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**UNIVERSITY OF SOUTHAMPTON**

FACULTY OF MEDICINE

Human Development and Health

**The influence of early life vitamin D deficiency on  
offspring skeletal muscle development, structure  
and function**

by

**Lisa Ellen Jones**

Thesis for the degree of Doctor of Philosophy

May 2018



# UNIVERSITY OF SOUTHAMPTON

## ABSTRACT

FACULTY OF MEDICINE

Human Development and Health

Doctor of Philosophy

### **THE INFLUENCE OF EARLY LIFE VITAMIN D DEFICIENCY ON OFFSPRING SKELETAL MUSCLE DEVELOPMENT, STRUCTURE AND FUNCTION.**

by Lisa Ellen Jones

Vitamin D deficiency (VDD) is highly prevalent in pregnant women, and may impair early life skeletal muscle development, and consequently influence muscle structure and function in adulthood and its rate of decline with aging. The profound decline in muscle mass and strength with age (sarcopenia) is associated with an increased risk of morbidity and mortality. Vitamin D status is determined by dietary intake, sunlight exposure and a deficiency in vitamin D is associated with obesity in humans. There is very little research on how VDD during pregnancy impacts the offspring's skeletal muscle development, structure and function across the life course. Thus, this thesis aimed to test the hypothesis that pregnancy VDD will impair offspring skeletal muscle development, structure and function across its life course, and this was investigated in animal models of obesity and dietary specific VDD.

In the first study, female C57BL/6J mice were fed a control (C; 7% kcal fat) or high-fat obesogenic (HF; 45% kcal fat) diet 6 weeks prior to mating and throughout pregnancy until weaning. Offspring were fed the C or HF diet postnatally. At 30 weeks of age, offspring isometric muscle contractile parameters, myofibre structure and mRNA levels of genes associated with muscle growth, contraction and insulin signalling were evaluated. In the second study, female C57BL/6J mice were fed a control (C; 1 IU/g vitamin D<sub>3</sub>) or VDD (0 IU/g vitamin D<sub>3</sub>) diet from 6 weeks prior to mating until weaning. All offspring were then weaned onto the C diet. At 15-weeks, offspring isometric muscle contractile force, grip strength, overall strength and open-field activity was assessed. In the third study, Welsh mountain ewes were fed a control (C, 2000 IU/kg vitamin D<sub>3</sub>), or a vitamin D deficient (VDD, 0 IU/kg vitamin D<sub>3</sub>) diet 17 days prior to conception until 127 days of gestation. In the late-gestation fetus, skeletal muscle radiolabelled glucose uptake, myofibre structure and mRNA levels of insulin signalling genes were quantified.

A pre-weaning obesogenic diet was associated with some effects on muscle contractile peak force and potential fatigability in the adult offspring, and vitamin D homeostasis was affected by maternal obesity. A gestational VDD diet did not alter the skeletal muscle peak force, grip strength or overall strength in the young adult mouse, or glucose uptake in the fetal muscle. However, activity levels were reduced in the VDD mouse adult offspring. Fetal muscle structure did not change following a VDD diet, but some changes to skeletal muscle mass were observed in adulthood in the VDD mouse model.

Overall, the results suggest that VDD during pregnancy is not as detrimental to the offspring's skeletal muscle as hypothesised at the outset of this thesis. However, reduction in activity levels following a prenatal VDD diet may predispose the offspring to health complications such as obesity and metabolic disorders in later life. Considering there are some changes in muscle mass of offspring following a prenatal VDD diet, determining any myofibre structural changes is required. These data are a novel addition to a small pre-existing body of evidence for a role of pregnancy vitamin D on later life skeletal muscle function, and pave the way for future analysis with the aim to understand how the underlying mechanisms in the offspring muscle are influenced following a VDD diet during pregnancy.



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## **Academic Thesis: Declaration of Authorship**

I, Lisa Ellen Jones,

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

‘The influence of early life vitamin d deficiency on offspring skeletal muscle development, structure and function’.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Signed: .....

Date: .....



## **Acknowledgements**

I'd firstly like to thank my primary supervisor Dr. Lucy Green for agreeing to have me as her PhD student, for her support and guidance throughout the project, and for encouraging me to think about my data in different ways. It's all about the flow diagrams! Thank you also for giving me the opportunity to do a science communication internship to help me financially whilst writing up and to help me with my desired career path. I would also like to acknowledge the invaluable role of my other supervisors; Prof. Philip Newland, Dr Jane Cleal, Dr Kirsten Poore and Dr Emiliano Rustighi, who have helped me get to where I am today through their feedback and support.

I would like to say a big thank you to The Gerald Kerkut Trust and the University of Southampton for the financial support of this project. I am truly grateful for the additional support The Gerald Kerkut Trust gave me academically and personally during my final year in the lab, which enabled me to push my thesis further and set up the vitamin D deficient mouse study.

Thank you to all of the other individuals for their help and support throughout my PhD including Dr Felino Cagampang, Prof. Clive Osmond, Dr Hugh Thomas, Dr. Filip Markiewicz, the Histochemical Research Unit and the Biomedical Imaging Unit. Particular thanks to my two (now qualified doctors!) master students Dr Vicky Reay and Dr Sarah Al-Rawi. You were both a pleasure to supervise and your hard work is greatly appreciated. Thank you to the staff at the Biomedical Research Facility for looking after my many mice and giving them a lovely home. Many mice were sacrificed for this project, and so it would be wrong to not acknowledge their unwilling, but extremely valuable, contribution.

To my fellow lab friends Emma, Claire and Brogan! Thank you for being there for me during the tough times and the good times. Thank you for listening to me rant and cry, and cry some more. Thank you for biscuit Fridays, hour-long tea breaks (Lucy I hope you aren't reading this!) and for making those longer lab days more bearable!

A massive appreciation for the 4 C's close to my heart: cookies, chocolate, caffeine and CrossFit. Thank you to all the Crossfit Goddesses! Especially Naomi, Fran and Lauren for being my rocks. For the deep chats, the fun adventures and helping me realise the wonders of being out in nature. So much love for you girls.

### *Acknowledgements*

Thank you to my housemate Sarah for making me feel at home, cooking me dinner, making me cake and cups of tea, and for talking things through when the thesis life got too much. I'd also like to thank all of my other friends who have supported me over the last four or so years.

An incredibly heartfelt thank you to my mentor Dr Jude Holloway. Jude is the one who picked me up and took me under her wing when the PhD got tough. She comforted, encouraged, and inspired me during my major PhD 'slump'. There are not enough words to truly express how grateful I am for her help and kindness. Jude –the thesis is in, I got to the top of Everest!

Lastly I would like to thank my family. Thank you to my sister Anna for her encouragement, and thank you to my Mum and Dad for their continual support for my many, many years as a student. They are of course the developmental origins of this thesis, and so without them this literally would not have been possible. I owe them so much, thank you.

## Definitions and Abbreviations

Abbreviation	Definition
<b>1,25(OH)<sub>2</sub>D</b>	1,25-dihydroxyvitamin D
<b>25(OH)D</b>	25-hydroxyvitamin D
<b>Acta</b>	Actin alpha
<b>ADP</b>	Adenosine diphosphate
<b>Akt</b>	Serine threonine-protein kinase
<b>ANOVA</b>	Analysis of variance
<b>ATP</b>	Adenosine triphosphate
<b>AUC</b>	Area under the curve
<b>BMI</b>	Body mass index
<b>BSA</b>	Bovine serum albumin
<b>C</b>	Control
<b>Ca</b>	Calcium
<b>cDNA</b>	Complementary deoxyribonucleic acid
<b>CSA</b>	Cross-sectional area
<b>CV</b>	Coefficient of variation
<b>CYP</b>	Cytochrome P450
<b>DBD</b>	DNA-binding domain
<b>DBP</b>	Vitamin D binding protein
<b>dGA</b>	Day of gestation
<b>DNA</b>	Deoxyribonucleic acid
<b>DOHaD</b>	Developmental Origins of Health and Disease
<b>EDL</b>	Extensor digitorum longus
<b>FGR</b>	Fetal growth restriction
<b>Fig.</b>	Figure
<b>FOV</b>	Field of view
<b>GH</b>	Growth hormone
<b>GLUT</b>	Glucose transporter
<b>HF</b>	High-fat
<b>HKG</b>	Housekeeper gene
<b>HRT</b>	Half-relaxation time
<b>IGF</b>	Insulin-like growth factor
<b>Igf1r</b>	Insulin-like growth factor 1 receptor
<b>IGFBP</b>	Insulin-like growth factor binding protein
<b>InsR</b>	Insulin receptor
<b>IPGTT</b>	Intraperitoneal glucose tolerance test
<b>IRS-1</b>	Insulin receptor substrate 1
<b>IU</b>	International units
<b>LBD</b>	Ligand-binding domain
<b>LC-MS</b>	Liquid chromatography tandem mass spectrometry
<b>LOQ</b>	Limit of quantification
<b>m.</b>	Muscle

*Definitions and Abbreviations*

<b>MHC</b>	Myosin heavy chain
<b>mRNA</b>	Messenger ribonucleic acid
<b>MyoD1</b>	Myogenic differentiation 1
<b>MYOG</b>	Myogenin
<b>NEC</b>	No enzyme control
<b>NTC</b>	No template control
<b>ORO</b>	Oil red O
<b>PARs</b>	Predictive adaptive responses
<b>PBS</b>	Phosphate-buffered saline
<b>PF</b>	Peak force
<b>PFA</b>	Paraformaldehyde
<b>Pi3K</b>	Phosphatidylinositol 3-kinase
<b>POST</b>	Postnatal
<b>PRE</b>	Prenatal
<b>PTH</b>	Parathyroid hormone
<b>RNA</b>	Ribonucleic acid
<b>ROI</b>	Region of interest
<b>RT</b>	Reverse transcription
<b>RXR</b>	Retinoid X receptor
<b>RyR</b>	Ryanodine receptor
<b>SEM</b>	Standard error of the mean
<b>SERCA</b>	Sarcoendoplasmic reticulum calcium ATPase
<b>SGCA</b>	A-sarcoglycan
<b>SR</b>	Sarcoplasmic reticulum
<b>TA</b>	Tibialis anterior
<b>Tnnt</b>	Troponin T
<b>TPT</b>	Time-to-peak tension
<b>TTN</b>	Titin
<b>UVB</b>	Ultraviolet B
<b>VDD</b>	Vitamin D deficiency
<b>VDR</b>	Vitamin D receptor
<b>VDRE</b>	Vitamin D response element

# Chapter 1: General introduction

## 1.1 Overview

Skeletal muscle health is important for an individual's metabolic function and strength in adult life. The risk of sarcopenia (loss of skeletal muscle mass and strength with age) and metabolic dysfunction both increase in later life and present many health consequences in terms of morbidity and mortality. Sarcopenia in itself is a major risk factor for falls and bone fractures in the elderly with considerable associated healthcare costs, and sarcopenia has also been linked to dysfunctional muscle metabolic function, including impaired glucose handling which is associated with type 2 diabetes.

It is well established that alterations in the postnatal environment (diet and lifestyle in adulthood) can have an impact on adult skeletal muscle function in terms of strength and glucose handling. Two key examples are dietary fat (and associated obesity) and vitamin D. Both are in the spotlight as potential 'drivers' of muscle function by virtue of the high prevalence of obesity and that vitamin D deficiency (VDD) is detected in many populations. Studies have found a positive correlation with adult vitamin D status and skeletal muscle mass, strength, performance and risk of falls. The observed range of mass and strength of individuals (Fig. 1.1) suggest that earlier life 'forces' are in action, and that this might also explain variable successes in adult lifestyle interventions (e.g. in diet or vitamin D) to improve muscle function or slow down the rate of muscle deterioration.

Research has suggested that the nutrient environment an individual is exposed to during pregnancy can influence skeletal muscle development and function in adult life. A newer concept is that this adult peak muscle mass may influence the decline with ageing, and therefore account for individual life course trajectories and responses to maternal lifestyle. Vitamin D deficiency and obesity (both highly prevalent in the population and known to impact pregnant women) are two potential driving factors that could lead to alterations in an individual's skeletal muscle development, and consequently influence the muscle's future trajectory and function. Such early life adaptive responses could alter an individual's receptivity to vitamin D or alter the effect of obesity in postnatal life.

Human studies have found an association between obesity and VDD, and so this thesis explores the effect of VDD and obesity in pregnancy on offspring skeletal muscle development and function. These findings may help contribute to the scientific basis upon which medical professionals offer advice on diet and lifestyle across the life course.

## **1.2 Aetiology of muscle disease**

### **1.2.1 Muscle diseases**

Glucose homeostasis is primarily maintained by three key insulin-sensitive tissues: muscle, liver and adipose tissue. Type 2 diabetes is a metabolic disorder characterised by insulin resistance and abnormally high blood glucose levels (hyperglycaemia). An early sign of developing type 2 diabetes is the resistance to insulin in skeletal muscle. Research shows that the muscle fibre composition in men and women with type 2 diabetes shifts towards a greater percentage of insulin-resistant fast-twitch (type II) fibres, with a reduction in insulin-sensitive slow-twitch (type I) fibres (Mårin *et al.* 1994). Skeletal muscle insulin resistance and type 2 diabetes has also been associated with sarcopenia (Sayer *et al.* 2005).

Sarcopenia is defined as the loss of skeletal muscle mass and strength with age and its prevalence is thought to range between 8.8% in women to 17.5% in old men (Morley *et al.* 2001). Sarcopenia is associated with increased risk of disease (metabolic dysfunction, risk of disability and falls) and associated healthcare costs, and increased mortality rate (Rantanen *et al.* 1999; Rantanen *et al.* 2000; Sayer *et al.* 2006). Risk factors for sarcopenia include age, gender, body composition, genetics and level of physical activity. Resistance exercise training has been found to be an effective intervention in slowing the loss of muscle mass and strength, and improving physical performance (Skelton *et al.* 1995; Vincent *et al.* 2002; Liu & Latham 2009). Studies also provide evidence that the maternal environment may increase the risk of type 2 diabetes in adulthood, associated with impaired muscle metabolic function (Hales *et al.* 1991), and also influences the rate of decline in muscle mass and strength, and therefore the risk of developing sarcopenia in older age (Sayer *et al.* 1998; Kuh 2002; Sayer *et al.* 2004), as discussed in subsequent sections.

## 1.2.2 Developmental Origins of Health and Disease Hypothesis

Epidemiological studies in human populations worldwide have found birth size is an important risk factor for developing various non-communicable diseases in adulthood. Low birth weight has been associated with an increased risk of developing cardiovascular disease. A study on British subjects found that low birth weight was associated with high systolic blood pressure (Barker *et al.* 1989a), a study on men in Hertfordshire observed an inverse relationship between birth weight and death rates from ischaemic heart disease (Barker *et al.* 1989b), and low birth weight was also found to increase the risk of coronary heart disease in men and women from the Hertfordshire cohort (Osmond *et al.* 1993). Type 2 diabetes (Hales *et al.* 1991) and osteoporosis (Cooper *et al.* 2001) have also been shown to be associated with low birth weight. These associations have led to the development of the Developmental Origins of Health and Disease (DOHaD) hypothesis, which proposes that the environment *in utero* affects long-term offspring health through physiological adaptations during pregnancy, which persist into adulthood.

During development there are critical windows of developmental plasticity, whereby the fetus changes its developmental trajectory in response to its environment at that time (Gluckman *et al.* 2005). It is thought that the fetus can adapt to the early environment for immediate benefit in order to increase its chances of survival, and predict the environment in postnatal life, these are termed predictive adaptive responses (PARs). If the developmental prediction of the postnatal environment is incorrect (i.e. there is a mismatch between the predicted and actual postnatal environment), the PAR may be detrimental and could lead to a higher risk of disease in later life (Gluckman *et al.* 2005). These principles could operate across a range of physiological systems, including skeletal muscle.

### 1.2.3 Maternal environment and skeletal muscle life course trajectory

Current concepts suggest that the intrauterine environment is important for muscle growth and development in determining adult peak muscle mass and strength, and its decline with ageing (Fig 1.1). In epidemiological studies, an association between low birth weight, an indicator of impaired *in utero* conditions, and a reduction in muscle strength was first observed in a study of 717 men and women (mean age 67.5 years) born in Hertfordshire UK between 1920 and 1930 (Sayer *et al.* 1998). This association has also been observed in other studies in both young and old age groups (Kuh 2002; Sayer *et al.* 2004; Inskip *et al.* 2007). These studies provide evidence for a suboptimal maternal environment increasing the risk of developing sarcopenia and therefore impaired skeletal muscle function in later life.

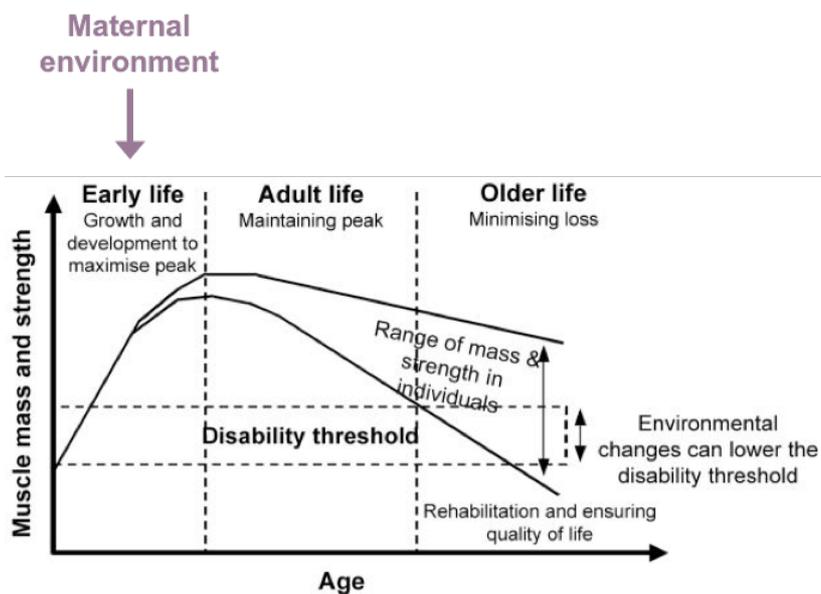


Figure 1.1: Life course model of muscle growth and decline with ageing. Adapted from Sayer *et al.* 2008.

## 1.3 Skeletal muscle

The idea put forward by epidemiological studies of a role for the early life environment consequently in affecting later muscle function makes very good sense because there are key windows in early life in which muscle structure is set up.

### 1.3.1 Skeletal muscle development and growth

#### 1.3.1.1 Myogenesis and muscle structure

The process of skeletal muscle development is known as myogenesis. Muscle fibres are mostly formed *in utero*, with muscle growth occurring in postnatal life. Small mononucleated cells named myoblasts proliferate in the presence of growth factors without differentiating. When there is a depletion of these growth factors, the myoblasts terminally differentiate, synthesise myofibrillar proteins and begin to fuse together. Isolation of myoblasts at different stages of development (embryonic, fetal and adult) (Pin *et al.* 2002) has shown that there are two or three distinct phases of myofibre formation. Embryonic myoblasts form the first wave of myofibres (primary myofibres). The fusion of fetal myoblasts creating long, cylindrical multinucleated myofibre, which lie in parallel along the length of the muscle, constitutes the second wave of myofibre formation (secondary myofibres) using the primary myofibres as a scaffold. These two phases occur in mice, but there is also a third wave of myofibre formation in humans and sheep that is thought to contribute to a larger muscle mass (Draeger *et al.* 1987; Wilson *et al.* 1992). In most mammalian species (including sheep and humans) myofibre formation is complete at birth, however in rodents it is complete in early postnatal life (Ontell & Kozeka 1984).

Each myofibre is made up of numerous myofibrils, which are in turn made up of repeated units called sarcomeres, and these are what contain the contractile proteins organised into thick and thin filaments. It is the crossover of these filaments that result in muscle contraction (see section 1.3.2). The sarcolemma (plasma membrane) surrounds the myofibres and sarcoplasm (cytoplasm). Mitochondria lay alongside myofibrils, which supply ATP for contraction. Each myofibril is surrounded by the sarcoplasmic reticulum

(SR), these are an intracellular muscle store for calcium ions which are released to allow for contraction. The muscle structure is displayed in Figure 1.2.

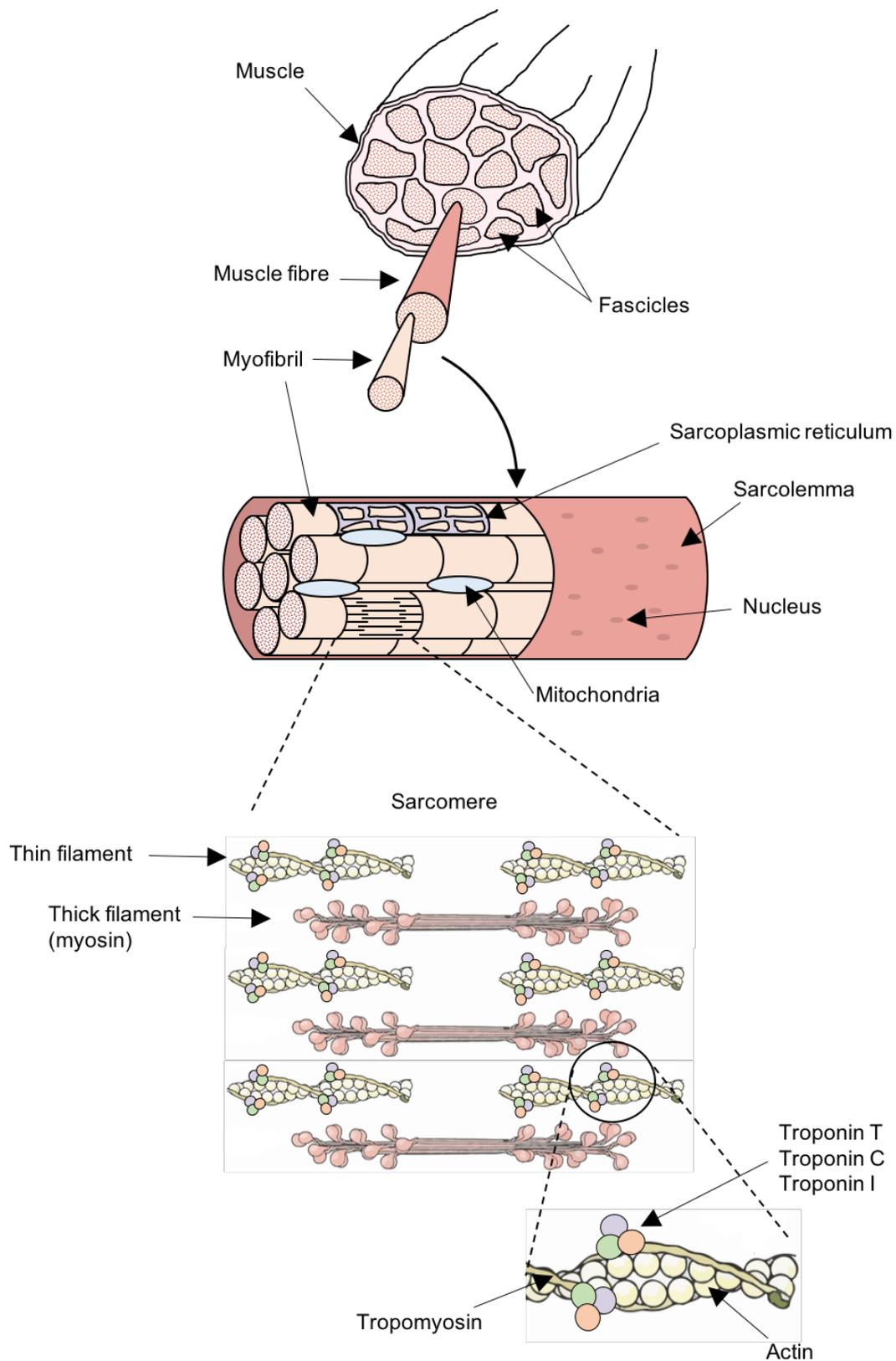


Figure 1.2: The structure of skeletal muscle fibres. Adapted from (Kandel *et al.* 2000).

### 1.3.1.2 Types of muscle fibres

Skeletal muscle fibres are classified based on structural and functional features. Different types of muscle fibres have different isoforms of myosin heavy chain (MHC) (Pette & Staron 2000). The protein myoglobin is the muscle's oxygen store. High myoglobin content gives the muscle a red colour, and the absence produces white muscle fibres. Muscle fibres are either slow-twitch or fast-twitch due to the speed in which they can break ATP down, and therefore contract. There are three main muscle fibre types: slow-twitch oxidative fibres (type I), fast-twitch oxidative fibres (type IIA) and fast-twitch glycolytic (type IIB) fibres.

#### *Type I fibres:*

- High myoglobin, mitochondria and blood capillary content (red in colour).
- High capacity to generate ATP from aerobic processes.
- Break down ATP at a slow rate, slow contraction speed and very resistance to fatigue.
- Main fibre type in postural muscles.
- Most sensitive to insulin.

#### *Type IIA fibres:*

- High myoglobin, mitochondria and blood capillary content (red in colour).
- High capacity to generate ATP from aerobic processes.
- Break down ATP at a faster rate, have faster contraction speed and less resistant to fatigue compared to type I fibres.
- Less sensitive to insulin compared to type I fibres.

#### *Type IIB fibres:*

- Low myoglobin and fewer mitochondria and blood capillaries (white in colour). Contain large amount of glycogen.
- Generate ATP through anaerobic processes.
- Break down ATP at the fastest rate, therefore have the fastest contraction speed but most prone to fatigue.
- Least sensitive to insulin.

In mammalian skeletal muscle there is a fourth fibre type called type IIX which expresses the MHCIIx isoform. This is considered an intermediate muscle fibre due to its contraction velocity being between that of type IIA and IIB fibres (Pette & Staron 2000). In the skeletal muscles of small mammals such as mice, all three fast MHC isoforms (IIa, IIX and IIB) are expressed. However, there are species differences and in the skeletal muscle of humans and sheep the MHCIIb isoform is not expressed (Smerdu *et al.* 1994; Tanabe *et al.* 1998). There is evidence that the fibres classified as IIB in fact express type IIX MHC transcripts (Smerdu *et al.* 1994) and so where literature refers to IIB fibres in humans and larger animals, they are more accurately IIX fibres. Skeletal muscles also constitute of hybrid fibres which express at least two MHC isoforms, for example type IIAX fibres which express both MHCIIa and MHCIIx.

### 1.3.1.3 Characterisation of skeletal muscles

The majority of skeletal muscles are composed of multiple types of muscle fibres, including both slow-twitch and fast-twitch. The proportion of each type is associated with the function of that muscle. Muscles required for fast, brief movements (for example muscles in the eye and muscles in the hands and feet for joint flexion and extension), are predominantly composed of fast-twitch myofibres as they are able to contract and relax much quicker. Muscles with the purpose to maintain posture (for example many muscles in the back and calves), are predominantly made up of slow-twitch fibres as they have long lasting contractions and a high resistance to fatigue. Many of the muscles in the leg are used for walking and running as well as supporting the body, and so these have a large proportion of both slow- and fast-twitch fibres (Martini & Nath 2009). Often studies on skeletal muscle will use a combination of predominately slow-twitch and fast-twitch muscles. This is important in order to have a good understanding of how a treatment or condition affects the two different muscle groups. For this thesis the soleus muscle (*m.*) and extensor digitorum longus (EDL) *m.* were sampled in the mouse models (Chapter 3 and Chapter 4), and in the sheep model (Chapter 5) the soleus, gastrocnemius and vastus lateralis *m.* were analysed. These muscles were chosen for each study so that outcome measures could be compared between muscles of different myofibre compositions. The role of the soleus *m.* is to maintain posture when in an upright position and to extend the ankle for plantar flexion. The EDL *m.* is important for dorsiflexion of the ankle/foot and extension of the toes for movements such as walking and running. The gastrocnemius *m.* is

also involved in plantar flexion as well as knee flexion to bend the hind limb which is important for running and jumping. The function of the vastus lateralis *m.* is for extension at the knee and flexion at the hip to straighten the hind limb. In the mouse, the soleus *m.* is an oxidative mixed fibre muscle with a high percentage of type I slow-twitch myofibres and the EDL *m.* is fast-twitch (Augusto *et al.* 2004; Bloemberg & Quadrilatero 2012). In the sheep, the soleus *m.* consists of only slow-twitch type I myofibres and the gastrocnemius and vastus lateralis *m.* contain a mixture of slow- and fast-twitch fibres (Konno & Watanabe 2012).

#### 1.3.1.4 Postnatal muscle growth

Postnatal muscle growth is due to an increase in muscle fibre number (hyperplasia), an increase in muscle fibre size (hypertrophy), or both processes combined (White *et al.* 2010). Satellite cells mediate postnatal muscle growth, by providing new myonuclei which proliferate, differentiate into myoblasts and fuse onto existing or developing myofibres in response to a stimulus such as exercise or injury, which causes the release of various growth factors (Seale & Rudnicki 2000). Various hormones regulate postnatal skeletal muscle growth. Growth hormone (GH) induces release of insulin-like growth factor I (IGF-I) which has been shown to be important for muscle. Mice lacking the GH receptor and the IGF-I receptor exhibited reductions in muscle fibre number and size (Mavalli *et al.* 2010). Testosterone is another regulator, a study investigating the effect of testosterone administration in elderly men found increases in muscle strength, muscle protein synthesis and IGF-I mRNA levels (Urban *et al.* 1995). Resistance strength training in humans has been found to increase muscle mass and strength through the process of hypertrophy (McDonagh & Davies 1984), and testosterone concentrations increase with strength training (Kraemer *et al.* 1990).

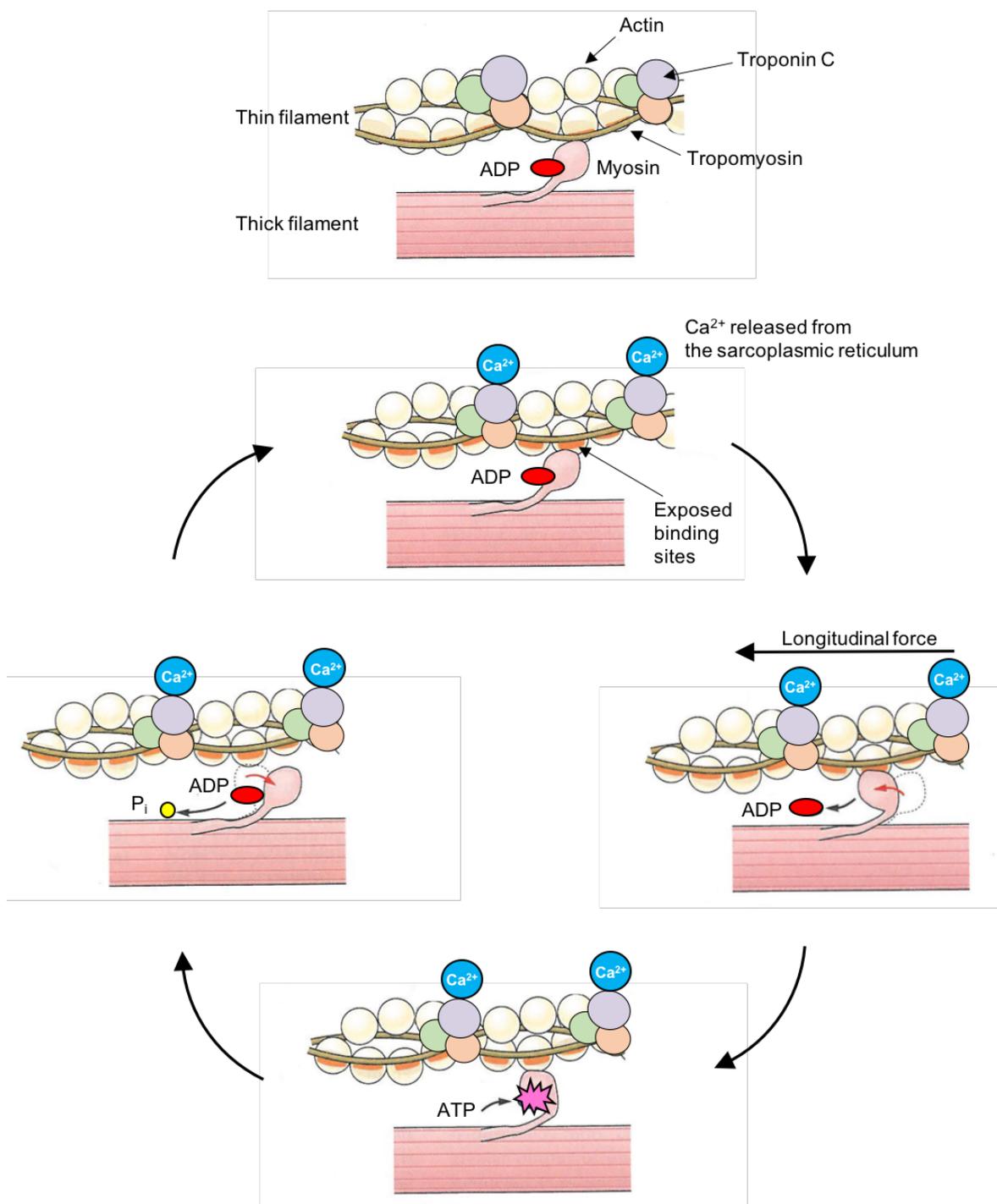
### 1.3.2 Skeletal muscle function: Muscle contraction

The process in which an action potential in the myofibre causes the myofibrils to contract is called “excitation-contraction coupling”. Calcium ions ( $\text{Ca}^{2+}$ ) and adenosine triphosphate (ATP) are both necessary for contraction. During relaxation, the

sarcoendoplasmic reticulum calcium ATPase (SERCA) pump actively transports  $\text{Ca}^{2+}$  into the SR, resulting in a high SR  $\text{Ca}^{2+}$  concentration and a low sarcoplasmic  $\text{Ca}^{2+}$  concentration. Upon arrival of an action potential from a motor neuron at the sarcolemma, it travels along the transverse tubules and activates the L-type calcium channels, allowing  $\text{Ca}^{2+}$  into the muscle cell. This channel also interacts with the type-I ryanodine receptor (RyR1), which sits in the SR membrane, induces a conformational change in RyR1, activating it and allows the transport of  $\text{Ca}^{2+}$  out of the SR, increasing the sarcoplasmic  $\text{Ca}^{2+}$  concentration (Block *et al.* 1988; Tanabe *et al.* 1988). At rest, the myosin heads of the myofibril thick filaments are bound to adenosine diphosphate (ADP). When the muscle fibre is stimulated, the  $\text{Ca}^{2+}$  released from the SR binds to troponin C, and displaces tropomyosin on the thin filaments, exposing the actin-binding sites of the thin filament. This exposure allows the myosin heads of the thick filament to bind to the thin filament, forming a cross bridge. The myosin head rotates and pulls the thick and thin filaments into greater overlap, resulting in a shortened (contracted) muscle fibre (Huxley & Hanson 1954), this is the sliding filament theory. ATP from mitochondria then attach to the myosin head, which is hydrolysed into ADP and inorganic phosphate, releasing energy to dissociate the myosin head from troponin C to allow for another power stroke. This process, (shown in Fig. 1.3) is repeated many times to produce a movement.

There is a lack of research into fetal skeletal muscle contraction properties. However, mammalian studies on neonatal rats and cats found that muscles had slower contraction and relaxation rates at birth, which then become faster over time (Buller *et al.* 1960; Close 1964). These findings were also found in human 12-15 weeks gestation fetal muscle due to a slower cross-bridge cycling and ATPase activity (Racca *et al.* 2013). These characteristics are similar to adult slow-twitch muscles. The reduced contraction and relaxation rates in fetal and neonatal life can be explained by the developmental changes in myosin heavy chains (MHC), whereby there is a large proportion of slow-twitch myofibres early on, with a temporal increase in fast-twitch myofibres (West *et al.* 1999).

It is important to note that very little is known about the underlying mechanisms and the skeletal muscle contractile regulators susceptible to change in sarcopenia. However, one human study reported a decline in MHC synthesis rate with age, which was associated with reduced muscle strength (Balagopal *et al.* 1997).



**Figure 1.3: The different stages of muscle contraction – the sliding filament theory.** Adapted from (Kandel *et al.* 2000).

### 1.3.3 Skeletal muscle function: Glucose homeostasis

Normal glucose homeostasis is important for optimal skeletal muscle function and vice versa. In the presence of increased blood glucose levels, the anabolic hormone insulin is secreted from the  $\beta$ -cells of the islets of Langerhans in the pancreas. Insulin binds to the insulin receptor (InsR) on the surface of muscle cells, phosphorylates insulin substrate 1 (IRS-1) (Sun *et al.* 1991), which then activates the phosphatidylinositol 3-kinase/serine/threonine-protein kinase (PI3K-Akt) pathway. Akt2 induces the translocation of insulin-sensitive glucose transporter GLUT-4 from the cytoplasm to the cell surface membrane (Kohn *et al.* 1996; Wang *et al.* 1996). GLUT-4 then allows for the transport of glucose into the muscle cell. GLUT1 is expressed at the cell surface under basal conditions and it is not mediated by insulin. Glucose uptake via GLUT-1 and GLUT-4 is important for metabolic processes in order to generate ATP for skeletal muscle contraction.

On a whole-body level, insulin signalling and glucose uptake has been shown to mature throughout gestation in the fetal sheep (Aldoretta *et al.* 1998). Over time, basal glucose concentration decreased and insulin concentrations increased in response to a hyperglycaemic clamp. However, during fetal/neonatal life GLUT-1 expression is higher than in the adult (He *et al.* 2003). It is also the main contributor for maintaining glucose uptake in skeletal muscle (Hay, W 1994), as opposed to the main contributor being GLUT-4 in adult muscle. As a result, the fetus is considered more insulin-insensitive and so glucose may be handled differently in fetal compared to adult muscle.

## 1.4 Vitamin D

Vitamin D is a pleiotropic steroid hormone with numerous functions including its role in calcium homeostasis and bone metabolism (Shin *et al.* 2010). There is also increasing evidence for its importance in skeletal muscle development and function. Vitamin D deficiency is a common problem in pregnant women and it is linked to obstetrical complications such as bacterial vaginosis (Bodnar *et al.* 2009), preeclampsia (Achkar *et al.* 2015; Akbari *et al.* 2018), gestational diabetes mellitus (Hu *et al.* 2018) and spontaneous pregnancy loss (Hou *et al.* 2016; Bärebring *et al.* 2018). Maternal vitamin D status is dependent upon three factors: sunlight exposure, dietary intake of vitamin D and maternal body composition as detailed in section 1.6. It is thought that the developing fetus acquires all of its vitamin D from the mother and therefore maternal VDD will impact the vitamin D status of the fetus.

### 1.4.1 Vitamin D biosynthesis

Vitamin D is obtained from two different sources. Vitamin D can be acquired via dietary intake in the form of vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (cholecalciferol) from foods such as oily fish, eggs, red meat, cheese and fortified products. Vitamin D can also be obtained from ultraviolet light exposure, which converts 7-dehydrocholesterol in the skin to form vitamin D<sub>3</sub>. In humans, vitamin D<sub>3</sub> is approximately three times more potent than D<sub>2</sub> in increasing 25(OH)D serum levels (Heaney *et al.* 2011). Following consumption and/or absorption, vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are hydroxylated in the liver by 25-hydroxylase (CYP27A1) to form 25-hydroxyvitamin D ((25(OH)D<sub>2/3</sub>)), the inactive form of vitamin D. This is further hydroxylated in the kidneys by the enzyme 1 $\alpha$ -hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D ((1,25(OH)<sub>2</sub>D<sub>2/3</sub>)), which is the biologically active form of vitamin D (Fig. 1.4). The kidney is a primary site of the gene CYP27B1 and consequently of 1 $\alpha$ -hydroxylase action, but CYP27B1 is also expressed in other tissues such as the placenta (Ma *et al.* 2012) and skeletal muscle (Girgis *et al.* 2014) which means that there is scope for local tissue production of 1,25(OH)<sub>2</sub>D from 25(OH)D. 24-hydroxylase activity (encoded by CYP24A1 gene) is increased by 1,25(OH)<sub>2</sub>D<sub>2/3</sub> and the enzyme functions to convert 25(OH)D<sub>2/3</sub> and 1,25(OH)<sub>2</sub>D<sub>2/3</sub> to 24,25(OH)<sub>2</sub>D<sub>2/3</sub> and

1,24,25(OH)<sub>2</sub>D<sub>2/3</sub>, respectively, thereby allowing for regulation of vitamin D levels (Shin *et al.* 2010).

Although 1,25(OH)<sub>2</sub>D is the biologically active form, it is usually 25(OH)D that is used clinically to assess vitamin D status. This is because 25(OH)D is the predominant form of circulating vitamin D (1,25(OH)<sub>2</sub>D is 1000 times lower concentration) and has a longer half-life of 2-3 weeks compared to the 4 h half-life of circulating 1,25(OH)<sub>2</sub>D. Vitamin D levels are tightly regulated in the blood via calcium, phosphate and parathyroid hormone (PTH), and so guidelines report that 1,25(OH)<sub>2</sub>D does not reflect accurate vitamin D reserves (Holick *et al.* 2011).

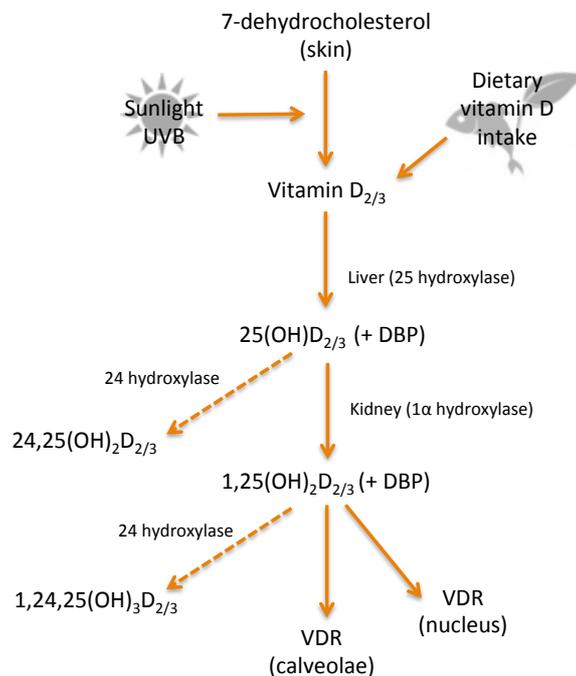


Figure 1.4: Vitamin D biosynthesis pathway.

Assessing vitamin D levels should also coincide with other factors such as calcium, phosphate and PTH due to their close relationship with vitamin D and their well-established role in the complex process of mineral homeostasis. Vitamin D in the form of 1,25(OH)<sub>2</sub>D increases calcium and phosphate absorption in the small intestine, consequently increasing their concentrations in the blood, which in turn reduces PTH release from the parathyroid gland. When calcium levels are low, PTH is released from the parathyroid gland and stimulates the release of calcium and phosphate from bone to normalise calcium levels in the blood (Khundmiri *et al.* 2016). Hyperphosphatemia and hypercalcemia are in turn thought to reduce 1,25(OH)<sub>2</sub>D synthesis as a result of negative feedback loops (Fukumoto 2014). There is evidence that calcium, phosphate and PTH

impact skeletal muscle development and/or function. In an embryonic stem line expressing the myogenic transcription factor MyoD, increased PTH and parathyroid hormone type 1 receptor (PTH1R) expression is associated with accelerated myocyte differentiation (Kimura & Yoshioka 2014). Hypophosphatemia has been associated with muscle weakness (Schubert & DeLuca 2010) and impaired muscle ATP synthesis (Pesta *et al.* 2016), and as ATP is fundamental for skeletal muscle contraction, it suggests that adequate phosphate levels are important for muscle function. With regards to calcium, calcium plays a critical role in muscle contraction and relaxation (as discussed in section 1.3.2) and is implicated in the action of vitamin D for muscle function (discussed in section 1.4.3) but its activity is also known to trigger cellular responses necessary for myogenesis and regulate myoblast proliferation and differentiation during development (Tu *et al.* 2016). One line of evidence is in mice with a homozygous mutation in the *RyR1* gene (involved in Ca<sup>2+</sup> release from the SR as discussed in section 1.3.2) die perinatally with severely impaired muscles made up of small myotubes and disarranged myofibrils (Zvaritch *et al.* 2007). Furthermore, the developing fetus is dependant on maternal calcium being transported across the placenta and previous studies suggest that vitamin D regulates this process as both calbindin D-9K (a calcium binding protein) and CaT1 (calcium transporter) possess VDREs within their promoter regions (Jeung *et al.* 1994; Wang *et al.* 2005). This alongside evidence for vitamin D action in skeletal muscle (section 1.4.3) emphasises the relationship between calcium and vitamin D.

## 1.4.2 Vitamin D transport

The inactive and active forms of vitamin D and their metabolites are primarily transported in the circulation bound to the vitamin D binding protein (DBP) with a small proportion bound to albumin and lipoprotein (Shin *et al.* 2010). It is thought that 88% of circulating 25(OH)D is bound to DBP, 11.97% bound to albumin and 0.03% is free of a carrier protein (Bikle *et al.* 1986). The same group found that for 1,25(OH)<sub>2</sub>D, 85% is bound to DBP, 14.6% bound to albumin and 0.4% is free (Bikle *et al.* 1986). It has previously been suggested that only non-bound 25(OH)D and 1,25(OH)<sub>2</sub>D enter cells via diffusion across the plasma membrane (the “free hormone hypothesis”) to then bind with VDR. However, considering there is only a small fraction of free 25(OH)D or 1,25(OH)<sub>2</sub>D, it is thought that their DBP-bound forms must be transported. Indeed there is evidence for the DBP-

mediated endocytic receptors megalin and cubulin to be expressed in the kidney (Nykjaer *et al.* 2001) and recently the placenta (Burke *et al.* 2013; Park *et al.* 2017) and skeletal muscle cells (Abboud *et al.* 2013) for the uptake of 25(OH)D.

### 1.4.3 Vitamin D action and skeletal muscle

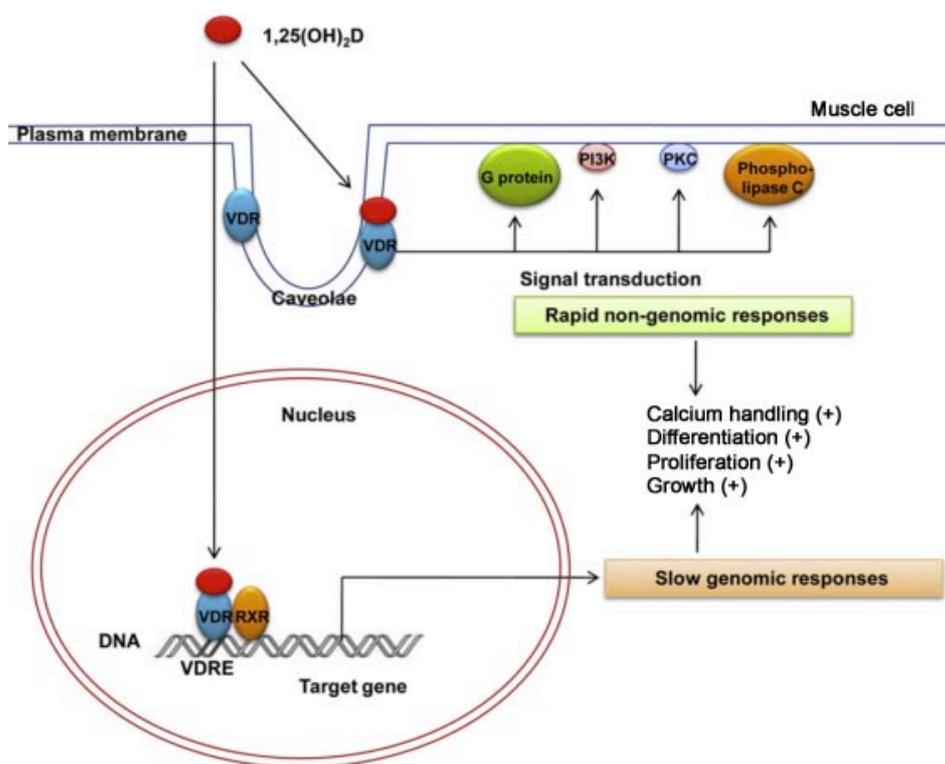
The actions of vitamin D are exerted through the biologically active 1,25(OH)<sub>2</sub>D binding to the vitamin D receptor (VDR) with high affinity. Vitamin D acts mainly via genomic signalling by acting as a nuclear transcription factor but there is evidence that it can also act via non-genomic signalling (Fig. 1.5), although details are not well known.

The genomic pathway involves the binding of vitamin D to a nuclear VDR. The nuclear VDR forms a heterodimer with the retinoid X receptor (RXR) causing a transformational change in VDR. This heterodimer then binds to specific genomic sequences, the vitamin D response element (VDRE) in the promoter of vitamin D target genes and activates mRNA transcription (Pojednic & Ceglia 2014). The VDR has two globular domains, the ligand-binding domain (LBD) which binds 1,25(OH)<sub>2</sub>D with a high affinity and aids dimerization, and the DNA-binding domain (DBD) which contains two zinc-finger motifs facilitating the binding of the VDR/RXR heterodimer to the VDRE (Shin *et al.* 2010).

The non-genomic pathways involve the binding of 1,25(OH)<sub>2</sub>D to the plasma membrane VDR in calveole, initiating the activation of various signalling pathways (Pojednic & Ceglia 2014). However, the type of VDR is not definite. Some researchers believe it is a novel membrane receptor (Nemere *et al.* 1998), some report it to be a membrane-associated calcium binding protein that acts as a calcium specific ion channel (Baran *et al.* 2000), and more recent evidence suggests that it is the nuclear VDR translocating from the cell nucleus to the plasma membrane (Capiati *et al.* 2002).

Previous studies provide evidence for the involvement of vitamin D in skeletal muscle development and function. The VDR and 1 $\alpha$ -hydroxylase are expressed in human muscle and mouse muscle cell lines (Bischoff *et al.* 2001; Ceglia *et al.* 2010; Girgis *et al.* 2014), suggesting a role for vitamin D in skeletal muscle. It has been suggested that vitamin D, via the genomic pathway, regulates the transcription of genes that code for sarcoplasmic,

contractile and cytoskeletal proteins involved in muscle function, such as calmodulin and calbindin D-9K (Brunner & de Boland 1990; Zanello *et al.* 1995) by binding to the VDRE. These proteins are important for skeletal muscle contraction as they regulate muscle cell  $\text{Ca}^{2+}$  uptake, an ion which is crucial for contraction (section 1.3.2). Vitamin D acting via non-genomic signalling is thought to involve the activation of signalling pathways and secondary messengers which promote an increase in  $\text{Ca}^{2+}$  influx via store-operated  $\text{Ca}^{2+}$  channels, voltage dependant  $\text{Ca}^{2+}$  channels and from the sarcoplasmic reticulum via ryanodine receptors (Hazell *et al.* 2012). Furthermore, the VDR is thought to mediate muscle cell differentiation, growth and regeneration (Endo *et al.* 2003; Srikuea *et al.* 2012; Tanaka *et al.* 2014). These findings all provide evidence for vitamin D having an important role in skeletal muscle physiology.



**Figure 1.5: Genomic and non-genomic signalling responses of 1,25(OH)<sub>2</sub>D binding to the vitamin D receptor.** Adapted from Shin *et al.*, 2010 and Pojednic & Ceglia, 2014.

Studies in humans have found a positive correlation between vitamin D status in adult life and skeletal muscle mass, muscle strength, performance and risk of falls (Bischoff-Ferrari *et al.* 2004a; Wicherts *et al.* 2007a; Harvey *et al.* 2014). A study looking at the lower extremity function of patients 60-90 years of age who participated in the third National

Health and Nutrition Examination Survey (NHANES) reported higher serum 25(OH)D concentrations were associated with faster gait speed and faster sit-to-stand speed (Bischoff-Ferrari *et al.* 2004b). Results from the Longitudinal Aging Study Amsterdam (LASA) found that in participants 65 years of age and above, lower 25(OH)D levels were associated with reduced grip strength and muscle mass (Visser *et al.* 2003). Data from this study also showed that participants with <50 nmol/L serum 25(OH)D has an increased risk of decline in physical performance (as determined by three physical performance tests) compared to participants with serum 25(OH)D levels >75 nmol/L (Wicherts *et al.* 2007a). These studies further add to the existing evidence suggesting that vitamin D is important for skeletal muscle function.

#### **1.4.4 Vitamin D deficiency and supplementation on skeletal muscle in adulthood.**

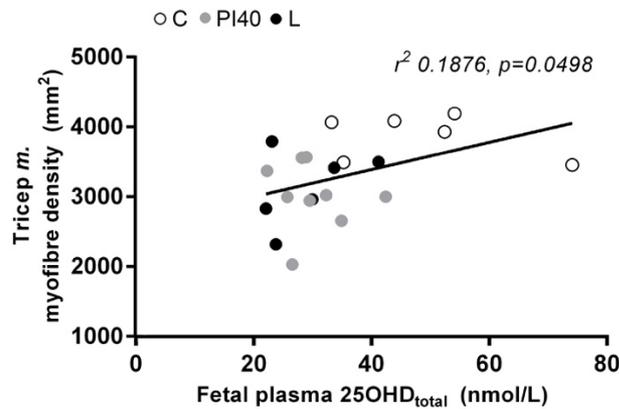
Studies in adulthood using histological analysis, found that human elderly patients with VDD had type II muscle fibre atrophy with necrosis and intramuscular fat and glycogen deposition (Yoshikawa *et al.* 1979), and it is these type II fibres which are the predominant type to be used in attempt to prevent a fall. Studies have also shown that such deterioration of human skeletal muscle of elderly patients was ameliorated by vitamin D supplementation (Sorensen *et al.* 1979; Sato *et al.* 2005; Ceglia *et al.* 2013). Sorensen *et al.* found that supplementation increased the fibre number and cross sectional area (CSA) of type II vastus lateralis muscle fibres in female patients 65-81 years old. Sato *et al.* also saw this effect on type II muscle fibres, but also an improvement in muscle strength and reduction in hip fractures in elderly women, and Ceglia *et al.* found a 10% increase in vastus lateralis muscle myofibre size of mobility-limited vitamin D deficient women, with a 30% increase in skeletal muscle intramyonuclear VDR concentration. Furthermore, a study looking at VDD during postnatal life in rabbits found that the ability of the SR in muscle cells was impaired for calcium uptake (Curry *et al.* 1974).

### 1.4.5 Gestational vitamin D deficiency and offspring skeletal muscle

Numerous studies have observed that a restricted nutrient environment *in utero* affects skeletal muscle development. One study shows that maternal nutrient restriction in peri-implantation (40% restricted) or in late-gestation (50% restricted) caused a reduction in total myofibre density of the triceps brachii *m.* of 127±1 dGA fetal sheep (Costello *et al.* 2008). Another sheep study found a reduction in myofibre number of the longissimus dorsi *m.* of 8-month old offspring following a 50% maternal nutrient restriction between 28-78 dGA (Zhu *et al.* 2006). In the guinea pig, a 60% nutrient restriction throughout gestation caused a reduction in neonatal bicep brachii *m.* fibre number (Dwyer *et al.* 1995) and in 11-month old rat offspring, protein restriction (80 g protein/kg vs. 200 g protein/kg) throughout pregnancy and lactation reduced muscle mass (Desai *et al.* 1996).

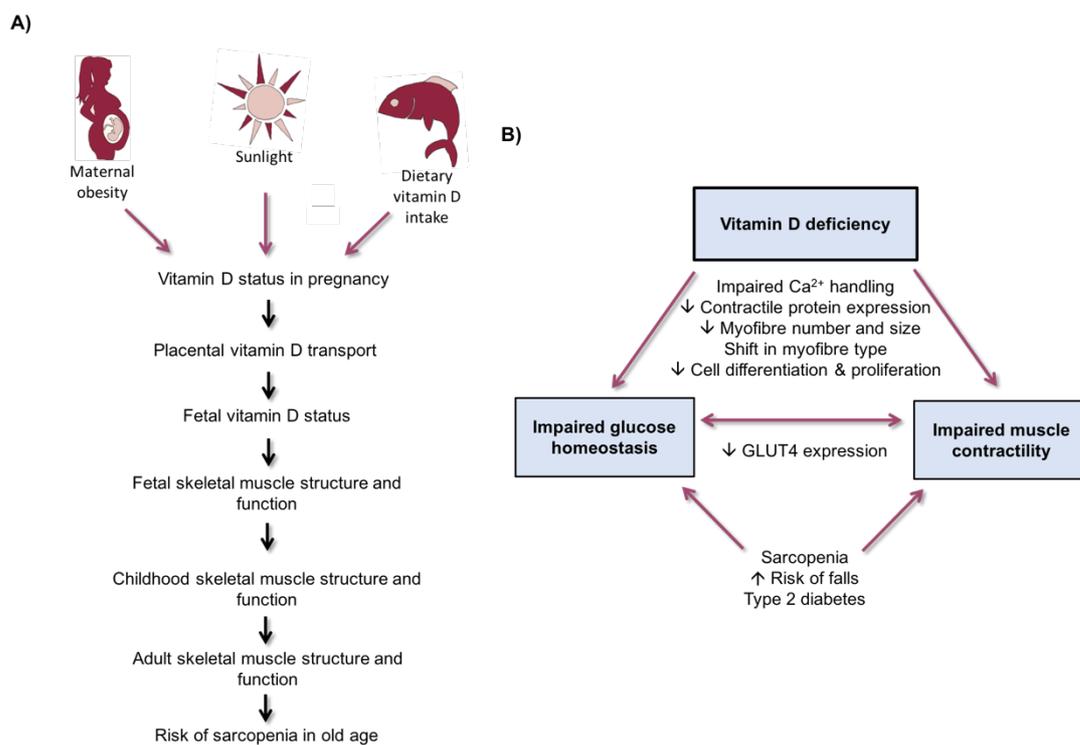
Pregnancies complicated by fetal growth restriction (FGR) have a reduced placental VDR expression which leads to enhanced placental apoptosis and therefore suboptimal placental and fetal growth (Murthi *et al.* 2016), and findings from a human birth cohort study report that gestational VDD is associated with impaired lung, brain and bone development in the offspring (Hart *et al.* 2015). These studies highlight the importance of adequate vitamin D during pregnancy on offspring development. In relation to skeletal muscle and vitamin D specifically, a study using fetal pigs found that a maternal vitamin D supplemented diet (500 IU vitamin D<sub>3</sub>/kg diet plus 50 µg 25(OH)D<sub>3</sub>/kg diet) fed from 43 days prior to mating resulted in a 9.3% increase in total muscle fibre number of the fetal longissimus *m.* at 90 days of gestation (Hines *et al.* 2013), although there was no change in myofibre CSA. In addition, a more recent vitamin D supplementation study during gestation in pigs found a 23.5% and 27.5% increase in longissimus dorsi *m.* total fibre number of newborn and weaning piglets, respectively (Zhou *et al.* 2016). A study using a total VDR knockout (VDR *-/-*) mouse model found that at 3 weeks old, muscle fibres of the quadriceps femoris *m.* were smaller in diameter compared to VDR *+/+* mice (Endo *et al.* 2003), and the effect of this VDR knockout spans early development into postnatal life. This reduction in size was thought to be due to an unusually high expression of the transcription factors myf5, myogenin, E2A and early life MHC isoforms as these are normally downregulated in the earlier stages of life. A rat study found that vitamin D depleted diets (0 IU vitamin D) fed during postnatal life from weaning lead to impaired soleus muscle excitation-contraction coupling 65-74 days post-weaning (Rodman & Baker 1978). In European sea bass, a dose of 3.5mg vitamin D<sub>3</sub>/kg of diet was fed to larvae from 9 days old, which increased the

recruitment of white muscle fibres in 44-day old larvae (Alami-Durante *et al.* 2011). In the same late gestation sheep fetuses as used by Costello *et al.* (Costello *et al.* 2008), lower myofibre density was associated with lower fetal plasma 25(OH)D concentrations ((Fig. 1.6; (Cleal *et al.* 2017)). In humans, a lower 25(OH)D status in the pregnant mother at 34-weeks gestation was associated with a reduction in grip strength of children at 4 years of age (Harvey *et al.* 2014). Newborn (day of parturition) rat offspring of dams fed a vitamin D deficient diet during pregnancy had reduced gastrocnemius *m.* cell size and enlarged interfibrillar spaces compared to control (Max *et al.* 2014). This was one of very few studies to our knowledge looking at maternal VDD on offspring postnatal muscle and further work is required to see if such effects on skeletal muscle extend beyond the newborn period into adult life.



**Figure 1.6: Association of fetal sheep total plasma 25(OH)D concentration (nmol/L) and triceps brachii *m.* total myofibre density.** Association of all diet groups. C, control; PI40, peri-implantation nutrient restriction; L, late-gestation nutrient restriction. Adapted from Cleal *et al.*, 2017.

Overall, evidence suggests that 1) there is a link between adult vitamin D and muscle structure, and 2) vitamin D deficiency during pregnancy impacts on offspring skeletal muscle (Fig. 1.7). However, there have been no studies that have followed the offspring up in later life to see if the skeletal muscle is still affected by vitamin D deficiency.



**Figure 1.7: Summary schematic diagram of the background knowledge. A)** The factors that affect maternal vitamin D status and **B)** the evidence linking vitamin D deficiency, muscle glucose homeostasis and muscle contractility together.

## 1.5 Obesity

### 1.5.1 Prevalence of obesity

Obesity is a major health concern and it is defined as a body mass index (BMI)  $\geq 30$  kg/m<sup>2</sup>. Worldwide, the prevalence of obesity nearly doubled from 1980 to 2014. In 1980, 5% and 8% of men and women, respectively, were obese, with an increase to 11% and 15% of men and women in 2014 (World Health Organisation 2014). Obesity increases the risk of mortality and 3.4 million people die annually due to being overweight or obese. Obesity leads to adverse metabolic effects including impaired insulin sensitivity and is a major risk factor for various non-communicable diseases such as type 2 diabetes (Abdullah *et al.* 2010), cardiovascular disease (Bogers *et al.* 2007) and some forms of cancer (Renegan *et al.* 2008).

### 1.5.2 Maternal obesity and offspring skeletal muscle

The prevalence of obesity during pregnancy is high, affecting approximately 20% of women of reproductive age in the UK (Poston *et al.* 2011). Obesity during pregnancy has doubled in England from 7.6% in 1989 to 15.6% in 2007 (Heslehurst *et al.* 2010). This issue is of importance as the maternal environment, and therefore obesity, impacts nutrient supply to the fetus. Maternal obesity is associated with many pregnancy complications and compromised health of the mother and offspring, in addition to increased risk of maternal death (Poston *et al.* 2011). Some of these complications include the increased risk of gestational hypertension, gestational diabetes mellitus, preeclampsia, large for gestational age infant, and congenital defects. The risk of stillbirth is also increased with obesity.

Previous studies have found that a high-fat obesogenic diet during pregnancy impairs offspring skeletal muscle development, structure and function. In rats, a high-fat/high-sugar diet during pregnancy and lactation impaired skeletal muscle development as shown by a reduction in myofibre number, CSA, the number of myonuclei, and an increase in intramuscular lipid deposition in the semitendinosus *m.* of 21 days-old pups (Bayol *et al.* 2005). A decrease in muscle fibre CSA and an increase in intramuscular lipid deposition

was also reported in the semitendinosus *m.* of 3 month-old lambs from ewes fed a diet 140% total digestible nutrients (Reed *et al.* 2014). Another rat study found an increase in intramuscular lipid deposition of the plantaris *m.* of offspring at weaning and 3 months of age following exposure to a maternal high-fat diet from 10 weeks prior to conception and throughout pregnancy and lactation (MacPherson *et al.* 2015). In the rat study from Bayol *et al.*, a reduction in insulin receptor mRNA levels and a trend for a decrease in *Glut4* mRNA levels in the gastrocnemius *m.* were also found with a maternal obesogenic diet, suggesting an impaired insulin sensitivity and glucose uptake in the muscle. More recent studies have also reported an effect on *Glut4* and glucose homeostasis. One study in male rat offspring at 70 days old, who were exposed to a maternal high-fat and high-sucrose diet from conception until parturition, found that mRNA expression of *Akt2*, *Raf1* (signalling molecules in the insulin signalling pathway) and *Glut4* in the gastrocnemius *m.* was downregulated compared to offspring exposed to a maternal control diet (Cuthbert *et al.* 2017). This suggests a potential reduction in the translocation of *Glut4* to the plasma membrane for skeletal muscle glucose uptake and a possible explanation for the higher fasting glucose concentrations in the offspring exposed to an obesogenic prenatal diet. In addition to this, in 3 month old female lambs exposed to maternal obesity during the periconceptual period there was a reduction in quadriceps *m.* GLUT-4 protein expression, therefore also suggesting impairments to offspring skeletal muscle insulin and glucose homeostasis (Nicholas *et al.* 2013). Interestingly, a recent study reported reduced glucose utilization in dam biceps femoris *m.* and in the whole fetus of dams fed a high-fat and high-sugar diet during pregnancy (Musial *et al.* 2017). This suggests the glucose utilisation of fetal skeletal muscles may also be impaired at this early life stage. Furthermore, another study found that 12-month old male rats from mothers fed a diet rich in saturated fat and sucrose had increased insulin levels, suggesting insulin resistance. They also performed next generation mRNA sequencing of the soleus *m.* and results showed upregulation of pathways involved in inflammatory responses and cytokine signalling whilst pathways associated with mitochondrial matrix, electron transport and mitochondrial complexes were downregulated (Latouche *et al.* 2014). Phosphorylation of protein kinase B in the insulin signalling pathway was also reduced. This study therefore suggests that maternal overnutrition leads to mitochondrial dysfunction and metabolic dysregulation, and in alignment with the aforementioned studies, predisposes the offspring to type 2 diabetes. Impairments to mitochondrial activity were also observed in the soleus *m.* of adult rat offspring following exposure to a maternal high-fat diet (Pileggi *et al.* 2016).

In addition to the aforementioned study, work from Bayol *et al.* also investigated the effect their maternal obesogenic diet had on rat muscle force of offspring at 10-weeks old. The skeletal muscle force of the gastrocnemius, soleus and plantaris all together (plantar group of muscles) was measured and the high-fat/high-sugar diet reduced the twitch and tetanic force produced by this set of muscles (Bayol *et al.* 2009). Another study found that maternal diet-induced obesity during pregnancy and lactation led to cardiovascular and metabolic dysfunction (Samuelsson *et al.* 2008). Offspring were reported to have endothelial dysfunction and hypertension, reduced locomotor activity, increased adiposity, a reduction in tibialis anterior *m.* mass and increased fasting plasma insulin and glucose levels of 3 to 6 month old offspring (Samuelsson *et al.* 2008), again suggesting that a maternal obesogenic diet alters skeletal muscle development and metabolic function. What is not known is to what extent an obesogenic diet during pregnancy impacts the muscle structure and contractile function in the mature adult offspring.

### 1.5.3 Postnatal obesity and skeletal muscle

As well as maternal obesity, postnatal obesity also has an impact on skeletal muscle structure and function. A study looking at the effect of a high-fat diet (58% kcal fat) from 8 weeks of age for 12-16 weeks on mouse skeletal muscle found that there was an accumulation of adipocytes in the gastrocnemius *m.* and a reduction in muscle fibre size in 24-week old mice (Pellegrinelli *et al.* 2015). This muscle atrophy was thought to be due to a reduction in expression of genes linked to myogenesis (MyoD1, myogenic differentiation 1; MYOG, myogenin; IGF-II, insulin-like growth factor 2; IGFBP-5, insulin-like growth factor binding protein 5) and also contractile proteins (TTN, titin; SGCA,  $\alpha$ -sarcoglycan) as observed in an *in vitro* cell model of human primary satellite cells cultured in the presence of adipocytes from obese patients. When these cells were differentiated, there was a reduction in myotube thickness as well as a downregulated expression of contractile proteins including myosin heavy chain (MF20), troponin and titin. A mouse study found that a high-fat diet (60% kcal fat) fed from 8 to 14 months of age led to a 15% reduction in forearm grip strength and a 11% reduction in sensorimotor coordination compared to control (Lee *et al.* 2015a). In addition, the peak force of twitch and tetanic muscle contractions of the mouse soleus *m.* were reduced in 17-week old mice fed a high-fat diet for 5 weeks (Ciapaite *et al.* 2015). A human study using 48-92 year old patients found that

a higher waist circumference (a clinical indicator of central obesity) is associated with a lower hand grip strength (Keevil *et al.* 2014). They found that for every 10 cm increase in waist circumference, there was a 3.56 kg and 1 kg reduction in grip strength in males and females, respectively. Taken together these studies from a range of species show a detrimental effect of a high-fat diet with obesity during adult life on skeletal muscle structure and function. However, to date there is little information on how this response to postnatal obesity depends on the prenatal exposure.

## 1.6 Obesity and vitamin D

High BMI in adults is associated with a lower 25(OH)D plasma concentration (Liel *et al.* 1988) and this observation has been repeated in subsequent human studies (Hyppönen & Power 2006; Vimalleswaran *et al.* 2013). This relationship has been suggested to be due to enhanced vitamin D uptake into adipose tissue of obese individuals and therefore a reduced bioavailability in the blood (Wortsman *et al.* 2000). The literature suggests that vitamin D status in the pregnant mother can subsequently impact their offspring's risk of developing obesity. Findings from the Southampton Women's Survey revealed that a low serum 25(OH)D status in the woman at 34 weeks of gestation was associated with increased adiposity in the offspring at 4 and 6 years old (Crozier *et al.* 2012). Data from a recent human study are consistent with these findings as a low maternal 25(OH)D concentration during the first trimester of pregnancy at 14 weeks was associated with an increased offspring waist circumference at 4 and 6 years of age (Daraki *et al.* 2018). In order to understand this association in more detail, one recent rodent study using Sprague-Dawley rats found that offspring born to mothers fed a vitamin D deficient diet during pregnancy until birth had an increase in proliferation and differentiation of pre-adipocytes and an increase in adipose tissue volume, leading to offspring obesity (Wen *et al.* 2018). These rat offspring also had increased plasma concentrations of total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein, which suggests that vitamin D deficiency during pregnancy impairs offspring lipid metabolism. Interestingly, Daraki *et al.* found no associations between maternal 25(OH)D status and blood lipid concentrations. Overall, these studies provide evidence for the relationship between obesity and vitamin D status in adults but also highlight that vitamin D status during pregnancy can influence adiposity in offspring. This relationship may contribute to the intergenerational cycle of obesity.

## 1.7 Summary

Recent evidence suggests that the *in utero* environment affects the development and growth of skeletal muscle, consequently impacting the peak muscle mass produced in adulthood and the rate of decline in muscle mass and strength. The studies reviewed in sections 1.4.5 and 1.5.2 provide evidence that vitamin D deficiency and obesity may be two potential drivers in altering muscle development and function during development and into adult life. This may also heighten the risk of developing muscle disease such as sarcopenia (Fig. 1.8). However, there is currently limited knowledge on the a) relationship between vitamin D and obesity as drivers of offspring skeletal muscle development and function; b) relationship between obesity in prenatal and postnatal life in determining muscle development and function, and c) the effect of specific alterations in maternal vitamin D on offspring muscle either during fetal life or into adulthood.

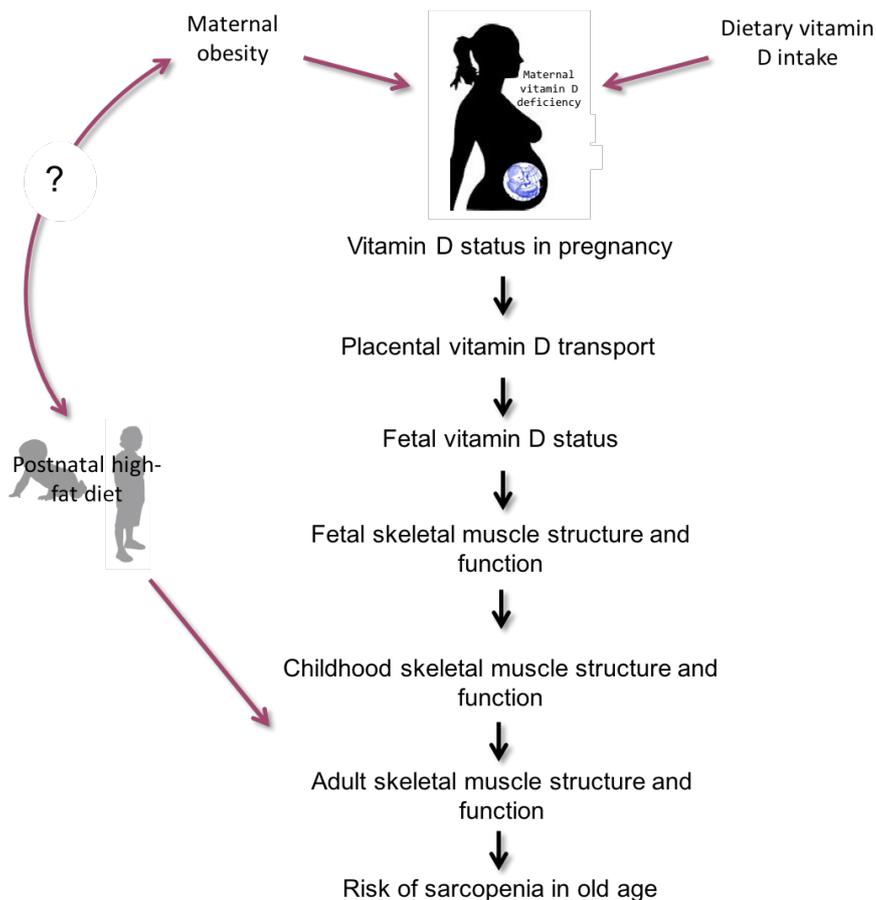


Figure 1.8: Schematic diagram showing the thesis flow of logic.

## 1.8 Hypothesis and aims

### Overall thesis hypothesis:

Vitamin D deficiency and obesity during early life development will impair offspring adult skeletal muscle function. This will be through impairments to a) muscle structure and factors regulating b) muscle glucose metabolism and c) muscle contraction.

### Aims:

In view of previous studies suggesting that the prenatal and postnatal environment independently affect skeletal muscle, and that there is a possible association between obesity and vitamin D status, the aims in the first results chapter of this thesis were as follows.

***Chapter 3:*** To initially investigate the effect of prenatal and post-weaning high-fat diet on a) mouse muscle contractile force, b) muscle structure and c) mRNA levels of genes involved in glucose regulation and muscle contractility. The second aim was to determine whether the effect of a postnatal high-fat diet was altered by exposure to a prenatal high-fat diet. The third aim was to relate the offspring measurements to markers of vitamin D status in order to see how a high-fat diet effects vitamin D homeostasis.

In this chapter I ascertained that a postnatal high-fat diet reduced adult offspring peak force in a sex- and muscle bed specific manner. This effect was not as profound with a prenatal high-fat diet, although data suggested that it had long term effects on vitamin D handling. However, it became apparent that as the high-fat diet used in this chapter contained a high vitamin D concentration, it was not a good model in which to explore further the influence of pregnancy vitamin D deficiency on long-term skeletal muscle function, and that a more direct manipulation of vitamin D was required. This lead to the aims in the second results chapter:

***Chapter 4:*** To create a mouse model of gestational vitamin D deficiency and to determine whether maternal vitamin D deficiency impairs young adult offspring muscle by analysing a) muscle force, b) grip strength, c) overall strength and d) open-field activity. A secondary

*Chapter 1:*

aim was to investigate any associations between these offspring outcome variables and maternal pregnancy vitamin D status through performing correlations.

A model of gestational vitamin D deficiency was successfully established, however there were very few effects in the young adult offspring. One reason could be that the control diet over the 12 weeks post-weaning could have corrected for any changes to early life skeletal muscle function and development. Consequently, this led to a model of gestational vitamin D deficiency focussing on the fetus, in which potential changes to the skeletal muscle at an earlier developmental stage could be examined. This led to the aims in the final results chapter:

***Chapter 5:*** Using a fetal sheep model, to test whether maternal vitamin D deficiency impairs fetal skeletal muscle by analysing changes to a) fetal muscle glucose uptake, b) muscle structure, and c) mRNA levels of genes associated with glucose metabolism and muscle contractility. A secondary aim was to determine how these fetal measurements relate to plasma vitamin D concentrations.

## Chapter 2: General methods

### 2.1 Animal models

All animal procedures were conducted under UK Home Office Licence and performed in accordance with the UK Animals (Scientific Procedures) Act of 1986. Three different animal models were used in this thesis, and full details are discussed in the relevant sections of Chapter 3 (section 3.2.1), Chapter 4 (section 4.2.1) and Chapter 5 (section 5.2.1).

### 2.2 mRNA levels

#### 2.2.1 RNA extraction

Skeletal muscle was ground up in a sterile 1.5 ml tube using a sterilised (autoclaved) plastic pestle to ensure full homogenisation prior to RNA extraction. Tubes and pestles were regularly cooled in liquid nitrogen to avoid the tissue thawing and the activation of RNA degrading ribonucleases. Crushed muscle tissue was stored at -80 °C.

