>Post</td>
<td>Δ</td>
<td></td>
<td></td>
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<tr>
<td>Systolic indices</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ea (mmHg/µL)</td>
<td>1.5 (±0.2)</td>
<td>1.2 (±0.4)</td>
<td>-0.3</td>
<td>1.5 (±0.2)</td>
<td>1.5 (±0.2)</td>
<td>-</td>
<td>1.2 (±0.1)</td>
<td>1.7 (±0.3)</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF (%)</td>
<td>65 (±5)</td>
<td>38* (±10)</td>
<td>-27</td>
<td>68 (±4)</td>
<td>57 (±9)</td>
<td>-11</td>
<td>62 (±4)</td>
<td>52 (±3)</td>
<td>-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt_max (mmHg/sec⁻¹)</td>
<td>8688 (±441)</td>
<td>4055* (±382)</td>
<td>-4633</td>
<td>8796 (±288)</td>
<td>4845* (±724)</td>
<td>-3951</td>
<td>8537 (±401)</td>
<td>4648* (±162)</td>
<td>-3889</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic indices</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt_min (mmHg/sec⁻¹)</td>
<td>-7735 (±483)</td>
<td>-3074* (±436)</td>
<td>4661</td>
<td>-7773 (±364)</td>
<td>-4351* (±882)</td>
<td>3422</td>
<td>-7440 (±313)</td>
<td>-3955* (±207)</td>
<td>3445</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>10.0 (±0.3)</td>
<td>15.0* (±2.0)</td>
<td>5</td>
<td>9.6 (±0.4)</td>
<td>14.4* (±1.9)</td>
<td>4.8</td>
<td>10.0 (±0.4)</td>
<td>14.4* (±1.3)</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM (Baseline n = 15 – 18, Post n = 6 – 8) *p < 0.05 vs baseline (One-way ANOVA comparing baseline vs post-ischaemic measures within diet conditions using a Bonferroni post-hoc test).

Abbreviations:
Post = post ischaemia; Δ = difference between baseline and post-ischaemia.
Systolic indices: Ea = arterial elastance; EF = ejection fraction; dP/dt_max = peak rate of pressure rise.
Diastolic indices: dP/dt_min = peak rate of pressure decline; Tau = relaxation time constant calculated by Glantz method (regression of dP/dt versus pressure).
6.4.3 The effect of fish oil supplementation on post-ischaemic preload-independent cardiac function

All groups displayed a significant reduction in stroke work compared to baseline resting measurements (Table 6.3). There were no significant changes to the ESPVR, preload-recruitable stroke work and dP/dt\text{max} – end-diastolic volume relationships following ischaemia when they were calculated for all groups. In addition, there were no clear changes observed within any of the groups when the slope of the diastolic index EDPVR was calculated.
Table 6.3: The effect of fish oil on baseline and post-ischaemia left ventricular preload-independent haemodynamic function.

<table>
<thead>
<tr>
<th>Systolic indices</th>
<th>Control</th>
<th>LowFO</th>
<th>ModFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW (mmHg*µL)</td>
<td>Baseline</td>
<td>Post</td>
<td>∆</td>
</tr>
<tr>
<td></td>
<td>10933 (±963)</td>
<td>4559 (±1020)</td>
<td>-6374</td>
</tr>
<tr>
<td>ESPVR</td>
<td>2.1 (±0.2)</td>
<td>1.9 (±0.2)</td>
<td>-0.2</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>115 (±8)</td>
<td>100 (±9)</td>
<td>-15</td>
</tr>
<tr>
<td>dP/dt_{max}−EDV</td>
<td>46 (±8.8)</td>
<td>32 (±7.6)</td>
<td>-14</td>
</tr>
</tbody>
</table>

Diastolic indices:

| EDPVR            | 0.038 (±0.01) | 0.036 (±0.01) | -0.002 | 0.041 (±0.01) | 0.042 (±0.01) | 0.001 | 0.036 (±0.01) | 0.041 (±0.01) | 0.005 |

Values are mean ± SEM (Baseline n = 15 – 18, Post n = 6 – 8) *p < 0.05 vs baseline (One-way ANOVA comparing baseline vs post-ischaemic measures within diet conditions using a Bonferroni post-hoc test).

Abbreviations:

Post = post ischaemia; ∆ = difference between baseline and post-ischaemia.

Systolic indices: SW = stroke work; ESPVR = end-systolic pressure volume relation; PRSW = preload recruited stroke work (slope of stroke work–EDV relationship); dP/dt_{max}−EDV = dP/dt_{max}−end diastolic volume relation

Diastolic indices: EDPVR = end-diastolic PV relation slope
6.5 DISCUSSION

The current study demonstrated that supplementing FO doses achievable within a typical Western-style human diet (high SFA and n-6 PUFA) modified *in vivo* ischaemic and post-ischaemic cardiac function. Importantly, a physiologically and clinically relevant ischaemic episode induced by regional blood flow restriction was used, typical of major vessel disease and myocardial infarction. Previously, the effects of FO on cardiac haemodynamic function during ischaemia and reperfusion have generally been restricted to *ex vivo* models and relied upon therapeutic and supra-therapeutical doses (Abdukeyum *et al.*, 2008; Goo *et al.*, 2014; Herrera *et al.*, 2015; McLennan *et al.*, 2012; Pepe & McLennan, 2002). Furthermore, with the exception of the study completed by Abdukeyum, *et al.*, 2008; ischaemia has been induced by globally restricting blood flow to the entire coronary circulation. As such, findings from this study offer novel and clinically relevant evidence to demonstrate that dietary achievable FO doses are associated with cardio-protective physiological actions akin to the protection provided by regular exercise training and remote or ischaemic pre-conditioning.

