THE EFFECTS OF OSTEOARTHRITIS, ACUTE VOLUNTARY INACTIVITY AND INJURY ON SKELETAL MUSCLE FUNCTION, Na⁺, K⁺-ATPase CONTENT AND ISOFORM ABUNDANCE

Submitted by

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ABSTRACT

Physical inactivity causes substantial maladaptations in each of skeletal muscle size, strength and endurance. In skeletal muscle, the Na⁺,K⁺-ATPase (NKA) enzyme is pivotal in the regulation of Na⁺ and K⁺ concentrations across the sarcolemmal and t-tubule membranes and hence in the maintenance of muscle excitability. The NKA content in skeletal muscle is increased by chronic physical activity and reduced by some chronic injuries and diseases in humans. Knee osteoarthritis, aging, and anterior cruciate ligament (ACL) injury adversely affects muscle mass, strength, and can lead to a reduction in physical activity. The detrimental functional effects seen in osteoarthritis and ACL injury may be partially attributable to physical inactivity. Hence, this thesis investigated the effects of several conditions and interventions that involve physical inactivity, including knee osteoarthritis (Study 1), voluntary limb unloading (Study 2) and ACL rupture (Study 3) on skeletal muscle function, muscle NKA content and NKA isoform abundance.

Study 1: This study examined the effects of osteoarthritis and aging, conditions associated with physical inactivity, on muscle NKA in thirty-six older-adults (range 55-81 yrs) including 19 with osteoarthritis (OA, 69.9±6.5 yrs, mean ± SD) and 17 asymptomatic controls (CON, 66.8±6.4 yrs). Participants completed knee extensor strength testing and a physical activity questionnaire. A vastus lateralis muscle biopsy was analysed for NKA content ([³H]ouabain binding sites), α₁-3 and β₁-3 isoform protein abundance (immunoblotting) and mRNA (real time RT-PCR). The association between age and NKA content was investigated within the OA and CON groups, and in pooled data. The NKA content was also contrasted between
sub-groups below and above the median age of 68.5 yr. OA had lower strength (-40.8%, p=0.005), but higher NKA α2 (~34%, p=0.006) and α3 (100%, p=0.016) protein abundance than CON and performed more incidental physical activity (p=0.035). No differences were found between groups for NKA content, abundance of other NKA isoforms or isoform gene expression. There was a negative correlation between age and NKA content within OA (r=-0.63, p=0.03) and with both groups combined (r=-0.47, p=0.038). The NKA content was 25.5% lower in the older (69-81 yrs) than in the younger (55-68 yrs) subgroup (p<0.05). Hence older age, but not knee osteoarthritis was related to lowered muscle NKA content in older adults.

**Study 2 (Part I):** This study examined the effects of three weeks of limb unloading using unilateral lower limb suspension (ULLS) and subsequent resistance training on thigh muscle mass, strength, power and balance in healthy young individuals, in both the unloaded (inactive) and weight-bearing legs. Six young adults (age: 22.4± 2.1 yrs; mean ± SD) underwent 23 d of ULLS followed by 4 weeks resistance training. Thigh muscle mass, *vastus lateralis* muscle fibre cross sectional area, knee extensor strength (0-360 °.s⁻¹), lower limb balance and vertical jump height were measured in both legs at baseline, post ULLS and after 4 weeks of resistance training. After ULLS, total thigh mass was reduced by 4.43% (p = 0.047) and average *vastus lateralis* muscle fibre cross sectional area tended to decrease (20.1%, p = 0.057). In the unloaded leg, strength was decreased by 24% (p=0.003) at 0 °.s⁻¹ and by 22% at 60 °.s⁻¹ (p= 0.002). Vertical jump height was reduced by 16% (p=0.041) and postural sway increased by 32-34% in the unloaded leg (p< 0.015). In the weight-bearing leg, strength decreased by 14% (p = 0.015) and 9% (p = 0.001) at 60 °.s⁻¹ and 120 °.s⁻¹,
respectively. All variables altered after ULLS returned to levels similar to baseline after resistance training. Hence, ULLS reduced skeletal muscle mass, strength, power and increased postural sway in the unloaded leg, which were rapidly reversible with subsequent resistance training and caused strength loss in weight-bearing limb. This suggests that not only does unilateral inactivity cause prominent localised impairments in muscle function, but promotes physical detraining manifesting in reduced strength in the weight-bearing limb.

**Study 2 (Part II):** This study investigated the effects of 23 d unilateral lower limb suspension (ULLS) and 4 wks resistance training (post-training) on exercise performance, $K^+$ regulation, skeletal muscle $Na^+$,$K^+$-ATPase content and NKA isoform abundance. Venous $[K^+]$ and $[Lac^-]$, heart rate and rating of perceived exertion (RPE) were measured during incremental one-legged cycling exercise continued until fatigue. Total NKA content ([$^3$H]ouabain binding site content) and NKA isoform abundance ($\alpha_1$, $\alpha_2$, $\alpha_3$, $\beta_1$, $\beta_2$ using immunoblotting) were measured in a resting biopsy from both the weight-bearing and unloaded legs at baseline, after ULLS and after 4 wks resistance training (post-training). Time to fatigue during one legged cycling was reduced by 22.5% after ULLS in the unloaded leg ($p=0.041$). During incremental exercise intensities, heart rate increased by 5.6-11% and RPE increased by 9.2%-17.1% in both the unloaded and weight-bearing leg after ULLS ($p<0.05$). There were no differences in plasma $[K^+]$ or $[Lac^-]$ during and following exercise, skeletal muscle NKA content, or abundance of any of the NKA isoforms, after ULLS or resistance training. After the resistance training, these variables impaired by ULLS were returned to baseline, except RPE. Despite greater fatiguability, short-term ULLS was not associated with impaired $K^+$
regulation or decreased skeletal NKA isoform abundance in healthy humans. This suggests that skeletal muscle NKA and plasma [K⁺] during exercise are resilient to impairment after short-term inactivity, demonstrating a complex and poorly understood regulation of muscle NKA content after inactivity.

**Study 3:** Six participants with anterior cruciate ligament injury (ACL; 4 females, 2 males; age: 25.0 ± 4.9 yrs; duration of injury: 15 ± 17 weeks; mean ± SD) and seven age and BMI matched control participants (CON; 5 females, 2 males; age: 23.3 ± 2.0 yrs; mean ± SD) completed the international physical activity questionnaire (IPAQ), subjective knee function questionnaire (International Knee Documentation Committee; IKDC) and were tested for knee extensor isometric strength, thigh cross sectional area (CSA; anthropometry) and postural sway in both injured and non-injured legs. A resting *vastus lateralis* muscle biopsy was performed on both legs in ACL, one leg in CON and analysed for muscle fibre type distribution and fibre cross sectional area (immunofluorescence), muscle NKA content ([³H]ouabain binding site content) and NKA isoform abundance (α₁-₃ and β₁-₂; immunoblotting). There was no difference in the weekly duration of overall or leisure physical activity between ACL and CON. Knee extensor isometric strength and thigh CSA were 21.7% and 7.1% lower in the ACL injured leg than in the non-injured leg, respectively (p<0.05). In ACL, two-legged postural sway was 43% higher than CON (p = 0.04), but postural sway did not differ between legs in ACL. Muscle NKA content was 20.1% lower in the injured than in the non-injured leg in ACL (p = 0.045) and 22.5% lower than CON (p =0.043). The NKA α₂ abundance was 63% lower in the injured compared to the non-injured leg in ACL (p = 0.032) and there was no difference in the abundance of the α₁,α₃, β₁ and β₂
isoforms between legs or groups. Hence, short-term ACL injury is accompanied by reduced muscle NKA content, \( \alpha_2 \) abundance, muscle function and subjective joint function. These maladaptations after ACL injury may be at least partially attributable to disuse, thus emphasizing the importance of exercise and rehabilitation after ACL injury.

**Conclusions:** This thesis investigated skeletal muscle function, muscle NKA content and isoform abundance in humans with each of osteoarthritis, ACL injury, older age, and after controlled unilateral inactivity (ULLS). Both older age and acute ACL injury, which are associated with longer term disuse, were associated with decreased muscle NKA content, whilst no impairments were found after short-term ULLS and knee osteoarthritis. In contrast, maximal voluntary strength was lower in each of ACL injury, osteoarthritis and ULLS. Hence, while the disuse associated with approximately 15 weeks of ACL injury lowered muscle NKA content with potential adverse implications for muscle function, 23 d of voluntary inactivity caused no change to skeletal muscle NKA content despite impairment of muscle strength, fatigue and mass.
DECLARATION

I, Ben Douglas Perry, declare that the PhD thesis entitled *The effects of osteoarthritis, acute voluntary inactivity and injury on muscle function, Na⁺, K⁺-ATPase content and isoform abundance* is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature       Date:
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This thesis, quite literally a work with blood, sweat and tears (and muscle!), would not be possible without the wealth of help, guidance and friendship I received throughout the time of my PhD candidature. No words I can write here can quite do all the people who helped me justice, but I shall try. Firstly I have to thank my principal supervisor, Prof. Michael McKenna. Your constant guidance, approachability, incredible expertise and focus on producing the highest quality research were invaluable, and I thank you for your patience and friendship. You will remain a mentor for my research life regardless of where I end up. I also must thank my co-supervisors Dr. Aaron Petersen and Dr. Itamar Levinger, who helped on data collection days, reading my many, many manuscripts and gave amazing insight throughout my thesis. Aaron, thank you for your many home brewed beers and friendship. I’m not sure they made the thesis better quality, but they certainly made me better! Itamar, your great presence in the lab and at times horrible jokes always helped.

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ABBREVIATIONS

[ ] concentration

$[^3]H$ouabain binding tritiated ouabain binding

1RM one repetition maximum

3-O-MFPase 3-O-methylfluorescein phosphatase

ACL anterior cruciate ligament

APSD anterior-posterior standard deviation

ATP adenosine 5′ triphosphate

BMC bone mineral content

BMD bone mineral density

BMI body mass index

$Ca^+$ calcium ion

cDNA complementary deoxyribonucleic acid

CGRP calcitonin gene related peptide

Cl$^-$ chloride ion

CON healthy control subjects

COX cytochrome C oxidase
CS  citrate synthase
CSA  cross-sectional area
CT  computerised axial tomography
DXA  dual X-ray absorptiometry
d  days
DHPR  Dihydropyridine receptor
DVT  deep vein thrombosis
EC  excitation-contraction coupling
EDL  extensor digitorum longus
FoxO1  Forkhead box protein
iEMG  integrated electromyography
IGF-1  insulin-like growth factor 1
IPAQ  international physical activity questionnaire
K+  potassium ion
[K+]e  extracellular K+ concentration
[K+]i  intracellular K+ concentration
KE  knee extensors
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>MAFbx</td>
<td>Atrogin 1</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>magnesium ion</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>Muscle ring finger 1</td>
</tr>
<tr>
<td>MVC</td>
<td>maximal voluntary contraction</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>sodium ion</td>
</tr>
<tr>
<td>[Na(^+)](_i)</td>
<td>intracellular Na(^+) concentration</td>
</tr>
<tr>
<td>[Na(^+)](_e)</td>
<td>extracellular Na(^+) concentration</td>
</tr>
<tr>
<td>Na(^+),K(^+)-ATPase</td>
<td>sodium-potassium adenosine 5’triphosphatase</td>
</tr>
<tr>
<td>NKA</td>
<td>Na(^+),K(^+)-ATPase</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>PF</td>
<td>plantar flexor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate ion</td>
</tr>
<tr>
<td>[Lac⁻]</td>
<td>lactate concentration</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>rubidium ion</td>
</tr>
<tr>
<td>RFD</td>
<td>rate of force development</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>t-tubules</td>
<td>tranverse tubules</td>
</tr>
<tr>
<td>ULLS</td>
<td>unilateral lower limb suspension</td>
</tr>
<tr>
<td>VJH</td>
<td>vertical jump height</td>
</tr>
<tr>
<td>( \dot{\text{VO}_2} )</td>
<td>oxygen consumption</td>
</tr>
<tr>
<td>( \dot{\text{VO}_2} ) peak</td>
<td>peak oxygen consumption during exercise</td>
</tr>
<tr>
<td>( \text{VO}_2 ) max</td>
<td>maximal oxygen consumption</td>
</tr>
<tr>
<td>WHO</td>
<td>world health organisation</td>
</tr>
<tr>
<td>wk</td>
<td>weeks</td>
</tr>
<tr>
<td>yrs</td>
<td>years</td>
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CHAPTER 1: INTRODUCTION

The World Health Organisation (WHO) estimates that approximately 60% of the world’s adult population does not perform enough physical activity (WHO, 2004). Physical inactivity is a prominent risk factor for a myriad of non-communicable diseases (Booth et al., 2002) and causes decreased muscle strength, cross-sectional area (CSA) and endurance which can impair quality of life (Kell et al., 2001; Narici et al., 2011). While physical inactivity is often simply due to a sedentary lifestyle, either injury or chronic health conditions can also force muscle disuse and reduced physical activity. The impairment in muscle function after injury may be partially attributable to inactivity-mediated maladaptations in skeletal muscle. Despite the well documented functional impairment of skeletal muscle after inactivity, injury and chronic conditions, the biochemical basis of such changes in human skeletal muscle remains incompletely understood.

The Na⁺,K⁺-ATPase (NKA) enzyme in skeletal muscle has a vital role in muscle excitability via the maintenance of Na⁺ and K⁺ concentrations across sarcolemmal and t-tubular membranes (Clausen, 2003). The NKA is a heterodimer comprising of an α (α₁-3) and β subunit (β₁-3), and the content of NKA is vital for the maintenance of muscle excitability. Skeletal muscle NKA content increases by around 10-20% after exercise training (Clausen, 2003) and decreases by 25-58% after chronic injury including spinal injury and shoulder impingement syndrome (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012). Hence injury, via inactivity, may reduce skeletal NKA content, which may consequently impair muscle excitability, contractility and adversely affect muscle fatigue (McKenna et al., 2008).
However, skeletal muscle NKA content in accompaniment with measures of muscle function have not been investigated after inactivity or injury in humans. This thesis investigated three models of impaired physical activity, these being knee osteoarthritis, voluntary unilateral inactivity and ACL injury in order to investigate the effects inactivity on skeletal muscle NKA and muscle function.

It is unknown whether the impairment of muscle function and reduced physical activity in knee osteoarthritis is associated with reduced skeletal muscle NKA content. Knee osteoarthritis increases in prevalence with older age (Dillon et al., 2006). Hence, Study One investigated the concomitant effects of age and osteoarthritis on skeletal muscle function, NKA content ([³H] ouabain binding site content) and NKA isoform relative abundance (immunoblotting) in mature-aged and elderly knee osteoarthritis patients and age-matched controls.

Inactivity leads to functional impairment of strength, endurance, power and balance (Kouzaki et al., 2007; Narici et al., 2011). Unilateral lower limb unloading (ULLS) is a model of disuse which simulates the disuse seen with injury requiring crutches (Berg et al., 1991). While the impairment of strength and endurance is well described in the unloaded leg, there is sparse research investigating changes in muscle function in the weight-bearing leg and the changes in balance and dynamic exercise endurance after ULLS and re-ambulation in order to better reflect the practical outcomes of disuse seen after short-term use of crutches. In addition, skeletal muscle NKA content has not been investigated after a controlled inactivity intervention and may have some role in the impairment in muscle function seen after disuse. Hence, Study Two (Part I) investigated impairments in muscle
mass, strength, power and balance after 23 d of ULLS and four weeks of subsequent resistance training in both the unloaded and weight-bearing legs, in healthy young adults. In Study 2 (Part II), time to fatigue, exercise heart rate, rating of perceived exertion, muscle NKA content and isoform abundance were investigated following ULLS and subsequent resistance training.

Finally, ACL injury is common during competitive sport and causes substantial impairment in both knee and muscle function, which reduces physical activity and knee-based quality of life (Frobell et al., 2010). Part of the decline in muscle function in ACL injury may be due to muscle disuse, which could cause a reduction in skeletal muscle NKA content. While chronic injury such as spinal injury or shoulder impingement syndrome lowered skeletal muscle NKA content (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012), it is unclear whether shorter term injury (<1 year) lowers NKA content or isoform relative abundance, and whether these changes are due to reduced overall physical activity or disuse specific to the ACL injured leg. Hence, Study 4 investigated knee extensor strength, thigh cross-sectional area, balance, muscle NKA content and relative isoform abundance in both the injured and non-injured leg in young patients with acute ACL injury and in healthy BMI- and age-matched controls.
CHAPTER 2: LITERATURE REVIEW

SECTION I: THE EFFECTS OF PHYSICAL INACTIVITY ON HEALTH, SKELETAL MUSCLE FUNCTION AND EXERCISE PERFORMANCE

This literature review comprises three sections. Section I covers the prevalence, impact and effects of physical inactivity on humans with emphasis on skeletal muscle. Section II discusses the prevalence and musculoskeletal effects of osteoarthritis and anterior cruciate ligament (ACL) injury in humans. In Section III, the function, activation, modulation and practical implications of the Na⁺,K⁺-ATPase (NKA) in skeletal muscle will be discussed with emphasis on exercise and physical inactivity in humans. This literature review focuses on data from humans; although research using animal models has been included when appropriate.