Frozen crushed samples were homogenised in 1 ml of peqGOLD TriFast™ (VWR, USA) until no visible fragments remained, and incubated at room temperature for 5 min. Chloroform (0.2 ml/ml TriFast™; VWR, USA) was added to allow phase separation, samples were shaken vigorously by hand for 15 sec and incubated at room temperature for 5 min. After centrifugation (10 min at 12,000 x g at 4°C), the aqueous phase containing RNA was transferred into a fresh sterile tube. The DNA and protein phases were also collected and stored at -80°C. For RNA precipitation, 500 µl isopropanol (0.5 ml/ml TriFast™; Fisher Scientific, UK) was added to the aqueous phase, mixed and stored at -20°C overnight. Then, samples were centrifuged (10 min at 12,000 x g at 4 °C) to form an RNA pellet and supernatant removed. To wash the RNA pellet, 1 ml 75% ethanol was

added and the tube was vortexed and centrifuged (10 min at 12,000 x g at 4 °C) twice. Supernatant was removed and the RNA pellet was air dried for 30 min and dissolved in 30 µl nuclease-free H<sub>2</sub>O (Fisher Scientific, UK) by incubating at 60 °C for 10 min.

## 2.2.2 Determination of RNA yield and quality

### RNA yield and purity

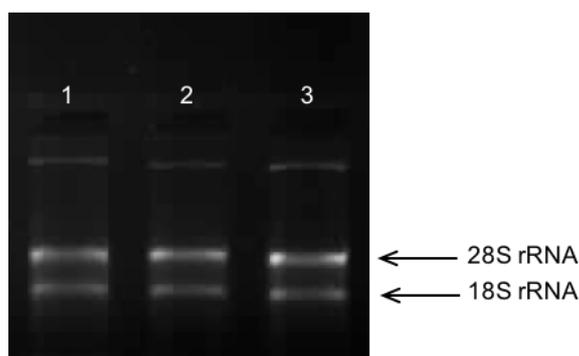
RNA yield was assessed through using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA). The NanoDrop measures the amount of RNA in 1 µl sample by measuring light absorbance at the 260 nm wavelength. The 260/280 ratio (the ratio of absorbance at 260 nm and 280 nm) is used to assess the purity of the RNA. A ratio of ~2.0 is accepted as a 'pure' RNA sample. If this value is much lower it suggests that there may be contamination of protein, phenol or other contaminants that absorb at or near 280 nm. The 260/230 ratio is used as a secondary measure of nucleic acid purity. Firstly, nuclease-free water was used to 'blank' the NanoDrop, and the two ratios were calculated to ensure there was no RNA detected. The RNA sample was then loaded onto the pedestal and the absorbance at the specific wavelengths was calculated.

### RNA quality

The RNA quality was determined by gel electrophoresis, which separates molecules based on their size and charge. RNA is negatively charged due to the presence of phosphate groups. When an electric current is passed through the agarose gel containing the RNA samples, the negatively charged RNA molecules move towards the positive electrode. The shorter the RNA fragment the faster it travels through the gel towards the positive electrode.

A 1% agarose gel was prepared by heating 1 g of agarose (Sigma-Aldrich, USA) in 100 ml 1x TBE buffer (Fisher Scientific, UK). GelRed™ (Biotium, USA) was then added and poured into a mould to set for 40 min. GelRed™ is a nucleic acid dye that allows the

nucleic acid bands to be visualised under UV light. The gel was placed in an electrophoresis tank filled with 1x TBE buffer. RNA samples were prepared by combining ddH<sub>2</sub>O, deionised formamide, loading dye and neat RNA. This was mixed, loaded into the wells of the agarose gel and run at 100 V for 30 min. The gel was placed under UV light in order to visualise the nucleic acid bands. Two clear bands (28S and 18S ribosomal RNA) show that the RNA is intact (Fig. 2.1).



**Figure 2.1: RNA gel.** Representative image of an RNA gel showing the 28s and 18s rRNA bands in three different samples of mouse skeletal muscle.

### 2.2.3 DNase treatment and cDNA synthesis

#### DNase treatment

Following RNA extraction, RNA samples were DNase-treated using Precision<sup>TM</sup> DNase (PrimerDesign, UK) to remove any contaminating genomic DNA. 2  $\mu$ l of 10x reaction buffer and 1  $\mu$ l of DNase was added to 1  $\mu$ g of RNA, and the overall volume was made up to 20  $\mu$ l by the addition of nuclease-free water. The samples were incubated at 30 °C for 10 min then at 55 °C for 15 min and cooled to 4 °C in the Veriti 96 well thermal cycler (Applied Biosystems, California, USA). RNA quality was assessed by running an agarose gel to ensure no genomic DNA was present (section 2.2.2), which is seen as a band higher up on the gel.

## **Reverse transcription (RT)**

mRNA was reverse-transcribed into cDNA by the GoScript™ Reverse Transcription System kit (Promega, UK). RNA (250 ng) was added to 1 µl of random primers and 5 µl of nuclease-free water, and heated for 5 min at 70 °C in the thermal cycler. Samples were then cooled on ice for 5 min. A reaction mixture containing 4 µl GoScript™ 5x reaction buffer, 2.4 µl MgCl<sub>2</sub> (3mM), 1 µl PCR nucleotide mix, 0.5 µl RNasin, 1 µl reverse transcriptase and 1.1 µl of nuclease-free water was added to each RNA sample. This was then heated to 25 °C for 5 min (annealing), 42 °C for 60 min (extending) and 70 °C for 15 min (inactivating reverse transcriptase). All cDNA was diluted 1:10 with nuclease-free water and stored at -80 °C.

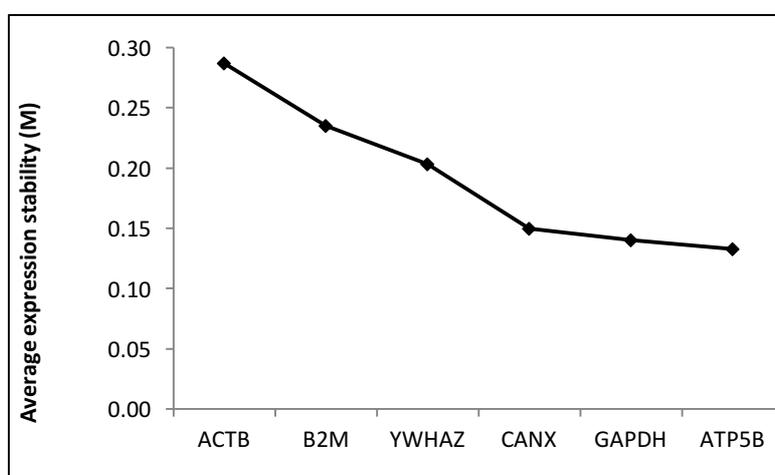
Alongside test RNA samples, several samples were run through the RT reaction to generate standards and controls. A ‘standard’ was created by pooling samples together. Four ‘no enzyme controls’ (NECs) were put through the RT reaction, by substituting 1 µl nuclease-free water for the reverse transcriptase, to ensure the results were valid and there was no contamination of genomic DNA during RNA preparation. A ‘no-template control’ (NTC), which contained nuclease-free water and no RNA, was produced as a general control to check for any nucleic acid contamination. Coefficient of variation (CV) controls to assess sample variation were produced from an RNA stock by pooling 2 µl of numerous RNA samples to give 6 identical samples.

### **2.2.4 Quantitative real-time PCR**

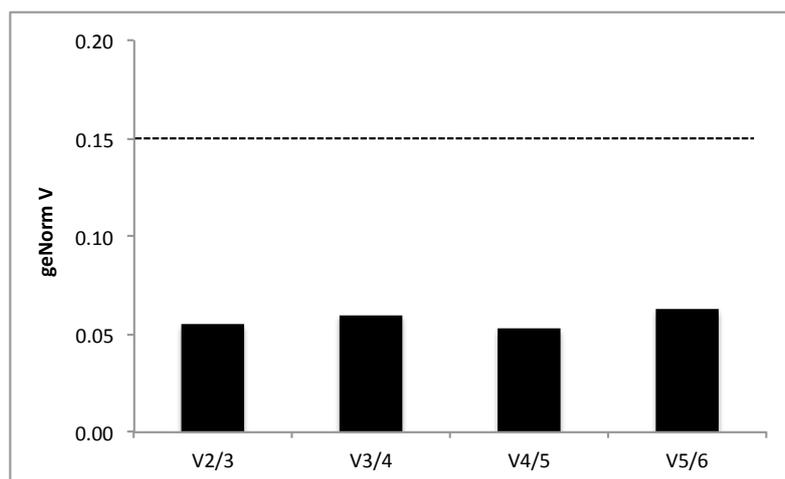
qRT-PCR was used to assess and compare the mRNA expression levels of various genes of interest in different samples. These were in relation to specific housekeeper genes (HKGs), selected from geNorm analysis.

## Housekeeper gene selection

The Primerdesign geNorm™ Reference Gene Selection Kit with Double-Dye (hydrolysis) probe for mouse was used to determine the optimal, most stable HKGs across all samples. A 6-gene geNorm was performed using YWHAZ, B2M, CANX, ATP5B, ACTB and GAPDH HKGs. Three samples per diet group per muscle type were used and an NEC and NTC were included. All samples were plated out in triplicate and all 6 HKGs were tested using the protocol below. After qRT-PCR reaction had taken place, the crossing point (cp) values were analysed using qbase+ 3.0 (Biogazelle, Belgium), and from this analysis, the software generated the average expression stability value (M) and the pairwise variation value (V). The M value shows how stable the HKGs are, with a lower M value representing a more stable HKG (Fig. 2.2). The V value determines the optimum number of reference genes to use. The graph in Figure 2.3 shows the effect of including an extra HKG and how the normalisation factor changes with the addition of another HKG. The optimal number of reference genes is when the V score drops below 0.15, indicating that there is no significant effect of adding another HKG.



**Figure 2.2:** A graph showing the M values for the tested HKGs from geNorm analysis. GAPDH and ATP5B have the lowest M score and therefore the most stable.



**Figure 2.3:** A graph showing the V score from the geNorm analysis. The use of two HKGs have a V score of below 0.15 and therefore the optimal number to use for normalisation of target genes.

### qRT-PCR protocols

Selected HKG expression was measured using the double-dye qPCR assay and the expression of target genes were measured using Roche UPL qPCR assay. Reaction mixtures (Table 2.1) and cycling conditions (Table 2.2) for both assays are shown below.

**Table 2.1:** Concentrations of PCR reaction components for the Roche UPL and Double-dye hydrolysis qPCR assays.

PCR component	Genes of interest	geNorm/housekeepers
	(Roche UPL)	(Double-dye hydrolysis)
Lightcycler 480 Probe Master Mix	5 $\mu$ l	5 $\mu$ l
Double-dye primer/probe mix (300nM)	-	0.5 $\mu$ l
Forward primer (33nM)	0.33 $\mu$ l	-
Reverse primer (33nM)	0.33 $\mu$ l	-
Probe (133nM)	1.33 $\mu$ l	-
cDNA (4ng)	3 $\mu$ l	3 $\mu$ l
ddH <sub>2</sub> O	-	To final volume
Final volume	10 $\mu$ l	10 $\mu$ l

**Table 2.2: Cycling conditions for Roche UPL and Double-dye hydrolysis qPCR assays.** \* data collection step.

<b>Cycling conditions</b>	<b>Genes of interest (Roche UPL)</b>	<b>geNorm/housekeepers (Double-dye hydrolysis)</b>
Polymerase activation	95°C for 10 min	95°C for 10 min
Product amplification	45 cycles of: 95°C for 10 sec 60°C for 30 sec 72°C for 1 sec*	40 cycles of: 95°C for 15 sec 60°C for 30 sec*
Cooling	40°C for 30 sec	40°C for 30 sec

### Calculation of values

A 7-point standard curve, produced by performing 1:2 serial dilutions from the original standard sample (see section 2.2.3), was used to determine the concentration of genes of interest for each test sample. The log concentration of each standard was plotted against the Cp value (Fig. 2.4) obtained from the Second Derivative Maximum method (Roche, UK). The Cp value is identified as the point in which the acceleration of the fluorescence signal is at its maximum (when the fluorescence of the reaction reaches the maximum of the second derivative of the amplification curve). As a result, the Cp value should always be located in the middle of the log-linear part of the qPCR amplification plot. Plotting the log concentration of each standard against its Cp value generates a straight line. The cDNA concentration of each test sample was determined from its Cp value and standard curve parameters using the equation below.

$$x = 10^{(y - c)/m}$$

$x$  = concentration,  $m$  = gradient of the line,  $c$  =  $y$  intercept,  $y$  = Cp value of the test sample

The cDNA concentration of the CV controls was determined to test for experimental variability using the equation  $CV = (\text{Standard deviation} / \text{mean}) \times 100$ . All cDNA samples were normalised by dividing the gene of interest concentration by the geometric mean of the selected HKGs for that sample.

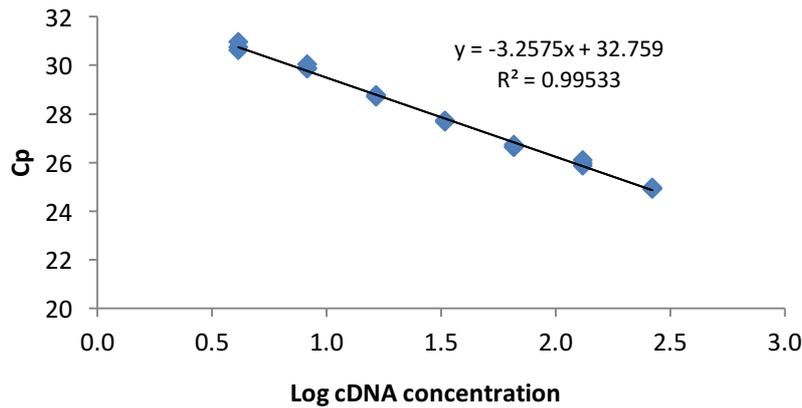


Figure 2.4: An example of a qPCR standard curve.

## 2.3 Immunofluorescence staining

An immunofluorescent staining protocol was established to assess the different types of muscle fibres in a sample. The procedure was adapted from Bloemberg and Quadrilatero's method (Bloemberg & Quadrilatero 2012).

### 2.3.1 Sectioning of muscle samples

Soleus and EDL *m.* samples previously slow-frozen in freezing isopentane were cut using a cryostat (Leica Biosystems, Germany) maintained at -20 °C. The sample was cut into 10µm thick transverse sections at the mid-belly. The quality of the muscle tissue was assessed by staining the sections with Mayers hematoxylin (Sigma-Aldrich, USA) for 60 sec and observing the histology under a light microscope. Sections for staining were then cut onto APES covered glass slides, left to air-dry for 30 min and stored at -80 °C for a maximum of two weeks until staining.

### 2.3.2 Primary antibody optimisation

Primary antibodies against the myosin heavy chain (MHC) proteins MHCI (BA-F8), MHCIIA (SC-71), MHCIIB (BF-F3) and MHCIIIX (6H1; DSHB, University of Iowa, USA) found in different types of myofibres were titrated. This was to find the optimal concentration of each primary antibody for maximal strength of staining with minimal background. The dilutions tested were based around those used by Bloemberg & Quadriatero. All antibodies were stored in 50:50 glycerol (Sigma-Aldrich, USA) and phosphate-buffered saline (PBS; Fisher Scientific, UK) at -20 °C.

In order to block non-specific protein interactions, 10% goat serum (Sigma-Aldrich, USA) in PBS was used. There was a high level of background staining, therefore 1% bovine serum albumin (BSA; Sigma-Aldrich, USA) was used as a protein buffer for blocking instead, and 0.1% Tween 20 was also added to lower background fluorescence.

**Table 2.3: Concentrations of the primary and secondary antibodies used for immunofluorescence staining.**

Primary antibody			Secondary Antibody		
Type	Concentration	MHC Reactivity	Type	Concentration	Colour
Cocktail # 1					
BA-F8	1:25	I	Alexa Fluor 350 IgG <sub>2b</sub>	1:500	Blue
SC-71	1:400	IIA	Alexa Fluor 488 IgG <sub>1</sub>	1:500	Green
BF-F3	1:50	IIB	Alexa Fluor 555 IgM	1:500	Red
Cocktail #2					
6H1	1:50	IIX	Alexa Fluor 555 IgM	1:500	Red
SC-71	1:400	IIA	Alexa Fluor 488 IgG <sub>1</sub>	1:500	Green

Following titration, staining for MHCIIIX with the primary antibody 6H1 still showed high background staining, making it hard to define myofibres. A new batch of 6H1 was tested and 0.1% Tween was taken out of the 1% BSA protein buffer solution. Staining of type IIX fibres with the new 6H1 antibody and altered protein buffer improved with a reduction in

background fluorescence. The new 6H1 primary antibody was titrated. As a result of the optimisations, primary antibodies were made up into two different cocktails as displayed in Table 2.3.

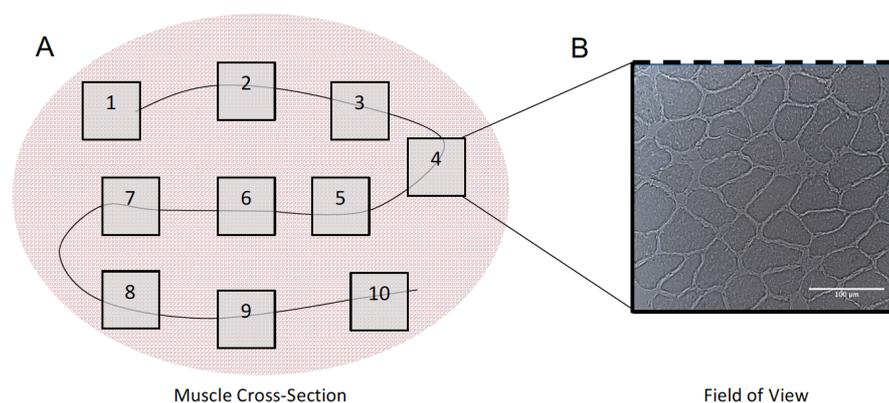
### **2.3.3 Staining procedure**

For all blocking, washing, antibody dilutions and negative controls, two solutions were made up for the two primary antibody cocktails. Cocktail 1 included 1% BSA in PBS with 0.1% Tween 20 and cocktail 2 included 1% BSA in PBS alone. All steps were carried out at room temperature.

Muscle cryosections stored at -80 °C were left to air dry for 60 min and then incubated in the relevant cocktail solution for a further 60 min in order to block non-specific protein interactions. Sections were incubated with the primary antibodies (Table 2.3) for 120 min. The first slide was used for cocktail 1 (BA-F8, SC-71, BF-F3), the second slide was used for cocktail 2 (6H1, SC-71), and the third and fourth for the corresponding negative controls (protein buffer solution only). All sections were washed 3x 5 min. The secondary antibodies (Table 2.3; Life Technologies, USA) were applied and incubated for 60 min in the dark. Sections were washed again 3x 5 min and slides mounted with Mowiol anti-fade reagent (made by David Johnston, Biomedical Imaging Unit, University of Southampton). Slides were stored in the dark to protect the fluorescent dye from the light.

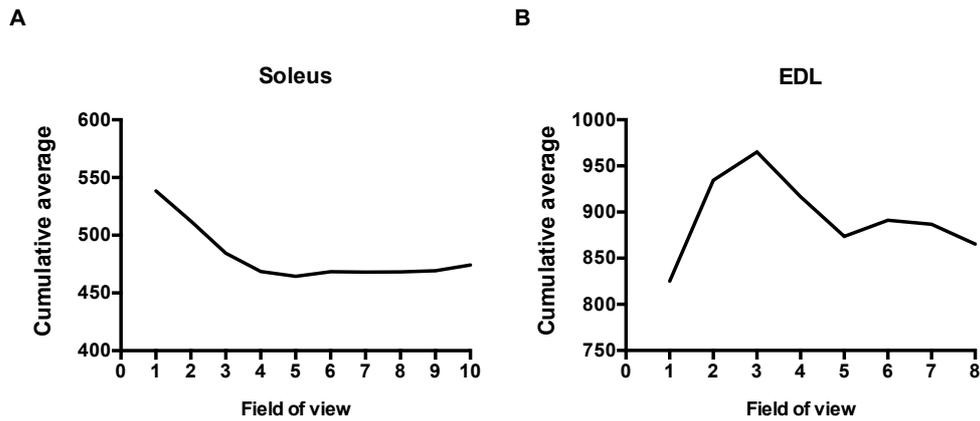
### **2.3.4 Image capture and and analysis**

All images were taken using the Zeiss Axioskop 2 microscope with Axiocam (Zeiss, UK) and KS400 software (Image Associates, UK). For each myofibre type/secondary antibody a specific microscope filter was used to visualise each isoform of MHC. Initially, up to 10 images at x40 magnification across the muscle section were taken as shown in Figure 2.5 in order to validate the optimal number of fields of view (detailed below). All images were anonymised to ensure there was no bias during the image analysis process.



**Figure 2.5: Image capture.** A) The pattern for image capture of different fields of view for a muscle section. B) Unbiased counting frame. Only fibres within the box or crossing the solid lines were counted. Scale bar represents 100  $\mu\text{m}$ .

Fiji Image J was used for image analysis on each field of view. Images were calibrated using a x40 graticule. In order to assess myofibres density, an image stack was created and myofibres were counted using the cell counter plugin. An inclusion/exclusion criteria was used where only the myofibres completely in field of view or crossing the solid borders were counted (Figure 2.5). The area of countable myofibres was determined by subtracting the areas of artefact and poor histology from the total image area. Density was calculated as number of myofibres per  $\text{mm}^2$  tissue. This analysis protocol was validated by two processes. The first was by checking the coefficient of variation ((COV; (standard deviation/mean)\*100)) between the repeated fields of view by repeating the density analysis from one control soleus and EDL *m.* sample six times on different days. Mean COV for the soleus and EDL *m.* control samples were 1.42% and 0.94%, respectively. The second validation step was to determine the optimal number of fields of view by calculating the cumulative average of myofibre density across ten fields of view for the soleus *m.* and eight (due to a smaller cross-section) for the EDL *m.* The myofibre density plateaued at five fields of view for both muscles (Fig. 2.6), and so this was the number of images used for all immunofluorescent myofibre image analysis.



**Figure 2.6: Cumulative total myofibre density averages.** A) Soleus and B) EDL muscle samples. EDL, extensor digitorum longus.

To calculate cross-sectional area (CSA) the myofibres completely in view were drawn around and the area was measured in  $\mu\text{m}^2$ . As a result of the cumulative density validation, 5 fields of view were analysed. The COV was also determined for myofibre CSA for the soleus (2.29%) and EDL (2.25%) *m*.

## 2.4 Isometric skeletal muscle contraction

### 2.4.1 Load cell calibration

At the start of each experimental day, a 1 g weight (held inside a paper basket) was attached to a piece of thread and connected to the load cell (1N S100 load cell; Strain Measurement Devices, USA) by a crocodile clip for calibration. The load cell was then connected to a transducer amplifier (FLYDE, UK). The thread and weighted basket were hung over a support bar in a way that mimicked the direction of the force applied to the load cell when the muscle was being contracted. The change in voltage produced by applying the weight to the load cell was measured and converted to a force reading using the equation below.

$$F = mg$$

$F$ = force (N),  $m$ = mass (kg),  $g$  = gravitational acceleration ( $9.81\text{m/s}^2$ )

## 2.4.2 Experimental set-up

The soleus and EDL *m.* from the right leg were used for the skeletal muscle isometric contraction experiments. The soleus was always experimented with first to ensure consistency between animals. The dissected muscle was placed into a flow-through organ bath containing oxygenated Krebs buffer (119 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO<sub>4</sub>, 25 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 11 mmol/L D-glucose and 2.5 mmol/L CaCl<sub>2</sub>, pH 7.55 before oxygenation with 95% O<sub>2</sub>, 5% CO<sub>2</sub>; Sigma-Aldrich, USA) at  $35\pm 0.5^\circ\text{C}$ . One end of the muscle was held in place by modified forceps, attached to the tendon, connected to a V101 shaker (Ling Dynamic Systems, UK), and the other end was held at the bone with a crocodile clip connected to the load cell. Once attached, the muscles were left in the organ chamber to acclimatise for 15 min. Silver electrodes (5 mm long, 0.37 mm diameter), which apply the electrical stimuli for contraction, were placed parallel to the muscle (but not in contact with the muscle) at the mid-belly of the muscle. Experimental set up is shown in Figure 2.7.

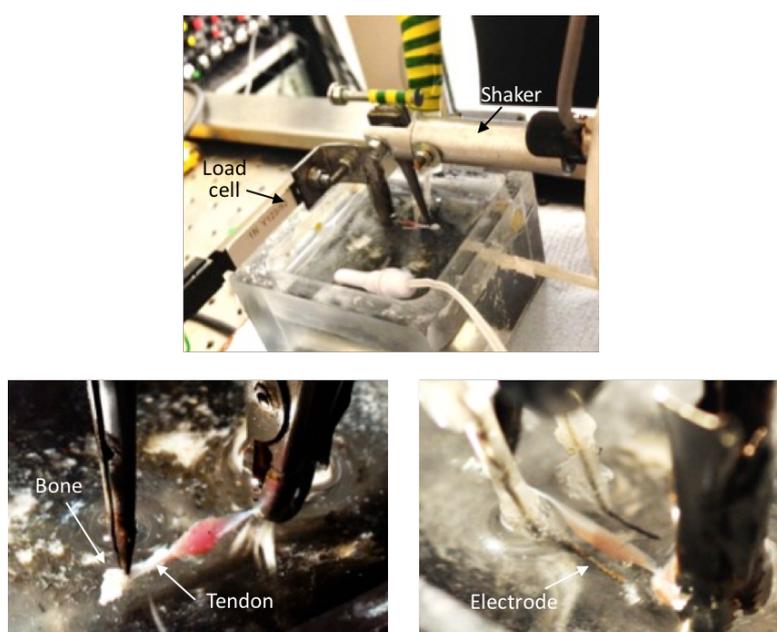


Figure 2.7: Skeletal muscle isometric contraction set up.

During contraction, the signal from the load cell was passed through the transducer amplifier. The transducer amplifier had a high-frequency cut-off of 10 kHz, and a Neurolog NL125 filter system (Digitimer, UK) applied a low pass filter of 400 Hz and notch filter of 50 Hz to the signal. The signal generated from the load cell was sampled at 10 kHz through the use of a CED1401 A/D interface (Cambridge Electronic Design, CED; UK). Force generated by the contracting muscle was recorded using Spike2 version 7.05b (CED).

### **Length optimisation**

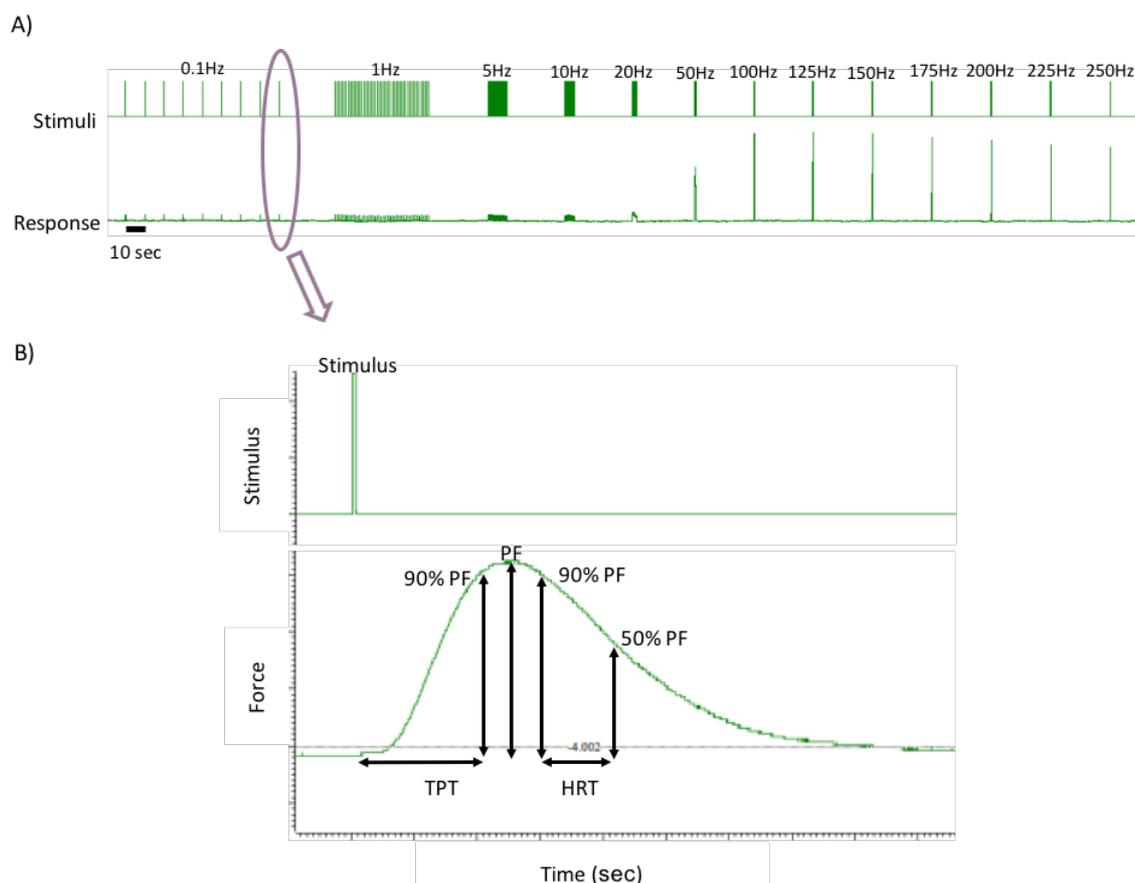
After acclimatisation in the organ chamber for 15 min, the optimal muscle length for muscle contraction was determined. The muscle was increased in 0.2 mm increments until the optimal length was found. The length at which the muscle produced a large active force with a minimal increase in passive force determined the optimal muscle length.

### **Isometric protocol**

The muscles were stimulated at several frequencies (0.1, 1, 5, 10, 20, 50, 100, 125, 150, 175, 200, 225 and 250 Hz) and the muscle force response was recorded in Spike2 (Fig. 2.8A). The stimulation protocol was designed in Spike2 with 1ms square wave pulse stimuli and a 30 second rest period in between each different frequency. The signal generated from Spike2 was delivered to a Micro1401 DAC (CED) that then passed to the input signal to a Master 8 stimulator (A.M.P.I, Israel). A pulse with a maximum of 10 V was produced and sent to a Neurolog NL800 Stimulus Isolator, which limited the stimuli current to 10 mA. The stimuli voltage produced by the Master 8 Stimulator was set to 10 V as this generated maximal muscle contractile force.

Following each experiment, the Spike2 files were exported to Matlab and the peak force (PF; maximum force generated) was determined and analysed (Fig. 2.8B). Time-to-peak tension (TPT; time taken from stimulation to 90% of PF) and half-relaxation time (HRT; time taken from 90% to 50% PF) will be determined and analysed in the near future following the parameters used previously (Law & Shields 2005). PF was measured for the individual responses at 0.1, 1, 5 and 10 Hz, and then an average at each frequency was

calculated. A single measurement was determined for the response at 20-250 Hz due to the summation of individual responses forming a tetanic response across the pulse train.



**Figure 2.8: Representative diagram of the numerous electrical stimuli and muscle response. A)** The pattern of electrical stimuli applied to the muscle. **B)** The muscle isometric contraction response (volts) over time of one stimulus at 0.1 Hz and the three different parameters which will be analysed: peak force (PF), time-to-peak tension (TPT) and half-relaxation time (HRT).

## 2.5 Blood plasma analysis

Blood plasma was analysed for 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub>, total 25(OH)D, 24,25(OH)<sub>2</sub>D<sub>2</sub>, 24,25(OH)<sub>2</sub>D<sub>3</sub> and total 24,25(OH)<sub>2</sub>D by high performance liquid chromatography tandem mass mass spectrometry (LC-MS) as detailed previously (Owens *et al.* 2014; Tang *et al.* 2017). Where dietary vitamin D has been specifically manipulated in this thesis it is always manipulation of vitamin D<sub>3</sub>. However, D<sub>2</sub> and D<sub>3</sub> metabolites contribute to overall vitamin D status and originate from different sources (discussed section 1.4.1). Therefore quantifying if manipulation to vitamin D<sub>3</sub> had an effect downstream in the vitamin D

pathway, and checking whether levels of the D<sub>2</sub> isoform were at an unexpected level was important. The 1,25-Dihydroxy Vitamin D enzymeimmunoassay kit (AC-62F1, Immunodiagnostic Systems, UK) was used when possible to measure plasma 1,25(OH)<sub>2</sub>D concentration in order to determine downstream effects of vitamin D manipulation on the vitamin D biosynthesis pathway, however this assay was not able to distinguish between the D<sub>2</sub> and D<sub>3</sub> isoforms. Calcium, albumin, and phosphate were measured using standard commercial assays (Roche Diagnostics, UK). The above plasma analysis was performed at The University of East Anglia. Serum ionized calcium was measured using an automated analyser (electrode E733, ABL800 Flex, Radiometer, UK) and parathyroid hormone (PTH) was analysed by a chemiluminescent immunoassay (A16972, Beckman Coulter, UK) at the University Hospital Southampton Clinical Biochemistry Department. Albumin was measured due to its involvement in binding to vitamin D for vitamin D transport as discussed in section 1.4.2. Calcium, phosphate and PTH were measured due to their close association with vitamin D in mineral homeostasis, as discussed in section 1.4.1. Fasting blood glucose levels were also obtained at post-mortem using the Aviva Blood Glucose Meter System (Accu-Chek, UK).

## **2.6 Statistical analysis**

All data were statistically analysed with SPSS Statistics version 22 (IBM, UK) and all graphs were produced in Prism 7 (GraphPad Software, USA). Prior to statistical modelling, all data were tested for normal distribution by visually assessing histograms and calculating skewness (accepted as normally distributed if between -1 and +1). Data not normally distributed were log-transformed and reassessed.

### **2.6.1 Maternal data analysis**

All maternal data was statistically analysed using an independent t-test. The mixed effects model analysis (detailed section 2.6.2) was not required as each dam originated from separate litters. All maternal data are presented as mean ± SEM. In Chapter 5, maternal data was adjusted for gestational age.

## 2.6.2 Offspring data analysis

In Chapter 3 and 4, a mixed effects model was used to statistically analyse the offspring data and statistical advice on this model was provided by Prof. Clive Osmond. A two-way ANOVA could be used to determine interactions between the prenatal and postnatal diet, however, it does have fundamental weaknesses. A two-way ANOVA assumes independence between data entries (offspring) and was therefore not appropriate for the analysis of mouse offspring data. Mouse models were used in these two chapters and have multiple pups in one pregnancy. Where two offspring born to the same dam were used in the analysis they will have shared the same treatment exposure and are more likely to be similar in outcome measures than two offspring from different dams. This therefore defies the assumption that each offspring has independence. The litter-of-origin should therefore be considered as the ‘experimental unit’ instead of the individual offspring, whereas a two-way ANOVA regards each individual offspring as the experimental unit. To take ‘litter-of-origin effects’ into consideration, which is beyond the scope of a two-way ANOVA, a mixed effects model was applied.

This mixed effects model takes the hierarchical nature of the data sets and, by adding the litter-of-origin as a ‘random effect’, the dam effects (within-mother and between-mother) are taken into account. This model also allows for litter size effects and difference in sampling age to be accounted for, and so all data and statistics reported are independent of litter-of-origin, litter size and age at post-mortem. Due to multiple offspring regularly coming from the same litter, it would be inaccurate to present the observed values as it would not reflect the true variation in data. Consequently, data presented are the adjusted ‘predicted’ group means  $\pm$  SEM derived from the maximal models containing all of the ‘fixed’ effects: diet group, litter size and age. This statistical model has previously been advocated (Osmond *et al.* 2005; Lazic & Essioux 2013). Prior to statistical analysis all data was z-transformed in order to standardise variables across the chapter and to allow for the predicted data to be compared directly. Correlations between two variables in Chapter 3 and 4 were also analysed by the mixed effects model, and the data are represented as the correlation coefficient  $r^2$ .

In Chapter 5, fetal sheep data were statistically analysed using an independent t-test or one-way ANOVA. The mixed effects model (detailed above) was not required for the fetal sheep data as each offspring came from a separate mother. All fetal sheep data were age-

adjusted prior to statistical analysis in order to account for slight differences in age at post-mortem. Data are presented as age-adjusted mean  $\pm$  SEM. Linear regression was used to determine the relationship between two age-adjusted variables.

### **2.6.3 Statistical power**

Power calculations could not be performed before the onset of this thesis in order to determine appropriate experimental group sizes for each chapter due to the absence of suitable pilot data from previous studies. In Chapter 3, the sample sizes used (n=7-8 per group) were dictated by the availability of animals as part of an ongoing mouse study on glucose tolerance and liver function (Thomas Ph.D Thesis 2017). In Chapter 4, sample size was restricted (n=3-9) as a subset of male offspring developed abnormal livers and were excluded from the study (discussed in section 4.2.2). In Chapter 5, the number of pregnant sheep was limited to n=9 in both diet groups, and with no appropriate pilot fetal sheep data to perform power calculations on, all animals were included in this study. Post-hoc power calculations were not carried out due to the very limited data set on which to derive a meaningful standard deviation.

# Chapter 3: The effect of prenatal and post-weaning high-fat diet on adult mouse offspring skeletal muscle

## 3.1 Introduction

Optimal muscle function is important for metabolic function and an individual's strength. Current concepts suggest that the intrauterine environment is important in determining peak adult muscle mass and its decline with ageing (Sayer *et al.* 2008). There are numerous early life triggers for altered muscle development (and therefore function) including undernutrition, overnutrition, and more specifically deficiencies in micronutrients such as vitamin D. There is a link between obesity and vitamin D, and to date, the relationship between obesity and vitamin D in driving skeletal muscle development and later muscle function is little investigated. There is evidence that a high BMI in adults is associated with a lower 25(OH)D blood plasma concentration (Liel *et al.* 1988). It is thought that the increased adipose tissue of obese individuals may sequester vitamin D, leading to a reduced bioavailability in the blood (Wortsman *et al.* 2000). Obese individuals also tend to have reduced levels of physical activity, and if they spend more time indoors they won't be exposed to the UVB rays from the sun which convert 7-dehydrocholesterol in the skin to vitamin D, contributing to the reduction in plasma 25(OH)D. Given the evidence for a reduction in vitamin D levels with obesity, and that VDD and obesity are highly prevalent in pregnancy, this chapter investigates the potential relationship between vitamin D status and skeletal muscle function in the offspring of an established model of high-fat-induced maternal obesity.

Studies analysing undernutrition during pregnancy have found that a lower body condition score (LBCS) in the mother during pregnancy resulted in a reduced total myofibre density and altered glucose/insulin signalling in fetal sheep (Costello *et al.* 2008), which may

influence skeletal muscle health and metabolism in later life. Obesity is highly prevalent in the UK and worldwide. The high rate of maternal obesity is problematic as it leads to pregnancy complications, such as issues in labour, and it compromises the health of the mother (e.g. increasing the risk of gestational diabetes). Moreover, maternal obesity affects the health of the offspring (Poston *et al.* 2011). Studies have shown that obesity is a factor in altering skeletal muscle function and development, both in prenatal and postnatal life. In rats fed a high-fat diet during pregnancy and lactation, there was an alteration in offspring muscle structure at 21 days of age as shown by a reduction in myofibre number and an increase in lipid deposition within the muscle (Bayol *et al.* 2005). When these rats were older at 10 weeks of age, the peak force and peak force relative to muscle weight for both twitch and tetanic contraction were lower compared to rats fed a control diet throughout pregnancy (Bayol *et al.* 2009), indicating that high-fat feeding during pregnancy and lactation leads to altered offspring skeletal muscle structure and force. Another mouse study found reduced muscle mass and locomotor activity in offspring of obese dams (Samuelsson *et al.* 2008).

Glucose uptake and insulin sensitivity in muscle are also important for muscle contractile function. Obesity is a major risk factor for type 2 diabetes, a condition associated with impaired glucose metabolism. Bayol *et al.* also found reduced insulin receptor mRNA levels and a trend for a decrease in glucose transporter type 4 mRNA levels in the muscle of offspring with a maternal obesogenic diet, two genes involved in insulin signalling and glucose uptake (Bayol *et al.* 2005). Human and mouse studies show that obesity in adult life affects skeletal muscle structure with obesity inducing increased intramuscular lipid deposition and a reduction in the expression of myogenic and contractile proteins leading to muscle atrophy (Pellegrinelli *et al.* 2015). In adult mice, a chronic high-fat diet reduced grip strength and lean mass (Lee *et al.* 2015a) and in humans (48-92 years old) a higher waist circumference, a marker of central obesity, was associated with lower grip strength (Keevil *et al.* 2014).

However, there is little evidence overall to date as to whether an obesogenic environment with a potentially altered VDD environment prenatally will influence how the offspring skeletal muscle responds to the postnatal obesogenic environment. It has been suggested that the long-term effects of maternal nutrition may be detrimental to the offspring if

postnatal nutrition does not match that predicted by the fetus (Gluckman & Hanson 2004). This concept of predictive adaptive responses (PARs), as discussed in section 1.2.2, could mean that changes to the maternal obesogenic/VDD environment influences the way in which an individual's muscle responds to the presence or absence of an obesogenic/VDD diet postnatally.

Vitamin D during pregnancy is another potential driver for changes in skeletal muscle function and structure in various species including humans, pigs and sheep (Harvey *et al.* 2014; Hines *et al.* 2013; Cleal *et al.* 2017). Vitamin D status in adult life has been positively correlated with skeletal muscle mass, strength and risk of falls (Bischoff-Ferrari *et al.* 2004a; Wicherts *et al.* 2007a; Harvey *et al.* 2014), suggesting the importance of vitamin D for skeletal muscle function. However, more understanding of the mechanisms linking changes to vitamin D handling and muscle development and function is required. The prenatal period may be able to change how muscles metabolise vitamin D and how they respond to changes in blood glucose and fatty acid concentrations in postnatal life. The placenta is the interface between mother and fetus, and with it being the only route for nutrients such as vitamin D, amino acids, fats and glucose to reach the fetus, it's an important factor to take into account as it is responsive to maternal and fetal nutritional cues (Lewis *et al.* 2012). In fact, research from our group found that maternal undernutrition affects the placenta through altered handling of vitamin D, amino acids and fatty acids, with lower levels of 25(OH)D associated with a reduction in fetal tricep muscle myofibre density in a sheep model of undernutrition (Cleal *et al.* 2017). This study states “the placenta may integrate nutritional cues in the face of maternal dietary challenges and alter fetal physiology”, and so provides evidence of how a ‘poor’ maternal diet can affect how the offspring's nutrient handling and muscle structure is impacted in adulthood.

This thesis chapter therefore investigates how a high-fat diet in prenatal and postnatal life affects the adult offspring's vitamin D homeostasis and skeletal muscle development, structure and function across the life course (Fig. 3.1).

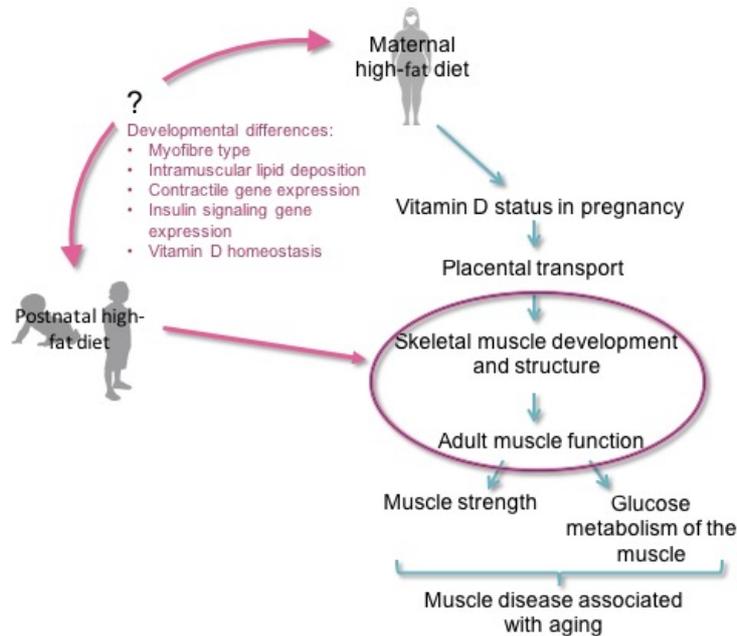


Figure 3.1: Schematic diagram of Chapter 3 flow of logic.

### 3.1.1 Hypothesis and aims

#### Hypothesis

Prenatal high-fat diet will alter the deleterious effect of a post-weaning high-fat diet on offspring skeletal muscle contraction. This effect will be linked to vitamin D status, changes in muscle fibre composition and also alterations in mRNA expression of genes associated with muscle contractility, glucose handling and insulin signalling.

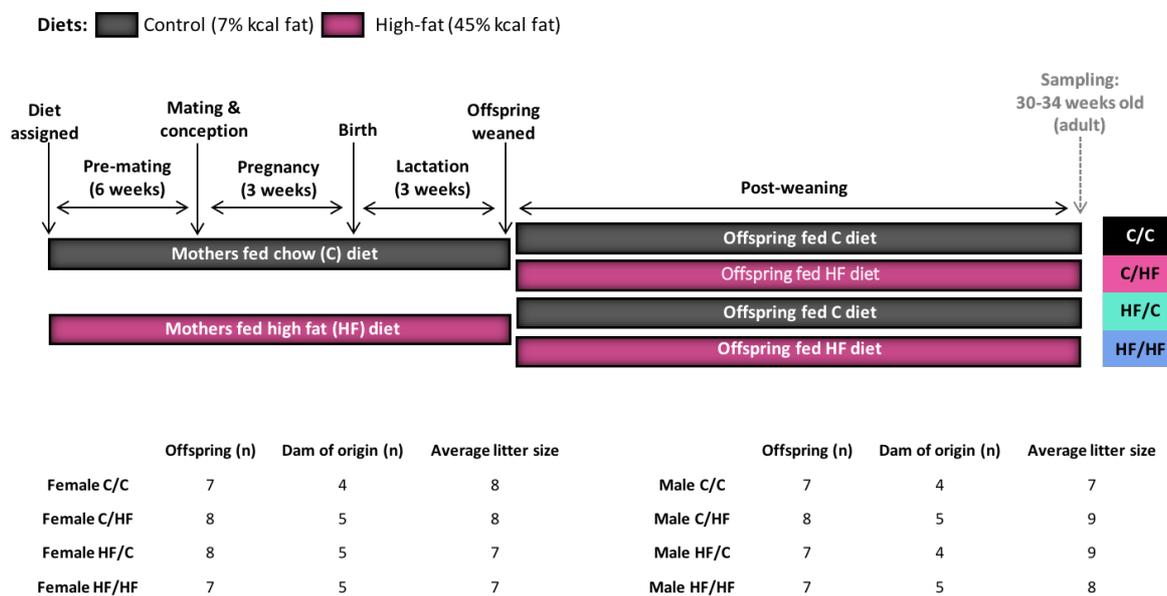
#### Aims

To investigate whether the effect of a postnatal high-fat diet is altered by an exposure to a prenatal high-fat diet on adult mouse skeletal muscle function by analysing contractile force and contractile kinetics. Mechanisms helping to explain these functional findings will involve analysis of a) myofibre structure and b) mRNA levels of genes associated with skeletal muscle contractility and glucose regulation. The secondary aim is to determine how these offspring measurements relate to plasma vitamin D status.

## 3.2 Methods

### 3.2.1 High-fat mouse model

Female C57BL/6 mice, who were proven breeders, were randomly assigned to be fed either a control (C; 7% kcal fat; RM1 – Special Diet Services, UK; Appendix A) or high-fat (HF; 45% kcal fat; 824053 – Special Diet Services, UK; Appendix A) diet *ad libitum* 6 weeks prior to mating and throughout lactation. Female and male offspring were randomly assigned to be weaned onto the C or HF diet creating 4 different diet groups: C/C, C/HF, HF/C and HF/HF (Fig. 3.2). Mice were housed under a 12hr light/dark cycle to maintain normal circadian rhythms and were given constant access to water. Offspring from different litters were used when possible, but this was not always achievable, and so multiple offspring from the same dam were regularly included. The number of offspring, dams of origin and average number of pups per litter for each diet group are displayed in Figure 3.2.



**Figure 3.2: High-fat fed obese mouse experimental model.** Female 30 wk offspring: C/C n=7, C/HF n=8, HF/C n=8, HF/HF n=7. Male 30 wk offspring: C/C n=7, C/HF n=8, HF/C n=7, HF/HF n=7. C, control diet; HF, high-fat diet.

### 3.2.2 Tissue collection

#### 30-week cohort

At 30-34 weeks of age, mice were fasted overnight and anaesthesia was induced by intraperitoneal sodium pentobarbitone (80 mg/kg or 90 mg/kg for a C or HF diet post-weaning groups, respectively). The foot was pinched to check for a foot withdrawal response to test the depth of anaesthesia. A blood sample was taken via a cardiac puncture whilst the heart was still beating, and centrifuged at 2000 xg for 10 min at 4 °C in a LiHep tube. The plasma was stored at -80 °C for future analysis. Whole blood glucose was determined by the Aviva Blood Glucose Meter System (Accu-Chek, UK). The heart, a section of left lobe from liver, kidneys and a sample of abdominal fat were dissected under sterile conditions and immediately snap-frozen in liquid nitrogen and stored at -80 °C for future analysis. Mice were culled by cervical dislocation, hind legs skinned and cut off, and the left leg placed in sterile foil (molecular work). The right leg was placed in ice-cold oxygenated Krebs buffer (119 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO<sub>4</sub>, 25 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 11 mmol/L D-glucose and 2.5 mmol/L CaCl<sub>2</sub>, pH 7.55 before oxygenation of 95% O<sub>2</sub> and 5% CO<sub>2</sub>) for analysis of muscle contraction. The soleus, EDL, gastrocnemius and tibialis anterior (TA) *m.* were dissected from both legs without dissection tools coming into contact with the muscle itself. Muscles from the left leg were snap-frozen in liquid nitrogen for gene expression analysis. Right leg gastrocnemius and TA *m.* were slow-frozen in freezing isopentane for histology, and the soleus and EDL *m.* were used for analysis of muscle contraction and then, at the completion of these experiments, they were slow-frozen in freezing isopentane. All frozen muscles were stored at -80 °C.

### 3.2.3 Blood plasma analysis

As described in full in section 2.5, blood plasma was analysed for 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D via LC-MS for both maternal and offspring samples. Some plasma

samples had 25(OH)D<sub>2</sub> levels lower than the limit of quantification (<0.1 nmol/L, LOQ), and these samples are classed as 0.1 nmol/L, as detailed in the appropriate graphs. In offspring plasma samples alone, standard assays were used to quantify calcium, phosphate and albumin concentrations.

### 3.2.4 Isometric skeletal muscle contraction

Full details are presented in section 2.4. Briefly, the load cell was calibrated prior to experiments each day and the muscle was then attached by metal clips to the force transducer via its tendons in an organ chamber. The organ chamber contained oxygenated Krebs buffer heated to  $35 \pm 0.5$  °C for 15 min to allow the muscle to acclimatise. The optimal muscle length was determined and then the muscle was stimulated at numerous frequencies 0.1 - 250 Hz. The peak force was determined at each stimulus frequency, and then a frequency for tetanic and single twitch contraction was chosen for each muscle. For single twitch analysis 10 Hz was used for both muscles. The peak force plateaued at 150 Hz for the soleus *m.*, and so this was the chosen frequency for tetanic contraction. A plateau in peak force was not reached in the EDL *m.*, therefore 250 Hz (the highest frequency) was analysed for tetanic contraction. At these frequencies, peak force, time-to-peak tension and half-relaxation time were measured.

### 3.2.5 Immunofluorescent staining

An immunofluorescent staining technique (fully described in section 2.3) was used to determine the different types of myofibres in the slow-frozen soleus and EDL *m.* samples. Muscle sections of 10 µm thickness were left to air dry for 60 min and incubated in a buffer with BSA to prevent any non-specific protein interactions for a further 60 min. Primary antibodies (BA-F8, SC-71, BF-F3 and 6H1; Table 2.3) were applied on the muscle sections for 120 min, washed and incubated in the dark for 60 min with the secondary antibodies

(Alexa Fluor 350, 488 and 555; Table 2.3). Images were captured (x40 magnification), and the density and CSA of the different fibre types were quantified (section 2.3.4).

### 3.2.6 Oil red O staining

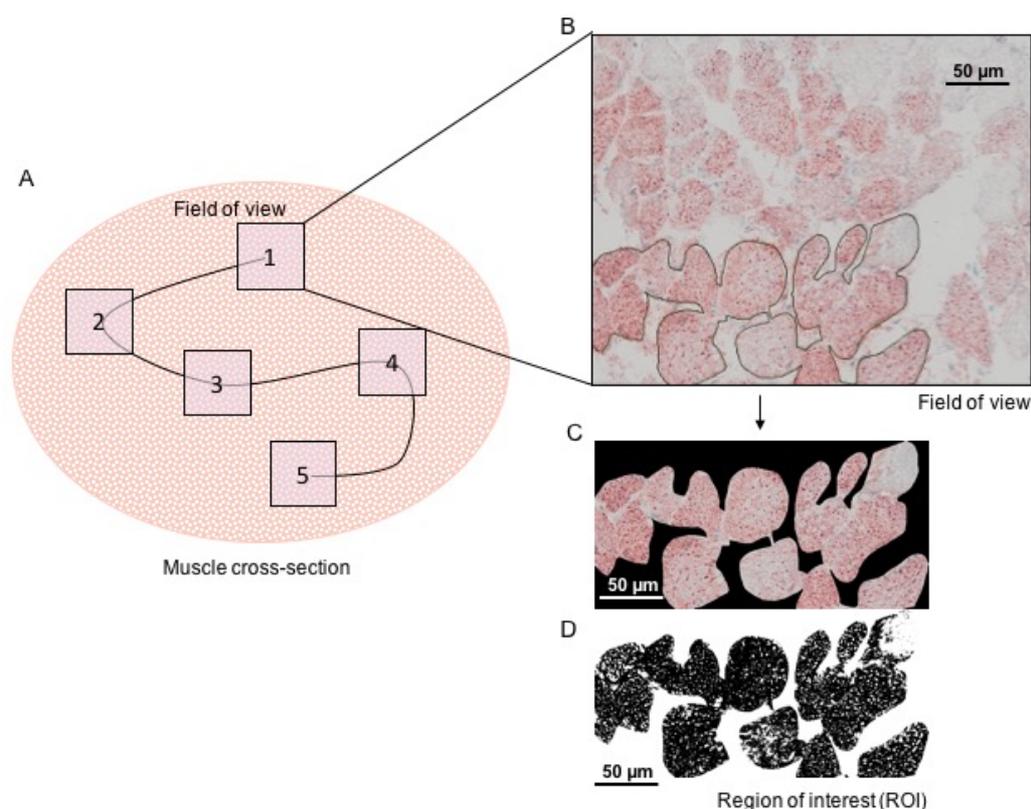
Intramuscular neutral lipid accumulation was quantified using a method adapted from Mehlem's oil red O (ORO) staining protocol (Mehlem *et al.* 2013).

#### Staining protocol

Slow-frozen muscle samples were cut into 10 µm thick transverse sections using a cryostat (as described in section 2.3.1) and stored at -80 °C. An ORO stock solution ((0.25 g ORO powder (BDH, UK) 40 ml isopropyl alcohol (Sigma-Aldrich, USA)) was prepared on a magnetic stirrer for 2 hours at room temperature and filtered through Whatman number 1 filter paper. On the day of staining, slides were defrosted at room temperature for 15 min, and a circle of liquid blocker pen was drawn around each muscle section and left to dry for a further 10 min. Just prior to use, an ORO working solution was made by mixing thoroughly 1.5 parts ORO stock with 1 part deionised water, and kept at 4 °C for 10 min to allow the solution to thicken. The working stock was filtered through a 0.45 µm syringe filter (Merck Millipore, UK) to remove ORO precipitates. Slides of muscle sections were rinsed in deionised water 3x 30 sec and the ORO working solution was applied to each section for 10 min. Slides were rinsed under flowing deionised water to prevent any stain sticking to the slides or blocker pen and further washed for 3x 30 sec. Sections were counterstained by dipping in Mayer's hematoxylin solution for 15 sec and washed in running tap water for 30 mins. Aqueous mounting medium (Bio-Rad Laboratories, USA) was applied to each section and allowed to solidify before the slides were mounted with pertex (Histolab Products AB, Sweden).

## Image acquisition and quantification

Bright-field images of the muscle sections were captured at x40 magnification using the dotSlide Virtual Slide BX51 microscope (Olympus, Japan). Five fields of view (section 2.3.4) were captured in a snaking pattern across the muscle section (Fig. 3.3A) for image processing and data quantification. In each field of view (FOV) using Fiji Image J, all of the regions of interest (ROI), excluding areas of artefact, were drawn around (Fig. 3.3B) and the total area for each ROI in each FOV was recorded ( $\mu\text{m}^2$ ). The surrounding area of each ROI was cleared causing it to turn black so it was not included in the analysis (Fig. 3.3C). A macro was written and applied to all of the ROI images which set the colour threshold to the desired minimum and maximum hue, saturation and brightness limits in order to detect the red stain alone. The stained area was recorded for each FOV and an image showing the measured area in black was created as a reference (Fig. 3.3D). ORO staining was expressed as a percentage of the tissue area from all ROIs in each FOV.



**Figure 3.3: Oil red O image analysis protocol.** **A)** Muscle cross-section showing locations of 5x field of view (FOV) for analysis. **B)** Example of one region of interest (ROI) in one FOV selected in black. **C)** ROI image for macro with lipid droplets stained red. **D)** Analysed area shown in black for reference. Scale bars represent 50  $\mu\text{m}$ .

### 3.2.7 Skeletal muscle mRNA analysis

Full details are given in section 2.2. Briefly, snap-frozen skeletal muscles were crushed, homogenised and total RNA was extracted using TriFast™. RNA quantity and quality were determined by spectrophotometry and the integrity was assessed by gel electrophoresis. RNA was DNase treated and reverse transcribed into cDNA using the GoScript™ Reverse Transcription System kit (Promega). qRT-PCR was used to measure the levels of various genes of interest (Table 3.1) in each sample. GeNorm analysis showed that GAPDH and ATP5B were the most stable housekeepers for soleus and EDL *m.* gene expression to be normalised to (section 2.2.4).

**Table 3.1: Primer sequences.** Primer sequences (5' to 3') used to measure mRNA levels of genes of interest by qRT-PCR and associated probe number.

Gene	Accession number	Primer	Sequence 5'-3'	Probe number
<i>Myh1</i>	NM_030679.1	Forward Reverse	CCT-CGC-TAG-TAA-CAT-GGA-GGTC TCCTCCTCCTTGGTCTTCAG	47
<i>Myh2</i>	NM_001039545.2	Forward Reverse	AGG-CGG-AAA-GAA-GCT-ACC-AT GTG-GTG-ATC-AGC-AGC-ATT-TC	106
<i>Myh4</i>	NM_010855.2	Forward Reverse	TGG-CCG-AGC-AAG-AGC-TAC TTG-ATG-AGG-CTG-GTG-TTC-TG	25
<i>Tnnt1</i>	NM_011618.1	Forward Reverse	GAA-GCC-TCA-GAC-TCC-CTC-TAC-A CAC-CGC-TTC-CTC-ATC-TTC-TG	63
<i>Tnnt3</i>	NM_001163664.1	Forward Reverse	CACAGCCAAGGGCAAAGT TGCTGCAGTGCACCTCTCT	11
<i>RyR1</i>	NM_009109.2	Forward Reverse	CTG-TGG-AAC-ATG-AAT-CCC-ATC GTG-GCC-TCC-AGT-CAC-AAA-G	106
<i>Acta1</i>	NM_009606.2	Forward Reverse	AAT-GAG-CGT-TTC-CGT-TGC ATC-CCC-GCA-GAC-TCC-ATA-C	11
<i>GLUT4</i>	NM_009204.2	Forward Reverse	GAC-GGA-CAC-TCC-ATC-TGT-TG GCC-ACG-ATG-GAG-ACA-TAG-C	5
<i>Insr</i>	NM_010568.2	Forward Reverse	GAT-TCC-CCA-GAT-GAG-AGG-TG AAC-GAT-GTC-ATC-TGC-CTT-AGC	5
<i>Irs1</i>	NM_010570.4	Forward Reverse	CTA-TGC-CAG-CAT-CAG-CTT-CC TTG-CTG-AGG-TCA-TTT-AGG-TCT-TC	106
<i>Igf1</i>	NM_001111274.1	Forward Reverse	GAC-CGA-GGG-GCT-TTT-ACT-TC CAT-CCA-CAA-TGC-CTG-TCT-GA	67
<i>Akt1</i>	NM_009652.3	Forward Reverse	TCG-TGT-GGC-AGG-ATG-TGT-AT ACC-TGG-TGT-CAG-TCT-CAG-AGG	45

### 3.2.8 *In vivo* strength test

Prior to post-mortem, mouse offspring underwent an established *in vivo* strength test (Deacon 2013). There were seven weights that consisted of a ball of tangled fine gauge stainless steel wire (12 g) attached to different numbers of steel chain links (7 g each). The lowest weight was placed in the cage, the mouse was held by the tail and lowered to grip onto the wire ball with its front paws. The mouse was raised up, and a stop clock started. If the mouse held on for 3 sec, the mouse rested for 30 sec and tested the next weight. However, if the mouse dropped the weight then it was given a maximum of three attempts with a 10 sec rest in between. If the mouse failed on the third attempt, the score was either calculated including the maximum weight or the maximum time achieved as detailed below:

E.g. 1: Completed 4 links but not 5 in any three attempts = (4 links x 3 sec) = 12

E.g. 2: Heaviest weight (e.g. 5 links) dropped at 2 sec = ((4 links x 3 sec) + (2 sec)) = 14

### 3.2.9 Statistical analysis

Offspring data were analysed using a mixed effects model (full details in section 2.6.2). This was so variability in dam effects (within-mother and between-mother) could be taken into account. It also allows for differences in litter size and age to be accounted for. As a result, all statistical differences displayed between diet groups are independent of the dam of origin, litter size and age. It is important to note that following statistical analysis, in the instance of this thesis chapter, litter size did not significantly influence offspring outcome measurements. Correlations were also analysed with the mixed effects model and data presented as the correlation coefficient  $r^2$  value.

Graphed data are the predicted means and SEM derived from maximal models (containing all mixed factors including diet group and litter size) applied to the observed data. For the statistical analysis, all data were Z-transformed before applying the mixed effects model in

order to standardise the dependant variables thereby allowing the effect estimates to be compared directly.

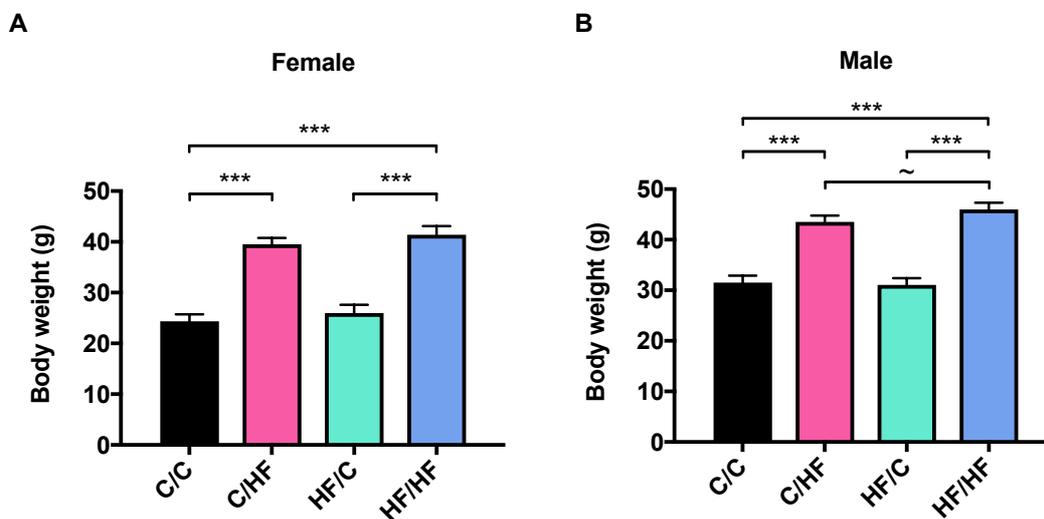
Maternal (dam) data were analysed using an independent t-test, and the data reported are the observed data. Unfortunately, any relationships between maternal and offspring variables could not be analysed as it is not known which offspring came from which dam. The maternal samples were from a subset of dams in the HF study, and not necessarily associated with all offspring used in the study.

Details of statistical analysis are given in section 2.6. SPSS version 22 was used for all analysis.

### 3.3 Results

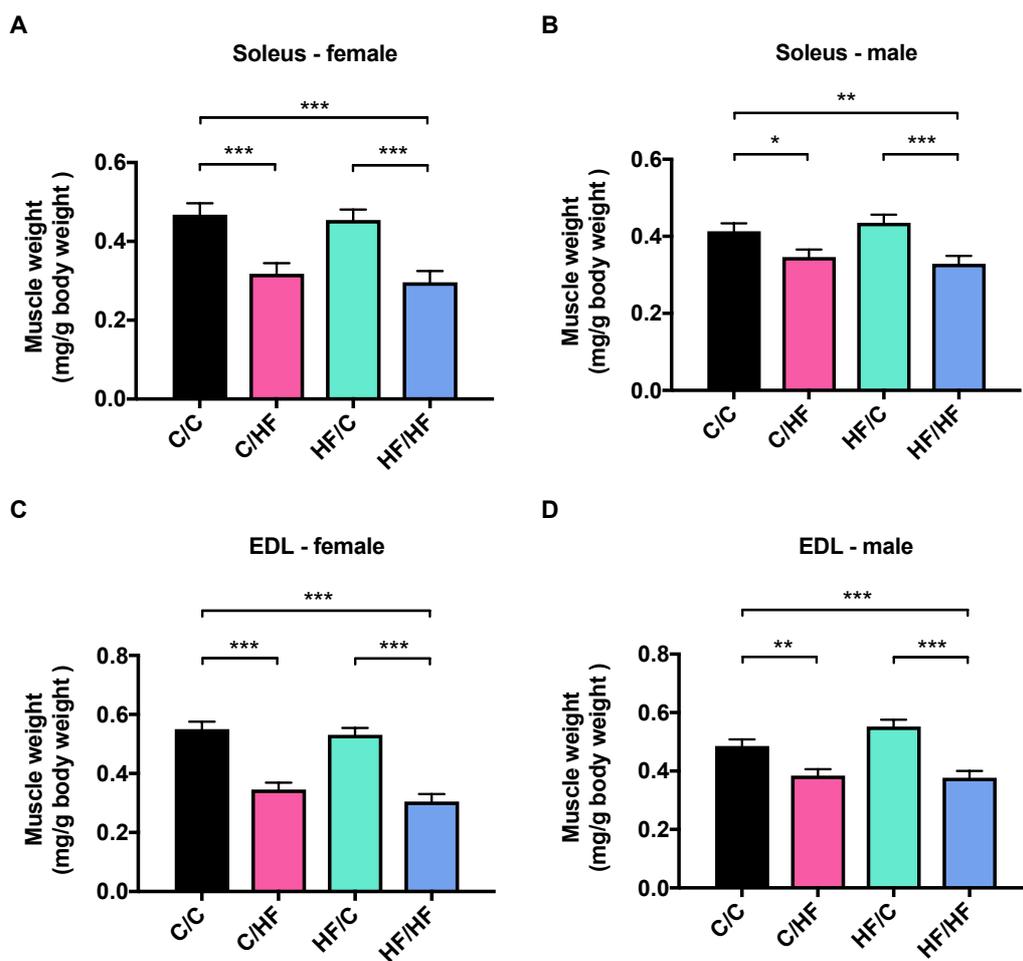
#### 3.3.1 Body and muscle weights

In both sexes (Fig. 3.4), the POST HF diet resulted in an increased body weight ( $P < 0.001$ ) regardless of PRE diet (C/C vs. C/HF; HF/C vs. HF/HF). Both sexes increased in body weight ( $P < 0.001$ ) in the HF/HF group compared with C/C. A PRE HF diet had no effect on offspring body weight.



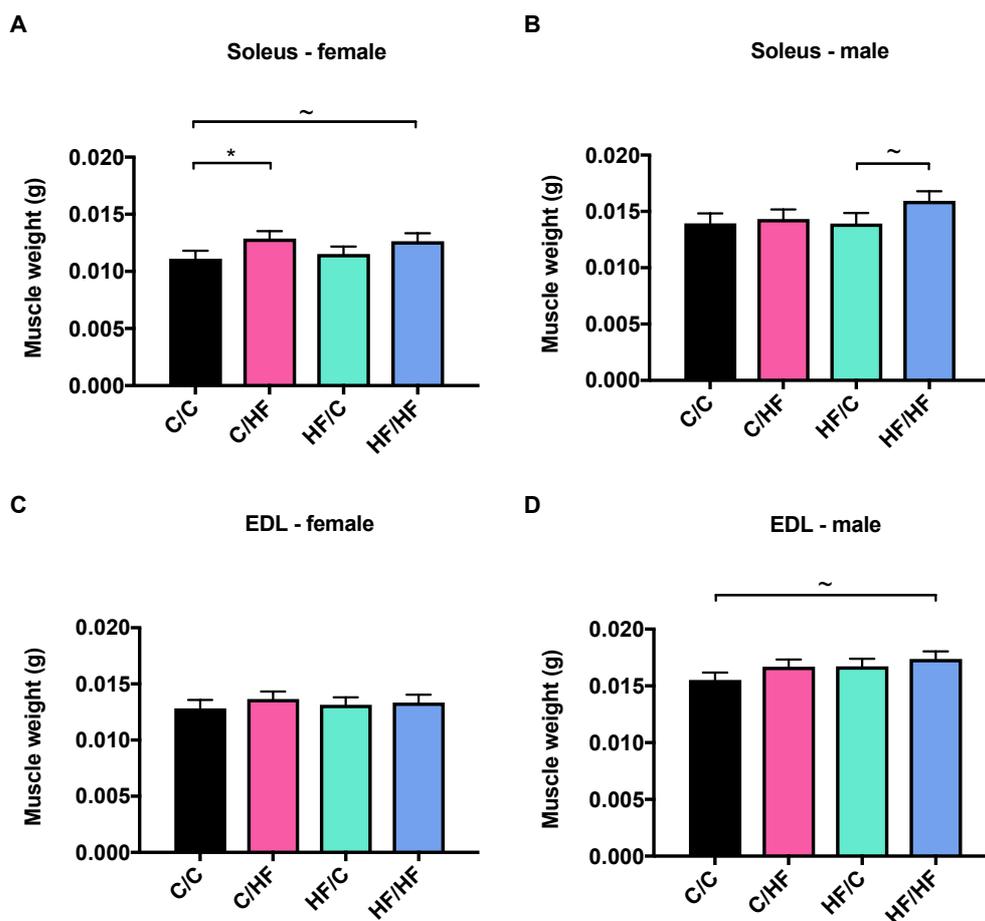
**Figure 3.4: Offspring body weight at 30 weeks of age.** Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \*\*\* $P < 0.001$ , ~  $P < 0.1$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

In both sexes, soleus and EDL muscle (*m.*) weight relative to body weight was lower in POST HF groups regardless of PRE diet ( $P < 0.05$  -  $P < 0.001$ , Fig 3.5). There was also a significant reduction in muscle weight with the HF/HF diet group compared with C/C offspring ( $P < 0.01$  -  $P < 0.001$ ). There was no effect of PRE HF diet on muscle weight.



**Figure 3.5: 30-week old offspring normalised muscle weights.** A) Female soleus, B) male soleus, C) female EDL and D) male EDL. All muscle weights are normalised for body weight (mg/g body weight). Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

Raw muscle weights (Fig. 3.6) do not show the same differences. EDL *m.* weight did not change with diet group for either sex. There were also no significant differences in male soleus *m.* raw weight between diet groups, however, there was an increase in female soleus *m.* weight with a POST diet (C/C vs C/HF,  $P < 0.05$ ).



**Figure 3.6: 30-week old offspring raw muscle weights.** A) Female soleus, B) male soleus, C) female EDL and D) male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ ,  $\sim P < 0.1$ .  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

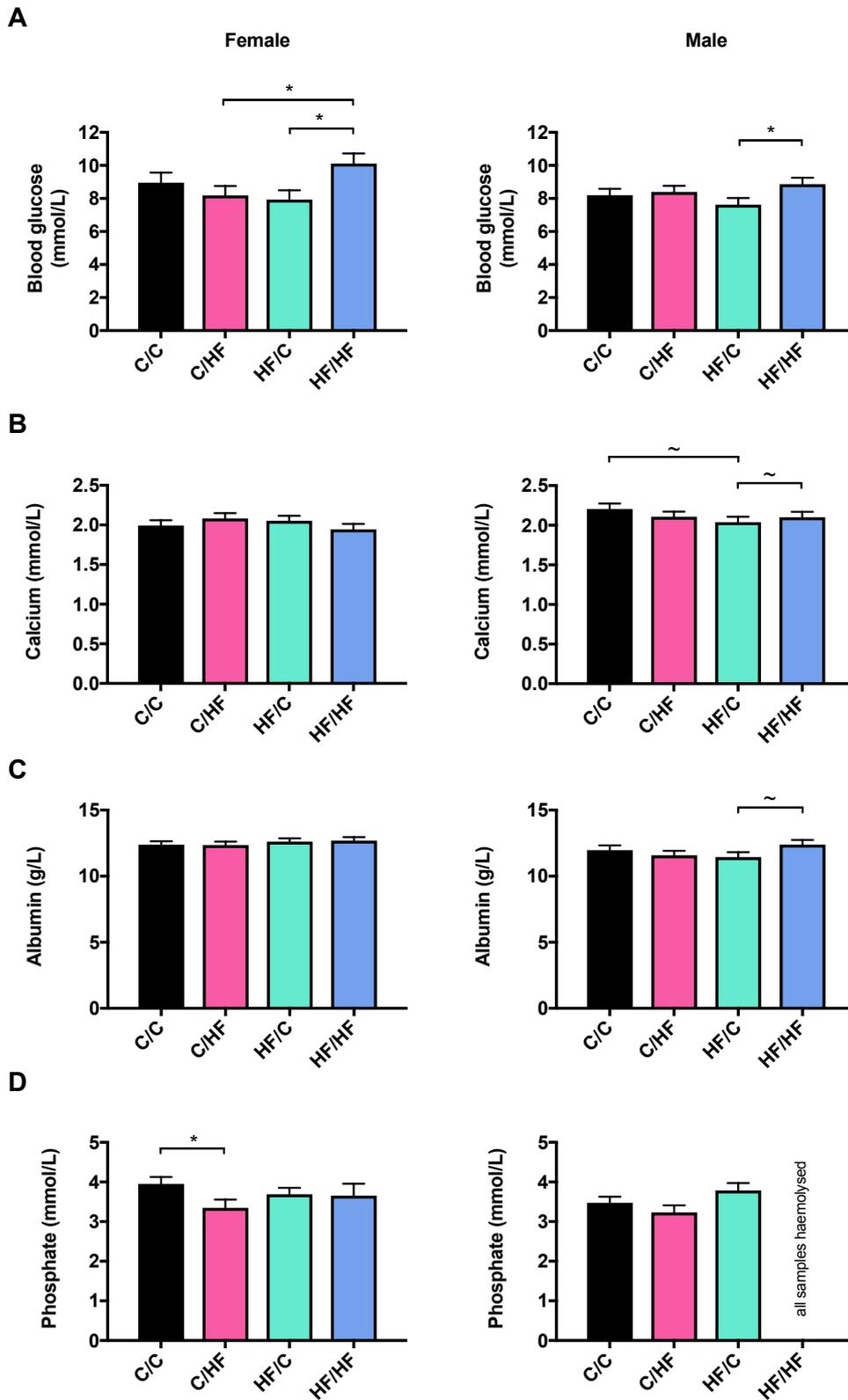
### 3.3.2 Blood analysis

#### 30-week offspring

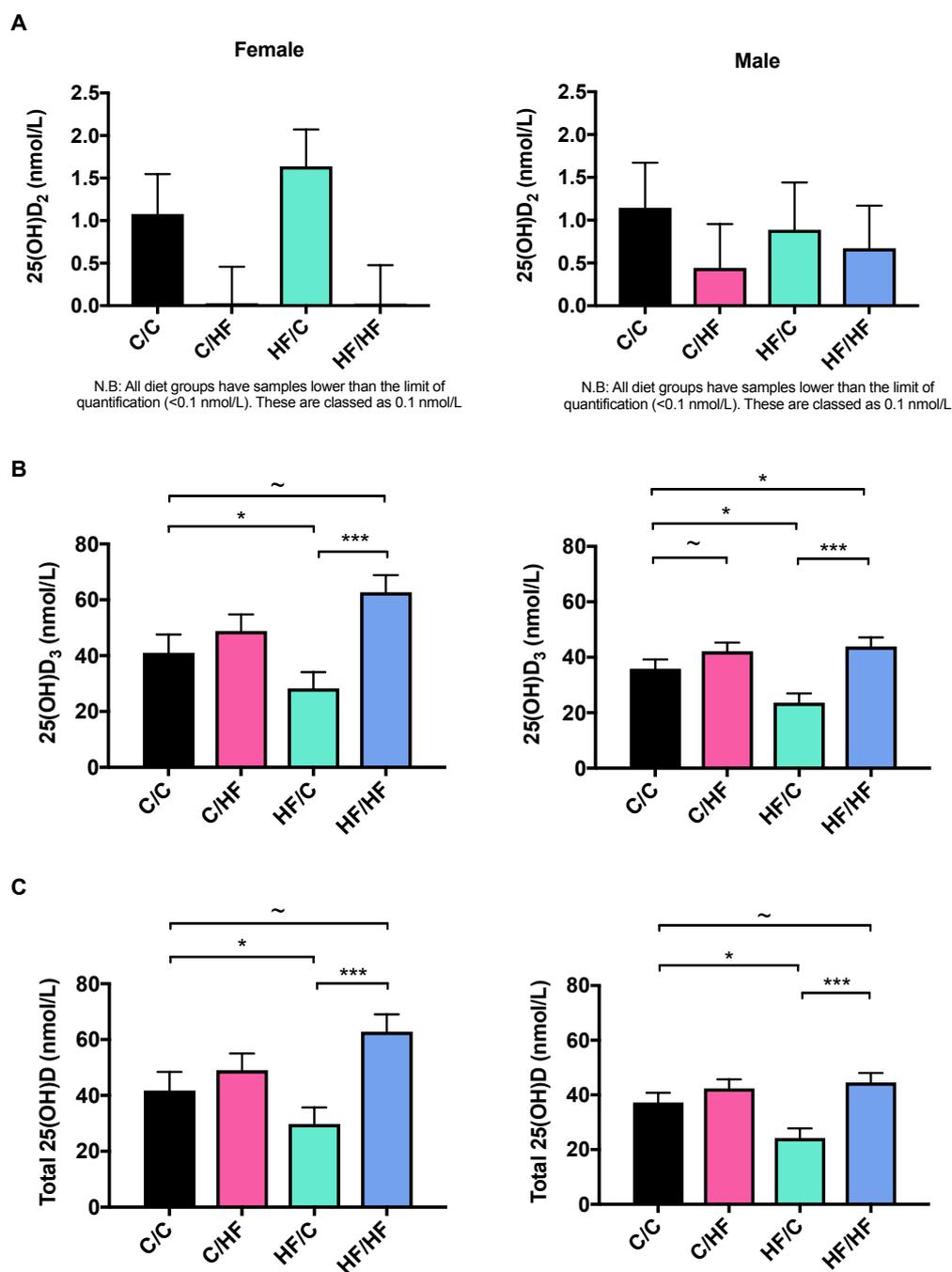
In female and male offspring, there was an increase in fasting blood glucose (Fig. 3.7A) with a POST HF diet when the PRE diet was HF (HF/C vs. HF/HF,  $P < 0.05$ ). Females in the HF/HF group also had an increase in blood glucose levels compared with C/HF ( $P < 0.05$ ).