6.5.1 Fish oil effects on cardiac dysfunction induced by regional ischaemia

Shortly after the onset of the regional ischaemic stimulus before cellular necrosis occurred, cardiac function was depressed in a manner which fits with the previously described physiological outcomes of non-infarcted ischaemic damage to the myocardium (Indolfi & Ross, 1993; Moens *et al.*, 2005). Physiologically, this was observed as acute reductions to heart rate, systolic blood pressure and as a consequence of these changes, rate pressure product was lower. Supplementation of dietary achievable FO doses did not influence this initial reduction to heart rate, systolic blood pressure or rate pressure product. However, at the onset of ischaemia, the decline in cardiac function observed across all groups is a normal compensatory mechanism. During ischaemia, myocardial substrate and oxygen supply is reduced, it is well established that in an attempt to compensate the reduced sub-endocardial perfusion, reductions in transmural wall thickening or segment shortening occur (Canty & Suzuki, 2012). This is often referred to as ‘perfusion-contraction matching’ (Indolfi & Ross, 1993; Ross, 1991) or ‘hibernation’ of the myocardium (Heusch *et al.*, 1997). This response of the myocardial tissue occurs as an effort to preserve the viability of
myocytes and delay the occurrence of myocyte necrosis by maintaining substrate availability. A major factor influencing transmural blood flow and ultimately substrate availability is heart rate. Sub-endocardial perfusion only occurs during diastole when coronary vasodilation is maximal (Spaan et al., 2008). For this reason, using beta-blockers to reduce heart rate is a common therapeutical intervention during ischaemia (López-Sendó et al., 2004) and during heart failure (Cullington et al., 2012) based on the rationale of indirectly modulating MVO$_2$ during periods of mismatched oxygen supply and demand to reduce myocyte necrosis. Therefore, the initial reduction in heart rate, systolic blood pressure and rate pressure product observed across all groups at the onset of ischaemia was a normal intrinsic cardiac mechanism for maintaining myocardial perfusion-contraction matching. Heart rate slowing also reflects another mechanism to maintain cardiac output by increasing the diastolic filling phase between beats. As such, FO modification of cardiac function, particularly heart rate during this response would be detrimental to the preservation of viable myocytes during ischaemia.

Within ten minutes of the onset of ischaemia heart rate, systolic blood pressure and rate pressure product started recovering in all groups and this gradual recovery continued throughout the remainder of ischaemia towards pre-ischaemic levels. The recovery in cardiac function observed during ischaemia in this study highlights some important differences between in vivo regional ischaemia and ex vivo global ischaemia experimental models. The typical ex vivo response to global ischaemia can be understood by detailed experiments completed using a erythrocyte-perfused isolated working heart model (Pepe & McLennan, 1993). The experiments demonstrated external work, heart rate and the pressure-time integral per heart beat (dP/dt$_{max}$) to be significantly reduced after five minutes of ischaemia, similar to the physiological changes observed in this study. In contrast to this study, these indices of cardiac function remained depressed or progressively deteriorated following twenty minutes of global ischaemia in the ex vivo model (Pepe & McLennan, 1993). This highlights the dubious physiological relevance of using ex vivo global ischaemia models to assess cardiac interventions or treatments.

Global ischaemia models have no non-ischaemic tissue capable of compensatory responses during ischaemia. Whereas, the region at risk of becoming infarcted due to ischaemia in this study was ~45%, meaning ~55% of the heart tissue remained viable.
Importantly, zone at risk was not different between any of the groups, indicating an equivalent ischaemic insult and the proportion of ventricular wall that was ischaemic was comparable to previous studies using similar surgical protocols (Abdukeyum et al., 2008; Curtis et al., 1987; Ertracht et al., 2011; Spadaro et al., 1980; Xue et al., 2016). Interestingly, the location of staining consistently demonstrated that the free left ventricular wall was at risk of ischaemia rather than the interventricular septum (Figure 6.2). In humans the LAD coronary artery usually (90%) supplies the anterior two-thirds of the interventricular septum. However, there is evidence to demonstrate that in the rat the interventricular septum is supplied by a branch that originates close to the origin of the left coronary artery (Spadaro et al., 1980). Thus, occlusion of the LAD coronary artery in the rat would not obstruct flow to the interventricular septum and explains why staining consistently showed that the free wall of the left ventricle was at risk of ischaemia.

Cardiac function in ex vivo models are devoid of neural, humoral and blood pressure feedback regulatory mechanisms. Thus, the heart’s response to decreased perfusion during ischaemia in an isolated system is to depress contractile function in order to preserve myocardial tissue while oxygen supply is not adequate. Whereas, the recovery in heart rate and systolic blood pressure observed across all groups in this study are linked to the important regulatory role of the baroreceptor reflex in maintaining physiological homeostasis. The baroreceptor reflex acts to maintain mean arterial pressure at all times, to do so it exerts potent control over the heart and vasculature (Cowley et al., 1973). The sudden decrease in arterial blood pressure at the onset of ischaemia triggers the baroreceptors to stimulate the medulla leading to vagal withdrawal and increased sympathetic nervous tone to the heart. This response explains how heart rate and systolic blood pressure partially recovers in the in vivo heart during ischaemia but not in the ex vivo heart. This makes the in vivo experimental model used in this study more clinically relevant and provides a strong basis to assess the effects of FO on cardiac function during ischaemia.

The current study demonstrated, for the first time, that there was a distinct accelerated recovery of heart rate, systolic blood pressure and rate pressure product in the ModFO group compared the Control group. Chapter 4 of the current thesis demonstrated dietary FO to slow in vivo resting heart rate, supporting previous in vivo (Hartog et al., 1987;
Lortet & Verger, 1995) and ex vivo research (Abdukeyum et al., 2008; Pepe & McLennan, 1996). Interestingly, in contrast to the slowed resting heart rate consistently observed with FO, this study demonstrated that heart rate rapidly recovered to pre-ischaemic rates and remained stable for the duration of the ischaemic episode in the ModFO group. This provides evidence to suggest that FO does not act solely to slow heart rate. Rather, it appears FO acts in a manner to adjust contractile function of the heart to a physiologically appropriate rate and force. Further support for this notion is found in a study which demonstrated that slowing of heart rate driven by oxygen deficits during ischaemia were attenuated by FO in blood perfused isolated hearts devoid of neural feedback (Pepe & McLennan, 1996).