2.1 Impact and prevalence of physical inactivity

The World Health Organisation (WHO) estimates that approximately 60% of the world’s adult population does not perform sufficient physical activity (2004). Physical inactivity is a prominent risk factor for a myriad of non-communicable diseases such as diabetes mellitus, various cardiovascular diseases and certain forms of cancer, as well as contributing to several other prominent disease risk factors including hypertension, high blood glucose levels, high cholesterol and obesity (Booth et al., 2002). Hence, inactivity not only has direct adverse effects on health and health spending (Allender et al., 2007), but contributes to the development of diseases such as type II diabetes and cardiovascular disease. The WHO
concluded that: “Unhealthy diets and physical inactivity are thus among the leading causes of the major noncommunicable diseases” (WHO, 2004)

In Australia, 66.9% of adults were considered physically inactive in results from the national health survey conducted by the Australian Bureau of Statistics (ABS, 2012), which is similar to other developed nations (Oldridge, 2008; Troiano et al., 2008). In Australia, physical inactivity directly contributed to 6.6% of disease and injury in 2003 (Begg et al., 2007). Further, inactivity is a common risk factor among the most prominent mortality causes (Figure 2.1). Physical inactivity has been estimated to cost the Australian economy up to 13.8 billion dollars annually, as well as an average 1.8 day loss of productivity per worker (Medibank-Private, 2008). Hence, inactivity is a prominent burden to health in Australia and worldwide.

**Figure 2.1:** Health burden attributable to physical inactivity by cause expressed as: (A) proportion of diseases caused by inactivity, (B) proportions by sex and (C) fatal and non-fatal outcome proportions in Australia 2003. Figure created and reported by Begg et al. (2007)
2.1.1 Classifying inactivity

An important question to consider in regards to physical inactivity is to establish the volume of exercise/activity that is beneficial for good health in humans. More specifically, how do we classify someone as inactive? The American College of Sports Medicine and the American Heart Association recommend that 30 minutes of moderate intensity exercise five times per week, or 3 intense 20 minute sessions per week are needed to acquire health benefits of exercise and thus not be “inactive” (Haskell et al., 2007). However, a flaw with the methodology of simply classifying a population as “active” based solely on their time exercising is well demonstrated by Hamilton et al., (2008), where hypothetically an office worker attaining this level of exercise (30 minutes of moderate intensity exercise per day) could still be seated for ~15.5 hours per day. Thus, a person who is sedentary for ~23 hours a day would still be labelled as “physically active” if they walked for 30 minutes per day, five d per week. The Australian Diabetes, Obesity and Lifestyle study (Healy et al., 2008b) reported that even in physically active adults who obtained 2.5+ hours of exercise per week, the volume of television watched correlated with systolic blood pressure, waist circumference and fasting plasma glucose (p <0.05). Even intermittent bouts of standing between periods of being sedentary have been shown to be beneficial for systolic blood pressure, waist circumference and fasting blood glucose (Healy et al., 2008a). Thus, the amount of sedentary behaviour, as well as physical activity should be considered for public health policies and guidelines (Hamilton et al., 2008).
2.1.2 Scientific models of inactivity

To characterise the effects of an inactive lifestyle in humans is methodologically difficult; the degree of inactivity and various extraneous factors such as diet and any pre-existing medical conditions can make it difficult to establish inactivity alone as a true cause of various non-communicable diseases. Studies using cross sectional data can draw correlations on attributable causes (Begg et al., 2007) and establish practical risk factors and public health policy, but a true cause and effect relationship requires the use of a valid model to cause a controlled period of physical inactivity.

Several research models in humans have been used to simulate inactivity or microgravity. including bed rest (Taylor et al., 1945; Taylor et al., 1949; Saltin et al., 1968; LeBlanc et al., 1992; Convertino et al., 1997), limb immobilisation (Seki et al., 2001; Farthing et al., 2009; Suetta et al., 2009) and unilateral lower limb suspension (Berg et al., 1991; Hather et al., 1992; Clark et al., 2007). The primary difference in the three approaches used is the affected area; unilateral lower limb suspension and immobilisation typically only affect a single lower limb. The participant is still mobile and the compensatory ambulation using crutches seems sufficient to prevent systematic, whole body cardiovascular and metabolic adaptations seen in complete bed rest (Taylor et al., 1945; Taylor et al., 1949; Chobanian et al., 1974; Hamburg et al., 2007). Space travel research provides fascinating insights into the effects of inactivity (Narici et al., 2011), but the available data and sample size is comparatively limited due to the obvious substantial financial burden attached to spaceflight and restricted access to astronauts. Head-down bed rest has been used to mimic spaceflight (Pavy-Le Traon et al., 2007) as well as forced inactivity as a consequence of
chronic disease. In animal studies, hind-limb suspension and limb immobilisation are the most popular methods to elicit inactivity (Grichko et al., 2000; Siu et al., 2008; Heinemeier et al., 2009; Yang et al., 2009).

2.1.2.1 Unilateral Lower Limb Suspension as a model of inactivity

Unilateral Lower Limb Suspension (ULLS) was first used in research as a model of inactivity in 1991 (Berg et al., 1991). This first model involved the participant completing all ambulatory activity on crutches, while one leg was suspended at slight knee flexion (~10°) using a strap around the shoulder and hip which attached to around the sole of the foot (Figure 2.2A, (Berg et al., 1991)). The suspension of the leg eliminated its weight-bearing role, localising muscular inactivity to the suspended lower limb. This model is not capable of simulating whole body inactivity as done in bed rest, but it does offer a viable alternative research method to investigate localised musculoskeletal and vascular adaptations of inactivity in humans (Berg et al., 1991; Hather et al., 1992; Hotta et al., 2006). Importantly, the ULLS model does offer several distinct advantages compared to bed rest. ULLS allows participants to complete most of their daily activities, hence, it is logistically and financially more viable for research in human volunteers and permits direct comparison of inactive and “active” muscle via contrasting the suspended and non-suspended legs. An alternate “strapless” version of ULLS was first used by Hather et al., (1992); this uses a raised sole (8-12 cm) on the foot of the weight-bearing leg, allowing the suspended leg to hang freely. Figure 2.2b shows the strapless version of ULLS (Tesch et al., 2004).
Figure 2.2: Representations of ULLS (A) represents the original model of ULLS as created by Berg et al. (1991) and (B) represents the alternate “strapless version as depicted by Tesch et al. (2004)

2.1.2.2 Criticisms and risks of ULLS as a model of inactivity

As a model of inactivity, ULLS replicates musculoskeletal maladaptations as seen in bed rest studies, but does not cause the same degree of atrophy or strength loss as observed in limb immobilisation (Adams et al., 2003). Widrick et al. (2002) argued that ULLS causes different contractile adaptations compared to spaceflight and more significantly, bed rest. The group reported that skinned soleus muscle fibres in humans undergoing ULLS had different adaptations in contractile performance, such as altered shortening velocity, compared to individuals who underwent bed rest or spaceflight. This finding, however, is contentious due to inconsistency between the inactivity protocols; the spaceflight participants underwent exercise testing and interventions while the participants undergoing ULLS did not. Additionally the ULLS protocol was done for five d less than the spaceflight and bed
rest protocols. These criticisms and further issues in data interpretation by Widrick et al. (2002) are detailed by Adams et al., (2002). However a vast body of literature supports ULLS as eliciting musculoskeletal and vascular maladaptations in a similar timeframe and magnitude to bed rest (Berg et al., 1991; Adams et al., 2003; Bleeker et al., 2005a; Hotta et al., 2006; Rittweger et al., 2006; de Boer et al., 2007a). One limitation of ULLS as a model of inactivity is its localisation to a single lower limb. This means that many whole body systematic effects seen in bed rest, such as decreased plasma volume (Taylor et al., 1945) and VO₂ peak (Taylor et al., 1949) are unlikely to be seen in this model due to the participants retaining mobility with the aid of crutches and not having a change in orthostatic pressure as noted during bed rest (Pavy-Le Traon et al., 2007).

A risk to participants undergoing ULLS is an increased incidence of deep or superficial vein thrombosis (Bleeker et al., 2004). While only a few participants undergoing ULLS have contracted deep vein thrombosis (DVT), this rate is larger than that of the general population (Bleeker et al., 2004). Thus the risk of DVT must be accounted for in any study utilising ULLS. Bleeker et al., (2004), suggested preventative measures such as compression garments, self massage and passive ankle movement. Further, the use of the blood clotting marker D-dimer and Doppler ultrasound have also been used to detect DVT in its pre-clinical stages (Rittweger et al., 2006). Finally, the ‘strapless’ version of ULLS where the leg hangs freely is likely to have decreased risk of DVT compared to the traditional method as the leg is not restrained at a set joint angle.
2.1.3 Physical inactivity, skeletal muscle function, morphology and architecture

2.1.3.1 Inactivity causes atrophy

Skeletal muscle in mammals comprises multiple fibre types with distinctive functions; Type I fibres are slower contracting, have a higher concentration of mitochondria and oxidative proteins beneficial for aerobic metabolism (Booth et al., 1991). Type II fibres have three sub-types; a, b and x. Type II(a) fibres the most oxidative, and Type II(b) the least oxidative. All Type II fibres have faster contraction speeds and greater concentration of glycolytic proteins compared to Type I fibres, and are more rapidly fatiguable (Hintz et al., 1984; Schiaffino et al., 1989; Booth et al., 1991). Because skeletal muscle enables movement, inactivity has profound adverse effects on skeletal muscle function and metabolism (Pavy-Le Traon et al., 2007; Hackney et al., 2011). One of the most researched and pronounced effects of physical inactivity on the musculoskeletal system is muscle atrophy (Adams et al., 2003; Narici et al., 2011). Whole muscle atrophy of approximately 5% occurs from as little as two weeks of ULLS (Adams et al., 1994; de Boer et al., 2007a), while ULLS for 3-4 weeks caused ~ 7-10% decrease in knee extensor (KE) cross sectional area (CSA) (Berg et al., 1991; Schulze et al., 2002; Clark et al., 2007; de Boer et al., 2007a). Over 5 weeks of ULLS caused a 9-16% reduction in muscle CSA (Dudley et al., 1992a; Hather et al., 1992; Tesch et al., 2004; Caruso et al., 2005); Figure 2.3. No published studies have investigated the effect of ULLS for longer than 42 days. Similar decreases in CSA have been reported in the plantar flexors, with an 8-11% decrease after 4-5 weeks of ULLS (Tesch et al., 2004; Clark et al., 2007) and up to 18-25% decrease after 42 d (Dudley et al., 1992a; Hather et al., 1992).
Figure 2.3: Percentage decrement in knee extensor (KE) cross sectional area (CSA) after different durations of unilateral lower limb suspension (ULLS), bed rest and cast immobilisation. Lines represent linear curve of ULLS (solid line), bed rest (dotted line) and immobilisation (dashed line). Data are mean values from: (Convertino et al., 1989; Berg et al., 1991; Dudley et al., 1992a; Hather et al., 1992; Veldhuizen et al., 1993; Berg et al., 1997b; Kubo et al., 2000; Akima et al., 2001; Hespel et al., 2001; Thom et al., 2001; Schulze et al., 2002; Alkner et al., 2004a; Alkner et al., 2004b; Tesch et al., 2004; Berg et al., 2007; Clark et al., 2007; de Boer et al., 2007a; Cook et al., 2010; Wall et al., 2013).

Bed rest studies have reported similar atrophy of the KE and plantar flexors as ULLS, with 7-10% decrease in CSA or muscle volume in the knee extensors reported after 3-4 weeks (Convertino et al., 1989; Kubo et al., 2000; Akima et al., 2001; Alkner et al., 2004a). Prolonged bed rest increases the degree of atrophy, with as much as 18% reduction in KE...
muscle CSA after 89 d of bed rest (Alkner et al., 2004b) and up to 30% reduction in CSA of the planter flexors (LeBlanc et al., 1992). Limb casting tends to accelerate atrophy compared to both ULLS and bed rest, with 10-14 d of limb immobilisation causing an ~11% decrease in KE CSA (Hespel et al., 2001; Thom et al., 2001), approximately double the amount of atrophy seen in ULLS for similar periods (de Boer et al., 2007a). Further, 28 d of limb immobilisation led to a 21% decrease in KE CSA (Veldhuizen et al., 1993). The increased atrophy noted with immobilisation may be linked to the increased stringency of restriction which limb casts place on participants and potentially the angle that the limb is cast, which is usually ~130-170° knee angle (Booth, 1977).

The degree of atrophy is not uniform across muscle groups during inactivity; Hather et al., (1992) reported over 9% less atrophy in the knee flexors compared to the knee extensors after 6 weeks of ULLS (7% vs 16%, respectively). While this could reflect the role of hamstring activation in stabilising the unloaded limb during ambulation with ULLS, this has also been found with bed rest (LeBlanc et al., 1992). Very prolonged (119 d) bed rest caused no change in upper body mass and no significant change in upper body maximal strength despite substantial decreases in both measures in the lower limbs (LeBlanc et al., 1992). This finding has also been repeated in other bed rest studies, suggesting that because the knee extensors and planter flexors have a more prominent role in resisting gravity, they undergo a greater magnitude of atrophy during inactivity (Hather et al., 1992; LeBlanc et al., 1992; Belavý et al., 2009; Clark, 2009).
2.1.3.2 CT, MRI, DXA and anthropometric measurements of muscle atrophy after inactivity

Most ULLS and bed rest studies have utilised Computerised Tomography (CT), or more commonly in recent years, Magnetic Resonance Imaging (MRI) of the leg to assess muscle cross sectional area (CSA), as they are accurate (Mitsiopoulos et al., 1998) and allow analysis of CSA of multiple muscle groups, but usually only from one site (usually the thickest part of the thigh or calf) of the scanned limb. This limitation of single site CT or MRI scans can be amended by performing serial scans over the entire limb segment to calculate volume (LeBlanc et al., 1992; Ferrando et al., 1995; Kubo et al., 2004). However, studies using thigh volume have reported a similar degree of atrophy as studies that have utilised CSA analysis from a mid-thigh single site (Akima et al., 2001; Kawakami et al., 2001); confirming that thigh CSA measured from a single site is a valid measure of muscle atrophy. Other commonly available scans which can be used to detect muscle loss, such as Dual X-Ray Absorpiometry (DXA scan) cannot differentiate mass of individual muscle groups, but evaluate the change in lean mass of the entire limb segment. A further method used to determine changes in muscle size is cross sectional analysis of individual muscle fibres using histochemical techniques, which allows for individual Type I, Type IIa and Type IIx changes in fibre size, but give no direct measurement of the effect on the whole muscle group. Finally, anthropometric-based estimates of thigh CSA, where thigh circumference and limb adiposity are measured is an inexpensive and convenient method which correlates well (r = 0.97) with CT scan findings (Knapik et al., 1996), but tends to overestimate thigh muscle
CSA. Further these estimates are less reliable than MRI or CT scans due to the possibility of researcher bias and error.

2.1.3.3 **Fibre type specific adaptations to inactivity**

Traditionally, inactivity studies using rodents suggest preferential atrophy of Type I fibres (Thomason et al., 1990; Rennie et al., 2010). However, studies in humans, primarily with the *vastus lateralis* muscle report mixed findings regarding muscle fibre-specific atrophy, with the length and method used to induce inactivity likely to play an important role. Short term (<42 d) inactivity induced by ULLS, bed rest and immobilisation seems not to induce any clear preferential atrophy of Type I or Type IIa/IIx fibres. Short term ULLS studies (16-42 d) reported no significant difference in CSA between Type I, Type IIa and Type IIx despite the differing duration of unloading in each study (Hather et al., 1992; Berg et al., 1993; Adams et al., 1994). Short-term limb casting of 10-14 d also produced no fibre type specific changes, with similar atrophy across muscle fibre types (Hespel et al., 2001; Thom et al., 2001). Conversely, a selective Type II fibre atrophy has been reported after 10 d of cast immobilisation in older participants (Hvid et al., 2010), and during spaceflight (Widrick et al., 1999). Only one report suggests selective Type I muscle fibre atrophy during inactivity; where 37 d of bed rest caused a significant decrease in only Type I fibres (Berg et al., 1997b). Because of these mixed findings it is difficult to establish whether short term inactivity promotes preferential muscle fibre type atrophy in humans. The effect could also be muscle specific, as 12 d of ULLS caused a 7% decrease in Type I fibres, but not Type II fibres in the soleus (Widrick et al., 2002).
Preferential atrophy is more apparent with longer term (over 55 d) inactivity; 84 d of bed rest caused greater reduction in Type I fibres in both the *vastus lateralis* and soleus muscles (Rudnick *et al.*, 2004; Trappe *et al.*, 2004), as did four months of bed rest (Ohira *et al.*, 2000). Hence, longer term inactivity may be associated with preferential atrophy of type I fibres in both the soleus and *vastus lateralis* muscle. However, in humans, muscle CSA loss is likely to be homogenous between muscle fibre types for inactivity periods of less than 5-6 weeks.