Fasting plasma calcium (Fig. 3.7B) and albumin concentrations (Fig. 3.7C) were not significantly different between diet groups. In the females, plasma phosphate concentrations (Fig. 3.7D) decreased with a HF POST diet (C/C vs. C/HF,  $P < 0.05$ ). Fasting plasma phosphate concentrations were not affected by diet group in the males, although quantification was not possible for HF/HF male offspring due to haemolysis of the samples.

There were no significant differences in  $25(\text{OH})\text{D}_2$  between the diet groups for either sex (Fig. 3.8A). Many of the samples were below the LOQ ( $< 0.1$  nmol/L), and so these were classed as 0.1 nmol/L. In female and male offspring,  $25(\text{OH})\text{D}_3$  (Fig. 3.8B) was significantly elevated in the HF/HF group compared with HF/C ( $P < 0.001$ ), and decreased with a PRE HF compared with a PRE C diet (C/C vs. HF/C,  $P < 0.05$ ). HF/HF male offspring had higher  $25(\text{OH})\text{D}_3$  concentration compared with C/C ( $P < 0.05$ ), but this was only a trend ( $P < 0.1$ ) in the females. In both female and male offspring there was an increase in total  $25(\text{OH})\text{D}$  (Fig. 3.8C) in the HF/HF vs. HF/C group ( $P < 0.001$ ). Both sexes had a reduction in fasting plasma total  $25(\text{OH})\text{D}$  in the HF/C group versus C/C ( $P < 0.05$ ), and a trend for an increase ( $P < 0.1$ ) with the HF/HF compared with the C/C offspring.

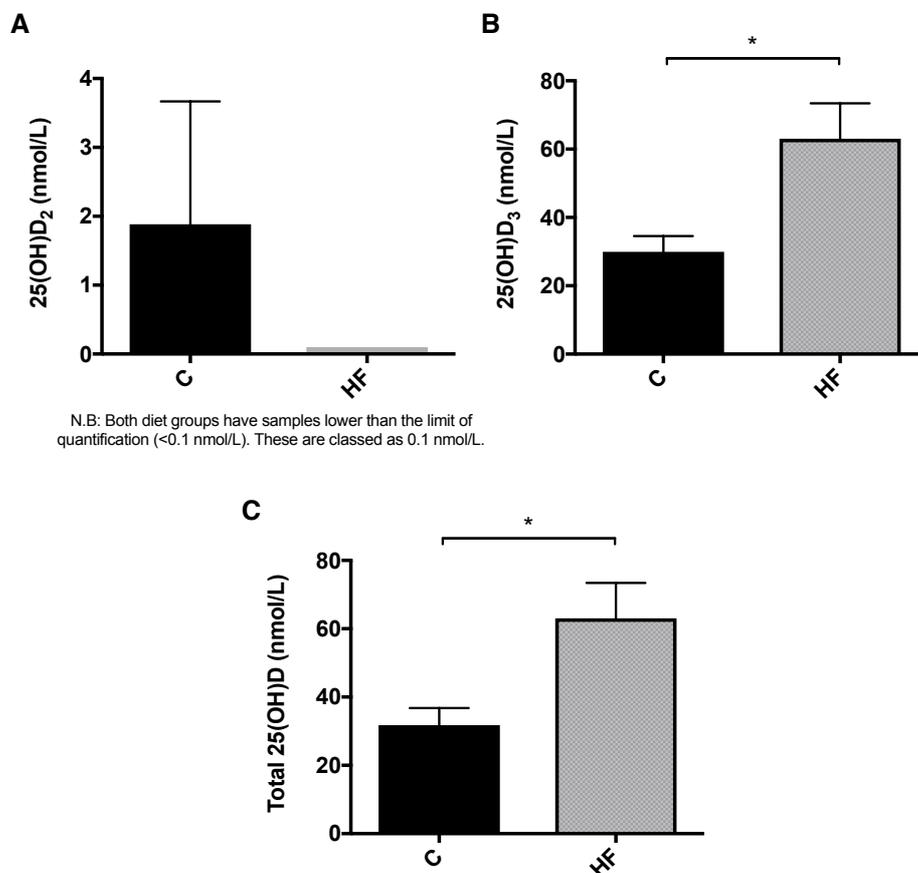


**Figure 3.7: 30-week old offspring blood analysis.** **A**) Fasted blood glucose (mmol/L) and fasted plasma levels of **B**) calcium (mmol/L), **C**) albumin (g/L) and **D**) phosphate (mmol/L) in male and female 30-week mouse offspring. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.



**Figure 3.8: 30-week old offspring plasma vitamin D concentrations.** A) Plasma 25(OH)D<sub>2</sub> concentration. Some samples were under the limit of detection (<0.1 nmol/L), and so these were classed as 0.1 nmol/L. B) Plasma 25(OH)D<sub>3</sub> concentration. C) Plasma total 25(OH)D concentration. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, n=7-8 per group. C, control diet; HF, high-fat diet.

E16 dams



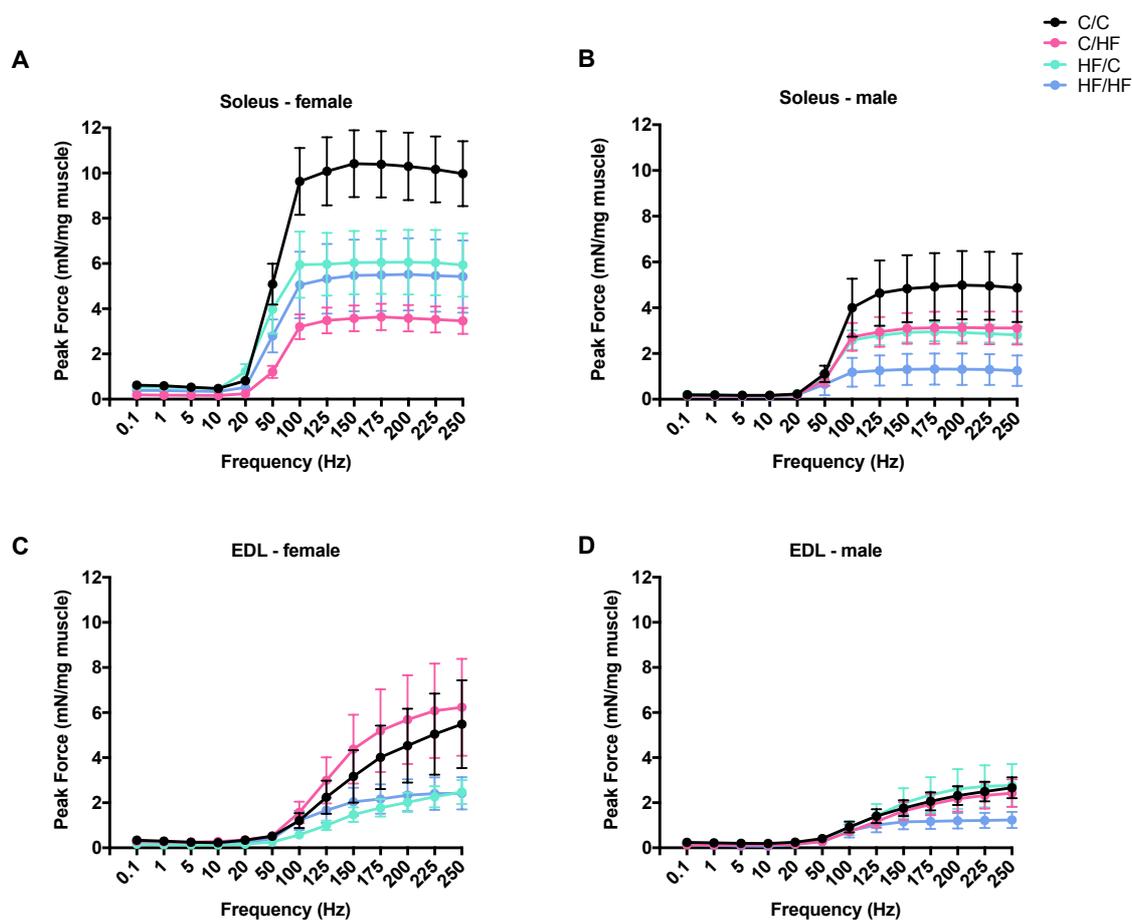
**Figure 3.9: E16 vitamin D plasma concentrations.** A) 25(OH)D<sub>2</sub>, 5x C and 6x HF were below LOQ; B) 25(OH)D<sub>3</sub> and C) total 25(OH)D plasma concentrations from pregnant dams at E16 of pregnancy either fed a high-fat or control diet. Data represented as mean ± SEM and statistically analysed by an independent t-test. \*P<0.05. E, embryonic day; C, control diet; HF, high-fat diet; LOQ, limit of quantification.

There was no significant difference between groups in maternal 25(OH)D<sub>2</sub> plasma concentration at embryonic day 16 (E16). The majority of samples were, however, below the LOQ for 25(OH)D<sub>2</sub> which is 0.1 nmol/L, and so these samples were assigned the value of 0.1 nmol/L. Plasma concentration of 25(OH)D<sub>3</sub> and total 25(OH)D at E16 were significantly higher in the dams fed a HF diet compared with those fed the C diet (P<0.05).

### 3.3.3 Isometric skeletal muscle contraction

#### 3.3.3.1 Peak force

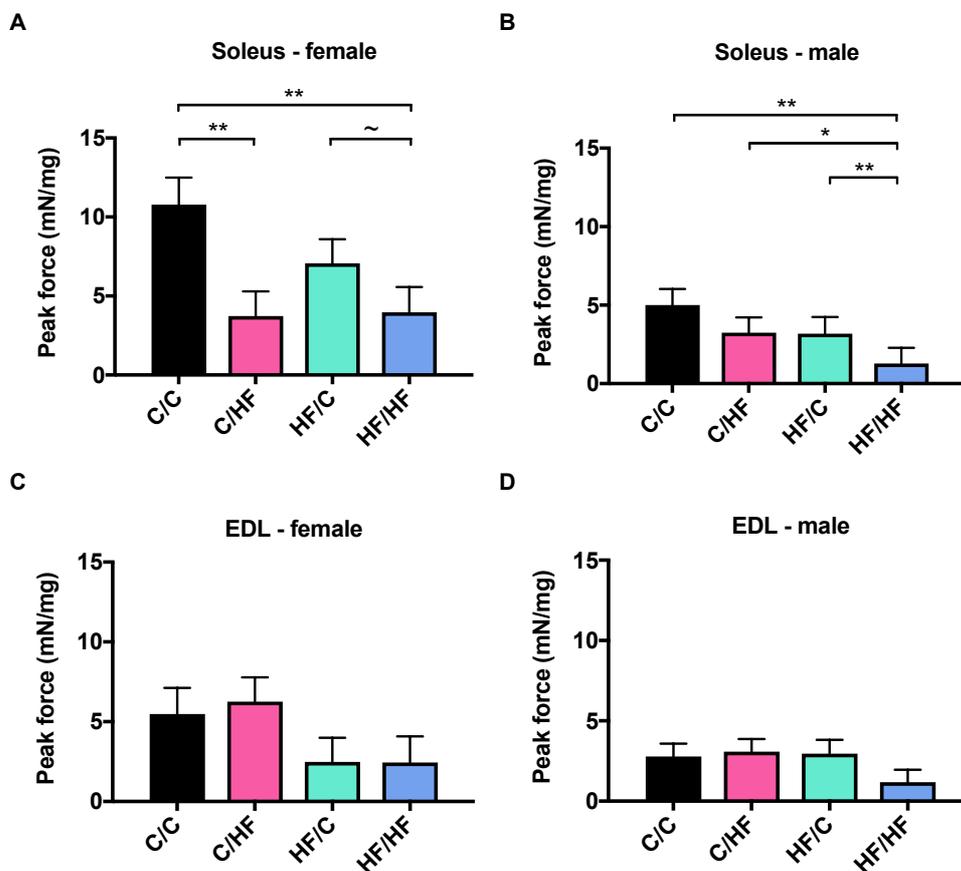
Figure 3.10 displays the peak force (PF) produced by the soleus and EDL *m.* (both sexes) for the 13 different frequencies 0.1-250 Hz. As explained in section 3.2.4, the chosen frequencies for the single-twitch (10 Hz) and tetanic (150 Hz for the soleus *m.* and 250 Hz for the EDL *m.*) contraction analysis were taken from these graphs.



**Figure 3.10: Peak force response of isometric muscle contraction.** The peak force (mN/mg muscle weight) generated during muscle contraction in response to different stimulation frequencies in: **A)** Female soleus; **B)** male soleus; **C)** female EDL; and **D)** male EDL. Data are displayed as mean  $\pm$  SEM of raw data. These data are displayed to show the relationship between peak force and frequency only, and were not statistically analysed over all frequencies.

## Tetanic contraction

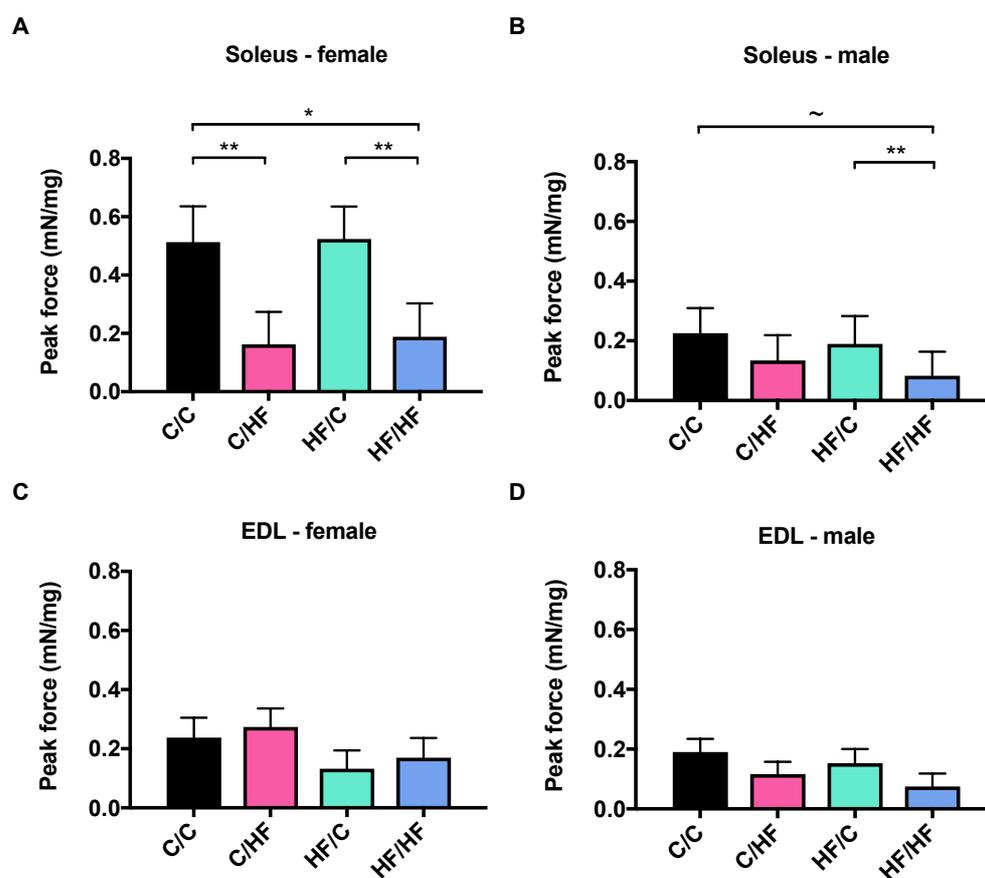
Tetanic peak force was greater in the female than male offspring for most diet groups in both muscles. In both the female and male soleus *m.* (Fig. 3.11A-B), the tetanic peak force at 150 Hz was significantly reduced ( $P < 0.01$ ) in the HF/HF group compared with C/C. The PF of the males was also lower in the C/HF ( $P < 0.05$ ) and HF/C ( $P < 0.01$ ) groups compared with HF/HF. The female soleus *m.* had a reduction in PF in the POST HF (alone) group (C/C vs. C/HF,  $P < 0.01$ ), but this was not observed in the males. There were no differences in tetanic PF at 250 Hz with diet group in the female or male EDL *m.* (Fig. 3.11C-D).



**Figure 3.11: Tetanic contraction peak force response.** Muscle peak force response (mN/mg muscle weight) of isometric tetanic muscle contraction at a stimulation frequency of 150 Hz for soleus *m.* and 250 Hz for EDL *m.* **A)** Female soleus, **B)** male soleus, **C)** female EDL and **D)** male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; *m.*, muscle.

### Single-twitch contraction

For single-twitch responses, 10 Hz was analysed for both muscle types. In the female soleus *m.* (Fig. 3.12A), there was a significant reduction in PF in the C/HF ( $P<0.01$ ) and HF/HF group ( $P<0.05$ ) compared with C/C, but the latter was only a trend ( $P<0.1$ ) in the male soleus *m.* (Fig. 3.12B). In both the female and male soleus *m.*, there was a reduction in PF ( $P<0.01$ ) in the HF/C group compared with HF/HF. There were no differences in the single-twitch PF response for the EDL *m.* (Fig. 3.12C-D).

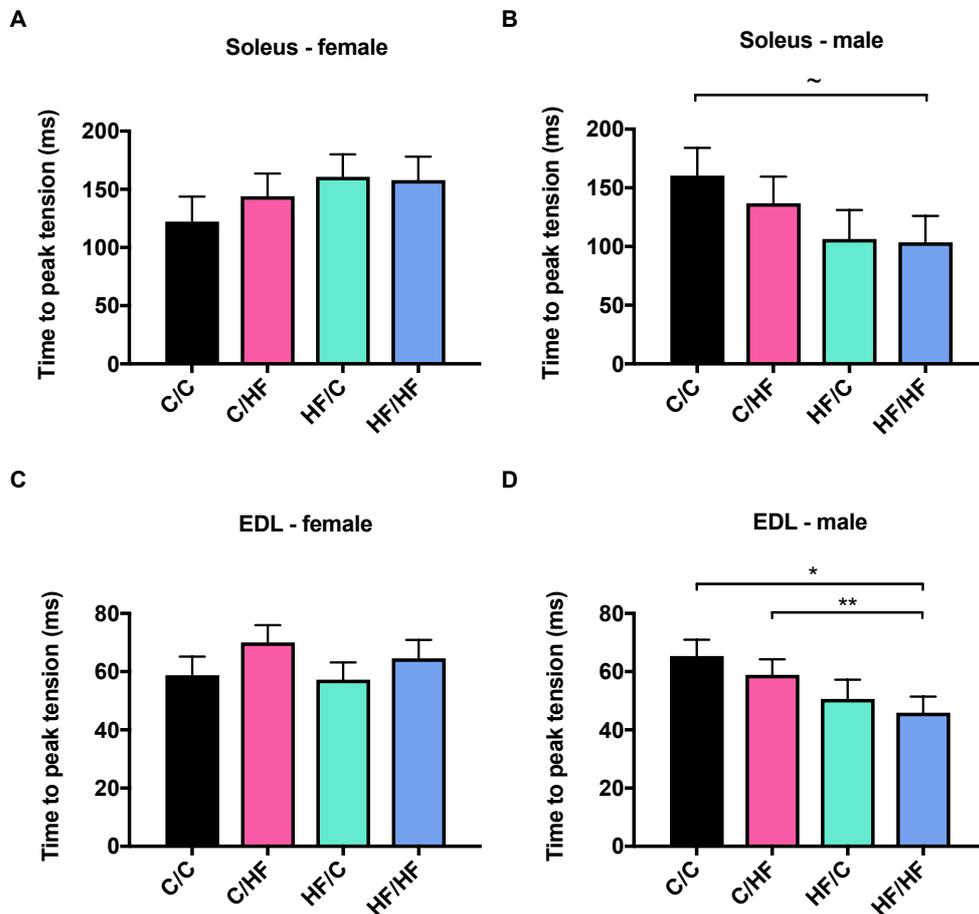


**Figure 3.12: Single-twitch contraction peak force response.** Muscle peak force response (mN/mg muscle weight) of isometric single-twitch muscle contraction at a stimulation frequency of 10 Hz. **A)** Female soleus, **B)** male soleus, **C)** female EDL and **D)** male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. ~P<0.1, \*P<0.05, \*\*P<0.01, n=7-8 per group. C, control diet; HF, high-fat diet.

### 3.3.3.2 Time-to-peak tension

#### Tetanic contraction

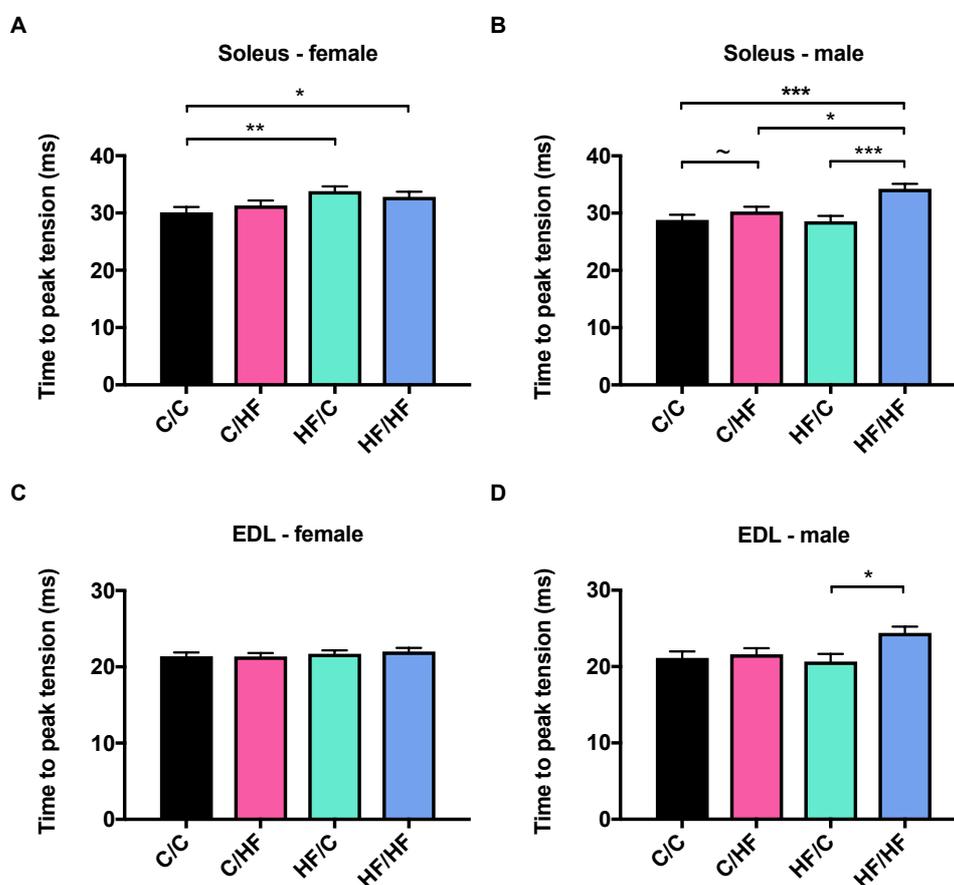
There were no significant differences in the TPT with diet group in the soleus *m.* of female or male offspring when stimulated at 150 Hz, however there was a trend ( $P < 0.1$ ) for a reduction in the HF/HF group compared with C/C (Fig. 3.13). The female EDL *m.* did not show any differences in TPT at a stimulation frequency of 250 Hz. Interestingly, the male EDL *m.* displayed a reduction in TPT in the HF/HF group when compared with C/C ( $P < 0.05$ ) and C/HF ( $P < 0.01$ ).



**Figure 3.13: Tetanic contraction time-to-peak tension response.** Muscle time-to-peak tension response (ms) of isometric tetanic muscle contraction at a stimulation frequency of 150 Hz for soleus *m.* and 250 Hz for EDL *m.* **A)** Female soleus, **B)** male soleus, **C)** female EDL and **D)** male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ , \* $P < 0.05$ , \*\* $P < 0.01$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; *m.*, muscle.

### Single-twitch contraction

At 10 Hz, the TPT for single-twitch contraction was shorter in the EDL *m.* compared with the soleus *m.* (Fig. 3.14). There was an increase in TPT duration with a PRE HF diet (C/C vs. HF/C;  $P < 0.01$ ) and with the HF/HF group (C/C vs. HF/HF;  $P < 0.05$ ) in the female soleus *m.* The male soleus *m.* also had a longer TPT in the HF/HF group compared with C/C ( $P < 0.001$ ), C/HF ( $P < 0.05$ ) and HF/C ( $P < 0.001$ ). There was also a trend ( $P < 0.1$ ) for an increase in TPT with C/HF compared with C/C diet group. No differences were found in the female EDL *m.*, but the EDL *m.* of male offspring had a longer TPT in the HF/HF group compared with HF/C ( $P < 0.05$ ).

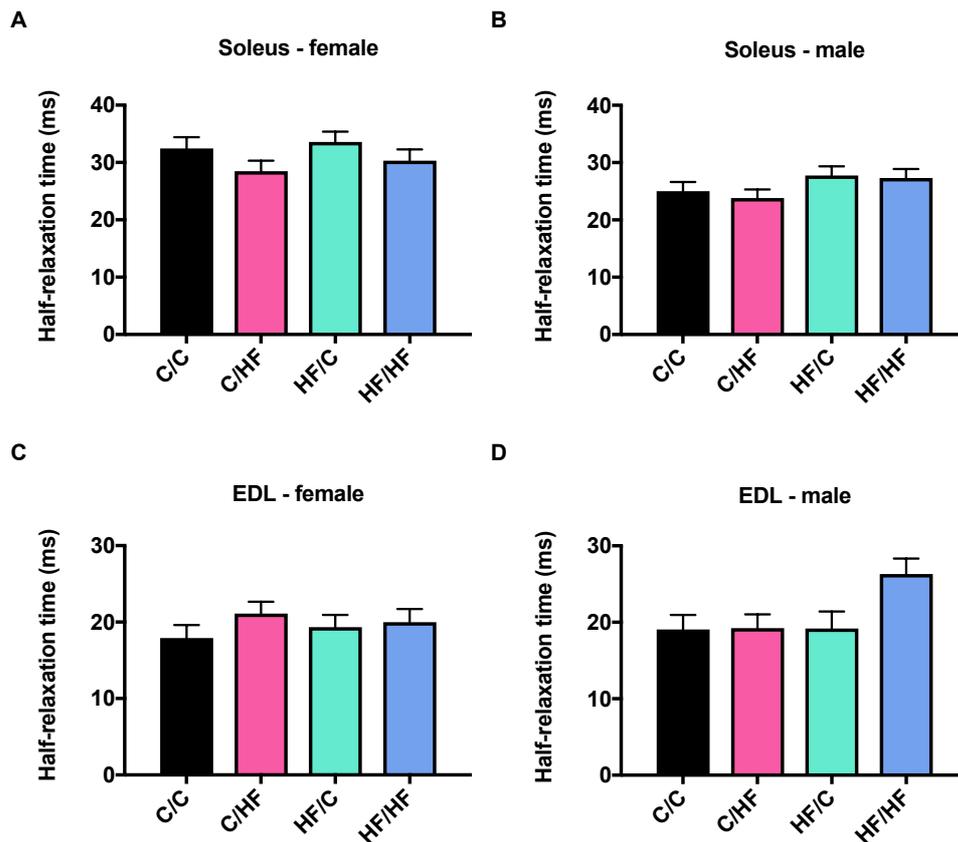


**Figure 3.14: Single-twitch contraction time-to-peak tension response.** Muscle time-to-peak tension response (ms) of isometric single-twitch muscle contraction at a stimulation frequency of 10 Hz. **A)** Female soleus, **B)** male soleus, **C)** female EDL and **D)** male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. ~ $P < 0.1$ , \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

### 3.3.3.3 Half-relaxation time

#### Tetanic contraction

There were no differences found in the half-relaxation time (HRT) with diet group in either sex when the soleus and EDL *m.* were stimulated at 150 Hz and 250 Hz, respectively (Fig. 3.15).

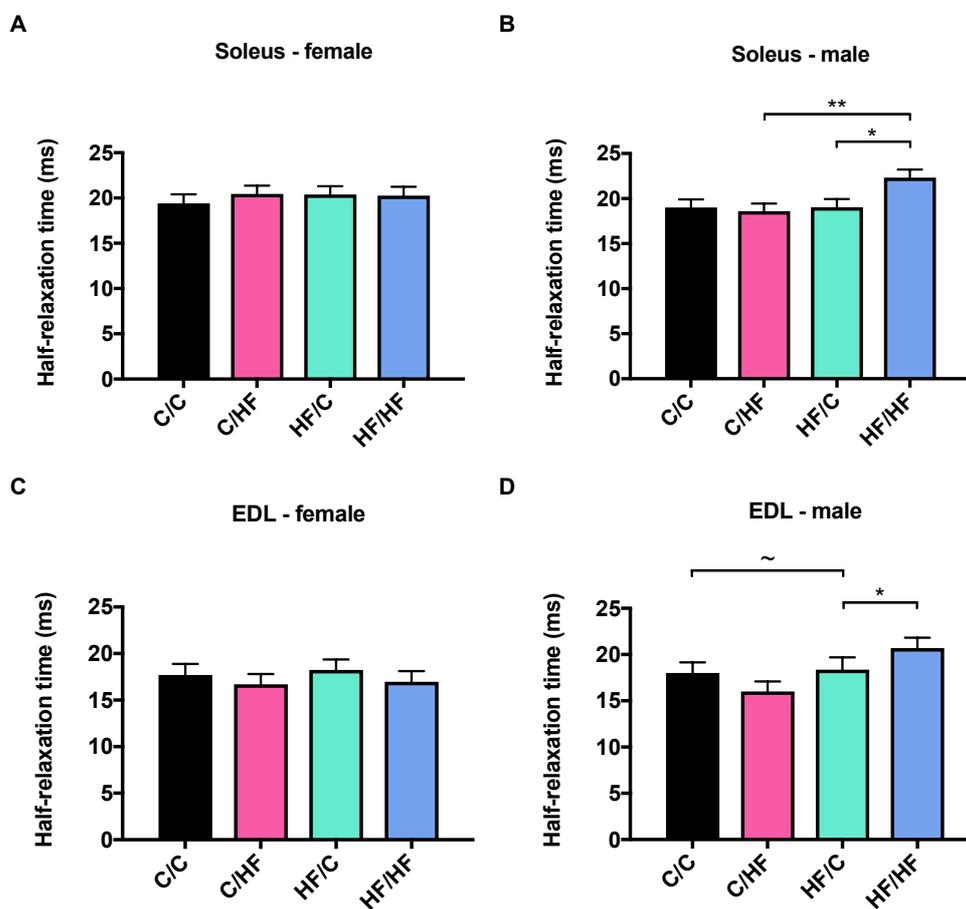


**Figure 3.15: Tetanic contraction half-relaxation time response.** Muscle half-relaxation time response (ms) of isometric tetanic muscle contraction at a stimulation frequency of 150 Hz for soleus *m.* and 250 Hz for EDL *m.* **A)** Female soleus, **B)** male soleus, **C)** female EDL and **D)** male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. Statistical significant determined at  $P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; *m.*, muscle.

#### Single-twitch contraction

There were no differences in the HRT of the female offspring for either muscle (Fig. 3.16) when stimulated at 10 Hz. The male soleus and EDL *m.* in the HF/HF group had a longer HRT compared with HF/C ( $P < 0.05$ ). There was also an increase in HRT with a PRE HF

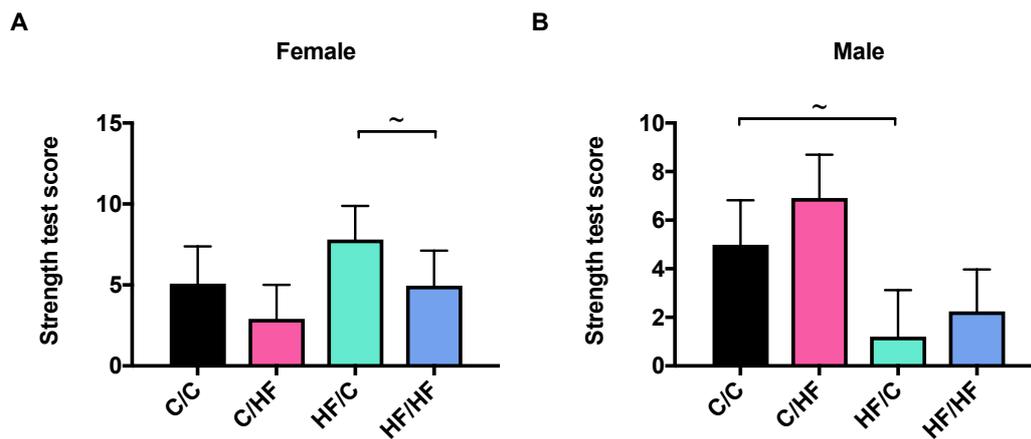
diet (C/HF vs. HF/HF;  $P < 0.01$ ) in the male soleus *m*. There was a trend for an increase ( $P < 0.1$ ) in HRT with a PRE HF diet (C/C vs. HF/C) in the male EDL *m*.



**Figure 3.16: Single-twitch contraction half-relaxation time response.** Muscle half-relaxation time response (ms) of isometric single-twitch muscle contraction at a stimulation frequency of 10 Hz. **A)** Female soleus, **B)** male soleus, **C)** female EDL and **D)** male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

### 3.3.4 *In vivo* strength test

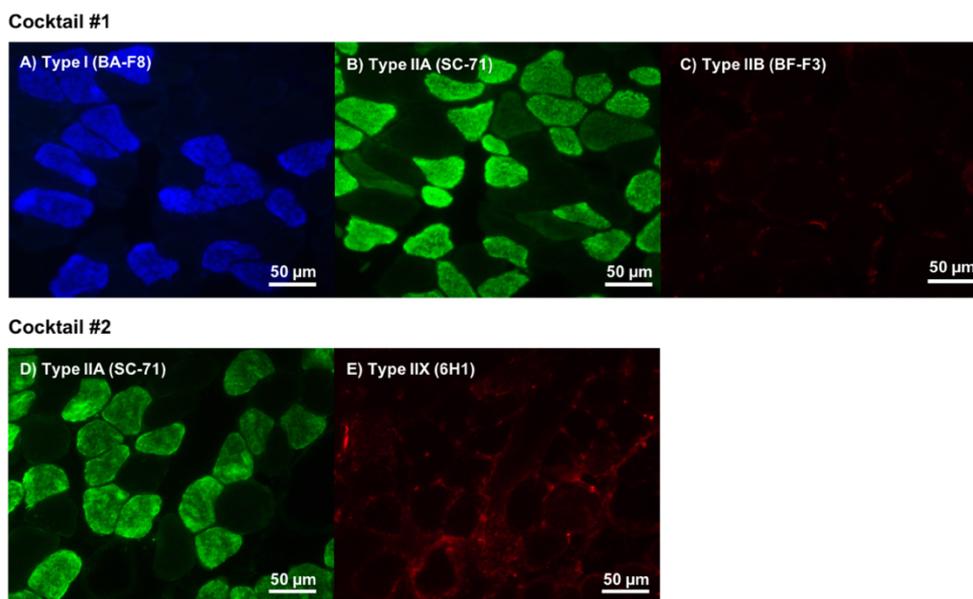
No significant changes in forearm strength were found with diet in the female or male offspring (Fig. 3.17). However, there was a trend for a reduction ( $P < 0.1$ ) in female strength with the HF/HF group compared with HF/C, and a trend for a reduction ( $P < 0.1$ ) in the HF/C group compared with the C/C group within the male offspring.



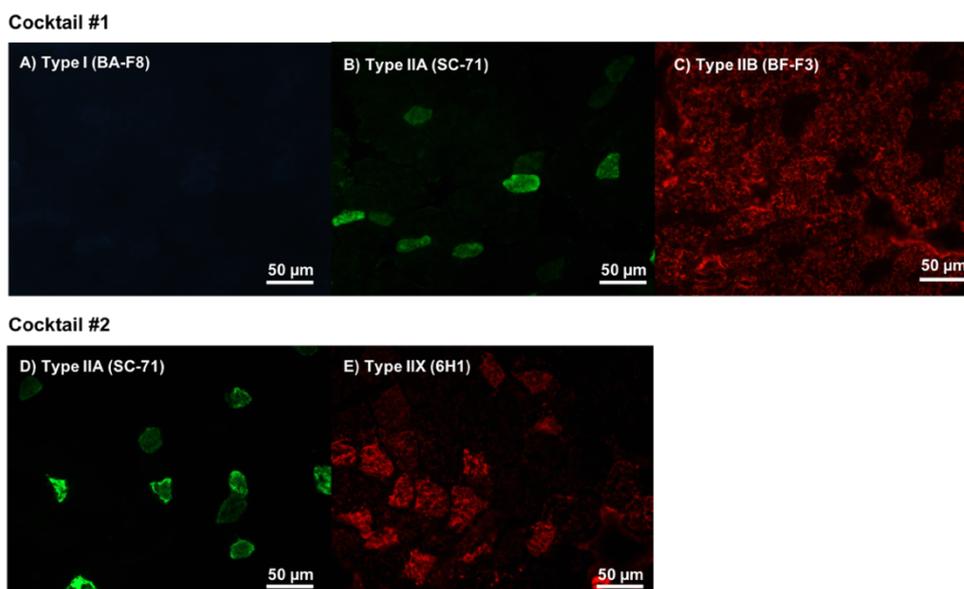
**Figure 3.17: In vivo strength test.** Forearm strength test for **A)** female and **B)** male 30-week offspring across all four diet groups. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

### 3.3.5 Myofibre typing

Representative images of the immunofluorescent staining of each myofibre for the soleus and EDL *m.* of 30-week offspring are displayed in Figures 3.18 and 3.19, respectively.



**Figure 3.18: Representative image of immunofluorescent staining of the soleus muscle.** Cocktail 1: **A)** Type I (BA-F8), **B)** Type IIA (SC-71) and **C)** Type IIB (BF-F3). Cocktail 2: **D)** Type IIA (SC-71) and **E)** Type IIX (6H1). Scale bars represent 50 µm, magnification is x40.



**Figure 3.19: Representative image of immunofluorescent staining of the EDL muscle.** Cocktail 1: **A)** Type I (BA-F8), **B)** Type IIA (SC-71) and **C)** Type IIB (BF-F3). Cocktail 2: **D)** Type IIA (SC-71) and **E)** Type IIX (6H1). Scale bars represent 50 µm, magnification is x40. EDL, extensor digitorum longus.

### 3.3.5.1 Myofibre density

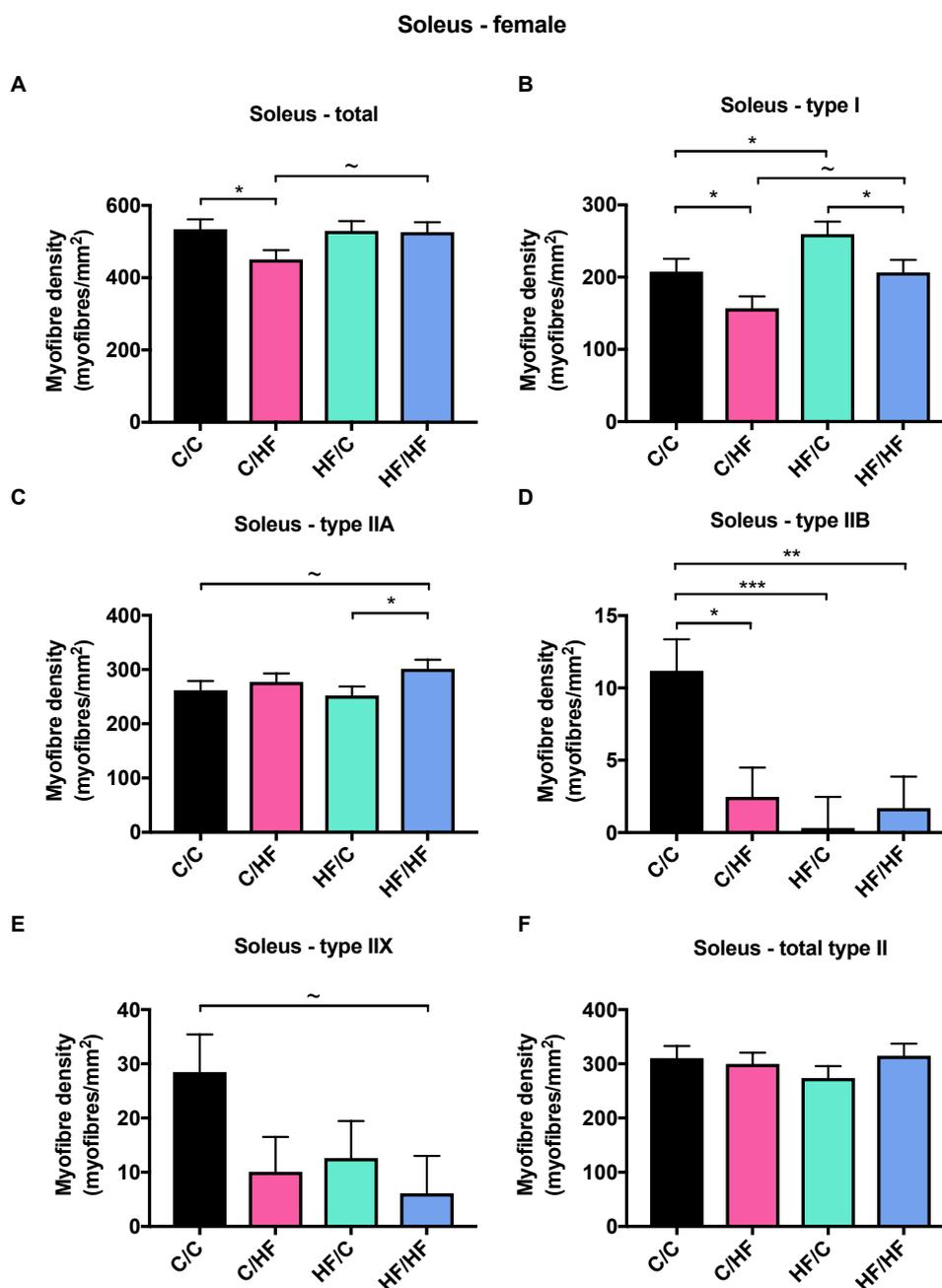
#### Soleus - female

Total myofibre density (Fig. 3.20A) of the female soleus *m.* was significantly reduced ( $P<0.05$ ) in the C/HF group compared with C/C with a trend for a reduction in the C/HF group compared with HF/HF ( $P<0.1$ ). The density of type I fibres (Fig. 3.20B) reduced with a POST HF diet regardless of PRE diet ( $P<0.05$ ). There was an increase in type I density ( $P<0.05$ ) with a PRE HF diet (C/C vs. HF/C), but this was only a trend ( $P<0.1$ ) when the POST diet was HF. Type IIA myofibre density (Fig. 3.20C) increased with a POST HF diet (HF/C vs HF/HF,  $P<0.05$ ) and there was a trend for an increase ( $P<0.1$ ) in the HF/HF compared with the C/C diet group. The myofibre density of type IIB fibres (Fig. 3.20D) was reduced in the C/HF ( $P<0.05$ ), HF/C ( $P<0.001$ ) and HF/HF ( $P<0.01$ ) diet groups when compared with C/C. There were no changes to female soleus *m.* myofibre density in the type IIX or total type II fibres, except for a trend in a reduction ( $P<0.1$ ) of type IIX density in the HF/HF compared with the C/C diet group. In terms of hybrid fibres, there were no changes with diet group in the number of type IIAB (Fig. 3.22B) or type IIAX (Fig. 3.22C) fibres. There was, however, a reduction in type I/IIA fibre density ( $P<0.05$ , Fig. 3.22A) in the HF/HF group compared with C/C.

#### Soleus – male

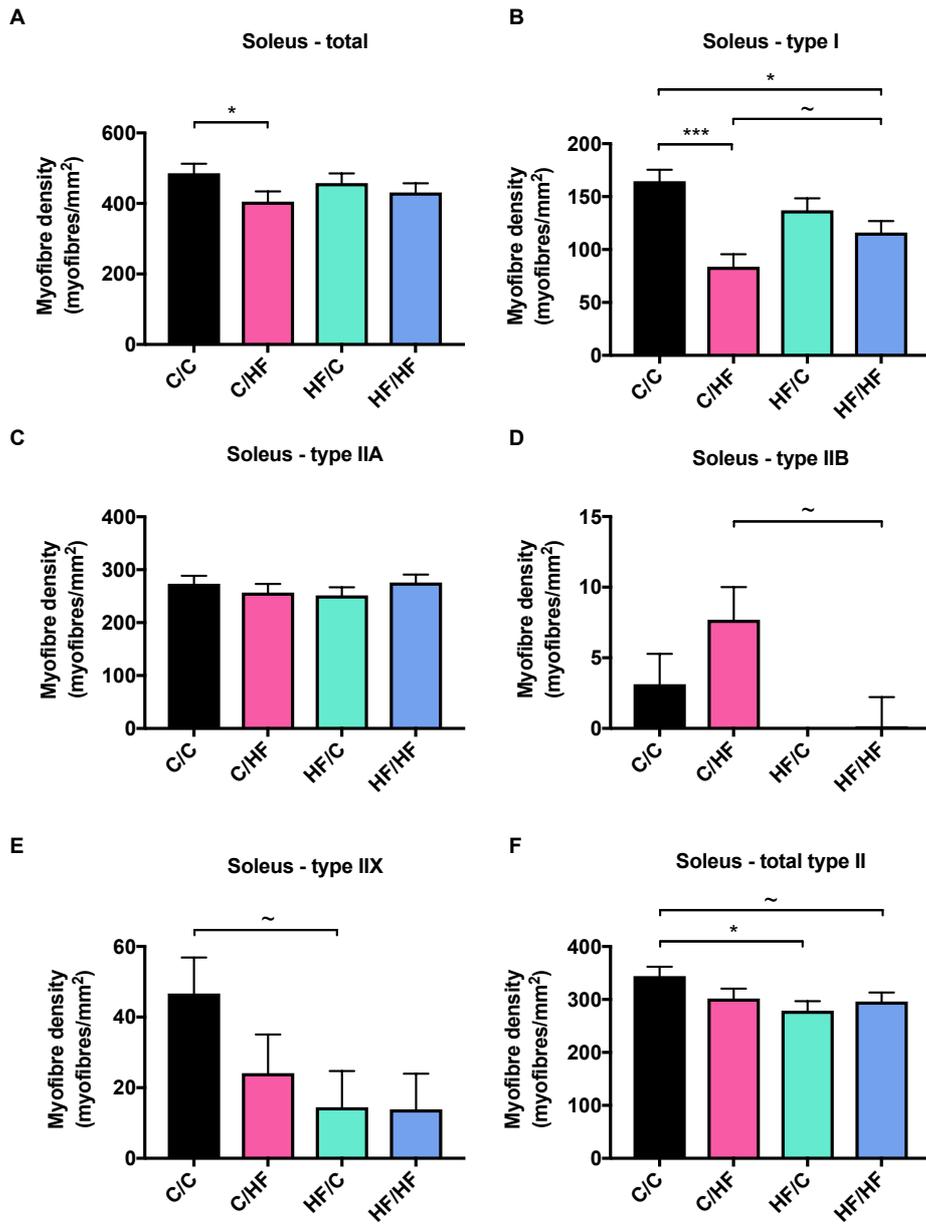
Myofibre density was reduced with a POST HF diet (C/C vs. C/HF) in total fibres ( $P<0.05$ , Fig. 3.21A) and type I fibres ( $P<0.001$ ; Fig. 3.21 B). Type I myofibre density also reduced ( $P<0.05$ ) in the HF/HF diet group when compared with control and a trend for an increase in density with a PRE HF diet (C/HF vs. HF/HF,  $P<0.1$ ). There were no changes in the density of type IIA (Fig. 3.21C), type IIB (Fig. 3.21D) or type IIX (Fig. 3.21E) fibres. There was, however, a trend for a reduction in density ( $P<0.1$ ) with a PRE HF diet in type IIB fibres (C/HF vs. HF/HF) and type IIX fibres (C/C vs. HF/C). Total type II myofibre density reduced ( $P<0.05$ ) with a PRE HF diet (C/C vs. HF/C,  $P<0.05$ ; Fig. 3.21F) and tended to decrease with the HF/HF diet (C/C vs. HF/HF,  $P<0.1$ ). The male soleus *m.* type

I/IIA hybrid fibre density (Fig. 3.22A) decreased with a post HF diet (HF/C vs. HF/HF,  $P < 0.05$ ) and there was no change in type IIAX (Fig. 3.22B) density.

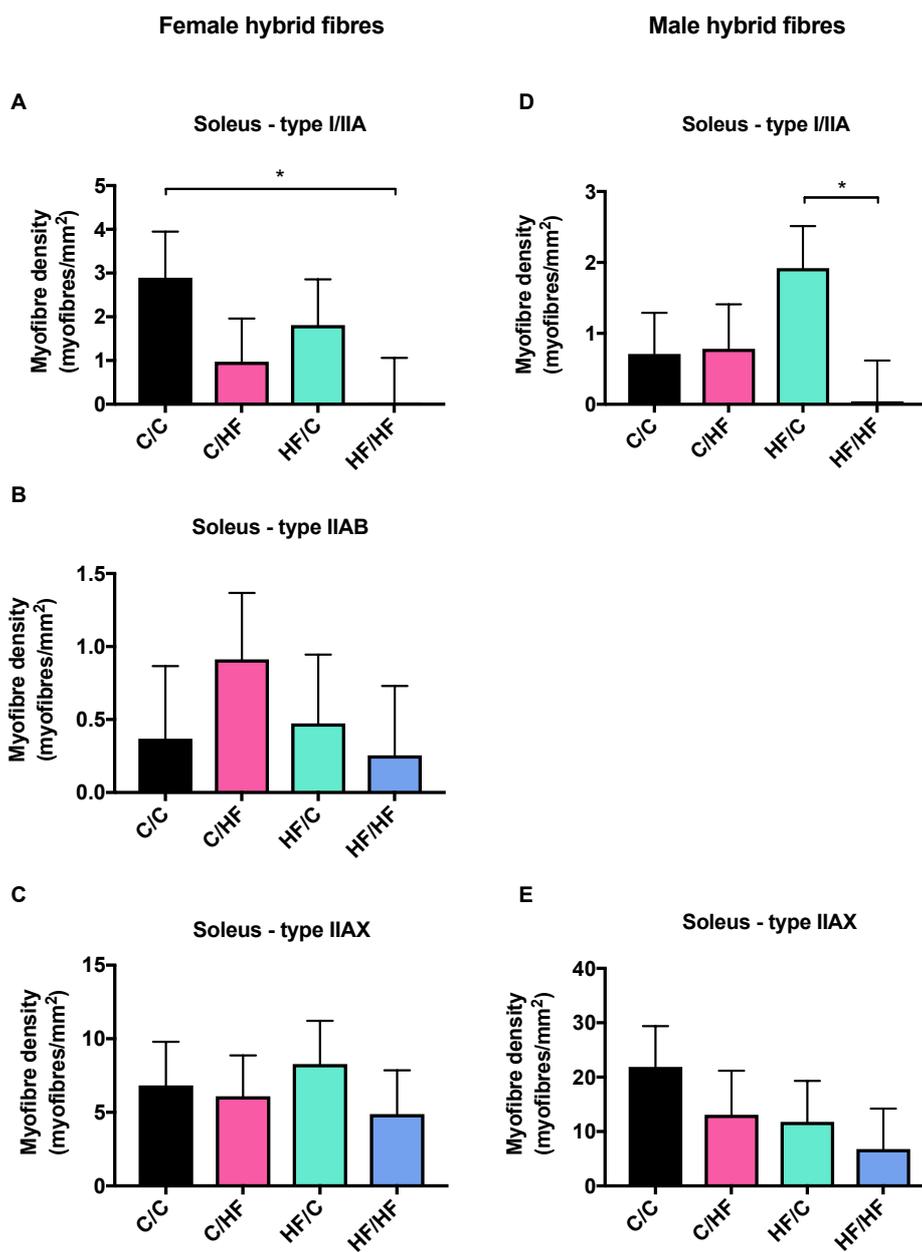


**Figure 3.20: Female soleus myofibre density.** Myofibre density (myofibres/mm<sup>2</sup>) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

Soleus - male



**Figure 3.21: Male soleus myofibre density.** Myofibre density (myofibres/mm<sup>2</sup>) for **A**) total, **B**) type I, **C**) type IIA, **D**) type IIB, **E**) type IIX and **F**) total type II fibres. Data are displayed as predicted means ± SEM from maximal models. All statistical analyses performed on Z-transformed data. ~P<0.1, \*P<0.05, \*\*\*P<0.001, n=7-8 per group. C, control diet; HF, high-fat diet.



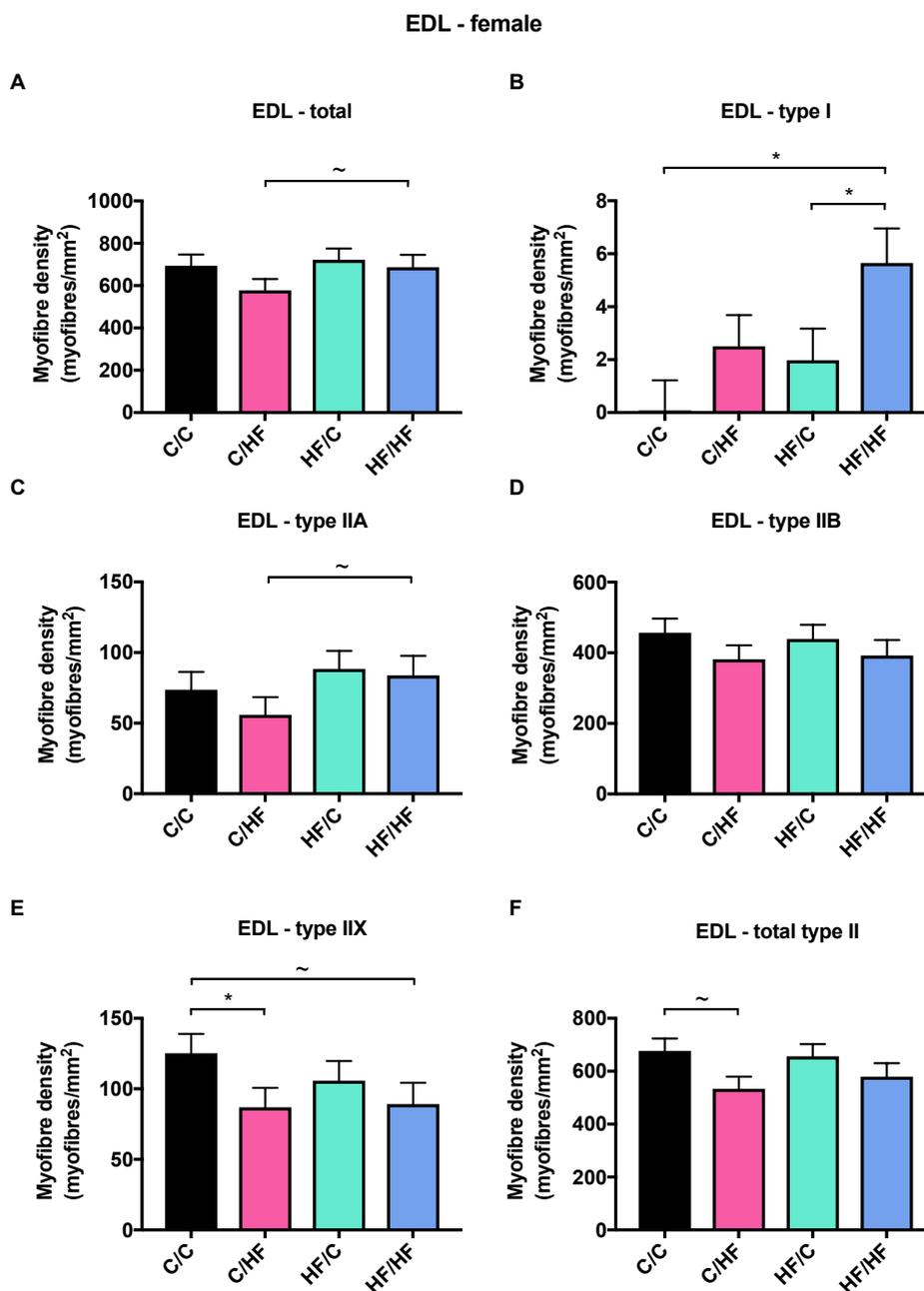
**Figure 3.22: Soleus hybrid myofibre density.** Myofibre density (myofibres/mm<sup>2</sup>) of hybrid fibres **A)** female type I/IIA, **B)** female type IIAB, **C)** female type IIAX, **D)** male type I/IIA and **E)** male type IIAX. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet.

### **EDL – female**

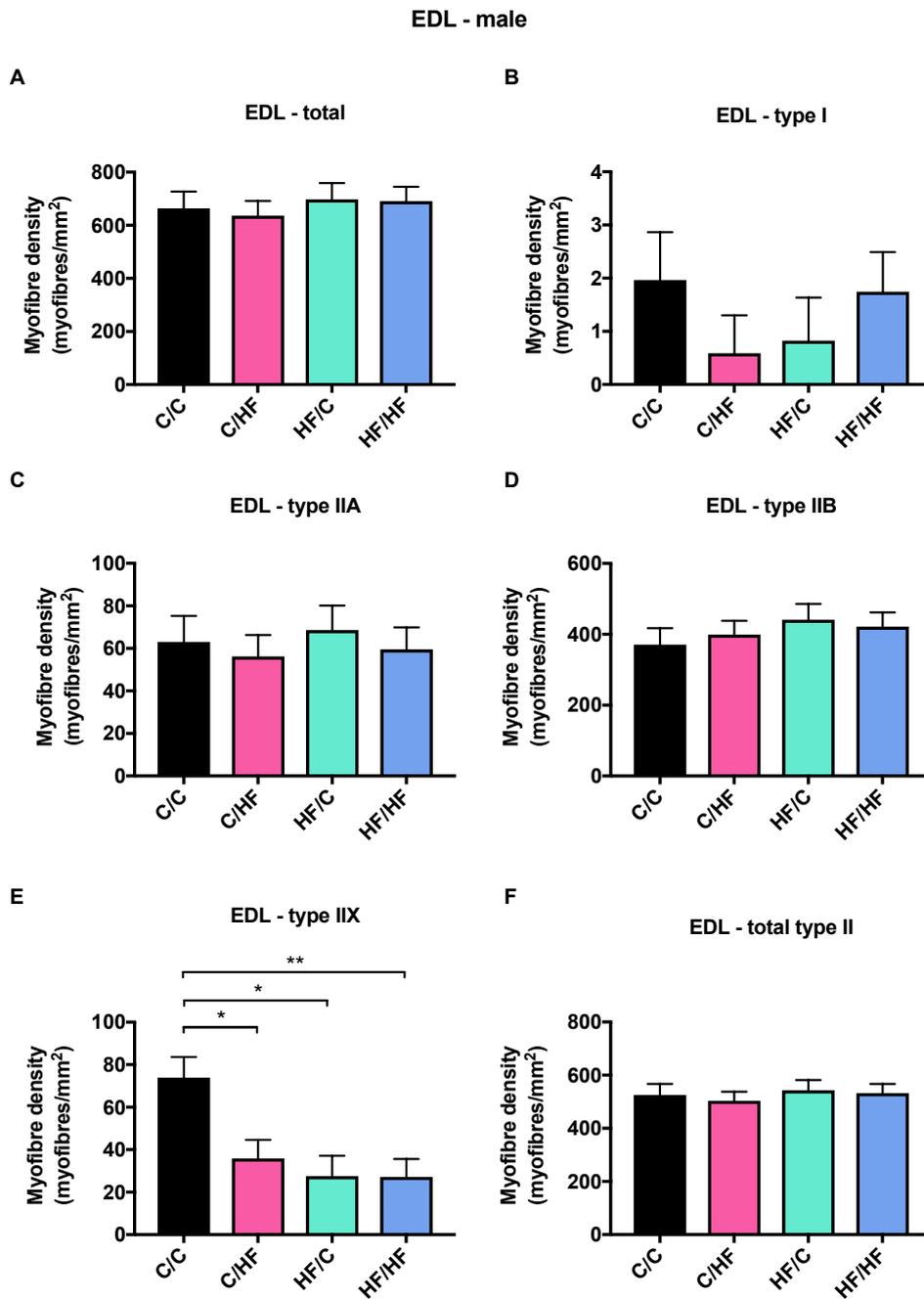
There were no significant changes in the density of total (Fig. 3.23A), type IIA (Fig. 3.23C), type IIB (Fig. 3.23D) or total type II (Fig. 3.23F) myofibres. However, there was a trend for an increase ( $P < 0.1$ ) in total and type IIA fibres with a PRE HF diet (C/HF vs. HF/HF), and total type II myofibre density tended to decrease ( $P < 0.1$ ) with a POST HF diet (C/C vs. C/HF). The number of type I myofibres (Fig. 3.23B) in the EDL *m.* of female offspring increased in the HF/HF group compared with C/C ( $P < 0.05$ ) and HF/C ( $P < 0.05$ ). Type IIX fibres density (Fig. 3.23E) decreased with a POST HF diet (C/C vs. C/HF,  $P < 0.05$ ) and tended to decrease in the HF/HF offspring (C/C vs. HF/HF,  $P < 0.1$ ). There was no change in the myofibre density for either the female EDL *m.* type IIAB (Fig. 3.25A) or type IIAX (Fig. 3.25B) hybrid fibres.

### **EDL - male**

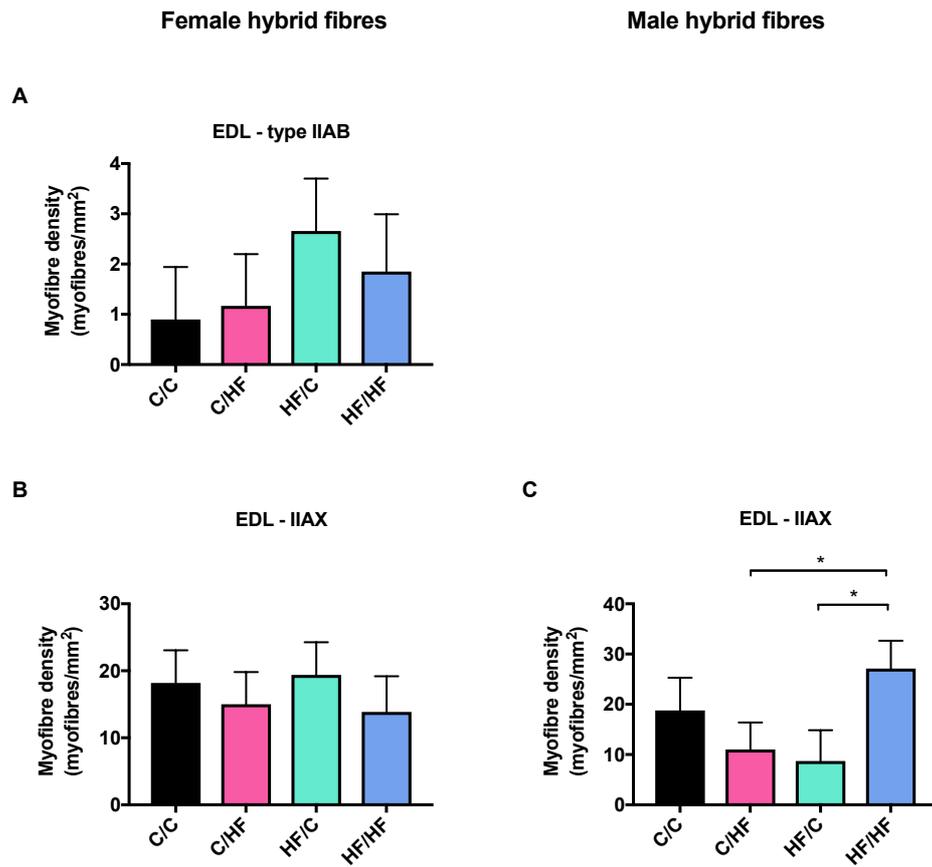
Myofibre density did not change for total (Fig. 3.24A), type I (Fig. 3.24B), type IIA (Fig. 3.24C), type IIB (Fig. 3.24D) or total type II (Fig. 3.24F) fibres. However, type IIX myofibres (Fig. 3.24E) from the EDL *m.* of male offspring did reduce in the C/HF ( $P < 0.05$ ), HF/C ( $P < 0.05$ ) and HF/HF ( $P < 0.01$ ) groups when compared with C/C. Type IIAX were the only hybrid fibres (Fig. 3.25) found in the male EDL *m.*, and their density was increased with a PRE HF diet (C/HF vs. HF/HF,  $P < 0.05$ ) and a POST HF diet (HF/C vs. HF/HF,  $P < 0.05$ ).



**Figure 3.23: Female EDL myofibre density.** Myofibre density (myofibres/mm<sup>2</sup>) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, n=7-8 per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus.



**Figure 3.24: Male EDL myofibre density.** Myofibre density (myofibres/mm<sup>2</sup>) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, \*\*\*P<0.001, n=7-8 per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus.



**Figure 3.25: EDL hybrid myofibre density.** Myofibre density (myofibres/mm<sup>2</sup>) of hybrid fibres **A)** female type IIAB, **B)** female type IIAX and **C)** male type IIAX. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus.

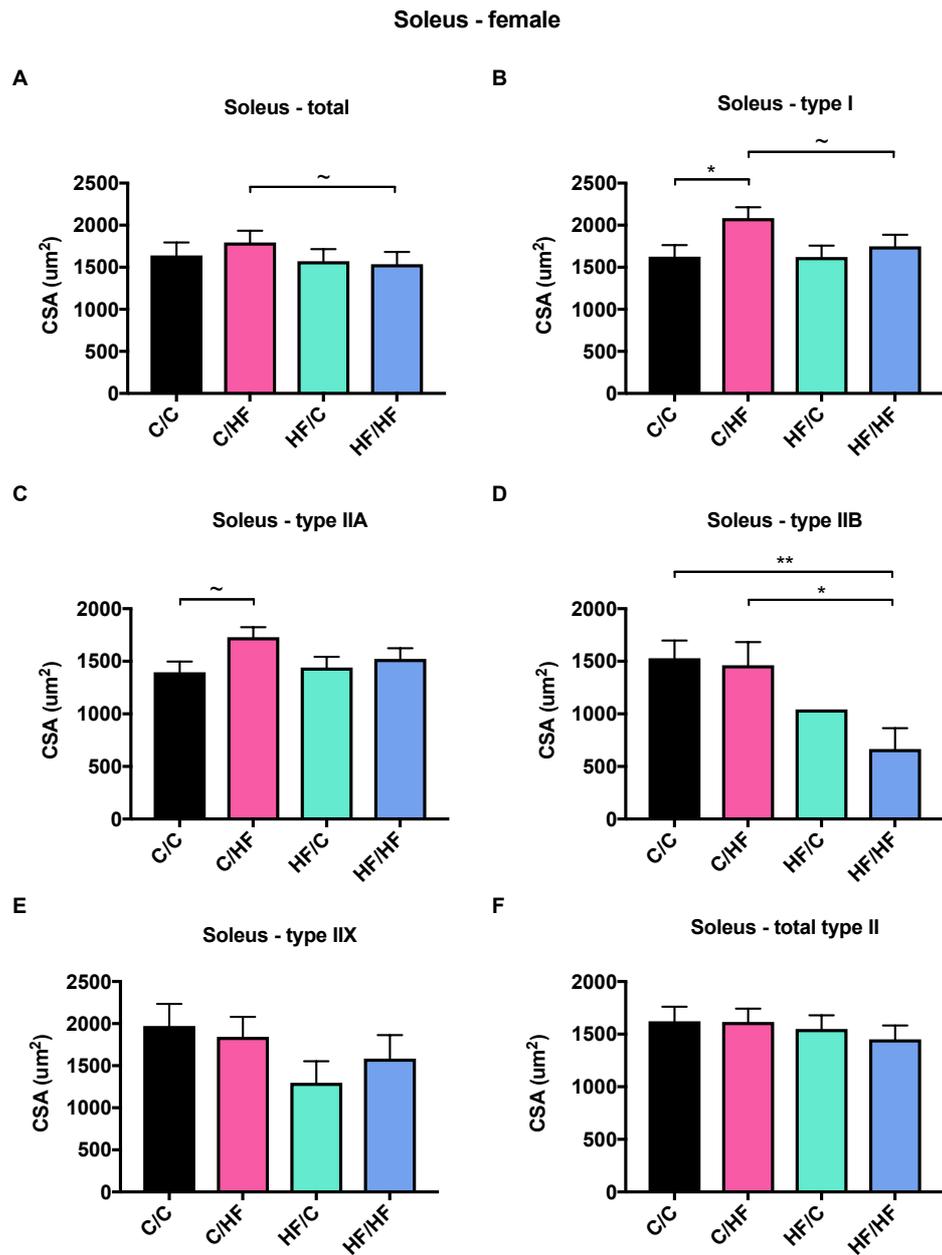
### 3.3.5.2 Myofibre cross-sectional area

#### Soleus – female

There were no differences in CSA with diet in the total (Fig. 3.26A), type IIA (Fig. 3.26C), type IIX (Fig. 3.26E) or total type II (Fig. 3.26F) fibres. However, there was a trend for a reduction ( $P < 0.1$ ) in total myofibre CSA with a PRE HF diet (C/HF vs. HF/HF) and there was a trend for an increase ( $P < 0.1$ ) in type IIA myofibres with a POST HF diet (C/C vs. C/HF). The CSA of type I fibres (Fig. 3.26B) increased ( $P < 0.05$ ) with a POST HF diet (C/C vs. C/HF), with only a trend for a reduction ( $P < 0.1$ ) in CSA with a PRE HF diet (C/HF vs. HF/HF). Type IIB fibre CSA (Fig. 3.26D) decreased in the HF/HF compared with C/C ( $P < 0.01$ ) and C/HF ( $P < 0.05$ ) diet groups. There were no differences in the CSA of type I/IIA or IIAX hybrid myofibres (Fig. 3.28A-B). Type IIAB hybrid fibres were not included in the CSA analysis as, by chance, they were not fully in the field of view, therefore could not be measured.

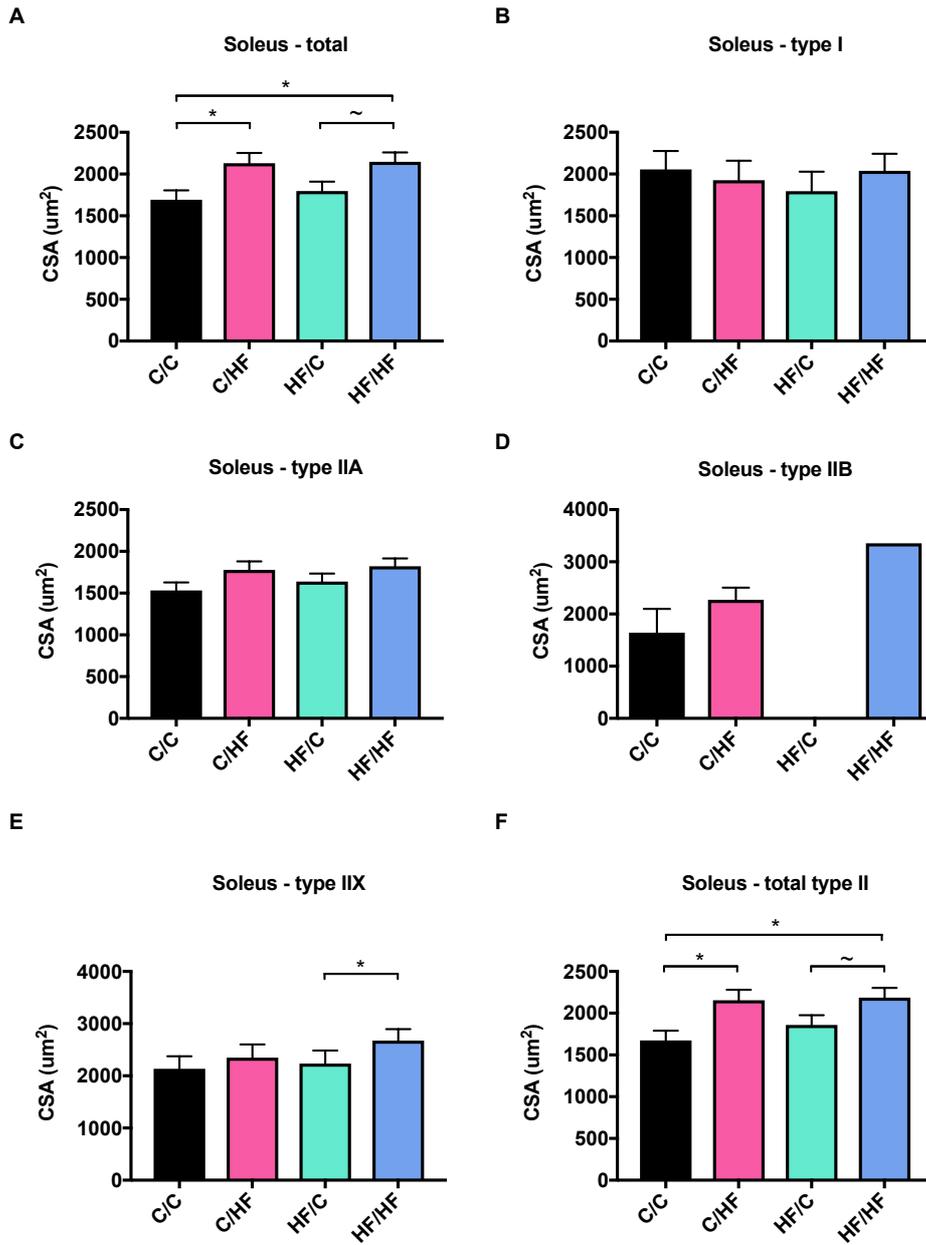
#### Soleus – male

For both total myofibres (Fig. 3.27A) and total type II myofibres (Fig. 3.27F), the CSA increased ( $P < 0.05$ ) in the HF/HF group compared with C/C. A POST HF diet was also associated with an increase in CSA (C/C vs. C/HF,  $P < 0.05$ ) but only a trend when the PRE diet was HF (HF/C vs. HF/HF,  $P < 0.1$ ). Type IIX fibres increased ( $P < 0.05$ ) with a POST HF diet (HF/C vs. HF/HF, Fig. 3.27E). Type IIAX hybrid fibre (Fig. 3.28D) CSA increased in the C/HF ( $P < 0.05$ ) and HF/HF ( $P < 0.05$ ) diet groups compared with C/C. The CSA did not significantly change with diet for the type I (Fig. 3.27B), type IIA (Fig. 3.27C), type IIB (Fig. 3.27D) or type I/IIA (Fig. 3.28C) fibres in the male offspring soleus *m*.