The rapid recovery of heart rate and systolic blood pressure in the ModFO group facilitated a higher rate pressure product. Heart rate, systolic blood pressure and the product of both, rate pressure product have been demonstrated to correlate strongly with in vivo MVO₂ (Kitamura et al., 1972), but this correlation was unable to be shown in ex vivo hearts (Aksentijevic et al., 2016). However, it is likely that the inability to demonstrate a correlation in the ex vivo model is because low-oxygen carrying perfusate was used instead of whole-blood leading to altered oxygen conductance. Therefore, the higher rate pressure product demonstrated in vivo in the current study indirectly suggests that MVO₂ is greater in the ischaemic heart following FO supplementation. Interestingly, during ex vivo ischaemia, it has been demonstrated that FO lowers MVO₂ when the heart is electrically paced to maintain a uniform beat rate, and left ventricular preload and afterload are strictly controlled, ensuring the same amount of work was conducted (Pepe & McLennan, 2002). In contrast, the present study demonstrated a faster heart rate and higher systolic blood pressure, indirectly suggesting a greater MVO₂ and indicative of greater cardiac work. The indirect observation of greater MVO₂ in this study in contrast to lowered MVO₂ in the study conducted by Pepe et al., 2002 demonstrates the important role FO plays in the efficient use of oxygen rather than exclusively as a depressor of oxygen use. This further highlights a role of FO in maintaining cardiac muscular contractile function, via the efficient use of available oxygen during ischaemia, in a similar manner to what has been demonstrated in skeletal muscle (Henry et al., 2015; Peoples & McLennan, 2010, 2014, 2017).
It has been demonstrated that during ischaemia, MVO2 can remain paradoxically high in the presence of abnormal wall motion and decreased contractile function in the canine heart (Dean et al., 1990) emphasizing the normal inefficient increases of MVO2 during ischaemia. The current study demonstrated that dietary achievable FO doses instigated productive increases of myocardial MVO2 during ischaemia. That is, when the heart is adapting to adverse conditions, FO enables ongoing function. Contradictory recent research has declared that FO does not improve the oxygen efficiency or mechano-energetic performance of the *ex vivo* working heart (Goo et al., 2014). The conflicting evidence described from the study conducted by Goo et al., 2014 and the current one can be clarified by comparing the experimental models used. The study conducted by Goo et al., 2014 used an *ex vivo* heart at low temperature (32°C), perfused with a tyrode solution bubbled with 100% oxygen, which despite very high PO2 carry much less oxygen than blood (Pepe & McLennan, 1993). In contrast, this study used an *in vivo* model where the heart was perfused by the animals own blood, core temperature was maintained at 37°C and all physiological feedback mechanisms remained intact. Neural and humoral influences, temperature regulation and most importantly, adequate oxygenation of the heart, are essential for optimal cardiac performance in a working physiological system (Kuzmiak-Glancy et al., 2015). Inadequate oxygenation of the myocardium through the use of red blood cell-free perfusate in *ex vivo* models has been demonstrated to diminish cardiac function, even in the absence of injury (Pepe & McLennan, 1993). It has been suggested that whole blood perfusate provides superior preservation of myocardial function during *ex vivo* heart preparations where *in vivo* methods are not available (White et al., 2015). This clarifies why whole blood perfused *ex vivo* models consistently demonstrate improved cardiac (Pepe & McLennan, 2002; Pepe & McLennan, 2007) and skeletal (Henry et al., 2015; Peoples & McLennan, 2010, 2017) muscle contractile function with FO supplementation that contrast the findings of Goo et al., 2014. It is therefore likely that the conflicting evidence demonstrated by Goo et al., 2014 is driven by the low temperature that experiments were conducted in and inadequate oxygenation of the heart as a result of non-whole blood perfusate being used in their *ex vivo* heart model.

Another factor which may have increased apparent oxygen consumption in the ModFO group is the possibility that there were more viable myocardial cells during the ischaemia. Although infarct size was not investigated directly in this study, previous
research has demonstrated that therapeutic and supra-therapeutical doses of FO can reduce infarct size in the rat heart (Abdukeyum et al., 2008; Pepe & McLennan, 2002; Pepe & McLennan, 2007). Less extensive damage resulting in a greater proportion of viable cells within the ischaemic region would also help explain why the ventricle was able to generate greater contractile function than the other groups during ischaemia. Contractile function has previously been demonstrated to improve in isolated hearts supplemented supra-therapeutical doses of FO (Abdukeyum et al., 2008; Pepe & McLennan, 2002). Although measured indirectly as systolic blood pressure, this study is the first to demonstrate improved contractile function during ischaemia in a clinically relevant in vivo model coupled with dietary achievable FO doses. The comparison of systolic blood pressure to left ventricular contractile function is appropriate in this study as the catheter was sitting in the aorta directly outside the left ventricle which is representative of the afterload or ‘tension’ generated by the left ventricle.

### 6.5.2 Fish oil effects on post-ischaemic cardiac dysfunction

Assessment of Control post-ischaemic in vivo pressure and volume relations, demonstrated the typical contractile dysfunction associated with the loss of viable myocardium caused by ischaemia. Acutely, myocardial ischaemia reduces total contractility (Pfeffer, 1995; Yang et al., 2004); this was reflected in Control animals by lower end-systolic pressure and the PV-loop shifting downwards on the y-axis. Reduced force of ventricular contraction results in decreased ventricular emptying and a greater end-systolic volume. Increased end-systolic volume means the diastolic filling phase of the cardiac cycle starts at a greater volume, therefore normal venous return added to an already partially filled ventricle results in a greater end-diastolic volume (preload). This is reflected by significant rightward shifting of PV-loops acutely following ischaemia in rats (Pfeffer et al., 1991). In comparison to their baseline PV-loop, the post-ischaemic Control PV-loop shifted rightwards on the x-axis, reflecting greater end-systolic volume and end-diastolic volume. Acutely, increased stretch of the myocytes caused by a greater end-diastolic volume generates a stronger contraction via the Frank-Starling law to ensure stroke volume is maintained and the increased blood volume in the ventricle is ejected into circulation rather than pooling within the ventricle (Glower et al., 1985). Although post-ischaemic stroke volume is maintained by this compensation, ejection fraction was lower in the Control group because of the greater end-diastolic volume.
The degree of post-ischaemic ventricular haemodynamic dysfunction has been demonstrated to be directly related to the extent of viable myocardium loss (Pfeffer et al., 1979). Together, the zone at risk measurements collected and the haemodynamic dysfunction observed confirms that the ischaemic episode was sufficient to cause typical post-ischaemic contractile dysfunction, consistent with what has been described in other studies. Therefore, the alterations observed between the Control baseline and post-ischaemic in vivo PV-loop provided a suitable basis to assess whether supplementing FO doses achievable within a typical Western-style human diet modified haemodynamic function after ischaemia.