The reason for the disparity between the rodent and human findings in regard to preferential muscle fibre atrophy is likely due to the metabolic differences between the species (Rennie *et al.*, 2010) and potentially the relatively limited time humans are exposed to inactivity models. It is worth noting that rodents have both a less stable metabolic environment and increased rate of protein catabolism during inactivity compared to humans (Rennie *et al.*, 2010). While some longer term bed rest studies report a shift to a faster muscle phenotype, this has sparsely been found with short-term inactivity. The findings from long-term studies, however, may be more applicable to chronic inactivity in humans, thus more long-term inactivity research is required assess the possibility of fibre type transition and preferential atrophy in humans.

**2.1.3.4 Changes in muscle fibre type distribution**

In addition to findings of muscle fibre selective atrophy during inactivity, studies using rodents report a prominent transition to a faster muscle phenotype, i.e. increased percentage distribution of hybrid and fast twitch fibres (Thomason *et al.*, 1990). Such fibre changes are of importance, as this could effect muscle metabolism and function
independent of decline in muscle CSA. However, in most studies in humans using short term inactivity (<42 d) via ULLS (Hather et al., 1992; Berg et al., 1993; Adams et al., 1994), immobilisation (Veldhuizen et al., 1993; Yasuda et al., 2005; Hvid et al., 2010) or bed rest (Berg et al., 1997b; Bamman et al., 1998; Desplanches et al., 1998; Ohira et al., 1999) do not support the findings in rodent muscle, with no significant change in Type I or Type IIa/x distribution reported in skeletal muscle. There are exceptions; after 35-day bed rest, a significant decrease in Type I fibres and a concomitant increase in Type IIx fibres was reported in the vastus lateralis muscle (Brocca et al., 2012). Conversely, another bed rest group from the same study which underwent 24 d of bed rest did not have any fibre type transition. The authors suggest this may have been due to the differing baseline percentages of Type I fibres; the 35-d bed rest group had almost double the percentage of Type I fibres compared to the 24-d bed rest group at baseline. Long-term bed rest (84 d) caused an approximate 20% decrease in the percentage of Type I fibres and a 10-20% increase in type II and hybrid fibres in the soleus muscle (Gallagher et al., 2005). Additionally, human single muscle fibres from the soleus muscle after 17 d of bed rest had increased peak force (Widrick et al., 1999) consistent with the concept of a conversion to a faster muscle phenotype. In summary, only longer term inactivity protocols in humans of more than ~2-4 months appear to cause detectable changes in fibre type distribution and muscle fibre specific atrophy of Type I fibres, while short term inactivity causes relatively equal decreases in CSA of both Type I and Type II fibres, with no change in fibre type distribution.
2.1.3.5 Mechanisms of disuse atrophy

On a molecular level, atrophy is caused by a decreased content of contractile proteins (Jackman et al., 2004) such as myosin (Canepari et al., 2009). The loss of contractile proteins in muscle atrophy seen during disuse is caused by a negative balance between protein synthesis and breakdown (Jackman et al., 2004; Zhang et al., 2007; Phillips et al., 2009; Rennie et al., 2010). Pathological atrophy as seen in cancer cachexia or muscle dystrophies are characterised by substantial protein catabolism, as is apparent with disuse atrophy in studies using rodents (Jackman et al., 2004; Rennie et al., 2010). However, in humans, decreased protein synthesis has a more prominent role in the etiology of disuse atrophy (Phillips et al., 2009; Rennie et al., 2010). A broad combination of proteins involved in both muscle synthesis and proteolysis change in response to physical inactivity (Chopard et al., 2009a; Marimuthu et al., 2011). Despite the substantial decline in muscle synthesis during human inactivity, the effect on key proteins for protein synthesis, such as protein kinase B (Akt) and mammalian target of rapamycin (mTOR) during inactivity is unclear. Increased Akt mediated phosphorylation of mTOR in response to stimuli such as insulin-like growth factor 1 (IGF-1) is vital for muscle hypertrophy (Chopard et al., 2009a). Akt and downstream proteins such as mTOR do not appear to be altered during long-term unloading. No change in Akt1 phosphorylation was reported after three (Gustafsson et al., 2010), 10 or 21 d of ULLS (De Boer et al., 2007b). In short term unloading however, the ratio of phosphorylated mTOR/total mTOR decreased after two d of limb immobilisation, but not after two weeks in the same participants (Abadi et al., 2009). Similarly, a decreased ratio of pAkt/Akt and ribosomal S6 phosphorylation was found after 2-4 d of immobilisation (Suetta et al., 2012).
Combined with research demonstrating disassociation between Akt signalling and protein turnover (Greenhaff et al., 2008), it is possible, although unconfirmed, that changes in Akt phosphorylation are transient and may occur in a specific timeframe during inactivity. Overall, the molecular mechanisms which lead to decreased protein synthesis in disuse atrophy are not well understood and are likely due to proteins other than the Akt signalling pathway (Marimuthu et al., 2011). Proteins involved in increased skeletal muscle catabolism such as muscle atrophy F-box (MAFbx; or atrogin 1) and muscle RING finger-1 (MuRF-1) are upregulated in pathological atrophy (Lecker et al., 2004) and in atrophy in rodent muscle (Bodine et al., 2001); but were not found to increase in response to 35 d (Brocca et al., 2012) or 60 d of bed rest in humans (Chopard et al., 2009a), nor were its upstream activating protein forkhead box gene group O (Foxo) altered after short-term ULLS (Sakuma et al., 2009; Gustafsson et al., 2010). However, several studies have reported upregulation of MAFbx and MuRF-1 with immobilisation in humans, primarily in the early stages (2-4 days) of immobilisation (De Boer et al., 2007b; Abadi et al., 2009; Bunn et al., 2011; Suetta et al., 2012), but the importance of increased MAFbx and MuRF-1 expression with disuse atrophy are likely to be minimal since human disuse atrophy is characterised predominantly by decreased protein synthesis (De Boer et al., 2007b; Mallinson et al., 2013).

**2.1.4 Effect of inactivity on muscle strength, endurance, power and proprioception**

**2.1.4.1 Inactivity reduces muscle strength**

One of the most prominent functional detrimental effects of inactivity is the decrement in maximal voluntary contraction (MVC), including both isometric MVC and isokinetic MVC...
measured at slow speeds of contraction (30-60°.s\(^{-1}\)). From as little as seven d of ULLS, knee extensor (KE) isometric strength at 95° knee flexion decreased by 15% (Deschenes et al., 2008). Longer durations of ULLS caused a greater decrement in strength, with 20-30 d of ULLS causing between 21-42\% decrease in isometric (knee angles of 95-60° knee flexion) and at 60°.s\(^{-1}\) isokinetic KE MVC (Dudley et al., 1992b; Ploutz-Snyder et al., 1995; Schulze et al., 2002; Clark et al., 2007; de Boer et al., 2007a). This is comparable to bed rest, where 20\% decrement in KE isometric MVC at 110-60° knee flexion was found with a similar timeframe (Dudley et al., 1989; Kubo et al., 2000; Kubo et al., 2004). Longer timeframes of inactivity accentuate strength decline, with 90 d of bed rest causing a 45\% decrease in isometric KE MVC (Alkner et al., 2004b), while in a separate study 119 d of bed rest caused only ~30\% decrease in isokinetic KE MVC at 90° knee flexion (LeBlanc et al., 1992). Together with the shorter term bed rest studies, it is apparent that the rate of strength decrement during bed rest is highest in the first few weeks of inactivity. Immobilisation seems to cause greater decrement in MVC compared to both ULLS and bed rest in short timeframes, with 22\%-52\% decrement in isometric (110-60°) and isokinetic KE MVC at 60°.s\(^{-1}\) seen from 10-28 d (Veldhuizen et al., 1993; Hortobagyi et al., 2000; Hespel et al., 2001; Thom et al., 2001). This may be due the increased restriction immobilisation places on the participant and the angle at which the knee is immobilised. Figure 2.4 reports voluntary strength decrements seen with ULLS, bed rest and immobilisation.

Most findings in regard to KE strength and inactivity report either isometric, slow speeds of concentric contraction (such as 60-120°.s\(^{-1}\)), or averaged strength decrement over several slower speeds. While peak torque has been noted to decline uniformly at KE speeds below
180 °.s⁻¹, the decline is not greater at any specified speed below 180 °.s⁻¹ after varied durations of ULLS (Berg et al., 1991; Deschenes et al., 2002), bed rest (Dudley et al., 1989; Berg et al., 1997b) or immobilisation (Labarque et al., 2002). Although, a ten day ULLS study did not find any significant reduction in KE strength at 120°.s⁻¹ (Deschenes et al., 2008), suggesting that at least in short-term unloading, decrements only occur at slower-speeds of muscle contraction. However, it is important to note that impairment of maximal KE torque at faster speeds of contraction may happen in older populations in response to inactivity. Four days of immobilisation and 10 d of ULLS caused strength loss at speeds over 120 °.s⁻¹ in participants over 65 yrs (Deschenes et al., 2008; Hvid et al., 2014).
Figure 2.4: Percentage decrement in knee extensor (KE) isometric MVC with different durations of unilateral lower limb suspension (ULLS), bed rest and immobilisation with MVC measured between the positions of 95-60° knee flexion. Lines represent linear curve of ULLS (solid line), bed rest (dotted line) and immobilisation (dashed line). Data are mean values from: (Berg et al., 1996; Bamman et al., 1998; Hortobagyi et al., 2000; Hespel et al., 2001; Kawakami et al., 2001; Deschenes et al., 2002; Schulze et al., 2002; Alkner et al., 2004b; Kubo et al., 2004; Tesch et al., 2004; Clark et al., 2007; de Boer et al., 2007a; Deschenes et al., 2008; Mulder et al., 2009; Wall et al., 2013).

Apart from the KE, MVC of other muscle groups are also impaired. The plantar flexors are an important postural muscle and undergo marked decline in MVC during inactivity. Three to four weeks of ULLS caused a 10-17% decrease in plantar flexor maximal strength (Schulze et al., 2002; Clark et al., 2006a; Clark et al., 2007; Seynnes et al., 2008b). A similar response
was seen in bed rest, with a 20-21% decrease in plantar flexor MVC after 20-28 d (Portero et al., 1996; Kubo et al., 2004). Immobilisation seems to have a slightly accelerated strength loss compared to both bed rest and ULLS, with 22-24% decrease in plantar flexor MVC seen after only 2-3 weeks of immobilisation (White et al., 1984; Davies et al., 1987). The knee flexors also undergo maladaptation in MVC with inactivity, but they are minimal compared to the declines in the KE and plantar flexors. Two weeks of ULLS and immobilisation caused an average 10% decrease in knee flexor strength across various isokinetic contraction speeds (Deschenes et al., 2002; Labarque et al., 2002) and 30 d of bed rest caused no significant change in knee flexor strength across a range of contraction speeds (Dudley et al., 1989). Atrophy of various other lower limb muscles also suggest strength decrements in the adductors, hip/gluteal muscles and dorsi flexors after inactivity (Belavý et al., 2009), while changes in upper body strength were minimal after inactivity; 119 d of bed rest caused less than a 5% change in both elbow flexor and extensor strength (LeBlanc et al., 1992).

In conclusion, short-and longer-term physical inactivity via immobilisation, ULLS or bed rest causes substantial maladaptations in KE and planter flexor strength, with more minor changes occurring in other muscle groups. As shown in Figure 2.4, the decrease in muscle strength is related to the duration of inactivity, but in the KE and plantar flexors muscle groups the rate of strength decline seems to slow after ~30 d of bed rest and ULLS (Narici et al., 2011).
2.1.4.2 Physiological mechanisms of decreased strength

The underlying cause of decreased strength/MVC during inactivity is multifaceted and likely dependent on the length of inactivity. An obvious cause of decreased strength with inactivity is muscle atrophy, but bed rest or ULLS for less than seven d generally causes no significant muscle atrophy yet results in reduced KE MVC (Berg et al., 1996; Deschenes et al., 2002; Deschenes et al., 2008). Even in longer durations of inactivity where significant atrophy occurs, the degree of strength loss is disproportionate to the decrease in muscle CSA. For example, 14 and 23 d of ULLS caused a 15% and 21% decline in KE MVC, respectively, despite only a 5% and 10% decrease in muscle CSA (de Boer et al., 2007a). Such disproportionate loss of strength, where strength loss is often double the magnitude of atrophy, has consistently been reported with ULLS with timeframes over 2 weeks (Berg et al., 1993; Schulze et al., 2002; Tesch et al., 2004; Clark et al., 2007; Cook et al., 2010), as well as bed rest (LeBlanc et al., 1992; Kawakami et al., 2001; Alkner et al., 2004b; Kubo et al., 2004) and immobilisation (Veldhuizen et al., 1993; Hespel et al., 2001; Thom et al., 2001). These findings indicate that atrophy alone does not account for all of the strength loss seen after inactivity and additional mechanisms must exist.

Impairment of the muscle contractile apparatus measured on a single fibre level provides insight into the causes of potential maladaptations to inactivity. Twelve days of ULLS caused a 5 and 8.7% decrease in normalised force (peak force/CSA) of Type I muscle fibres from the soleus and gastrocnemius muscles, respectively (Widrick et al., 2002). During bed rest, normalised force decreased by 40% after 42 d and 42% after 56 d of bed rest (Larsson et al., 1996a; Yamashita-Goto et al., 2001). Normalised peak force of single muscle fibres
decreased by 47% after 84 d of bed rest (Trappe et al., 2004) and similar findings have been reported with 3.5 months of immobilisation (D’Antona et al., 2003). These results indicate reductions in single fibre force can occur independently of changes to muscle fibre CSA during inactivity and may be due to reduced fibre density and myosin/cross bridge content (Larsson et al., 1996a; Widrick et al., 2002; D’Antona et al., 2003).

Decreased neural drive and excitability strongly contribute to the declined muscle strength seen during inactivity. Clark et al. (2006b) reported that 48% of the strength loss with 28 d of ULLS was due to neural factors; they reported lower limb-specific changes in the Hoffman reflex (H-reflex) and increased M-wave latency (altered neural conduction) of the plantar flexors, indicative of decreased central drive. This notion is supported by Seynnes et al. (2008a) who report increased H-reflex and decreased T-wave reflex sensitivity in response to 23 d of ULLS. Seven d of ULLS caused decreased central drive as measured via diminished plantar flexor integrated electromyography (iEMG) in both young and old participants (Deschenes et al., 2008), as did 14 d of ULLS in young participants (Deschenes et al., 2002; Seynnes et al., 2008b). Reduced central drive is also evident during bed rest, with a reduction in EMG amplitude after 42 and 90 d of bed rest (Berg et al., 1997b; Alkner et al., 2004b). Increased unsteadiness during movement, implicating decreased coordination was also found after 28 d of ULLS (Clark et al., 2007). Neural changes, including decreased central drive, are likely responsible for the majority of strength loss during short term (<14 d) unloading (Deschenes et al., 2002; Deschenes et al., 2008) and also play a prominent role in strength loss during longer term inactivity (Berg et al., 1996; Alkner et al., 2004b; Clark et al., 2006b).
Architectural changes to connective tissue such as tendons after unloading also contributes to decreased maximal torque generation. A decrease in muscle tendon stiffness will likely adversely affect the rate of force development, due to increased tendon elasticity during muscle contraction (Wilkie, 1949; Narici et al., 2011). Tendon stiffness was increased by 19% with chronic resistance training (Kubo et al., 2002), but during ULLS, tendon stiffness decreased by 10% and 29%, after 14 and 23 d, respectively (de Boer et al., 2007a). In the Achilles tendon, a 10.4% decrease was found in tendon stiffness and a 29% decrease in the distal aponeurosis stiffness after 28 d of ULLS (Kinugasa et al., 2010). Twenty d of bed rest caused similar maladaptations, with a 28-32% decrease in tendon stiffness (Kubo et al., 2000; Kubo et al., 2004), while 90 d of bed rest lead to a remarkable 57% decrease in tendon stiffness (Reeves et al., 2005). Increased pennation angle, or the angle which pennated muscle exerts force, increases in response long-term resistance training (Kawakami et al., 1995) and correlates to muscle mass in bodybuilders (Kawakami et al., 1993). A change to pennation angle changes the mechanical properties of the joint and would alter maximal torque (Kawakami et al., 1993; Kawakami et al., 1995); 14 d of ULLS caused 3% decrease in pennation angle (de Boer et al., 2007a; Seynnes et al., 2008b), while 23 d of ULLS caused a 7.6% decrease (de Boer et al., 2007a).

2.1.4.3 Inactivity reduces vertical jump height and rate of force development

Vertical jump height (VJH), which associates with muscle power (Buehring et al., 2011) and rate of force development declines after inactivity. Twenty-four days of ULLS caused a 28% decrease in single-leg jump height, whilst during bed rest jump height decreased by 28% after 56 d (Buehring et al., 2011) and 32.1% after 90 d (Rittweger et al., 2007). A simulated
special forces exercise which involved soldiers lying prone caused a 10% decrease in vertical jump height after only eight days (Thorlund et al., 2011). Inactivity also decreases the rate of force development (de Boer et al., 2007a; Rittweger et al., 2007; Thorlund et al., 2011). In single human fibres; 84 d of bed rest caused a 23% decrease in single fibre contraction velocity (Trappe et al., 2004). Together these findings demonstrate that inactivity decreases VJH and the rate of force development, likely due to an interplay of factors including atrophy, decreased strength, neural drive and changes in muscle architecture.