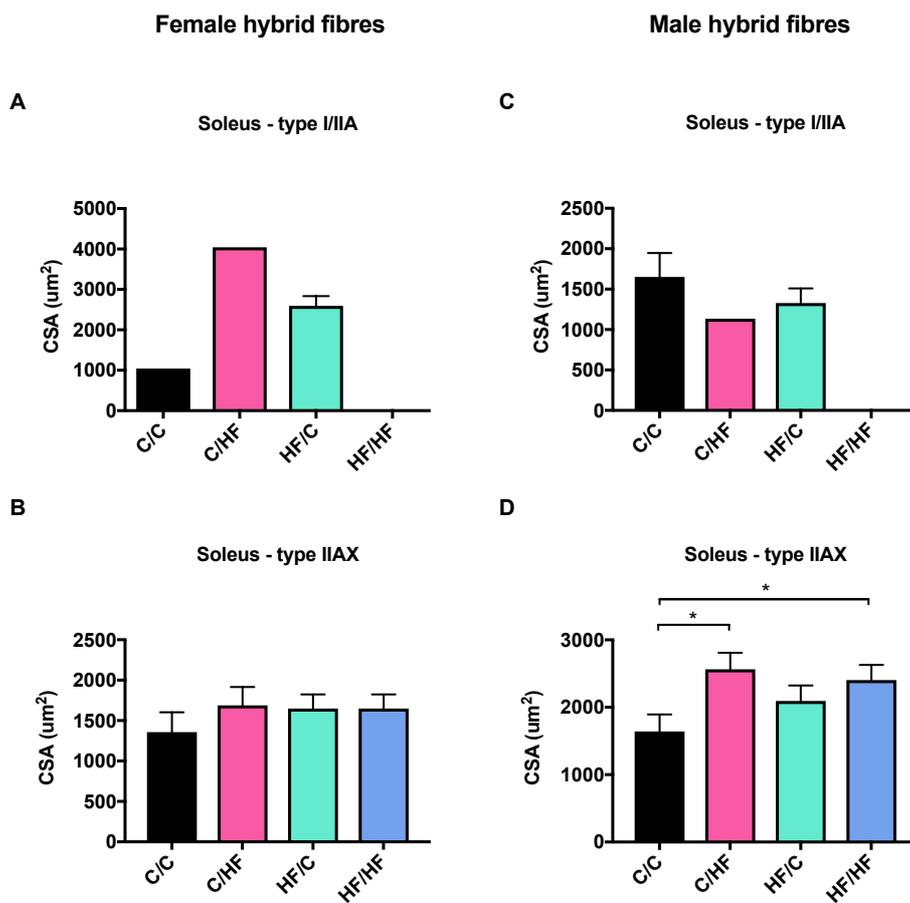


**Figure 3.26: Female soleus myofibre cross-sectional area.** Myofibre CSA ( $\mu\text{m}^2$ ) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; CSA, cross-sectional area.

Soleus - male



**Figure 3.27: Male soleus myofiber cross-sectional area.** Myofiber CSA ( $\mu\text{m}^2$ ) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. ~ $P < 0.1$ , \* $P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; CSA, cross-sectional area.



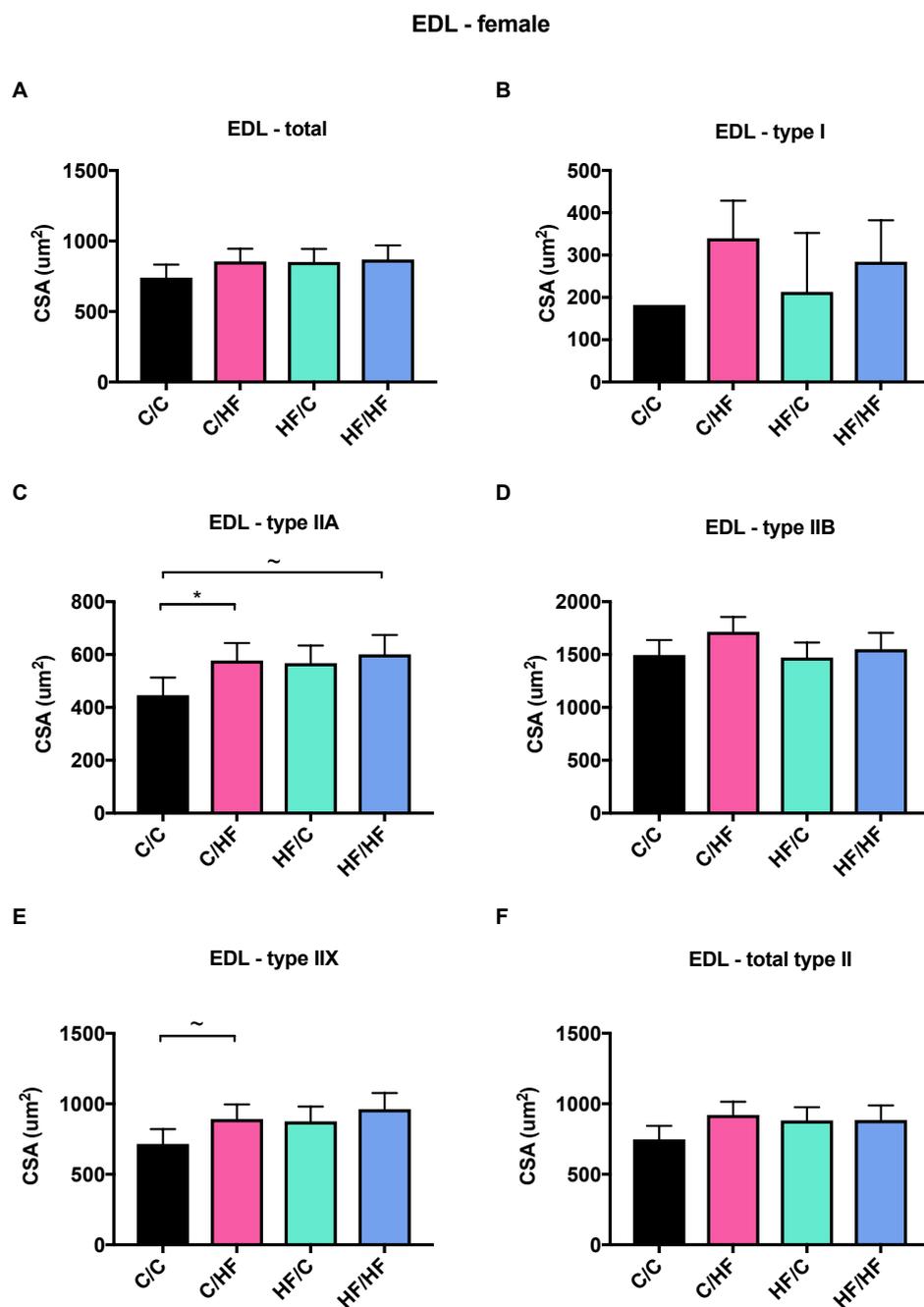
**Figure 3.28: Soleus hybrid myofibre cross-sectional area.** Myofibre CSA ( $\mu\text{m}^2$ ) of hybrid fibres **A)** female type I/IIA, **B)** female type IIAX, **C)** male type I/IIA and **D)** male type IIAX. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; CSA, cross-sectional area.

### **EDL – female**

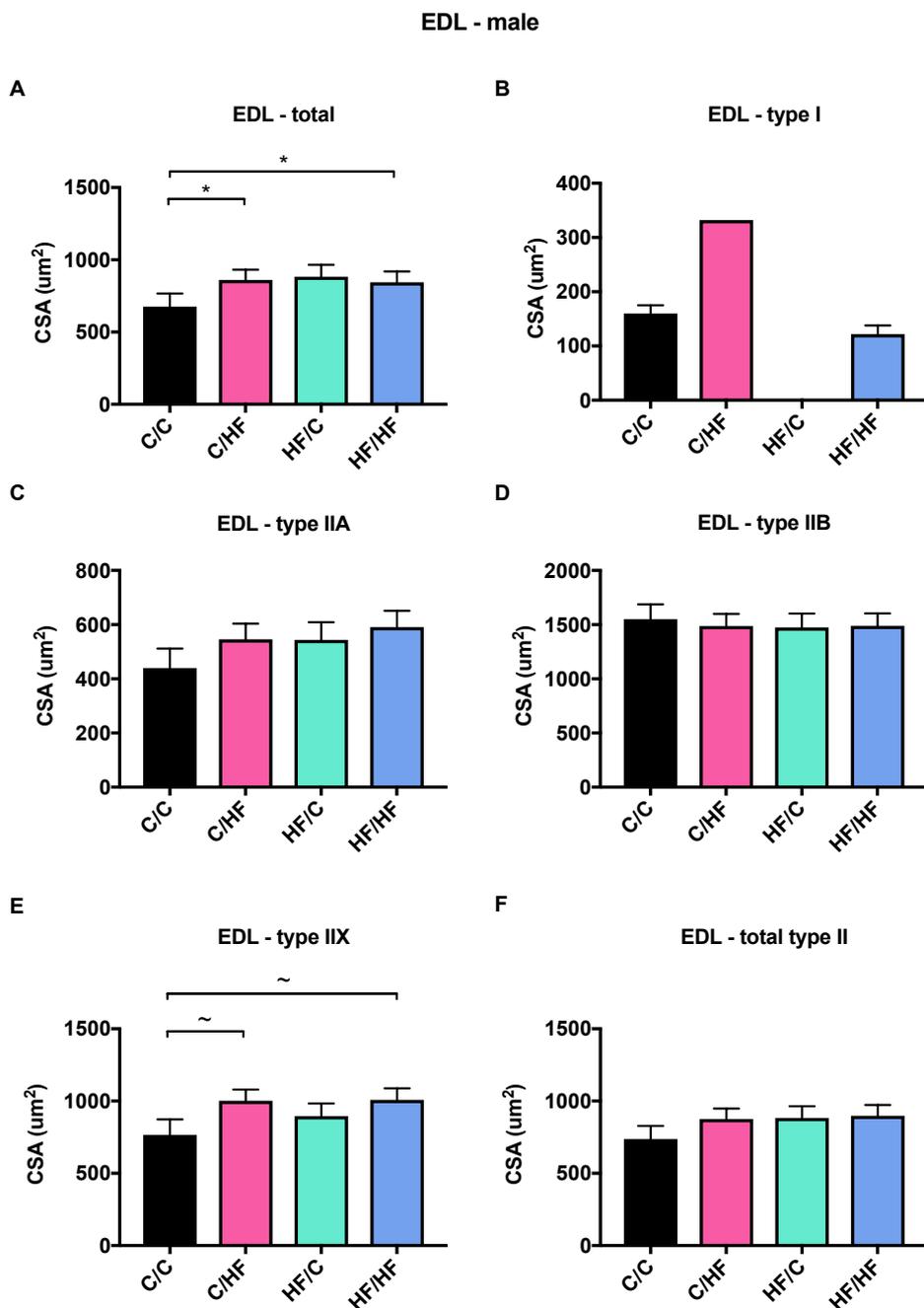
The CSA of female EDL *m.* total (Fig. 3.29A), type I (Fig. 3.29B), type IIB (Fig. 3.29D), type IIX (Fig. 3.29E) and total type II (Fig. 3.29F) fibres did not change with diet. However, there was a trend for an increase in type IIX CSA with a POST HF diet (C/C vs. C/HF,  $P < 0.1$ ). The type IIA EDL fibres (Fig. 3.29C) increased in CSA with a POST HF diet (C/C vs. C/HF,  $P < 0.05$ ) and a trend for an increase ( $P < 0.1$ ) in the HF/HF group. There were no differences in CSA of type IIAB hybrid fibres (Fig. 3.31A), but there was an increase ( $P < 0.05$ ) in type IIAX hybrid fibres (Fig. 3.31B) in the HF/HF group compared with C/C.

### **EDL – male**

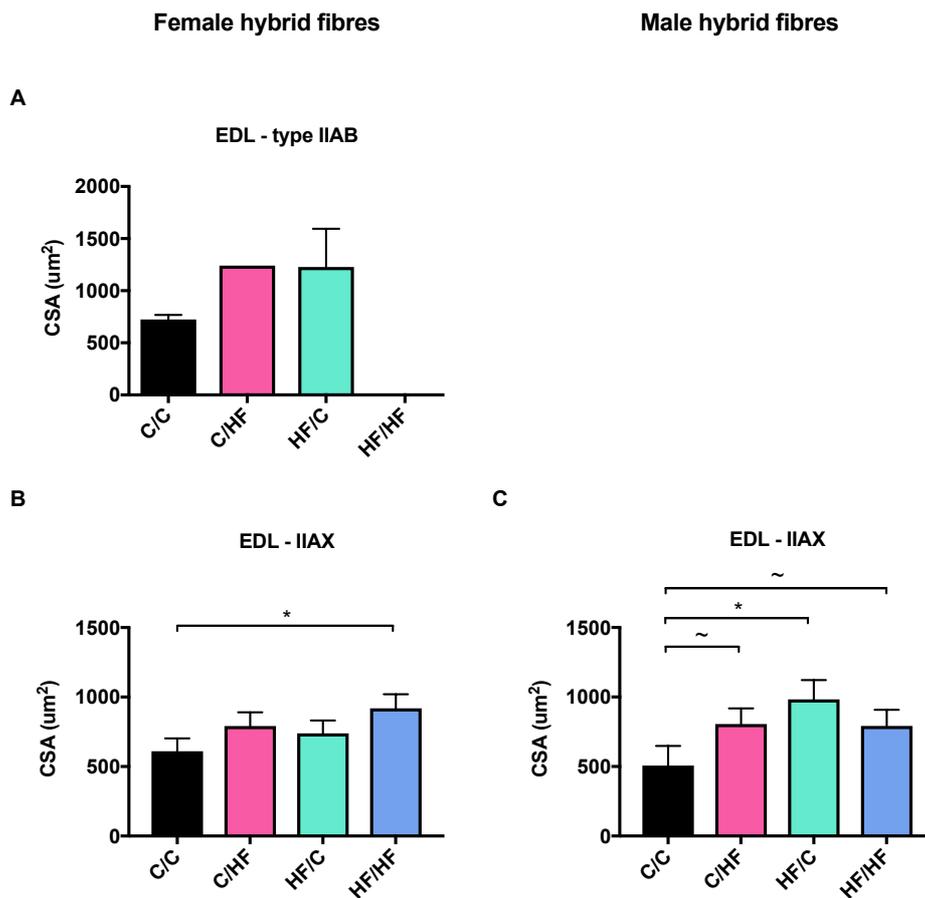
CSA of total myofibres (Fig. 3.30A) increased with a POST HF diet (C/C vs. C/HF,  $P < 0.05$ ) and with a HF/HF diet (C/C vs. HF/HF,  $P < 0.05$ ). There were no changes in male EDL *m.* fibre CSA of type I (Fig. 3.30B), type IIA (Fig. 3.30C), type IIB (Fig. 3.30D), type IIX (Fig. 3.30E) or total type II fibres (Fig. 3.30F), but there was a trend for an increase ( $P < 0.1$ ) in the type IIX CSA of C/HF and HF/HF offspring when compared with C/C. Regarding the type IIAX hybrid fibres (Fig. 3.31C), CSA significantly increased with a PRE HF diet (C/C vs. HF/C,  $P < 0.05$ ), but only with a trend ( $P < 0.1$ ) for an increase in CSA for the C/HF and HF/HF offspring.



**Figure 3.29: Female EDL myofibre cross-sectional area.** Myofibre CSA ( $\mu\text{m}^2$ ) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; CSA, cross-sectional area.



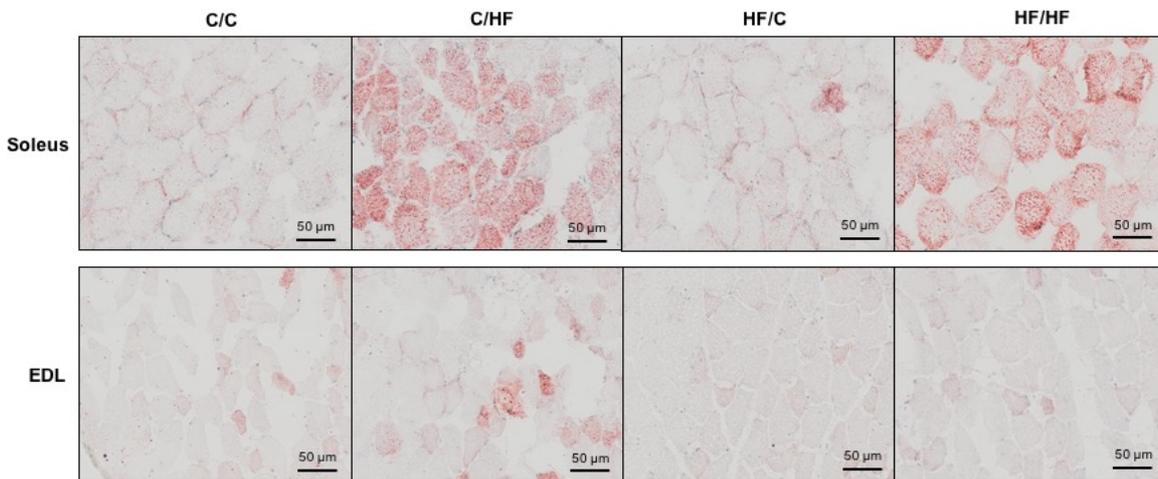
**Figure 3.30: Male EDL myofiber cross-sectional area.** Myofiber CSA ( $\mu\text{m}^2$ ) for **A)** total, **B)** type I, **C)** type IIA, **D)** type IIB, **E)** type IIX and **F)** total type II fibres. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; CSA, cross-sectional area.



**Figure 3.31: EDL hybrid myofibre cross-sectional area.** Myofibre CSA ( $\mu\text{m}^2$ ) of hybrid fibres **A)** female type IIAB, **B)** female type IIAX, **C)** male type IIAX. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; CSA, cross-sectional area.

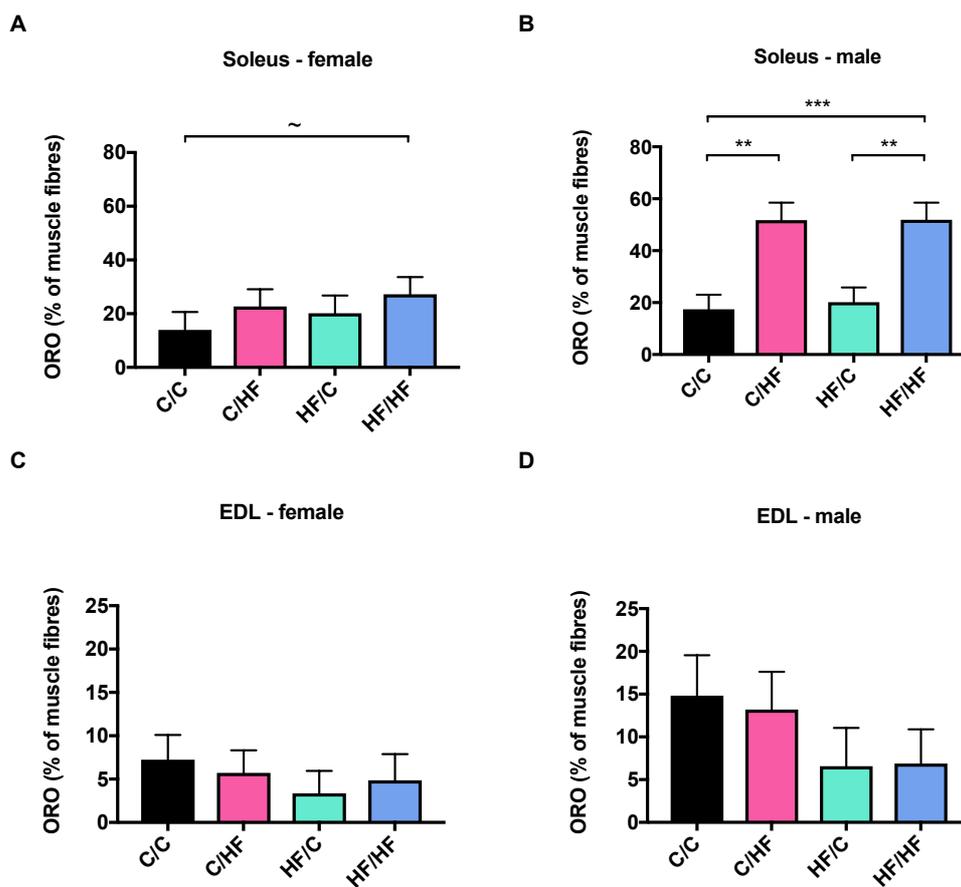
### 3.3.5.3 Intramuscular lipid accumulation

Representative oil red O (ORO) staining for the female soleus and EDL *m.* fibres across the four dietary groups are displayed in Figure 3.32.



**Figure 3.32: Lipid accumulation in 30-week offspring skeletal muscle.** Representative images of the ORO staining in the soleus *m.* and EDL *m.* from offspring of all dietary groups. Red staining shows areas of lipid accumulation. Scale bars represent 50 μm, (magnification x40). EDL, extensor digitorum longus; *m.*, muscle; C, control; HF, high-fat, ORO, oil red O.

There were no significant effects of diet group on the amount of lipid accumulation in the soleus *m.* of female offspring (Fig. 3.33A), however, there was a trend for an increase ( $P < 0.1$ ) in the HF/HF diet group compared with C/C. Conversely in male offspring, a POST HF diet caused an increase ( $P < 0.01$ ) in intramuscular lipid accumulation in the male soleus *m.* regardless of PRE diet (Fig. 3.33B). Diet did not lead to any changes in lipid accumulation in the female EDL *m.* (Fig. 3.33C) or male EDL *m.* (Fig. 3.33D).



**Figure 3.33: Oil red O analysis of offspring skeletal muscle.** Lipid accumulation (%) within the muscle fibres of **A)** female soleus, **B)** male soleus, **C)** female EDL, **D)** male EDL *m*. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*\*P<0.05, n=7-8 per group. C, control diet; HF, high-fat diet; EDL, extensor digitorum longus; *m*., muscle; ORO, oil red O.

### 3.3.6 Gene expression

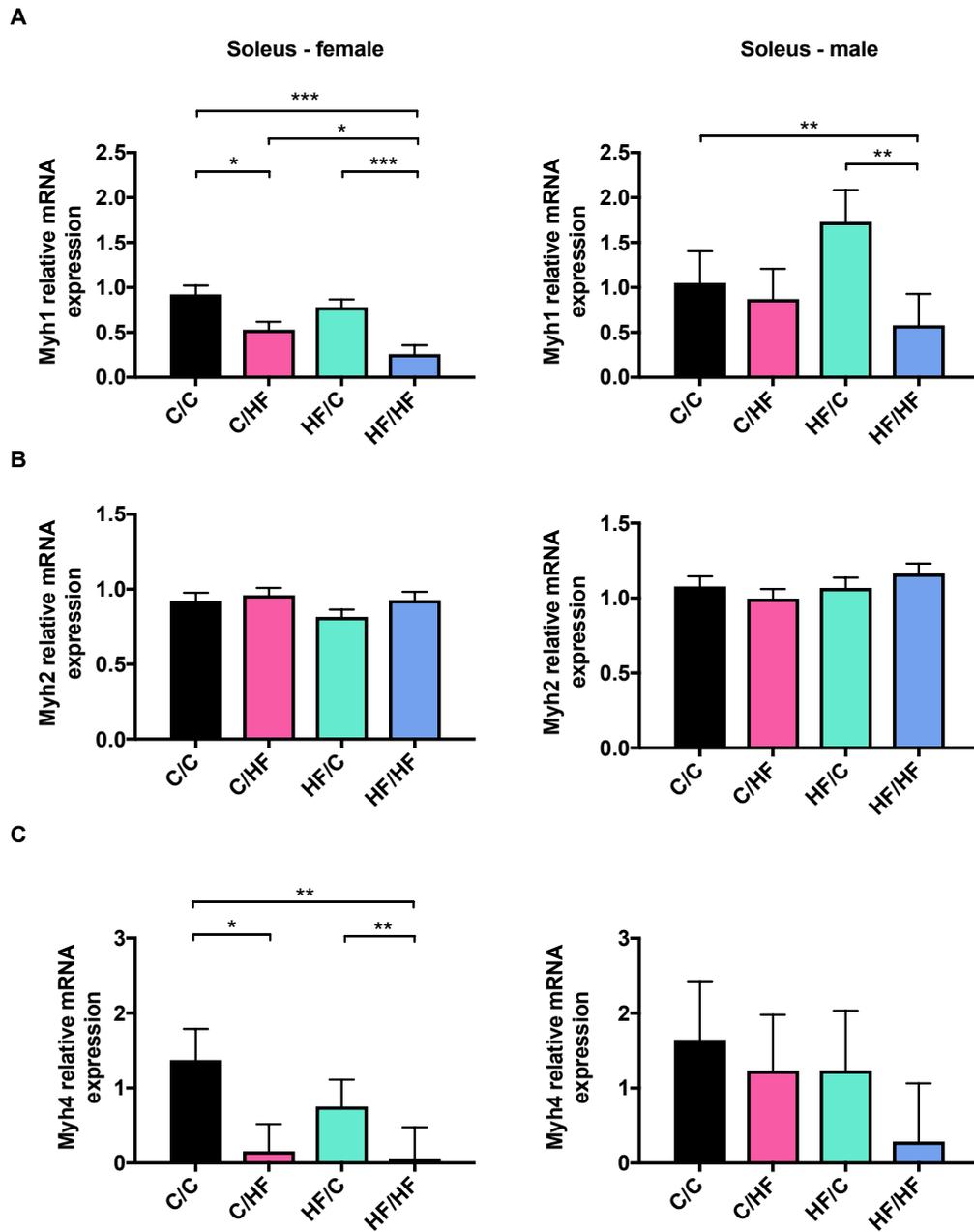
#### 3.3.6.1 30-week offspring

##### Contractile components

The genes encoding numerous components of the skeletal muscle contractile machinery were analysed for their mRNA expression (Fig. 3.34-3.37).

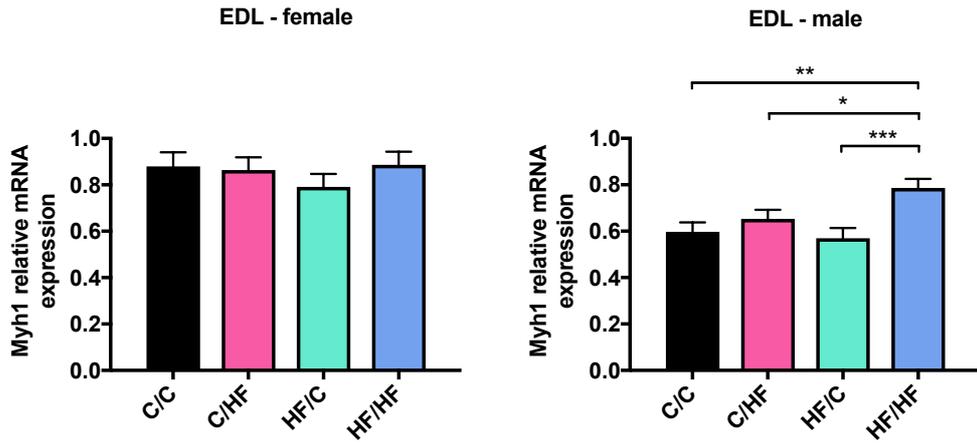
In the soleus *m.*, there was a reduction in *Myh1* expression (type IIx fibres; Fig. 3.34A) in the HF/HF compared with the C/C group (females,  $P < 0.001$ ; males,  $P < 0.01$ ) and compared with the HF/C diet group (females,  $P < 0.001$ ; males,  $P < 0.01$ ). Female *Myh1* expression reduced in the HF/HF compared with C/HF group ( $P < 0.05$ ) and in the C/HF compared with C/C group ( $P < 0.05$ ), but this was not found in the males. No significant differences were found in the soleus *Myh2* mRNA expression (type IIa fibres; Fig. 3.34B) in either female or male offspring. *Myh4* expression (type IIb fibres; Fig. 3.34C) was not different in the male offspring, however, there was a decrease in expression with a POST HF diet (C/C vs. C/HF,  $P < 0.05$ ; HF/C vs. HF/HF,  $P < 0.01$ ). Female soleus *m.* *Myh4* expression was reduced in the HF/HF group when compared with C/C ( $P < 0.01$ ).

For the EDL *m.*, no differences were found in the expression of *Myh1* (Fig. 3.35A), *Myh2* (Fig. 3.35B) or *Myh4* (Fig. 3.35C) mRNA in the female offspring, however, there was a trend for an increase ( $P < 0.1$ ) in *Myh2* with a POST HF diet compared with control (C/C vs. C/HF). In the male offspring, *Myh1* (Fig. 3.35A) was significantly higher in the HF/HF compared with C/C ( $P < 0.01$ ), C/HF ( $P < 0.05$ ) and HF/C ( $P < 0.001$ ) diet groups. The expression of *Myh2* (Fig. 3.35B) increased with a POST HF diet regardless of PRE diet ( $P < 0.05$ ) and in the HF/HF group (C/C vs. HF/HF,  $P < 0.001$ ). There were no differences in *Myh4* (Fig. 3.35C) between diet group in the males.

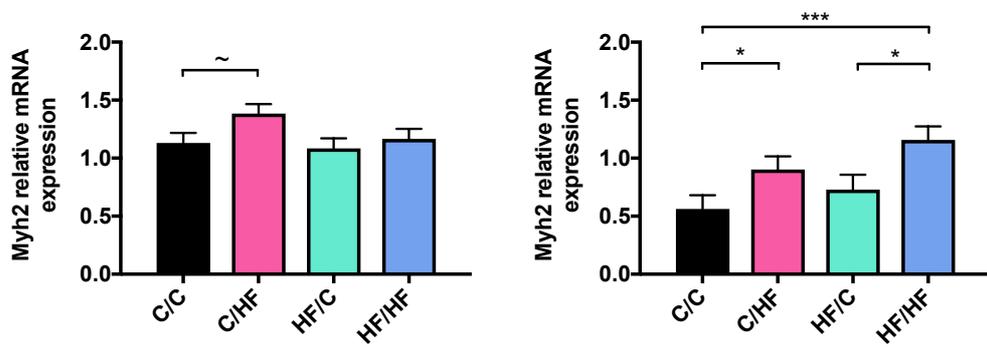


**Figure 3.34: 30-week soleus muscle mRNA expression of myosin heavy chain isoforms. A) *Myh1*, B) *Myh2*, C) *Myh4*.** Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; Myh, myosin heavy chain; mRNA, messenger ribonucleic acid.

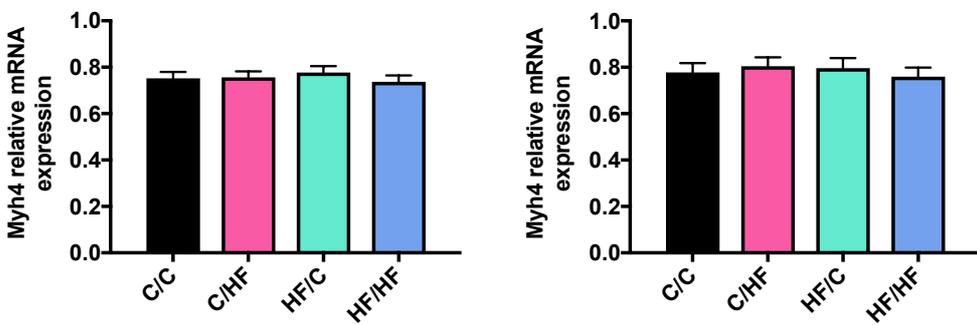
A



B



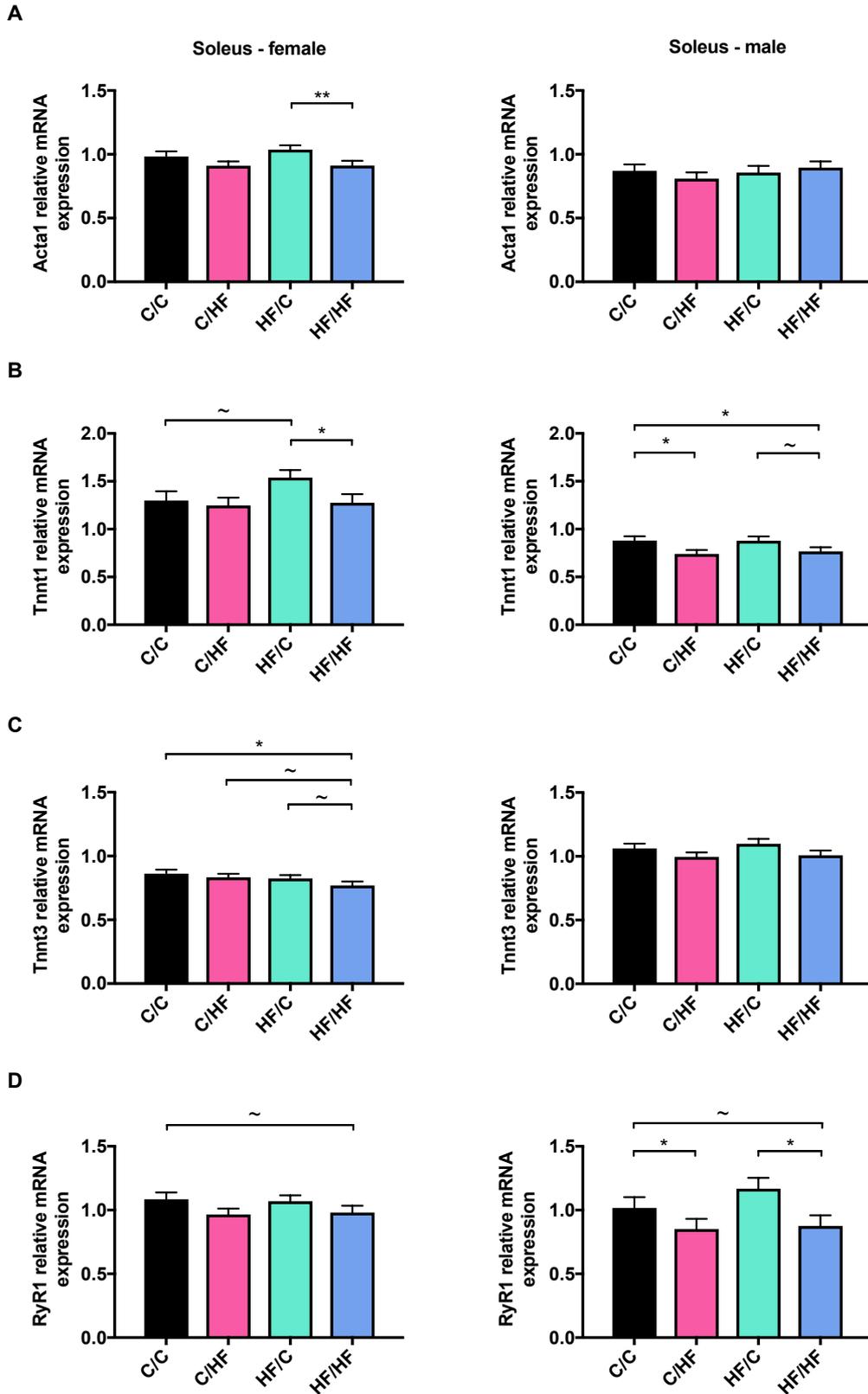
C



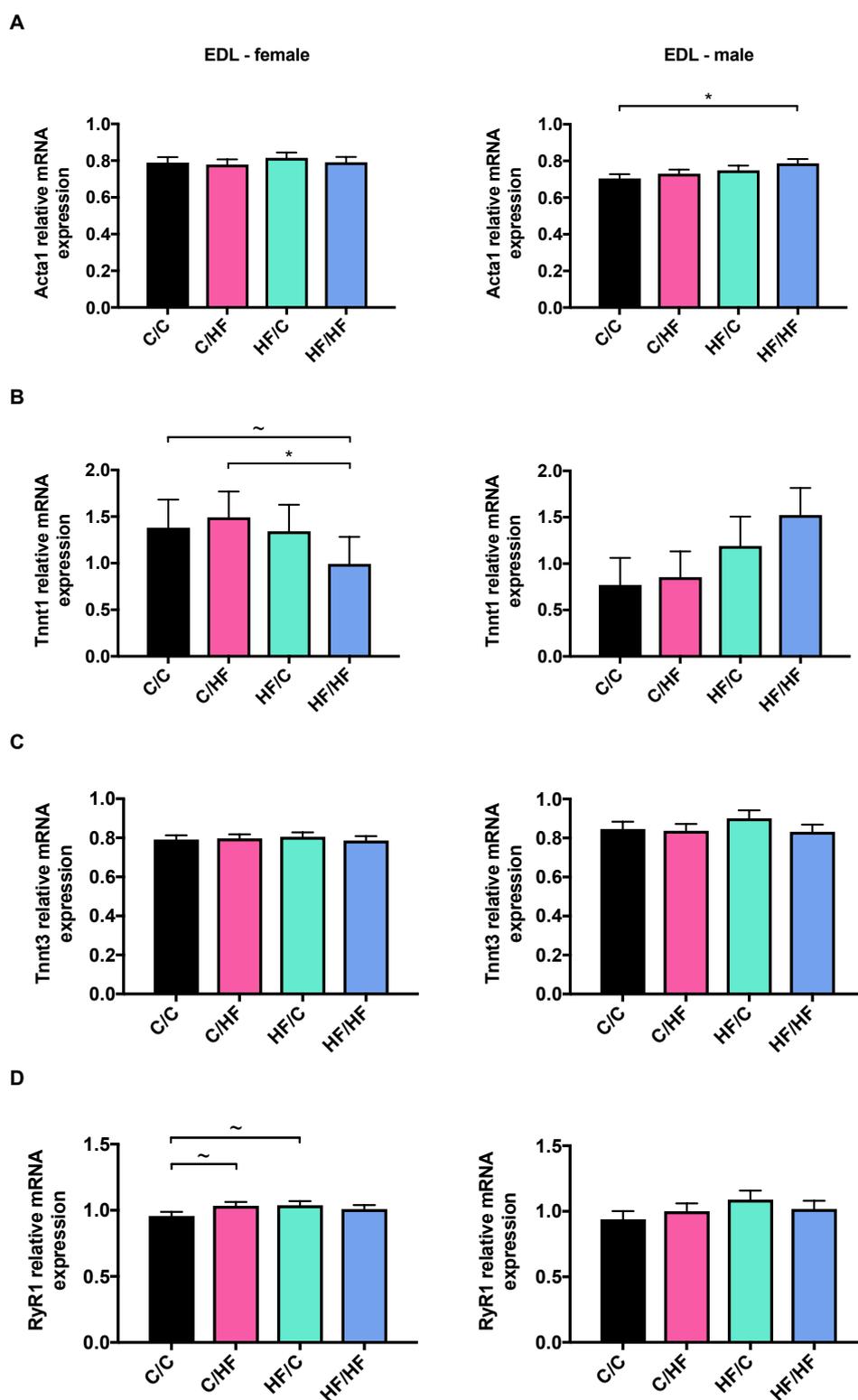
**Figure 3.35: 30-week EDL muscle mRNA expression of myosin heavy chain isoforms. A) *Myh1*, B) *Myh2*, C) *Myh4*.** Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, n=7-8 per group. C, control diet; HF, high-fat diet; Myh, myosin heavy chain; mRNA, messenger ribonucleic acid.

Other components as part of the contractile machinery were also analysed including the mRNA expression for actin alpha 1 (*Acta1*), troponin T1 (*Tnnt1*, slow-twitch muscle fibres), troponin T3 (*Tnnt3*, fast-twitch muscle fibres) and the ryanodine receptor (*RyR1*). In the soleus, *Acta1* (Fig. 3.36A) was reduced in the HF/HF group compared with HF/C ( $P<0.01$ ) in the female offspring, but no differences were observed in the males. The expression of *Tnnt1* (Fig. 3.36B) was significantly reduced ( $P<0.05$ ) in the HF/HF group compared with HF/C in the females, but only a trend ( $P<0.1$ ) in the males. The male soleus decreased in *Tnnt1* mRNA expression in the C/HF ( $P<0.05$ ) and HF/HF ( $P<0.05$ ) groups compared with control. Expression of *Tnnt3* (Fig. 3.36C), did not change with diet group in the male offspring, but there was a decrease ( $P<0.05$ ) in the HF/HF females, and a trend ( $P<0.1$ ) for a decrease in the HF/HF group when compared with C/HF and HF/C. Expression of *RyR1* mRNA (Fig. 3.36D), did not significantly change in the female soleus *m.*, apart from a trend ( $P<0.1$ ) for a decrease in the HF/HF compared with C/C group, which was also observed in the males. Regardless of PRE diet, there was a reduction ( $P<0.05$ ) in male soleus *m.* *RyR1* expression with a POST HF diet.

For the EDL *m.*, there was no change in *Acta1* (Fig. 3.37A) with diet in the females, however there was a significant increase in the male HF/HF offspring (C/C vs. HF/HF,  $P<0.05$ ). A reduction ( $P<0.05$ ) in *Tnnt1* (Fig. 3.37B) was found in the HF/HF group compared with C/HF for the female EDL *m.*, with a trend ( $P<0.1$ ) when compared with control (C/C vs. HF/HF). No differences in *Tnnt1* expression were found in the male EDL *m.*, and no differences were found in *Tnnt3* (Fig. 3.37C) for either female or male EDL *m.* There were also no significant changes in *RyR1* mRNA (Fig. 3.37D) levels in the female or male offspring, however there was a trend ( $P<0.1$ ) for an increase in the C/HF and HF/C groups when compared with C/C.



**Figure 3.36: 30-week soleus muscle mRNA expression of contractile components. A) *Acta1*, B) *Tnnt1*, C) *Tnnt3*, D) *RyR1*. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, \*\*P<0.01, n=7-8 per group. C, control diet; HF, high-fat diet; *Acta*, actin alpha 1; *Tnnt*, troponin T; *RyR1*, ryanodine receptor; mRNA, messenger ribonucleic acid.**



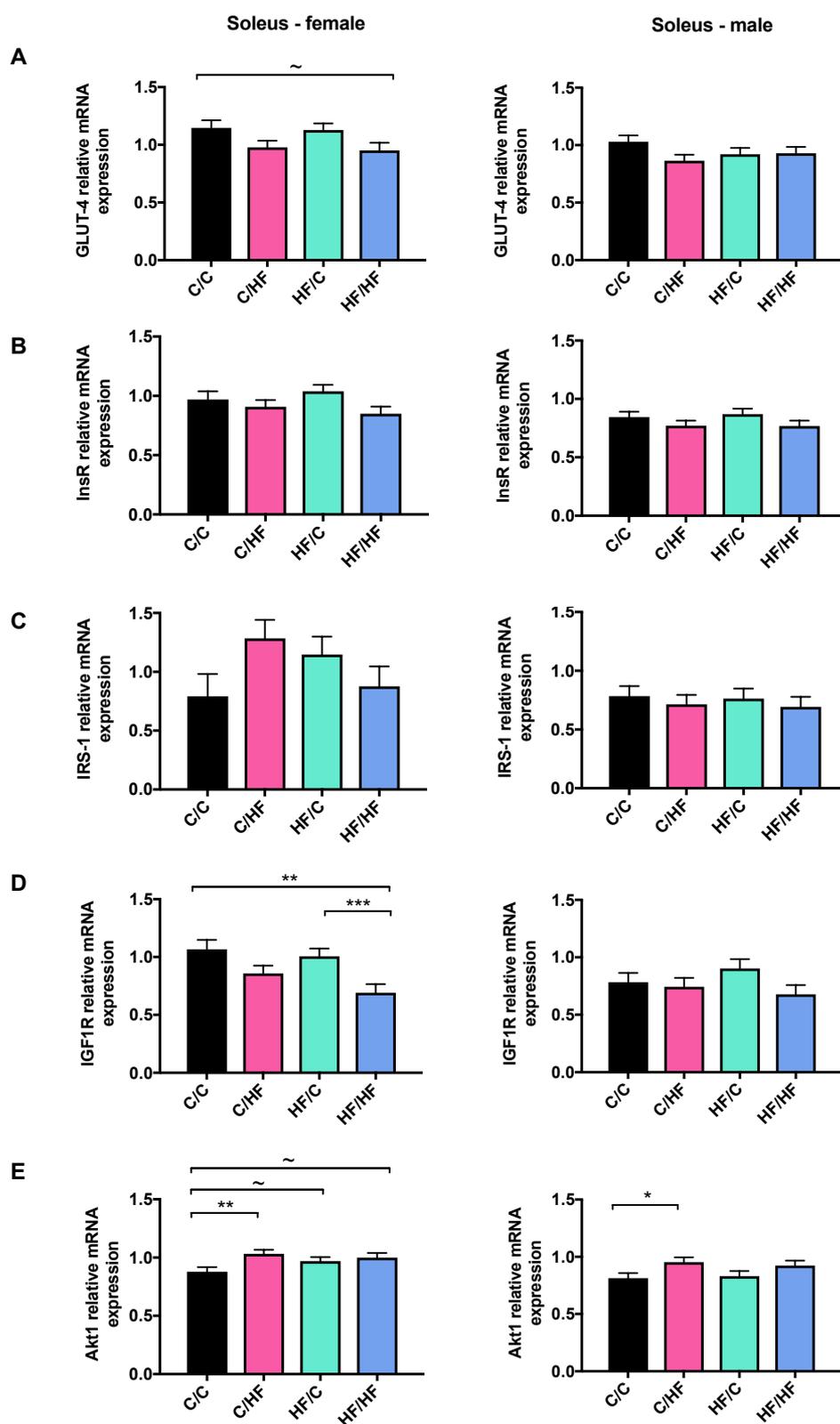
**Figure 3.37: 30-week EDL muscle mRNA expression of contractile components. A) *Acta1*, B) *Tnnt1*, C) *Tnnt3*, D) *RyR1*. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, n=7-8 per group. C, control diet; HF, high-fat diet; *Acta*, actin alpha 1; *Tnnt*, troponin T; *RyR1*, ryanodine receptor; mRNA, messenger ribonucleic acid.**

## Insulin signalling

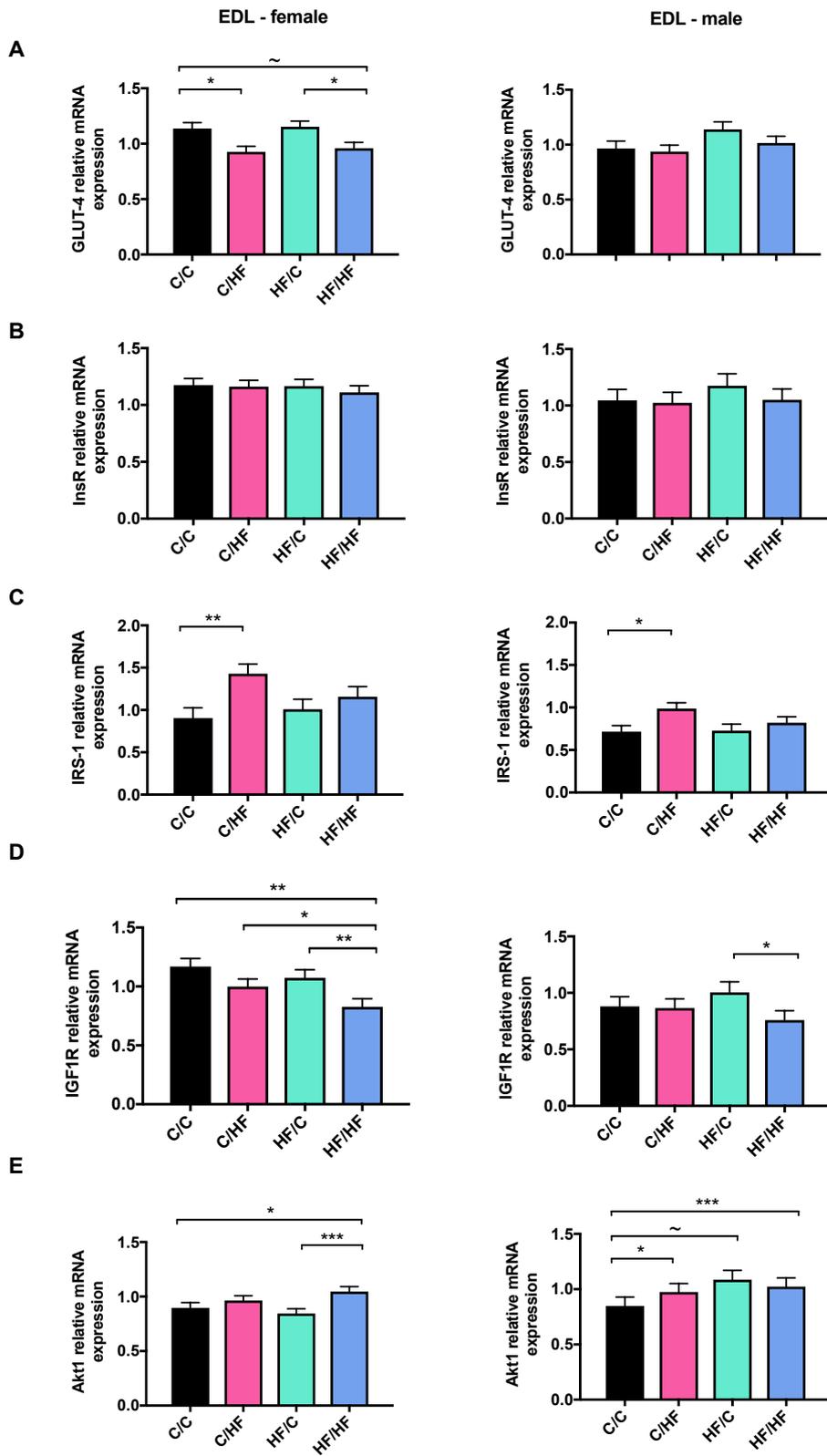
The expression of genes that encode for various proteins as part of the insulin-signalling pathway were quantified at the level of mRNA.

In the soleus *m.* of female and male 30-week offspring, there were no differences in the mRNA expression of *Glut4* (glucose transporter 4; Fig. 3.38A), *Insr* (insulin receptor; Fig. 3.38B) or *Irs1* (insulin receptor substrate 1; Fig. 3.38C), although there was a trend ( $P<0.1$ ) for a reduction in *Glut4* in the female soleus *m.* with the HF/HF group compared with C/C. The HF/HF diet group displayed a significant reduction in *Igflr* expression (insulin-like growth factor 1 receptor; Fig. 3.38D) when compared with C/C ( $P<0.01$ ) and HF/C ( $P<0.001$ ) in the females but not males. There was an increase in *Akt1* expression (serine/threonine kinase; Fig. 3.38E) in both the female and male C/HF offspring compared with control ( $P<0.01$  and  $P<0.05$ , respectively). There were also trends ( $P<0.1$ ) for an increase in female soleus *m.* *Akt1* expression in the HF/C and HF/HF groups when compared with control.

In the EDL *m.* of female offspring, a POST HF diet was associated with a reduction in *Glut4* expression regardless of PRE diet (C/C vs. C/HF,  $P<0.05$ ; HF/C vs. HF/HF,  $P<0.05$ ; Fig. 3.39A), with a trend ( $P<0.1$ ) for a reduction in the HF/HF group compared with control. There were no differences between dietary groups in *Glut4* with the male EDL *m.* Expression of *Insr* (Fig. 3.39B) did not change with diet group in either sex. EDL *m.* *Irs1* expression (Fig. 3.39C) increased with a POST HF diet (C/C vs. C/HF) in both female ( $P<0.01$ ) and male ( $P<0.05$ ) offspring. The expression of *Igflr* (Fig. 3.39D) decreased with a POST HF diet (HF/C vs. HF/HF) in the females ( $P<0.01$ ) and males ( $P<0.05$ ). The female EDL *m.* samples also displayed a reduction in *Igflr* expression with the HF/HF group when compared with C/C ( $P<0.01$ ) and C/HF ( $P<0.05$ ). In terms of *Akt1* expression (Fig. 3.39E), there was an increase in the HF/HF group compared with C/C in the female ( $P<0.05$ ) and male ( $P<0.001$ ) offspring. The female EDL *m.* *Akt1* expression increased ( $P<0.001$ ) with a POST HF diet (HF/C vs. HF/HF). The male offspring also increased ( $P<0.05$ ) in *Akt1* with a POST HF diet (C/C vs. C/HF). There was a trend ( $P<0.1$ ) for an increase in *Akt1* with a PRE HF diet compared with control in the male EDL *m.* (C/C vs. HF/C).



**Figure 3.38: 30-week soleus muscle insulin-signalling pathway mRNA expression.** A) *Glut4*, B) *InsR*, C) *Irs1*, D) *Igf1r*, E) *Akt1*. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim$ P<0.1, \*P<0.05, \*\*P<0.01, n=7-8 per group. C, control diet; HF, high-fat diet; *Glut*, glucose transporter; *InsR*, insulin receptor; *Irs*, insulin receptor substrate; *Igf1r*, insulin-like growth factor 1 receptor; *Akt*, serine/threonine kinase; mRNA, messenger ribonucleic acid.



**Figure 3.39: 30-week EDL muscle insulin-signalling pathway mRNA expression. A) *Glut4*, B) *InsR*, C) *Irs1*, D) *Igf1r*, E) *Akt1*.** Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $n = 7-8$  per group. C, control diet; HF, high-fat diet; *Glut*, glucose transporter; *InsR*, insulin receptor; *Irs*, insulin receptor substrate; *Igf1r*, insulin-like growth factor 1 receptor; *Akt*, serine/threonine kinase; mRNA, messenger ribonucleic acid.

## 3.4 Discussion

### 3.4.1 Skeletal muscle contractile force and kinetics

Skeletal muscle physiological function was assessed by determining the peak force (PF) and contractile kinetics (time-to-peak tension, TPT; half-relaxation time, HRT) of isometric tetanic and single-twitch contraction. The major findings (Table 3.2) were that PF was impaired in the soleus *m.* only by a POST HF diet and a combined PRE and POST HF diet, but with little effect of PRE HF diet alone. There were subtleties in the features of the effects according to diet; a HF diet affected PF, TPT and HRT differently based on the time of the HF challenge, and the muscle bed.

**Table 3.2: Overview of the main skeletal muscle contractile findings following a high-fat diet.** EDL, extensor digitorum longus; PF, peak force; TPT, time-to-peak tension; HRT, half-relaxation time; POST, postnatal; PRE, prenatal. ↓= change with tetanic and twitch contraction, ↓= change with tetanic contraction only, ↓= change with twitch contraction only, X= no change in parameter.

	Soleus			EDL		
Female	PF	TPT	HRT	PF	TPT	HRT
POST HF	↓	X	X	X	X	X
PRE HF	X	↑	X	X	X	X
PRE + POST HF	↓	↑	X	X	X	X
Male	PF	TPT	HRT	PF	TPT	HRT
POST HF	↓	↑	↑	X	↑	↑
PRE HF	↓	↑	↑	X	↓	X
PRE + POST HF	↓	↑	X	X	↓	X

#### 3.4.1.1 Peak force

The reduction in PF observed in this study is of physiological significance because it means a reduced capacity to produce maximal contraction. This is likely to consequently

impact on adult muscle strength and may have implications in later life, such as developing sarcopenia (Morley *et al.* 2001).

### ***Postnatal high-fat***

A POST HF diet reduced the PF of tetanic and single-twitch contraction in the female and male soleus *m.*, but not the EDL *m.* (although with several subtle differences between single-twitch and tetanic contraction), resulting in weaker muscles. Although the *in vivo* strength test score was not significantly different with a POST HF diet, there was a trend for a decrease in the female offspring's strength score in the HF/HF compared with HF/C group. The reduction in PF in the soleus *m.* and trend in the *in vivo* strength test is similar to a previous study in adult mice which showed a reduction in *in vivo* grip strength and lower raw soleus *m.* mass in adult 14-month-old mice (older than the current study) fed a POST HF diet (Lee *et al.* 2015a). In our study, there was also an increase in raw soleus *m.* weight with POST HF but this was only in female offspring, whereas Lee *et al.* studied males and their HF diet contained a higher percentage of fat (60% vs. 45% kcal fat). In the present study, all muscles of male and female offspring reduced in normalised muscle weight (muscle weight relative to body weight) with a POST HF diet, which may be one factor explaining the reduced PF. A human study of 48-92 year old men and women found that a higher waist circumference (indicator of central obesity) is associated with a lower grip strength (Keevil *et al.* 2014). These participants are older relative to lifespan than the 30-week mice, and unlike this chapter, the participants were not exposed to a HF diet specifically, however, the current study and work from Keevil *et al.* both show impairments in adulthood strength with obesity. For the current study, additional *in vivo* strength tests, such as grip strength, would have been useful to allow comparison with previous studies.

### ***Prenatal high-fat***

There was a trend for a decrease in male offspring *in vivo* strength in the HF/C group compared with C/C, however, this was not reflected in the PF data. There were no significant effects of a PRE HF diet on the isolated muscle PF of the female soleus or EDL

*m.* or male EDL *m.* Another study in fact observed that there was a reduction in muscle force with maternal obesity (Bayol *et al.* 2009). The differences in findings compared with this study are likely to be due to differences in the study paradigms. Bayol *et al.* used a group of muscles including the gastrocnemius, soleus and plantaris *m.* instead of a single muscle, so there will be a greater range of myofibre types involved in the contractile process. Although Bayol *et al.* did not analyse a HF diet specifically, both studies investigate a mouse model of maternal obesity. They also used an obesogenic mouse diet which was higher in carbohydrates: 47% carbohydrate versus 35% carbohydrate, and had lower % kcal fat than the current study (19% vs. 45% kcal fat). The increase in carbohydrate of the diet used by Bayol *et al.* may have had a greater PRE effect for muscle function than the diet used here. Bayol *et al.* also used rats at 10 weeks of age compared with the 30-week old mice used here. Therefore, the effect of a PRE obesogenic diet may be more significant at a younger age as there is less time to recover and adapt to the impact a PRE HF diet has on skeletal muscle. In this study there was a reduction in PF during tetanic contraction in the male soleus *m.* after a PRE HF diet (C/HF vs. HF/HF). The effect of a PRE obesogenic diet here (despite protocol differences) agrees with Bayol *et al.* We observed this effect in a muscle bed and sex-specific manner, which cannot be comparable to their study as they grouped male and female offspring together and did not look at the soleus *m.* in isolation. In this chapter there was no effect on offspring muscle weights with a PRE HF diet. With no change in the amount of muscle to body weight, it could help to explain why there were no changes to PF in these offspring.

### ***Combined pre- and postnatal high-fat***

In male and female soleus *m.*, a combined PRE and POST HF diet (HF/HF group) reduced PF, although this was just a trend for the male tetanic contraction. This general reduction in the soleus *m.* suggests that a HF diet over the life course is detrimental to skeletal muscle contractile function compared with a control diet over the life course. This is not surprising, since these offspring had a double ‘hit’ in HF and results in reduced muscle weight in relation to body weight. These results are similar with Bayol *et al.* as they found a reduction in specific muscle twitch and tetanic contraction and a reduction in muscle weight relative to body weight with their obesogenic diet during pregnancy and postnatally up until 10 weeks of age. In some ways this makes sense, but it goes against the theory of

predictive adaptive responses (PARs) which suggests that a mismatch in PRE and POST diet is more detrimental than when the diets are matched (Gluckman *et al.* 2005), but perhaps that is not the case with HF diets.

The EDL *m.* PF did not change, which is interesting as there was a decrease in muscle weight relative to body weight. Although we did not find an increase in PF with the EDL *m.*, we lost the significant reduction as found in the soleus *m.*, and hence the EDL *m.* may have better compensatory mechanisms related to mitochondrial enzyme activity to counteract the reduction in muscle weight relative to body weight.

### ***Muscle bed***

In contrast to the soleus *m.*, an altered HF diet PRE and/or POST did not alter EDL *m.* contractility. This agrees with another study of adult mice that showed a reduced twitch and tetanic contraction PF in the soleus *m.* but not in the EDL *m.* of C57BL/6J male mice fed a HF diet for 5 weeks at 12 weeks of age (Ciapaite *et al.* 2015). Indeed, another study of the gastrocnemius/plantaris *m.* also found no change in PF with a POST HF diet (Shortreed *et al.* 2009). Despite their diet containing more fat (60% vs. 45% kcal), they only fed the mice a HF diet for 8 weeks. Furthermore, the soleus *m.* is predominately composed of oxidative slow-twitch muscle fibres and previous studies in adult mice have found that these oxidative fibres are more susceptible to an obesogenic challenge than the more fast-twitch, glycolytic muscles (Lee *et al.* 2015a). The soleus *m.* is a postural muscle, so if a HF diet is affecting postural slow-twitch muscles more, it may provide evidence for how obesity may increase the risk of falls in the elderly population.

#### **3.4.1.2 Contractile kinetics**

##### ***Postnatal high-fat***

The TPT and HRT did not change with a POST HF diet in the female soleus *m.* for tetanic or twitch contraction. There was also no difference in the males with tetanic contraction despite reductions in the ability of these muscles to generate force. As these muscles had reduced contractile PF but had comparable contractile and relaxation speeds, it implies that the soleus *m.* may have increased fatigability as mentioned previously (Bayol *et al.* 2009).

The TPT and HRT from male soleus *m.* twitch contraction, however, was prolonged in the HF/HF compared with HF/C offspring, which is associated with a reduction in twitch contractile PF. This combination of slower contraction and relaxation times, and reduced ability to generate force indicates muscle fatigue. The regulation of calcium release from the sarcoplasmic reticulum (SR), important for muscle contraction, might also be impaired explaining the unchanged and longer contraction time of tetanic and twitch contraction, respectively. Interestingly, Ciapaite *et al.* found that the time to contract did not change in male muscle with a POST HF diet, but there was in fact a faster relaxation time after twitch contraction in the soleus *m.* Ciapaite *et al.* suggest it could be due to “altered cross-bridge detachment kinetics... unrelated to MHC composition”. Investigating cross-bridge kinetics would help to understand the effect a HF diet has on contraction in this current study in much more detail. Our results show that despite no change in male EDL *m.* PF, there was an increase in the TPT and HRT of twitch contraction only (HF/C vs. HF/HF), suggesting the POST HF diet may be having some underlying effects leading to impaired calcium release and increased fatigue in the male EDL *m.* as well as the soleus *m.*

### ***Prenatal high-fat***

There was no change in single-twitch PF with a PRE HF diet in the female or male soleus *m.*, however, the time in which it took to contract was slower. This could be due to an effect on the binding and activity of proteins involved in the myosin-actin-troponin interactions, as well as the rate of calcium release required for contraction. Interestingly, there was no change in tetanic TPT or HRT with the soleus *m.*, suggesting a PRE HF diet does not impair contraction and relaxation speeds for coordinated muscle movements in this predominantly slow-twitch muscle. The reduction in male soleus *m.* tetanic PF with a PRE HF diet, together with comparable contraction and relaxation speeds may be an indicator of fatigue as mentioned above when discussing the effect of a POST HF diet on contractile kinetics. These data are novel in terms of a PRE HF diet, but work from Bayol *et al.* found that a PRE obesogenic (increased carbohydrate) diet did not affect contractile or relaxation times. Generally, there was no effect of a PRE HF diet on the contraction and relaxation speeds in the EDL *m.*, which is consistent with the PF data.

### ***Combined pre- and postnatal high-fat***

The soleus *m.* of offspring in the HF/HF group took longer to reach peak force (TPT) in twitch contraction compared with the C/C offspring. This is interesting as their PF was reduced, which may indicate that the regulation of calcium handling might be impaired, leading to a slower release of calcium from the SR for muscle contraction. There were no changes to the speed of relaxation of either muscle in the HF/HF offspring group. In the soleus *m.*, where we reported a decrease in PF, the fact that the time the muscle took to relax did not decrease as well could be indicative that the double hit in HF increased the muscle's susceptibility to fatigue. There was very little effect on TPT or HRT of the EDL *m.*, but this isn't surprising considering no changes to PF were found.

### **3.4.2 Muscle structure**

#### ***Postnatal high-fat***

The total myofibre density in the female and male soleus *m.* were lower with a POST HF diet, especially in the C/HF group. This reduction was mainly due to a lower density of type I myofibres. A human study looking at fibre composition and ATPase enzyme activity in the vastus lateralis *m.* (which has a different myofibre composition to the soleus *m.*) in patients with type 2 diabetes found that they had a lowered oxidative capacity as a result of a decrease in type I myofibre density (Oberbach *et al.* 2006).

One reason for a reduction in overall myofibre density in the present study could be a result of obesity-induced inflammation; a study in humans showed that adipocytes from obese patients can induce inflammation within the muscle, which is associated with muscle atrophy as a result of impaired myogenic and contractile protein expression (Pellegrinelli *et al.* 2015). In this study we observed an increase in neutral lipid staining within the muscle fibres of male offspring fed a POST HF diet. This agrees with previous studies looking at HF-fed mice and obese patients (Choi *et al.* 2016; Pellegrinelli *et al.* 2015). This increase in intramuscular fat deposition could therefore be a reason for the reduction in myofibre density and PF in the male soleus *m.*, and while this is not investigated in this thesis, it may

result in the accelerated decline of muscle mass with age and onset of sarcopenia. Abdominal fat is known to secrete hormones and cytokines known as adipokines (e.g. leptin and adiponectin), which are involved in inflammatory responses and glucose and fatty acid metabolism. Studies suggest that skeletal muscle in obese individuals are resistant to these adipokines, resulting in insulin resistance and reduced muscle strength (Bucci *et al.* 2013; Choi *et al.* 2016).

There was no effect of a POST HF diet on female soleus *m.* lipid accumulation, which suggest that their lowered contractile response is not a result of increased lipid infiltration, but perhaps these inflammatory mediators from adipose tissue deposited in other tissues are having a local effect on the muscle, despite no increase in intramuscular fat. An increase in reactive oxygen species (ROS), which occurs in obesity and type 2 diabetes is associated with muscle wasting in adulthood. This could therefore be another mechanism involved causing a reduction in soleus *m.* myofibre density with a POST HF diet (Espinosa *et al.* 2016).

Alongside a decrease in type I myofibre density, there was an increase in their CSA. This might have been a compensatory mechanism to increase the muscle's oxidative capacity. Type IIA is the other most common myofibre in the soleus *m.* There was an increase in their density in the female soleus *m.* of HF/HF vs. HF/C offspring. The fact that these myofibres did not decrease in density with a POST HF like the type I fibres, may be another way of trying to increase the oxidative capacity. Research shows that skeletal muscle has the ability to remodel following disease states (Bassel-Duby & Olson 2006), so it could be that there is a subtle switching of fibres (type I to IIA fibres) as the muscle potentially becomes less oxidative and more insulin resistant. In contrast, the main changes to myofibres in the EDL *m.* was a reduction in type IIX density in the C/HF group. This is to be expected as the EDL *m.* is primarily composed of type II fibres, but interestingly the reduction in type IIX density alone was not associated with a reduction in PF. Furthermore, the POST HF diet did not increase lipid deposition in the EDL *m.* The differences in myofibres affected between the soleus and EDL *m.* supports the notion that oxidative fibres have a higher sensitivity to a post HF diet. Some studies did not find any differences in the protein expression of the different MHC isoforms (which dictate the fibre type), or the myofibre density in either adult soleus or EDL *m.* (Shortreed *et al.* 2009; Ciapaite *et al.*

2015). However these mice were only fed a HF diet for a much shorter time of 8 and 5 weeks, respectively in adulthood compared with our 30 week old mice.

### ***Prenatal high-fat***

The soleus *m.* exhibited a higher type I myofibre density after exposure to a PRE HF diet, although this was only a trend in the males. This is interesting as the PRE HF diet is having the opposite effect on type I myofibres compared with what we saw with after a POST HF diet, however these changes did not relate to a change in PF. These results differ to a previous study where a PRE HF diet was associated with a reduction in the tibialis anterior *m.* mass (Samuelsson *et al.* 2008). Interestingly, in this study, a PRE HF diet did not change the level of lipid accumulation in the muscles. This is inconsistent with previous work which found that a maternal obesogenic diet resulted in an increase in intramuscular lipid accumulation and a reduction in myofibre density (Bayol *et al.* 2005). As previously discussed, the diet they used was very different with an increased percentage of carbohydrate so this could have elicited a more significant effect prenatally. These discrepancies in outcome and the diets used to generate a pregnancy model of obesity highlights that more research needs to be done to look into the mechanisms underpinning differences in muscle development and function after maternal high-fat feeding. In terms of the EDL *m.*, there was a reduction in type IIX myofibre density only (common EDL myofibre type), and an increase in male type IIAX fibre density in the HF/HF compared with C/HF offspring, but again, these changes to myofibre density were not associated with any changes to the PF response in the EDL *m.*

### ***Combined pre- and postnatal high-fat***

In the male soleus *m.*, total myofibre and total type II myofibre CSA increased in the HF/HF offspring, and type I density was reduced. The decrease in soleus *m.* type I myofibre density may be due to previous studies reporting that a HF diet had a greater impact on oxidative fibres. This, and the fact that there was an increase in intramuscular lipid accumulation in HF/HF male offspring could explain the reduction in PF with the soleus *m.* of HF/HF male offspring compared with C/C. Soleus *m.* type IIB myofibre density was the only one to be affected in the female HF/HF group when compared with C/C, with a reduction in both density and CSA. However, this wasn't reflected in total type

II fibre density. A lack of change in female soleus *m.* type I density (predominant myofibre) or ORO staining despite reductions in PF may suggest that a change in myofibre structure is not the cause of its impaired ability to contract. No changes to the total myofibre density in either male or female soleus *m.* may indicate the presence of PARs; the HF/HF offspring may have been ‘adapted’ for a POST HF diet as they experienced a HF diet prenatally, and so minimising changes in myofibre number, and so the reduction in PF could have been due to alternative mechanisms.

Type I density increased in the female EDL *m.* and type IIX myofibre density increased in the male EDL *m.* of HF/HF offspring compared with C/C. It is not surprising the HF diet affected the soleus and EDL *m.* in different ways, as their respiratory mechanisms in order to produce energy are very different; slow-twitch fibres are more prevalent in the soleus *m.* and are more dependant on aerobic metabolism, while fast-twitch fibres are more prevalent in the EDL *m.* and are more dependent on anaerobic metabolism. There were no changes to the density or CSA of the fibres predominantly in the EDL *m.*, which may explain why there was no reduction in EDL *m.* PF.

When offspring of all diet groups were analysed together, correlations (Appendix D) revealed that as lipid accumulation within the muscle fibres increased, the PF of both tetanic and twitch contraction decreased in the male offspring. Furthermore, as total myofibre density increased so did tetanic and twitch contractile PF in both the male and female soleus *m.* These findings show a clear relationship between muscle structure and the ability of the muscle to generate force in the predominantly slow-twitch muscle. The EDL *m.* on the other hand did not show the same relationship, but as type IIB myofibre density increased, so did PF, although not enough to bring about significant differences.

### 3.4.3 Gene expression

#### 3.4.3.1 Contractile components

##### *Postnatal high-fat*

The mRNA levels of *Myh1*, which codes for the myosin in type IIX fibres, reduced in the male and female soleus *m.* with a POST HF diet. However, this decrease was not associated with a change in type IIX myofibre density itself. This discrepancy between the mRNA and protein levels could be a result of the numerous regulatory processes which happen after transcription including post-transcriptional modification, translational regulation and protein degradation (Vogel & Marcotte 2012). The female soleus *m.* also had decreased *Myh4* mRNA levels which was consistent with the observed reduction in type IIB myofibre density. Levels of *Tnnt1* mRNA (troponin isoform in slow-twitch muscle) were lower with a POST HF diet in the soleus *m.*, which contrasts to another study (Ciapaite *et al.* 2015). Those authors found a decrease in soleus *m.* *Tnnt3* and an increase in *Tnnt1* mRNA, but the increase in *Tnnt1* was only found in a cohort of mice fed a 45% kcal fat diet from palm oil alone, and not in a cohort where the 45% kcal fat came from lard. In the current study, the female soleus *m.* had lower *Acta1* mRNA levels (which codes for the protein actin) and, together with *Myh1*, *Myh4* and *Tnnt1* mRNA level changes may contribute the decreased PF seen in the soleus *m.* with a POST HF diet. Levels of *RyR1* mRNA were reduced in the male soleus *m.* with a POST HF diet. As *RyR1* codes for the ryanodine receptor which is involved in the release of calcium into the cytoplasm for muscle contraction, it could therefore be a mechanism involved in the slower single-twitch contraction speed observed in these offspring.

The expression of contractile components at the mRNA level did not change in the female EDL *m.*, which fits with the fact that a POST HF diet did not cause a change in PF in the EDL *m.* The male EDL *m.*, on the other hand, had increased levels of *Myh1* mRNA (but type IIX fibre density reduced) and *Myh2* mRNA (although the number of type IIA fibres did not change). As described above, these differences between mRNA and MYH protein (fibre type) could be a result of regulatory mechanisms after transcription. Despite these changes in mRNA levels, they were not enough to cause physiological change. There was

no effect of a POST HF diet on *Tnnt1* or *Tnnt3* mRNA levels in the EDL *m.* which is consistent with findings from Ciapaite *et al.*

### ***Prenatal high-fat***

A PRE HF diet resulted in a reduction in soleus *m.* *Myh1* mRNA levels and this was not associated with a decrease in type IIX fibres. Type IIX fibres are not a predominant type in the slow-twitch soleus *m.*, and this may be a reason why changes in this gene were not linked to changes in fibre density or impaired force production. The time for the soleus *m.* to reach PF was increased with a PRE HF diet, yet *RyR1* mRNA levels were unchanged. An analysis of the protein level would be a good next step since, as discussed, mRNA and protein levels do not always correlate, and it is the protein which is the functional component to elicit physiological changes. Unfortunately, analysing the mRNA expression of SERCA (sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase) or its regulators phospholamban and sarcolipin, which are responsible for the clearance of calcium from the sarcoplasm back into intracellular stores after contraction, were out outside the scope of this thesis. Analysing the expression of these genes and proteins would help to understand any changes in contractile relaxation times. Ciapaite *et al.* looked at the protein expression of all three components, but they did not change with a HF diet. However, as they only fed the mice a HF diet for 5 weeks in adulthood, it would be interesting to see whether there are any effects in our mouse model. Tetanic PF of the male soleus *m.* was reduced with a PRE HF diet (C/HF vs. HF/HF), but with no changes in the mRNA levels of genes coding for contractile proteins, it suggests that at this level of gene expression these do not contribute to the reduction in observed in PF.

Interesting there was a reduction in *Tnnt1* mRNA levels in the EDL *m.* of HF/HF female offspring compared with HF/C, yet PF and TPT did not change. The EDL *m.* is a fast-twitch muscle, and as *Tnnt1* is the gene encoding the troponin isoform in slow-twitch muscle, it does not perhaps seem surprising that the lowered *Tnnt1* mRNA levels had no functional effect. The mRNA levels of *Myh1* increased in the male EDL *m.*, which was associated with an increase in type IIX myofibres, however it appears that this was not enough to be associated with changes to force production or contractile kinetics.

### ***Combined pre- and postnatal high-fat***

The combined PRE and POST HF diet caused a reduction in female soleus *m. Myh1* levels (associated with a trend for fewer type IIX myofibres), reduced *Myh4* mRNA (associated with reduced type IIB fibres), and a reduction in *Tnnt3* mRNA. There was also a trend for lowered *RyR1* mRNA levels, suggesting a potential impairment in the amount of calcium released from the SR for muscle contraction. The male soleus *m.* of HF/HF offspring also had reduced *Myh1* mRNA levels, a trend for a decrease in *RyR1* and lowered *Tnnt1* mRNA levels. All of these changes to gene expression of genes that code for proteins important for contractility may have contributed to the reduced PF these offspring displayed.