Post-ischaemic stroke volume and cardiac output was decreased by ~15 - 20% and ~25 - 30% respectively across all groups compared to baseline measures. However, supplementation of dietary achievable FO doses maintained PV-loop integrity leading to attenuated declines in left ventricular ejection fraction, despite the decrease in stroke volume. Ejection fraction has been demonstrated to be increased as a result of enhanced ventricular filling in the resting in vivo marmoset heart following supra-therapeutical FO doses (McLennan et al., 1992a). This is the first study to show preservation of ejection fraction following ischaemia while using dietary achievable FO doses. Diastolic function was impeded in all groups indicated by increases in the relaxation constant Tau and the maximal rate of relaxation (dP/dt_{min}). The myocardium commonly becomes stiff and slower to or less able to relax during and following ischaemia resulting in impaired diastolic filling (Varma et al., 2003). Nevertheless, this study demonstrated post-ischaemic dP/dt_{min} to be ~1000mmHg.sec^{-1} faster in the FO supplemented animals compared to the Control group. This reflects observations made in an ex vivo study which also demonstrated supra-therapeutical doses of FO to improve maximum rate of ventricular relaxation (Abdukeyum et al., 2008). Therefore, it is likely that this in part contributed to the maintenance of left ventricular ejection fraction observed in FO supplemented animals of this study.

The lower stroke volume and cardiac output were likely related to the reductions in the maximal rate of pressure development (dP/dt_{max}) observed across all groups. This indicates the left ventricle was in a weakened systolic inotropic state following ischaemia (Kass et al., 1989). However, this study demonstrated that dP/dt_{max} was ~700mmHg.sec^{-1} faster in the FO supplemented animals compared to the Control group.
In addition, although not statistically significant, end-systolic pressure in FO supplemented animals was ~10-15mmHg higher during post-ischaemic measurements. These findings reflect a study which demonstrated supra-therapeutical doses of FO to preserve *in vivo* systolic heart function twenty four hours after permanent ligation of the LAD in the rat (Lescano de Souza Junior *et al.*, 2017). Furthermore, findings from this study support an *ex vivo* study which demonstrated that during reperfusion left ventricular developed pressure (equivalent to end-systolic pressure) is increased in hearts supplemented supra-therapeutical doses of FO (Abdukeyum *et al.*, 2008). As a result of these changes, the time for ejection would be greater, allowing stroke volume to be maintained and stopping rightward shift of the PV-loop. These changes were similar to an *in vivo* sheep heart model assessing the effect of ischaemic pre-conditioning on global ischaemia (Tanoue *et al.*, 2002). The PV-loops of Control groups in both studies shifted downwards and rightwards compared to baseline. The FO supplemented group in this study and the ischaemic pre-conditioned group in the study conducted by Tanoue *et al.*, 2002 did not show any rightward shifting compared to their baseline loops. The remarkable resemblance of the effects of human dietary achievable FO doses on cardiac function to the effects demonstrated with ischaemic pre-conditioning suggests they share a similar mechanism of action to provide cardio-protection. Such changes indicate that post-ischaemic left ventricular contractile function was improved independent of the Frank-Starling law. The Frank-Starling compensation observed in the Control group during post-ischaemic function would be causing increased contractility to maintain stroke volume. Therefore, systolic contractile dysfunction in the Control group may have been masked by the powerful Frank-Starling compensation, meaning the differences between groups could become more evident over longer time frames when the compensation diminishes.

### 6.5.3 Conclusions

Ischaemia and reperfusion induced cardiac dysfunction characterised by reduced rate pressure product during ischaemia and reduced post-ischaemic ejection fraction, was largely prevented by prior feeding with FO. The moderate dose was more effective than the low dose but many effects were observed with both indicating that the addition of FO doses achievable within a typical Western-style human diet can protect against ischaemia and reperfusion induced cardiac dysfunction. Until now this protective action
of FO was only demonstrated while using therapeutic and supra-therapeutic doses not nutritionally relevant to the human diet. Furthermore, the cardio-protective actions demonstrated mimicked both the potent ‘early’ phase and the long lasting ‘late’ phase protection previously only attributable to ischaemic pre-conditioning. The modification of ischaemic and post-ischaemic cardiac function described in this study, together with the demonstration of slowed resting heart rate and reduced arrhythmia generation, provides novel evidence to support increasing fish or FO intake for nutritional pre-conditioning of the heart.
CHAPTER 7

Discussion and conclusions
Pharmacological and therapeutic strategies aimed at reducing and treating cardiovascular disease are important to improve patient quality of life. The majority of studies to advance cardiovascular outcomes are pharmacologically based. Notwithstanding the success such treatment has on decreasing the impact of cardiovascular disease on healthcare systems, research targeting modification of lifestyle related risk factors, such as diet and exercise (Eckel et al., 2014), are just as essential in reducing mortality and morbidity rates and underpinning the prevention of disease burden. Regular fish intake is consistently associated with a cardio-protective action in human population based studies (Bang & Dyerberg, 1972; Burr et al., 1989; Hirai et al., 1980; Kagawa et al., 1982; Kromhout et al., 1985). Now for the first time, this thesis provides physiological evidence of heart rate slowing, reduced arrhythmia generation and attenuated post-ischaemic contractile dysfunction, all of which would contribute to reducing the risk of cardiovascular disease (Fox et al., 2008; Gorenek et al., 2015). Importantly, the modification of in vivo heart function was attributable to supplementing FO doses achievable within a typical Western-style human diet (high SFA and n-6 PUFA). This highlights that in the typical Western-style diet which contains very small amounts of LC n-3 PUFA (Micha et al., 2014), as also could be implied from global erythrocyte EPA+DHA levels (Stark et al., 2016), and high amounts of SFA and n-6 PUFA, the cardiovascular benefits of FO can be produced solely through increasing LC n-3 PUFA intake without the need to concurrently reduce n-6 PUFA intake as previously postulated by Simopoulos, A.P., 2008, Lands, E.M., 2003 & Rupp et al., 2004. As such, this research emphasises the importance of nutritional interventions aimed at increasing fish or FO intake in the Western-diet for optimal cardiac function and provides further support for the recommendation to establish a dietary reference intake (Flock et al., 2013).