2.1.4.4 Muscle function and CSA after re-ambulation from inactivity

The recovery from maladaptations caused by inactivity, such as decreases in muscle mass, strength and power have been investigated with various durations of ULLS, bed rest and immobilisation. The first ULLS study by Berg et al. (1991) found that 50% of the strength which was lost after 28 d of ULLS returned within 4 d of re-ambulation, while both strength and thigh CSA fully returned within seven weeks of re-ambulation. A 45% decrease in knee extensor strength after immobilisation was only 12% lower than pre-inactivity levels after two weeks re-ambulation and training, while muscle CSA returned after 12 weeks (Hortobagyi et al., 2000). Such rapid recovery of strength supports the concept that strength loss during inactivity is only partially caused by atrophy; ten d of ULLS caused 13.6% decrease in KE strength, which was completely ameliorated after 4 d of re-ambulation (Berg et al., 1996). This recovery was rapid as 10 d of ULLS was not sufficient to cause significant thigh muscle atrophy. The impairment of muscle strength, power and CSA after 2-3 weeks of immobilisation and ULLS were completely returned to baseline after only three weeks of re-ambulation and resistance training (Hespel et al., 2001; Campbell et al.,
Conversely, power lost after 90 d of bed rest required 140 d of re-ambulation to recover to baseline (Rittweger et al., 2007) and strength lost after 117 d of bed rest did not return within eight weeks of ambulation (LeBlanc et al., 1992). Thus, after short-term inactivity without atrophy, complete strength return is rapid, suggesting neural adaptations (Berg et al., 1996), whilst in longer term disuse, the restoration of muscle CSA and muscle architecture (pennation angle, tendon stiffness and fascicle length) is likely a substantially longer process (Hather et al., 1992; LeBlanc et al., 1992; Rittweger et al., 2007). Together these findings suggest that the rate of recovery is slower after long-term bed rest compared to short-term ULLS or immobilisation, although there is a lack of long-term inactivity research. It is worth noting that the rate of muscle CSA and strength increase caused by re-ambulation/training after inactivity (Berg et al., 1991; LeBlanc et al., 1992; Hespel et al., 2001) is substantially greater than that of training alone in healthy young individuals (Folland et al., 2007).

2.1.5 Inactivity impairs balance

The effect of inactivity on ambulative function such as balance and gait is sparsely researched. Increased postural sway was found in two participants after 30 d bed rest (Dupui et al., 1992). Later research with a larger sample size confirmed that 20 d bed rest increased postural sway by 29-40%. Furthermore, these changes were actually unrelated to muscle mass, as participants who performed resistance training during bed rest had the same maladaptation to postural sway (Kouzaki et al., 2007). Similarly, 14 d of bed rest without countermeasures increased postural sway in both the anterior-posterior and medio-lateral directions (Sarabon et al., 2013). Despite these changes in postural sway after
inactivity, knee proprioception was not altered after 30 d of bed rest (Bernauer et al., 1994), suggesting that proprioception at the ankle joint could be altered by inactivity (Sarabon et al., 2013). No research has investigated the effect of single limb immobilisation or ULLS on postural sway; thus it is unknown whether such localised inactivity models produce the same results as bed rest.

2.1.6 Inactivity, muscle fatiguability and exercise performance

Inactivity reduces skeletal muscle endurance, potentially due to increased muscle fatigability. Twenty-eight to thirty days of ULLS caused a 17-24% decline in knee extensor endurance using two separate endurance protocols (Berg et al., 1993; Cook et al., 2010). Cook et al. (2010) tested muscle endurance via a time to fatigue test using constant pace (180 °.s⁻¹) knee extension at 40% of MVC, while Berg et al. (1993) reported the decrease in power over three sets of knee extensions. Further, 21 d of ULLS caused 13% decrease in work performed during 30 maximal knee extensions (Schulze et al., 2002). Similar findings have been found after bed rest; 28 d bed rest reduced time to fatigue by 43% of the plantar flexors at 50% of MVC (Portero et al., 1996) and after seven weeks of bed rest, fatiguability measured by the decrease in maximal isometric torque per minute of exercise was increased by 29% (Mulder et al., 2007). Similar reductions have been found with more complex exercise performance after ULLS; a 10.4% decrease in one-legged cycling duration and 11% decrease in VO_2 peak was found after 20 d ULLS (Sato et al., 2010). The impairment of muscle endurance after inactivity is relatively prolonged; seven weeks of re-ambulation after four weeks of ULLS was not sufficient to decrease knee extensor endurance to pre-inactivity levels, despite knee extensor maximum torque and muscle cross
sectional area returning to baseline (Berg et al., 1991). Hence, while it is apparent that ULLS decreases muscle endurance at the same absolute torque or workload, it is less clear whether there is a direct decrease in muscle fatiguability per se after ULLS. Further, the potential biomolecular mechanisms behind decreased muscle endurance and potentially fatiguability after inactivity are not well understood.

2.1.6.1 Cardiovascular effects of physical inactivity

The cardiovascular effects of inactivity vary depending on the model used, specifically whether the inactivity is whole-body or localised to a single limb. During complete unloading such as bed rest and spaceflight, profound cardiovascular changes occur including reduced plasma volume (Taylor et al., 1945; Greenleaf, 1984; Fortney et al., 1994) stroke volume (Perhonen et al., 2001) and red blood cell mass (Fortney et al., 1994). These ultimately cause reduced $\dot{V}O_2$ max (Taylor et al., 1949; Convertino, 1997). Changes in plasma volume particularly contribute to the decreased $\dot{V}O_2$max after bed rest; the thoraco-cephalic fluid shift and resulting increase in diuresis and natriuresis caused by 6° head-down bed rest ultimately leads to a 10-15% decrease in plasma volume (Pavy-Le Traon et al., 2007). While bed rest causes similar musculoskeletal adaptation to both ULLS and limb immobilisation, the maintenance of an upright posture and ability to ambulate with crutches during ULLS and immobilisation makes cardiovascular adaptations, such as changes in plasma volume seen during bed rest, less likely to occur with these inactivity models. A detailed review of cardiovascular adaptations to bed rest and the underlying mechanisms can be found elsewhere (Pavy-Le Traon et al., 2007).
Despite the localisation of ULLS and immobilisation to a single limb, which preclude most central cardiovascular effects seen during bed rest or spaceflight, some subtle maladaptations to peripheral and central cardiovascular function have been noted. Twenty days of ULLS caused a 11% decrease in one-legged VO2peak (Sato et al., 2010) and an elongated fast phase of oxygen uptake during incremental exercise (Hotta et al., 2006). The findings were accompanied by no change in heart rate, leading the authors to suggest that fibre type changes and less oxidative metabolism in the muscle potentially caused such changes; reduced citrate synthase activity in skeletal muscle has been reported after both ULLS and bed rest (Hikida et al., 1989; Berg et al., 1993). Localised inactivity also causes peripheral vascular maladaptations. Femoral artery diameter was reduced by 12% after four weeks of ULLS and was accompanied by reduced calf muscle blood flow at rest (Bleeker et al., 2005a). These changes were reversed four weeks after ULLS and are consistent with changes to femoral artery diameter after bed rest (Bleeker et al., 2005b). The vascular maladaptations were specific only to the unloaded limb, as 12 d of immobilisation decreased popliteal and femoral artery diameters only in the immobilised leg (Rakobowchuk et al., 2011). It is unlikely that muscle microvasculature, such as capillary density, is diminished after localised inactivity, with research either showing no change (Berg et al., 1993), or increased (Hather et al., 1992) capillary density after ULLS or bed rest. The concomitant decrease in muscle fibre size may mask potential changes in capillary density after localised inactivity (Hather et al., 1992).
2.1.6.2 Underlying mechanisms for impaired muscle endurance and exercise performance after inactivity

Physical inactivity has drastic effects on skeletal muscle leading to decreased endurance and potentially increased muscle fatigability (Berg et al., 1993; Mulder et al., 2007; Cook et al., 2010). Inactivity impairs oxidative metabolism in skeletal muscle; mitochondrial activity such as measured by Citrate Synthase (CS) and Cytochrome C oxidase (COX) abundance was reduced after 14 d of immobilisation, as was several various transcriptional genes involved in mitochondrial biogenesis and function (Abadi et al., 2009). Similarly, 10 d of immobilisation caused a 19% decrease in mitochondrial volume (Nielsen et al., 2010). The effect on metabolic fuel sources in the muscle are less clear; while some studies report no decrease in total glycogen or creatine after short term inactivity in humans (Stanish et al., 1982; Hespel et al., 2001), a 54% decrease in intramuscular glycogen stores was found after 10 d of immobilisation (Nielsen et al., 2010). The change in oxidative and anaerobic metabolism in muscle after inactivity affects substrate utilisation during exercise. Increased venous blood [Lac\(^{-}\)] and respiratory exchange ratio (RER) were seen after as little as 3 d of immobilisation and 10 d of bed rest (Katkov et al., 1979; Convertino et al., 1986; Smorawiński et al., 2001). Lactate transport capacity in skeletal muscle may also be impaired, as reported in rats after 4 weeks hindlimb suspension (Dubouchaud et al., 1996); although this would theoretically reduce or slow the accumulation venous blood [Lac\(^{-}\)] in response to exercise, not lead to an increased blood [Lac\(^{-}\)]. Hence, further human research is required to confirm how inactivity causes increased blood [Lac\(^{-}\)] during exercise.
Inactivity may also impair several of the important steps of excitation-contraction (EC) coupling in skeletal muscle; the physiological series of events responsible for muscle contraction. As little as three d of immobilisation in humans caused a decrease in skeletal muscle sarcoplasmic reticulum Ca\(^{2+}\) uptake with no change in Ca\(^{2+}\)-ATPase activity (Thom et al., 2001). The Na\(^+\),K\(^+\)-ATPase (NKA) enzyme, which is vital for the maintenance of intracellular and extracellular Na\(^+\) and K\(^+\) gradients required for muscle contraction, decreases in content in animals undergoing both inactivity and denervation (Clausen et al., 1983; Kjeldsen et al., 1986; Leivseth et al., 1992; Jebens et al., 1995) and was lowered by 25-58% in humans with spinal injury and shoulder impingement (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012). The role of inactivity alone in humans via any controlled inactivity intervention, including bed rest, immobilisation or ULLS on NKA content and its potential association to muscle function and plasma [K\(^+\)] regulation during exercise have yet to be investigated. Hence, the effects of ULLS and subsequent resistance training on skeletal muscle NKA content, muscle function and exercise performance are investigated in Chapter 5.
SECTION II: INACTIVITY, OSTEOARTHRITIS AND ANTERIOR CRUCIATE LIGAMENT INJURY

2.2 Physical inactivity in osteoarthritis and ACL injury

A prominent cause of physical inactivity is chronic disease or injury. Short-term injuries such as broken bones, acute and chronic illness and spinal injury all substantially decrease physical activity. Hence, it is important to understand the degree to which physical activity is reduced in illness and injury, and the impacts this inactivity could have on health, muscle function and physiology additional to the injury or illness alone. This section will describe the prevalence and potential effects of two conditions associated with inactivity, short-term ACL injury in young adults and knee osteoarthritis in older adults. These common disorders generally present various degrees of functional limitation to physical activity and populations with either ACL injury or osteoarthritis are more sedentary than the general population. Hence, these conditions present a clinically relevant insight into the effects of physical inactivity in skeletal muscle across young and mature aged humans.

2.2.1 Osteoarthritis

2.2.1.1 Etiology, prevalence, health burden and risk factors of knee osteoarthritis

Osteoarthritis is a highly prevalent joint condition which can cause joint pain, stiffness and in some cases disability, usually due to complex inflammatory responses or catabolic degradation of articular cartilage (Sandell et al., 2001; Lawrence et al., 2008; Michael et al., 2010). The knee is the most common joint affected by osteoarthritis (Michael et al., 2010) and osteoarthritis is the leading cause of inactivity and disability in the elderly (Felson et al., 1998; March et al., 2004). In Australia, osteoarthritis affects around 3 million people, or
approximately 15% of the adult population and the incidence increases to 47% of adults over the age of 65 yrs (March et al., 2004). This is similar to the prevalence of OA in other developed nations such as the USA (Felson et al., 1987; Dillon et al., 2006). Knee osteoarthritis can have a dramatic effect on daily functional tasks such as walking up and down stairs, sitting and standing (Creamer et al., 2000). Hence, knee osteoarthritis increases the prevalence of physical inactivity (Creamer et al., 2000; Naal et al., 2010) and subsequently increases co-morbidity from various factors, including cardiac disease and obesity (Kadam et al., 2004; van Dijk et al., 2008). This leads knee osteoarthritis to be a prominent financial burden on the health system, with estimates that musculoskeletal injuries including osteoarthritis account for between 1-2.5% of gross national product in developed nations such as Australia, USA, France and UK (March et al., 1997).

The development of knee osteoarthritis has been associated with multiple lifestyle, health and pathophysiological factors. The most prevalent lifestyle risk factors for osteoarthritis (OA) include higher body mass index/obesity, previous knee injury, gender, occupation and sporting physical activity (Felson et al., 1997; Cooper et al., 2000; Murphy et al., 2008b). Obesity, or increased BMI, has been repeatedly shown to be one of the most prominent risk factor for knee osteoarthritis, likely due to the increased load placed on the joint (Felson et al., 1997; Cooper et al., 2000; Murphy et al., 2008b). For instance, a BMI of over 36 has been estimated to increase the risk of knee osteoarthritis by 13.6% (Coggon et al., 2001). While a less prominent factor, previous knee trauma also increases the risk of OA, with 6% higher incidence of osteoarthritis after hip or knee surgery when participants were over 65 years of age (Gelber et al., 2000). Females not only have a higher incidence of osteoarthritis
(7% more likely than males), but will also tend to have greater severity of symptoms (O'Connor, 2006; Murphy et al., 2008b). The role of prior physical activity in the development of osteoarthritis is unclear; former elite weightlifting and soccer athletes had greater incidence of knee osteoarthritis compared to a non-athletic population, independent of surgery (Kujala et al., 1995), as do those who worked in an occupation with heavy physical load (Sandmark et al., 2000; Manninen et al., 2002; Yoshimura et al., 2004). Moderate amounts of prior physical activity, however, do not seem related to osteoarthritis, suggesting that only heavy competitive exercise or occupation-based joint loading increase knee osteoarthritis risk rather than physical activity per se (Cooper et al., 1999; Hootman et al., 2003; Bosomworth, 2009).

2.2.1.2 Osteoarthritis, physical limitation and exercise

Knee osteoarthritis can greatly limit joint function and ability to perform daily tasks (Creamer et al., 2000). Hence, exercise prescription in knee osteoarthritis patients is challenging; while intense exercise may temporarily worsen symptoms of osteoarthritis, a properly prescribed and monitored exercise program actually decreases pain and increases physical functioning in people with knee osteoarthritis (Fransen et al., 2008). Patients with knee osteoarthritis have to overcome functional limitations to exercise, as they typically have decreased muscular strength, flexibility and increased acute pain during exercise (Messier et al., 1992; Slemenda et al., 1997; Focht et al., 2002). A 20% lowering of knee extensor strength standardised for muscle mass seen in a large sample (n = 462) of knee osteoarthritis participants suggests that at least part of the weakness in knee osteoarthritis may be due to joint dysfunction, although the role of prior physical inactivity also has to be
considered (Slemenda et al., 1997). This joint dysfunction and reduced strength also impair basic gait parameters, such as gait cadence and velocity, suggesting joint strength is of functional significance (Messier et al., 1992; Al-Zahrani et al., 2002). While pain may acutely increase in knee osteoarthritis patients in some forms of exercise, especially high intensity exercise (Focht et al., 2002), long term training actually decreases knee osteoarthritis related pain and various exercise modalities are well tolerated by osteoarthritis patients (Fransen et al., 2008).

Despite the clear benefits of exercise to patients with knee osteoarthritis, they remain less active than the general population (Creamer et al., 2000; Naal et al., 2010; Lee et al., 2012). A comprehensive systematic review by Naal et al. (2010) indicated that both knee and hip osteoarthritis patients performed inadequate exercise time per week, including substantially less than 10,000 steps per day. However, there were exceptions, with four studies using accelerometers reporting over 10,000 steps per day in osteoarthritis patients (Wallbridge et al., 1982; Walker et al., 2002; Heisel et al., 2004; Heisel et al., 2008). This suggests that while osteoarthritis patients are generally less active than the general population, each cohort of participants should be assessed for physical activity, as many osteoarthritis participants may indeed be physically active. There are multiple factors which are associated with physical inactivity in knee osteoarthritis patients, the most commonly reported and prominent factors being increased age, obesity/BMI, and greater knee dysfunction and pain (Lee et al., 2012; Veenhof et al., 2012). Hence, osteoarthritis patients are at particular risk of poor health outcomes, due to the effects of both the condition itself and the increased likelihood of physical inactivity. This decreased physical activity
associated with knee osteoarthritis has important implications for aerobic power and cardiovascular health (Creamer et al., 2000; van Dijk et al., 2008; Naal et al., 2010). Thirty-seven end-stage knee osteoarthritis patients had 37.5% lower VO₂ peak compared to matched controls and a trend for increased manifestation of coronary heart disease (Philbin et al., 1995), which is in agreement with epidemiological data reporting increased cardiovascular co-morbidities in knee osteoarthritis patients (Kadam et al., 2004; van Dijk et al., 2008).