The female EDL *m.* following a PRE and POST HF diet did not exhibit any changes in mRNA levels of the contractile components analysed, which is consistent with the lack of effect on PF or contractile and relaxation speeds. Interestingly, the male EDL *m.* increased in *Myh1* (but a decreased in type IIX fibre density), *Myh2* (but no change in type IIA fibre density) and *Acta1* mRNA levels. The muscles did not increase in PF but it could explain why they had a faster TPT for tetanic contraction following a PRE and POST HF diet.

#### **3.4.3.2 Insulin-signalling pathway**

Optimal skeletal muscle glucose uptake is important for muscle contractility. Glucose is necessary to produce ATP required for myofibril filament cross-bridge formation during contraction (section 1.3.2). As previously mentioned, myofibre type by virtue of their different energy systems and oxidative properties (section 1.3.1) affects glucose metabolism, and consequently muscle contractility differently. Therefore, analysing components of the insulin-signalling pathway is important in understanding the effect of a HF diet on skeletal muscle physiological function. A more thorough understanding of the metabolic adaptations would be very insightful. Measuring insulin and fatty acid concentrations in the blood and muscle samples would give a better understanding of the effects a HF diet has on a larger physiological scale.

***Postnatal high-fat***

There was no effect in the soleus *m. Glut4* mRNA levels of a POST HF diet, despite an increase in blood plasma glucose levels in HF/HF offspring compared with the HF/C group. The *Glut4* gene codes for a glucose transporter found in skeletal muscle (as well as other tissues), and it is involved in insulin-mediated glucose uptake. Although there is no change in *Glut4* mRNA levels, we do not know whether the translocation of GLUT-4 protein from intracellular vesicles to the plasma membrane was improved, as found in a previous study looking at maternal undernutrition on GLUT-4 translocation in skeletal muscle of suckling rats (Gavete *et al.* 2005). The *Igf1r* mRNA levels decreased in the HF/HF females compared with HF/C, demonstrating an impairment in insulin signalling. It could explain the increase in blood glucose levels in these offspring as research shows *Igf1r*<sup>+/-</sup> mice are insulin resistant (Bokov *et al.* 2011). In the female and male C/HF offspring compared with C/C, there was an increase in soleus *m. Akt1* mRNA levels, which is an inhibitor of apoptosis and a key signalling protein in skeletal muscle growth within the insulin-signalling pathway (Zhou *et al.* 2000). This increase could be a compensatory mechanism to help counteract the decrease in total myofibre density found in the C/HF group.

We found many mRNA changes of insulin-signalling components in the EDL *m.* of offspring fed a POST HF diet. In female EDL *m.*, *Glut4* mRNA levels were lower following a POST HF diet regardless of PRE diet. This reduction in skeletal muscle *Glut4* mRNA may in part mediate the glucose intolerance previously observed in this mouse model by Cagampang *et al.* (unpublished data), who showed that POST HF diet regardless of PRE results in an increased blood glucose area under the curve (impaired glucose tolerance). The fact that we found differences in *Glut4* in the EDL *m.* but not the soleus *m.* is interesting since previous work found that it was the more insulin-sensitive slow-twitch fibres that had a significant decrease in *Glut4* in obese or diabetic patients. (Gaster *et al.* 2001). The mRNA levels of *Irs1* and *Akt1* increased in the female and male EDL *m.* following a POST HF diet which could, if acting in a compensatory way, help explain why there were no reductions in EDL *m.* total myofibre density, CSA or impairments in contractile function. However, EDL *m. Igf1r* mRNA levels did decrease in the HF/HF group compared with HF/C, which may also contribute to the glucose intolerance as mentioned with regards to the soleus *m.*

There were no changes to *Insr* mRNA levels in either muscle type, following a POST HF diet despite one study reported a link between the InsR and contractility, whereby muscle insulin receptor knockout mice (MIRKO) had a reduced maximal twitch force compared with control mice (O'Neill *et al.* 2010). One study has found that the ablation of mesodermal transcription factor *Tbx15* (specifically expressed in type IIB glycolytic fibres) caused a reduction in glycolytic fibre number with a small increase in oxidative fibres, resulting in reduced contraction rates and an increase in whole-body glucose tolerance due to a reduction in total muscle size (Lee *et al.* 2015b). This shows a clear link between muscle contractility and glucose metabolism, but the link needs to be explored further.

### ***Prenatal high-fat***

A PRE HF diet had very little effect on the mRNA levels of insulin-signalling components investigated. There were no effects in the soleus *m.* or male EDL *m.*, but there was a decrease in female EDL *m. Igflr* mRNA levels in the HF/HF compared with C/HF offspring. This may explain the increase in baseline blood glucose, and therefore potential glucose intolerance/insulin resistance between these diet groups. This effect on blood glucose is in agreement with another study looking at maternal obesity, in which impaired glucose tolerance was observed in 3 and 6 month-old female offspring when a HF diet was fed 6 weeks before mating until weaning (Samuelsson *et al.* 2008). By contrast, another study found that a PRE obesogenic diet resulted in an increase in gastrocnemius *m. Igflr* mRNA levels, which the authors suggest could be a mechanism in maintaining insulin sensitivity (Bayol *et al.* 2005). The reasons for discrepancies between our results and these previous studies could be due to them using a higher percentage of carbohydrate, using rats instead of mice, and looking at a much younger offspring age (21 days as opposed to 30 weeks).

### ***Combined pre- and postnatal high-fat***

A PRE and POST HF diet caused a decrease in *Igf1r* mRNA levels in the female soleus and EDL *m*. This may explain why there was a trend for a decrease in *Glut4* mRNA levels in these muscles, however there were no effects on the mRNA levels of downstream signalling targets like *Irs1*, and these observations were not associated with any significant effects to blood glucose levels. The levels of *Akt1* mRNA in both the HF/HF male and female EDL *m*. increased, which may contribute to the observed increase in female type I myofibre density and type IIAX CSA, and male total myofibre CSA. Similarly to the POST HF diet, these changes in *Akt1* mRNA levels may help explain why there were no impairments in EDL *m*. contractile function.

Analysing the protein expression (in addition to our mRNA) of contractile and insulin-signalling pathway components would provide a more thorough understanding of how a HF diet alters the mechanisms underlying changes to isometric muscle contraction. In terms of glucose handling, quantifying the fractions of GLUT-4 within intracellular membranes and in the plasma membrane to assess translocation, would be useful information.

#### **3.4.4 Plasma vitamin D concentrations**

Plasma 25(OH)D<sub>3</sub> concentration was higher in the HF/HF vs. HF/C group of female and male offspring and there was an increase in the concentration in HF/HF compared with C/C males, with a trend for this increase in females. In human studies, obesity has been associated with vitamin D deficiency (section 1.6). Although the HF-fed mice in this study developed an obese phenotype, the impact of this on blood vitamin D results are likely to be confounded by the HF diet containing a greater amount of vitamin D from the lard compared with the control diet (Appendix A). It is important to note however, that despite the inclusion of more vitamin D in the HF diet (through the increased lard content), the model is of relevance to human obesity since it too is likely to include increased vitamin D consumption due to a fatty diet.

Despite the increase in plasma 25(OH)D<sub>3</sub> concentrations in the HF/HF diet vs. C/C and HF/C offspring, there were still reductions in PF which could be a result of the altered myofibre structure. Interestingly, there was a decrease in 30-week plasma 25(OH)D<sub>3</sub> concentration with a PRE HF diet (HF/C vs. C/C) in female and male offspring. This suggests that an early life HF-high-vitamin D diet has long term implications on vitamin D homeostasis. This effect did not, however, correspond with changes in muscle contraction between the two groups. Correlations (Appendix D) show no associations with blood plasma 25(OH)D<sub>3</sub> concentrations and peak force in the EDL *m.* or the male soleus *m.* However, in the female soleus *m.* the positive correlation observed in the C/C offspring for tetanic contraction was lost with a PRE, POST and PRE-POST combined HF diet. For soleus *m.* twitch contraction, there was in fact a significant inverse relationship between 25(OH)D<sub>3</sub> concentration and PF in the HF/C female offspring which was not observed in the other diet groups. Overall these correlations suggest that altered vitamin D homeostasis may play a part in the effect of a prenatal HF- high-vitamin D diet on the regulation of muscle contraction, but this requires further investigation. Due to technical issues, it was not possible to quantify mRNA or protein levels of components involved in vitamin D signalling. This would be valuable information in order to unpick the relationship between the HF diet and vitamin D signalling on muscle contraction.

### 3.4.5 Conclusion

In conclusion, a POST HF diet and a combined PRE and POST HF diet were associated with reductions in adult skeletal muscle isometric contraction force in a muscle bed-specific manner. A reduced ability to contract optimally was found in the slow-twitch soleus *m.* but there was no effect in the fast-twitch EDL *m.* Since the soleus *m.* is a postural muscle, it may help to explain mechanisms underlying why some obese humans develop sarcopenia in later life, and therefore why there is an increase in falls in the elderly, especially those with obesity. There was also very little change in muscle PF with a maternal HF diet. The soleus *m.* of male and female offspring fed a POST HF diet and a combined PRE and POST HF diet reduced in muscle weight per gram of body weight, there was a lack of change in half-relaxation time (which indicates muscle fatigue), and their total and type I myofibre density was reduced. A POST HF diet and a combined PRE

and POST HF diet was also associated with an increase in intramuscular lipid deposition in the males, but not females. On a molecular level, mRNA levels coding for MYH proteins were reduced in the male and female offspring fed a POST HF diet and combined PRE and POST HF diet. The combination of these impairments to genes encoding the myosin vital for the contractile process, the reduction in myofibre density and the increase in lipid deposition are most likely to be the reasons behind the reductions in PF. Both sexes had altered *Tnnt1* mRNA levels with a POST HF diet, which could have impacted the ability to contract. However, only the male soleus *m.* had reduced *RyR1* mRNA levels with a POST HF diet, which linked to slower contraction speeds also only found in the male offspring.

Thus, while the force production during contraction was impaired in both sexes, the pathway to these alterations in function appear to be slightly different between females and males. Of course, the main outcomes are similar (muscle weight, myofibre density, genes coding for some of the proteins important for contractility and insulin signalling) but the increase in fat deposition within the muscles was only observed in the male offspring. This may not be surprising as it is well known that there are male and female differences in the pattern of fat deposition. In females, fat around the reproductive organs is a reproductive strategy due to its importance for fertility (Power & Schulkin 2008) and may explain why there was no increase in intramuscular lipid deposition in the female soleus or EDL *m.*

In this chapter we have mainly observed that a POST HF diet and a combined PRE and POST HF diet lead to impaired skeletal muscle contractile function, and that there are sex differences in the underlying mechanism. Despite this, there are still some modest effects of a PRE HF diet. The current data suggest that a PRE diet alters the homeostasis of vitamin D in later life and that the relationship between vitamin D and muscle contraction is altered. The flooding of the dam and/or offspring with dietary vitamin D<sub>3</sub> in the HF-fed groups may be a 'real' human issue. It does, however, means that this isn't the ideal model in which to further investigate the impact of vitamin D deficiency (VDD) on skeletal muscle development and lifelong consequences. As a result, the next steps in this thesis involved two models of pregnancy VDD and the impact it has on offspring (Chapter 4) and fetal (Chapter 5) skeletal muscle.

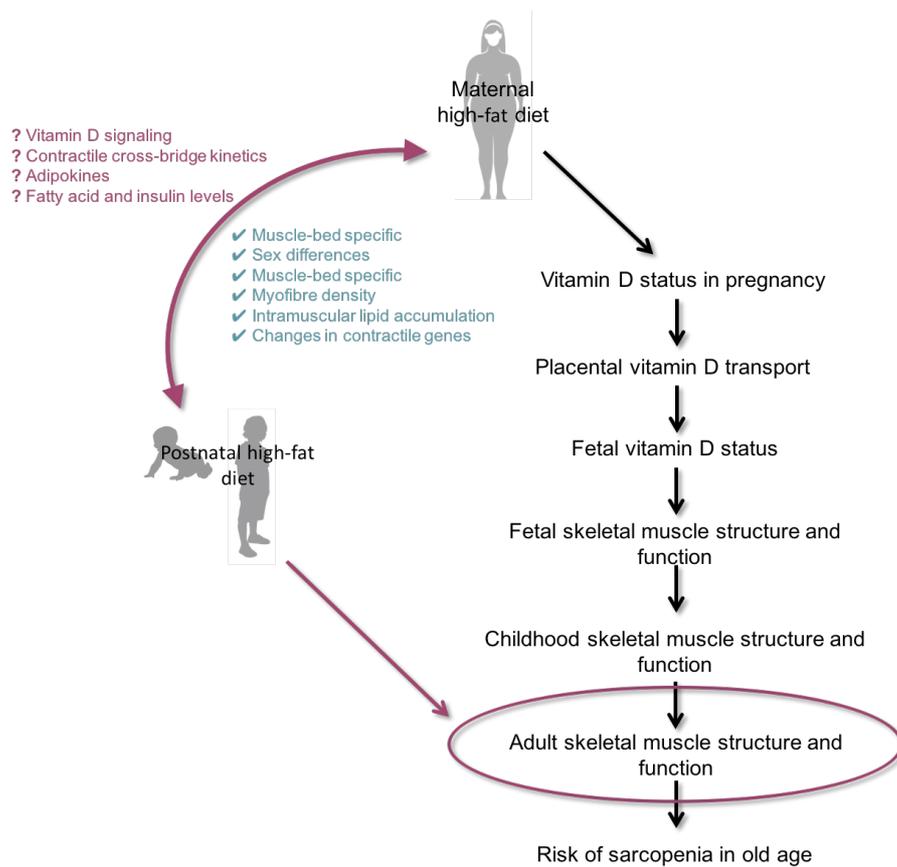


Figure 3.40: Schematic diagram showing thoughts after Chapter 3 results.

# Chapter 4: The effect of prenatal vitamin D deficient diet on young adult mouse offspring strength and activity

## 4.1 Introduction

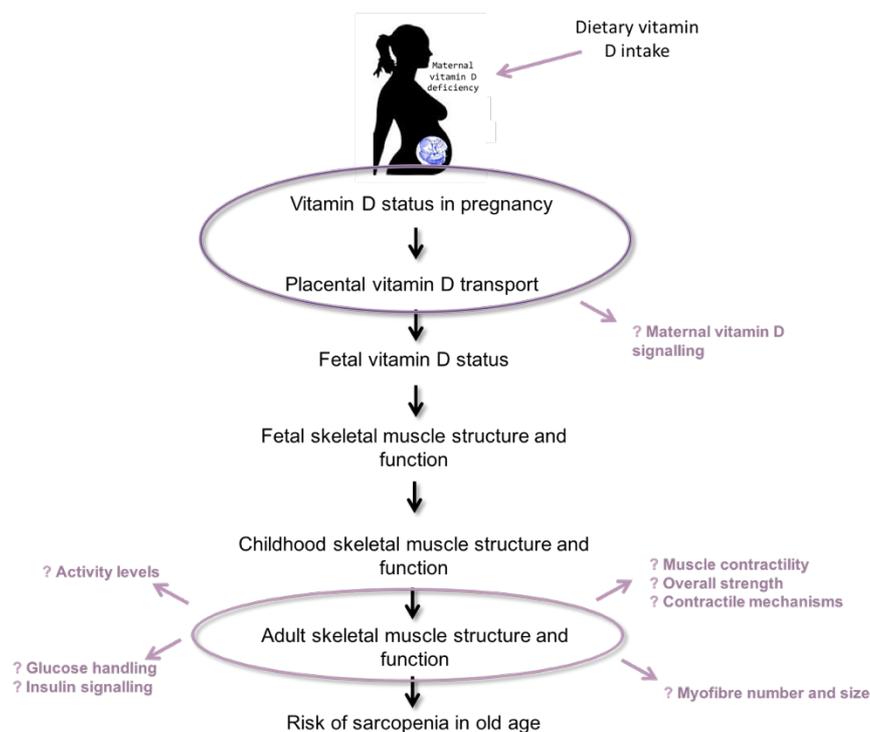
In Chapter 3 the effect of a prenatal and/or postnatal high-fat (HF) obesogenic diet on skeletal muscle was explored in the adult offspring. Previous studies have reported an association of vitamin D deficiency (VDD) with obesity in humans, but it became clear in Chapter 3 that the high-fat diet fed to induce pregnancy obesity in the mice was also high in vitamin D<sub>3</sub> and so even if this may apply to some obese human pregnancies, it was not an ideal model in which to address the thesis hypothesis concerning the impact of VDD on skeletal muscle development, structure and function across the life course. Despite this, there appeared to be some effects of a prenatal HF diet, rich in vitamin D<sub>3</sub>, on the 30-week offspring, and it altered vitamin D homeostasis in adulthood, which suggested that the relationship between vitamin D and muscle contraction is altered with a prenatal HF diet. This supports indirectly part of the hypothesis that a change in early life vitamin D leads to altered adult muscle function. The next logical step in this thesis was to investigate the effect of specific pregnancy vitamin D deficiency in a mouse model without other confounding factors.

Vitamin D deficiency is highly prevalent in pregnant women, e.g. 83% of pregnant US black women and 47% of pregnant white women were vitamin D deficient, with variation between populations (Liu & Hewison 2012). Research suggests that vitamin D is important for skeletal muscle development and function in adult life as detailed in sections 1.4.2 and 1.4.3. VDR and 1 $\alpha$ -hydroxylase are expressed in adult human and mouse muscle (Bischoff *et al.* 2001; Girgis *et al.* 2014). Furthermore, VDD has been reported to cause type II myofibre atrophy in VDD elderly patients (Yoshikawa *et al.* 1979), and vitamin D supplementation of women aged 65-81 years of age led to an increased number of type IIA fibres in the vastus lateralis *m.* (Sorensen *et al.* 1979) and an increase in percentage and fibre diameter of type II muscle fibres (Sato *et al.* 2005). Another study in mobility-limited VDD women found vitamin D supplementation increased vastus lateralis *m.* fibre size by

10% and intramyonuclear VDR concentration by 30% (Ceglia *et al.* 2013). Furthermore, one human study found that plasma 25(OH)D concentration was positively associated with muscle strength (maximal isometric contraction), vertical jump, and standing broad jump in 125 physically active children (Bezrati *et al.* 2016), and they suggest that VDD in adulthood may “limit exercise performance”. There are numerous environmental factors leading to VDD in adult life, and it may be having an impact on women during pregnancy, and their offspring.

There is a real paucity of information on the effect of prenatal vitamin D or VDD on offspring muscle function but several lines of evidence led to the work carried out in this chapter. A study using a VDR knock-out mouse model found that the absence of VDR impaired muscle development at 3-weeks of age with a down-regulation of myoregulatory transcription factors that would normally be important for muscle development and growth (Endo *et al.* 2003). This is interesting data but of course it is a lifelong influence and not just limited to a gestational exposure. One previous human study reported that lower maternal serum 25(OH)D concentrations were associated with a reduction in offspring hand grip strength at 4 years of age (Harvey *et al.* 2014), but while the data were statistically controlled for potential confounders, the VDD was not directly manipulated. A rat study looking at the effect of maternal VDD on newborns muscle structure (Max *et al.* 2014) is one of the very few studies to our knowledge looking at maternal VDD and offspring muscle. They found that the gastrocnemius *m.* of newborns (21 days postpartum) from mothers fed a prenatal VDD diet 8 weeks prior to mating and during pregnancy and lactation had smaller muscle cells compared with dams fed a vitamin D<sub>3</sub> adequate diet and that the interfibrillar space was increased. Following transcriptome analysis of the gastrocnemius *m.*, they reported that genes involved in protein catabolism, cell differentiation and proliferation, muscle cell development, and cytoskeleton organization were differentially expressed in the newborns exposed to maternal VDD versus adequate vitamin D levels. In addition, work from our laboratory showed that maternal 40-50% restriction of total calorific intake during critical early and late gestation windows in sheep was linked to a reduction in late gestation (127±1 dGA) fetal myofibre density of the triceps brachii *m.* at (Costello *et al.* 2008). In these fetuses, lower myofibre density was associated with a reduced fetal total 25(OH)D concentration (Cleal *et al.* 2017). Indeed another study looking at vitamin D supplementation as opposed to VDD found an increase in total muscle fibre number in fetal pigs (90 dGA) with greater maternal 25(OH)D<sub>3</sub> concentrations (Hines *et al.* 2013).

In summary, there is insufficient certainty of a link between maternal VDD and offspring skeletal muscle structure and a gap in knowledge of its impact on the offspring muscle function. Therefore, this chapter focuses on the effect of dietary VDD in the pregnant mouse and how it affects the 15-week (young adult) offspring's skeletal muscle function, general strength and activity levels.



**Figure 4.1: Schematic diagram of Chapter 4 flow of logic.**

### 4.1.1 Hypothesis and aims

#### Hypothesis

A prenatal vitamin D deficient diet will reduce offspring strength and activity levels in adulthood.

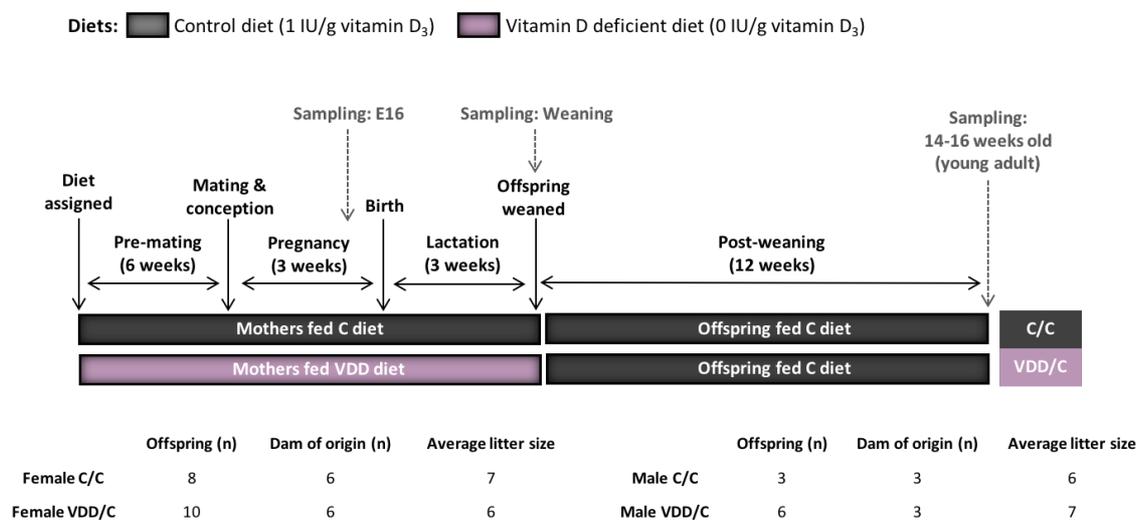
#### Aims

To investigate whether there is an effect of a prenatal VDD diet on adult mouse strength and activity levels by assessing a) isometric skeletal muscle contractile peak force, b) *in vivo* strength tests, 3) open-field activity monitoring and c) analysis of whole-body glucose tolerance. The secondary aim is to then test for any associations between the offspring outcome variables and maternal pregnancy plasma vitamin D status.

## 4.2 Methods

### 4.2.1 Vitamin D deficient mouse model

Female C57BL/6 mice (3-4 months old, 1 previous pregnancy), were randomly selected to be fed either a control (C; 1 IU/g vitamin D<sub>3</sub>; 58M1 - Test Diet, USA; Appendix B) or a vitamin D deficient (VDD; 0 IU/g vitamin D<sub>3</sub>; 5B7Q - Test Diet, USA; Appendix B) semi-purified ‘maintenance’ diet *ad libitum* for 6 weeks before mating. In groups of 5, C and VDD-fed dams were mated overnight with C-fed C57BL/6 male mice (total of 3 male proven breeders approximately 12 weeks old). Pregnancy was confirmed by visualisation of a copulation plug the next morning which was termed day 0.5 of gestation (E0.5). Males were then removed and pregnant female mice were transitioned onto the equivalent ‘gestation’ version of the C or VDD diet (57W5 and 5SL1- Test Diet, USA, respectively; Appendix B) throughout pregnancy and lactation. Dam weight was recorded every week from diet assignment at 6 weeks prior to mating until E16 of pregnancy. Dams were split into individual cages for littering purposes 1 week prior to delivery. At three weeks of age, the litter size was standardised to 3 female and 3 male offspring. These offspring were assigned to the C maintenance diet thereby creating two experimental groups: C/C and VDD/C (Fig. 4.2), and male and female offspring were separated. The final number of experimental offspring, dams of origin and average litter size for each diet group are displayed in Figure 4.2.



**Figure 4.2: Vitamin D deficient mouse experimental model.** Female 15 wk offspring: C/C n=8; VDD/C n=10. Males: 15 wk offspring: C/C n=3; VDD/C=6; C, control; VDD, vitamin D deficient; IU, international units.

Throughout the protocol, mice were housed in a laminar flow ventilated cabinet (Tecniplast, Italy) held at 21 °C with ultraviolet B (UVB) filters over interior fitted lights to block UVB rays which promote vitamin D production in the skin. The scintainer was programmed for a 12hr light/dark cycle to maintain normal circadian rhythms and constant access to water was provided.

## 4.2.2 Tissue collection

### Dam cohort

A subset of non-fasted dams was sampled at E16 (C n=6, VDD n=6). Body weight of dams was measured and anaesthesia was induced by inhalation of isoflurane and the foot was pinched to check the depth of anaesthesia by observing an absence of a foot withdrawal response. Blood was sampled by cardiac puncture whilst the heart was still beating, and centrifuged at 2000 x g for 10 min at 4 °C in a LiHep tube. Plasma was stored at -80 °C. Blood samples from dams at weaning were also obtained in this way but no other tissues were taken. Non-fasted blood glucose levels were measured using a glucometer (Accu-Chek, UK). Dams were culled via cervical dislocation. Embryos were culled by cutting through the brainstem. The number of embryos was recorded, and all embryos and their corresponding placenta were weighed. From these data, the fetal:placental ratio was calculated. The fetal crown-rump length was measured and tail tips were snap frozen in liquid nitrogen in order to determine the sex at a later date. A sample of pooled amniotic fluid was taken and snap frozen. Every other embryo was slow-frozen in a tube on dry ice and the others fixed in 4% paraformaldehyde (PFA). Placentas were cut in half; one half was slow-frozen, the other fixed in 4% PFA. The heart, a section of left lobe from the liver, kidneys, sample of abdominal fat and left side skeletal muscles (soleus *m.*, EDL *m.*, TA *m.* and gastrocnemius *m.*) were dissected under sterile conditions and immediately snap-frozen in liquid nitrogen. All snap- and slow-frozen samples were stored at -80 °C and all fixed samples were incubated at room temperature in 4% PFA (24-hours) and then transferred to 70% ethanol and paraffin wax-embedded.

## 15-week offspring

At 14-16 weeks of age, mice (females: C/C n=8, VDD/C n=10; males: C/C n=3, VDD/C n=6) were fasted and anaesthesia was induced by inhalation of isoflurane. Fasted whole blood glucose levels were measured and a sample of blood was collected by cardiac puncture. The blood was centrifuged at 2000 x g for 10 min at 4 °C in a LiHep tube to separate the plasma which was then stored at -80 °C. Offspring were culled by cervical dislocation. Various offspring tissues were collected under sterile conditions and snap frozen in liquid nitrogen: a section of the left liver, aorta, heart, adrenals, kidneys, section of abdominal fat and the pancreas. The ovaries and testes were collected from female and male offspring, respectively. The soleus, EDL, gastrocnemius and TA *m.* were collected from both legs. From the left leg of female and male offspring, all muscles were snap frozen in liquid nitrogen and stored at -80 °C for future analysis. From the right leg, all muscles of female offspring were slow-frozen in freezing isopentane for histology. From the right leg of the male offspring, the TA and gastrocnemius *m.* were also slow-frozen immediately, whereas the soleus and EDL *m.* were kept in ice-cold oxygenated Krebs buffer (recipe in section 2.4.2) for muscle contraction experiments (section 4.2.5) and then slow-frozen in freezing isopentane (Sigma-Aldrich, USA). All snap and slow-frozen muscles were stored at -80 °C. All offspring carcasses were stored at -20 °C for potential future micro-CT scanning.

The sample size was smaller than expected for this chapter. A subset of the C/C male offspring were found to have abnormal livers at post-mortem that were fatty and sclerotic in appearance. These offspring were therefore excluded from further analysis. In an attempt to understand the reason behind this, plasma and liver samples were sent to histology, and results came back negative for the mouse hepatitis virus antibodies and so hepatitis was ruled out. Considering no abnormalities were observed in the E16 dams, it was thought that the control diet fed from weaning may have elicited this effect. Therefore, a subset of mice was used to determine any effects of the C (58M1) diet compared to the control diet used in Chapter 3 from weaning onwards, but no abnormalities to their livers were found.

### 4.2.3 Blood plasma analysis

Full details in section 2.5. Blood plasma was analysed for 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub>, 24,25(OH)<sub>2</sub>D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> by LC-MS. Total 25(OH)D and total 24,25(OH)<sub>2</sub>D were calculated by adding the D<sub>2</sub> and D<sub>3</sub> isoforms together. Standard commercial assays were used to quantify calcium, albumin, and phosphate concentrations. Some plasma samples had concentrations lower than the limit of quantification (LOQ) for 25(OH)D<sub>2</sub>, 24,25(OH)<sub>2</sub>D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> (<0.1 nmol/L, <0.8 nmol/L and <0.9 nmol/L, respectively), and those samples are classed as 0.1 nmol/L, 0.8 nmol/L or 0.9 nmol/L as detailed in the appropriate graphs.

### 4.2.4 Glucose tolerance test

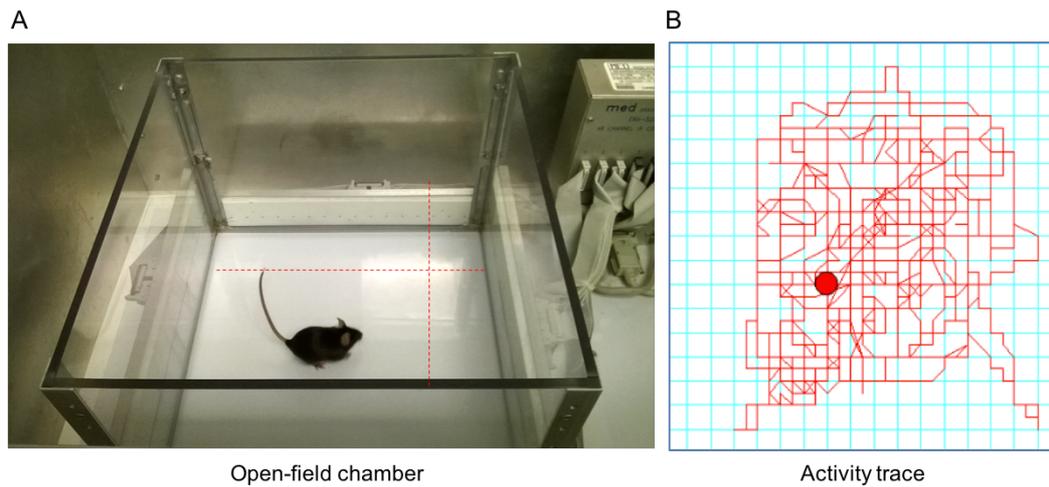
An intraperitoneal glucose tolerance test (IPGTT) was performed a week before tissue collection, open-field activity monitoring and *in vivo* strength tests (13-15 weeks of age). Offspring (females: C/C n=7, VDD/C n=10; males: C/C n=2, VDD/C=6) were fasted overnight and prior to the IPGTT, the tail was locally anaesthetised using lidocaine (Teva, UK). A basal blood glucose concentration was obtained from tail blood using a glucometer (Accu-Chek, UK) and mice were injected intraperitoneally with a 20% glucose solution (2g/kg body weight; Baxter Healthcare Ltd). Blood glucose levels were quantified at 15, 30, 60 and 120 min after glucose bolus. A “Hi” reading on the glucometer was noted as 34 mmol/L and any “Lo” readings were noted at 0.55 mmol/L as these are the highest and lowest concentrations detected by the device, respectively. Area under the curve (AUC) was calculated from baseline glucose using GraphPad Prism 7 (GraphPad Software, Inc, California, USA) in order to assess glucose tolerance.

#### 4.2.5 Isometric skeletal muscle contraction

Male offspring only (C/C n=3, VDD/C n=6) were used for the isometric muscle contraction experiments using the soleus and EDL *m.* following overnight fasting. Briefly (full details in section 2.4), the load cell was calibrated at the start of each experimental day and the muscle was attached by metal clips to the force transducer via its tendons in an organ chamber filled with oxygenated Krebs buffer ( $35 \pm 0.5$  °C). Muscles acclimatised in the organ bath for 15 min prior to data collection. Optimal muscle length was determined (details in section 2.4.2) and the muscle was stimulated at 13 different frequencies 0.1 - 250 Hz. Based on the data and analysis protocol from Chapter 3 (details in section 3.2.4), peak force (PF) of both muscles was determined at 10 Hz for single-twitch contraction, and at 150 Hz and 250 Hz with tetanic contraction for the soleus and EDL *m.*, respectively. Due to time restraints, time-to-peak tension (TPT) and half-relaxation time (HRT) could not be quantified for this thesis, however, it will be part of the future work.

#### 4.2.6 Open-field activity

Open-field activity was assessed in female and male 15-week offspring (females: C/C n=5, VDD/C n=7; males: C/C n=2, VDD/C=5) using an activity monitor (Med Associates Inc., Vermont) composed of a clear perspex chamber (27.3 x 27.3 x 20.3 cm) with multiple lasers on various levels to detect movement (Fig. 4.3). The chamber was surrounded by an aluminium case to ensure the mouse was not distracted by any movement in the room. The mouse was placed into the middle of the chamber and open-field activity was measured for 5 min in a silent room. Mice were acclimatised the day before by introducing them to the open-field chamber for 5 min. On the day, distance travelled, time ambulatory (period in which a mouse crossed more than 3 photo-beams in any two sec), average velocity, vertical counts (number of times offspring reared onto hind limbs) and number of jumps were recorded which is detected by the mouse crossing the laser beams. An example activity trace is shown in Figure 4.3B with the mouse represented as the red dot. Presence or absence of urination and the number of faecal boli were noted. The chamber was fully cleaned before and after each mouse to remove the scent of any other mice which would disrupt behaviour and therefore bias the results.



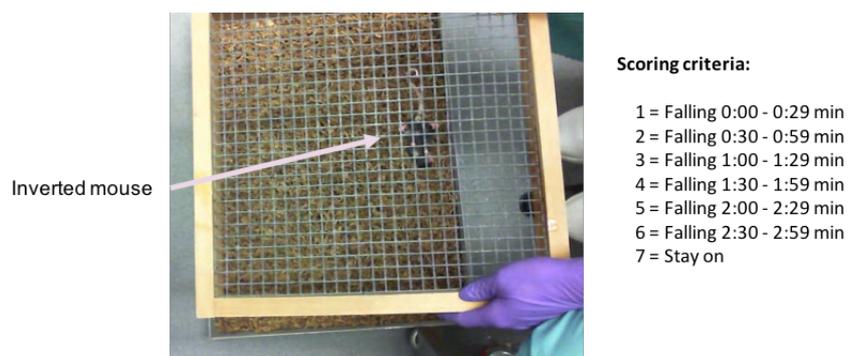
**Figure 4.3: 15-week offspring open-field activity.** **A)** The open-field chamber. Dotted red lines denote examples of the laser beams which formed a grid in the chamber. **B)** a representation of the activity trace produced with the mouse represented as the red dot.

#### 4.2.7 *In vivo* strength tests

All *in vivo* strength tests were performed on female and male 15-week offspring (females: C/C n=8, VDD/C n=10; males: C/C n=3, VDD/C=6).

##### **Inverted screen**

A wire mesh screen (43 x 43 cm) made up of 12 mm<sup>2</sup> squares and 1 mm thick wire was used with a wooden border to prevent the mouse climbing over to the other side (Fig. 4.4). The screen was held 50 cm above a layer of mouse bedding to ensure a soft landing for when the mouse let go of the wire. Each mouse was placed on the middle of the screen, which was inverted over a period of 2 sec. The time that the mouse fell off was noted and the experiment was stopped at 3 min if the mouse remained on the screen. This was repeated three times with 1 min between repeats. Performance for the inverted screen test was scored (as shown in Fig. 4.4 and an average summary measurement for the three repeats was used in analysis.



**Figure 4.4: Aerial view of the 15-week offspring inverted screen test with scoring criteria.**

### Hang-wire test

The hang-wire test was made up of a 38 cm long and 2 mm thick horizontal metal bar connected by two wooden supports either end and held at a height of 49 cm above the laboratory bench (Fig. 4.5). The mouse was held by the tail and placed onto the centre of the horizontal bar so that it gripped with the front paws only. The tail was released and the time taken for the mouse to pull up onto the bar and reach one of the end wooden poles (first contact with front paw) was measured. Performance of the hang-wire test was determined by the criteria shown in Figure 4.5. The apparatus was cleaned before and after each mouse to remove scent.



**Scoring criteria:**

- 1 = Fall off
- 2 = Reach pole in 50 - 60 s
- 3 = Reach pole in 40 - 49 s
- 4 = Reach pole in 30 - 39 s
- 5 = Reach pole in 20 - 29 s
- 6 = Reach pole in 10 - 19 s
- 7 = Reach pole in 0 - 9 s

**Figure 4.5: 15-week offspring hang-wire test and scoring criteria.**

## Grip strength

Front and hind limb grip strength was assessed using a wide-range force transducer (MLT1030/D, ADInstruments, UK) and bridge AMP (Quad Bridge Amp, ADInstruments, UK) connected to a wire grid platform for the mouse to grip hold of (Fig. 4.6). Before each mouse, the equipment was calibrated using a 20g weight. In order to assess front grip strength, the mouse was held by the tail and allowed to grip onto the handle with its front paws only. The mouse was gently pulled back until it let go. To determine hind limb strength, the mouse was restrained and positioned so it would grip onto the handle via its back legs. The mouse was gently pulled backwards until it released its grip. All measurements were recorded using the programme LabChart Reader 8 (ADInstruments, UK). For each mouse, five repeats were recorded for the front and back limbs. The lowest and highest values were discarded and an average summary measurement (g) of the remaining values was used in analysis.

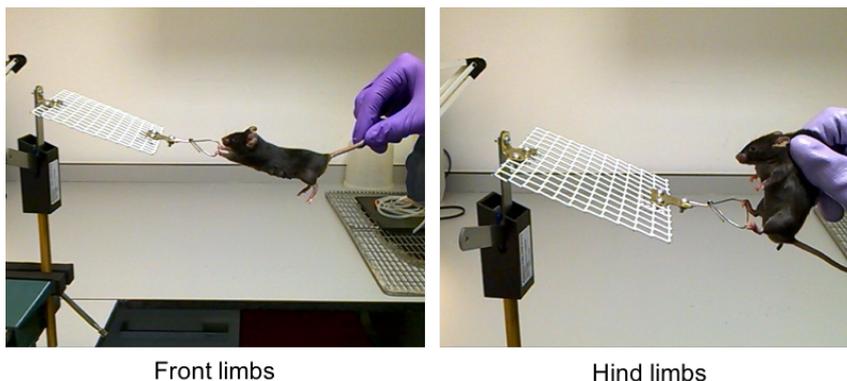


Figure 4.6: 15-week offspring grip strength test of the front and hind limbs.

### 4.2.8 Immunofluorescent staining

An immunofluorescent staining protocol (fully described in section 2.3) was used to image the different types of myofibres in the soleus and EDL *m.* samples. Briefly, muscle sections of 10  $\mu\text{m}$  thickness were incubated for 60 min in a buffer with BSA to prevent any non-specific protein interactions. Primary antibodies (BA-F8, SC-71, BF-F3 and 6H1; Table 2.3) were applied to the muscle sections for 120 min, washed and incubated in the dark for 60 min with the secondary antibodies (Alexa Fluor 350, 488 and 555; Table 2.3).

Images (x40 magnification) were taken using a fluorescent microscope (section 2.3.4) but due to time pressures, the myofibre density and CSA could not be quantified in this thesis.

#### **4.2.9 Statistical analysis**

All maternal data (at E16 and weaning) were statistically analysed using an independent t-test, and the data reported are the mean and SEM of the observed data.

Data from the E16 and 15-week offspring were analysed using a mixed effects model (full details in section 2.6.2). It was not possible to have one offspring per dam, and so this model takes into account any variability in dam effects (within-mother and between-mother). It also allows for differences in litter size and offspring age if necessary. Consequently, all statistical differences between diet groups are independent of the dam of origin, litter size and age. It is important to note that in this chapter, litter size did not have a significant impact on offspring outcome measurements. Graphed data are displayed as the predicted means and SEM from maximal models (containing all mixed factors including diet group, litter size and age) applied to the observed data. For the statistical analysis, all data were Z-transformed before applying the mixed effects model in order to standardise the dependant variables. This allows the effect estimates to be compared directly. In order to determine any relationships between two parameters, correlations were also analysed on the Z-transformed data using the mixed effects model. Tabulated data are correlation coefficient  $r^2$ . Due to small sample sizes, correlations with the mixed effects model could only be applied when data from both sexes were combined.

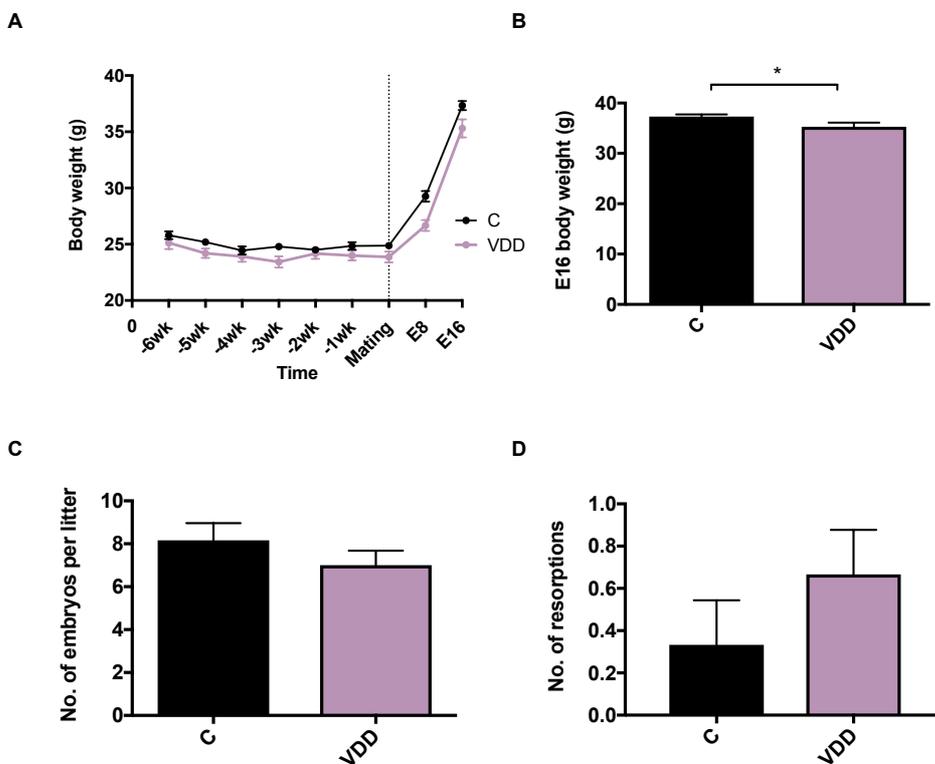
Details of all statistical analysis are given in section 2.6. SPSS version 22 was used for all analysis.

## 4.3 Results

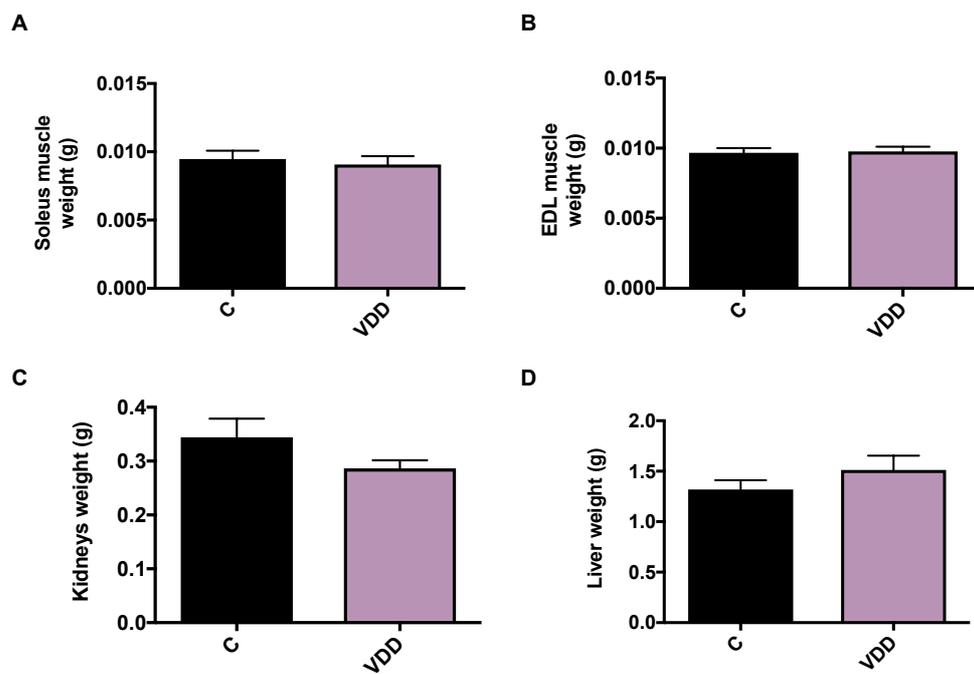
### 4.3.1 Body, muscle and organ measurements

#### Dam measurements

Dam body weight (Fig. 4.7A) was measured from 6 weeks prior to mating (when they were assigned a diet) and throughout pregnancy until embryonic day (E) 16. Body weights of all dams from the E16 cohort and those whose offspring were studied at 15 weeks were included. A maternal VDD diet caused a small reduction in dam body weight at E16 (Fig. 4.7B ( $P < 0.05$ )). Litter size (Fig. 4.7C) and the number of resorptions/miscarriages (Fig. 4.7D) were not affected by the VDD diet. Weight of the maternal soleus *m.* (Fig. 4.8A) and EDL *m.* (Fig. 4.8B) did not change with a prenatal VDD diet. There was also no change in maternal kidney weight (Fig. 4.8C) or liver weight (Fig. 4.8D); two organs involved in vitamin D biosynthesis.



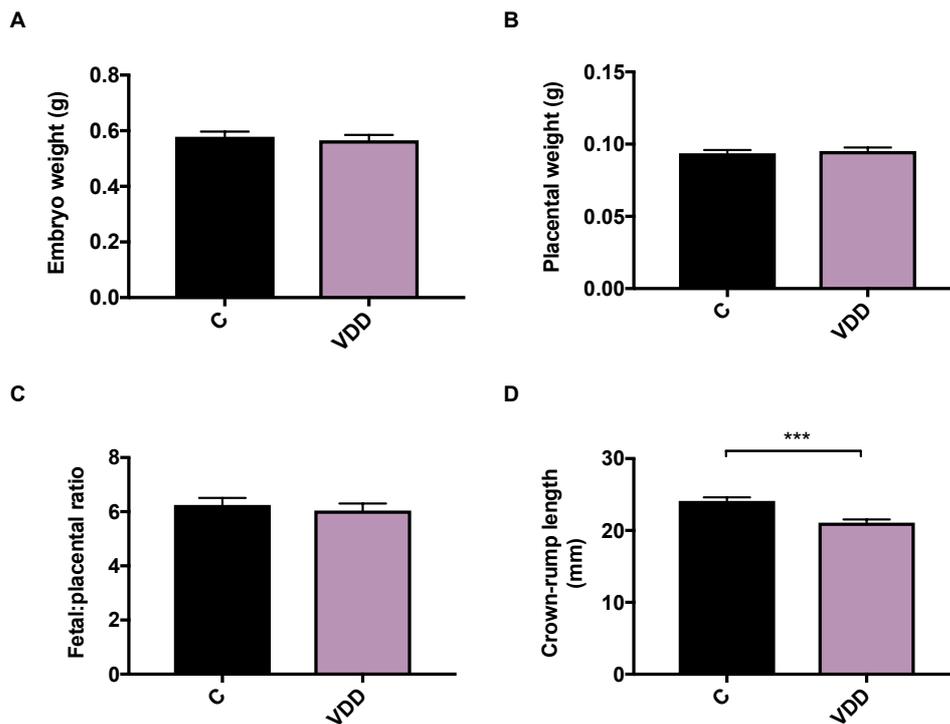
**Figure 4.7: Dam body weight, litter size and resorptions.** A) Dam body weight trajectory from 6 weeks prior to mating until E16, B) E16 body weight (g), C) litter size from E16 dams and D) number of resorptions from E16 dams. Data represented as mean  $\pm$  SEM and statistically analysed by an independent t-test. \* $P < 0.05$ . Body weight data C  $n=12$ ; VDD  $n=12$ , litter size and number of resorptions C  $n=6$ ; VDD  $n=6$ . E, embryonic day; C, control diet; VDD, vitamin D deficient.



**Figure 4.8: Dam muscle and organ weights at E16.** Weight (g) of **A**) soleus *m.*, **B**) EDL *m.*, **C**) kidneys and **D**) liver. Data represented as mean  $\pm$  SEM and statistically analysed by an independent t-test. C n=6; VDD n=6. E, embryonic day; C, control diet; VDD, vitamin D deficient; *m.*, muscle; EDL, extensor digitorum longus.

## E16 offspring

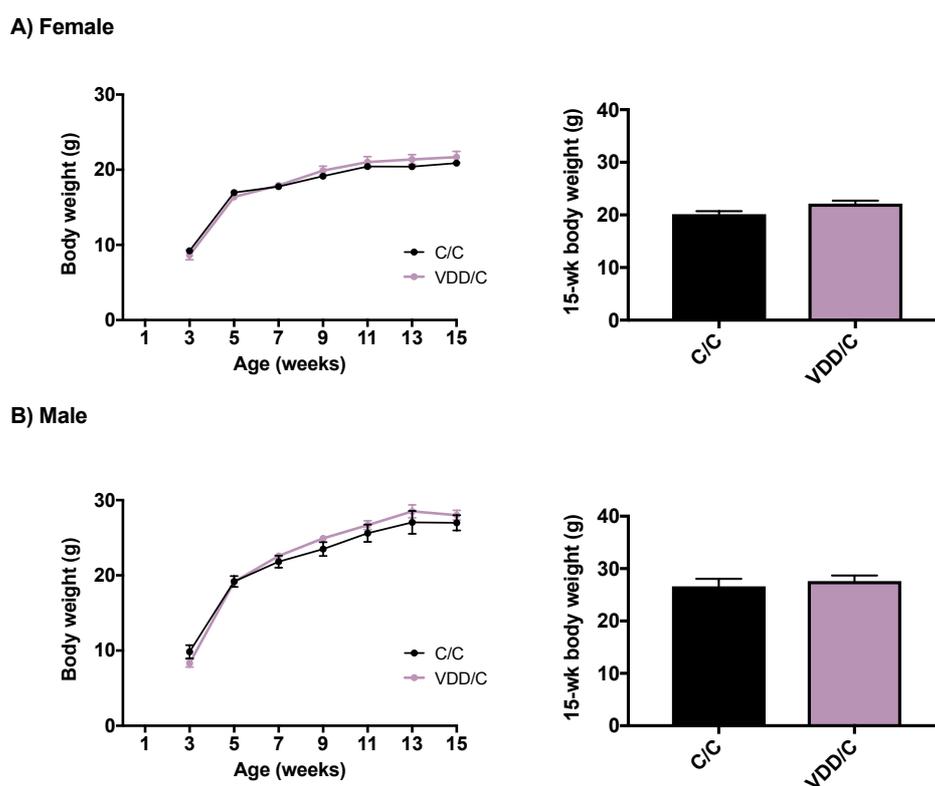
In the E16 cohort a maternal VDD diet was not associated with a change in embryo and placenta weight nor in fetal-placental ratio (Fig. 4.9A-C). However, the crown-rump length (Fig. 4.9D) of the E16 embryos from dams fed a VDD diet was significantly reduced ( $P < 0.001$ ).



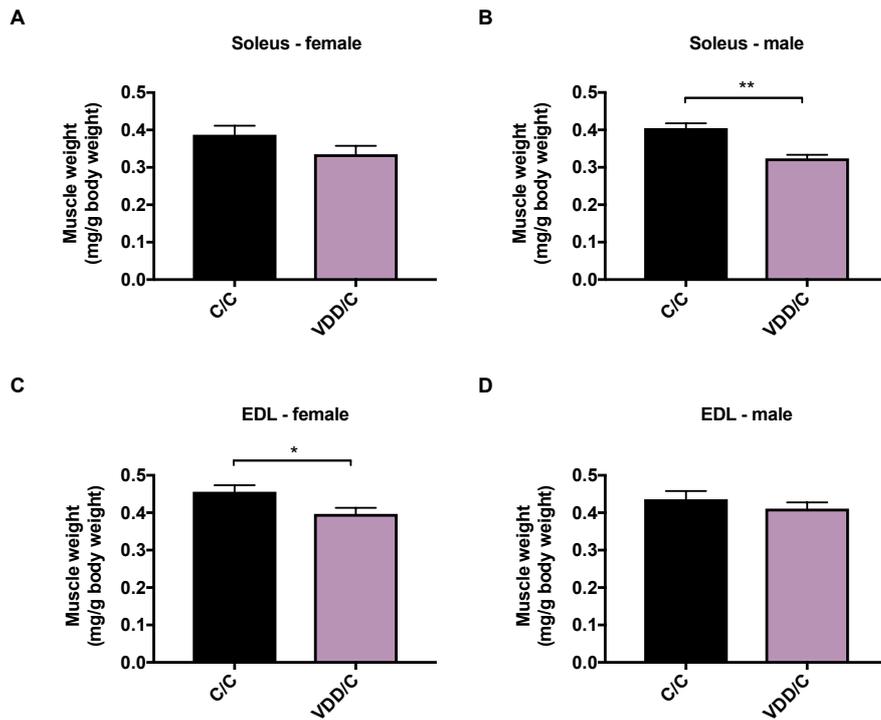
**Figure 4.9: E16 offspring measurements.** A) Embryo weight (g), B) placental weight (g), C) fetal-placental ratio and D) crown-rump length (mm). Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \*\*\* $P < 0.001$ . C n=6; VDD n=6. C, control diet; VDD, vitamin D deficient.

## 15-week offspring

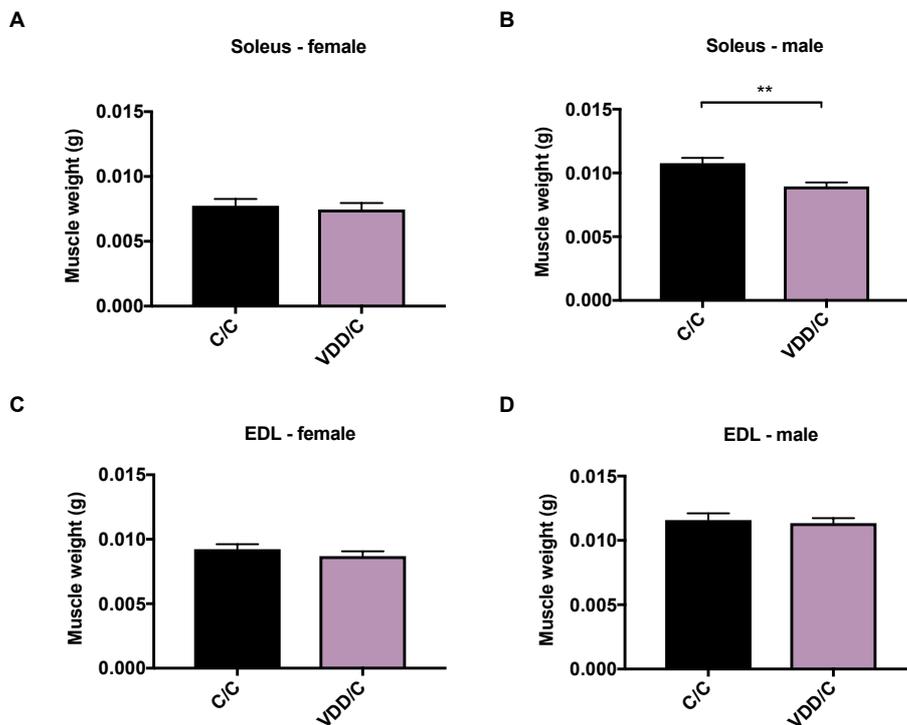
Body weight at 15 weeks was not affected by a prenatal VDD diet in the female or male offspring (Fig. 4.10). Soleus *m.* weight, raw and when relative to body weight, was lower in the male VDD/C offspring ( $P<0.01$ ; Fig. 4.12B and Fig 4.11B, respectively). There was no effect of a maternal VDD diet on the female soleus *m.* weight. EDL *m.* weight relative to body weight was reduced ( $P<0.01$ ) with a prenatal VDD diet in the female offspring (Fig. 4.11C). There was no effect of diet on the male EDL *m.* weight.



**Figure 4.10: Offspring body weight.** Body weight (g) trajectory from 3 to 15 weeks of age and offspring body weight (g) at 15-weeks for **A)** female and **B)** male offspring. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient; wk, week.

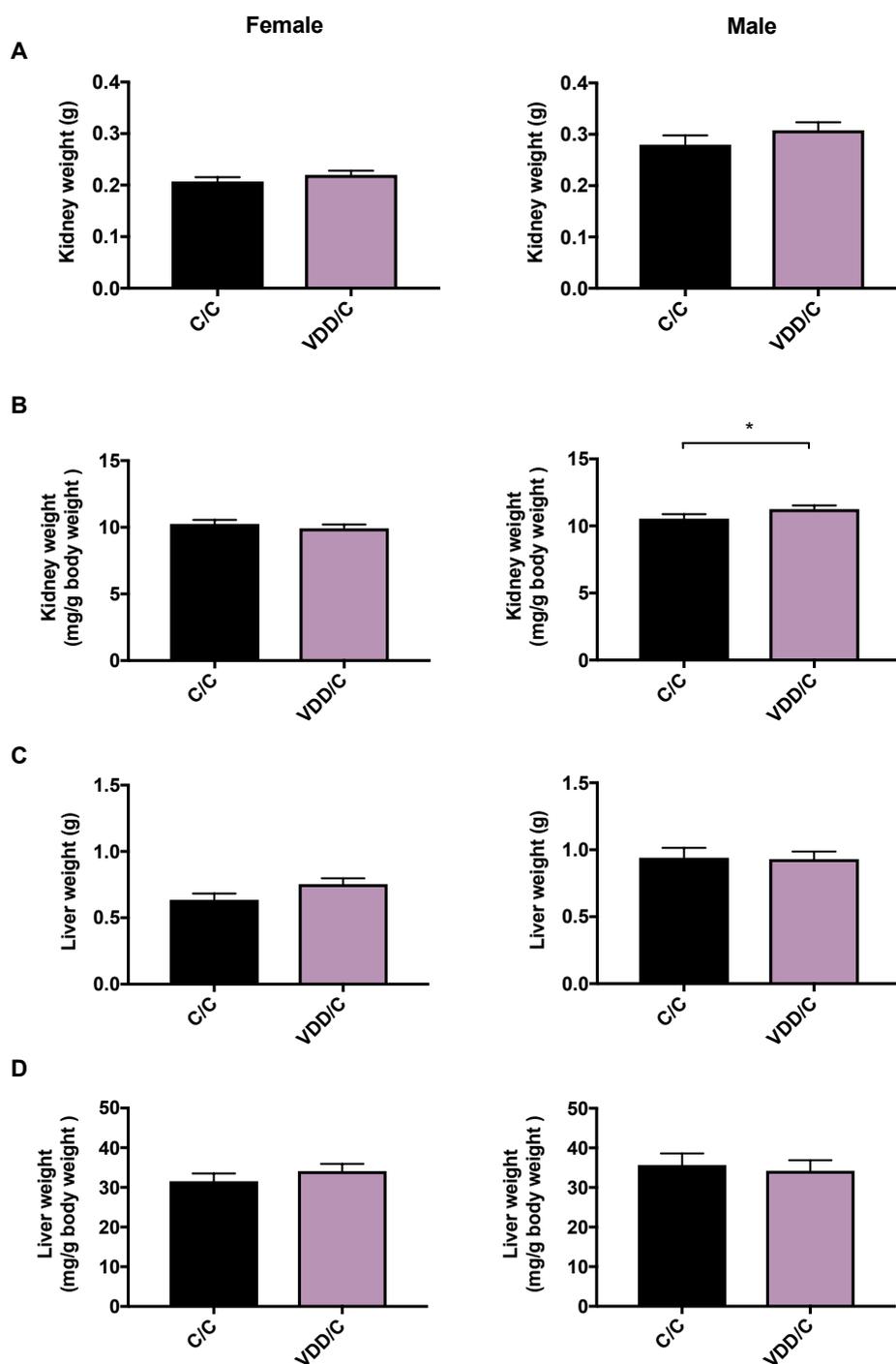


**Figure 4.11: 15-week old offspring normalised muscle weights.** A) Female soleus, B) male soleus, C) female EDL and D) male EDL *m*. All muscle weights are normalised for body weight (mg/g body weight). Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ , \*\* $P < 0.01$ . Females: C/C  $n=8$ ; VDD/C  $n=10$ . Males: C/C  $n=3$ ; VDD/C  $n=6$ . C, control diet; VDD, vitamin D deficient; EDL, extensor digitorum longus.



**Figure 4.12: 15-week old offspring raw muscle weights.** A) Female soleus, B) male soleus, C) female EDL and D) male EDL. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \*\* $P < 0.01$ . Females: C/C  $n=8$ ; VDD/C  $n=10$ . Males: C/C  $n=3$ ; VDD/C  $n=6$ . C, control diet; VDD, vitamin D deficient; EDL, extensor digitorum longus.

There were no changes in kidney weight as a result of a maternal VDD diet in the female offspring, and no changes to liver weight in the males (Fig. 4.13). There was a small increase in kidney weight relative to body weight ( $P<0.05$ ) in the male offspring.

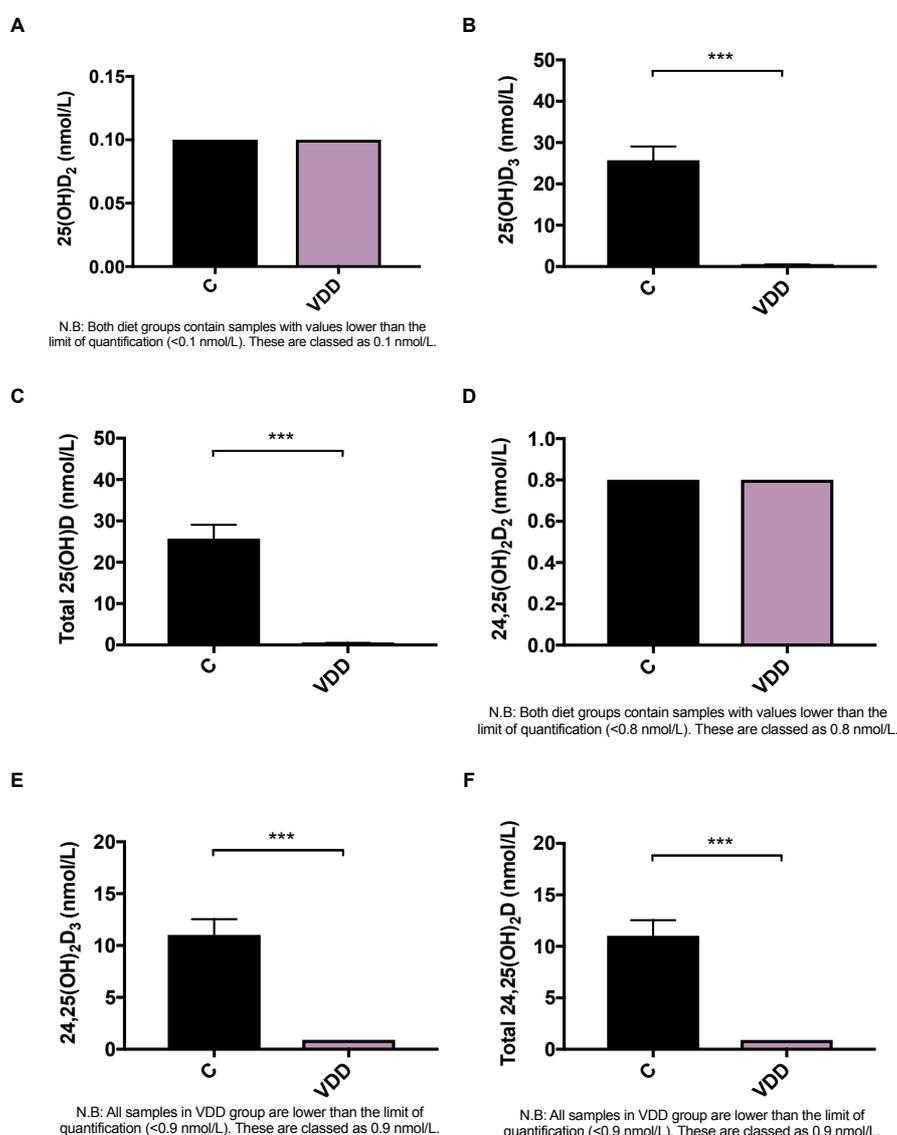


**Figure 4.13: 15-week offspring organ weights.** A) weight (g) of both kidneys, B) kidney weight relative to body weight (mg/g), C) liver weight (g) and D) liver weight relative to body weight (mg/g). Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P<0.05$ . Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient.

### 4.3.2 Blood analysis

#### E16 dams

There was a significant reduction ( $P < 0.001$ ) in fasting blood plasma 25(OH)D<sub>3</sub> (Fig. 4.14B) and total 25(OH)D (Fig. 4.14C) concentrations of dams at E16. No difference was observed in 25(OH)D<sub>2</sub> (Fig. 4.14A) which could be a result of many samples being below the LOQ in both diet groups. E16 dams fed the VDD diet also had lower 24,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 4.14 E) and total 24,25(OH)<sub>2</sub>D (Fig. 4.14F) concentrations ( $P < 0.001$ ). There was no



**Figure 4.14: Maternal E16 fasted vitamin D plasma analysis.** Fasted plasma concentrations (nmol/L) of **A**) 25(OH)D<sub>2</sub>, **B**) 25(OH)D<sub>3</sub>, **C**) total 25(OH)D, **D**) 24,25(OH)<sub>2</sub>D<sub>2</sub>, **E**) 24,25(OH)<sub>2</sub>D<sub>3</sub> and **F**) total 24,25(OH)<sub>2</sub>D. Some samples were below the LOQ as detailed on the graph, and so these samples were classed as that concentration. Data represented as mean ± SEM and statistically analysed by an independent t-test. \*\*\* $P < 0.001$ , C n=6; VDD n=6. E, embryonic day; C, control diet; VDD, vitamin D deficient.

change in E16 24,25(OH)<sub>2</sub>D<sub>2</sub> plasma concentration (Fig. 4.14D) which may also be explained by both diet groups containing samples below the LOQ. The concentration of calcium, phosphate and albumin in the plasma (Fig. 4.15) did not change in the E16 dams with a maternal VDD diet.

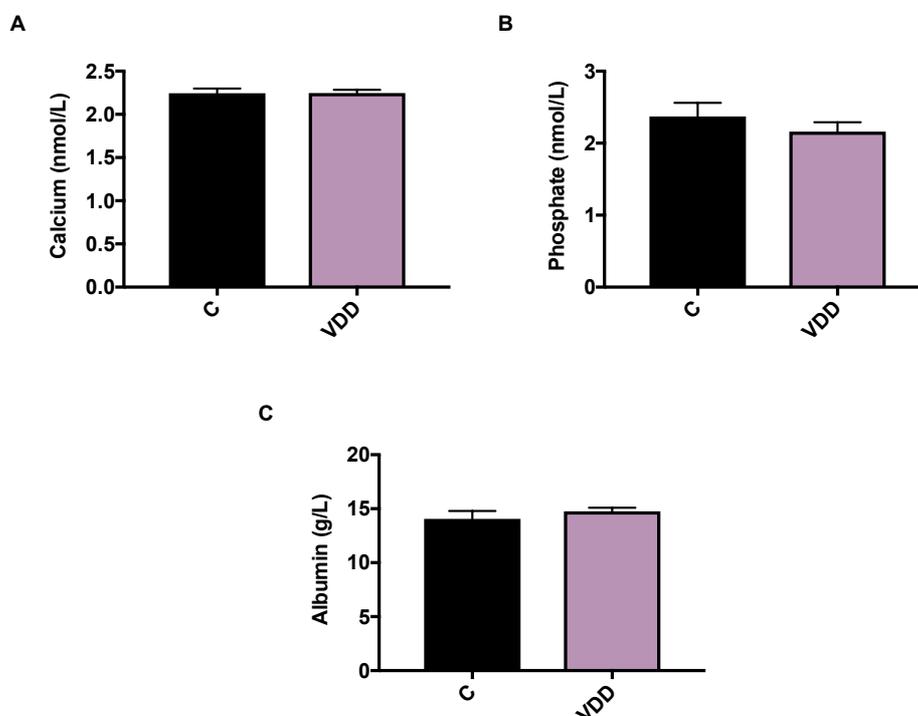
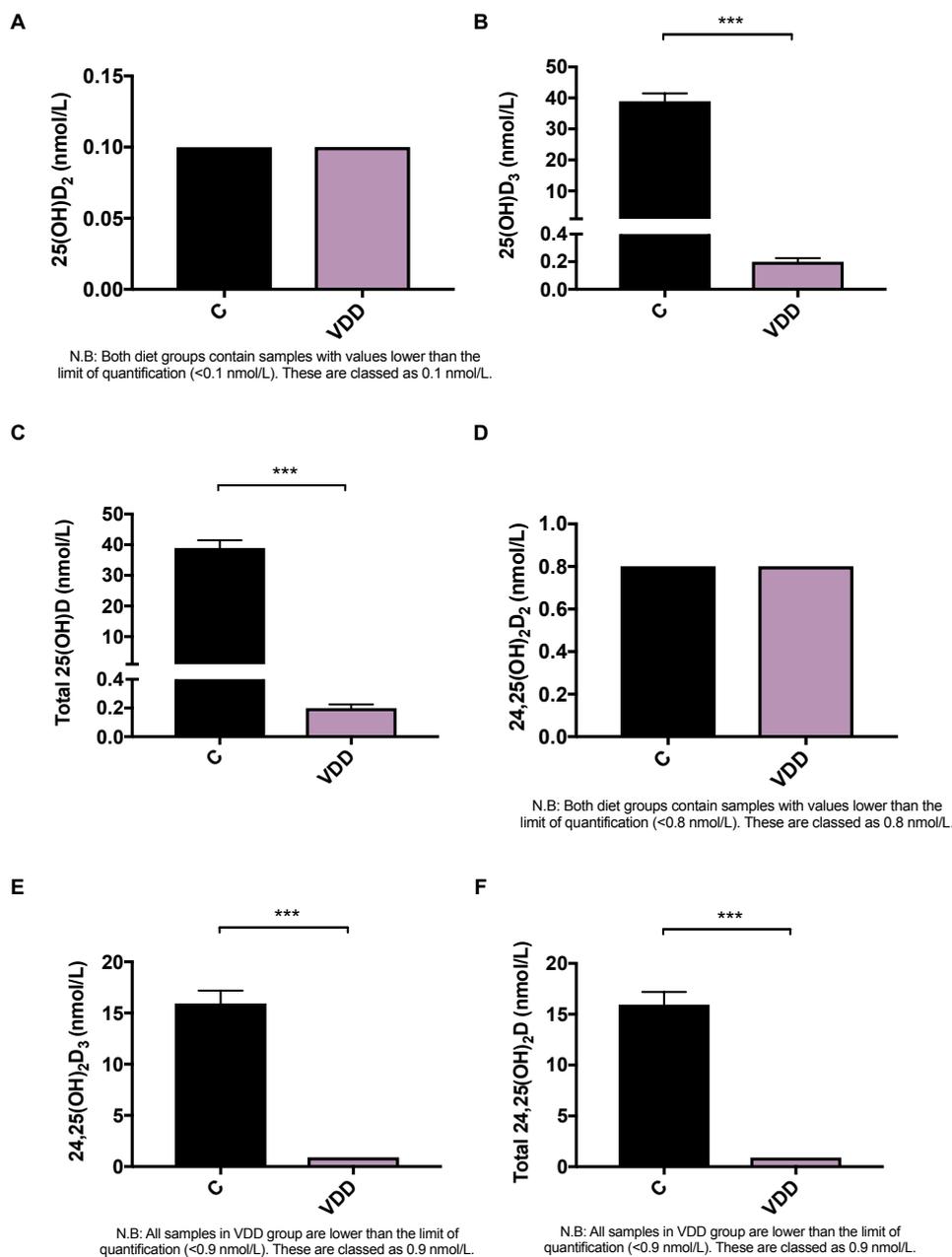


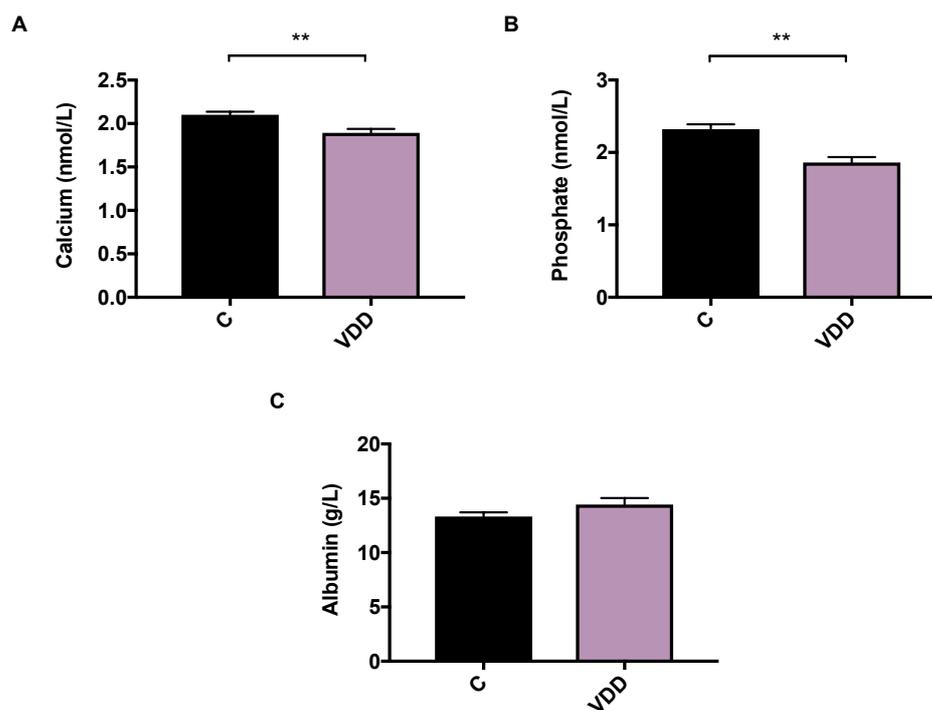
Figure 4.15: **Maternal E16 fasted plasma analysis.** A) Calcium (nmol/L), B) phosphate (nmol/L) and C) albumin (g/L). Data are displayed as mean  $\pm$  SEM and statistically analysed by an independent t-test. Statistical significance was classed as  $P < 0.05$ . C n=6; VDD n=6. E, embryonic day; C, control diet; VDD, vitamin D deficient.

## Weaning dams

Similar to the E16 dams, plasma concentration of 25(OH)D and 24,25(OH)<sub>2</sub>D metabolites were reduced in fasted dams at weaning ( $P < 0.001$ , Fig. 4.16A-F). Plasma concentrations of calcium and phosphate reduced ( $P < 0.01$ ) in the VDD dams at weaning, but albumin was unchanged (Fig. 4.17).