7.1.1 Direct effect of dietary achievable fish oil doses on heart function

The heart is remarkably adaptive to both acute and chronic stimuli. This research demonstrated that the preferential incorporation of DHA into myocardial phospholipids influences both chronotropic and inotropic heart function. Specifically, the physiological changes observed included i) modification of heart rate independent of
well-defined autonomic tone changes, suggestive of an intrinsic mechanism, ii) less severe and reduced incidence of arrhythmias (including fatal) during simulated acute coronary syndrome and iii) improved left ventricular post-ischaemic contractile function.

7.1.1a Heart rate

In each cardiac cycle, the beat to beat interval is controlled, along with stroke volume and together they are responsible for cardiac output. Control of cardiac output is essential as it contributes to maintaining mean arterial pressure for the adequate perfusion of organs and tissues in the body. This research demonstrated that supplementing FO to a typical Western-style diet slows resting heart rate, in a similar fashion to exercise training (Blomqvist & Saltin, 1983; D'Souza et al., 2015b). Previously heart rate slowing had only been demonstrated in animals using FO doses in the therapeutic or supra-therapeutical range (McLennan, 2014). The dietary achievable doses used in this series of projects provides a compelling physiological link to explain the consistent observation of FO supplementation being associated with lower heart rates in humans (Dallongeville et al., 2003; Geelen et al., 2005; Grimsgaard et al., 1998; Mozaffarian. et al., 2005). Increased resting heart rate in patient and healthy populations is associated with a greater risk of CVD and all-cause mortality (Aune et al., 2017; Cullington et al., 2012; Hartaigh et al., 2014; McAlister, 2009; Zhang & Zhang, 2009). As such, the cardio-protective action of regular fish consumption is likely to be associated with its ability to slow resting heart rate when physiological strain is absent.

In contrast to FO-induced slowing during rest, heart rate (initially depressed by ischaemia) rapidly recovered in FO supplemented animals. This suggests that during physiological strain (ischemia), the heart is protected against a loss of mechanical function and as a consequence sustained reduction in heart rate is attenuated. Support for this hypothesis can be found in previous studies which have demonstrated FO to slow resting heart rate but not impair peak heart rate during exercise in humans (Buckley et al., 2009; Macartney et al., 2014; Peoples et al., 2008). Interestingly, beta-blockers, commonly used in the treatment of hypertension and heart failure (Lechat et al., 1998), inhibit peak heart rate responses by up to 30beats.min⁻¹, and slow resting heart rates by up to 10beats.min⁻¹ through blocking sympathetic input to the heart.
(Epstein et al., 1965). Heart rate reserve (the difference between resting and peak heart rate) is reduced by these changes, which can limit exercise tolerance in patients prescribed beta-blockers (Hradec et al., 2013; Piña et al., 2003). This highlights a potential clinical role for FO to modulate heart rate without compromising heart rate reserve and exercise tolerance.

Resting heart rate was slowed without any well-defined changes to autonomic tone, suggestive of an intrinsic mechanism. Slowing of resting heart rate is commonly attributed to increased vagal tone, particularly in studies investigating exercise training induced bradycardia (Billman et al., 2015; Coote & White, 2015). It is likely that vagal tone contributes to slowing of resting heart rate but the exclusive action of increased vagal tone is to slow heart rate. In addition to heart rate slowing, supplementation of FO was shown to modulate heart rate and allow it to increase during ischaemia. Exercise training can also cause resting bradycardia without compromising peak heart rate (Blomqvist & Saltin, 1983; Scheuer & Tipton, 1977). Furthermore, both exercise training and dietary FO slow resting heart rate of hearts studied ex vivo (Abdukeyum et al., 2008; D'Souza et al., 2014; Pepe & McLennan, 2002) and even in cardiac transplant patients (Harris et al., 2004), none of which would receive vagal stimulation. Given the findings from the current project and what is observed in exercise training induced slowing of resting heart rate, it is reasonable to suggest that both stimuli modify heart rate intrinsically, as was originally hypothesised for exercise training decades ago (Sutton et al., 1967).

It is well established that the sino-atrial node is responsible for intrinsic pace-setting of the rhythmic electrical excitation patterns of the heart (Mangoni & Nargeot, 2008). Originally it was theorised that sarcolemma membrane ion channels were responsible for the ignition of action potentials in the sino-atrial node (Weidmann, 1955). However, it has now been postulated that the control of sino-atrial node firing is tightly regulated by two coupled processes (Yaniv et al., 2015). The first being, the hyperpolarisation activated sarcolemma cation current, commonly referred to as the ‘funny current’ and the second being, the spontaneous release of calcium from the sarcoplasmic reticulum, otherwise referred to as the ‘calcium clock’ (Maltsev & Lakatta, 2009; Yaniv et al., 2015). It has been hypothesised that the funny current is responsible for stabilizing the beat to beat rate, whereas the calcium clock is the main source of beat to beat
variability, independent of autonomic tone (Zaniboni et al., 2014). Supplementation of FO has been demonstrated to slow the funny current in rabbit sino-atrial cells (Verkerk et al., 2009), in a similar fashion to exercise training induced electrical remodelling of the sino-atrial node (Boyett et al., 2017a; D'Souza et al., 2014; D'Souza et al., 2015a). It has also been demonstrated that LC n-3 PUFA can modulate other ion channel activities including voltage-gated Na\(^+\) (Xiao et al., 2005), K\(^+\) (Farag et al., 2016), Ca\(^{2+}\) channels (Pepe & McLennan, 2002) and the spontaneous release of Ca\(^{2+}\) from the sarcoplasmic reticulum which is closely linked to the calcium clock (Siddiqi et al., 2008). Analysis of cellular mechanisms responsible for the heart rate slowing was beyond the scope of the experiments described in this thesis. However, the cellular evidence in conjunction with the physiological findings presented provides support for the hypothesis that LC n-3 PUFA mediated slowing of heart rate is closely linked to intrinsic beat rate modification at the sino-atrial node.