Age is another important factor to consider when investigating the physical activity, health and exercise capacity in people with knee osteoarthritis. Increased age not only increases the incidence of knee osteoarthritis, but is also associated with more severe osteoarthritis symptoms (Felson et al., 1997; Cooper et al., 2000; Murphy et al., 2008b). Independent of knee osteoarthritis, dramatic declines in muscle strength, size and cardiovascular endurance are seen with ageing, especially above 60 years of age (Deschenes, 2004). Muscle strength decreases by around 15% per decade after 60 years of age, mostly due to sarcopenia (Larsson et al., 1979; Lindle et al., 1997; Deschenes, 2004). This sarcopenia also partially accounts for the decline with VO₂ max seen with age (Fleg et al., 1988), where a 5-10% decline occurs per decade, depending on training history (Dehn et al., 1972; Fleg et al., 1988; Rogers et al., 1990). Interestingly, not all the strength decline with age is due to sarcopenia, suggesting other neural and/or biomolecular changes in muscle with age (Deschenes, 2004). Hence, an elderly end-stage knee osteoarthritis patient may have complex concomitant detrimental effects on skeletal muscle strength and function from
ageing, osteoarthritis and inactivity; highlighting the potential complexity in investigating the effect of knee osteoarthritis on skeletal muscle.

### 2.2.1.3 Osteoarthritis and biochemical changes to skeletal muscle

The detrimental effects of osteoarthritis on skeletal muscle are likely predominantly because of physical inactivity. The varied effects of inactivity on skeletal muscle and muscle biochemistry are detailed in Section I. Research investigating maladaptive molecular changes in skeletal muscle in response to osteoarthritis is sparse. In patients with unilateral hip osteoarthritis, the rate of force development (RFD) and maximal voluntary contraction (MVC) was substantially less on knee extensors of the arthritic limb, which was negated when RFD and MVC were normalised to leg CSA, suggesting disuse mediated this effect (Suetta et al., 2007). This strength loss and atrophy of relevant muscle groups due to disuse is supported in both knee and hip osteoarthritis (Fisher et al., 1997; Stevens et al., 2003; Rasch et al., 2007). Vastus lateralis muscle fibres from knee osteoarthritis patients exhibited selective atrophy of Type II fibres, typical of long-term disuse or immobilisation (Sirca et al., 1980; Fink et al., 2007) and had smaller muscle fibres than standard muscle fibre CSA guidelines (Reardon et al., 2001; Fink et al., 2007). At a molecular signalling level, increased myostatin and IGF-1 mRNA content (signalling associated with disuse) were substantially higher in hip osteoarthritis patients compared to controls (Reardon et al., 2001). Intriguingly, vastus lateralis muscle from knee OA patients requiring surgery exhibited higher inflammatory factors which could be associated with atrophy, including JNK1/2, IL-6 and STAT 3 (Levinger et al., 2011a). It is not possible to ascertain whether this increased
inflammation was due to disuse, osteoarthritis or a combination of both (Levinger et al., 2011a).

To conclude, it is apparent that osteoarthritis of the knee and hip exhibit prominent hallmarks of disuse and inactivity in skeletal muscle on both a molecular and whole muscle scale. There is no research however investigating what effect osteoarthritis has on contractile proteins vital for excitation-contraction coupling in skeletal muscle to the author’s knowledge; including NKA content. Hence, while osteoarthritis likely has some degree of effect on muscle mass, strength and fatiguability due to inactivity; how this manifests on a biomolecular level in skeletal muscle is poorly understood and is investigated in Chapter 3.

2.2.2 Anterior Cruciate Ligament Injury

2.2.2.1 Prevalence, causes and functional effects of ACL injury

The anterior cruciate ligament (ACL) is an important ligament required for the stability of the knee during movement (Figure 2.5). Acute damage or complete rupture of the ACL has considerable effects on knee stability and lower limb function (Miyasaka et al., 1991). ACL injury is relatively common in Australia and while the true incidence of ACL injury is unknown, approximately 10,000 people per year have ACL reconstructive surgery after sustaining an ACL injury, many of which occur during popular sporting or physical activities such as skiing, football, netball and soccer (Janssen et al., 2012). A recent study from New Zealand report that over 65% of ACL injuries were sustained playing sport (Gianotti et al., 2009), with other common causes including vehicle trauma, falls and workplace associated
trauma (Miyasaka et al., 1991; Gianotti et al., 2009). While the biomechanical mechanism for ACL damage can vary considerably, the conventional mechanism for ACL tear is rapid deceleration or turning of the knee (Noyes et al., 1989). ACL injury commonly occurs with other trauma to the knee such as meniscus damage, potentially exacerbating the physical limitation placed on the patient (Smith et al., 2001) and also increasing the risk of knee osteoarthritis in later life (Daniel et al., 1994; Roos, 2005; Murphy et al., 2008b).

Figure 2.5: Basic knee anatomy showing ACL ligament and related structures. Image from the American Academy of Orthopaedic Surgeons (http://orthoinfo.aaos.org/topic.cfm?topic=a00297l; accessed on February 27th 2013)
The functional limitations placed on a knee with ACL injury varies, but may include near full functioning (termed ‘copers’), including the ability to hop and perform pivoting or agility tasks (Barber et al., 1990; Rudolph et al., 2000) and in rare instances patients can still play some competitive sports without reconstructive surgery (Kostogiannis et al., 2007). Conversely, ACL injury can also heavily restrict knee function and patient mobility (termed ‘non-copers’), with many participants being unable to perform agility or turning tasks, hop and they also display altered gait (Berchuck et al., 1990; Wexler et al., 1998; Rudolph et al., 2000; Chmielewski et al., 2005; Roberts et al., 2007). Prominent functional restriction caused by ACL injury is common, with two-thirds of ACL injured athletes not returning to their competitive sport within 12 months of injury.

2.2.2.2 Factors influencing the functional outcomes of ACL injury

A complex interplay of factors including secondary knee injury, neuromuscular control of the knee and quadriceps strength dictate the functional outcomes of an ACL-injured or deficient participant (Williams et al., 1988; Smith et al., 2001; Williams et al., 2005b). One of the most important considerations on the functional effect of ACL injury is secondary injuries to the knee, such as damage to meniscus and articular cartilage (Finsterbush et al., 1990; Daniel et al., 1994; Murrell et al., 2001). Secondary meniscus and cartilage damage is common, with approximately one-third of ACL injured patients having some degree of secondary damage (Finsterbush et al., 1990). This is important as the meniscus have an important role in stabilisation and proprioception of the knee (Aagaard et al., 1999).

An important consideration for the functional coping of a patient with ACL injury is the stabilisation of the knee. While joint laxity measures alone are a poor indicator of function
after ACL injury per se (Snyder-Mackler et al., 1995; Patel et al., 2003; Kocher et al., 2004), patients with poor functional coping, such as decreased strength and inability to perform hopping typically exhibit poor knee stability (Chmielewski et al., 2005; Roberts et al., 2007). The strength of the quadriceps is critical for knee stabilisation. In a cohort of ‘copers’ and ‘non-copers’, the function of the knee in the ‘non-copers’ was associated with decreased quadriceps strength; while the ‘copers’ had only minimal strength decrement in the injured leg (Williams et al., 2005b). This finding was supported by Roberts et al. (2007), who reported greatly decreased strength relative to the non-injured leg in ‘non-copers’ who were unable to perform a hopping task. Strength training of the quadriceps in rehabilitation after ACL injury also improves knee function and ability to return to physical activity (Mikkelsen et al., 2000; Palmieri-Smith et al., 2008). The decrement in quadriceps strength during ACL injury is likely due to disuse atrophy caused by the injury (Lorentzon et al., 1989; Williams et al., 2005b) and decreased neural activation (Urbach et al., 1999; Urbach et al., 2001; Konishi et al., 2002; Williams et al., 2005b), which is also commonly seen during physical inactivity (Narici et al., 2011). These inactivity-induced maladaptations may be further exacerbated by the avoidance of quadriceps activation during gait in ACL injured patients (Berchuck et al., 1990; Wexler et al., 1998). Knee extensor strength, however, may also be decreased due to reduced motor recruitment, specifically increased antagonist (hamstring) activation (Alkjaer et al., 2012). Taken together, these findings suggest that knee extensor strength is a strong mediator of the functional impact of ACL injury (Palmieri-Smith et al., 2008).
While it is estimated that 84% of ACL injured patients show decreased quadriceps strength compared to the contra-lateral leg (Wilk et al., 1994), the degree of strength loss is highly variable with ACL injury. In a cohort of 16 participants, the injured leg produced 37.5% less isometric maximal torque at 90° knee flexion than the non-injured leg, but this decrease was less as the relative knee flexion joint angle decreased (Makihara et al., 2006). Similarly, a 25% decrease in knee extensors strength in ‘non-copers’ was measured at 90° knee flexion (Williams et al., 2005a), but only an 8% decrease in knee extensor isokinetic strength measured at 60 °.s$^{-1}$ (Keays et al., 2001). Knee extensor weakness after ACL injury is accompanied by muscle atrophy, with between 4.5%-13% lesser quadriceps CSA in the ACL injured leg (Lorentzon et al., 1989; Williams et al., 2005a). Further, a tendency was reported ($p = 0.1$) for decreased CSA of type II muscle fibres and significantly lower ratio of Type II/Type I CSA in eight ACL injured patients who had undergone muscle atrophy compared to a cohort of non-atrophied ACL deficient participants (Baugher et al., 1984). ACL-injured participants also have greater atrophy of both Type I and Type II muscle fibres when compared to more minor knee injuries (Nakamura et al., 1986), and when compared to the non-injured leg (Lorentzon et al., 1989).

A secondary factor which may reduce knee stability and knee function after ACL injury is the proprioception of the knee. ACL injury is well documented to cause maladaptation in knee proprioception (Barrack et al., 1989; MacDonald et al., 1996) and decreased knee proprioception after ACL injury is associated with decreased strength, knee stability and performance in hopping tasks in ‘non-copers’ (Roberts et al., 2007). Decreased knee proprioception also has functional consequences to postural balance; balance during single-
legged stance balance was worse in an ACL injured leg compared to the non-affected limb and was associated decreased knee proprioception, greater joint laxity and decreased quadriceps strength (Ageberg et al., 2005; Lee et al., 2009). However, the extent to which knee extensor strength could mediate changes in proprioception is not known and a recent review concluded that decreased knee proprioception has only a low to moderate effect on knee function after ACL injury (Gokeler et al., 2012).

2.2.2.3 Biomolecular changes to skeletal muscle in response to ACL injury

No research has directly characterised the effect of ACL injury on contractile or mitochondrial-related genes or proteins; likely due to the great variability in physical functioning and difficulty standardising physical activity between ACL injured patients. While such adaptations to skeletal muscle are likely to be similar to those seen during bed rest or immobilisation (see Section I), the inflammation in the ACL injured knee (Higuchi et al., 2006; Cuellar et al., 2010) may have some additional effects. Further, the degree of functional limitation with ACL injury will likely have some role in the skeletal muscle maladaptations. Characterising such maladaptations could be vital to establish a threshold of physical activity which could preserve muscle contractile function for those with ACL injury and allow for greater mechanistic insight into the biochemical changes that occur in skeletal muscle response to inactivity. This thesis therefore investigated the effects of ACL injury and the consequential muscle disuse on skeletal muscle NKA and muscle function in Chapter Six.
SECTION III: ROLE OF MUSCLE Na⁺, K⁺-ATPase IN FATIGUE, TRAINING AND INACTIVITY

2.3 The process of muscle contraction: E-C coupling

In mammalian cells, the initiation of muscle contraction occurs via a series of events initiating from the central nervous system. After an action potential (AP) propagates down the axon from a higher nerve centre such as the brain or spinal cord, it reaches the synaptic gap, where an influx of Ca²⁺ causes the release of the neurotransmitter acetylcholine in order to cause excitation of the subsequent motor end plate and thus the skeletal muscle (Ríos et al., 1991). The AP is then further propagated throughout the muscle along the sarcolemma by the end plate potential and via by the t-tubules, where the voltage sensitive dihydropyridine (DHPR) receptors interact with the ryanodine (RyR) receptors in the terminal cisternae of the sarcoplasmic reticulum (SR), causing the release of Ca²⁺ into the cytoplasm of the muscle fibre (Franzini-Armstrong et al., 1997), allowing muscle contraction via the sliding filament process and cross bridge cycling involving the myosin and actin proteins as described elsewhere (Huxley, 2004). There are several complex steps in the peripheral nervous system preceding motor end plate depolarisation in skeletal muscle which directly mediates skeletal muscle contraction, such as the frequency of action potentials, e.g. temporal summation, and the number of motor units recruited, which directly impact the amount of muscle cells used in skeletal muscle contraction. More specific description of the process of muscle contraction can be found elsewhere (McArdle et al., 2010). An AP is propagated by changes to the charge of the muscle cell, which is dictated by the intracellular and extracellular concentrations of sodium (Na⁺), chloride (Cl⁻)
and potassium (K⁺). In skeletal muscle, during resting conditions, Na⁺ channels are normally closed in order to maintain resting membrane potential (~90 mV). The opening of voltage gated Na⁺ channels causes a rapid depolarisation of the membrane, which then propagates the action potential along the membrane. After the Na⁺ channels have closed, the voltage-gated K⁺ channels open to cause K⁺ efflux into the extracellular space in order to return the membrane potential to a resting state.

2.3.1 Physiological importance of potassium

Potassium is a vital element in biological systems. In humans, the vast majority of K⁺ is located intracellularly, with skeletal muscle containing 225 times the content of K⁺ than that of plasma (Kjeldsen, 2010). The maintenance of intracellular ([K⁺]ᵢ) and extracellular ([K⁺]ₑ) concentration is vital to maintain membrane potential, which is subsequently important for muscle contraction. In humans and other mammals, normal plasma [K⁺] is between 4 to 4.5 mmol/L. Clinically low [K⁺], or hypokalaemia is defined as below 3.5 mmol/L (Kjeldsen, 2010) and high [K⁺], or hyperkalemia is over 5 mmol/L (Evans et al., 2005). Skeletal muscle plays a vital role in the regulation of K⁺, with muscle storing ~96% of the body’s K⁺ (Kjeldsen, 2010). This skeletal muscle ‘reservoir’ of K⁺ allows diversion of K⁺ content to vital tissues such as the brain and heart when dietary K⁺ intake is low and the ability of the muscle to uptake K⁺ is vital for the short term maintenance of plasma K⁺ homeostasis when diet or exercise-induced increases in K⁺ occur. Over approximately 6 hours plasma K⁺ regulation is primarily meditated via renal K⁺ reabsorbton or excretion and dietary intake.

Short term challenges to K⁺ homeostasis such as that presented during physical exercise require a rapid cellular response. In skeletal muscle, an individual AP only increases [K⁺] in
the extracellular space by approximately 2-10 µM\(^+\) depending on muscle fibre type (Sejersted et al., 2000; Clausen, 2003; Allen et al., 2008). However, the frequency of AP combined with the volume of muscle cells recruited during muscle contraction leads to an efflux of K\(^+\) which can rapidly increase \([K^+]_e\) in the muscle interstitium to levels between 11-15 mmol/L during intense muscle contraction (Green et al., 1999b; Green et al., 2000; Juel et al., 2000b; Mohr et al., 2004). Because of the potential for rapid K\(^+\) accumulation, various cellular mechanisms exist in order to reverse the accumulation of K\(^+\). The most responsive and important protein underlying these cellular mechanisms is the ubiquitously expressed Na\(^+\),K\(^+\)-ATPase, or Na\(^+\)/K\(^+\) pump which was first described by Jens Skou in 1957 (Skou, 1957). The Na\(^+\),K\(^+\)-ATPase (NKA) actively transports 3 Na\(^+\) ions into the extracellular space, while transporting 2 K\(^+\) ions into the cell and is activated within seconds (Clausen, 2003). Apart from maintaining muscle excitability, NKA also plays a vital role in regulating cellular volume and assists in transport of glucose and lipids against their transport gradients in other tissues (Skou et al., 1992).