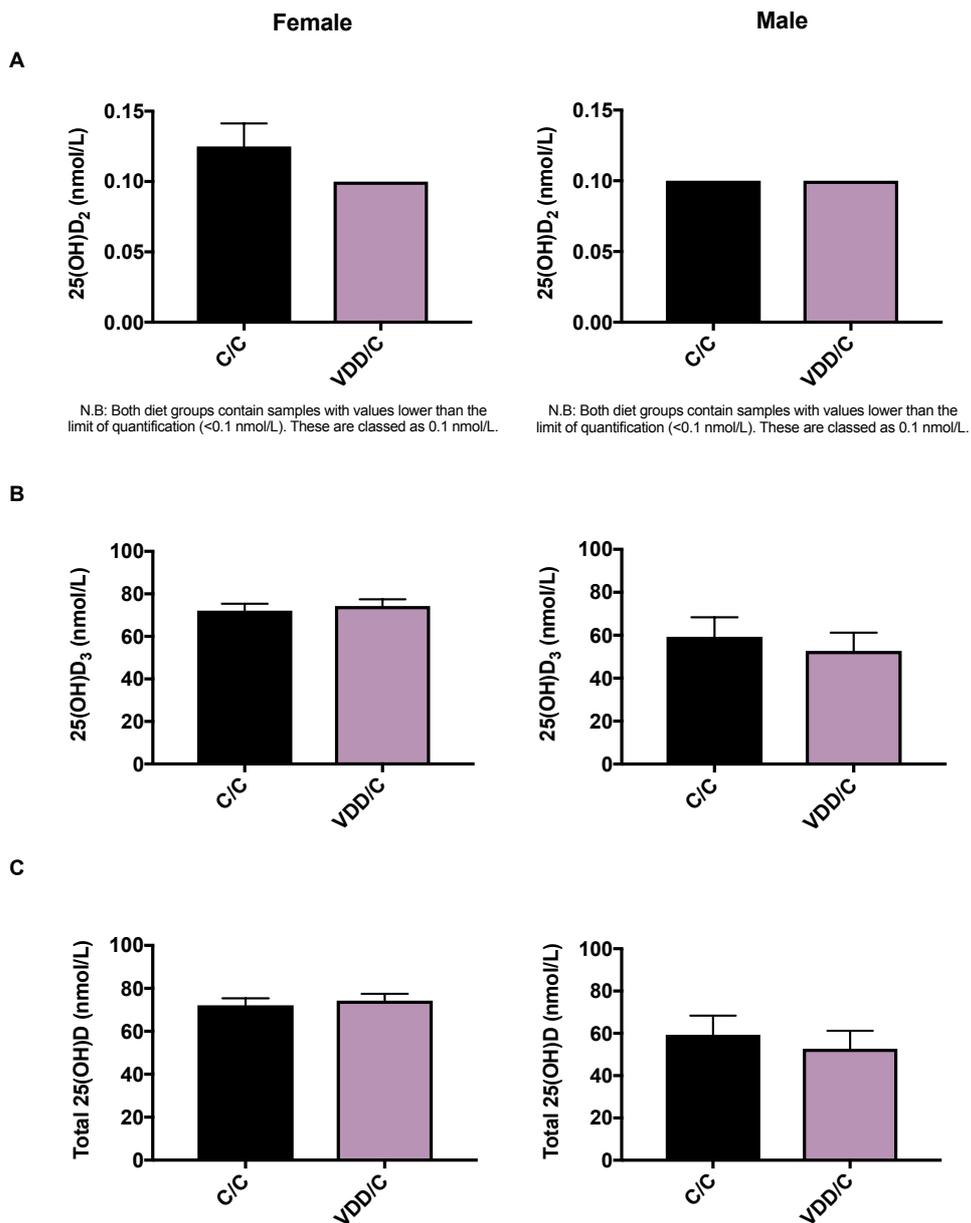
**Figure 4.16: Maternal weaning vitamin D plasma analysis.** Fasted plasma concentrations (nmol/L) of **A**) 25(OH)D<sub>2</sub>, **B**) 25(OH)D<sub>3</sub>, **C**) total 25(OH)D, **D**) 24,25(OH)<sub>2</sub>D<sub>2</sub>, **E**) 24,25(OH)<sub>2</sub>D<sub>3</sub> and **F**) total 24,25(OH)<sub>2</sub>D. Some samples were below the limit of quantification as detailed on the graph, and so these samples were classed as that concentration. Data represented as mean ± SEM and statistically analysed by an independent t-test. \*\*\*P<0.001. C n=5; VDD n=6. C, control diet; VDD, vitamin D deficient.



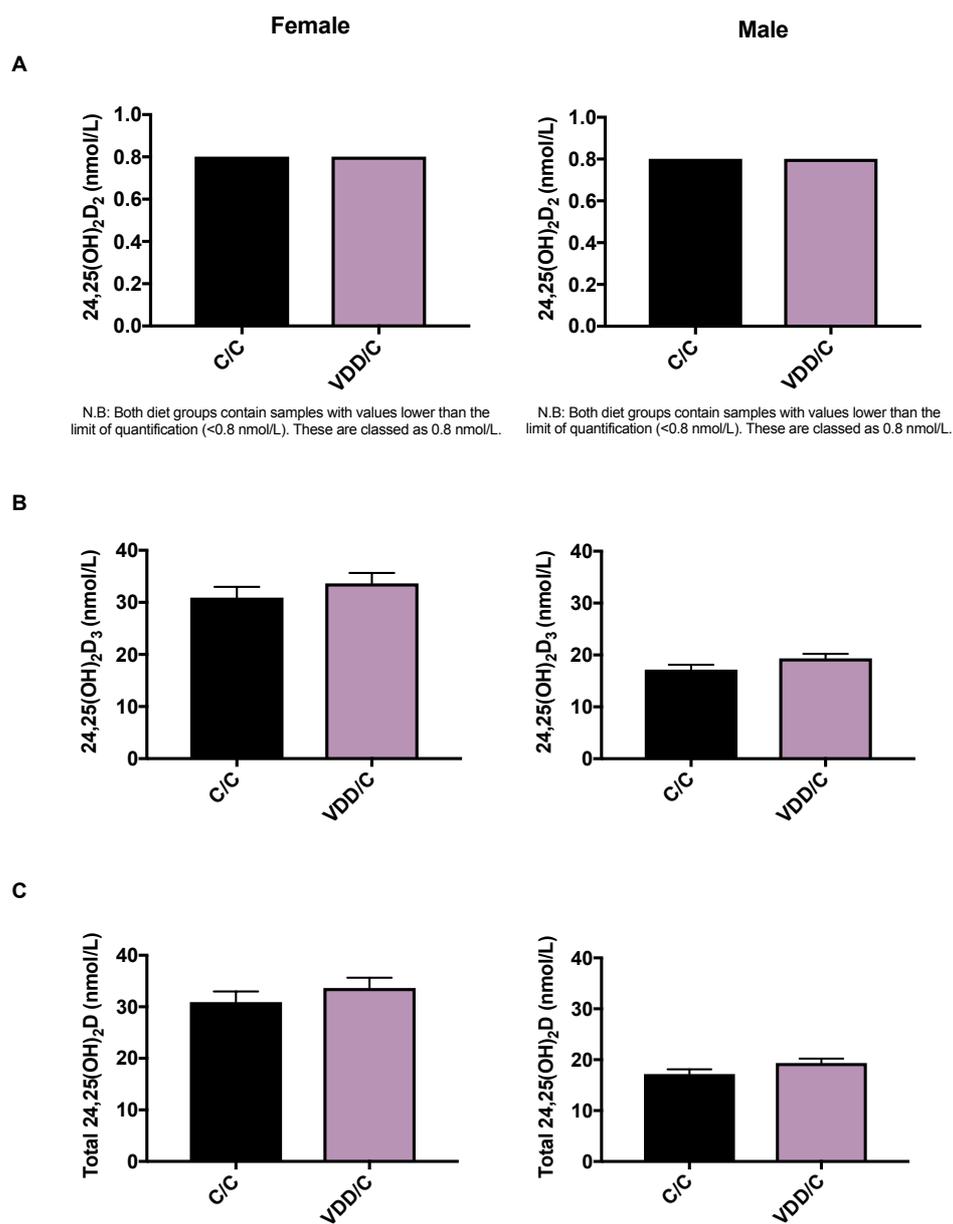
**Figure 4.17: Maternal weaning plasma analysis.** A) Calcium (nmol/L), B) phosphate (nmol/L) and C) albumin (g/L). Data are displayed as mean  $\pm$  SEM and statistically analysed by an independent t-test. \*\* $P < 0.01$ . C n=5; VDD n=6. C, control diet; VDD, vitamin D deficient.

### 15-week offspring

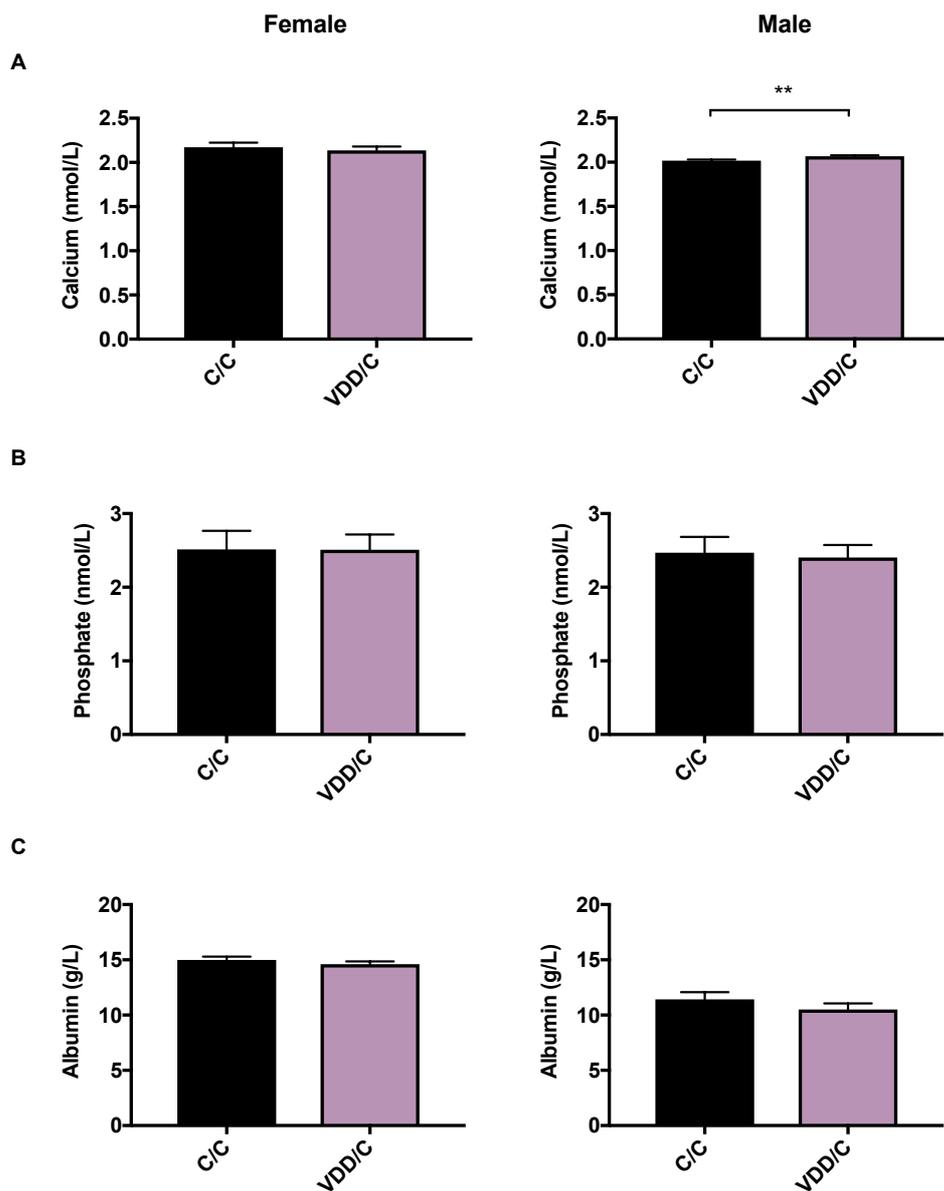
There were no differences in the 15-week offspring fasted plasma concentrations of vitamin D metabolites in the VDD/C offspring compared with C/C (Fig. 4.18 and Fig. 4.19). Blood plasma calcium levels of the VDD/C male offspring increased ( $P < 0.01$ ) compared with C/C, whereas the females did not (Fig. 4.20A). Phosphate (Fig. 4.20B) and albumin (Fig. 4.20C) concentrations were not different between dietary groups in the female or male offspring.



**Figure 4.18: 15-week old offspring fasted plasma vitamin D concentrations part 1.** Fasted plasma concentrations (nmol/L) of **A**) 25(OH)D<sub>2</sub>, **B**) 25(OH)D<sub>3</sub> and **C**) total 25(OH)D of female and male offspring. Data are displayed as predicted means ± SEM from maximal models. All statistical analyses performed on Z-transformed data and statistical significance was classed as P<0.05. Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient.



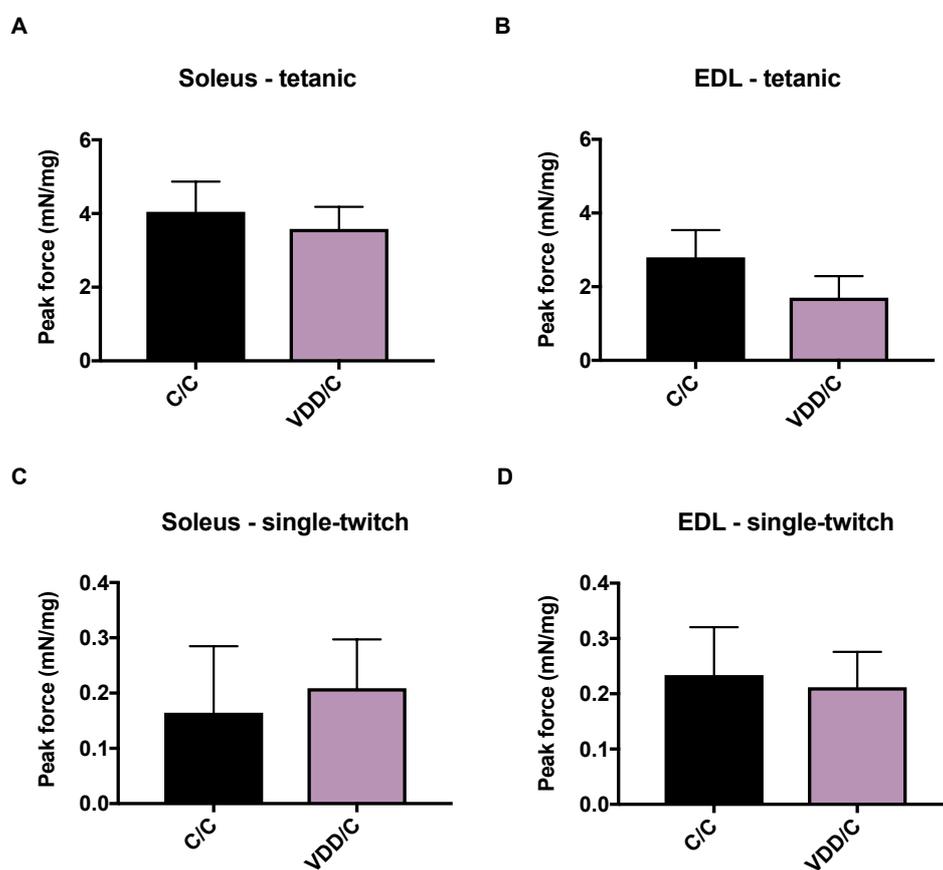
**Figure 4.19: 15-week old offspring fasted plasma vitamin D concentrations part 2.** Fasted plasma concentrations (nmol/L) of A) 24,25(OH)<sub>2</sub>D<sub>2</sub>, B) 24,25(OH)<sub>2</sub>D<sub>3</sub> and C) total 24,25(OH)<sub>2</sub>D of female and male offspring. Data are displayed as predicted means ± SEM from maximal models. All statistical analyses performed on Z-transformed data and statistical significance was classed as P<0.05. Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient.



**Figure 4.20: 15-week offspring fasted plasma analysis.** A) Calcium (nmol/L), B) phosphate (nmol/L) and C) albumin (g/L) of female and male offspring. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \*\*P<0.01. Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient.

### 4.3.3 Isometric skeletal muscle contraction peak force

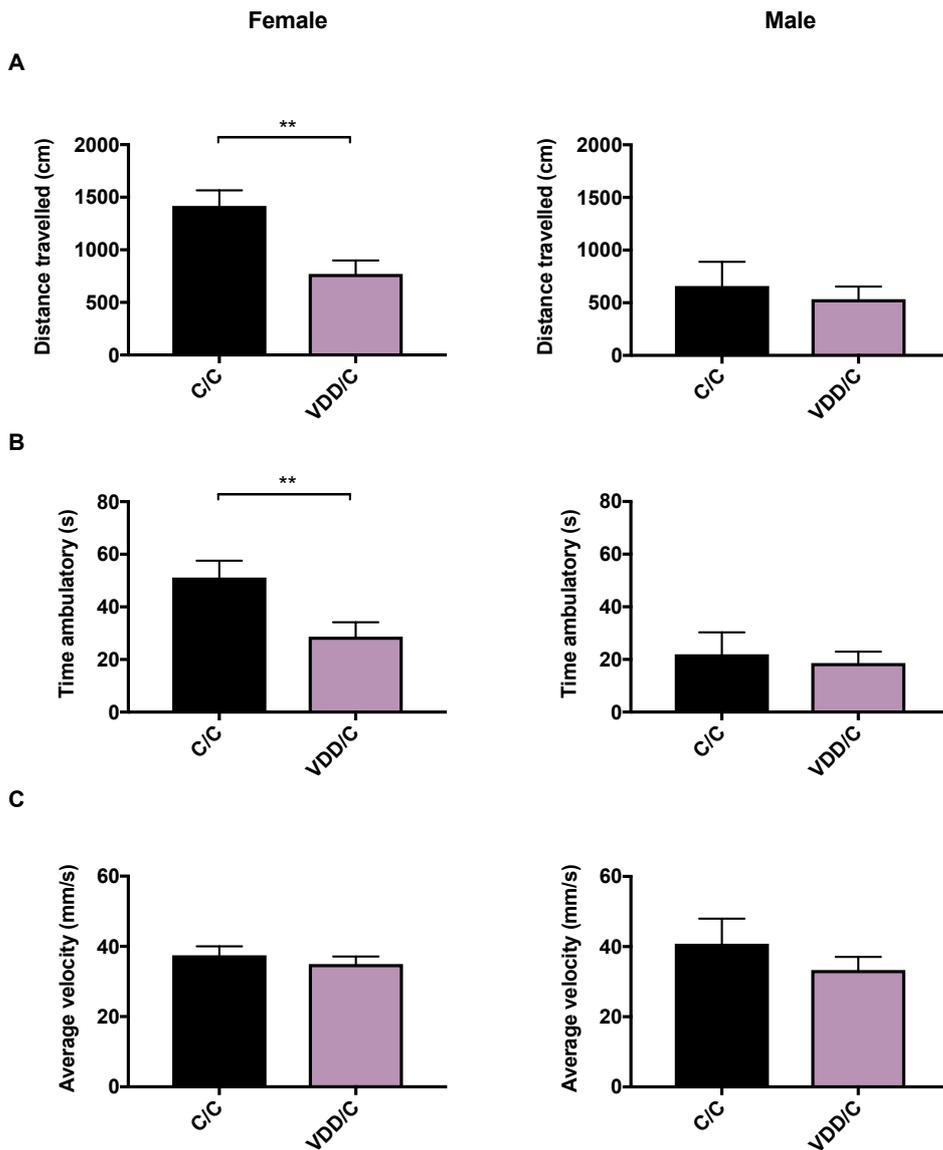
In male offspring soleus and EDL *m.* there was no difference in PF (tetanic or single-twitch contraction) between C/C and VDD/C group offspring (Fig. 4.21).



**Figure 4.21: Isometric skeletal muscle contraction peak force response.** Male 15-week offspring muscle peak force response (mN/mg muscle weight) of **A)** soleus *m.* tetanic contraction (150Hz stimulation frequency), **B)** EDL *m.* tetanic contraction (250Hz), **C)** soleus *m.* single-twitch contraction (10Hz) and **D)** EDL *m.* single-twitch contraction (10Hz). Data are displayed as the predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. Statistical significance determined as  $P < 0.05$ . C/C  $n=3$ ; VDD/C=6. C, control diet; VDD, vitamin D deficient diet; EDL, extensor digitorum longus; *m.*, muscle.

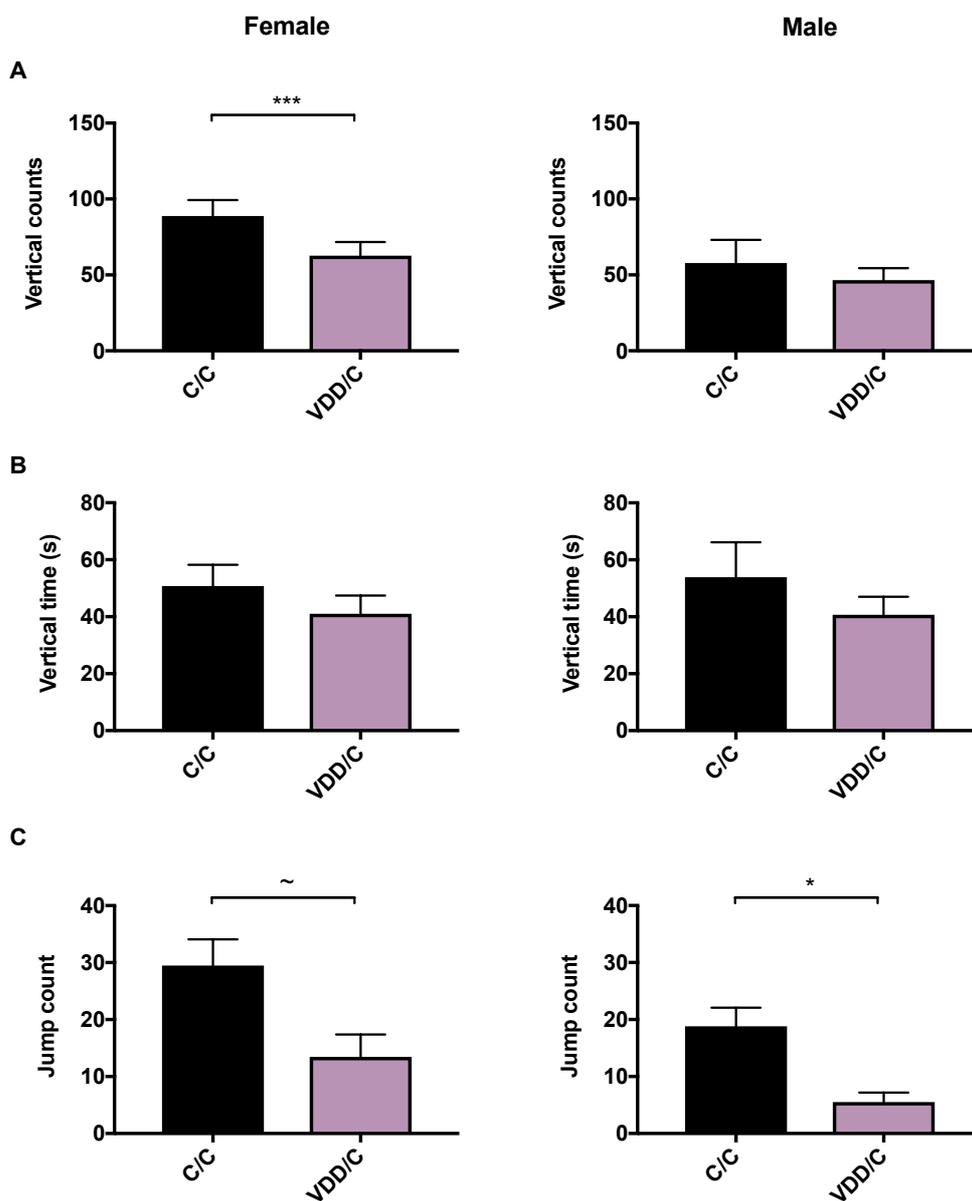
### 4.3.4 Open field activity

In female offspring the distance travelled ( $P<0.01$ ) and time spent in ambulatory movements ( $P<0.01$ ) in the horizontal plane was significantly lower in VDD/C compared with C/C female offspring (Fig. 4.22). However, there was no change in female offspring average velocity. A prenatal VDD diet had no effect on male offspring distance travelled, time ambulatory or average velocity.



**Figure 4.22: 15-week old offspring open-field horizontal activity.** A) Distance travelled (cm), B) time ambulatory (s) and C) average velocity (mm/s). Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \*\* $P<0.01$ . Females: C/C n=5; VDD/C n=7. Males: C/C n=2; VDD/C=5. C, control diet; VDD, vitamin D deficient diet.

In female but not male offspring, the amount of rearing onto hind legs was lower in the VDD/C compared with the C/C group (Fig. 4.23A,  $P < 0.001$ ). A maternal VDD diet did not change the time in which the male or female offspring spent on their hind legs (Fig. 4.23B). In males ( $P < 0.05$ ) and female ( $P < 0.1$ ) offspring there was a lower jump count in the VDD/C compared with C/C group (Fig. 4.23C).

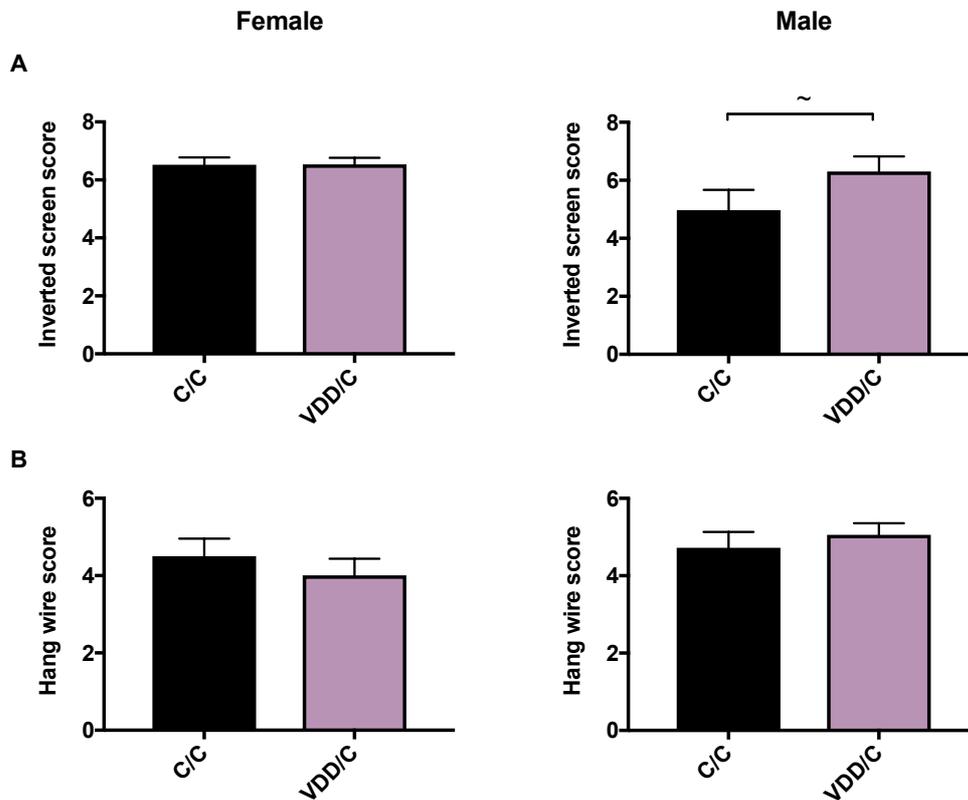


**Figure 4.23: 15-week old offspring open-field vertical activity.** A) Vertical counts, B) vertical time (s) and C) jump count. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \*\*\* $P < 0.001$ , \* $P < 0.05$ , ~  $P < 0.1$ . Females: C/C  $n=5$ ; VDD/C  $n=7$ . Males: C/C  $n=2$ ; VDD/C  $n=5$ . C, control diet; VDD, vitamin D deficient diet.

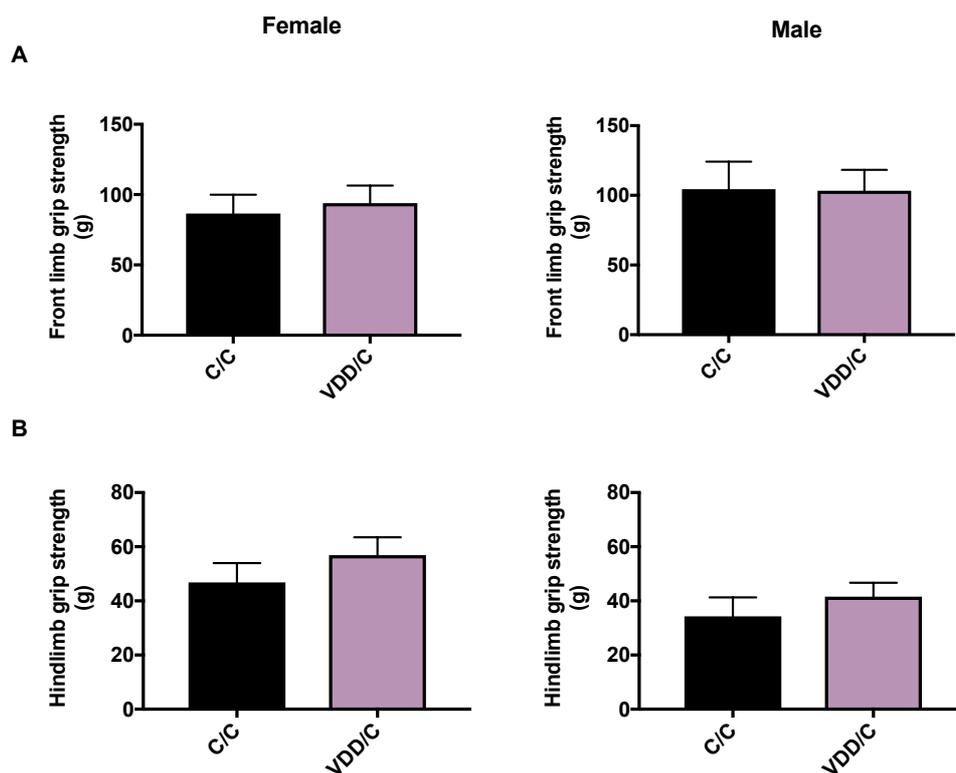
### 4.3.5 *In vivo* strength tests

In female and male offspring there was no effect of a prenatal VDD diet on the inverted screen test (Fig. 4.24A) or hang wire test (Fig. 4.24B) scores. However, there was a trend for an increase ( $P < 0.1$ ) in the time in which the male VDD/C offspring held onto the wire grid during the inverted screen test compared with C/C.

The grip strength of the front (Fig. 4.25A) and back (Fig. 4.25B) limbs were also measured. There was no effect of exposure to a maternal VDD diet on the front or hind limb grip strength in either the female or male offspring. This lack of effect persisted when grip strength was assessed relative to body weight (data not shown).



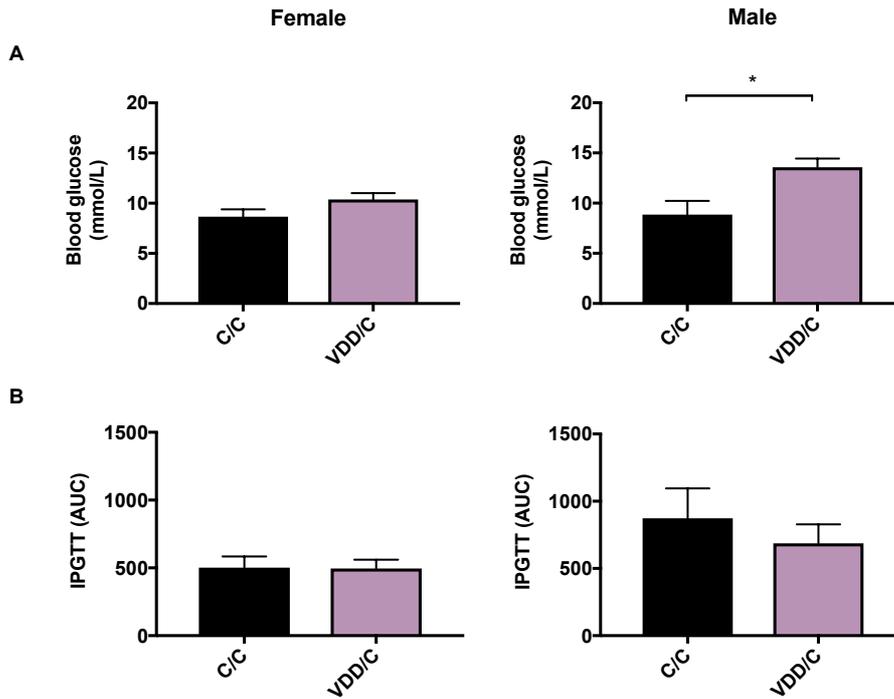
**Figure 4.24: *In vivo* strength tests of 15-week old offspring.** A) Inverted screen test and B) hang wire test. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ . Females: C/C  $n=8$ ; VDD/C  $n=10$ . Males: C/C  $n=3$ ; VDD/C  $n=6$ . C, control diet; VDD, vitamin D deficient diet.



**Figure 4.25: Grip strength of 15-week old offspring.** A) Front limb grip strength and B) hind limb grip strength. Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data.  $\sim P < 0.1$ . Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient diet.

### 4.3.6 Glucose tolerance

Fasting blood glucose was higher ( $P < 0.05$ ) in the male but not the female offspring from mothers fed a VDD diet compared with control diet (Fig. 4.26A). In both sexes, AUC of the intraperitoneal glucose tolerance test, was unaltered by maternal VDD diet compared with control (Fig. 4.26B).



**Figure 4.26: Glucose homeostasis of fasted 15-week offspring.** **A)** Fasted blood glucose (mmol/L) and **B)** glucose tolerance assessed by IPGTT (AUC). Data are displayed as predicted means  $\pm$  SEM from maximal models. All statistical analyses performed on Z-transformed data. \* $P < 0.05$ . Females: C/C  $n=7$ ; VDD/C  $n=10$ . Males: C/C  $n=2$ ; VDD/C  $n=6$ . C, control diet; VDD, vitamin D deficient diet; IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve.

### 4.3.7 Offspring plasma vitamin D versus offspring measurements

There was no correlation between 15-week offspring plasma 25(OH)D<sub>3</sub> concentration and isometric skeletal muscle PF (Table 4.1) or activity (Table 4.3). Interestingly, when the two diet groups were combined, there was a negative correlation between offspring plasma 25(OH)D<sub>3</sub> concentration to hang wire test score and front limb grip strength (Table 4.2), and baseline blood glucose levels (Table 4.4).

**Table 4.1: The correlation of 15-week old offspring plasma 25(OH)D<sub>3</sub> to muscle peak force.** 15-week male offspring plasma 25(OH)D<sub>3</sub> was correlated against the soleus and EDL *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. Statistical significance determined as  $P < 0.05$ . C/C n=3; VDD/C n=6. C, control diet; VDD, vitamin D deficient diet; PF, peak force; EDL, extensor digitorum longus; *m.*, muscle.

15-week male offspring				
Plasma 25(OH)D <sub>3</sub>				
	Soleus tetanic PF	Soleus twitch PF	EDL tetanic PF	EDL twitch PF
All offspring	0.031	0.017	0.292	0.130

**Table 4.2: The correlation of 15-week old offspring plasma 25(OH)D<sub>3</sub> to in vivo strength test results.** 15-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against inverted screen score, hand wire score, front limb grip strength and hind limb grip strength. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\* $P < 0.01$ , \* $P < 0.05$ , ~  $P < 0.1$ . Females: C/C n=8; VDD/C n=10. Males: C/C n=3; VDD/C n=6. C, control diet; VDD, vitamin D deficient diet.

15-week female + male offspring combined				
Plasma 25(OH)D <sub>3</sub>				
	Inverted screen	Hang wire	Front limb grip strength	Back limb grip strength
All offspring	0.295	-0.405*	-0.459*	0.192
C/C	0.644 *	0.123	-0.299	-0.119
VDD/C	0.165	-0.697**	-0.479~	0.380

**Table 4.3: The correlation of 15-week old offspring plasma 25(OH)D<sub>3</sub> to open-field data.**

15-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against distance travelled, time ambulatory, average velocity, vertical counts, vertical time and jump count. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \* $P < 0.05$ . Females: C/C n=5; VDD/C n=7. Males: C/C n=2; VDD/C n=5. C, control diet; VDD, vitamin D deficient diet.

15-week female + male offspring combined						
Plasma 25(OH)D <sub>3</sub>						
	Distance travelled	Time ambulatory	Average velocity	Vertical counts	Vertical time	Jump count
All	0.290	0.295	0.177	0.263	0.137	0.137
C/C	0.334	0.275	0.702*	0.522	0.577	0.566
VDD/C	-0.029	-0.021	-0.005	-0.112	-0.320	-0.168

**Table 4.4: The correlation of 15-week old offspring plasma 25(OH)D<sub>3</sub> to glucose tolerance.** 15-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against IPGTT AUC and baseline blood glucose concentration. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*P<0.01, \*P<0.05, ~ P<0.1. Females: C/C n=7; VDD/C n=10. Males: C/C n=2; VDD/C n=6. C, control diet; VDD, vitamin D deficient diet; IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve.

15-week female + male offspring combined		
Plasma 25(OH)D <sub>3</sub>		
	IPGTT AUC	Baseline blood glucose
All	-0.242	-0.406 **
C/C	-0.682 ~	-0.575
VDD/C	-0.135	-0.538 *

#### 4.3.8 Dam plasma vitamin D versus offspring measurements

At E16, embryo weight was positively associated with dam 25(OH)D<sub>3</sub> concentration in the mothers fed a C diet (P<0.01), but this significance was lost in the embryos from VDD mothers and when both groups were combined. Crown-rump length was positively correlated to dam 25(OH)D<sub>3</sub> concentration in the embryos of C-fed dams (P<0.01) and when embryos of both diet groups were grouped together (P<0.001), but not in the VDD group alone. There were no correlations in placental weight or the fetal-placental ratio with dam 25(OH)D<sub>3</sub> concentration.

There were no significant correlations between the dam 25(OH)D<sub>3</sub> plasma concentration and 15-week offspring body weight, muscle weight or 25(OH)D<sub>3</sub> concentration.

**Table 4.5: The correlation of maternal plasma 25(OH)D<sub>3</sub> on E16 offspring measurements.** Maternal plasma 25(OH)D<sub>3</sub> was correlated with E16 embryo weight, placental weight, fetal-placental ratio and crown-rump length. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*\*P<0.001, \*\*P<0.01. C dams n=6; VDD dams n=6. C embryo n=49, VDD embryo n=42. C, control diet; VDD, vitamin D deficient diet; E, embryonic day.

	E16 offspring			
	Maternal plasma 25(OH)D <sub>3</sub>			
	Embryo weight	Placental weight	Fetal-placental ratio	Crown-rump length
All offspring	0.335	-0.003	0.213	0.729 ***
C	0.731 **	0.230	0.352	0.657 **
VDD	-0.261	-0.298	0.168	-0.259

**Table 4.6: The correlation of maternal plasma 25(OH)D<sub>3</sub> on 15-week offspring measurements.** Maternal plasma 25(OH)D<sub>3</sub> was correlated with 15-week old offspring body weight, normalised and raw soleus and EDL *m.* weights and plasma 25(OH)D<sub>3</sub> concentration. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. ~ P<0.1. C dams n=6, VDD dams n=6. Female offspring: C/C n=8; VDD/C n=10. Male offspring: C/C n=3; VDD/C=6. C, control diet; VDD, vitamin D deficient diet; EDL, extensor digitorum longus; *m.*, muscle.

	15-week female + male offspring combined					
	Maternal plasma 25(OH)D <sub>3</sub>					
	Offspring body weight	Offspring soleus <i>m.</i> normalised weight	Offspring EDL <i>m.</i> normalised weight	Offspring soleus <i>m.</i> raw weight	Offspring EDL <i>m.</i> raw weight	Offspring plasma 25(OH)D <sub>3</sub>
All	-0.208	0.413	0.514 ~	0.209	0.188	0.121
C/C	0.092	-0.260	-0.050	-0.199	-0.011	0.241
VDD/C	0.317	-0.055	-0.012	0.268	0.238	-0.265

**Table 4.7: The correlation of maternal plasma 25(OH)D<sub>3</sub> to 15-week offspring muscle peak force.** Maternal plasma 25(OH)D<sub>3</sub> was correlated with the 15-week offspring soleus and EDL *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. Statistical significance determined as P<0.05. C dams n=3; VDD dams n=3. C/C offspring n=3; VDD/C offspring n=6. C, control diet; VDD, vitamin D deficient diet; PF, peak force; EDL, extensor digitorum longus; *m.*, muscle.

	15-week male offspring			
	Maternal plasma 25(OH)D <sub>3</sub>			
	Soleus tetanic PF	Soleus twitch PF	EDL tetanic PF	EDL twitch PF
All offspring	0.123	-0.115	0.427	0.104

**Table 4.8: The correlation of maternal plasma 25(OH)D<sub>3</sub> to 15-week in vivo strength test results.** Maternal plasma 25(OH)D<sub>3</sub> was correlated with 15-week offspring inverted screen score, hand wire score, front limb grip strength and hind limb grip strength. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. Statistical significance determined as  $P < 0.05$ . C dams  $n=6$ ; VDD dams  $n=6$ . Female offspring: C/C  $n=8$ ; VDD/C  $n=10$ . Male offspring: C/C  $n=3$ ; VDD/C  $n=6$ . C, control diet; VDD, vitamin D deficient diet.

15-week female + male offspring combined				
Maternal plasma 25(OH)D <sub>3</sub>				
	Inverted screen	Hang wire	Front limb grip strength	Back limb grip strength
All offspring	-0.117	0.108	-0.166	-0.314
C/C	0.098	-0.129	-0.479	-0.322
VDD/C	0.000	-0.114	0.052	-0.335

**Table 4.9: The correlation of maternal plasma 25(OH)D<sub>3</sub> to 15-week offspring open-field data.**

Maternal plasma 25(OH)D<sub>3</sub> was correlated with 15-week offspring activity data: distance travelled, time ambulatory, average velocity, vertical counts, vertical time and jump count. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , ~ $P < 0.1$ . C dams  $n=6$ ; VDD dams  $n=6$ . Female offspring: C/C  $n=5$ ; VDD/C  $n=7$ . Male offspring: C/C  $n=2$ ; VDD/C  $n=5$ . C, control diet; VDD, vitamin D deficient diet.

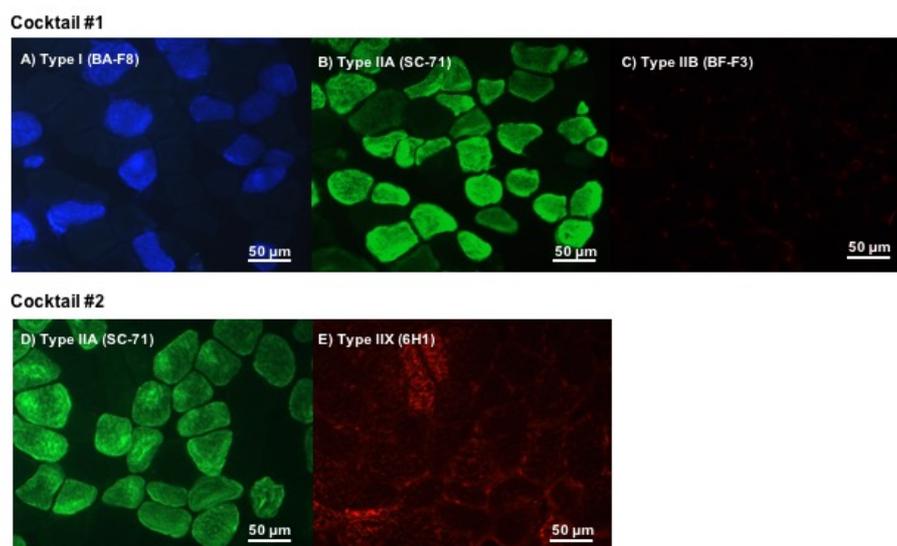
15-week female + male offspring combined						
Maternal plasma 25(OH)D <sub>3</sub>						
	Distance travelled	Time ambulatory	Average velocity	Vertical counts	Vertical time	Jump count
All	0.711***	0.684**	0.131	0.585*	0.535*	0.745*
C/C	0.036	-0.024	0.677~	-0.041	-0.118	0.147
VDD/C	-0.084	-0.090	0.128	0.330	0.405	-0.245

**Table 4.10: The correlation of maternal plasma 25(OH)D<sub>3</sub> with 15-week offspring glucose tolerance.** Maternal plasma 25(OH)D<sub>3</sub> was correlated with 15-week offspring IPGTT AUC and baseline blood glucose concentration. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \* $P < 0.05$ . C dams:  $n=6$ , VDD  $n=6$ . Female offspring: C/C  $n=7$ ; VDD/C  $n=10$ . Male offspring: C/C  $n=2$ ; VDD/C  $n=6$ . C, control diet; VDD, vitamin D deficient diet; IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve.

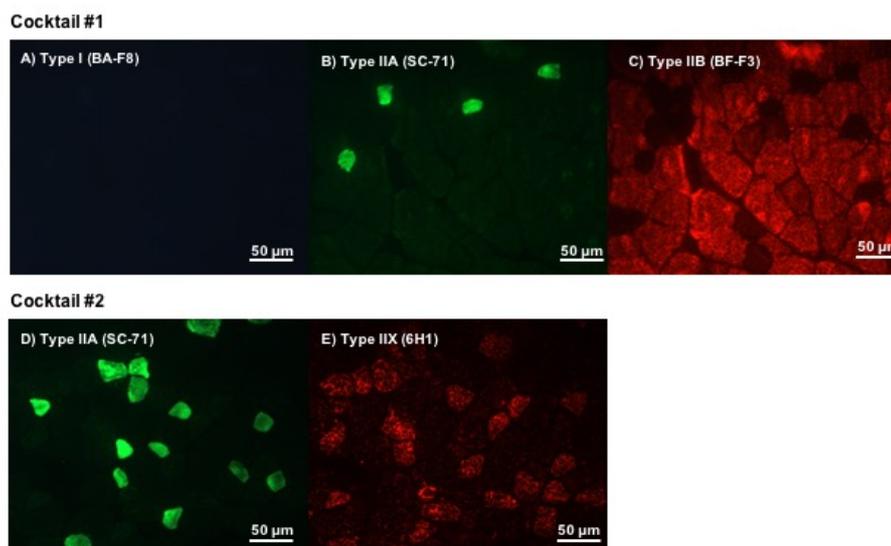
15-week female + male offspring combined		
Maternal plasma 25(OH)D <sub>3</sub>		
	IPGTT AUC	Baseline blood glucose
All	0.174	-0.585*
C/C	-0.233	-0.017
VDD/C	0.156	0.197

### 4.3.9 Myofibre typing

Representative images of the immunofluorescent staining of each type of myofibre for the soleus and EDL *m.* of Chapter 4 15-week offspring are shown in Figures 4.27 and 4.28, respectively. Unfortunately, the analysis of the images captured could not be achieved within the scope of this thesis due to time pressures. However, this will be completed in the near future.



**Figure 4.27: Representative immunofluorescent staining of the soleus muscle.** Cocktail 1: **A)** Type I (BA-F8), **B)** Type IIA (SC-71) and **C)** Type IIB (BF-F3). Cocktail 2: **D)** Type IIA (SC-71) and **E)** Type IIX (6H1). Scale bars represent 50 µm, magnification is x40.



**Figure 4.28: Representative of immunofluorescent staining of the EDL muscle.** Cocktail 1: **A)** Type I (BA-F8), **B)** Type IIA (SC-71) and **C)** Type IIB (BF-F3). Cocktail 2: **D)** Type IIA (SC-71) and **E)** Type IIX (6H1). Scale bars represent 50 µm, magnification is x40. EDL, extensor digitorum longus.

## 4.4 Discussion

### 4.4.1 A model of pregnancy vitamin D deficiency

As a result of the increased levels of vitamin D<sub>3</sub> in the high-fat diet in Chapter 3, it was important that the next chapter of this thesis focussed specifically on a deficiency in vitamin D<sub>3</sub> during pregnancy without confounding factors of a high-fat diet. Results from the LC-MS blood plasma analysis informed us that the prenatal vitamin D<sub>3</sub> deplete diet did induce gestational VDD as reflected by a significant decrease in maternal plasma total 25(OH)D and 25(OH)D<sub>3</sub> in the mothers at E16 and weaning. Due to the small blood volume in the offspring at E16 and weaning, fetal or weaning plasma vitamin D concentrations could not be analysed by LC-MS, however with hindsight, a cardiac puncture could have been performed and the blood samples analysed for vitamin D metabolites by a radioimmunoassay (Liu *et al.* 2013). Considering that 25(OH)D is the inactive form of vitamin D, it would have been interesting to quantify the concentration of the active form 1,25(OH)<sub>2</sub>D as well. However, as previously discussed (section 1.4.1) it is 25(OH)D that is used to clinically assess an individual's vitamin D status. Due to the small plasma volumes obtained from each mouse we could not get this measured. However, as 25(OH)D gets converted into 1,25(OH)<sub>2</sub>D by 1 $\alpha$ -hydroxylase (Fig. 1.4), the lower 25(OH)D concentration in the VDD dams compared with C dams suggests that the active form would also be reduced in the VDD-fed mothers. The blood plasma total 25(OH)D and 25(OH)D<sub>3</sub> concentrations were not reduced in the VDD/C female or male offspring compared with the C/C group, which is not surprising considering all offspring were assigned to the C diet at weaning until 15-weeks of age. This may have been enough time to replete vitamin D levels. Another reason for this lack of reduction in 15-week plasma 25(OH)D<sub>3</sub> concentration of VDD/C offspring could be a result of components in the vitamin D signalling pathway being upregulated to compensate for the lower maternal vitamin D levels during development. Overall it can be concluded that this was a successful mouse model of gestational VDD.

## 4.4.2 Offspring strength

### 4.4.2.1 Skeletal muscle contractile peak force

A prenatal VDD diet did not change the PF produced by the soleus or EDL *m.* of the male offspring and there was no correlation between offspring muscle PF and maternal plasma 25(OH)D<sub>3</sub>. In this study we would have ideally analysed TPT and HRT to have a more detailed analysis on how the contractile kinetics are affected with a prenatal VDD diet and to carry on from Chapter 3. It is important to note that the male C/C offspring was only n=3, which is a very small number for physiological tests. It could be that with a more ideal group size to match that in Chapter 3, some differences would emerge with diet group for the PF of tetanic contraction. Ideally the muscle contractile function of the female offspring would have also been measured to investigate whether there were any sex differences as previous human studies suggest that the higher estrogen levels in females increase skeletal muscle force (Heikkinen *et al.* 1997; Skelton *et al.* 1999). Furthermore, one study has shown that 4-month old female mice with low estrogen levels (result of ovariectomy) were associated with a reduction in maximal isometric tetanic force and strong-binding myosin (Moran *et al.* 2007), which were recovered following estrogen replacement. Despite the lack of effect in offspring contractile PF, these are novel data.

Although there is evidence to suggest a role of vitamin D in skeletal muscle development, structure and function (section 1.4.3 and 1.4.4), very little is known about the effect of VDD during pregnancy on offspring muscle function. One study found that a lower maternal 25(OH)D plasma concentration during pregnancy was associated with a reduction in offspring grip strength at 4 years of age (Harvey *et al.* 2014). Furthermore, previous work investigating postnatal VDD on skeletal muscle contraction found reductions in the skeletal muscle force produced (Pleasure *et al.* 1979; Cielien *et al.* 2016) and another study found that VDD impaired rat soleus *m.* excitation-contraction coupling (Rodman & Baker 1978). There is a clear gap in the literature of research investigating the effect of gestational VDD on offspring skeletal muscle contractile mechanisms, and no previous studies investigating the effect of VDD during pregnancy on offspring skeletal muscle contractility itself.

The current data imply that VDD may not be an important driving factor for the development of offspring skeletal muscle, but interestingly the VDD/C male offspring in

the current study had increased plasma calcium concentrations at 15 weeks of age, despite the mothers having reduced calcium concentrations at weaning. There may have been compensatory effects in components important for calcium handling and therefore ability to contract, which could be one potential reason why the PF was not affected. Indeed, Cielen *et al.*, reported a reduction in the specific maximal tetanic PF of the soleus and EDL *m.* in male C57BL/6 12 and 18-week male mice fed a VDD diet (0 IU/g VD; control group 1 IU/g) from 8 weeks of age (Cielen *et al.* 2016). However, their VDD diet contained 20% w/w lactose, 2% w/w calcium, and 1.25% w/w phosphorus in order to “maintain normal calcium and phosphorus serum levels”, which was not done for this thesis and may account for the observed reduction in maternal weaning plasma calcium concentration. Furthermore, a study by Pleasure *et al.*, looking at VDD in 3-week-old chicks found that the force produced by the triceps surae *m.* was reduced in the VDD group (Pleasure *et al.* 1979). They also reported a slower relaxation time of tetanic contraction and *in vitro* calcium transport into the SR with the VDD group, along with a reduction in the mitochondrial calcium content from leg muscles.

Ceilen *et al.*, found a reduction in soleus and EDL *m.* VDR protein expression in 18-week-old mice, but no changes to fibre size. It would be interesting to investigate how the prenatal VDD diet impacts the expression of genes and proteins involved in vitamin D signalling. Furthermore, despite no effect of the VDD diet on PF, there were reductions in the male soleus *m.* weight and the female EDL *m.* weight of VDD/C offspring when relative to body weight. This suggests that maternal VDD is having some effect on the muscle, and so assessing its effect on the muscle composition and expression of contractile proteins would be another logical step for future work. Measurement of myofibre density and of vitamin D receptivity in the current study was not possible in the timeframe of this thesis but should be done in future analysis.

#### 4.4.2.2 *In vivo* strength tests

In this chapter, the lack of difference between dietary groups for either female or male offspring in the inverted screen score, the hand wire score or the grip strength of the front and hind limb grip strength suggests that VDD during pregnancy does not impact the young adult offspring's strength. This concurs with the finding in individual isolated muscles (discussed above) of no effect on PF. There have been no studies that have looked specifically at maternal VDD on offspring strength, but one study has reported low maternal vitamin D 25(OH)D concentrations to be associated with reduced grip strength in offspring at 4 years of age (Harvey *et al.* 2014). However, we did not see any correlations between maternal plasma 25(OH)D<sub>3</sub> and mouse offspring grip strength, or any of the other *in vivo* strength tests. The discrepancy in results could be due to the age of the offspring; 15-week old mice are relatively a lot older than 4-year old human offspring. However, Harvey *et al.* used a dynamometer fitted to the hand size of the child, which when compared to our *in vivo* strength test protocols is a much more accurate method of measuring muscle strength. In hindsight, a hind limb involuntary contraction assay could have been used whereby the mouse is anaesthetised and electrodes are placed at the knee and heel, and when stimulated, the plantar flexor muscles exert force onto a force transducer .

#### 4.4.3 Activity levels

Activity levels (distance travelled, time doing short burst of fast movement and vertical counts) in the female adult offspring were impaired more than in the male offspring from mothers fed a VDD diet. Only the jump count was reduced in the VDD/C male offspring. However, across all sexes and diet groups a lower maternal plasma 25(OH)D<sub>3</sub> was associated with a reduction in distance travelled, time spent in ambulatory movements, vertical counts, vertical time and jump count in adult offspring. Thus overall these data suggesting that VDD during pregnancy does impair offspring physical activity in adulthood.

The lack of any observed effects of the prenatal VDD diet on offspring muscle contractility or overall strength suggests that the reduction in activity levels may not be a result of changes in skeletal muscle development. Instead, the reduced activity could be driven by changes in neurological development. Previous studies have shown that VDD during pregnancy is associated with a reduction in cortical thickness and nerve growth factor in the rat brain at birth (Eyles *et al.* 2003), and with a reduction in neurogenesis of the hippocampal dentate gyrus in adulthood (Keilhoff *et al.* 2010) and impaired dopamine signaling and dopamine-mediated behaviours (Kesby *et al.* 2009; Kesby *et al.* 2010). It could be that the 15-week old offspring in this chapter had a reduced motor cortex thickness (area of the brain responsible for movement), reduced hippocampal neurogenesis (involved in exploration) and impaired dopaminergic signaling of motor neurons, all contributing to the reduction observed in activity levels. Reasons for the sex differences in the activity data are not clear. One study observed a reduction in lateral ventricular volume of adult male offspring (but not the females) following a prenatal VDD diet, and a reduction in hippocampal volume was only observed in the female offspring at birth, and not in the males (Harms *et al.* 2012).

Interestingly studies have reported hyperlocomotion in offspring from mothers fed a VDD diet (Burne *et al.* 2004; Harms *et al.* 2008), which contrasts to the results in this thesis. The differences could be a result of their use of younger offspring (10-weeks vs. 15-weeks) and that the offspring in these two previous studies were transferred onto a C diet at birth, and not at weaning. In fact, Burne *et al.* found that although the offspring exposed to a VDD diet until birth had increased activity levels, the activity levels of the subset of offspring exposed to a VDD diet until weaning remained unchanged compared with C/C, thereby partially supporting our lack of observed hyperlocomotion. A difference in species may also explain the disparity between results; Burne *et al.* used a rat model and Harms *et al.* used two mouse models. The hyperlocomotion reported by Harms *et al.* was observed in the 129/SvJ mice alone and not the C57BL/6 mice. The combination of results in this chapter and these previous studies suggest that inter- and intra-species differences may exist in the way a pregnancy VDD diet affects offspring locomotion.

#### 4.4.4 Glucose tolerance

There was no difference in the IPGTT AUC, a marker of glucose tolerance, in the 15-week offspring from mothers fed a prenatal VDD diet. There was also no relationship between maternal 25(OH)D<sub>3</sub> plasma concentration and offspring glucose tolerance. However, fasted baseline blood glucose levels were increased in the young male offspring, and perhaps they are not old enough to have developed full glucose intolerance but have an early phase dysfunction. When all offspring were combined (regardless of sex or diet group), a lower maternal 25(OH)D<sub>3</sub> plasma concentration was associated with a higher baseline blood glucose concentration in the offspring in adulthood. There is very little research investigating the link between vitamin D and glucose tolerance, especially during pregnancy on offspring outcome. However, previous data report an inverse relationship between maternal blood 25(OH)D concentration and fasting glucose levels during late gestation in women with gestational diabetes (Senti *et al.* 2012). Furthermore, two rodent studies have reported an impaired glucose tolerance in the offspring of mothers fed a VDD diet (Reichetzeder *et al.* 2014; Zhang *et al.* 2014), suggesting that VDD in early life may 'programme' the offspring to have impaired glucose metabolism in adulthood and could be a result of increased inflammation. These two studies looked at offspring at a younger age; Zhang *et al.* used 15-week rats (which have a longer lifespan than mice) and Reichetzeder *et al.* used mice of 8-9 weeks of age. In contrast to the idea above that the male offspring may not be old enough to have developed glucose intolerance despite raised blood glucose levels, perhaps an impaired glucose tolerance is present at an earlier age, but over time this is not so apparent. In fact, work from this research group found that maternal undernutrition during pregnancy was associated with reduced blood glucose levels, mild glucose intolerance and impaired insulin secretion in young male adult sheep offspring, but his glucose intolerance had resolved in the mature adult sheep and could be a result of a compensatory increase in GLUT-4 (Costello *et al.* 2013).

#### 4.4.5 Conclusion

In conclusion, we have successfully established a mouse model of gestational vitamin D deficiency. There was no change in the PF generated by muscles of young adult offspring whose mothers were fed a VDD diet compared to a C diet. We also did not find any changes in the overall strength or glucose tolerance in these animals, however, activity levels were impaired. The strength and activity data in a model of maternal VDD are novel and warrant further investigation.

The data so far in this model suggest that maternal VDD is having a rather limited effect on the skeletal muscle function. When all animals were combined, none of the strength data correlated with maternal plasma 25(OH)D<sub>3</sub> concentration. However, there are reductions in the 15-week offspring male soleus *m.* weight and female EDL *m.* weight relative to body weight, which while not translated into impaired muscle function, do suggest that the maternal VDD diet may be causing some changes to muscle structure. Immunofluorescent staining for fibre-typing has been done, and future work will involve analysing the density and CSA of the different myofibre types to understand whether the prenatal VDD diet is causing changes to muscle composition as previously reported in other studies focussing on postnatal VDD (Max *et al.* 2014). Further analysis of the contractile data to analyse the contractile and relaxation speeds (TPT and HRT) will also provide more in depth information of how the muscles of young adult offspring are contracting after exposure to a maternal VDD diet.

Generally, the data so far do not support the hypothesis that a prenatal VDD diet will reduce offspring strength in adulthood. This is surprising considering the evidence for a role of vitamin D in skeletal muscle. Work from Harvey *et al.* contrast with the current findings as they imply that maternal vitamin D status during pregnancy is positively associated with offspring muscle function (Harvey *et al.* 2014). The power of the approach in this chapter is a really clear and strong disruption of the vitamin D supply to the developing fetus. This approach therefore has advantages over epidemiological approaches such as Harvey *et al.* in which the control over the exposure is more limited and can only be dealt with statistically.

Data in this chapter suggest that altered skeletal muscle may not be the primary cause for the reduced activity levels in the VDD/C offspring. Combined with the finding that lower maternal plasma 25(OH)D<sub>3</sub> concentration is associated with lower open-field activity, this chapter suggests that maternal VDD is clearly having a profound effect on the activity of the offspring in later life. If not due to changes in skeletal muscle then this effect on activity may be a result of neurological impairments as previous studies have reported maternal VDD to lead to altered brain development, although this line of investigation by molecular and histological analysis of the brains from the 15-week offspring was beyond the scope of this thesis. In humans, a reduction in physical activity may lead to health complications such as obesity. The observed reduction in activity in adult offspring of this prenatal VDD model could therefore increase the propensity for adiposity in an obesogenic postnatal environment and may suggest a potential pathway underlying the propensity to obesity in humans.

In the context of other published work at younger developmental ages, it is possible that the lack of effect may be having more of an impact at an earlier offspring age than our 15-week old (young adult) mice. In this chapter there were no overt effects of maternal VDD on skeletal muscle, however it could be that impairments would have been detectable at a younger age and perhaps be ‘rescued’ over time by the postnatal control diet. Indeed, there is evidence to suggest that maternal diet can drive changes in prenatal 25(OH)D concentration in sheep associated with fetal myofibre density (Cleal *et al.* 2017). Having a subset of newborn mouse offspring to analyse would be a good way to explore this idea. So, the next steps in this thesis involved determining the impact of maternal vitamin D restriction during pregnancy on late gestation fetal skeletal muscle (Chapter 5).

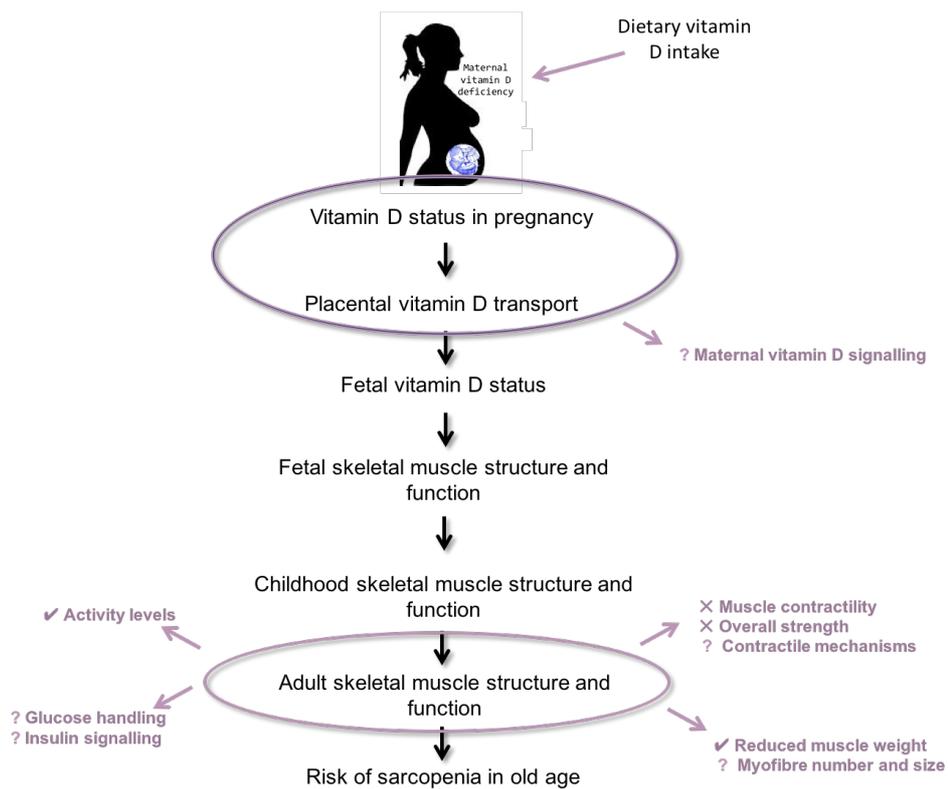


Figure 4.29: Schematic diagram showing thoughts after Chapter 4 results.

# Chapter 5: The impact of maternal dietary vitamin D deficiency on fetal sheep muscle development and function

## 5.1 Introduction

In Chapter 4 it was shown that a prenatal vitamin D deficient (VDD) diet was associated with reduced activity levels in the young adult mouse offspring despite no changes in isometric skeletal muscle contractile peak force, overall strength or glucose tolerance. The lower activity levels were associated with lower maternal plasma 25(OH)D<sub>3</sub> concentrations which in part supports the overarching hypothesis of this thesis that a prenatal VDD diet will reduce offspring strength and activity levels in adulthood. All of these findings are novel but they seem to imply either that maternal VDD is having very little effect on offspring muscle development or that any changes during development are compensated for with a return to normal vitamin D status over time. Indeed, the weight of certain muscles of the VDD/C group offspring were lower and further investigation at earlier developmental stages would help to understand how VDD during pregnancy may impact the offspring's muscle development. Therefore, the next logical step in this thesis is to look specifically at the effect of maternal VDD during pregnancy on offspring skeletal muscle during fetal life.

Vitamin D has been suggested to be an important hormone in regulating muscle structure and function during adult life ((sections 1.4.2, 1.4.3 and 4.1), Sørensen *et al.* 1979; Sato *et al.* 2005; Ceglia *et al.* 2013)). Notwithstanding the findings in Chapter 3, a few lines of evidence suggest that changes in vitamin D levels during pregnancy may alter later life muscle function. In humans, lower maternal blood 25(OH)D concentration at 34 weeks gestation was associated with lower childhood grip strength at 4 years of age (Harvey *et al.* 2014). In a vitamin D receptor (VDR) knock-out mouse model, the offspring at 3 weeks of age had impaired muscle development and a reduction in the expression of transcription factors present during muscle development (Endo *et al.* 2003). However these studies do not focus specifically on muscle development and function during fetal life.

Sheep are a well-established model for studying fetal physiology. Their pregnancies are more comparable to humans than to mice as they have a high rate of singleton births and similar ontogeny of many organs including a full complement of cardiomyocytes and renal glomeruli at term. Fetal blood sampling is also feasible. Sheep and humans, unlike mice, appear to have a third wave of myofibre formation (Wilson *et al.* 1992) and myofibre formation is complete at birth (Ontell & Kozeka 1984), as discussed in section 1.3.1.1.

Previous studies in a sheep model of maternal 50% calorific restriction showed that lower fetal total 25(OH)D plasma concentration was associated with decreased triceps brachii *m.* myofibre density in the late gestation fetus (Costello *et al.* 2008; Cleal *et al.* 2017), however levels of vitamin D were not specifically manipulated. There appears to only be one study looking directly at vitamin D on fetal skeletal muscle in a porcine model, however it is investigating vitamin D supplementation as opposed to a deficiency during pregnancy. They observed that supplementation of 25(OH)D<sub>3</sub> during pregnancy was associated with an increase in fetal pig total myofibre density (Hines *et al.* 2013). Thus, there is a need for further clarification of the effects of VDD on muscle development during fetal life.

Glucose is important for whole-body metabolic function and it is a fundamental source of energy for skeletal muscle growth and contractile function. Skeletal muscle, along with liver and fat, is one of the key tissues influencing whole-body glucose homeostasis. Vitamin D in adult life may be associated with the regulation of glucose metabolism since low circulating 25(OH)D concentrations have been associated with type 2 diabetes in 40-64 year olds (Scragg *et al.* 1995) and 70-88 year old men (Baynes *et al.* 1997), however the mechanism by which they are associated is not well defined. Adult mice of 10-12 weeks old which express a functionally inactive mutant VDR have reduced insulin secretion and a lower glucose tolerance (Zeitz *et al.* 2003). Furthermore, adult 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment altered the expression of insulin receptor substrate-1 (IRS-1) and VDR in adult mouse skeletal muscle tissue (Alkharfy *et al.* 2010), suggesting it may improve insulin sensitivity. Little is known about the association between VDD and glucose handling in fetal life.

In summary, little is known about the effect of maternal VDD during pregnancy and the effect it has on fetal skeletal muscle development, structure and function. This chapter therefore focuses on using a model of gestational VDD to explore the effect of dietary

vitamin D status in the ewe, how it effects fetal vitamin D status and how it impacts fetal skeletal muscle.

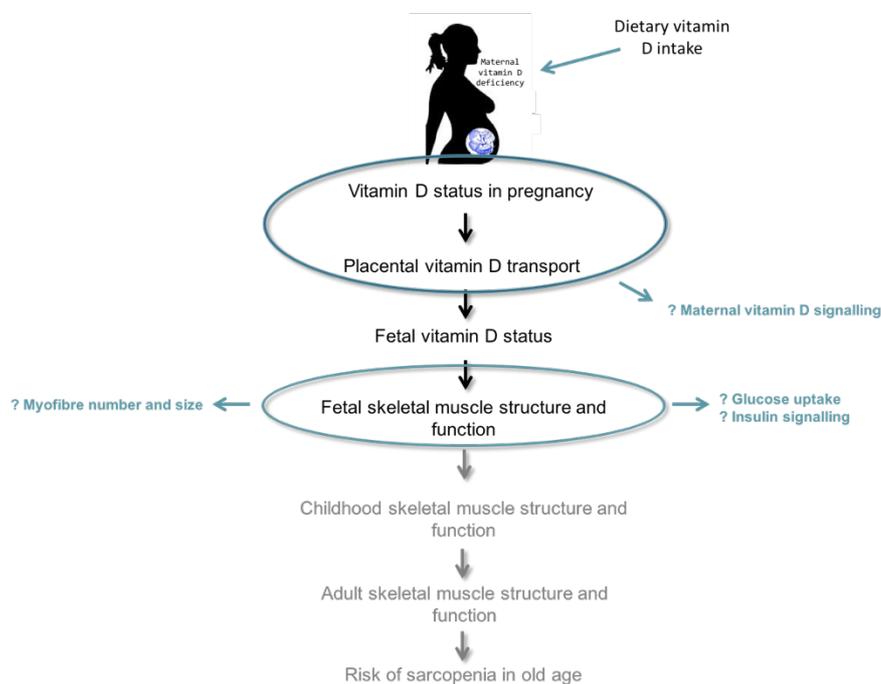


Figure 5.1: Schematic diagram of Chapter 5 flow of logic.

## 5.1.1 Hypothesis and aims

### Hypothesis

The maternal vitamin D deficient diet will decrease skeletal muscle glucose uptake and myofibre density. This effect will be related to insulin-resistance and changes to the expression of genes which code for important components of the insulin signalling pathway.

### Aims

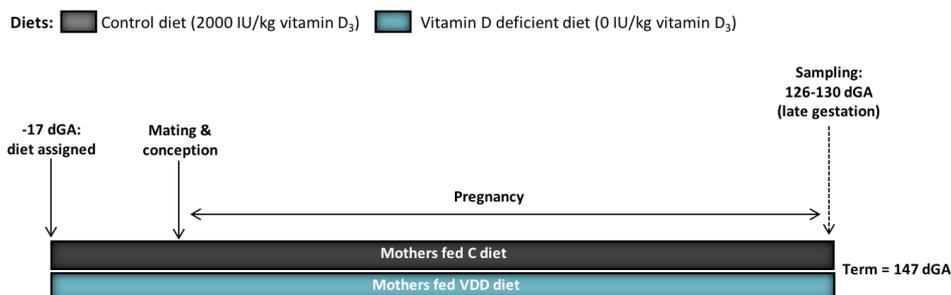
To investigate whether there is an effect of maternal VDD on fetal sheep skeletal muscle by assessing a) glucose uptake, b) myofibre structure and c) mRNA levels of genes associated with glucose metabolism and muscle growth. The secondary aim is to determine how these fetal measurements relate to plasma vitamin D concentrations.

## 5.2 Methods

### 5.2.1 Vitamin D deficient fetal sheep model

First parity pregnant Welsh Mountain ewes and a Welsh Mountain ram of uniform body condition score (BCS; 2.5-3.0) were group housed at The Royal Veterinary College in an open-fronted barn on wheat straw, and fed a diet of hay with supplementary feed. From 24 days prior to conception, ewes were housed in a closed-side barn with high glass side windows without being exposed to direct sunlight, and all ewes were acclimatized onto the control pelleted diet providing 100% of metabolizable energy requirements (2000 IU/kg vitamin D<sub>3</sub>, Appendix C, Charnwood Milling Company Ltd., UK).

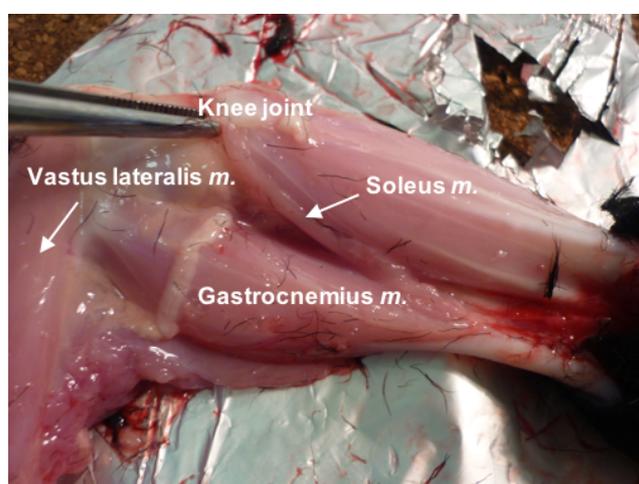
At 17 days prior to mating, ewes were weighed and either remained on the control diet (C, n=9) or without the vitamin D<sub>3</sub> supplement (vitamin D deficient, VDD; n=9; Appendix C, Charnwood Milling Company Ltd., UK) until post-mortem at 126-130 dGA (Fig. 2.2). Water was available *ad libitum*. Feed and straw samples were analysed for vitamin D<sub>2</sub> and D<sub>3</sub> content (Eurofins, UK; Appendix C). Estrous cycles were synchronised by the withdrawal of a vaginal medoxyprogesterone acetate impregnated sponge (Chronogest CR Sponge 25, MSD Animal Health, UK) 2 days prior to mating. The weight and BCS of the ewes were measured throughout pregnancy. Twin-bearing and non-pregnant ewes as confirmed by an ultrasound scan were removed from the study. At 123 days of gestation (dGA) the animals were transported to the University of Southampton Biomedical Research Facility and housed individually in temperature-controlled rooms on a 12-hour light/dark cycle but lamps were not sheathed to block ultraviolet wavelengths.



**Figure 5.2: Vitamin D deficient fetal sheep experimental model.** C, control; VDD, vitamin D deficient; IU, international units; dGA, days of gestation. Fetal offspring: C n=9, VDD n=9.

## 5.2.2 Tissue collection

Prior to post-mortem, food was withdrawn from the ewes overnight. At 126-130 dGA, the ewe and fetus (C fetuses: female n=3, male n=6; VDD fetuses: female n=5, male n=4) were killed by an intravenous overdose of sodium pentobarbitone (145 mg/kg; Animalcare Ltd, UK). Maternal blood samples were obtained from the jugular vein, and fetal blood was collected via cardiac puncture. Blood was collected in Lithium Heparin and serum vacutainer tubes and centrifuged ( $\leq 1300 \times g$  for 10 min at 4 °C). Maternal and fetal blood glucose concentrations were measured by a glucometer (Accu-Chek, UK). Fetal legs were cut off, skinned and the soleus, gastrocnemius and vastus lateralis *m.* were dissected (Fig. 5.3). Left leg mid-belly muscle samples were snap-frozen in liquid nitrogen for further molecular work and slow-frozen in freezing isopentane for histology. Right leg mid-belly samples of the soleus and gastrocnemius were added to ice-cold modified Tyrode's buffer (137 mmol/L NaCl, 5 mmol/L D-glucose, 5 mmol/L KCl, 12 mmol/L NaHCO<sub>3</sub>, 1 mmol/L MgCl<sub>2</sub>, 1.5 mmol/L CaCl<sub>2</sub>, 10 mmol/L HEPES, 2 mmol/L Na Pyruvate and 0.1% bovine serum albumin, pH 7.4; Sigma, USA) for radioactive glucose uptake experiments that day. All snap and slow-frozen muscles were stored at -80 °C.



**Figure 5.3: Anatomical location of the fetal sheep gastrocnemius, soleus and vastus lateralis muscles.**

### 5.2.3 Blood plasma analysis

Ewe and fetal blood plasma were analysed for 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D via high performance LC-MS. An enzymeimmunoassay kit was used to measure 1,25(OH)<sub>2</sub>D and standard assays were used to quantify calcium, albumin, and phosphate concentrations. Serum ionized calcium was determined using an automated analyser and PTH was measured using a chemiluminescent immunoassay. See section 2.5 for details.