7.1.1b Arrhythmias

Ischaemic episodes as short as five minutes in the myocardium can lead to electrical instability and arrhythmias even in the absence of cell necrosis and reperfusion can exacerbate the instability (Bolli, 1988). This project demonstrated a potent anti-arrhythmic action of dietary achievable FO doses during ischaemia and reperfusion, previously only described using therapeutic and supra-therapeutic doses (Abdukeyum et al., 2008; Abeywardena et al., 2016; Hartog et al., 1987; Hock et al., 1990; McLennan et al., 1988; McLennan et al., 1993; McLennan et al., 2007). This finding provides strong evidence, for the first time, to justify how increasing dietary FO intake in a typical Western-style diet containing high amounts of SFA and n-6 PUFA leads to the prevention of sudden (arrhythmic) cardiac death (Burr et al., 1989; Valagussa et al., 1999) and addresses the confusion regarding the minimum dose required (Reiffel & McDonald, 2006).

The protection afforded by FO supplementation can be rationalised by comparing to ischaemic pre-conditioning of the myocardium, as both have been demonstrated to provide an effective anti-arrhythmic action (Abdukeyum et al., 2008). The cardio-protective actions of ischaemic pre-conditioning are closely linked to alteration of intracellular signalling cascades, particularly Ca\(^{2+}\) regulation (Das & Das, 2008; Otani, 2008; Yellon & Downey, 2003). Abnormalities in Ca\(^{2+}\) regulation can provide an
Intracellular Ca\(^{2+}\) overload is associated with spontaneous depolarisation of non-pacemaking cells and the generation of arrhythmias (Brooks et al., 1995; Rubart & Zipes, 2005), including the initiation of VT, VF and triggered activity (Antzelevitch & Burashnikov, 2011; Undrovinas et al., 1992). It was hypothesised that the slowing of heart rate was likely linked to modified Ca\(^{2+}\) regulation in pacemaker cells of the sino-atrial node. Thus, it is plausible that the anti-arrhythmic action of myocardial DHA incorporation is also attributable to stabilisation of Ca\(^{2+}\) movement within non-pacemaking myocytes.

Paradoxically, increased incorporation of DHA into myocardial phospholipids would increase the peroxidation index, potentially leading to the production of arrhythmogenic oxidative products. However, when the peroxidation index is increased by phospholipid DHA incorporation persistent basal lipid oxidation occurs, as a consequence, antioxidant enzymes such as mitochondrial superoxide dismutase (MnSOD) are continually upregulated (Abdukeyum et al., 2016). This observation is consistent with the up-regulation of anti-oxidant systems that occurs with the regular oxidative stress of exercise training (Hoshida et al., 2002) or ischaemic pre-conditioning of the myocardium (Otani, 2008; Yellon & Downey, 2003). Furthermore, increased ADP sensitivity and reactive oxygen species generation are physiological consequences of DHA incorporation in skeletal muscle mitochondria, yet there is no evidence of oxidative damage (Herbst et al. 2014). It has also been demonstrated that cyclooxygenase and lipoxygenase enzymes are up-regulated in the myocardium during inflammatory events such as ischaemia and reperfusion (Jenkins et al., 2009). Enzymatic oxygenation of membrane bound EPA and DHA, via cyclooxygenases and lipoxygenases, leads to the formation of a range of unique PUFA-derived bioactive compounds involved in the resolution of inflammation, including: resolvins; neuroprostanes; maresins; protectins; and lipoxins (Serhan & Petasis, 2011). Furthermore, non-enzymatically generated endogenous lipid mediators have been shown to have the same anti-arrhythmic properties as DHA, suggesting they are also directly involved in the process (Roy et al., 2016; Roy et al., 2015). Although cellular mechanisms were not investigated in this research, the anti-arrhythmic action of dietary achievable FO doses described is almost certainly linked to intrinsic changes of Ca\(^{2+}\).
regulation, anti-oxidant systems and the resolution of inflammation and in many ways holds similarities to ischaemic preconditioning and exercise preconditioning.

7.1.1c Contractile performance

In addition to heart rate and rhythm, inotropy and lusitropy are critical for maintaining cardiac output and are therefore tightly controlled within the working physiological system. This project has provided evidence, for the first time, that dietary achievable FO doses attenuate post-ischaemic myocardial contractile dysfunction, previously described using therapeutic doses (Abdukeyum et al., 2008; Pepe & McLennan, 2002) and in a similar manner to ischaemic pre-conditioning (Efstathiou et al., 2001; Lasley et al., 1993; Przyklenk & Kloner, 1998). Improved post-ischaemic myocardial contractile performance provides evidence that helps link the observation of reduced heart failure incidence amongst populations that regularly consume fish (Levitan et al., 2010).

The modulation of myocardial post-ischaemic contractile dysfunction is remarkably similar to the contractile changes FO supplementation produces in skeletal muscle, particularly muscles susceptible to fatigue (Henry et al., 2015) and muscles acutely subjected to hypoxia (Peoples & McLennan, 2017). A common mechanism of action hypothesised to account for the physiological actions of FO on contractile performance is enhanced oxygen efficiency across organs, including the healthy or failing rat heart (McLennan et al., 2012; Pepe & McLennan, 2007), skeletal muscle (Peoples & McLennan, 2010, 2014, 2017) and confirmed as lower whole body oxygen consumption during exercise in healthy humans (Peoples et al., 2008). This hypothesis is strengthened by evidence that when rats are supplemented DHA they have increased chances of surviving asphyxia-induced cardiac arrest, suggestive of enhanced myocardial oxygen efficiency (Kim et al., 2016). Attributing enhanced contractile oxygen efficiency to increased phospholipid DHA concentration from FO is reinforced by environmental adaptations found in comparative physiology. Phospholipid DHA incorporation of excitable tissue increases in response to chronic hypoxia exposure in rats, presumably as a protective response (Jezkova et al., 2002). Equally, DHA is concentrated in the swimming muscles of the deep diving seal where contractile function is maintained in a low oxygen environment during breath hold periods of exceeding 12 minutes (Trumble & Kanatous, 2012). Likewise, phospholipid n-3 PUFA concentrations are high in migrating birds which are exposed to high altitude for
prolonged periods of time while maintaining wing contractile performance (McWilliams et al., 2004). Notably, DHA concentrations were shown to be highest in muscles with high proportions of fast oxidative glycolytic muscle fibres responsible for powerful yet sustained contraction in this thesis. Fast oxidative glycolytic muscles contain an extensive network of sarcoplasmic reticulum and mitochondria (Berchtold et al., 2000). Double packing of the mitochondrial membrane has been proposed as an explanation for such high LC n-3 PUFA concentrations (Infante et al., 2001). This suggests that phospholipid incorporation of DHA from FO is integral to maintaining contractile performance, particularly during conditions where oxygen availability is challenged.