### 2.3.2 Ionic regulation during exercise

Muscle contraction causes a displacement of ions such as K\(^+\), Na\(^+\) and Cl\(^-\) in skeletal muscle (McKenna et al., 2008). Muscle [K\(^+\)] decreases by ~22% during intense exercise (Sjøgaard et al., 1985), with accompanying increases in interstitial K\(^+\) as high as 11-15 mmol/L from a resting value of ~4 mmol/L (Green et al., 1999b; Green et al., 2000; Juel et al., 2000b; Mohr et al., 2004). The increase in [K\(^+\)]_e in arterial plasma is to a lesser magnitude, with [K\(^+\)] doubling from resting levels up to ~8 mmol/L during brief and very high intensity exercise (Medbø et al., 1990). Conversely, while plasma Na\(^+\) concentration ([Na\(^+\)]) increases during
exercise (Barchi et al., 1977), a concomitant loss of plasma volume could mask an actual decrease in [Na\(^+\)] by ~ 8% in the interstitial space during exercise in humans (McKenna, 1992; Street et al., 2005). Intracellular Na\(^+\) concentration ([Na\(^+\)]\(_{i}\)) can increase up to 4-fold (from 6 to 24 mmol/L) after intense exercise (Sjøgaard et al., 1985). Drastic increases in [Na\(^+\)], have also been reported in electrically stimulated isolated rat muscle preparations (Everts et al., 1992; Everts et al., 1994). Due to plasma fluid loss during exercise, there is little change in plasma Cl\(^-\) concentration during exercise (Lindinger et al., 1992; Böning et al., 2007) and measures of interstitial Cl\(^-\) during exercise have not been reported to the authors knowledge. Conflicting evidence exists however in regards to intracellular Cl\(^-\) concentration during and after exercise (Lindinger et al., 1987; Kowalchuk et al., 1988; Lindinger et al., 1988), with concentration seeming to vary depending on muscle fibre types and activation patterns (McKenna et al., 2008).

2.3.3 Potassium and fatigue

Extracellular accumulation of K\(^+\) during muscle contraction may have an inhibitory effect on muscle excitability, implicating high muscle [K\(^+\)]\(_{e}\) during exercise as a potential cause of muscle fatigue. Several in vitro studies using isolated rat muscle report that incubation in high [K\(^+\)]\(_{e}\), such as over 10 mmol/L, causes an inhibitory effect on tetanic force compared to control samples incubated at 4 mmol/L, for both EDL and soleus muscles (Cairns et al., 1997; Nielsen et al., 1998). In isolated rat muscle, force decline caused by one minute of 60 Hz stimulation at 10 mmol/L [K\(^+\)]\(_{e}\) was ameliorated when NKA was activated via epinephrine, salbutamol or pre-loading of [Na\(^+\)]\(_{i}\) (Clausen et al., 2007). The role of K\(^+\) fluxes in the development of fatigue in humans is more complex and likely affected by other
cellular adaptations to exercise. For example, mechanically skinned single fibres from rat EDL muscle treated with 20 mmol/L lactate increased the critical \([K^+]_e\) at which force decline began to occur by 2 mmol/L or 15%, suggesting lactic acid accumulation, or more precisely \(H^+\) accumulation could offset fatigue caused by high \([K^+]_e\) (Pedersen et al., 2004; Pedersen et al., 2005). This was due to reduced \(Cl^-\) channel opening and thus reduced \(Cl^-\) conductance, allowing higher \([K^+]_e\) before stimulated muscle force was inhibited. While such findings highlight the complexity and potential multifaceted cause of fatigue in muscle, they do not eliminate perturbations in ions such as \(K^+\) as a potential contributor to fatigue in humans during exercise. In humans, acute leg exercise preceded by arm exercise led to a more rapid accumulation of interstitial \(K^+\) and development of fatigue (Nordsborg et al., 2003). The high interstitial \(K^+\) seen during exercise of up to 12-15 mmol/L is similar to the critical \([K^+]_e\) for muscle force decline noted in isolated muscle preparations. It is unknown, however, how much muscle \(Cl^-\) conductance is altered during exercise in humans and a critical \([K^+]_e\) in exercising muscle is not known in vivo, as fatigue has been demonstrated to occur at a broad range of interstitial \([K^+\)]. Hence, in humans high interstitial \(K^+\) or \([K^+]_e\) alone is unlikely to directly cause fatigue, but may still be important when considered with other ionic, environmental and metabolic factors (McKenna et al., 2008). For example, in rat soleus muscle, when extracellular \(Na^+\) ([Na+]_e) was decreased and \([K^+]_e\) increased simultaneously there was a 50% decrease in tetanic force (Overgaard et al., 1999), where as only a 10% decline was seen when either [Na+]_e was increased, or [K+]_e was increased to the same extent. Importantly, the alterations in [Na+]_e and [K+]_e used by the above research were within the ionic concentrations seen during fatigue in skeletal muscle (Green et al.,
In conclusion, the inhibiting effects of high [K\(^+\)]\(_e\) can be reversed or even enhanced by ionic fluxes of Na\(^+\) and Cl\(^-\); combined fluxes of ions need to be taken into consideration rather than investigated separately. Since NKA plays a primary role in the regulation of both Na\(^+\) and K\(^+\) concentration inside and outside muscle cell, NKA is likely to have an important contribution to muscle fatigue. However, it is important to acknowledge that there are several other potential causes of fatigue in muscle, such as impaired sarcoplasmic reticulum function due to accumulation of inorganic phosphates, which are beyond the scope of this review and discussed elsewhere (Allen et al., 2008).

### 2.3.4 Structure and function of NKA

The Na\(^+\),K\(^+\)-ATPase (NKA) is a P-type ATPase heterodimer protein consisting of an α and β subunit (some tissues express also express a γ subunit), which translocates 3 Na\(^+\) ions out of the cell and 2 K\(^+\) ions into the cell per molecule of ATP hydrolysed. The α subunit is referred to as the catalytic subunit where both the Na\(^+\) and K\(^+\) are exchanged and ATP is hydrolysed. The β subunit has a regulatory role in modulating NKA activity and is also implicated in mobilisation and routing of NKA into the cell membrane (Clausen, 2003). The NKA also has a site for binding of cardiac glycosides, presumably for endogenous digitalis-like compounds (Hamlyn et al., 1996; Crambert et al., 2002), which can also be targeted pharmacologically (Gheorghiade et al., 1997; Clausen, 2003). The structure of NKA is described in Figure 2.6.
2.3.4.1 Subunits and isoforms of NKA

The catalytic α subunit of NKA constitutes the majority of the NKA heterodimer (Figure 2.6), at a molecular weight of 112 kDa. The process of how NKA exchanges Na⁺ and K⁺ is well established (Post et al., 1972; Glynn, 1993). Before activation, NKA is accessible to the interior of the cell and have a higher affinity for Na⁺ ions, which with ATP present causes 3Na⁺ to be trapped in the NKA. The energy produced from the hydrolysis of an ATP then causes a conformational change in the shape of the protein. After this conformational change, the NKA is now accessible to the extracellular space and the pump loses its affinity
for Na⁺, causing the release of Na⁺ ions into the extracellular space. When the NKA is accessible to the extracellular space, it has a higher affinity for K⁺ ions, causing 2K⁺ ions to become trapped in the protein. The NKA then undergoes another conformational change reversing then to be accessible to the cell interior, then releasing the 2K⁺ ions into the cell and returning the NKA to its original state. Figure 2.7 is a simplified diagram of the above procedure as described by Glynn (1993).

![Figure 2.7: Schematic of the processes involved in Na⁺ and K⁺ exchange in the Na⁺,K⁺-ATPase (NKA) (Glynn, 1993).](image)

While the β subunit (~38 kDa) of NKA is not directly involved in the process of Na⁺ and K⁺ occlusion and exchange, it does have a vital regulatory role in NKA and other P-type ATPases. The β subunit of NKA is responsible for the transport of the maturing NKA to and
insertion into the cell membrane and plays a role in preventing cellular degradation of the NKA (Geering, 2001). The correct routing and unfolding into the cell membrane seems to influence the enzymatic properties of the α unit (Hasler et al., 1998; Geering, 2001; Kristensen et al., 2010). The γ subunit, or FXDY accessory protein(s) is present in most tissues; and is associated with activation of NKA (Béguin et al., 1997). In skeletal muscle, FXYD1 (phospholemman) has an important regulatory role on NKA activity (Crambert et al., 2002; Crambert et al., 2003).

Both α and β subunits of NKA have several distinct isoforms with four α isoforms (α₁, α₂, α₃ and α₄) and three β isoforms: (β₁, β₂ and β₃) known to exist in mammalian tissues (Blanco et al., 1998). The α isoforms are expressed tissue-specifically, with each isoform seeming to have a tissue specific function (Blanco et al., 1998; Kristensen et al., 2010). In skeletal muscle, the existence of α₁ and α₂ has been conclusively shown in humans (Hundal et al., 1994; Murphy et al., 2008a; Kristensen et al., 2010) and in rats (Fowles et al., 2004). The α₃ isoform is found in low abundance in human skeletal muscle (Hundal et al., 1994; Murphy et al., 2004; Kristensen et al., 2010). It should be noted that the α₄ isoform is expressed primarily in the testes (Shamraj et al., 1994; Lingrel et al., 2003) and sperm (Hlivko et al., 2006), although some evidence suggests it does exist in human muscle (Keryanov et al., 2002). Further discussion on the α₄ isoform is beyond the scope of this review due to its extremely low, if any, content in skeletal muscle.

The β₁, β₂ and β₃ isoforms of NKA are all present in skeletal muscle in rats (Arystarkhova et al., 1997; Fowles et al., 2004). Whilst β₁ and β₂ are consistently reported in human skeletal muscle (Hundal et al., 1994; Juel et al., 2000a; Murphy et al., 2007), some studies did not
detect the expression of the β2 isoform (Hundal et al., 1994; Juel et al., 2000a; Kristensen et al., 2010). This has been refuted though by several studies which have reported the expression and content of both the β2 and β3 isoform in human skeletal muscle (Murphy et al., 2004; Murphy et al., 2008a). The family of FXYD (1-7) proteins is expressed tissue specifically when associated with the NKA. In skeletal muscle FXYD1 (phospholemman; PLM) is the only FXYD protein present and is an important acute activator of NKA during muscle contraction.

The function of each individual isoform is poorly understood. In Xenopus oocytes, a cell from a genus of frog, all of the isoform combinations which were expressed were found to be functional (Crambert et al., 2000). The α1 subunit has traditionally been thought to play a ‘housekeeping’ role, due to its location primarily in the sarcolemma and its higher affinity for both Na+ and K+ compared to the α2 isoform during resting conditions (Crambert et al., 2000). Further, the α1 isoform is the most abundant isoform in cardiac muscle, whereas the α2 isoform is the most abundant in skeletal muscle (Orlowski et al., 1988; Hansen, 2001). However, partial knockout of α1 in mice decreased muscle contractile strength (Lingrel et al., 2003), indicating some role in muscle contraction. Conversely, the α2 isoform makes up ~75-85% of NKA in rat EDL muscle (Hansen, 2001) and muscle NKA content is increased by chronic physical exercise in humans and rodents (Kjeldsen et al., 1986; Green et al., 1993; McKenna et al., 1993; Green et al., 1999a). Research using mice confirms the importance of the α2 isoform for exercise and contractile force. Partial global knockout which caused a 50% reduction in α2 impaired contractile force in mouse isolated muscle (Lingrel et al., 2003), while mice with muscle-specific knockout of α2 had remarkably decreased muscle
strength, endurance and exercise tolerance (Radzykevich et al., 2013). In mice with complete global knockout of either α₁ or α₂ isoform, the mice had either invalid formation of the embryo or death moments after birth (Lingrel et al., 2003). The role of the α₃ isoform is unknown in skeletal muscle.

The function of the individual β isoforms with NKA is not clear. The β₁ isoform tends to have a higher affinity to Na⁺ compared to β₂, regardless of the α isoform of the NKA (Crambert et al., 2000), suggesting that like α₁, the β₁ may be primarily utilised in basal conditions. It should be noted however that the above differences in Na⁺ and K⁺ affinity with NKA isoforms are altered by muscle contraction/exercise, likely due to the phosphorylation of PLM (Juel, 2009; Cirri et al., 2011; Juel et al., 2013). Hence, investigating the function of each NKA isoform via Na⁺ affinity alone without the presence or phosphorylation of PLM is not appropriate in vivo. Overall, there is very limited evidence conclusively demonstrating the separate functions of the β NKA isoforms in skeletal muscle.

2.3.5 Localisation of NKA

Using vanadate facilitated [³H]ouabain, which only detects functional NKA heterodimers, the presence of NKA is extensive in both the sarcolemma and the t-tubules of skeletal muscle in various species (Clausen, 2003). While a high concentration of NKA has been confirmed in the sarcolemma (Clausen et al., 1974; Venosa et al., 1981; Clausen, 2003), the methodological problems in isolating the t-tubules for vanadate facilitated ouabain binding have led to some uncertainty quantifying the content of NKA in t-tubules of skeletal muscle. Initial research suggested the existence of NKA in t-tubules using isolated frog single fibres, where Na⁺ and K⁺ gradients were maintained despite the removal of the sarcolemma.
(Costantin \textit{et al}., 1967). Lau \textit{et al}.
(1979) used isolated t-tubular membranes from rabbit and confirmed an ATP-driven transport of Na\(^+\) into the t-tubules, congruent with the concept of the presence of NKA in the t-tubules. Venosa \textit{et al}.
(1981) attempted to quantify the amount of NKA in t-tubules using vanadate facilitated ouabain binding and only found a 20% decrease in ouabain binding when the t-tubule NKA were inhibited, suggesting that 20% of NKA were in the t-tubules. However, the methodology used by Venosa and Horowicz (1981) involving glycerol pre-treated muscle has been argued to have underestimated the quantity of NKA in the t-tubules (Clausen, 2003). Several studies support the concept of similar overall content of NKA in the t-tubules compared to the sarcolemma in various animal muscles (Barchi \textit{et al}., 1977; Mitchell \textit{et al}., 1983; Hidalgo \textit{et al}., 1986). Further, western blotting of the \(\alpha_2\) isoform, the most abundant isoform in skeletal muscle NKA (Hansen, 2001) has confirmed that approximately 50% of the \(\alpha_2\) isoforms are located in the t-tubules when measured using membrane fractionation (Kristensen \textit{et al}., 2010). When the considerably larger area of the t-tubules is taken into account however, the sarcolemma does have a much higher total content of NKA in comparison to the t-tubules (Clausen, 2003).

\subsection*{2.3.5.1 Localisation of NKA isoforms}

The NKA \(\alpha_1\) isoform is expressed almost entirely in the sarcolemma of both soleus and EDL in rats (Kristensen \textit{et al}., 2010) and also in human soleus muscle as confirmed by histological measures (Hundal \textit{et al}., 1994). In addition, \(\alpha_1\) has consistently been reported to be 2-4 fold higher in rat oxidative muscle compared to glycolytic muscle (Thompson \textit{et al}., 1996; Juel \textit{et al}., 2001; Fowles \textit{et al}., 2004; Kristensen \textit{et al}., 2010). This result was not
confirmed in humans, with no difference in \( \alpha_1 \) abundance found between human Type I and Type II single fibres (Thomassen et al., 2013).

The NKA \( \alpha_2 \) isoform constitutes ~75-85% of all \( \alpha \) isoforms of the NKA in rat EDL muscle (Hansen, 2001). Several studies using rats have found greater \( \alpha_2 \) abundance in the EDL muscle compared to the soleus by as much as 50% (Clausen et al., 1982; Bundgaard et al., 2002; Musch et al., 2002). In human single fibres, Type I fibres had 37% less \( \alpha_2 \) abundance (Thomassen et al., 2013). Conversely, other studies found either no difference, or contradictory findings in regard to \( \alpha_2 \) abundance between fast and slow twitch muscle fibres in rats (Chin et al., 1993; Fowles et al., 2004). Fowles et al. (2004) found that the EDL muscle had ~40% greater \([^3]H\)ouabain binding site content compared to the soleus, yet puzzlingly reported no significant difference using western blotting, despite that the \([^3]H\)ouabain binding assay only detects the \( \alpha_2 \) isoform in rat muscle. There was a significant decrease in \( \alpha_2 \) abundance using both methodologies in the white gastrocnemius compared to the red portion (Fowles et al., 2004), although the rats were older than those used by many of the studies finding higher \( \alpha_2 \) abundance in the EDL (Clausen et al., 1982; Bundgaard et al., 2002; Musch et al., 2002). With only limited human research, conflicting findings in rats, different methodologies of quantifying NKA and varying ages of rodents used; it is not at this time definitively clear whether the \( \alpha_2 \) isoform is preferentially expressed between skeletal muscle fibre type. It is likely however that Type II muscle fibres have a higher relative \( \alpha_2 \) abundance, as this has been reported in human single muscle fibres (Thomassen et al., 2013) and has been consistently supported by findings in rat muscle (Clausen et al., 1982; Bundgaard et al., 2002; Musch et al., 2002). Within skeletal
muscle, approximately half of the α₂ isoforms were found in the sarcolemma and calveolae membranes, while the remaining α₂ isoforms were found throughout the t-tubule system (Hundal et al., 1994; Lavoie et al., 1995; Williams et al., 2001; Kristensen et al., 2010).