### 5.2.4 Skeletal muscle glucose uptake

#### Glucose uptake protocol

Fresh fetal skeletal muscle from the soleus and gastrocnemius *m.* was placed in cold modified Tyrode's buffer (recipe section 5.2.2) at post-mortem, transferred to the lab and cut into small strips (~50 mg). Muscle strips were attached to hooks via silk suture to allow them to be moved between the incubation steps (all at 38 °C in a shaking water bath) as follows: 1) 5 ml Tyrode's buffer (20 min); 2) 5 ml Tyrode's buffer in the absence (basal) or presence of 16 or 160 nM insulin (20 min; Novo Nordisk, UK); 3) the muscle strips were quickly blotted on filter paper to remove any residual insulin, then 4) a final incubation in 3 ml Tyrode's containing 0.75 µCi [<sup>3</sup>H]-2-deoxyglucose (Sell *et al.* 2012) and 0.089 µCi [<sup>14</sup>C] L-glucose to account for extracellular glucose uptake (30 min; PerkinElmer, USA). Muscle strips were shaken in ice-cold Tyrode's buffer for 15 sec to wash off any residual radioactive buffer and to stop further glucose uptake. Strips were blotted on filter paper, weighed, snap-frozen in liquid nitrogen and stored at -80°C. There were numerous steps in trying to optimise the protocol, as detailed in section 5.3.3.1.

In the gastrocnemius *m.* of C diet group only, some investigation of the effect of vitamin D on insulin stimulated glucose uptake was carried out at the second incubation stage (with or without insulin) for. This involved the addition of 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (Bayer HealthCare Pharmaceuticals, Germany) with or without 100 nM VDR antagonist ZK159222 (Bayer HealthCare Pharmaceuticals, Germany).

## **Quantifying glucose uptake**

Following snap freezing, the muscle strips were homogenised in 500  $\mu\text{l}$   $\text{dH}_2\text{O}$  and centrifuged at 10,000 x g for 5 min. Supernatant (300  $\mu\text{l}$ ) was added to glass scintillation vials with 1 ml Optiphase 'Hisafe' II scintillation fluid (PerkinElmer, USA) and the radioactivity of the supernatant for  $^3\text{H}$  and  $^{14}\text{C}$  was determined by liquid scintillation counting for 5 min using channels for simultaneous quantification (Tri-carb 2100TR, PerkinElmer, USA). L-glucose uptake was subtracted from deoxyglucose uptake to account for extracellular uptake.

## **5.2.5 Immunohistochemistry**

### **5.2.5.1 Sectioning of muscle samples**

Slow-frozen sheep fetal muscles were cut through the mid-belly in 10  $\mu\text{m}$  thick transverse sections using a cryostat (Leica Biosystems, Germany) kept at  $-20\text{ }^\circ\text{C}$ . Sections were transferred onto APES covered glass slides, left to air-dry for 30 min and stored at  $-20\text{ }^\circ\text{C}$  for a maximum of two weeks until staining.

### **5.2.5.2 Primary antibody optimisation**

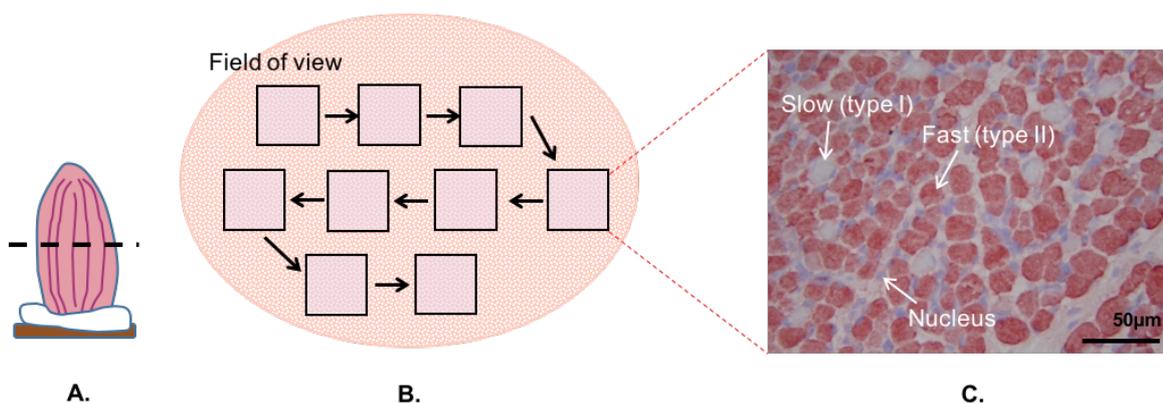
In order to distinguish between fast- and slow-twitch myofibres, the MY32 monoclonal anti-skeletal fast myosin primary antibody was used to positively stain fast-twitch fibres (Sigma, USA). Titration of the MY32 antibody (1:250, 1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:4000, dilutions in tris-buffered saline [TBS]) determined that the optimal antibody dilution with maximal staining strength and minimal background was 1:3000.

### 5.2.5.3 Muscle staining

The cryosections were removed from -20 °C storage, left to air dry for 30 min and fixed in water-free acetone for 15 min at room temperature. Acetone was allowed to evaporate for 10 min and endogenous peroxidase activity was inhibited by incubating the muscle sections with 0.3% hydrogen peroxide in 1% sodium azide solution for 15 min. Sections were rinsed in TBS 3x 5 min. Avidin solution (Vector, UK) was applied to the muscle sections for 20 min and sections were washed in TBS 3x 2 min. Biotin solution (Vector, UK) was then applied for 20 min, rinsed 3x 2 min in TBS. Non-specific protein interactions were blocked with Dulbecco's Modified Eagles Medium (DMEM) containing 20% fetal calf serum and 1% BSA for 20 min. Sections were incubated with MY32 (1:3000 diluted in TBS) for 60 min at room temperature. Muscle sections were washed 3x 5 min in TBS. Sections were incubated with the anti-mouse biotinylated secondary antibody (Vector, UK) for 30 min. Muscle sections were washed in TBS 3x 5 min and treated with avidin biotin-peroxidase complex for 30 min and washed 3x 5 min in TBS again. Sections were treated with 3-Amino-9-ethylcarbazole (Biogenex, USA) for 10 min, rinsed with TBS and washed for 5 min in running tap water. Finally, sections were counterstained with Mayer's haematoxylin (for nucleus visualisation) for 1.5 min and washed in running tap water for 5 min. Slides were baked with aquamount (Add-Serotec, UK) for 20 min before being mounted with pertex (Histolab Products AB, Sweden). All chemicals were from Sigma, USA unless stated otherwise. Fast-twitch fibres were stained red and the slow-twitch fibres remained unstained (pale blue).

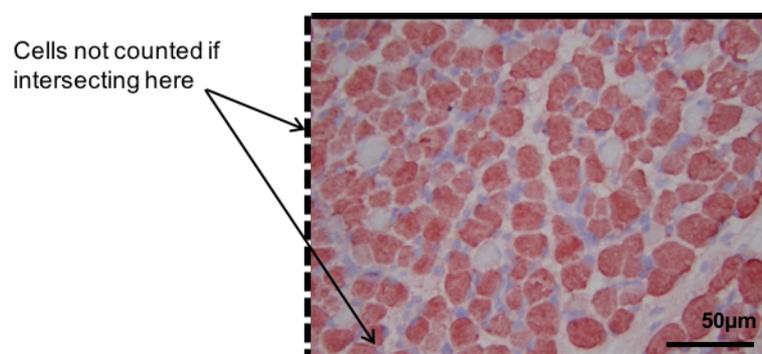
### 5.2.5.4 Image capture and analysis

All images (x40 magnification) were captured using the DMLS microscope (Leica, Germany). Initially, ten images (field of view, FOV) were taken in a snaking fashion across the section to ensure there was no overlap (Fig. 5.4.) and to validate the optimal number of FOVs for this protocol (detailed below). All image file names were anonymised by a third party to prevent bias.



**Figure 5.4: Sample cutting and imaging technique.** A) Black line shows where the muscle was cut at mid-belly. B) Images (field of view) taken in a snaking motion starting at the top left corner and C) example of field of view photograph.

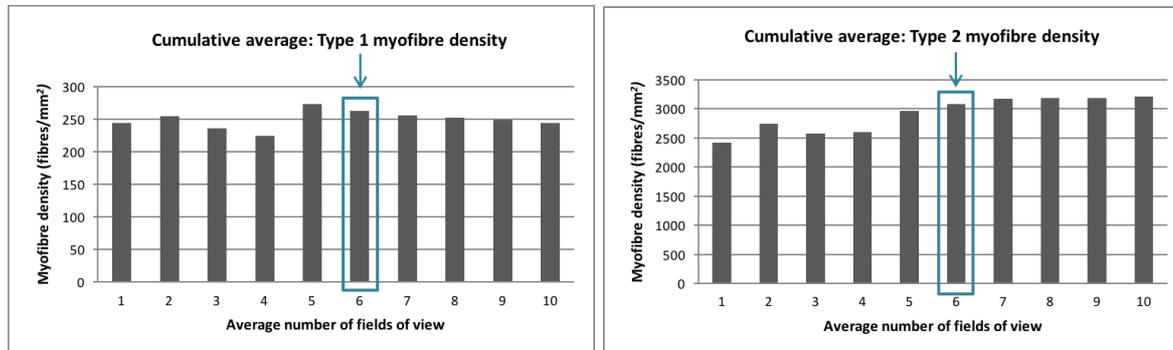
Fiji Image J (<http://fiji.sc/>) was used to quantify the myofibre density of slow-twitch (type I) and fast-twitch (type II) myofibres in each FOV and all images were calibrated using a x40 graticule. In each FOV, the area of total countable myofibres ( $\mu\text{m}^2$ ) was measured by subtracting areas of artefact, large vessels or areas of poor morphology. An inclusion/exclusion criteria was put in place whereby only the myofibres completely in FOV or intersecting the top or right-hand borders (solid line) were counted (Fig. 5.5).



**Figure 5.5: Diagrammatic representation of a field of view.**

Myofibre density was measured as number of myofibres per  $\text{mm}^2$  ‘countable’ area of muscle section tissue. An average density of six FOVs was calculated for each muscle sample. This image analysis procedure was validated by two methods. First, the cumulative average of myofibre density across ten FOVs was calculated (Fig. 5.6). Myofibre density plateaued at about six FOVs and so this was the number of images used for immunohistochemistry myofibre image analysis. Secondly, the coefficient of variation

((COV; (standard deviation/mean)\*100)) was calculated by repeating the density analysis for both type I and type II fibres for six FOVs, on six different occasions. Mean COV for the type I and type II fibres were 1.91% and 1.27%, respectively.



**Figure 5.6: Cumulative average plot of the fetal total myofibre density. A)** Vastus lateralis *m.* type I myofibres and **B)** vastus lateralis type II myofibres.

The cross-sectional area (CSA) of type-II fibres was determined. Fiji ImageJ software randomly selected a quarter of a FOV to analyse and only myofibres completely within the FOV were drawn around to measure myofibre CSA ( $\mu\text{m}^2$ ). The area of a total of 100 myofibres were measured which were selected from the 1st, 3rd and 5th FOVs in order to account for potential differences in morphology across a muscle section.

## 5.2.6 Skeletal muscle mRNA analysis

Full details on mRNA analysis are given in section 2.2. Briefly, snap-frozen skeletal muscles started at  $-80\text{ }^\circ\text{C}$  were crushed, homogenised and total RNA was extracted using TriFast™. Spectrophotometry was used to assess RNA quantity and quality, and gel electrophoresis was used to determine the integrity of the RNA. Unlike Chapter 3, the RNA was not DNase treated. RNA was reverse transcribed into cDNA using the GoScript™ Reverse Transcription System kit (Promega). qRT-PCR was used to quantify the mRNA levels of selected genes (Table 5.1) in each sample and mRNA levels were normalised to the geometric mean of GAPDH and  $\beta$ -Actin housekeeper genes.

**Table 5.1: Primer sequences.** Primer sequences (5' to 3') used to measure mRNA levels of genes of interest by qRT-PCR.

Gene	Primers/probe	Sequence 5' - 3'
InsR	Forward primer Reverse primer Probe	ACC-GCC-AAG-GGC-AAG-AC AGC-ACC-GCT-CCA-CAA-ACT-G AAC-TGC-CCT-GCC-ACT-GTC-ATC-AAC-G
GLUT-4	Forward primer Reverse primer Probe	CCG-TGG-CAG-GAC-ATT-TGA-C TTC-CTG-CTC-CAG-AAG-AGA-AGG-T ATC-TCA-GCC-GTC-TTC-CGC-CGG
IGF1R	Forward primer Reverse primer Probe	CTG-CAG-CGC-CTC-TAA-CTT-TGT CAC-TGG-CCC-AGG-AAT-GTC-A CAA-GAA-CCA-TGC-CTG-CAG-AAG-GAG-CA
GAPDH	Forward primer Reverse primer Probe	CTA-GGC-TAC-ACT-GAG-GAC-CAG-GTT CCC-AGC-ATC-GAA-GGT-AGA-AGA TCT-CCT-GCG-ACT-TCA-ACA-GCG-ACA-CT
$\beta$ -Actin	Forward primer Reverse primer Probe	GAG-GCA-TCC-TGA-CCC-TCA-AG TCT-CCA-TGT-CGT-CCC-AGT-TG CCC-CAT-TGA-GCA-CGG-CAT-TGT-CA

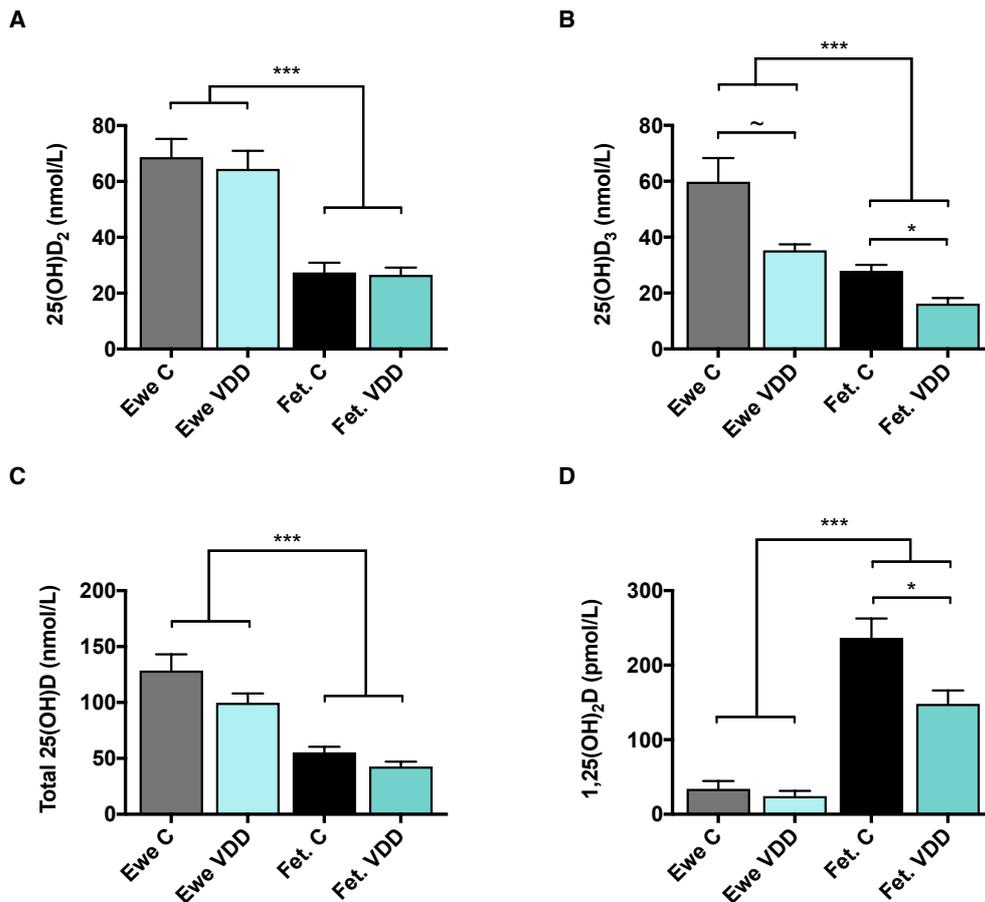
### 5.2.7 Statistical analysis

The fetal measurements were age-adjusted for gestational age at post-mortem prior to statistical analysis using SPSS version 22. All data were plotted and visualised on a histogram and were accepted as normally distributed if the skewness value was between -1 and +1. If the data were not normally distributed the raw values were log-transformed prior to analysis. Data were analysed using an independent t-test, one-way ANOVA and linear regression. Data are displayed as age-adjusted mean  $\pm$  SEM and the data following linear regression are displayed as  $R^2$  and B values. Statistical significance was accepted if  $P < 0.05$ .

## 5.3 Results

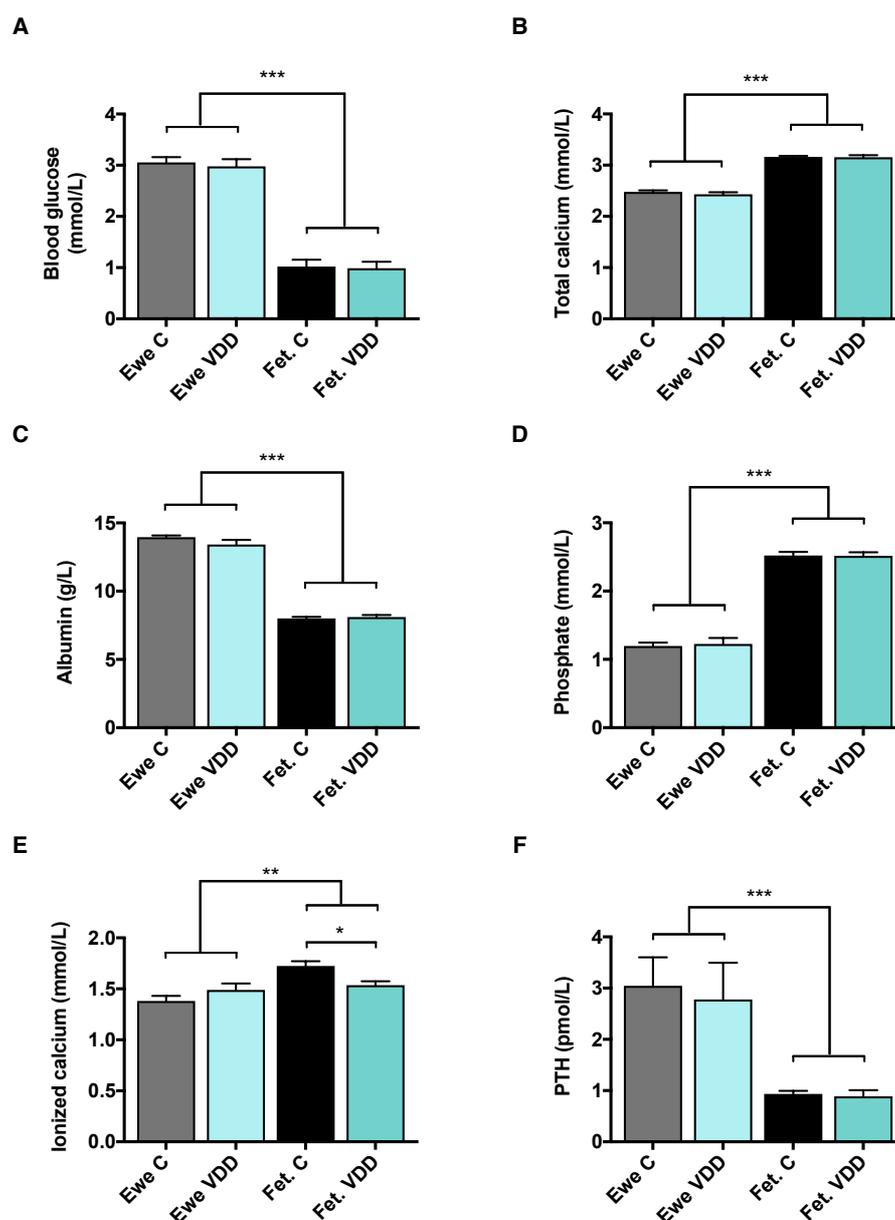
### 5.3.1 Blood analysis

There were no significant differences between VDD and C group ewe plasma 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> (P<0.1, trend only), total 25(OH)D or 1,25(OH)<sub>2</sub>D concentrations (Fig. 5.7). Fetal plasma 25(OH)D<sub>2</sub> and total 25(OH)D concentrations were not different between diet groups, but fetal 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D concentrations were significantly lower in the VDD compared with the C group (P<0.05). When diet groups were combined, plasma 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D concentrations were significantly lower in the fetus compared with the ewe (P<0.001), whereas plasma 1,25(OH)<sub>2</sub>D was significantly higher in the fetuses compared with the ewe (P<0.001).



**Figure 5.7: Ewe and fetal plasma vitamin D concentrations.** A) 25(OH)D<sub>2</sub>, B) 25(OH)D<sub>3</sub>, C) total 25(OH)D and D) 1,25(OH)<sub>2</sub>D fasted blood plasma concentrations in the ewe and fetus. Data presented as age-adjusted mean ± SEM. All data were statistically analysed by an independent t-test. \*\*\*P<0.001, \*P<0.05, ~P<0.1. Ewe C n=9, VDD n=9; fetal C n=9, VDD n=9. C, control; VDD, vitamin D deficient.

There were no differences between VDD and C groups in fasted maternal or fetal blood glucose or plasma total calcium, albumin, phosphate and parathyroid hormone concentration (Fig. 5.8). Ionized calcium concentration was significantly lower ( $P<0.05$ ) in the VDD compared with C group fetuses, but not in the ewe. When both diet groups were combined, blood glucose, albumin and PTH concentrations were significantly lower ( $P<0.001$ ) in the fetus compared with the ewe. Plasma concentrations of total calcium, phosphate and ionized calcium were higher in the fetus compared with the ewe ( $P<0.001$ ,  $P<0.001$  and  $P<0.01$ , respectively).



**Figure 5.8: Ewe and fetal blood plasma analysis.** A) fasted blood glucose (mmol/L) and fasted plasma levels of B) total calcium (mmol/L), C) albumin (g/L), D) phosphate (mmol/L), E) ionized calcium (mmol/L) and F) PTH (pmol/L) in the ewe and fetus. Data presented as age-adjusted mean  $\pm$  SEM. All data were statistically analysed by an independent t-test. \*\*\* $P<0.001$ , \* $P<0.05$ . Ewe C n=9, VDD n=9; fetal C n=9, VDD n=9. C, control; VDD, vitamin D deficient; PTH, parathyroid hormone.

### 5.3.2 Body and muscle data

There were no differences between the C and VDD fetuses in fetal body weight, muscle weights, normalised muscle weights (muscle weight in relation to body weight) or muscle circumferences (Table 5.2).

**Table 5.2: Fetal body and skeletal muscle measurements.** Fetal body weight, soleus and gastrocnemius *m.* weight, and soleus and gastrocnemius *m.* circumference. Data displayed as age-adjusted mean  $\pm$  SEM. All age-adjusted data were statistically analysed by an independent t-test. Statistical significance deemed as  $P < 0.05$ . C n=9, VDD n=9. C, control; VDD, vitamin D deficient; *m.*, muscle.

	C n=9	VDD n=9	P
<b>Fetal body weight (kg)</b>			
	3.04 $\pm$ 0.07	3.00 $\pm$ 0.08	0.67
<b>Fetal muscle weights (g)</b>			
Soleus <i>m.</i>	0.22 $\pm$ 0.02	0.24 $\pm$ 0.02	0.55
Gastrocnemius <i>m.</i>	8.18 $\pm$ 0.22	8.36 $\pm$ 0.41	0.76
<b>Normalised fetal muscle weight (mg muscle weight/g body weight)</b>			
Soleus <i>m.</i>	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.44
Gastrocnemius <i>m.</i>	2.70 $\pm$ 0.07	2.75 $\pm$ 0.22	0.67
<b>Fetal muscle circumference (mm)</b>			
Soleus <i>m.</i>	13.18 $\pm$ 0.44	12.79 $\pm$ 0.53	0.58
Gastrocnemius <i>m.</i>	67.18 $\pm$ 1.14	68.01 $\pm$ 1.75	0.71

There were no significant associations between fetal plasma 25(OH)D<sub>3</sub> concentration and fetal weight, muscle weight or muscle circumference of the soleus or gastrocnemius *m*. (Table 5.3). There were also no associations with fetal total 25(OH)D concentrations (data not shown).

**Table 5.3: Age-adjusted fetal plasma 25(OH)D<sub>3</sub> concentration and fetal measurements.** The association between fetal fasting 25(OH)D<sub>3</sub> concentration and fetal body weight, muscle weights (raw and normalised to body weight) and muscle circumference. Statistical analysis was performed using linear regression. Data are linear regression R<sup>2</sup> and B values from age-adjusted (all data) and log-transformed (as required) data. Statistical significance determined as P<0.05. C n=9; VDD n=9. C, control diet; VDD, vitamin D deficient diet; BW, body weight.

	Age-adjusted fetal plasma 25(OH)D <sub>3</sub>					
	All		C		VDD	
	R <sup>2</sup>	B	R <sup>2</sup>	B	R <sup>2</sup>	B
Age-adjusted fetal weight	0.045	-0.006	0.066	-0.012	0.066	-0.011
Age-adjusted soleus weight (raw)	0.120	-0.005	0.038	-0.003	0.214	-0.010
Age-adjusted soleus weight (normalised to BW)	0.077	-0.004	0.002	-0.001	0.149	-0.008
Age-adjusted gastrocnemius weight (raw)	0.025	-0.001	0.003	0.000	0.122	-0.004
Age-adjusted gastrocnemius weight (normalised to BW)	0.021	0.001	0.168	0.002	0.000	-3.22E <sup>-05</sup>
Age-adjusted soleus circumference	0.000	0.001	0.007	-0.014	0.017	-0.036
Age-adjusted gastrocnemius circumference	0.011	-0.056	0.269	-0.231	0.063	0.229

### 5.3.3 Radioactive glucose uptake

Various optimisations were carried out for the radioactive glucose uptake experiments on fresh fetal sheep muscle tissue. All details are described below.

#### 5.3.3.1 Optimisations

##### 1. *Time course for radiation incubation*

The muscle samples were originally incubated with or without 16 nM insulin for 20 min (Costello *et al.* 2013) and then each muscle type was put under a time course for the incubation supplemented with radiation, either 5, 10 or 15 min. This showed that uptake increased over time, however, no difference was found in insulin-stimulated glucose uptake with increasing time in glucose.

##### 2. *Change insulin concentration*

The next step was to test whether increasing the insulin concentration would increase glucose uptake *vs.* basal. For the soleus and gastrocnemius *m.*, two different concentrations were tested: 16 nM and 160 nM. However, increasing insulin concentration did not alter glucose uptake from basal levels.

##### 3. *Tested quality of [<sup>3</sup>H]-2-deoxyglucose*

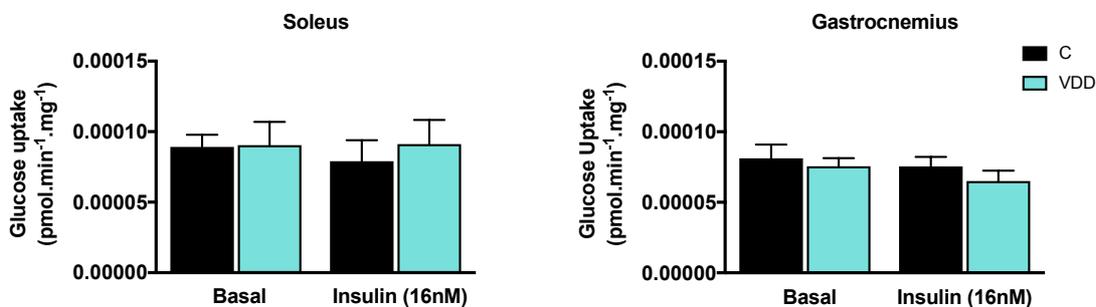
The quality of the [<sup>3</sup>H]-2-deoxyglucose was tested to ensure it was not defective. To do this an extra fetal gastrocnemius *m.* sample was used, which was incubated in [<sup>3</sup>H]-methyl glucose (another marker of cellular glucose uptake) and L-glucose. There was no increased glucose uptake with [<sup>3</sup>H]-methyl glucose compared with [<sup>3</sup>H]-2-deoxyglucose, and so all future experiments continued to use [<sup>3</sup>H]-2-deoxyglucose.

##### 4. *Testing the interference in measurement by L-glucose*

In initial experiments, the muscle strips were blotted with filter paper at the end of the incubations to remove any residual L-glucose from the outside. It was possible that this may not have been sufficient to remove residual L-glucose and that glucose uptake would have continued and potentially confounded the analysis. Therefore, with all further experiments (as described in section 5.2.4) muscle strips were transferred to ice-cold Tyrode's buffer and shaken for 15 sec prior to blotting.

### 5. Longer time course

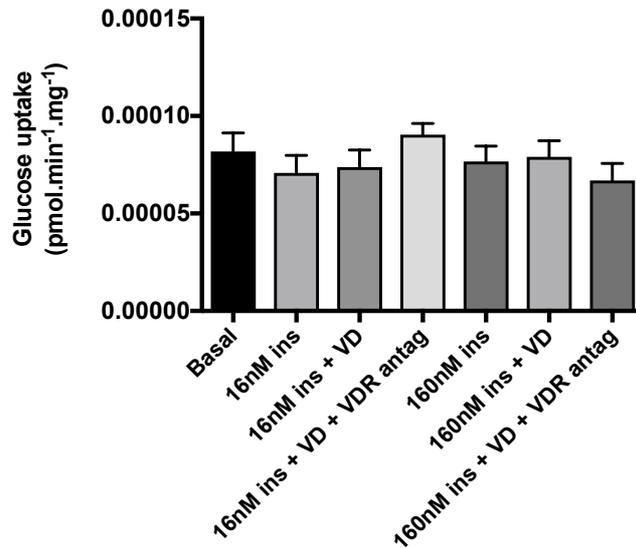
Due to the persistent lack of difference in glucose uptake between basal and insulin conditions, a longer time course of 20, 30 and 40 min in radiation was tested. A shaking water bath was also introduced to the method to ensure thorough mixing of buffer, insulin and glucose. Results showed insulin-stimulated glucose uptake continued to increase over this extended time period, so a 30-minute incubation containing the radiation was chosen for all future experiments. However, there was still no difference in basal compared with insulin-stimulated uptake at this 30-minute incubation time for either diet group in the soleus or gastrocnemius *m.* (Fig. 5.9). There was also no difference between diet groups in the rate of glucose uptake in either muscle.



**Figure 5.9: Basal vs. insulin-mediated glucose uptake.** Radioactive glucose uptake (pmol.min<sup>-1</sup>.mg<sup>-1</sup>) of the soleus and gastrocnemius *m.* in the absence and presence of 16nM insulin. Data displayed as age-adjusted mean  $\pm$  SEM and were statistically analysed by one-way ANOVA. C n=6, VDD n=5. C, control; VDD, vitamin D deficient; *m.*, muscle.

### 5.3.3.2 Adding 1,25(OH)<sub>2</sub>D<sub>3</sub> and a VDR antagonist

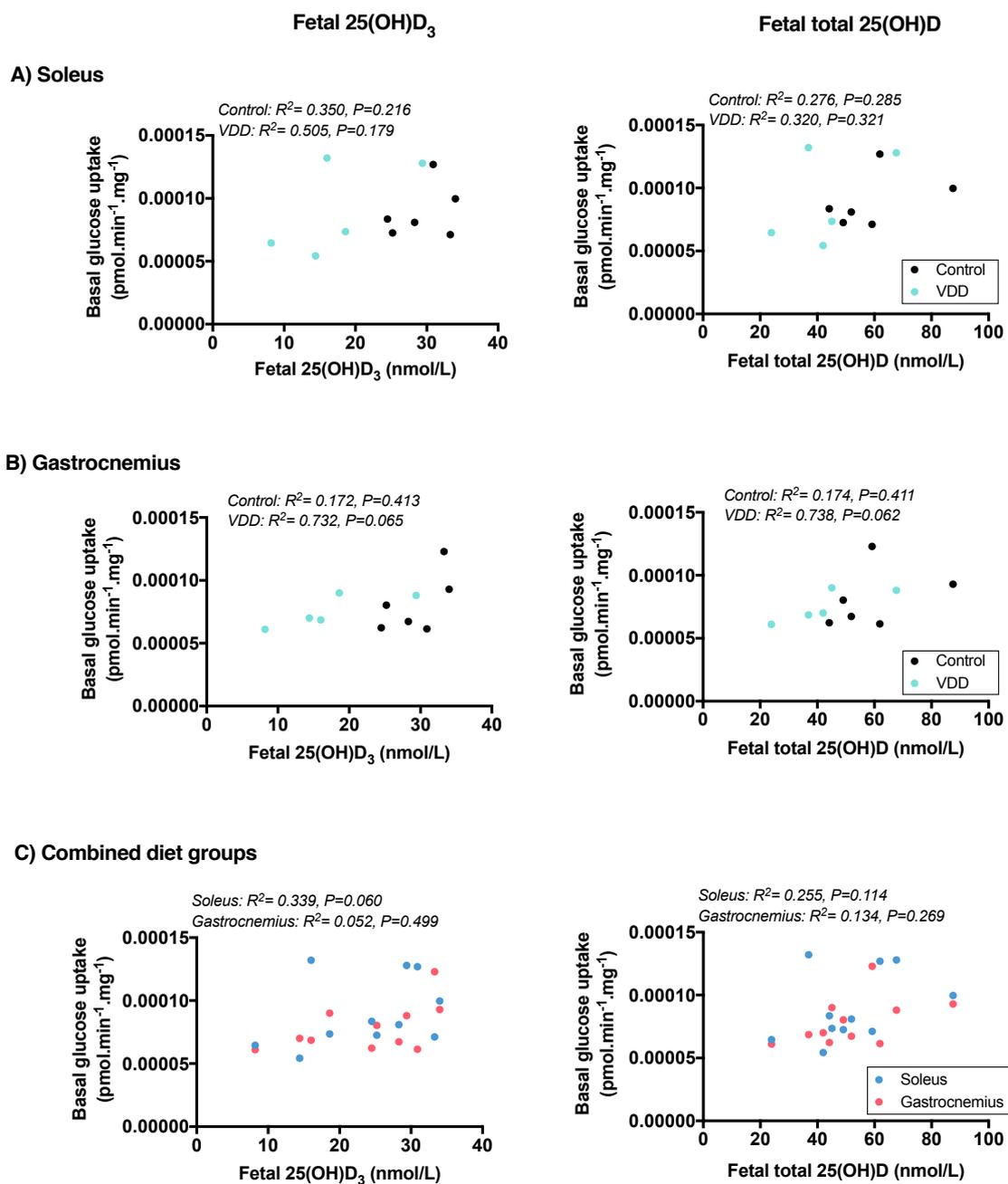
There was no effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> or the VDR antagonist on glucose uptake (Fig. 5.10) of the gastrocnemius *m.* in the presence of insulin compared with basal conditions.



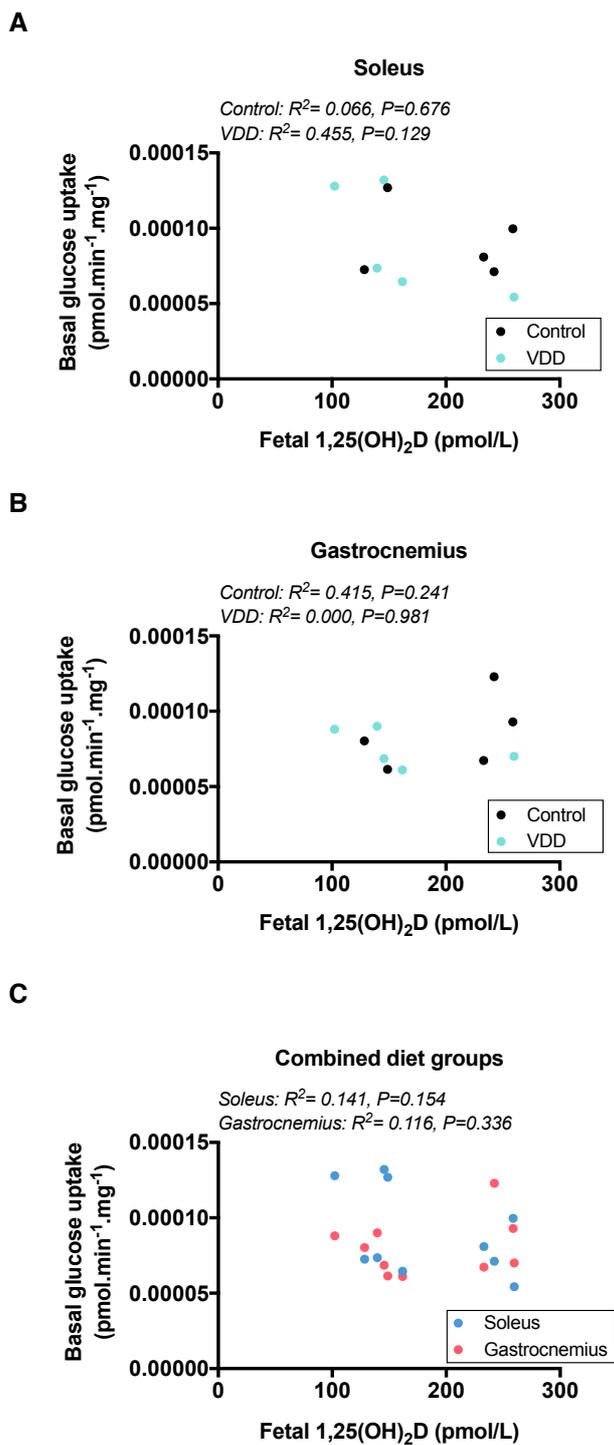
**Figure 5.10: Effect of vitamin D and a vitamin D receptor antagonist on glucose uptake.** Strips of gastrocnemius *m.* were incubated in the presence of insulin, 1,25(OH)<sub>2</sub>D<sub>3</sub> and vitamin D receptor antagonist. Data displayed as age-adjusted mean ± SEM and statistically analysed by one-way ANOVA; n=6. Ins, insulin; VD, 1,25(OH)<sub>2</sub>D<sub>3</sub>; VDR, vitamin D receptor; antagon, antagonist; *m.* muscle.

### 5.3.3.3 Fetal plasma vitamin D concentrations vs. basal glucose uptake

There was no association between fetal plasma 25(OH)D<sub>3</sub> or total 25(OH)D concentration and basal glucose uptake in either soleus or gastrocnemius *m.* for both C and VDD diet group (Fig. 5.11). However, there was a trend for an increased basal glucose uptake in the gastrocnemius *m.* to be associated with increased fetal plasma 25(OH)D<sub>3</sub> ( $R^2=0.73$ ,  $B=9.40e^{-7}$ ,  $P<0.1$ ) and increased fetal plasma total 25(OH)D ( $R^2=0.74$ ,  $B=4.45e^{-7}$ ,  $P<0.1$ ) in the VDD group. In both diet groups combined, there was a trend for an association with fetal plasma 25(OH)D<sub>3</sub> and basal glucose uptake in the soleus *m.* ( $R^2=0.34$ ,  $B=2.27e^{-6}$ ,  $P<0.1$ ; Fig 5.11C), but not in the gastrocnemius *m.* There were no associations between fetal plasma 1,25(OH)<sub>2</sub>D and basal glucose uptake (Fig. 5.12).



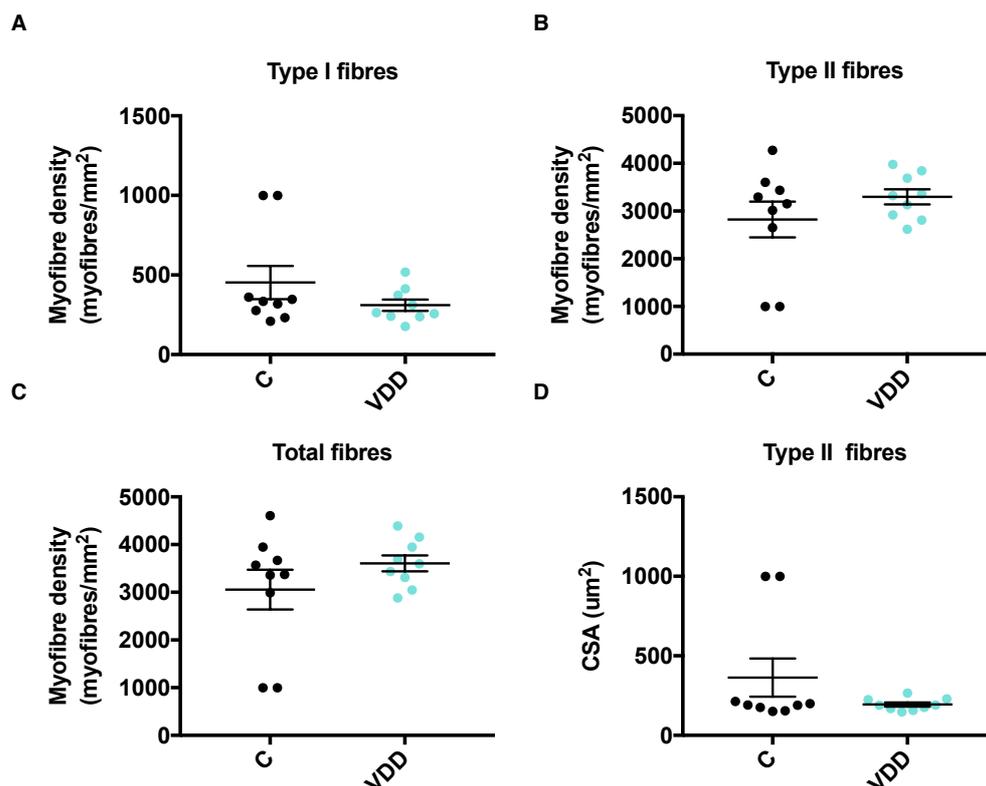
**Figure 5.11: Fetal vitamin D concentration and basal glucose uptake.** Fetal 25(OH)D<sub>3</sub> (left) and total 25(OH)D (right) plasma concentration versus basal glucose uptake for **A)** soleus *m.*, **B)** gastrocnemius *m.* and **C)** both muscles when diet groups were combined. Data represented as individual values of age-adjusted data. Statistical analysis was performed by linear regression. C, control; VDD, vitamin D deficient; *m.* muscle.



**Figure 5.12: Fetal 1,25(OH)<sub>2</sub>D concentration and basal glucose uptake.** Fetal 1,25(OH)<sub>2</sub>D<sub>3</sub> plasma concentration versus basal glucose uptake for **A)** soleus *m.*, **B)** gastrocnemius *m.* and **C)** both muscles when diet groups were combined. Data represented as individual age-adjusted values. Statistical analysis was performed by linear regression. C, control; VDD, vitamin D deficient; *m.* muscle.

### 5.3.4 Myofibre typing

There were no differences between VDD and C groups in fetal total, type I or type II myofibre density or type II myofibre CSA in the fetal vastus lateralis *m.* (Fig. 5.13). There were no associations between myofibre density or CSA with fetal 25(OH)D<sub>3</sub> concentration (Table 5.4), or fetal total 25(OH)D concentration (data not shown).



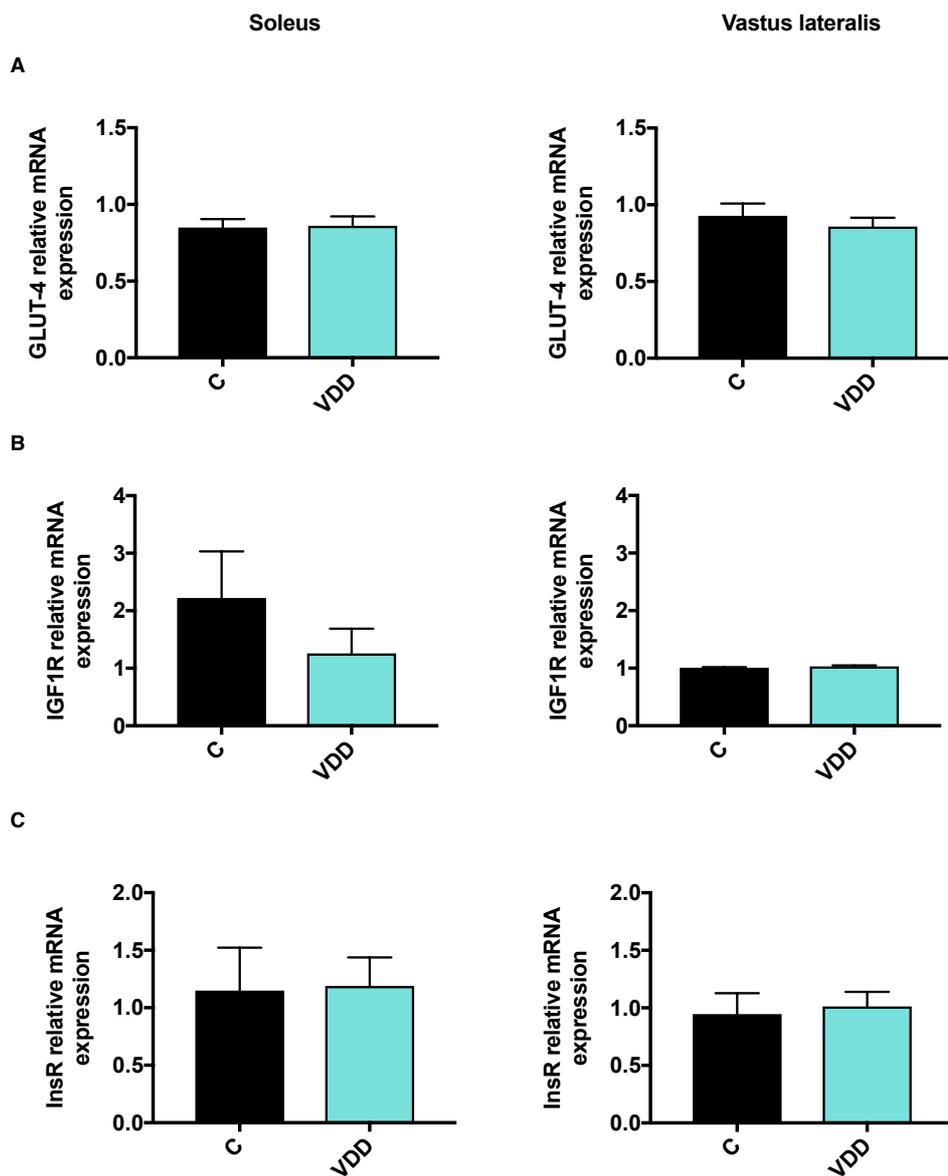
**Figure 5.13: Fetal myofibre density and cross-sectional area in vastus muscle.** Myofibre density (myofibres/mm<sup>2</sup>) of **A)** type I fibres, **B)** type II fibres and **C)** total fibres, and **D)** cross-sectional area (µm<sup>2</sup>) of type II fibres in the fetal vastus lateralis *m.* Data presented as age-adjusted mean ± SEM. All data were statistically analysed by an independent t-test. Statistical significance  $P < 0.05$ . C n=9, VDD n=9. C, control; VDD, vitamin D deficient; CSA, cross-sectional area.

**Table 5.4: Fetal plasma 25(OH)D<sub>3</sub> concentration and myofibre structure.** Association between fetal fasting 25(OH)D<sub>3</sub> concentration and type I density, type II density, total density and type II CSA of fetal vastus *m.* Statistical analysis performed using linear regression. Data are linear regression R<sup>2</sup> and B values from age-adjusted data. Statistical significance determined as P<0.05. C n=9; VDD n=9. C, control diet; VDD, vitamin D deficient diet; CSA, cross-sectional area; *m.*, muscle.

	Fetal plasma 25(OH)D <sub>3</sub>							
	Type I density		Type II density		Total density		Type II CSA	
	R <sup>2</sup>	B	R <sup>2</sup>	B	R <sup>2</sup>	B	R <sup>2</sup>	B
All	0.001	0.355	0.024	9.011	0.024	9.302	0.136	-1.451
C	0.139	-3.077	0.072	18.870	0.047	15.348	0.071	-0.762
VDD	0.102	5.857	0.005	30.719	0.019	11.912	0.111	-2.245

### 5.3.5 Gene expression

There was no difference between VDD and C group mRNA levels of GLUT-4, IGF1R or InsR in the fetal soleus and vastus lateralis *m.* (Fig. 5.14). Changes in fetal 25(OH)D<sub>3</sub> concentration were not associated with changes in the mRNA expression of GLUT-4, IGF1R or InsR (Table 5.5), and neither were fetal total 25(OH)D concentrations (data not shown).



**Figure 5.14: Fetal soleus and vastus lateralis muscle insulin-signalling pathway mRNA expression.** A) GLUT-4, B) IGF1R and C) InsR mRNA expression in the fetal soleus (left) and vastus lateralis *m.* (right). Data presented as age-adjusted mean  $\pm$  SEM and were log transformed prior to statistical analysis. All data were statistically analysed by an independent *t*-test. Statistical significance  $P < 0.05$ . C n=9, VDD n=9. C, control; VDD, vitamin D deficient; GLUT, glucose transporter; IGF1R, insulin-like growth factor 1 receptor; InsR, insulin receptor; mRNA, messenger ribonucleic acid.

**Table 5.5: Fetal plasma 25(OH)D<sub>3</sub> concentration and skeletal muscle insulin-signalling mRNA expression.**

Association between fetal fasting 25(OH)D<sub>3</sub> concentration and the mRNA expression of GLUT-4, IGF1R and InsR in the fetal soleus and vastus lateralis *m*. Statistical analysis was performed using linear regression. Data are linear regression R<sup>2</sup> and B values from age-adjusted and log-transformed data. Statistical significance determined as P<0.05. C n=9; VDD n=9. C, control diet; VDD, vitamin D deficient diet; *m*., muscle; GLUT, glucose transporter; IGF1R, insulin-like growth factor 1 receptor; InsR, insulin receptor; mRNA, messenger ribonucleic acid.

	Fetal plasma 25(OH)D <sub>3</sub>					
	Soleus GLUT-4		Soleus IGF1R		Soleus InsR	
	R <sup>2</sup>	B	R <sup>2</sup>	B	R <sup>2</sup>	B
All	0.001	0.000	0.096	-0.018	0.019	0.005
C	0.024	0.002	0.428	-0.043	0.047	0.011
VDD	0.009	0.002	0.239	-0.036	0.054	0.010
	Vastus GLUT-4		Vastus IGF1R		Vastus InsR	
	R <sup>2</sup>	B	R <sup>2</sup>	B	R <sup>2</sup>	B
	All	0.013	0.001	0.056	-0.001	0.077
C	0.005	0.001	0.110	0.001	0.203	0.013
VDD	0.269	0.006	0.180	-0.001	0.081	0.008

## 5.4 Discussion

### 5.4.1 A model of fetal vitamin D deficiency

In this model, feeding ewes a diet without any supplementary vitamin D<sub>3</sub> (0 IU/kg, VDD diet group) caused a very small decrease in fetal plasma 25(OH)D<sub>3</sub> concentration and only a trend for a reduction in the ewes. It shows that the diet was having an effect and indeed analysis of the feed (post-production) revealed that vitamin D<sub>3</sub> was below the limit of quantification (<100 IU/kg; Appendix C). This model however does contrast with the Chapter 4 mouse model which had a harder ‘hit’ of gestational vitamin D<sub>3</sub> deficiency. The reduction in plasma 25(OH)D<sub>3</sub> concentration in the VDD group may not have been as significant in this current study due to vitamin D<sub>3</sub> coming from other sources. Although exposure to sunlight was controlled for in the barns in which they were housed, UVB light filters were not fitted, and so vitamin D synthesis as a result of UVB exposure may have been a confounder.

Plasma 25(OH)D<sub>2</sub> concentration was not affected by the VDD diet, which is not surprising considering the experimental diet manipulated only supplementary vitamin D<sub>3</sub>. However, concentrations in the sheep were much more detectable than in humans and mice. This is most likely due to the ergosterol in fungi of plant matter, which when exposed to UVB synthesises vitamin D<sub>2</sub> (Jäpelt & Jakobsen 2013). As a result, the greater vitamin D<sub>2</sub> levels may have come from the soya or grass meal that was present in both diets in the same quantities, and so this needs to be taken into consideration for future studies.

The higher concentration of 25(OH)D (overall) in the mother compared to the fetus is similar to that in human (Salle *et al.* 2000) and previous sheep studies (Cleal *et al.* 2017), and is consistent with research suggesting that 25(OH)D is able to readily cross the placenta (Kovacs 2012). Total 1,25(OH)<sub>2</sub>D concentration was reduced in the VDD fetal group and was higher in fetal versus maternal plasma. The higher fetal 1,25(OH)<sub>2</sub>D concentration in the sheep is thought to be due to an increased production rate and clearance, allowing for rapid fetal growth (Ross *et al.* 1989) associated with the stimulation of calcium absorption.

## 5.4.2 Fetal skeletal muscle size and structure

There were no differences in fetal muscle weight or circumference between the C and VDD diet groups. Assuming there would be a link between vitamin D and muscle during fetal life, perhaps this was not surprising given the lack of a significant difference in total 25(OH)D between diet groups. The lack of association between fetal 25(OH)D<sub>3</sub> and fetal muscle weights and circumferences would appear to suggest it does not alter muscle morphology in the late gestation fetus. In addition, there were no differences in fetal vastus lateralis *m.* type I, type II or total myofibre density and no difference in type II myofibre CSA with a prenatal VDD diet. There were also no associations with fetal 25(OH)D<sub>3</sub> or total 25(OH)D concentrations and these parameters, suggesting 25(OH)D does not affect myofibre structure in the fetal sheep.

There are no previous studies looking at VDD on fetal skeletal muscle weight or circumference, but these results do contradict other studies looking at vitamin D and myofibre structure. A rat model of pregnancy VDD found that a maternal VDD diet was associated with smaller myofibre size in the newborn offspring (Max *et al.* 2014). One study found that 25(OH)D<sub>3</sub> supplementation increased porcine fetal total muscle fibre number and Pax7+ myoblasts, indicative of prolonged proliferation (Hines *et al.* 2013). Although not in a model of VDD, a lower fetal plasma total 25(OH)D concentration was associated with a lower triceps brachii *m.* myofibre density in a sheep model of gestational nutrient restriction (Cleal *et al.* 2017), and interestingly the range of fetal total 25(OH)D concentration was very similar to the current study, in which no changes to myofibre structure were observed. These previous studies suggest that vitamin D is important for fetal muscle growth. These contrasting findings in relation to this thesis could partially be due to the different species used and different muscle beds investigated. Type II myofibre CSA did not change with diet group in this current study, which is similar to another fetal sheep study, although focussing on undernutrition (Costello *et al.* 2008). They found that the myofibre CSA of the fetal sheep triceps brachii and soleus *m.* did not change with undernutrition during pregnancy. As previously described, a reason why a changes to fetal myofibres were not observed in this thesis may have been due to the levels of vitamin D<sub>2</sub> in the diet not being manipulated as well as vitamin D<sub>3</sub>, and meaning that the ewe and fetal total 25(OH)D plasma concentration was unaltered in the VDD group compared with C (see section 5.4.1).

It is also important to note that the offspring samples in this chapter are from late gestation fetuses 12-21 days before birth compared to the work by Hines *et al.* and Max *et al.* observing changes to myofibre structure in the newborn. Sheep myofibre development occurs in stages as mentioned in section 1.3.1. A study looking at the sheep tibialis cranialis *m.* observed that primary myotubes were identified 32-38 dGA, secondary myotubes were present from 38 dGA and a third wave of myofibre formation from 62 dGA (Wilson *et al.* 1992). They stopped observations at 72 dGA at which stage the tibialis cranialis *m.* was still made up of immature secondary myofibres. This indicates that muscle development was still occurring into the later stages of pregnancy. It may therefore be possible that studying fetuses before term rather than at birth may not fully reflect how 25(OH)D<sub>3</sub> influences myofibre number and size in fetal life.

### 5.4.3 Fetal skeletal muscle glucose uptake

Basal glucose uptake increased over incubation time as expected. However there was no difference between basal and insulin-stimulated glucose uptake into the fetal muscle samples, nor was there a difference between diet groups. There are no other *ex-vivo* fetal studies looking at radiolabelled glucose uptake into skeletal muscle, but there are numerous studies using adult skeletal muscle. These adult studies have found an increase in skeletal muscle radiolabelled glucose uptake in the presence of insulin (Ozanne *et al.* 1996; Costello *et al.* 2013). A reason for this difference in findings might be the way in which glucose is handled in the body of a late gestation fetus compared to an adult. Skeletal muscle expresses two members of the facilitated glucose transporters (GLUTs) as detailed in section 1.3.3. In adult skeletal muscle under basal conditions, GLUT-1 (insulin-independent) is expressed in the sarcolemma whilst GLUT-4 (insulin-stimulated) is sequestered in intracellular compartments. In the presence of insulin, GLUT-4 is translocated to the sarcolemma allowing for increased glucose uptake (Wang *et al.* 1996). GLUT-1 expression is greater in the fetus compared to the adult (He *et al.* 2003), and during fetal life GLUT-1 is the main contributor for maintaining glucose uptake in skeletal muscle (Aldoretta *et al.* 1998). Therefore, GLUT-1 may be most accountable for fetal glucose uptake in muscles examined in this chapter, and in view of its insulin insensitivity may explain why there was no effect of insulin on glucose uptake. In addition, GLUT-4

translocation is lower in the fetus/newborn compared to adults (He *et al.* 2003), perhaps another reason why there was no increase in glucose uptake with insulin.

In this chapter, the length of time the muscle was incubated in radiolabelled glucose was 30 min, and although this was the same as another study (Pittner *et al.* 1995) and other studies used a shorter incubation time (Ozanne *et al.* 1996; Costello *et al.* 2013), they were all based on adult tissue. It might be that fetal muscle is more insensitive to its environment and that a longer incubation is needed. In contrast to my findings, *in vivo* fetal sheep studies have found an increase in fetal whole body glucose uptake (Hay & Meznarich 1986) and hind limb skeletal muscle glucose utilisation in the presence of insulin (Anderson *et al.* 2001), albeit at a lower level compared to the adult. Anderson *et al.* found that GLUT-4 protein expression did increase with insulin in fetal sheep muscle, with a peak at 2.5 hours of hyperinsulinemia, and this was associated with an increase in glucose utilisation rate (Anderson *et al.* 2001). These data suggest that the fetal muscle is relatively insensitive to insulin (vs. adult) and needs more time to respond, therefore the time period used in this current study may have been too acute, and a 1 hour incubation (for example) as used in the *in vivo* experiment by Anderson *et al.* may have been more appropriate.

There were no significant associations between fetal vitamin D levels and basal glucose uptake. These are novel observations and even in adult life there is a paucity of knowledge on linkages between vitamin D status and muscle glucose uptake. One study in adult mice showed that 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment altered IRS-1 (insulin receptor substrate) expression in mouse skeletal muscle tissue (Alkharfy *et al.* 2010) which is involved in insulin signalling and consequently glucose uptake. If, as this work suggests, vitamin D mainly regulates glucose uptake via insulin signalling pathways, it may explain why basal (non insulin-mediated) glucose uptake did not change with increased or decreased vitamin D in the fetal sheep.

## 5.4.4 Gene expression

### 5.4.4.1 Muscle growth

There were no differences in IGF1R (insulin-like growth factor 1 receptor) mRNA levels in the fetal sheep soleus or vastus lateralis *m.* with a maternal VDD diet, and there were no associations with fetal plasma 25(OH)D<sub>3</sub> concentration. There are no previous studies looking into the effect of specific vitamin D deficiency during pregnancy on fetal muscle IGF1R expression. The current data suggest that vitamin D may not play a role in IGF-I related muscle development and are consistent with the lack of association between fetal vitamin D status and myofibre density and CSA analysis. IGF1R is a marker of muscle growth and it acts through the binding of IGF-1 to elicit myofibre proliferation and hypertrophy, and previous studies in sheep have shown that during fetal life undernutrition, IGF-1 peptide and its receptor are linked to muscle size and myofibre composition (Osgerby *et al.* 2002; Costello *et al.* 2008). However, considering that the promoter region of IGF binding protein (which regulate the action of IGF-I) contains a vitamin D response element (VDRE), it would suggest that if our model was more profoundly deficient in vitamin D, then there may be more changes to the gene expression of proteins important for muscle growth.

### 5.4.4.2 Insulin-signalling pathway

There was no effect of GLUT-4 (glucose transporter 4) or InsR (insulin receptor) mRNA levels in the soleus or vastus lateralis *m.* of fetuses from the VDD mothers, and there was no association between these genes and fetal 25(OH)D<sub>3</sub> plasma concentration.

There are no known previous studies looking at the impact VDD has on fetal InsR expression. In the sheep model of gestational undernutrition, Costello *et al.* reported a decrease in triceps brachii *m.* InsR mRNA level in the late gestation fetus as well as a reduction in myofibre density (Costello *et al.* 2008). The increase in InsR mRNA levels could be a compensatory mechanism to increase glucose tolerance when myofibre density is reduced. A previous study found that 3-month mouse offspring from mothers fed a VDD

diet had reduced IRS-1 protein expression in the pancreatic islet cells (Maia-Ceciliano *et al.* 2016). Furthermore, a study using U-937 human promonocytic cells found  $1,25(\text{OH})_2\text{D}_3$  increased transcription of the InsR gene (Maestro *et al.* 2000). These suggest that there is a link between vitamin D and InsR expression, and therefore insulin-mediated glucose transport. However, further investigation within skeletal muscle is required, and although data suggests that the fetus is relatively insulin insensitive and skeletal muscle glucose uptake was not altered in the VDD compared with C offspring, there may be alterations in InsR gene or protein expression.

GLUT-4 is the predominant glucose transporter expressed in skeletal muscle (section 1.3.3). We may not have seen a difference in fetal GLUT-4 mRNA levels in response to a maternal VDD diet as GLUT-1 has a greater role in glucose uptake compared with GLUT-4 in fetal skeletal muscle as previous explained in section 5.4.3. Although there are no previous studies looking into the effect of maternal VDD on fetal muscle GLUT-4 expression, one study reported a decrease in rat myofibre GLUT-4 mRNA expression when cultured in a high-glucose environment which increased with a 24-hour treatment of  $1,25(\text{OH})_2\text{D}_3$  (Tamilselvan *et al.* 2013). It would therefore be interesting to explore this relationship further, and also assess GLUT-1 expression in a model with a greater severity of VDD.

### 5.4.5 Conclusion

There were no significant changes in fetal muscle size, glucose uptake, myofibre structure of mRNA levels of genes important in muscle growth and insulin signalling in response to a small reduction in fetal plasma  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  concentrations. However, this model was not an ideal model of pregnancy VDD as the diet had high levels of vitamin  $\text{D}_2$ , and therefore resulting in total  $25(\text{OH})\text{D}$  plasma concentration not being reduced in the VDD ewes or fetuses. More investigation is required into how a VDD diet during pregnancy can impact the fetal skeletal muscle structure, development and function, but a model to ensure  $25(\text{OH})\text{D}_2$  levels are also low to produce a greater VDD challenge is required. In addition, analysing the myofibre structure, muscle mRNA expression of genes involved in myogenesis, muscle growth, muscle contractile function and insulin-mediated

glucose uptake would be very informative in helping us to understand the role of VDD in early life on muscle and metabolic-related disease in adulthood.

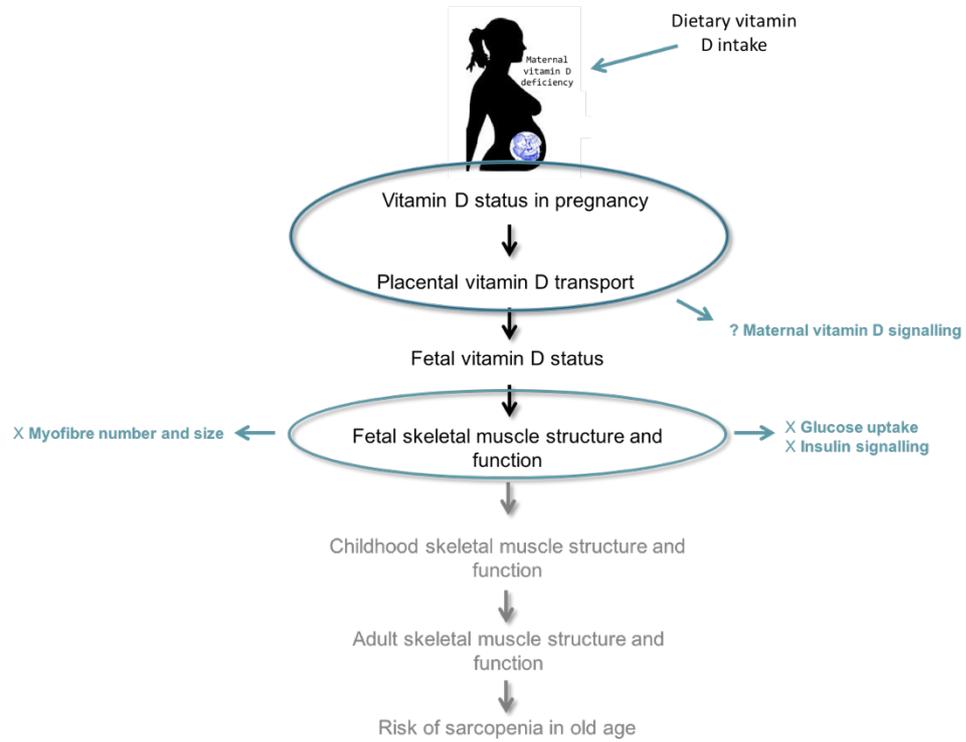


Figure 5.15: Schematic diagram showing thoughts after Chapter 5 results.

## Chapter 6: General discussion

### 6.1 Overview of findings

In Chapter 3, an established mouse model of obesity as a result of high-fat (HF) feeding was used to exploit the known association between obesity and vitamin D deficiency (VDD). A reduction in isometric contraction peak force (PF) was following a post-weaning (POST) and also a combined prenatal (PRE) and POST HF diet, and this was only observed in the soleus *m.* and not the EDL *m.* In these diet groups of both male and female offspring, the relative muscle mass was lower and the total myofibre and type I myofibre density of the soleus *m.* was reduced compared with control. The time-to-peak tension (TPT) and half-relaxation time (HRT) increased or remained unchanged despite lower PF in the female and male soleus *m.* and both parameters increased despite no change to PF in the male EDL *m.*, all indicative of muscle fatigue. Some differences were observed between males and females; intramuscular lipid infiltration in the soleus *m.* only was higher in the male but were unchanged in the female muscle. As the main differences were found in the soleus *m.* (a postural muscle), it may help to explain why obese individuals have an increased propensity to develop sarcopenia in older age and have an increased risk of falls. A PRE HF diet alone elicited some effects on the offspring's skeletal muscle. There was a reduction in male tetanic contractile PF, a slower contractile speed and consequently potential fatigue. In relation to the central hypothesis of this thesis, a key observation was that adult offspring plasma 25(OH)D<sub>3</sub> was increased in the POST and PRE/POST diet groups, but a PRE HF diet was associated with a reduction in 25(OH)D<sub>3</sub> plasma concentration. This suggested that a PRE HF diet alters the long-term homeostasis of vitamin D which could influence muscle function in later life.

In Chapter 4, a mouse model of VDD during pregnancy was successfully established as a result of specifically removing dietary vitamin D<sub>3</sub> during pregnancy and lactation, leading to reductions in late gestation maternal total 25(OH)D and 25(OH)D<sub>3</sub> plasma concentrations. This PRE VDD diet did not cause any impairments to isometric skeletal muscle contractile PF, *in vivo* strength test scores or grip strength of the front and hind limbs in 15-week (young adult) mouse offspring. These data suggest that maternal VDD does not directly impact the function of skeletal muscle, but there were reductions in open-field activity which were associated with lower maternal 25(OH)D<sub>3</sub>. These effects could be

a result of more neurological effects as opposed to skeletal muscle specific as studies have found early life VDD to impair the offspring's cortical thickness (Eyles *et al.* 2003) and therefore potentially the motor cortex, hippocampal neurogenesis (Keilhoff *et al.* 2010) and dopamine signalling (Kesby *et al.* 2009), as discussed in section 4.4.3. Despite the lack of effect on muscle function, the maternal VDD diet was associated with a reduced male soleus *m.* weight and a reduced muscle weight relative to body weight for the female EDL *m.*, which could indicate structural changes to the muscle fibres.

In Chapter 5, vitamin D content of maternal diet was manipulated directly by exclusion of the standard vitamin D<sub>3</sub> supplement from the pelleted feed formulation given to the VDD group. In order to determine the effect of a VDD diet on skeletal muscle development and potential function, an earlier time point (late-gestation fetus) was studied. There was a reduction in fetal plasma 25(OH)D<sub>3</sub> in the VDD diet group compared to C fetuses, however there were no changes in fetal plasma 25(OH)D<sub>2</sub> or total 25(OH)D concentrations. The 'hit' of VDD was not hard enough as the fetal plasma 25(OH)D<sub>2</sub> concentrations remained high, and following feed post-production analysis, vitamin D<sub>2</sub> levels were very similar in both pelleted diets (Appendix C). There were no significant changes in fetal skeletal muscle basal glucose uptake, fetal weight, fetal muscle weight, fetal muscle circumference, myofibre structure or mRNA levels associated with muscle growth or insulin-mediated glucose uptake between control and VDD diet groups. The fetal 25(OH)D<sub>3</sub> plasma concentration ranged from 8.2 to 36.6 nmol/L (across all groups) but further analysis revealed no association of fetal plasma 25(OH)D<sub>3</sub> with any of the aforementioned muscle measurements. These data, albeit in a mild vitamin D<sub>3</sub> deficient sheep model, do not support a relationship between vitamin D status and muscle structure or muscle function, as assessed by glucose uptake, in fetal life.

The cross-cutting themes from sections 6.3, 6.4, 6.5, and the strengths, limitations and future direction of work are discussed in the following sections.

## 6.2 Suitability of the animal model and nutritional challenges

### 6.2.1 Animal model

Mouse models were used in both chapters 3 and 4 due to their shorter pregnancies compared to humans and sheep. A shorter gestation period and shorter lifespan means that investigating the effects across the life course is more easily achievable. In order to quantify muscle PF and contractile times, which were the primary functional outcome measurement in these two chapters, only small animal muscles could be used with the isometric skeletal muscle contraction set-up. As a result, a mouse model was a very appropriate choice of animal in order to assess muscle function in later life in response to nutritional challenges. On the other hand, the small size of a mouse can make tissues are harder to dissect and mean that only small volumes of blood can be obtained. There are also some drawbacks to using rodents as a model for human development since, unlike humans, rodents always give birth to multiple offspring and nutrient delivery and growth profile is not equivalent in singleton versus multiple offspring and hence between the two species. Muscle fibre formation is thought to be complete in early postnatal life in the rodent (Ontell *et al.* 1993) whereas in humans and most other mammals it is set at birth (Brameld *et al.* 1998). In both chapters 3 and 4, the soleus and EDL *m.* were sampled. The mouse soleus *m.* is mainly made up of oxidative fibres with a high percentage of type I slow-twitch fibres and its function is to maintain posture, whereas the mouse EDL *m.* consists of fast-twitch fibres and its important for extension of the toes and dorsiflexion of the foot for movements such as walking (Augusto *et al.* 2004; Bloemberg & Quadri 2012). Choosing these two muscles based on their differences in function and myofibre composition was a strength of this thesis as it enabled a more in depth understanding of how the two diets affect different muscle beds.

A sheep model was used in Chapter 5 to assess the skeletal muscle structure and function in fetal life as they are a well-established model of fetal developmental physiology. Their pregnancies are longer, making it more relevant to a human pregnancy than using a mouse model. Welsh Mountain sheep were chosen for this study as they are more likely to have singleton pregnancies rather than twins. This is important as it is believed that these two types of pregnancies have different fetal growth patterns due to the differential metabolic demands (Blickstein 2005). The effect of suboptimal nutritional challenges investigated in this thesis (in particular VDD) on the fetus would therefore be more severe with twins or

multiple pups and this is why only singleton births were included in this thesis. Parity of the ewe enrolled on the study was controlled (only first parity used) in this Chapter as differences in size at birth between the first born and subsequent offspring have been reported (Ong *et al.* 2002). The large size of the fetal sheep is a huge benefit as it means tissue collection is much easier and a sufficient amount of blood can be obtained from the fetus which is not possible in rodent models. This allows for more extensive blood plasma analysis to be performed. As an example, in Chapter 5, 1,25(OH)<sub>2</sub>D was quantified but the small volume of blood extracted from the mouse offspring in Chapter 3 and Chapter 4 meant that this was not possible. Due to the large muscle size, fetal contractile PF could not be quantified in the set up available for this thesis, and so other measurements of muscle function were assessed. Sheep are a good model of human skeletal muscle development since like humans (but unlike rodents) they have a third wave of myogenesis which is thought to contribute to a larger muscle mass and is completed during prenatal life (Draeger *et al.* 1987; Maier *et al.* 1992; Wilson *et al.* 1992). Furthermore, similar to the two mouse models as detailed above, the different skeletal muscles chosen to be sampled were a strength of this chapter. In the sheep, the soleus *m.* consists entirely of slow-twitch type I fibres and the gastrocnemius and vastus lateralis *m.* are made up of a mixture of slow- and fast-twitch fibres (Konno & Watanabe 2012). Together with their differences in myofibre composition and function (see section 1.3.1.3), it allowed for very different fetal muscle beds to be analysed. It is important to note that sheep digestion is profoundly different to humans. Sheep are ruminants and digestion occurs in two steps, and carbohydrates are fermented in the rumen meaning very little glucose is obtained from their foods. As a result, sheep rely on gluconeogenesis to produce glucose, and so there are differences in glucose metabolism (Leng 1970).

### **6.2.2 Nutritional challenge**

In both mouse models (Chapter 3 and 4) the nutrient challenge was given to the mothers 6 weeks prior to mating, throughout pregnancy (18-22 days) and during lactation (3 weeks), before the offspring were weaned onto their specific diet throughout postnatal life. As explained in section 1.6, VDD is associated with obesity and the reason for using an established gestational HF-fed obesity model in Chapter 3. Previous data suggest that the dams fed a 45% fat (predominantly from lard) diet have increased body weight and reduced glucose tolerance (Thomas Ph.D Thesis 2017). This model therefore reflects the

common features associated with obesity during pregnancy of increased maternal adiposity and excess nutrient intake. Although this model does induce obesity, there were not as many PRE HF effects on offspring skeletal muscle function as anticipated. Other studies have used obesogenic models to investigate the impact on skeletal muscle, but these are mainly based on diets high in sugar (20-40% sucrose vs. 10.5% sucrose in the present study) as opposed to mainly high in fat (Bayol *et al.* 2005; Bayol *et al.* 2009; Latouche *et al.* 2014; Cuthbert *et al.* 2017). It could be that an obesogenic diet containing higher fat and carbohydrate combined is more of a reflection of the modern diet, and therefore looking at the combination during prenatal life may have more of an effect on the offspring's muscle. It is also important to note that the HF diet had increased levels of vitamin D<sub>3</sub> compared to the C diet (discussed in section 6.4), so a 'flooding' of the prenatal system with vitamin D may have caused any potential negative effects of an obesogenic exposure prenatally on skeletal muscle to be overridden, especially considering that VDD supplementation during pregnancy causes an increase in myofibre density of fetal, newborn and weaning pigs (Hines *et al.* 2013; Zhou *et al.* 2016).

Chapters 4 and 5 both used a VDD diet by removing the vitamin D<sub>3</sub> in the feed. Vitamin D<sub>3</sub> can be synthesised in the skin from UVB rays (section 1.4.1) and therefore in Chapter 4 all mice were housed with UVB filters fitted over the lighting lamps to prevent any vitamin D synthesis in the skin. Indeed, dam plasma total 25(OH)D and 25(OH)D<sub>3</sub> were significantly reduced by late pregnancy in this model which confirmed that a deficiency in vitamin D had been established. However, in Chapter 5 the modest differences in dam plasma 25(OH)D<sub>3</sub> between diet groups show that despite removal of the vitamin D<sub>3</sub> supplement in the diet, 25(OH)<sub>3</sub> was still present. It was not possible to house the sheep in the same conditions as the mice in Chapter 4, but they were housed in barns with double-glazed windows and out of direct sunlight. Not having filters to block any UVB rays may have meant these preventative measures were not enough to stop vitamin D<sub>3</sub> production, and consequently detection of 25(OH)D<sub>3</sub> in the plasma. Total 25(OH)D and 25(OH)D<sub>2</sub> plasma concentrations were not altered between the control and VDD group, and feed analysis revealed that vitamin D<sub>2</sub> remained at a similar level in both diets, and therefore could explain why a lack of differences were observed.