Regulation of Ca\(^{2+}\) recurs (already discussed in reference to heart rate and arrhythmia) as a unifying cellular mechanism to help justify the hypothesis that enhanced oxygen efficiency allows maintained contractile performance. Abnormalities in excitation-contraction coupling as a result of Ca\(^{2+}\) regulation have been shown to cause inefficient oxygen use leading to contractile dysfunction (Brooks et al., 1995; Kristian & Siesjo, 1998; Pepe & McLennan, 2002; Rubart & Zipes, 2005). In fact, it has been shown that the attenuation of post-ischaemic contractile dysfunction associated with ischaemic preconditioning is partly due to optimised Ca\(^{2+}\) regulation (Das & Das, 2008). Research has identified that LC n-3 PUFA alters both sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) mediated Ca\(^{2+}\) reuptake and ryanodine receptor (RyR) mediated Ca\(^{2+}\) release in isolated ventricular and atrial myocytes (Honen et al., 2003; Negretti et al., 2000; O'Neill et al., 2002). Of physiological consequence, the maximal contractile rate of developed tension and relaxation in skeletal muscle has been shown to be partially protected during acute hypoxia in rats supplemented FO (Peoples & McLennan, 2017). Furthermore, from within a fatigued state, hind-limbs of animals fed FO demonstrated an augmented response to a caffeine bolus facilitates sarcoplasmic reticulum Ca\(^{2+}\) release (Fryer & Neering, 1989). For the reasons discussed, myocardial phospholipid DHA incorporation, caused by the provision of dietary achievable FO doses is essential for attenuating post-ischaemic contractile dysfunction demonstrated in this project.

7.1.1d Linking the effects of fish oil in the heart to phospholipid composition

The dose-dependent and preferential incorporation of DHA into cardiac and skeletal muscle phospholipids when made available via the diet highlights the important role LC
n-3 PUFA have in the healthy physiological functioning of excitable tissue, particularly the heart. Historically, animal studies supplementing FO have used doses which are mostly unachievable in the equivalent human diet, making extrapolation of the physiological findings to human health ambiguous. Dose response studies (Slee et al., 2010), covering the range used in the current research show that previous supra-therapeutical doses of FO maximally increased myocardial DHA phospholipid fatty acid composition (Abdukeyum et al., 2008; Hock et al., 1990); the ModFO diet contributed 1/5 these doses or less, yet myocardial phospholipid DHA concentration was close to maximal. Remarkably, the LowFO diet, which supplemented FO at roughly 1/20th the dose of those previous studies, still resulted in about half maximal myocardial phospholipid DHA incorporation. Thus, the dietary equivalence of the doses used in this study (Human equivalence: LowFO ≈ EPA+DHA 570mg/d or ≈ 2 salmon [100g] serve/week; ModFO ≈ EPA+DHA 2.3g/d or ≈ 6 capsules per/day) allows translation of the cardiac physiological effects described to the reduced risk of cardiovascular disease consistently described in populations that regularly consume fish (Bang & Dyerberg, 1972; Burr et al., 1989; Hirai et al., 1980; Kagawa et al., 1982; Kromhout et al., 1985) ranging up to supplemental intake (Valagussa et al., 1999). Furthermore, phospholipid DHA incorporation and cardiac physiology was modified despite n-6 PUFA and SFA concentrations being held constant in the diet, in-line with relative concentrations consumed in a typical Western-style diet (Broadhurst et al., 1998; Simopoulos, 1991; USDA, 2016). This supports and strengthens the notion that the addition of small amounts of FO to the diet exerts a protective effect directly on the heart (McLennan, 2014), similar to exercise training and ischaemic pre-conditioning. Therefore, nutritional interventions aiming to increase fish or FO intake could prove effective in improving heart function and reducing the risk of cardiovascular disease in high risk sedentary populations unable to exercise. The effectiveness of the LowFO diet suggests it is near threshold, thus supporting the suggestion that a dietary reference intake may be derived on the basis of healthy heart function (Flock et al., 2013).

7.1.2 Future directions

This series of experiments provided physiological evidence of FO modifying intrinsic heart rate and cardiac inotropic state. There was no well-defined indication that autonomic tone was altered to account for these changes. Modified autonomic input to
the heart provides a justification for the slowing of resting heart rate observed, however the contradicting evidence of stabilised sinus rhythm and faster recovery of heart rate during ischaemia alludes to the possibility that other processes were involved. As such, optimisation of cardiac function observed with FO feeding is likely related to an intrinsic action on the myocardium and pacemaker regions of the heart. Studies in heart transplant patients (Harris et al., 2004) and in ex vivo animal heart preparations provide further support to this hypothesis (Abdukeyum et al., 2008; Pepe & McLennan, 1996). However, the surgical and methodological process involved in setting up an ex vivo heart preparation, including the choice of perfusate or whole blood can significantly alter baseline cardiac function. It is even possible that the brief periods of ischaemia during setup may actually impart an ischaemic pre-conditioning like effect on the heart. Such investigations would benefit from less invasive surgical protocols such as echocardiography tracking of cardiac haemodynamic function. Therefore, further investigation of the influence of FO on intrinsic cardiac function is warranted using a pithed in vivo animal preparation to remove the influence of the autonomic nervous system while maintaining the heart in a working physiological system.