The β₁ NKA isoform is found in a higher abundance in oxidative compared to glycolytic muscle fibres, as well as in soleus compared to the EDL in rats (Fowles et al., 2004; Zhang et al., 2006), while the β₂ isoform has a higher relative abundance in the EDL and glycolytic fibres compared to the soleus and oxidative fibres, respectively. The localisation of phospholemman, which regulates the activity of the NKA heterodimer, is distributed throughout both the muscle sarcolemma and the t-tubules and seems to be distributed similarly between muscle types in rats (Rasmussen et al., 2008) and humans (Thomassen et al., 2013). Although one study in rat muscle reported higher content of phospholemman in the sarcolemma of oxidative fibres compared to glycolytic fibres (Juel, 2009). Within skeletal muscle fibres, both β₁ and β₂ were found to be expressed in both the muscle sarcolemma and throughout the t-tubules (Hundal et al., 1992; Lavoie et al., 1996). There are approximately 6 times more β isoforms in the cell membrane compared to in the t-tubules (Lavoie et al., 1997). In addition, there was approximately 5-fold more β isoforms in the cell membrane than α isoforms and 2.5 fold more β than α isoforms in the t-tubules (Lavoie et al., 1997). Considering a functional NKA heterodimer requires both α and β subunit, this finding suggests there is a substantial surplus of β subunits in both the membrane and t-tubules of skeletal muscle (Lavoie et al., 1997). The physiological function of these excess β subunit isoforms is not known (Kristensen et al., 2010). The β₁ isoform is the most abundant of all the β NKA isoforms, with approximately 4-fold higher abundance
than $\beta_2$ (Lavoie et al., 1997). It should be noted though that it could not be determined what fraction of $\beta_1$ or $\beta_2$ isoforms are actually functional and thus it is unknown how accurate this ratio of $\beta_1: \beta_2$ is when functional subunits are considered.

While well described in rats (Lavoie et al., 1997; Fowles et al., 2004; Zhang et al., 2006), the fibre type specificity of NKA content is less clear in humans. Only a single study has investigated NKA isoform abundance in human single muscle fibres and reported 37% lower $\alpha_2$ abundance in Type I fibres than Type II fibres, with no fibre-type difference for the $\alpha_1$ and $\beta_1$ isoforms (Thomassen et al., 2013). This is consistent with most rodent studies using vanadate-facilitated ouabain binding, with 20-50% higher content NKA in rodent EDL in comparison to the soleus (Clausen et al., 1982; Kjeldsen et al., 1984; Bundgaard et al., 2002; Musch et al., 2002). However, it should be emphasized that no assay is available to quantify total functional pumps in single human fibres, as in contrast to the protein abundance of single fibres relative to another as reported by Thomassen et al. (2013). Thus, further research is required to definitively establish the fibre type specificity of NKA isoforms in human skeletal muscle.

In conclusion, the key findings in regards to localisation of Na$^+$/K$^+$-ATPase are:

1) Within the muscle cell, the NKA $\alpha_1$ isoform is expressed only in the sarcolemma, while the $\alpha_2$ isoform is expressed approximately equally between the sarcolemma and t-tubular membranes.

2) The $\alpha_2$ isoform represents 75-85% of all NKA heterodimers in rat EDL muscle.
3) Most evidence suggests that Type II fibres have a higher abundance of NKA, although this is not definitive and the \( \alpha_3 \) isoform is 2-4 more abundant in slow twitch compared to fast twitch muscles.

4) While the \( \alpha_3 \) and \( \beta_3 \) isoforms have an increased affinity for Na\(^+\) compared to the \( \alpha_2 \) and \( \beta_2 \) isoforms in basal conditions, muscle excitation and phospholemman increases the Na\(^+\) affinity of all of the NKA heterodimers.

2.3.6 Content of functional NKA in skeletal muscle

The overall content of functional NKA in skeletal muscle is \(~300\) pmol.g\(^{-1}\) wt.weight or 180,000 billion Na\(^+\),K\(^+\)-ATPase pump molecules per gram wet weight (Hansen et al., 1988; Clausen, 2008b). The vanadate facilitated \([^3H]ouabain\) binding site content is the most accepted method to quantify the content of functional NKA in muscle. Unlike western blotting, the \([^3H]ouabain\) binding site content only assesses complete and functional heterodimers of NKA, as opposed to individual isoforms. In addition the \([^3H]ouabain\) binding site content measure also allows absolute quantitative assessment of NKA, whereas immunoblotting provides a quantity relative to a control. The quantification of the content of labelled ouabain in a sample will be a direct indication of the content of NKA, as each functional NKA enzyme has a single ouabain binding site (Clausen et al., 1982; Nørgaard et al., 1984; Kjeldsen, 1986). In brief, the measure requires the incubation of approximately 20 mg of muscle separated into 3-4 segments to be incubated in a vanadate solution with \([^3H]ouabain\), followed by washout of the unbound ouabain and then liquid scintillation counting to determine the content of ouabain in each muscle segment. This value can then be standardised for sample mass and corrected for non-specific binding. In rat skeletal
muscle, the $\alpha_1$ isoform of NKA has a low affinity to ouabain, thus the vanadate-facilitated ouabain binding site content is unable to detect $\alpha_1$ heterodimers (Clausen, 2003). In human skeletal muscle, there is no significant difference in ouabain affinity between the $\alpha_1$, $\alpha_2$ and $\alpha_3$ isoforms (Müller-Ehmsen et al., 2001; Wang et al., 2001). Thus, the $[^3]$H]ouabain binding site content in human muscle samples allows for quantification of all NKA $\alpha$ isoforms. Table 2.1 shows baseline, or pre-intervention, $[^3]$H]ouabain binding site content from multiple laboratories with differing age groups and training status. Typical $[^3]$H]ouabain binding site content varies between 250-360 pmol.g$^{-1}$ wet weight, varying possibly due to training status and potentially age. The highest values recorded, of up to 425 pmol. g$^{-1}$ wet weight have been seen after intensified exercise training in already trained individuals. Caution must be applied, however, when comparing $[^3]$H]ouabain binding site content between laboratories, due to the likelihood of subtle differences in the methodology of the assay.
Table 2.2: Baseline $[^3]$Houabain binding site content in trained and untrained participants

<table>
<thead>
<tr>
<th>Study</th>
<th>Age (Mean yrs)</th>
<th>n (sex)</th>
<th>Training status/Level</th>
<th>$[^3]$Houabain content (pmol.g$^{-1}$ wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Klitgaard et al., 1989)</td>
<td>28</td>
<td>6 (M)</td>
<td>Untrained</td>
<td>278</td>
</tr>
<tr>
<td>(Green et al., 1993)</td>
<td>19</td>
<td>9 (M)</td>
<td>Untrained</td>
<td>339</td>
</tr>
<tr>
<td>(McKenna et al., 1993)</td>
<td>19</td>
<td>6 (M)</td>
<td>Untrained</td>
<td>333</td>
</tr>
<tr>
<td>(Fraser et al., 2002)</td>
<td>26</td>
<td>8 (M)</td>
<td>Untrained</td>
<td>311</td>
</tr>
<tr>
<td>(McKenna et al., 2012)</td>
<td>24</td>
<td>16 (8M, 8F)</td>
<td>Untrained</td>
<td>350</td>
</tr>
<tr>
<td>(Green et al., 1999a)</td>
<td>21</td>
<td>16 (M)</td>
<td>Untrained</td>
<td>284</td>
</tr>
<tr>
<td>(Madsen et al., 1994)</td>
<td>30</td>
<td>39 (M)</td>
<td>Endurance trained/Recreational</td>
<td>307</td>
</tr>
<tr>
<td>(Evertsen et al., 1997)</td>
<td>18</td>
<td>11 (M)</td>
<td>Endurance trained/Elite</td>
<td>343</td>
</tr>
<tr>
<td>(Medbø et al., 2001)</td>
<td>19</td>
<td>8 (M)</td>
<td>Endurance trained/Elite</td>
<td>356</td>
</tr>
<tr>
<td>(Fraser et al., 2002)</td>
<td>27</td>
<td>8 (7M, 1F)</td>
<td>Resistance trained/Recreational</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>8 (M)</td>
<td>Endurance trained/Recreational</td>
<td>357</td>
</tr>
<tr>
<td>(Aughey et al., 2006)</td>
<td>27</td>
<td>33 (M)</td>
<td>Endurance Trained/Semi elite</td>
<td>318</td>
</tr>
<tr>
<td>(Dørup et al., 1988)</td>
<td>67</td>
<td>25 (10M, 15F)</td>
<td>Untrained</td>
<td>251</td>
</tr>
<tr>
<td>(McKenna et al., 2012)</td>
<td>67</td>
<td>17 (9M, 8F)</td>
<td>Untrained</td>
<td>352</td>
</tr>
<tr>
<td>(Klitgaard et al., 1989)</td>
<td>68</td>
<td>6 (M)</td>
<td>Untrained</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>16 (M)</td>
<td>Trained/Recreational</td>
<td>317</td>
</tr>
</tbody>
</table>
2.3.7 Acute activation of NKA

2.3.7.1 Excitation induced activation of NKA

While the NKA content is an important factor in the capacity of muscle to maintain Na\(^+\) and K\(^+\) gradients required to maintain muscle excitability, the NKA activity can increase acutely via activation of several factors including muscle excitation, increased intracellular [Na\(^+\)], hormones such as adrenaline and insulin, \(\beta_2\) agonists and via the phosphorylation of phospholemman (PLM). The most rapid and prominent activation of NKA occurs with muscle excitation. In rat isolated muscle preparations, stimulation at 60 Hz caused a rise of \([K^+]_e\) from 4 to 10 mmol/L within 10 s in the soleus and within 2 s in the EDL muscle (Clausen et al., 2004). While such a rapid increase in \([K^+]_e\) may not occur in humans during exercise, a high magnitude of \(K^+\) efflux does occur in humans, with several studies reporting interstitial \([K^+]\) concentration up to ~10-14 mmol/L after intense muscle contraction (Green et al., 1999b; Green et al., 2000; Juel et al., 2000b; Mohr et al., 2004; Nielsen et al., 2004). Such rapid release of \(K^+\) from skeletal muscle and increase in \([K^+]_e\) requires prompt increase of NKA activity in order to maintain muscle excitability (Clausen, 2003). In rat isolated soleus muscle, high frequency unloaded stimulation at 60 Hz for 10 s caused a 58% increase in \([Na^+]_i\), which returned to baseline within 2 minutes (Everts et al., 1994). Importantly, the recovery of baseline intracellular \([Na^+]\) after stimulation was prevented when ouabain, a specific NKA inhibitor, was pre-incubated into the muscle, thus indicating that the NKA was the cause of the accelerated post-excitation Na\(^+\) efflux (Everts et al., 1994). The same study also reported an approximate two-fold increase in labelled \(^{86}\text{Rb}^+\) influx and \(^{22}\text{Na}^+\) efflux at lower intensity stimulation, despite no increase in intracellular Na\(^+\); suggesting that
contraction without increased Na⁺ accumulation still activates NKA (Everts et al., 1994). The NKA activity varies between fibre types in rats; when 2 Hz electrical stimulation was performed for 5 minutes, ⁸⁶Rb⁺ influx was 2.5 fold higher in the soleus than the EDL muscle (138% vs 58% respectively) and with only the EDL having a 70% increase in [Na⁺]ᵢ content (Everts et al., 1992). This finding implicates soleus NKA having a greater sensitivity to excitation than the EDL in vivo. The rate of activation of NKA is extremely rapid; 10 s of 120 Hz contractions elicited a ~22-fold increase in NKA activity (Nielsen et al., 1997). Because the frequency of electrical stimulation used by Nielsen et al. (1997) is non-physiological, it is unknown whether such activation of NKA can occur in humans. Human exercise studies do, however, strongly support excitation-induced NKA activation; rapid increases in plasma [K⁺] to 5-8 mmol/L at the immediate completion of high intensity exercise is consistently followed by a rapid recovery of K⁺ to baseline levels, usually within ~2 minutes after intensive exercise, using a variety of exercise intensities, durations and modalities (Medbø et al., 1990; Fraser et al., 2002; Medved et al., 2003; Petersen et al., 2005; Sostaric et al., 2006). Intriguingly, some studies have reported post-exercise hypokalemia where plasma venous [K⁺] can decrease by as much as 0.5-1.0 mmol/L below resting [K⁺] at 5-10 minutes post-exercise (Hallen et al., 1994; Petersen et al., 2005), suggesting that the increased excitation induced activity of NKA is sustained well after exercise, potentially due to the combined effect of muscle contraction and exercise-induced sympathoadrenal activation and the subsequent release of epinephrine (Clausen et al., 1987; Everts et al., 1988). This is supported in vitro where intracellular Na⁺ was still reduced by ~30% below rest 10 minutes after the end of electrical stimulation (Nielsen et al., 1998).
2.3.7.2 Hormonal and \( \beta_2 \) receptor activation of NKA

Another important regulator of acute NKA activity is \( \beta_2 \) adrenoreceptors agonists, including endogenously produced epinephrine and synthetic \( \beta_2 \) agonists including isoproteranol and salbutamol, which are used in the treatment of asthma. In denervated rat diaphragm, isoproteranol caused a 30% decrease in intracellular \( \text{Na}^+ \) (Evans et al., 1973); additionally isoproteranol caused hyperpolarisation in avian slow twitch muscle, which was preventable by pre-incubation with ouabain (Somlyo et al., 1969). In rat soleus muscle, isoproteranol caused a 12% increase in \( ^{42}\text{K}^+ \) influx, 40% increase in \( ^{22}\text{Na}^+ \) efflux and up to a 67% decrease in intracellular \( \text{Na}^+ \), which was suppressed by ouabain incubation (Clausen et al., 1977a).

Structurally similar to salbutamol and isoproteranol, epinephrine (adrenaline) is produced endogenously and has a marked effect on acute NKA activation (Clausen et al., 1977a). Epinephrine caused an ouabain-suppressible increase in \( ^{22}\text{Na}^+ \) efflux by 83% and \( ^{42}\text{K}^+ \) influx by 34% in rat soleus muscle \textit{in vitro} (Clausen et al., 1977a) and may induce a greater activation of NKA in soleus muscle compared to EDL muscle (Pfliegler et al., 1983; Everts et al., 1988). Importantly, epinephrine at concentrations commonly seen in plasma during intense exercise in humans (Galbo et al., 1975) caused hyperpolarisation and activation of NKA in isolated human intercostal muscles (Ballanyi et al., 1988). Brachial arterial infusion of the \( \beta_2 \) agonist terbutaline into the forearm of humans caused a 7% decrease in arterial plasma [\( \text{K}^+ \)], supporting that \( \beta_2 \) agonists activate NKA in humans \textit{in vivo}. Adrenalectomy also caused decreased tolerance to KCl infusion in cats (Lockwood et al., 1977) and rats (Bia et al., 1982), whilst the exercise induced hyperkalemia was enhanced when the \( \beta \) blocker propranolol was given before incremental cycling (Hallen et al., 1994), suggesting that
epinephrine via the activation of NKA has an important physiological role in muscle \( K^+ \) uptake during and immediately after exercise. The common mechanism behind the activation of NKA by catecholamines and other \( \beta_2 \) agonists is via activation of \( \beta_2 \) adrenoreceptors, which cause downstream activation of cAMP and subsequent phosphorylation of protein kinase A (Clausen, 2003; Clausen, 2010). Unlike excitatory, ion-induced activation of NKA which occurs within seconds, epinephrine takes minutes to have effect (Clausen, 2010). Thus while epinephrine has an important role in regulating \( [K^+] \) during rest and exercise, excitatory activation of skeletal muscle NKA is likely to be the key regulator of \( K^+ \) during sustained muscle contractions.

Insulin and other hormones such as insulin like growth factor 1 (IGF-1), calcitonin gene-related peptide (CGRP), calcitonin and amylin also regulates acute NKA activity. Insulin is a well-established acute activator of NKA, both \textit{in vivo} and \textit{in vitro}. \textit{In vitro}, insulin caused a significant increase in \(^{86}\text{Rb}\) uptake in cultured human fibroblasts and a 70% increase in NKA current in rabbit cardiac myocytes (Hansen \textit{et al.}, 2000). Insulin induced increased NKA activity was suppressible using ouabain (Clausen \textit{et al.}, 1977b; Clausen \textit{et al.}, 1987). The effect of insulin on NKA is independent of translocation of potential NKA to the sarcolemma (Longo \textit{et al.}, 2001) and does not necessarily increase intracellular Na\(^+\) (Kitasato \textit{et al.}, 1980; Lytton, 1985); thus insulin-mediated NKA activity is likely due to different mechanism(s) than muscle excitation or epinephrine. \textit{In vivo} studies also support the role of insulin activating NKA; infusion of insulin into the forearm increased \( K^+ \) uptake, which was reversed when ouabain was infused (Ferrannini \textit{et al.}, 1988) and insulin has been extensively shown to decrease plasma \([K^+]\) (DeFronzo \textit{et al.}, 1980). Like epinephrine, the activation of NKA by
insulin is much slower than excitatory activation of NKA and while insulin activation of NKA has profound implications during basal conditions and in clinical populations requiring insulin, it is unlikely that the insulin-mediated NKA activation is important during or immediately post-exercise as plasma insulin decreases during heavy exercise (Pruett, 1970). Each of IGF-1, CGRP, calcitonin and amylin also cause acute NKA activation, but these peptide hormones do not heavily contribute to K\(^+\) regulation during or immediately after (within 5-10 minutes) exercise and thus are beyond the scope of this review.