### 6.3 How important are nutritional challenges during early developmental windows to offspring skeletal muscle?

At the outset of this thesis, previous studies suggested that the environment a fetus is exposed to during pregnancy impacts the rate of muscle development and growth in early life, and it was postulated that this would consequently affect the peak muscle mass achieved in adulthood, and the rate of decline in muscle mass and strength with age (Sayer *et al.* 2008). Previous studies indicate that inadequate intrauterine nutrition reduces the blood supply to the periphery of the fetus, which impacts on the growth and development of peripheral tissues (Costello *et al.* 2008) in the fetus. Subsequent studies highlighted that the impact of diet-driven reductions in maternal body condition on offspring skeletal muscle structure can persist into mature adult life (Costello *et al.* 2013). The reduction in blood flow to the fetal periphery during maternal undernutrition appears to involve a disruption of endothelial function in femoral arteries (Ozaki *et al.* 2000; Nishina *et al.* 2003). It is therefore of great interest that work by others on the Chapter 5 sheep fetuses showed that fetal femoral artery endothelial function was impaired in the VDD group (Torrens *et al.* 2015). As discussed in the next section (6.4), this potential marker of impaired blood flow to the periphery does not appear to be coupled to changes in skeletal muscle structure.

In Chapter 3 the results show that a high-fat (and high-vitamin D) diet mainly has an effect on muscle function and structure when it is imposed in postnatal life. However, some effects of PRE HF diet are evident in the male offspring. In the male soleus *m.* there was a reduction in PF of tetanic contraction and an increase in the TPT and HRT of twitch contraction, indicative of increased fatigue. The potential muscle fatigue could be a result of impairments to mitochondrial activity as reported by previous studies investigating the effects of a maternal obesogenic diet on offspring skeletal muscle function (Latouche *et al.* 2014; Pileggi *et al.* 2016). One reason why these effects were only observed in the males could be due to their muscles being more susceptible to membrane damage following contraction (Bär *et al.* 1988). Despite these changes in contraction and contractile kinetics, there was no difference in adult muscle mass with a PRE HF diet, as reported by a previous study (Samuelsson *et al.* 2008) and very little change to myofibre density. There was a reduction in soleus *m.* mRNA levels of *Myh1* (but not reflected by changes in type IIX myofibre number or size) and EDL *m.* *Tnnt1* (coding for slow-twitch troponin), both important for muscle contractility. Even though these do not bring about major changes to

muscle function, it is still evidence to show that there is some effect of a suboptimal diet during pregnancy on the offspring's muscle, and this is further discussed in section 6.4. Of course a central theme to this thesis is to investigate the effect of vitamin D, and a PRE HF diet was associated with a reduction in plasma 25(OH)D<sub>3</sub> concentration in both sexes in adult life. This could be due to a disruption in the adult biosynthesis and metabolism of vitamin D<sub>3</sub> to 25(OH)D<sub>3</sub> within the liver, kidney and/or placenta by the prenatal challenge. Long-term consequences for skeletal muscle of offspring having reduced 25(OH)D<sub>3</sub> are possible, but actually were not revealed in the analysis to date in this thesis (discussed section 6.4). Further analysis may highlight some changes to the muscle on a molecular level.

Chapters 4 and 5 used animal models of specific dietary VDD. In Chapter 4 a gestational VDD diet led to some reductions in muscle mass relative to body weight of 15-week (young adult) offspring. However, there were no observed impairments to muscle function as further discussed in section 6.4. This early life nutritional challenge did however lead to reduced activity levels in the offspring, but whether the skeletal muscle is involved in this reduction is unclear and more investigation is required. It is possible that any effects a PRE VDD diet was having on the offspring in early life had been compensated for by the control diet over the postnatal period into adulthood. In view of previous work in undernutrition models, and the observation of impaired fetal femoral artery endothelial function following a maternal VDD diet (Torrens *et al.* 2015), this might lead us to expect a corresponding reduction in skeletal muscle myofibre density. However, data from Chapter 5 focussing on changes as a result of dietary VDD to skeletal muscle during fetal life, found no links between levels of vitamin D in fetal life and fetal skeletal muscle structure, mRNA levels or function (further discussed in section 6.4).

Thus, despite clear evidence from previous studies demonstrating that a suboptimal environment during pregnancy does impact the offspring's muscle at various ages, the findings in this thesis suggest that how (and if) the muscle responds will depend on the type and extent of nutritional challenge during early life development.

## 6.4 Does vitamin D affect skeletal muscle?

The results from this thesis suggest that vitamin D is not having a profound effect on the offspring's skeletal muscle in terms of its development, structure or function.

A link between obesity and vitamin D deficiency in adulthood (Liel *et al.* 1988; Wortsman *et al.* 2000; Hyppönen & Power 2006; Vimalleswaran *et al.* 2013) and also between mother and offspring (Crozier *et al.* 2012; Daraki *et al.* 2018; Wen *et al.* 2018) has been reported (see section 1.6), and both of these conditions are highly prevalent in pregnant women. However the mouse model of obesity in Chapter 3 was not able to mimic this relationship since HF diet used to induce obesity was 'flooded' with higher levels of vitamin D<sub>3</sub>. The higher levels of vitamin D<sub>3</sub> in the mouse diet were most likely due to the amount in the lard, and this could actually be a good model of modern humans diets in which more foods high in saturated fats are consumed. The fact that vitamin D is higher in these fatty foods, but vitamin D status is also influenced by adipose tissue and therefore body composition, makes it more complicated to determine the effects of vitamin D on muscle function in our HF mouse model. Regardless of this increase in vitamin D in the HF diet, 30-week offspring plasma 25(OH)D<sub>3</sub> concentration was reduced with a PRE HF diet. This indicates that a PRE high-fat and high-vitamin D diet prenatally impacts the way the vitamin D is handled in the offspring long-term. When assessing the relationship between adult offspring plasma 25(OH)D<sub>3</sub> concentration and muscle structure and isometric contraction, lower plasma 25(OH)D<sub>3</sub> concentration was associated with higher PF of twitch contraction in the female soleus *m.* only, and this relationship was mainly a result of that in the HF/C group. This is surprising considering the evidence for reduced vitamin D status being associated with a reduced hand grip strength (Harvey *et al.* 2014). Plasma 25(OH)D<sub>3</sub> did not appear to affect the PF in the EDL *m.* or in the male offspring. A lack of a relationship between vitamin D status and muscle functional measurements for the most part suggests vitamin D is not having a direct effect on the muscle function, but the fact that there is a relationship in the HF/C female offspring strengthens the suggestion that a PRE HF diet has to ability to alter vitamin D homeostasis and its physiological function into adulthood. There were also very few significant relationships between plasma 25(OH)D<sub>3</sub> concentration and myofibre structure, although lower 25(OH)D<sub>3</sub> was associated with a higher density in all of the different myofibre types apart from type I fibres. This is interesting as these findings contrast to previous studies which report a positive

relationship between vitamin D and type II myofibre size in the adult (Yoshikawa *et al.* 1979; Sorensen *et al.* 1979; Ceglia *et al.* 2013).

In Chapter 4, a mouse model of dietary VDD during pregnancy was successfully developed with reductions in total 25(OH)D and 25(OH)D<sub>3</sub> plasma concentration in the mothers and 15-week offspring. There were no changes to isometric skeletal muscle contractile PF, front limb grip strength, hind limb grip strength or overall strength in the VDD/C offspring compared with C/C offspring. However, there were reductions in male soleus *m.* and female EDL *m.* mass when relative to body weight of 15-week (young adult) VDD/C offspring. This may signify that a VDD diet in early life does impact the offspring's muscle structure (see section 6.5 for further discussion). There were also no associations between maternal plasma 25(OH)D<sub>3</sub> and muscle weight or these strength measurements. These results suggest that this model of gestational VDD is not impacting on muscle function and disagree with the results from a human study which found that lower maternal vitamin D levels were associated with a lower offspring handgrip strength at 4 years of age (Harvey *et al.* 2014). However, the work by Harvey *et al.* was an epidemiological study in which the observed associations cannot be ascribed with certainty to vitamin D alone. These data from Chapter 4 are novel in themselves and therefore more investigation is required. For this thesis the contractile kinetics (TPT and HRT) have not been analysed, and although there was no change to PF, it would be interesting to see if the TPT or HRT changes to indicate whether there were any signs of fatigue as found in a study looking at VDD during the postnatal period alone (not prenatally) on chicks (Pleasure *et al.* 1979).

Even though there were no changes to muscle strength in Chapter 4 mice, there was a reduction in open-field activity, which was also associated with lower maternal plasma 25(OH)D<sub>3</sub> concentrations for all parameters except average velocity, suggesting that the VDD diet is having an impact on the offspring. Results in this chapter do suggest that this reduction in activity may not be a result of impairments to skeletal muscle but perhaps they are more closely linked to neurological defects (discussed in section 4.4.3) as reported in previous studies on the rat brain (Eyles *et al.* 2003; Kesby *et al.* 2009; Kesby *et al.* 2010; Keilhoff *et al.* 2010), especially considering the lack of change in the PF data in these VDD/C offspring. Interestingly, as part of the open-field activity analysis, vertical counts of the female offspring, and jump count in the males (trend in females) were significantly reduced in those exposed to a PRE VDD diet. Considering that these two forms of activity require hind limb activation, there may be some underlying effects of the pregnancy VDD

diet on muscle, perhaps on a neuromuscular level. In view of these data, investigating cortical thickness and neurogenesis in the offspring brains, and also dopaminergic signalling on a skeletal muscle level may help to understand the reasons behind reduced activity levels in these VDD/C offspring.

In Chapter 5, a VDD diet during pregnancy did not lead to alterations in muscle structure, mRNA levels of genes associated with muscle growth or insulin-mediated glucose uptake, or function as assessed by radiolabelled glucose uptake. Of course this was not an ideal model of VDD as the plasma 25(OH)D<sub>2</sub> concentration remained higher than expected (as discussed in section 5.4.1), and even when linear regression was applied across all groups to the outcome measurements, a lack of relationship between vitamin D and outcome variable persisted. There are very little data on the effect of VDD during pregnancy on skeletal muscle in the fetus but previous studies, although not focussed on VDD, do suggest that there is a relationship between vitamin D and fetal/newborn myofibre density (Hines *et al.* 2013; Zhou *et al.* 2016; Cleal *et al.* 2017). Furthermore, one study investigating gestational VDD found the rat newborn had smaller myofibres (Max *et al.* 2014). All three studies suggest that vitamin D levels are associated with changes in early life offspring muscle. It could be that the fetal stage is too early to see differences as a direct result of VDD in pregnancy, but ideally a model whereby the 25(OH)D<sub>2</sub> and total 25(OH)D concentration are significantly reduced in the VDD group would be required to determine any differences as the ‘hit’ of vitamin D deficiency was not hard enough.

As mentioned, improvements to the dietary models in Chapter 3 and 5 would be ideal for future work. Although only studied in adulthood, previous data suggest vitamin D does have an important role in muscle due to the expression of the vitamin D receptor (VDR) and 1 $\alpha$ -hydroxylase in muscle (Bischoff *et al.* 2001; Ceglia *et al.* 2013; Girgis *et al.* 2014) and that low vitamin D levels are associated with reduced strength (Harvey *et al.* 2014) and performance (Wicherts *et al.* 2007b), and an increased risk of falls (Heike A Bischoff-Ferrari *et al.* 2004). A deeper understanding of the impact of vitamin D and its deficiency during pregnancy on vital components of the vitamin D signalling pathway such as VDR and 1 $\alpha$ -hydroxylase would be ideal, but due to the low mRNA expression of these genes and the required protocol optimisations, this could not be completed in the scope of this thesis. Investigating how their expression on an mRNA or protein level changes would give a more detailed picture.

Taking all chapters into account, VDD during pregnancy would appear to be less detrimental to skeletal muscle structure and function than initially thought. What has not been investigated is whether any effects of a prenatal diet deficient in vitamin D persist into elderly life, or which specific time points in early life (e.g. periconceptual, late gestation, weaning) result in the biggest effect following VDD. Consequently, in order to understand the effect that early life VDD has on offspring skeletal muscle, additional time points and more specific nutritional challenge timeframes would be interesting to explore.

## **6.5 Does a change in muscle structure influence muscle function?**

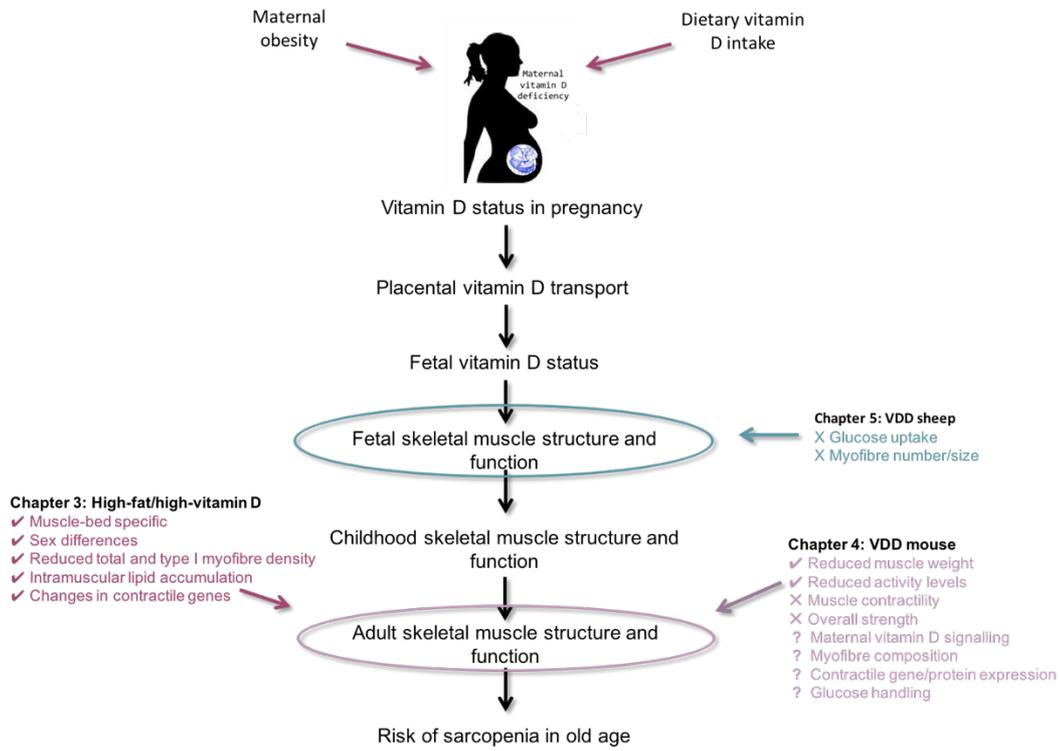
The relationship between skeletal muscle structure and function was explored in Chapter 3 and 5. In Chapter 3, reductions to PF were observed in the soleus *m.* alone of female and male 30-week offspring following a POST HF (with high-vitamin D) diet. Muscle structure was assessed by myofibre density, CSA and lipid accumulations into the muscle. The POST HF diet also caused reductions in total and type I myofibres in the soleus *m.*, which lowers the oxidative capacity (Oberbach *et al.* 2006) and when PF and myofibre density were correlated, the PF generated during tetanic and single-twitch contraction was lower with a reduction in total myofibre density when all diet groups were combined. This provides evidence that myofibre density does influence muscle function. Intramuscular lipid accumulation was increased in the male offspring following a POST and combined PRE and POST HF diet, which could further explain the reduction in male soleus *m.* PF and prolonged contractile times indicative of fatigue. Other studies have found a similar association between lipid accumulation and impaired muscle force and provide data to suggest that the increased inflammation within the muscle can induce muscle atrophy due to a reduction in proteins important for muscle development and contraction (Bayol *et al.* 2005; Ciapaite *et al.* 2015; Pellegrinelli *et al.* 2015).

In Chapter 5 there were no differences in fetal muscle function, as assessed by radiolabelled glucose uptake, or skeletal muscle structure. There were also no significant relationships between muscle structure and function following linear regression. Generally, the lack of association between structure and function does support the importance of structure determining function in skeletal muscle. Ideally the CSA of type I fibres would

have been determined (outside the scope of this thesis) and the density and CSA of all type II myofibre isoforms (IIA, IIB and IIX) and hybrid fibres would have been quantified to investigate this in more detail. Vitamin D supplementation in human adults increased myofibre structure and muscle strength (Sato *et al.* 2005). A reason for not seeing any significant relationship in Chapter 5 between fetal muscle structure and function could be that the myofibres in the sheep fetus are still developing and there is no real requirement of muscle function that early on in life. Other studies have found that VDD impacts on muscle structure in the newborn rat (Max *et al.* 2014) and the fetal and newborn pig (Hines *et al.* 2013; Zhou *et al.* 2016), but there are no previous studies looking at fetal muscle function in relation to structure.

Further investigation is now warranted on any potential relationship between muscle structure and function in the VDD mouse model (Chapter 4). Despite the lack of effect on muscle PF, grip strength and overall strength, there were reductions in muscle weight relative to body weight in the male soleus *m.* and female EDL *m.* mass following a prenatal VDD diet, and this could indicate structural changes. Myofibre structure could not be quantified in this thesis for Chapter 4, but image analysis of the immunofluorescent fibre typing to determine myofibre density and CSA of the type I, different type II isoforms and hybrid fibres is a component of future work. Further investigation into myofibre structure and levels of mRNA encoding for proteins associated with muscle growth and contractility may provide an insight into whether any molecular or structural changes could lead to dysfunction in later life.

Thus in this thesis, there is some evidence for a relationship between muscle structure and function, but this is more evident in certain dietary models, in certain muscle beds and at certain developmental time points. This thesis has looked at structure in terms of myofibre number and size, but deeper molecular biology investigation at the protein level into the machinery for myogenesis, muscle growth, contractility and glucose uptake would be the best next step.



**Figure 6.1: Thesis overview.** A summary of the flow of logic with results from each chapter.

## 6.6 Limitations and future work

The data from three animal models in this thesis provide novel insight and a contribution to the understanding of the effect vitamin D deficiency on offspring skeletal muscle. There are several areas which would be of benefit to explore further and which were outside the scope of this thesis.

Considering that this thesis is centred on vitamin D deficiency, it would be very beneficial to gain an understanding of how the vitamin D signalling pathway is affected by investigating the impact of vital components at the mRNA and protein level. Analysing the relationship between their expression and muscle structure and function would add additional insight. Measurement of gene expression at the mRNA level of VDR and CYP27B1 genes was attempted on muscle samples from Chapter 3, but as these genes are expressed in low levels in skeletal muscle the PCR assays need optimisation. Alternative techniques to detect expression could also be performed, for example, immunofluorescence to assess the localisation of the proteins (Ceglia *et al.* 2010) or western blotting to quantify protein expression.

The quantification of the capillary density in the muscle samples from all three results chapters would add to the story regarding an impaired vasodilatory response and reduced blood flow in the femoral artery in models of maternal undernutrition (Ozaki *et al.* 2000; Burrage *et al.* 2009; Torrens *et al.* 2015). It would help to understand whether blood perfusion is altered in the muscle itself and not just the hind limb of offspring exposed to PRE VDD, and whether the nutrient delivery to the muscle was impaired.

As previously explained, the intention of using an established mouse model of obesity in Chapter 3 to investigate effect of obesity-driven VDD on skeletal muscle was confounded by the high levels of vitamin D<sub>3</sub> found in the HF diet. In effect the experimental diet was a high-fat/high-vitamin D diet and led to setting up the VDD mouse model in Chapter 4 which was a successful model of dietary specific VDD. The postulated relationship between obesity and vitamin D could be explored in future work by developing a pregnancy-obese model that doesn't have the confounding effect of the high dietary vitamin D intake. Considering one of the angles in this thesis to investigate VDD on skeletal muscle included a model of obesity, it would be interesting to measure inflammatory mediators and fatty acid metabolites to see whether they alter vitamin D

receptivity and handling in the muscle, and also contractile regulators. Previous studies have reported an increase in EDL *m.* long-chain fatty acid metabolites after HF feeding (Ciapaite *et al.* 2015), which have been reported to inhibit the calcium pump SERCA (Dumonteil *et al.* 1994) and therefore affect calcium handling within the muscle, potentially leading to muscle fatigue. However, there is no known studies looking into the relationship between fatty acid metabolites and vitamin D in muscle, and their effects on muscle structure or function.

A subset of mice in Chapter 4 had abnormal livers (discussed in section 4.2.2), and so the number of male offspring were limited, especially for the control group (C/C n=3, VDD/C n=6). This meant that the sample size used for the isometric skeletal muscle contraction experiments was low. In addition, due to time restrictions, only the male offspring muscles were assessed and future work should be on both male and female offspring in order to see any sex differences.

Chapters 3 (HF) and 4 (VDD) both used mouse models. Due to their shorter pregnancies and shorter lifespan it would be interesting to generate subsets of offspring in each study and analyse them at various ages (e.g. young adult, mature adult and elderly). This would provide a greater understanding of how maternal VDD effects the offspring across the life course as the different ages can be tracked back to the same mother. Looking at an age in older life would help to understand the link between maternal VDD and development of sarcopenia in more detail.

Further understanding of the impact that vitamin D has on the contractile properties in the offspring skeletal muscle would be beneficial and would require additional analysis of the isometric contractile traces to determine the contractile time and relaxation time in Chapter 4 mice. In Chapter 3 and 4 skeletal muscle samples, it would be interesting to investigate calcium clearance and cross-bridge detachment kinetics by looking into the expression/activity of calcium pumps involved in muscle contraction to understand in much more detail the effect of a HF/high-vitamin D and a VDD diet on muscle contraction.

The nutritional challenge in Chapter 5 sheep was not ideal due to the lack of change in maternal and fetal total 25(OH)D plasma concentration as a result of the high 25(OH)D<sub>2</sub> levels. For future work, if this study is to be repeated then the ingredients (e.g. the grass meal) should be altered in the pellets to get a harder 'hit' on total 25(OH)D blood plasma concentrations. In Chapter 5, it is unclear why there was no association between fetal

vitamin D concentration and muscle glucose uptake. In this thesis glucose uptake was assessed *ex vivo* and could differ from the *in vivo* situation. Furthermore, despite the insulin-insensitive GLUT-1 being the predominant glucose transporter during fetal life (Aldoretta *et al.* 1998), the finding that insulin did not stimulate glucose uptake *ex vivo* did contrast with previous *in vivo* fetal sheep work (Hay & Meznarich 1986; Anderson *et al.* 2001; Anderson *et al.* 2005). Therefore, it would be interesting to set up *in vivo* sheep studies of skeletal muscle glucose uptake (Anderson *et al.* 2001) to see whether increased blood insulin levels have a differential effect on glucose uptake in the skeletal muscle between control and VDD fetal sheep. Analysing insulin levels in the maternal and fetal sheep plasma would also provide useful information as it would allow a more thorough understanding into insulin sensitivity and glucose homeostasis in the fetal sheep in relation to fetal vitamin D status. Additionally, in this chapter, muscle composition was analysed but only for type I and type II density and type II CSA. There are of course multiple isoforms of type II fibres and also hybrid fibres, so a protocol to quantify these different types in the sheep would provide a more extensive understanding on how VDD may affect fetal muscle structure. Quantifying the amount of intramuscular fat (as in Chapter 3) would be interesting to investigate considering it has been reported to reduced glucose tolerance and impair glucose uptake into cells (Boden & Chen 1995). Current work in association with this chapter is that some fetal sheep vastus lateralis *m.* samples (4 samples with a higher 25(OH)D and 4 with a lower 25(OH)D concentration) have been sent to Dr Spiros Garbis (University of Southampton) for proteomic analysis. These data could provide some interesting information in the pathways altered with varying vitamin D levels. This type of analysis would also be interesting in the muscles of the mouse models in Chapter 3 and 4.

Although the muscles sampled for each chapter were selected due to their differences in slow-twitch to fast-twitch myofibre composition (detailed section 1.3.1.3), for all chapters, it would be interesting to investigate the effect maternal VDD has on a larger range of muscle beds throughout the body and also to understand the changes in mechanistic components important in muscle development, for example, genes and proteins involved in myogenesis, which could help to detect any early markers which might influence myofibre type, number and size. It is also important to note that the observed differences in this thesis are based on relatively small experimental group sizes, even though these are generally in line with other publications in this field. Where trends were reported it remains possible that significant differences would have been found with bigger group numbers. Due to a paucity of previous relevant data, power calculations to determine

optimal group sizes could not be performed and they were dictated by animal availability, as explained in section 2.6.3.

## **6.7 Conclusion**

The data reported in this thesis show some effect of suboptimal nutrition in early life on the offspring's skeletal muscle mass (Chapter 4), contraction peak force and potential fatigability (Chapter 3) and open-field parameters including some activity involving hind limb activation (Chapter 4). The results suggest that vitamin D handling in the adult offspring may be altered by maternal obesity/body composition (Chapter 3). Maternal VDD during pregnancy is not associated with changes to fetal muscle structure or function (Chapter 5), it does seem to be associated with changes to muscle mass in adulthood, although changes to myofibre structure requires further investigation. A maternal VDD diet did lead to reduced activity levels in offspring and this could predispose the offspring to further complications such as obesity and metabolic conditions (Chapter 4). VDD is highly prevalent in pregnant women, and epidemiological studies have suggested an association with maternal vitamin D status and offspring health. Despite previous evidence to suggest vitamin D has a role in skeletal muscle structure and function, a deficiency in it during pregnancy may not be as detrimental to the offspring's muscle as hypothesised at the outset of this thesis. These previous epidemiology studies can only look at associations and therefore observations in skeletal muscle cannot be attributed solely to vitamin D levels alone with certainty. The data in this thesis adds to a limited previous research base, and more investigation is needed in order to confirm whether VDD during pregnancy has a profound effect on offspring skeletal muscle or not.

# Appendix A Chapter 3 mouse model diets

## A.1 Control diet feed formulation

### Rat and Mouse No.1 Maintenance

#### Calculated Analysis

NUTRIENTS	Total	Supp (9)
<b>Proximate Analysis</b>		
Moisture (1)	%	10.00
Crude Oil	%	2.71
Crude Protein	%	14.38
Crude Fibre	%	4.65
Ash	%	6.00
Nitrogen Free Extract	%	61.73
<b>Digestibility Co-Efficients (7)</b>		
Digestible Crude Oil	%	2.47
Digestible Crude Protein	%	12.92
<b>Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)</b>		
Total Dietary Fibre	%	17.05
Pectin	%	1.52
Hemicellulose	%	10.17
Cellulose	%	4.32
Lignin	%	1.68
Starch	%	44.97
Sugar	%	4.05
<b>Energy (5)</b>		
Gross Energy	MJ/kg	14.74
Digestible Energy (15)	MJ/kg	11.90
Metabolisable Energy (15)	MJ/kg	10.74
Atwater Fuel Energy (AFE)(8)	MJ/kg	13.75
AFE from Oil	%	7.42
AFE from Protein	%	17.49
AFE from Carbohydrate	%	75.09
<b>Fatty Acids</b>		
<b>Saturated Fatty Acids</b>		
C12:0 Lauric	%	0.02
C14:0 Myristic	%	0.14
C16:0 Palmitic	%	0.31
C18:0 Stearic	%	0.04
<b>Monounsaturated Fatty Acids</b>		
C14:1 Myristoleic	%	0.02
C16:1 Palmitoleic	%	0.09
C18:1 Oleic	%	0.77
<b>Polyunsaturated Fatty Acids</b>		
C18:2(ω6) Linoleic	%	0.69
C18:3(ω3) Linolenic	%	0.06
C20:4(ω6) Arachidonic	%	0.13
C22:5(ω3) Clupanodonic	%	
<b>Amino Acids</b>		
Arginine	%	0.91
Lysine (6)	%	0.66
Methionine	%	0.22
Cystine	%	0.24
Tryptophan	%	0.18
Histidine	%	0.35
Threonine	%	0.49
Isoleucine	%	0.54
Leucine	%	0.98
Phenylalanine	%	0.66
Valine	%	0.69
Tyrosine	%	0.49
Taurine	%	
Glycine	%	1.11
Aspartic Acid	%	0.67

NUTRIENTS	Total	Supp (9)
Glutamic Acid	%	3.17
Proline	%	1.20
Serine	%	0.56
Hydroxyproline	%	
Hydroxylysine	%	
Alanine	%	0.16
<b>Macro Minerals</b>		
Calcium	%	0.73
Total Phosphorus	%	0.52
Phytate Phosphorus	%	0.24
Available Phosphorus	%	0.28
Sodium	%	0.25
Chloride	%	0.38
Potassium	%	0.67
Magnesium	%	0.23
<b>Micro Minerals</b>		
Iron	mg/kg	159.30
Copper	mg/kg	11.50
Manganese	mg/kg	72.44
Zinc	mg/kg	35.75
Cobalt	µg/kg	634.10
Iodine	µg/kg	1202.69
Selenium	µg/kg	298.99
Fluorine	mg/kg	10.49
<b>Vitamins</b>		
β-Carotene (2)	mg/kg	0.16
Retinol (2)	µg/kg	2566.38
Vitamin A (2)	iu/kg	8554.27
Cholecalciferol (3)	µg/kg	15.54
Vitamin D (3)	iu/kg	621.70
α-Tocopherol (4)	mg/kg	76.45
Vitamin E (4)	iu/kg	84.10
Vitamin B <sub>1</sub> (Thiamine)	mg/kg	8.58
Vitamin B <sub>2</sub> (Riboflavin)	mg/kg	4.33
Vitamin B <sub>3</sub> (Pyridoxine)	mg/kg	4.81
Vitamin B <sub>12</sub> (Cyanocobalamin)	µg/kg	7.49
Vitamin C (Ascorbic Acid)	mg/kg	2.59
Vitamin K (Menadione)	mg/kg	10.17
Folic Acid (Vitamin B <sub>9</sub> )	mg/kg	0.79
Nicotinic Acid (Vitamin PP) (6)	mg/kg	61.32
Pantothenic Acid (Vitamin B <sub>3/5</sub> )	mg/kg	20.17
Choline (Vitamin B <sub>4/7</sub> )	mg/kg	1080.14
Inositol	mg/kg	2369.59
Biotin (Vitamin H) (6)	µg/kg	277.13

#### Notes

- All values are calculated using a moisture basis of 10%. Typical moisture levels will range between 9.5 - 11.5%.
- a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene.  
b. Retinol includes the Retinol equivalents of β-Carotene.  
c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity  
d. 1 µg Retinol = 3.33\* iu Vitamin A activity  
e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene  
f. The standard analysis for Vitamin A does not detect β-carotene
- 1 µg Cholecalciferol (D<sub>3</sub>) = 40.0 iu Vitamin D
- 1 mg all-*rac*-α-tocopherol = 1.1 iu Vitamin E activity  
1 mg all-*rac*-α-tocopherol acetate = 1.0 iu Vitamin E activity
- 1 MJ = 239.23 Kcalories = 239.23 Calories = 239.230 calories
- These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
- Based on in-vitro digestibility analysis.
- AF Energy = Atwater Fuel Energy = ((CO%/100)\*9000)+((CP%/100)\*4000)+((NFE%/100)\*4000)/239.23
- Supplemented nutrients from manufactured and mined sources.
- Calculated.

## A.2 High-fat diet feed formulation

Special Diets Services



### Data Sheet: 824053 - '45% AFE FAT'



Diet Code: **824053**

Diet Name:

**RM AFE45%FAT 20%CP 35%CHO (P)**

Description:

**Purified laboratory rodent diet.**

'High Fat' diet for use with 829050 or other 'Rodent AFE' diets in this series.

**Useful for studying obesity, diabetes and other fat/energy induced diseases.**

#### BASIC INFORMATION

DIET	824053 - '45% AFE Fat'
INGREDIENT	g% (w/w)
Casein	26.533
Choline Bitartrate	0.296
L-Cystine	0.399
Lard	17.895
Rice Starch	28.344
Cellulose	6.171
Soya Oil	4.319
Sucrose	10.490
Mineral Mix	4.319
Vitamin Mix	1.234
Total	100.000

SPECIFICATION	% (w/w)	kcal/g	% kcal
Crude Fat	22.6	2.03	45
Crude Protein	23.0	0.92	20
Crude Fibre	4.6	/	/
Ash	4.2	/	/
Carbohydrate	39.8	1.59	35
Total AFE		4.54	100

#### OTHER INFORMATION

##### Storage:

This is a perishable material. Please store in a cool dry place. If possible store refrigerated or even freeze to reduce nutrient oxidation.

##### Shelf Life:

3 months from date of manufacture.  
6 months if stored refrigerated.  
9 months if stored frozen.

##### Feeding Directions:

Feed ad-libitum. Clean drinking water should be available at all times.

##### Product Form, Packaging & Net Weight:

Standard: 10 mm diameter pellets. Packed in sealed plastic buckets. **3 kg net weight.**

##### Nutrient Information:

**AFE = ATWATER FUEL ENERGY =** Decimal fractions of Fat, Protein, Carbohydrate multiplied by 9, 4 & 4 respectively to give kcal AFE / g of diet.

1 MJ = 239.23 Kcal

Nutrient figures on a fresh weight basis unless otherwise stated

Contact SDS for further details.

Tel: +44 (0) 1376 511 260

Fax: +44 (0) 1376 511 247

e-mail: [info@sdsdiets.com](mailto:info@sdsdiets.com)

824053 COM 050209



## DIET FORMULATION AND SPECIFICATION DATA

### BASIC DIET INFORMATION:

Code:	824018
Name:	RM AFE 45% FAT SY(P)
Date:	04/02/2011

### CALCULATED ANALYSIS:

		FRESH	10% H2O
TOTAL	%	100.00	100.00
MOISTURE	%	4.11	10.00
CRUDE OIL	%	22.61	21.22
CRUDE PROTEIN	%	22.97	21.56
CRUDE FIBRE	%	4.50	4.22
ASH	%	4.42	4.15
NFE	%	40.38	37.90
PECTIN	%	0.00	0.00
HEMICELLULOSE	%	0.12	0.11
CELLULOSE	%	5.92	5.56
LIGNIN	%	0.00	0.00
STARCH	%	15.85	14.88
SUGAR	%	22.66	21.27
GROSS ENERGY	MJ/kg	20.30	19.05
DIGESTIBLE ENERGY	MJ/kg	18.61	17.47
METABOLISABLE ENERGY	MJ/kg	17.19	16.13
AF ENERGY	kcal/kg	4568.36	4287.75
C14 1 MYRISTOLEIC	%	0.02	0.02
C16 1 PALMITOLEIC	%	0.03	0.03
C18 1 W9 OLEIC	%	6.46	6.06
C18 2 W6 LINOLEIC	%	3.76	3.53
C18 3 W3 LINOLENIC	%	0.39	0.37
C20 4 W6 ARICHIDONIC	%	0.01	0.01
C22 5 W3 CLUPANODONIC	%	0.00	0.00
C12:0 LAURIC	%	0.03	0.03
C14:0 MYRISTIC	%	0.31	0.29
C16:0 PALMITIC	%	4.30	4.04
C18:0 STEARIC	%	1.92	1.80
ARGININE	%	0.73	0.69
LYSINE	%	1.46	1.37
S LYS	%	0.00	0.00
METHIONINE	%	0.57	0.53
S METH	%	0.00	0.00
CYSTINE	%	0.45	0.42
S CYST	%	0.39	0.37
CL	%	0.28	0.26
S CL	%	0.19	0.18
K	%	0.45	0.42
S K	%	0.44	0.41
MG	%	0.08	0.08
S MG	%	0.06	0.06
FE	mg/kg	58.86	55.24
S FE	mg/kg	52.92	49.67
CU	mg/kg	8.76	8.22
S CU	mg/kg	7.43	6.97
MN	mg/kg	13.29	12.47
S MN	mg/kg	11.96	11.23
ZN	mg/kg	68.90	64.67
S ZN	mg/kg	37.09	34.81
CO	µg/kg	0.00	0.00
S CO	µg/kg	0.00	0.00
I	µg/kg	254.59	238.95
S I	µg/kg	254.59	238.95
SE	µg/kg	197.20	185.09
S SE	µg/kg	197.20	185.09
F	mg/kg	1.24	1.16
VIT A	iu/kg	4931.57	4628.65
S VIT A	iu/kg	4931.57	4628.65
VIT D3	iu/kg	7863.40	7380.39
S VIT D3	iu/kg	1232.89	1157.16
VIT E	iu/kg	100.64	94.46
S VIT E	iu/kg	96.39	90.47
VIT B1 THI	mg/kg	7.36	6.91
S VIT B1	mg/kg	7.25	6.80
VIT B2 RIB	mg/kg	6.37	5.98
S VIT B2	mg/kg	5.92	5.56
VIT B6 PYR	mg/kg	8.65	8.12
S VIT B6	mg/kg	8.54	8.02
VIT B12 CY	µg/kg	30.82	28.93

This information is intended as a guide only. For actual data we recommend that analysis work is carried out to confirm the nutrient parameters precisely



TRYPTOPHAN	%	0.20	0.19
S TRYPT	%	0.00	0.00
HISTIDINE	%	0.52	0.49
THREONINE	%	0.80	0.75
S THREO	%	0.00	0.00
ISOLEUCINE	%	1.16	1.09
LEUCINE	%	1.75	1.64
PHENYLALAN	%	0.96	0.90
VALINE	%	1.39	1.30
TYROSINE	%	0.96	0.90
TAURINE	%	0.00	0.00
GLYCINE	%	0.91	0.85
ASPARTIC A	%	1.30	1.22
GLUTAMIC A	%	3.76	3.53
PROLINE	%	1.57	1.47
SERINE	%	0.87	0.82
HYD PROLIN	%	0.00	0.00
HYD LYSINE	%	0.00	0.00
ALANINE	%	0.74	0.69
CA	%	0.63	0.59
S CA	%	0.62	0.58
TOTAL P	%	0.37	0.35
S PHOS	%	0.29	0.27
PHYTATE P	%	0.00	0.00
AVAIL P	%	0.27	0.25
NA	%	0.35	0.33
S NA	%	0.12	0.11

S VIT B12	µg/kg	30.82	28.93
VIT C ASCO	mg/kg	0.00	0.00
S VIT C	mg/kg	0.00	0.00
VIT K MENE	mg/kg	0.95	0.89
S VIT K	mg/kg	0.95	0.89
FOLIC ACID	mg/kg	2.37	2.22
S FOLIC	mg/kg	2.34	2.20
NICOTINIC	mg/kg	36.62	34.37
S NICOTIN	mg/kg	36.25	34.02
PANTOTHENI	mg/kg	18.46	17.33
S PANTOTH	mg/kg	17.69	16.60
CHOLINE	mg/kg	1330.10	1248.40
S CHOLINE	mg/kg	1216.13	1141.43
INOSITOL	mg/kg	0.00	0.00
S INOSITOL	mg/kg	0.00	0.00
BIOTIN	µg/kg	246.58	231.43
S BIOTIN	µg/kg	246.58	231.43

S = Supplemented nutrients from manufactured and mined sources. The TOTAL nutrient level including theoretical natural contribution for the diet pre-processing is found immediately above the SUPP nutrient.

#### INGREDIENTS:

NAME	% INCLUSION
CASEIN	26.51
SUCROSE	20.34
RICE STARCH	18.43
LARD	18.00
CELLULOSE	6.16
SOYA OIL	4.32
MINERAL MIX	4.32
VITAMIN MIX	1.23
L-CYSTINE	0.40
CHOLINE BITARTRATE	0.30

This information is intended as a guide only. For actual data we recommend that analysis work is carried out to confirm the nutrient parameters precisely

# Appendix B Chapter 4 mouse model diets

## B.1 Maintenance: Control diet feed formulation

### AIN-93M Maintenance Purified Diet (also known as #5801-M) 58M1

DESCRIPTION		NUTRITIONAL PROFILE <sup>1</sup>													
<p>TestDiet® AIN-93M Maintenance Purified Diet is the maintenance diet for rodents recommended by the American Institute of Nutrition. It is formulated to substitute for the previous version (AIN-76A) to improve animal performance.</p> <p>Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.</p>		<p><b>Protein, %</b> <b>13.0</b></p> <p>Arginine, % 0.49 Histidine, % 0.36 Isoleucine, % 0.67 Leucine, % 1.21 Lysine, % 1.02 Methionine, % 0.36 Cystine, % 0.23 Phenylalanine, % 0.67 Tyrosine, % 0.71 Threonine, % 0.54 Tryptophan, % 0.15 Valine, % 0.80 Alanine, % 0.39 Aspartic Acid, % 0.90 Glutamic Acid, % 2.86 Glycine, % 0.27 Proline, % 1.65 Serine, % 0.77 Taurine, % 0.00</p> <p><b>Fat, %</b> <b>4.1</b></p> <p>Cholesterol, ppm 0 Linoleic Acid, % 2.04 Linolenic Acid, % 0.31 Arachidonic Acid, % 0.00 Omega-3 Fatty Acids, % 0.31 Total Saturated Fatty A 0.60 Total Monounsaturated Fatty Acids, % 0.88 Polyunsaturated Fatty Acids, % 2.16</p> <p><b>Fiber (max), %</b> <b>5.0</b></p> <p><b>Carbohydrates, %</b> <b>73.0</b></p> <p><b>Energy (kcal/g) <sup>2</sup></b> <b>3.81</b></p> <table border="1"> <thead> <tr> <th>From:</th> <th>kcal</th> <th>%</th> </tr> </thead> <tbody> <tr> <td>Protein</td> <td>0.518</td> <td>13.6</td> </tr> <tr> <td>Fat (ether extract)</td> <td>0.368</td> <td>9.7</td> </tr> <tr> <td>Carbohydrates</td> <td>2.922</td> <td>76.7</td> </tr> </tbody> </table>		From:	kcal	%	Protein	0.518	13.6	Fat (ether extract)	0.368	9.7	Carbohydrates	2.922	76.7
From:	kcal	%													
Protein	0.518	13.6													
Fat (ether extract)	0.368	9.7													
Carbohydrates	2.922	76.7													
<p>Product Forms Available* Catalog #</p> <p>1/2" Pellet 7603 1/2" Pellet, Irradiated 1810541 3/16" Pellet 1812811 Meal 1810540</p> <p>*Other Forms Available On Request</p> <p><b>INGREDIENTS (%)</b></p> <p>Corn Starch 46.5692 Maltodextrin 15.5000 Casein - Vitamin Tested 14.0000 Sucrose 10.0000 Powdered Cellulose 5.0000 Soybean Oil 4.0000 AIN 93M Mineral Mix 3.5000 AIN 93 Vitamin Mix 1.0000 Choline Bitartrate 0.2500 L-Cystine 0.1800 t-Butylhydroquinone 0.0008</p>		<p><b>Minerals</b></p> <p>Calcium, % 0.50 Phosphorus, % 0.31 Potassium, % 0.36 Magnesium, % 0.05 Sodium, % 0.13 Chloride, % 0.20 Fluorine, ppm 1.0 Iron, ppm 39 Zinc, ppm 35 Manganese, ppm 11 Copper, ppm 6.0 Cobalt, ppm 0.0 Iodine, ppm 0.21 Chromium, ppm 1.0 Molybdenum, ppm 0.14 Selenium, ppm 0.22</p> <p><b>Vitamins</b></p> <p>Vitamin A, IU/g 4.0 Vitamin D-3 (added), IU/g 1.0 Vitamin E, IU/kg 78.8 Vitamin K, ppm 0.75 Thiamin Hydrochloride, ppm 6.0 Riboflavin, ppm 6.5 Niacin, ppm 30 Pantothenic Acid, ppm 16 Folic Acid, ppm 2.1 Pyridoxine, ppm 5.8 Biotin, ppm 0.2 Vitamin B-12, mcg/kg 28 Choline Chloride, ppm 1,250 Ascorbic Acid, ppm 0.0</p> <p>1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated. 2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9.4 kcal/gm respectively.</p>													
<p><b>FEEDING DIRECTIONS</b></p> <p>Feed ad libitum to mice and rats. Plenty of fresh, clean water should be available at all times.</p> <p><b>CAUTION:</b> Perishable - store properly upon receipt. For laboratory animal use only; NOT for human consumption.</p> <p>8/7/2013</p>		<p>ISO 9001:2000 CERTIFIED</p> <p><b>TestDiet</b> www.testdiet.com</p>													

## B.2 Maintenance: Vitamin D deficient diet feed formulation

### Mod AIN-93M w/ No Vitamin D

5B7Q

#### DESCRIPTION

Modification of TestDiet® AIN-93M Semi-Purified Diet with Vitamin D removed.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location.

Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

#### Product Forms Available\*

Product Forms Available*	Catalog #
1/2" Pellet	1811911
1/2" Pellet, Irradiated	1812015

#### \*Other Forms Available By Request

INGREDIENTS (%)	
Corn Starch	46.5692
Dextrin	15.5000
Casein - Vitamin Free	14.0000
Sucrose	10.0000
Powdered Cellulose	5.0000
Soybean Oil	4.0000
AIN 93M Mineral Mix	3.5000
AIN-93M Vitamin Px/No Vit D	1.0000
Choline Bitartrate	0.2500
L-Cystine	0.1800
t-Butylhydroquinone	0.0008

#### FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

#### CAUTION:

Perishable - store properly upon receipt.  
For laboratory animal use only, NOT for human consumption.

11/16/2006

#### NUTRITIONAL PROFILE <sup>1</sup>

<b>Protein, %</b>	<b>13.2</b>	<b>Minerals</b>	
Arginine, %	0.49	Calcium, %	0.50
Histidine, %	0.36	Phosphorus, %	0.31
Isoleucine, %	0.67	Phosphorus (available), %	0.11
Leucine, %	1.21	Potassium, %	0.36
Lysine, %	1.02	Magnesium, %	0.05
Methionine, %	0.36	Sodium, %	0.12
Cystine, %	0.23	Chloride, %	0.20
Phenylalanine, %	0.67	Fluorine, ppm	1.0
Tyrosine, %	0.71	Iron, ppm	35
Threonine, %	0.54	Zinc, ppm	35
Tryptophan, %	0.15	Manganese, ppm	11
Valine, %	0.80	Copper, ppm	6.0
Alanine, %	0.39	Cobalt, ppm	0.0
Aspartic Acid, %	0.90	Iodine, ppm	0.21
Glutamic Acid, %	2.86	Chromium, ppm	1.0
Glycine, %	0.27	Molybdenum, ppm	0.14
Proline, %	1.65	Selenium, ppm	0.17
Serine, %	0.77		
Taurine, %	0.00	<b>Vitamins</b>	
<b>Fat, %</b>	<b>4.1</b>	Vitamin A, IU/g	4.0
Cholesterol, ppm	0	Vitamin D-3 (added), IU/g	0.0
Linoleic Acid, %	2.04	Vitamin E, IU/kg	78.2
Linolenic Acid, %	0.31	Vitamin K (as menadione), ppm	0.29
Arachidonic Acid, %	0.00	Thiamin Hydrochloride, ppm	6.0
Omega-3 Fatty Acids, %	0.31	Riboflavin, ppm	6.0
Total Saturated Fatty A	0.59	Niacin, ppm	30
Total Monounsaturated		Pantothenic Acid, ppm	15
Fatty Acids, %	0.84	Folic Acid, ppm	2.0
Polyunsaturated Fatty Acids, %	2.36	Pyridoxine, ppm	5.8
		Biotin, ppm	0.2
<b>Fiber (max), %</b>	<b>5.0</b>	Vitamin B-12, mcg/kg	25
		Choline Chloride, ppm	1,250
<b>Carbohydrates, %</b>	<b>73.0</b>	Ascorbic Acid, ppm	0.0
<b>Energy (kcal/g) <sup>2</sup></b>	<b>3.79</b>		
<b>From:</b>	<b>kcal</b>	<b>%</b>	
Protein	0.529	13.8	
Fat (ether extract)	0.368	9.6	
Carbohydrates	2.922	76.5	

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.

2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9.4 kcal/gm respectively.



**TestDiet**  
www.testdiet.com

## B.3 Gestational: Control diet feed formulation

## AIN-93G Growth Purified Diet (also known as #5801-G)

57W5

## DESCRIPTION

TestDiet® AIN-93G Growth Purified Diet is the growth diet for rodents recommended by the American Institute of Nutrition. It is formulated to substitute for the previous version (AIN-76A) to improve animal performance.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. If long term studies are involved, store the diet at -20° C or colder. Be certain to keep in air tight containers.

Product Forms Available*	Catalog #
1/2" Pellet	7597
1/2" Pellet, Irradiated	1810393
Meal	1810538
Meal, Irradiated	1810539

\*Other Forms Available By Request

## TYPICAL ANALYSIS

Protein.....	18.7%
Fat.....	7.0%
Fiber.....	5.0%
Carbohydrate.....	64.7%
Metabolizable Energy.....	3.97 kcal/gm

## INGREDIENTS (%)

Corn Starch	39.7486
Casein - Vitamin Free	20.0000
Maltodextrin	13.2000
Sucrose	10.0000
Soybean Oil	7.0000
Powdered Cellulose	5.0000
AIN 93G Mineral Mix	3.5000
AIN 93 Vitamin Mix	1.0000
L-Cystine	0.3000
Choline Bitartrate	0.2500
t-Butylhydroquinone	0.0014

## FEEDING DIRECTIONS

Feed ad libitum to mice and rats. Plenty of fresh, clean water should be available at all times.

## CAUTION:

Perishable, upon receipt store in a cool dry place, refrigeration recommended.

For laboratory animal experimental use only. NOT for human consumption.

8/12/2010

NUTRITIONAL PROFILE <sup>1</sup>

<b>Protein, %</b>	<b>18.3</b>	<b>Minerals</b>	
Arginine, %	0.70	Calcium, %	0.51
Histidine, %	0.52	Phosphorus, %	0.32
Isoleucine, %	0.96	Potassium, %	0.36
Leucine, %	1.73	Magnesium, %	0.05
Lysine, %	1.45	Sodium, %	0.13
Methionine, %	0.52	Chlorine, %	0.22
Cystine, %	0.37	Fluorine, ppm	1.0
Phenylalanine, %	0.96	Iron, ppm	39
Tyrosine, %	1.01	Zinc, ppm	35
Threonine, %	0.77	Manganese, ppm	11
Tryptophan, %	0.22	Copper, ppm	6.0
Valine, %	1.14	Cobalt, ppm	0.0
Alanine, %	0.55	Iodine, ppm	0.21
Aspartic Acid, %	1.29	Chromium, ppm	1.0
Glutamic Acid, %	4.08	Molybdenum, ppm	0.14
Glycine, %	0.39	Selenium, ppm	0.24
Proline, %	2.36		
Serine, %	1.10	<b>Vitamins</b>	
Taurine, %	0.00	Vitamin A, IU/g	4.0
		Vitamin D-3 (added), IU/g	1.0
<b>Fat, %</b>	<b>7.1</b>	Vitamin E, IU/kg	81.6
Cholesterol, ppm	0	Vitamin K (as menadione), ppm	0.29
Linoleic Acid, %	3.58	Thiamin Hydrochloride, ppm	6.1
Linolenic Acid, %	0.55	Riboflavin, ppm	6.7
Arachidonic Acid, %	0.00	Niacin, ppm	30
Omega-3 Fatty Acids, %	0.55	Pantothenic Acid, ppm	16
Total Saturated Fatty Acids, %	1.05	Folic Acid, ppm	2.1
Total Monounsaturated Fatty Acids, %	1.54	Pyridoxine, ppm	5.8
Polyunsaturated Fatty Acids, %	3.78	Biotin, ppm	0.2
		Vitamin B-12, mcg/kg	29
<b>Fiber (max), %</b>	<b>5.0</b>	Choline Chloride, ppm	1,250
		Ascorbic Acid, ppm	0.0
<b>Carbohydrates, %</b>	<b>63.2</b>		
<b>Energy (kcal/g) <sup>2</sup></b>	<b>3.89</b>		
<b>From:</b>	<b>kcal</b>	<b>%</b>	
Protein	0.731	18.8	
Fat (ether extract)	0.637	16.4	
Carbohydrates	2.528	65.1	

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.  
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.



**TestDiet**  
www.testdiet.com

## B.4 Gestational: Vitamin D deficient diet feed formulation

## TestDiet® AIN-93G w/ No Added Vitamin D

5SL1

## DESCRIPTION

Modified TestDiet® AIN-93G Semi-Purified Diet 57W5 with No Added Vitamin D.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

**Product Forms Available\***      **Catalog #**  
1/2" Pellet, Irradiated              1813534

\*Other Forms Available On Re  
INGREDIENTS (%)

Com Starch	39.7486
Casein - Vitamin Free	20.0000
Maltodextrin	13.2000
Sucrose	10.0000
Soybean Oil	7.0000
Powdered Cellulose	5.0000
AIN 93G Mineral Mix	3.5000
AIN-93M Vitamin Px/No Vit D	1.0000
L-Cystine	0.3000
Choline Bitartrate	0.2500
t-Butylhydroquinone	0.0014

NUTRITIONAL PROFILE <sup>1</sup>

<b>Protein, %</b>	<b>18.3</b>	<b>Minerals</b>	
Arginine, %	0.70	Calcium, %	0.51
Histidine, %	0.52	Phosphorus, %	0.32
Isoleucine, %	0.96	Phosphorus (available), %	0.16
Leucine, %	1.73	Potassium, %	0.36
Lysine, %	1.45	Magnesium, %	0.05
Methionine, %	0.52	Sodium, %	0.13
Cystine, %	0.37	Chloride, %	0.22
Phenylalanine, %	0.96	Fluorine, ppm	1.0
Tyrosine, %	1.01	Iron, ppm	36
Threonine, %	0.77	Zinc, ppm	35
Tryptophan, %	0.22	Manganese, ppm	11
Valine, %	1.14	Copper, ppm	6.0
Alanine, %	0.55	Cobalt, ppm	0.0
Aspartic Acid, %	1.29	Iodine, ppm	0.21
Glutamic Acid, %	4.08	Chromium, ppm	1.0
Glycine, %	0.39	Molybdenum, ppm	0.14
Proline, %	2.36	Selenium, ppm	0.24
Serine, %	1.10		
Taurine, %	0.00	<b>Vitamins</b>	
		Vitamin A, IU/g	4.0
<b>Fat, %</b>	<b>7.1</b>	Vitamin D-3 (added), IU/g	0.0
Cholesterol, ppm	0	Vitamin E, IU/kg	81.6
Linoleic Acid, %	3.58	Vitamin K (as menadione), ppm	0.29
Linolenic Acid, %	0.55	Thiamin Hydrochloride, ppm	6.1
Arachidonic Acid, %	0.00	Riboflavin, ppm	6.7
Omega-3 Fatty Acids, %	0.55	Niacin, ppm	30
Total Saturated Fatty A	1.05	Pantothenic Acid, ppm	16
Total Monounsaturated		Folic Acid, ppm	2.1
Fatty Acids, %	1.54	Pyridoxine, ppm	5.8
Polyunsaturated Fatty Acids, %	3.78	Biotin, ppm	0.2
		Vitamin B-12, mcg/kg	29
<b>Fiber (max), %</b>	<b>5.0</b>	Choline Chloride, ppm	1,250
		Ascorbic Acid, ppm	0.0
<b>Carbohydrates, %</b>	<b>63.2</b>		
<b>Energy (kcal/g) <sup>2</sup></b>	<b>3.89</b>		
<b>From:</b>	<b>kcal</b>	<b>%</b>	
Protein	0.731	18.8	
Fat (ether extract)	0.637	16.4	
Carbohydrates	2.528	64.9	

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.

2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9.4 kcal/gm respectively.

## FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

## CAUTION:

Perishable - store properly upon receipt.  
For laboratory animal use only; NOT for human consumption.

2/13/2009



**TestDiet**  
www.testdiet.com



## C.2 Vitamin D deficient diet feed formulation

### Customer Print

CHARNWOOD MILLING  
 CHARNWOOD  
 DEM:1:CURRENT  
 07 November 2013 7:19 AM [FM]  
 Created : 27/11/2007 Amended: 06/11/2013

Page:1

Prepared For: DEM

0

1

2

3

4

5

6

7

Ration : P316A : NO VIT D RVC EXP SHEEP NUTS

Sell £

0.00

Stock SHEEP Type: COMPLEMENTARY Pack: 25KG BAGS Form: 02 - CUBES Group: 11 - ANIMAL FEED

#### Diet Ingredients

		<u>%</u>	<u>Kilos</u>
1	BARLEY	5.00	50.00
3	WHEAT	10.00	100.00
70	GM HIPRO SOYA	13.50	135.00
78	GM FULL FAT SOYA MEAL	5.00	50.00
95	GRASS MEAL	15.00	150.00
102	MOLASSES	5.00	50.00
107	PTS STRAW	35.00	350.00
553	MICRONIZED WHEAT	10.00	100.00
1051	TRIAL NO VIT D SHEEP SUPPLEMENT	1.50	15.00
<b>Totals</b>		<b>100.000</b>	<b>1000.000</b>

<u>Nutrient</u>	<u>Level</u>	<u>Nutrient</u>	<u>Level</u>	<u>Nutrient</u>	<u>Level</u>
VOLUME	100.000 gm	M+C	0.445 %	Iodine	4.110 mg/kg
Dry Matter	88.985	Threonine	0.614	Cobalt	0.033 mg/kg
Crude Prot	14.800 %	Prot Degra	12.800 %	Selenium	0.283 mg/kg
OIL (A)	2.328 %	RDP	7.343 %	Zinc	79.200 mg/kg
Crude Oils	2.772 %	Calcium	0.713 %	Manganese	79.350 mg/kg
Starch	15.200 %	Phosphorus	0.316 %	Iron	194.250 mg/kg
Crude Fibr	17.698 %	Phosphorus	0.134 %	Molybdenum	0.053 mg/kg
Crude Ash	8.298 %	Sodium	0.667 %	Vitamin A	7999.995 iu/kg
ME [Rumina	9.768 mj/kg AF	Chloride	0.631 %	Vitamin D3	0.000 iu/kg
DE HORSE	9.702 mj/kg	Salt	1.029 %	Vitamin E	24.990 mg/kg (as alph
Lysine(herol)	0.767 %	Magnesium	0.156 %		
Methionine	0.224	Copper	6.525 mg/kg		

NOTES

## C.3 Post-production sheep feed analysis for vitamin D

	vitamin D3 (IU/kg)	vitamin D2 (IU/kg)
Vitamin D3 deficient diet (P316a)	<100 (LOQ) IU/kg	598 IU/kg
control diet (P316)	1150 IU/kg	586 IU/kg
wheat straw	<100 (LOQ) IU/kg	<100 (LOQ) IU/kg

## Appendix D Chapter 3 correlations

### D.1 Offspring plasma 25(OH)D<sub>3</sub> vs. peak force

**Table A 1: The correlation of 30-week old offspring plasma 25(OH)D<sub>3</sub> to soleus muscle peak force.** 30-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against the soleus *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*P<0.01. \*P<0.05, ~P<0.1; n=7-8 per group. C, control; HF, high-fat; PF, peak force.

	Blood plasma 25(OH)D <sub>3</sub>			
	Female		Male	
	Soleus tetanic PF	Soleus twitch PF	Soleus tetanic PF	Soleus twitch PF
All	-0.300 ~	-0.421 *	-0.170	-0.174
C/C	0.916 **	0.577	0.758	0.917 ~
C/HF	0.117	0.401	-0.476	-0.406
HF/C	-0.102	-0.777 *	0.327	0.587
HF/HF	-0.383	-0.297	0.044	0.005

**Table A 2: The correlation of 30-week old offspring plasma 25(OH)D<sub>3</sub> to EDL muscle peak force.** 30-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against the EDL *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. ~P<0.1; n=7-8 per group. C, control; HF, high-fat; PF, peak force; EDL, extensor digitorum longus.

	Blood plasma 25(OH)D <sub>3</sub>			
	Female		Male	
	EDL tetanic PF	EDL twitch PF	EDL tetanic PF	EDL twitch PF
All	0.014	-0.001	-0.190	-0.079
C/C	0.268	0.298	0.438 ~	0.663 ~
C/HF	0.162	0.233	0.275 ~	0.671 ~
HF/C	0.201	0.439	-0.326	-0.120
HF/HF	-0.422	-0.436	-0.019	-0.007

## D.2 Offspring myofibre density vs. peak force

**Table A 3: The correlation of 30-week old offspring soleus myofibre density to muscle peak force.** 30-week female and male offspring soleus myofibre density was correlated against the soleus *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*P<0.01. \*P<0.05, ~P<0.1; n=7-8 per group. C, control; HF, high-fat; PF, peak force.

Soleus - female								
	Total density		Type I density		Type IIA density		Total type II density	
	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF
All	0.451*	0.555**	0.371 ~	-0.489**	0.066	0.253	0.138	0.199
C/C	0.214	-0.087	0.236	-0.293	-0.078	0.176	-0.090	0.023
C/HF	0.010	0.245	0.037	0.310	0.005	-0.158	-0.034	-0.061
HF/C	0.146	0.169**	0.763	0.104	0.148	0.138	0.116	0.107
HF/HF	0.416	0.802*	0.351	0.518	0.460	0.771*	0.393	0.709 ~
Soleus - male								
	Total density		Type I density		Type IIA density		Total type II density	
	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF
All	0.423*	0.451*	0.269	0.273	0.216	0.308	0.391~	0.379~
C/C	0.456	0.391	0.041	0.147	0.290	0.247	-0.004	-0.057
C/HF	-0.182	-0.406	0.039	-0.243	-0.150	-0.222	0.039	0.055
HF/C	0.336	0.009	0.325	0.098	-0.072	-0.239	0.579	0.490
HF/HF	0.971***	0.981***	0.585	0.624	0.926**	0.891**	0.850*	0.776*

**Table A 4: The correlation of 30-week old offspring EDL myofibre density to muscle peak force.** 30-week female and male offspring EDL myofibre density was correlated against the EDL *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \* $P < 0.05$ , ~ $P < 0.1$ ;  $n = 7-8$  per group. C, control; HF, high-fat; PF, peak force; EDL, extensor digitorum longus.

EDL - female										
	Total density		Type I density		Type IIB density		Type IIX density		Total type II density	
	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF
All	-0.306	-0.490*	-0.031	-0.091	-0.144	-0.303	0.330	0.181	-0.132	-0.319
C/C	0.696	0.275	0.624	0.206	0.557	0.278	0.691*	0.783*	0.744~	0.408
C/HF	-0.721~	-0.803*	0.593	0.473	-0.640	-0.907~	0.431	0.412	-0.497	-0.569
HF/C	-0.143	-0.395	0.223~	0.120	-0.142	-0.312	-0.035	0.180	-0.143	-0.287
HF/HF	-0.616	-0.665	-0.794~	-1.095~	-0.343	-0.385	-0.135	-0.315	-0.497	-0.514
EDL - male										
	Total density		Type I density		Type IIB density		Type IIX density		Total type II density	
	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF	Tetanic PF	Twitch PF
All	0.305	0.249	-0.381~	-0.200	0.400*	0.397*	-0.120	0.008	0.317	0.300
C/C	-1.076	0.454	-0.801	0.000	-0.065	0.402	-0.920	-0.937*	-0.638*	-0.027
C/HF	0.389	0.135	-0.042	-0.009	0.338	0.181	0.120	0.065	0.361	0.298
HF/C	0.346	0.552	-0.799	-0.607	0.692	0.867~	-0.266	-0.063	0.544	0.701
HF/HF	-0.118	-0.318	0.135	0.232	-0.090	-0.249	-0.103	-0.217	-0.134	-0.349

### D.3 Offspring intramuscular lipid deposition vs. peak force

**Table A 5: The correlation of 30-week old offspring soleus intramuscular lipid deposition to muscle peak force.** 30-week female and male offspring soleus intramuscular lipid deposition was correlated against the soleus *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*P<0.01. \*P<0.05, ~P<0.1; n=7-8 per group. C, control; HF, high-fat; PF, peak force.

	Oil red O staining			
	Female		Male	
	Soleus tetanic PF	Soleus twitch PF	Soleus tetanic PF	Soleus twitch PF
All	-0.082	-0.100	-0.609**	-0.517**
C/C	-0.098	0.378	-0.796*	-0.821*
C/HF	0.176*	-0.562	0.499	0.356
HF/C	0.112	-0.060	-0.657	-0.426
HF/HF	0.646	0.104	-1.076*	-0.989~

**Table A 6: The correlation of 30-week old offspring EDL intramuscular lipid deposition to muscle peak force.** 30-week female and male offspring EDL intramuscular lipid deposition was correlated against the EDL *m.* peak force of tetanic and single-twitch contraction. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*P<0.01. \*P<0.05, ~P<0.1; n=7-8 per group. C, control; HF, high-fat; PF, peak force; EDL, extensor digitorum longus.

	Oil red O staining			
	Female		Male	
	EDL tetanic PF	EDL twitch PF	EDL tetanic PF	EDL twitch PF
All	0.066	-0.058	-0.084	-0.109
C/C	0.148	-0.159	-1.035~	-0.679
C/HF	-0.097	-0.097	-0.006	0.240
HF/C	-0.186	-0.781*	0.235	0.560
HF/HF	-0.179	-0.140	0.180	0.256

## D.4 Offspring intramuscular lipid deposition vs. 25(OH)D<sub>3</sub>

**Table A 7: The correlation of 30-week old offspring intramuscular lipid deposition to plasma 25(OH)D<sub>3</sub>.** 30-week female and male offspring soleus and EDL intramuscular lipid deposition was correlated against plasma 25(OH)D<sub>3</sub>. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. ~P<0.1; n=7-8 per group. C, control; HF, high-fat; EDL, extensor digitorum longus.

	Soleus oil red O staining		EDL oil red O staining	
	Plasma 25(OH) <sub>2</sub> D <sub>3</sub>		Plasma 25(OH) <sub>2</sub> D <sub>3</sub>	
	Female	Male	Female	Male
All	-0.058	0.353~	-0.022	-0.073
C/C	-0.446	-0.438	-0.017	-0.200
C/HF	-0.371	-0.191	0.075	-0.036
HF/C	0.259	-0.662	-0.429	0.183
HF/HF	0.149	-0.086	-0.182	-0.515

## D.5 Offspring plasma 25(OH)D<sub>3</sub> vs. myofibre density

**Table A 8: The correlation of 30-week old offspring plasma 25(OH)D<sub>3</sub> to soleus myofibre density and CSA.** 30-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against the soleus *m.* myofibre density and CSA. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*\*P<0.001. \*P<0.05, ~P<0.1; n=7-8 per group. C, control; HF, high-fat; CSA, cross-sectional area.

Soleus - female					
Plasma 25(OH) <sub>2</sub> D <sub>3</sub>					
	Total density	Type I density	Type IIA density	Total type II density	Total CSA
All	-0.037	-0.318~	0.295	0.191	-0.066
C/C	0.346	0.181	0.030	0.039	0.073
C/HF	0.148	-0.011	0.670	0.243	0.092
HF/C	-0.745~	-0.833*	-0.446	-0.314	0.797*
HF/HF	0.618	0.035	1.004~	1.714***	-0.277
Soleus - male					
Plasma 25(OH) <sub>2</sub> D <sub>3</sub>					
	Total density	Type I density	Type IIA density	Total type II density	Total CSA
All	-0.112	-0.378*	0.209	0.104	0.315
C/C	0.599	0.537	0.569	-0.451	-1.768*
C/HF	-0.296	0.368	-0.227	-0.395	-0.310
HF/C	0.324	-0.339	-0.068	0.644	-0.023
HF/HF	0.175	-0.422	0.394	0.560	-0.036

**Table A 9: The correlation of 30-week old offspring plasma 25(OH)D<sub>3</sub> to EDL myofibre density and CSA.** 30-week female and male offspring plasma 25(OH)D<sub>3</sub> was correlated against EDL *m.* myofibre density and CSA. Data are correlation coefficient  $r^2$  values. All statistical analyses performed with the mixed effects model on Z-transformed data. \*\*\*P<0.001. \*P<0.05, ~P<0.1; n=7-8 per group. C, control; HF, high-fat; CSA, cross-sectional area; EDL, extensor digitorum longus.

EDL - female						
Plasma 25(OH) <sub>2</sub> D <sub>3</sub>						
	Total density	Type I density	Type IIB density	Type IIX density	Total type II density	Total CSA
All	-0.184	0.089	-0.146	-0.109	-0.256	-0.037
C/C	0.284	0.114	0.117	0.391	0.255	-0.258
C/HF	1.176	-0.305	0.564	0.201	1.420~	-0.856
HF/C	-0.784*	0.260	-0.585	-0.804*	-0.670~	0.573
HF/HF	0.603	-0.771	0.502	0.694	0.469	-0.563
EDL - male						
Plasma 25(OH) <sub>2</sub> D <sub>3</sub>						
	Total density	Type I density	Type IIB density	Type IIX density	Total type II density	Total CSA
All	-0.040	0.145	-0.075	0.059	-0.055	0.029
C/C	-0.409*	0.234	-0.419***	-0.211***	-0.737***	0.045
C/HF	-0.018	0.277	-0.010	0.233	0.140	0.035
HF/C	0.365	0.146	0.311	-0.114	0.274	-0.144
HF/HF	-0.197	0.086	-0.332	0.466	-0.169	0.587~



## Appendix E Abstract 1

*Fetal and Neonatal Physiological Society 2016, University of Cambridge*

### **The effect of a maternal vitamin D depleted diet during gestation on the behaviour and activity of young adult mouse offspring.**

Jones, L. E<sup>1</sup>, Newland, P.L<sup>2</sup>, Cagampang, F.R<sup>1,2</sup>, Poore, K.R<sup>1</sup>, Cleal, J.K<sup>1,2</sup>, Green, L.R<sup>1,2</sup>

<sup>1</sup> Inst. Developmental Sciences, University of Southampton, UK,

<sup>2</sup> Biological Sciences, University of Southampton, UK.

The fetus is reliant on maternal vitamin D, and vitamin D deficiency (VDD) affects a substantial proportion of the human population. Maternal VDD is linked to altered offspring brain development <sup>(1)</sup> and to impaired skeletal muscle structure and function <sup>(2, 3)</sup>. In mice, we tested the idea that a maternal VDD diet would impair activity in young adult offspring.

Female C57BL/6J mice were fed a control (C; 1 IU/g vitamin D<sub>3</sub>) or VDD (0 IU/g vitamin D<sub>3</sub>) diet 6 weeks prior to mating and throughout pregnancy and lactation. Offspring were weaned onto the C diet creating two diet groups: C/C and VDD/C (n=5 and n=7 per group, respectively). Open-field activity was assessed for 5 min in female 15wk offspring. Distance travelled, time ambulatory (period in which a mouse crossed more than 3 photo-beams in any two sec), average velocity, vertical counts (number of times offspring reared onto hind limbs) and number of jumps were recorded. Data are mean ± SEM and were analysed by independent t-test.

VDD/C female offspring displayed a reduction in distance travelled (P<0.001) and time spent in ambulatory behaviour (P<0.01) compared to C/C offspring, but average velocity was not different between groups. Vertical counts and the number of jumps in this time period were significantly lower in the VDD/C compared to C/C offspring (P<0.01).

The observed reduction in offspring activity with a maternal VDD diet may reflect altered anxiety or hind limb strength. Underlying mechanisms could include impaired brain development and neurological function, and altered skeletal muscle fibre type composition, respectively. These changes in response to a maternal VDD diet may have implications for offspring age-related degeneration in neurological function and skeletal muscle strength.

*Supported by The Gerald Kerkut Charitable Trust.*

- (1) Eyles *et al.* Neuroscience. 2003, 118(3):641-653
- (2) Max *et al.* Mol Nutr Food Res. 2014, 58(2):343-352
- (3) Harvey *et al.* J Clin Endocrinol Metab. 2013, 99(1):330-337

## Appendix F Abstract 2

*Physiology 2016 (Dublin, Ireland) (2016). Proc Physiol Soc 37, PCA215*

### **The effect of maternal and post-weaning high-fat diet on markers of skeletal muscle insulin-mediated glucose uptake and growth in adult mouse offspring**

Jones, L. E<sup>1</sup>, Cagampang, F.R<sup>1</sup> Poore, K.R<sup>1</sup>., Cleal, J.K<sup>1</sup>., Newland, P.L<sup>2</sup>., Green, L.R<sup>1</sup>

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Adult obesity is linked to skeletal muscle structure, metabolism and contraction. In the adult female mouse soleus muscle (postural muscle; 37% type I, 63% type II myofibres), the reduction in adult skeletal muscle isometric contraction force by post-weaning (POST) high-fat (HF) diet is minimized by a prior exposure of their mothers to a pregnancy and lactation (PRE) HF diet (1). The POST HF diet was also associated with reduced soleus muscle GLUT-4 and insulin receptor (InsR) mRNA levels (2), which may mediate in part the glucose intolerance previously observed in this model (2) and be linked to muscle contractile capacity. However, muscles are heterogeneous in fibre structure and function, for example, the extensor digitorum longus (EDL) muscle is 96% type II myofibres and is involved in dorsiflexion and extension of the foot and toes. Therefore in this study we determined the expression of GLUT-4 and InsR (markers of insulin-mediated glucose uptake), and Akt-1 (marker of muscle fibre growth and inhibitor of apoptosis) in the EDL muscle.

Female C57BL/6J mice were fed a control (C; 7% kcal fat) or high fat (HF; 45% kcal fat) diet 6 weeks prior to mating and throughout lactation (PRE). Offspring were weaned (POST) onto the same C or HF diet creating 4 different diet groups: C/C, C/HF, HF/C and HF/HF (n=6-8 per group). Female 30-week offspring EDL muscle GLUT-4, InsR, and Akt-1 mRNA levels were measured by RT-qPCR. Data are mean relative gene expression  $\pm$  SEM and were analysed by 2-way ANOVA.

Female offspring EDL muscle GLUT-4 mRNA levels were reduced in POST HF mice regardless of PRE diet (C/HF+HF/HF,  $0.95 \pm 0.02$  vs. C/C+HF/C,  $1.14 \pm 0.04$ ;  $p < 0.001$ ). There was no significant difference in InsR mRNA levels between groups. Akt-1 mRNA

levels were higher in POST HF mice regardless of PRE diet (C/HF+HF/HF,  $1.00 \pm 0.03$  vs. C/C+HF/C,  $0.86 \pm 0.03$ ;  $p < 0.01$ ).

Our finding of lower GLUT-4 mRNA in POST HF diet group EDL muscle is similar to previous observations in the soleus muscle (2) and may contribute to the glucose intolerance in these animals. Unlike the soleus muscle, InsR mRNA levels in EDL muscle were unaltered by diet, highlighting muscle bed heterogeneity in the insulin-mediated glucose uptake pathway. Increased Akt-1 mRNA levels in POST HF EDL muscle suggests an alteration in muscle growth which could impact on whole body glucose homeostasis and muscle contraction in these animals.

*Supported by The Gerald Kerkut Trust and Diabetes UK.*

(1) Jones, L (2015) Sex-specific effects of prenatal and post-weaning high fat diet on adult mouse offspring skeletal muscle contraction, FASEB, 29(1).

(2) Jones, L (unpublished) Pre-natal and post-weaning high fat diet effects on vitamin D status and skeletal muscle function. SRI 2016 conference.

(3) Cagampang, FR (unpublished).

## Appendix G Abstract 3

*Experimental Biology 2015 (Boston, USA)*

*The FASEB Journal, 2015, vol. 29 no. 1 Supplement 123.5*

### **Sex-specific effects of prenatal and post-weaning high fat diet on adult mouse offspring skeletal muscle contraction**

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Maternal obesity is linked to altered offspring skeletal muscle structure and function. We tested the idea that prenatal (PRE) high fat (HF) diet would alter the deleterious effect of post-weaning (POST) HF diet on offspring skeletal muscle contraction.

C57 mice were fed a control (C, 7% kcal) or HF (45% kcal) diet in PRE and/or POST periods (C/C, C/HF, HF/C, HF/HF). Male and female 30 wk offspring (n=7-8/group) soleus and extensor digitorum longus (EDL) muscle peak force (PF) of isometric contraction was measured in response to several electrical stimulation frequencies.

In soleus and EDL, PF was greater in females than males. In female soleus (not EDL), POST HF alone markedly reduced PF (C/HF vs. C/C,  $P < 0.01$ ). In female soleus and EDL there was only a trend for a reduction in PF in HF/C and HF/HF groups ( $P = 0.1$ ). In male soleus and EDL, combined PRE and POST HF tended to reduce PF (HF/HF vs. C/C,  $P = 0.1$ ). In male soleus (not EDL), POST HF tended to reduce PF in PRE HF animals (HF/HF vs. HF/C,  $P = 0.1$ ), and PRE HF tended to reduce PF in POST HF animals (HF/HF vs. C/HF,  $P = 0.1$ ).

Thus reduced adult skeletal muscle isometric contraction force by POST HF diet is modified by a prior PRE HF diet in a sex- and muscle bed-specific manner. This could reflect developmental differences in myofibre type composition or lipid deposition, and has potential implications for muscle health in adult/ageing obese individuals.

*Supported by The Gerald Kerkut Charitable Trust and Diabetes UK.*

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