Investigation of the mechanisms responsible for the physiological outcomes described was out of the scope of the current research. As such, cellular studies investigating sino-atrial node spontaneous activity, sarcoplasmic reticulum Ca$^{2+}$ regulation, anti-oxidant up-regulation and resolution processes are warranted. Studies aiming to investigate such outcomes would achieve more physiologically relevant outcomes through using cells obtained directly from animal’s fed dietary achievable FO doses, rather than using acute application of LC n-3 PUFA to the cell culture medium. This type of cellular experiment would allow a more direct translation of any observed changes to be specifically attributed to DHA phospholipid concentrations and the effects of diet as opposed to the effects of circulating DHA, solvents or non-specific detergent actions exposed in some studies when added acutely to the cell culture medium (refer to McLennan, P.L., 2014).

Finally, the experiments completed in this research demonstrated physiological effects of dietary achievable FO doses on cardiac function while using an appropriate in vivo animal model and a background diet relevant to the typical Western-style diet. Skeletal muscle contractile function has been investigated in the physiologically appropriate
auto-perfused rat hind-limb model using both dietary achievable (Henry et al., 2015) and supra-therapeutical FO doses (Peoples & McLennan, 2010, 2014, 2017); however never on the basis of muscle fibre content. The current research identified a strong association between increased DHA phospholipid concentrations in skeletal muscle with a high proportion of fast oxidative glycolytic fibres. Therefore, further investigation is warranted to determine if FO modifies contractile function uniformly across striated muscles (including heart), regardless of fibre type proportion, or is more effective in the presence of a high proportion of a particular fibre type.

7.1.3 Application of the findings and final conclusions: The importance of nutritional pre-conditioning with increased fish or fish oil intake

Cardiovascular disease persists as a principal contributing factor to worldwide morbidity and mortality. According to recent data released by the World Health Organisation almost one third of all deaths worldwide in 2008 were related to cardiovascular disease. Furthermore, the impact of cardiovascular disease is expected to increase substantially as the proportion of the aged population increases (Heidenreich et al., 2011). The physiological outcomes described in the current thesis are similar to the cardio-protective effects commonly observed with exercise training, remote and ischaemic pre-conditioning. The unpredictable onset of ischaemic/reperfusion injury and invasive methods required to implement remote or ischaemic pre-conditioning make it impractical in clinical practice (Iliodromitis et al., 2007). Additionally, the lack of specificity of potential therapeutic candidates drawn from ischaemic pre-conditioning further reduces the viability of such an approach (Otani, 2008). The shortfalls of implementing ischaemic pre-conditioning to prevent arrhythmias and cardiac contractile dysfunction induced by ischaemia and reperfusion injury could be overcome via ‘nutritional pre-conditioning’ with increased dietary fish or FO intake (Abdukeyum et al., 2008). Increased physical activity (exercise training) is well-established to reduce the risk of cardiovascular disease (Mozaffarian et al., 2016; Thompson et al., 2003). The evidence from this research suggests that nutritional pre-conditioning with FO offers cardio-protection in a similar manner. Importantly, a habitual dietary approach to prevent cardiovascular disease, like increased physical activity levels, overcomes the need to predict the onset of ischaemia and reperfusion. Pharmacological therapies are constrained to populations which are identified to be at risk or have already suffered a
form of cardiovascular disease, this makes dietary and exercise modifications a more effective preventative approach as they can be applied across vast populations with no prior indication of cardiovascular disease.

It may be argued that the studies conducted in the current thesis were completed in rats, making the findings less applicable to human health. However, one of the main design issues of current trials in humans, which have often been unable to replicate the epidemiological findings, is the failure to measure or control for how much LC n-3 PUFA an individual is consuming within and outside of the trial. A properly designed randomised controlled trial relies upon absolute separation of the treated and control group. However, it has been highlighted that in investigations of FO supplements there is often considerable overlap of LC n-3 PUFA red blood cell phospholipid concentrations between study groups (James et al., 2014), yet trials often conclude that FO is ineffective, when in fact the design is flawed and sound conclusions can not be made. For example, the ORIGIN trial (Bosch et al., 2012) showed equal median EPA+DHA intake in control and treated groups at baseline but the interquartile dietary range (40-568mg/d) extended beyond the 250mg EPA+DHA which has been identified in epidemiology and randomized trials for maximal reduction in the risk of cardiovascular death (Kris-Etherton et al., 2009; Mozaffarian et al., 2005a). Using an animal model in the current studies allowed for precise FO dosing and definitive separation of dietary groups to identify physiologically plausible mechanisms of cardio-protection. Thus, the current study was more akin to cohort and other observational studies that separate participants into quintiles or other percentiles according to estimated dietary intake or erythrocyte LC n-3 PUFA phospholipid concentrations (Siscovick et al., 1995). Human observational studies commonly describe differences in relative risk between those who eat no fish and those who regularly eat some fish or much fish (Kromhout et al., 1985; Levitan et al., 2010; Mozaffarian et al., 2005a; Mozaffarian et al., 2005b) making them like the control group in comparison to dietary FO groups.

Identification and correction of dietary deficiencies has led to considerable public health benefits in the prevention and treatment of a range of health problems (Blot et al., 1993; Holick, 2007). The applications of nutritional pre-conditioning with FO in the typical Western-style diet which contains very low amounts of LC n-3 PUFA (Micha et al.,
2014), as also could be implied from global erythrocyte EPA+DHA levels (Stark et al., 2016), far outreach those of ischaemic pre-conditioning. In fact, extensive savings in European health budgets have been predicted if dietary FO intake can be increased (Shanahan, 2016). Despite this, there is evidence to suggest that there is insufficient nutritional education amongst current cardiovascular specialists, leading to misinformation and reluctance to provide patients with dietary advice (Devries et al., 2017). Confusion surrounding the cardiovascular effects of dietary FO (Nestel et al., 2015) likely exacerbates this problem. This research provides further evidence to demonstrate FO as a safe and effective tactic for improving intrinsic heart health and providing protection from cardiovascular disease and stress, with a clear translation path from animal to human health due to the nutritional relevance and physiologically appropriate in vivo model used. As such, observations made in this study could form part of the evidence used to inform and set dietary guidelines for fish intake (Flock et al., 2013), offering real potential to improve heart health outcomes.
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