2.3.8 Mechanisms of increased NKA activation

2.3.8.1 Phospholemman (FXDY1) and NKA

Phospholemman phosphorylation is an important regulator of NKA activity in muscle (Crambert et al., 2002; Crambert et al., 2003; Rasmussen et al., 2008; Cirri et al., 2011). In *Xenopus oocytes*, Phospholemman associates with both the \(\alpha_1\) and \(\alpha_2\) isoform of NKA, although a stronger association was seen with the \(\alpha_1\) isoform (Crambert et al., 2002). Phosphorylation of phospholemman caused a small but significant decrease in affinity of NKA to K\(^+\) and an approximate two-fold decrease in NKA affinity to Na\(^+\) in *Xenopus oocytes* (Crambert et al., 2002), although Cirri et al., (2011) report only a 30% increase in affinity to Na\(^+\) in the \(\alpha_1\beta_1\) NKA heterodimer when phospholemman was associated with NKA *in vitro*, with no changes in K\(^+\) affinity. Knockout or inactivation of phospholemman in mice led to a \(~50\)% decrease in NKA activity in two studies using homogenised heart and skeletal muscle (Jia et al., 2005; Reis et al., 2005), however, other studies have found contradictory results where NKA activity was increased compared to controls in myocytes harvested from phospholemman knockout mice (Despa et al., 2005; Han et al., 2006; Pavlovic et al., 2007).
Phospholemman is activated during muscle contraction is via upstream activation of Protein Kinase A (PKA) and Protein Kinase C (PKC) (Palmer et al., 1991; Walaas et al., 1999; Pavlovic et al., 2007; Bibert et al., 2008; Rasmussen et al., 2008). While it is clear that phospholemman has an important role in the short-term regulation of NKA, the contradictory results which report both an increase (Despa et al., 2005; Han et al., 2006; Pavlovic et al., 2007) and decrease (Jia et al., 2005; Reis et al., 2005) in NKA activation when phospholemman is knocked out/removed demonstrates that the upstream activators and phosphorylation status/site of phosphorylation of phospholemman may alter the effects on NKA activity. Bibert et al. (2008) demonstrated that activation of phospholemman from PKA or PKC and the resultant difference in phosphorylation state of phospholemman, had divergent effects on either NKA activity or affinity. In cardiac myocytes, phospholemman in its inactive state decreased NKA ionic affinity, while phosphorylated phospholemman increased the ionic affinity of NKA to Na\(^+\) and K\(^+\) (Pavlovic et al., 2007). Hence the phosphorylation state and upstream activation of phospholemman may be of greater importance than the total phospholemman content. In humans, one-legged cycling caused a significant increase in phosphorylated phospholemman in only the exercising leg, demonstrating that the phosphorylation of phospholemman during exercise is caused by contractile activity and not systemic factors such as circulating epinephrine or insulin during exercise (Benziane et al., 2011). Further, various Na\(^+\) concentrations caused substantially increased NKA activation in muscle after exercise in humans, potentially due to the phosphorylation of phospholemman (Juel et al., 2013).
2.3.8.2 Translocation of NKA

Increased $^{22}\text{Na}^+$ efflux from rat soleus was reported when intracellular $\text{Na}^+$ was artificially increased in the muscle via cuts in the sarcolemma (Buchanan et al., 2002). Conversely, other studies have reported increased $^{42}\text{K}^+$ or $^{86}\text{Rb}^+$ influx and $^{22}\text{Na}^+$ efflux, synonymous via NKA activation, without any change in intracellular $\text{Na}^+$ content using a variety of stimulation frequencies (Everts et al., 1992; Everts et al., 1994; Buchanan et al., 2002). In light of such findings, a potential mechanism for excitation-mediated activation of NKA is an increased sensitivity of NKA to $\text{Na}^+$ ions (Crambert et al., 2000). A potential mechanism behind this phenomenon is the proposed translocation of NKA to the sarcolemma from intracellular stores. The first evidence of this phenomenon was reported when insulin incubation increased ouabain binding site content in frog muscle, which the investigators suspected as being due to an “unmasking” of inactive NKA (Grinstein et al., 1974; Erlij et al., 1976). This increased NKA content was then confirmed to occur during exercise and electrically stimulated muscle contraction (Sandiford et al., 2005b). In rat skeletal muscle, $\alpha_1$ and $\alpha_2$ isoforms increased in abundance by 20-58% in the fractionised membrane portion by increase after exercise/contraction, accompanied by a concomitant decrease in $\alpha_1$ and $\alpha_2$ abundance in the endosomal fraction of the homogenate (Sandiford et al., 2005b). This suggests that the increased sarcolemmal NKA isoform abundance was due to translocation from an intracellular source, but may also reflect imprecision in membrane isolation. Other laboratories have also been able to confirm a translocation paradigm of NKA $\alpha_2$ subunit using isolated giant sarcolemma vesicles in both rat and human muscle (Juel et al., 2000a; Juel et al., 2001). While centrifuge based fractionation and giant sarcolemma vesicle
methodologies have proven effective in demonstrating a translocation paradigm in NKA during muscle contraction, several studies using vanadate facilitated ouabain-binding site assessment have not been able to find an increased $[^3]$Houabain binding site content, after insulin incubation or muscle contraction (Clausen et al., 1977b; McKenna et al., 2003). Considering that $[^3]$Houabain binding site assessment reports only functional units compared to individual isoforms, such a finding does create some doubt over the legitimacy of the NKA translocation paradigm. Clausen and Kohn (1977b) argue that subcellular fractionation leads to a low yield of membrane which may not be representative of the entire sample, thus findings relying on subcellular fractionation are not a valid method to assess NKA translocation; they further asserted that the earlier frog muscle studies which used ouabain binding and supported the translocation paradigm did not adequately incubate/saturate the muscle sample, which caused an artificial increase in $[^3]$Houabain binding site content (Clausen et al., 1977b). Conversely, Benziane et al. (2008) contest that the ouabain binding methodology, while accurate for overall muscle ouabain binding sites, is not appropriate for detecting NKA translocation due to the long incubation time of muscle ex vivo which negates the timeframe when translocation could occur (Juel et al., 2001). Hence while there is substantial evidence for translocation of NKA in response to exercise and insulin (Tsakiridis et al., 1996; Juel et al., 2000a; Chibalin et al., 2001; Juel et al., 2001; Sandiford et al., 2005a; Benziane et al., 2008), the lack of data demonstrating changes in functional membrane NKA after exercise or insulin should be considered (Clausen et al., 1977b; McKenna et al., 2003). More research needs to be conducted to conclusively demonstrate the existence and potential mechanisms underlying NKA
translocation. In conclusion, the acute activation of NKA in skeletal muscle during muscle contraction may be caused by a numerous factors, including increased intracellular Na\(^+\) which directly stimulates NKA activity, increased Na\(^+\) sensitivity of NKA due to translocation of NKA to the sarcolemma from intracellular stores and the phosphorylation of the NKA associated protein phospholemman.

### 2.3.9 Quantifying activity of NKA

While NKA measured via vanadate-facilitated ouabain binding or specific isoforms via western blotting provides important data in the overall content and relative abundance of NKA, respectively, and indeed NKA content contributes significantly to NKA activity; measurement of NKA content does not directly assess the rate of activity of NKA. There are several methods to assess the *in vitro* activity of NKA. In whole muscle preparations, the efflux and influx of labelled ions such as \(^{22}\)Na or \(^{86}\)Rb can be used to quantify the activity of NKA and can be expressed as a percentage of maximal theoretical activity, if NKA content is known (Clausen *et al.*, 1977a; Clausen *et al.*, 1977b; Everts *et al.*, 1994). Changes in intracellular Na\(^+\) and K\(^+\) can also be used to indirectly quantify NKA activity (Clausen *et al.*, 2004). In isolated myocytes, the whole cell patch voltage clamp technique can be used to assess alterations in NKA electrical current (Han *et al.*, 2006; Pavlovic *et al.*, 2007). However, these in vitro techniques are of limited application in human research, as techniques such as labelled \(^{42}\)K or \(^{86}\)Rb cannot be used during non-steady state conditions such muscle contraction. A technique developed to measure NKA activity *ex vivo* uses K\(^+\) stimulated 3-O-methylfluorescein phosphatase activity (3-O-MFPase) (Fraser *et al.*, 1998). The assay allows
quantification of NKA activity via the fluorescent determination of 3-O-MFPase activity, activated via the addition of KCl in previously frozen muscle homogenate. The 3-O-MFPase activity in this assay was almost completely inhibited when the homogenate was treated with ouabain, demonstrating its specificity to NKA (Fraser et al., 1998). This assay has met criticism, as there is no ATP hydrolysis involved and the assay is only conducted at one [K+] without Na+ (Benziane et al., 2008). This is of particular concern with the phosphorylation of phospholemman, as phospholemman has been theorised to increase NKA activity via increasing the sensitivity of NKA to Na+ (Cirri et al., 2011). Hence, an assay which does not include variable levels of Na+ would miss the increased NKA activity potentially caused by phospholemman.

A more recent assay to assess NKA activity has been developed, which is performed at various [Na+] (Juel et al., 2013). Using this assay, increased NKA activity and increased NKA Na+ affinity were seen after both intense exercise in humans and after upstream activation of phospholemman (Juel et al., 2013). The assay measures inorganic phosphate (Pi) production from ATP hydrolysis, but uses only the fractionated membrane of the muscle sample, yielding only 2% of the total protein of the total sample and ~10-12% of NKA α1 and α2 abundance (Juel et al., 2013). While this more recent assay may be more valid for NKA activity quantification, the low total protein yield using fractionation questions whether the NKA recovered is representative of the whole muscle sample. Hence, more research is needed to determine the most appropriate method of analysing NKA activity ex vivo in humans, although the assay developed Juel et al. (2013) is promising, particularly if the total protein yield can be increased.
### 2.3.10 NKA activity and fatigue in humans

Multiple studies from two separate laboratories have investigated the effect of fatigue on NKA activity during exercise in humans, using primarily the 3-O-MFPase activity assay. Muscle contraction causes a marked increase in NKA activity, both *in vitro* and in human exercise studies (Clausen, 2003; Clausen, 2008a). Almost all of the studies investigating NKA activity when using the 3-O-MFPase activity assay with exercise in humans have found a significant decrease in maximal NKA activity during fatigue using various forms of physical activity. Submaximal cycling at ~75% VO$_2$ peak until fatigue decreased 3-O-MFPase maximal activity by 14% -18.9% (Leppik *et al.*, 2004; Murphy *et al.*, 2006) and incremental cycling to fatigue caused ~29-32% decrease in maximal 3-O-MFPase activity (Sandiford *et al.*, 2005a). Isometric exercise on an isokinetic dynamometer decreased 3-O-MFPase maximal activity by 35% (Fowles *et al.*, 2002), whereas a more modest 10.9% - 13.8% decrease was noted during fatiguing isokinetic contractions (Fraser *et al.*, 2002; Petersen *et al.*, 2005). High intensity exercise lasting approximately one minute caused a 12.9% decrease in 3-O-MFPase maximal activity (Aughey *et al.*, 2005), whilst repeated high intensity cycling caused a similar 12.7% decrease in 3-O-MFPase activity (Aughey *et al.*, 2007). This inactivation or decreased activity of NKA was similar between trained and non-trained participants (Fraser *et al.*, 2002). Conversely, no difference in 3-O-MFPase activity was found in humans exercising at 57% of VO$_2$ peak after glucose feeding, suggested to be due to an increase in pump activity via the translocation of NKA in response to the increase in insulin (Green *et al.*, 2007b).
While the findings using the 3-O-MFPase activity assay have been relatively consistent, a recent study utilising an assay for NKA activity which accounts for Na\(^+\) concentration contests the validity of the 3-O-MFPase activity assay results (Juel et al., 2013). With this assay, intense exercise caused an increase in NKA activity in both rats (Juel, 2009) and human skeletal muscle (Juel et al., 2013), which contrasts the findings using the 3-O-MFPase assay (McKenna et al., 2008). This suggests either that the 3-O-MFPase assay is insensitive to changes in exercise induced NKA Na\(^+\) affinity, likely mediated by phospholemman phosphorylation (Cirri et al., 2011), or the cellular fractionation method used by Juel et al. (2013) which recovered 2% of total protein is not representative of the total NKA pool. Further, while the exercise performed in the study by Juel et al. (2013) was intense, it was not maximal and does not demonstrate whether NKA activity is reduced at heavy fatigue. NKA activity was not measured in the studies of this thesis due to the methodological concerns regarding the 3-O-MFPase activity assay (Fraser et al., 1998) and the logistical challenges to perform the recently developed Juel et al. (2013) assay.

### 2.3.11 Aging and NKA content

Aging has been reported to lower skeletal muscle NKA content and some NKA isoform abundance in animals (Clausen et al., 1982; Kjeldsen et al., 1984; Sun et al., 1999; Ng et al., 2003). Early studies using \([^3]H\)ouabain binding found a 58% decrease in total skeletal muscle NKA in rat soleus and EDL muscle from 85 day old rats compared to infant rats (Clausen et al., 1982). In female guinea pigs, a 60% decline in NKA content was reported from birth to maturity in the EDL muscle (Kjeldsen et al., 1984). The NKA \(\alpha_1\) relative isoform abundance was increased in a fibre type-specific manner in 30 month old rats compared to 6, 18 or 16
month old rats (Sun et al., 1999; Ng et al., 2003; Zhang et al., 2006), with a 25% and 77% increase in $\alpha_1$ reported by Ng et al., (2003) in the white and red gastrocnemius respectively, while 1-5 fold increases were reported in $\alpha_1$ isoform abundance in red and white gastrocnemius and the soleus between 18 month to 30 month old brown Norway rats by Sun et al. (1999). The decrease in $\alpha_2$ isoform abundance to aging is more consistent, with a 20-40% decrease reported in 30 month old rats (Sun et al., 1999; Ng et al., 2003; Zhang et al., 2006).

While it is clear that aging alters skeletal muscle NKA using animal models, findings have not been conclusive in humans. Nørgaard et al. (1984) first investigated NKA skeletal muscle content in humans across different ages using 20 participants between 20-80 years old. No significant correlation was found between age and skeletal muscle NKA content from vastus lateralis biopsies, although physical activity levels, which may affect NKA content (Clausen, 2003), were not reported in this study. Klitgaard and Clausen (1989) reported no significant difference between old (mean age 68) and young untrained participants (mean age 28). The Klitgaard et al. (1989) study had a small sample size, with only six participants in the old and young untrained groups and while not statistically significant, a 14% difference was reported. These mixed findings suggests more research in conjunction with assessment of physical activity levels is required to better establish the effect of age on skeletal muscle NKA content, which is investigated in a mature aged and elderly participants in Chapter Three.
2.3.12 Exercise training and NKA content

Chronic physical activity is a potent regulator of skeletal muscle NKA content in mammalian tissue. Animal studies consistently report training increasing NKA content in skeletal muscle (Kjeldsen et al., 1986; Green et al., 1992; Suwannachot et al., 1999; Suwannachot et al., 2001). The first study to investigate the adaptation of NKA to exercise training found a 165% increase in dog gracilis NKA activity after 6 weeks of treadmill training (Knochel et al., 1985), however such a magnitude of change was likely affected by the small fraction of recovered NKA from the membrane due to centrifuge separation (Clausen, 2003). A later study reported a 46% increase in $[^{3}\text{H}]$ouabain binding sites in rat hindlimb muscle after six weeks of swim training, which was reversed by three weeks rest (Kjeldsen et al., 1986). High intensity running increased $[^{3}\text{H}]$ouabain binding site content in horse gluteus muscle by 23-32% in separate studies (McCutcheon et al., 1999; Suwannachot, 2001) as well as in the hindlimb muscles in foals by 20-30% (Suwannachot et al., 1999; Suwannachot et al., 2001). Increased $[^{3}\text{H}]$ouabain binding was noted equally in both EDL and soleus muscle after six weeks of swim training (Kjeldsen et al., 1986), demonstrating specific fibre type adaptations are unlikely in rodents. The time-course of NKA content adaptation is relatively short; detectable changes in $[^{3}\text{H}]$ouabain binding site content were reported from as little as four d of in vitro chronic muscle stimulation, reaching a peak 86% increase in $[^{3}\text{H}]$ouabain binding sites after 10 d of stimulation (Green et al., 1992).

In humans, the first evidence to suggest exercise increased skeletal muscle NKA content was when Klitgaard and Clausen (1989) reported a 30-40% higher NKA content in swim, run and strength trained older participants compared to age-matched sedentary controls.
Several studies since, using a broad range of controlled exercise programs, have shown increased NKA content by 13-22% in human skeletal muscle using $[^3]$Houabain binding site assessment (Green et al., 1993; McKenna et al., 1993; Madsen et al., 1994; Evertsen et al., 1997; Green et al., 1999a; Medbø et al., 2001), a comparable relative change to that reported in several animal studies (Kjeldsen et al., 1986; Suwannachot et al., 1999; Suwannachot et al., 2001). The increase in NKA content by physical training is remarkably fast; six consecutive d of prolonged sub-maximal exercise, cycling two hours per day at 68% of VO$_2$peak, caused a 13.6% increase in $[^3]$Houabain binding site content (Green et al., 1993), while a 9% increase in $[^3]$Houabain binding sites was reported after only 3 d of consecutive training (Green et al., 2004). Endurance training for 11 weeks, 5-6 times per week at ~68% of VO$_2$peak on a cycle ergometer caused a 22% increase in $[^3]$Houabain binding sites in muscle from the vastus lateralis in untrained male participants (Green et al., 1999a). These results taken together suggest that the majority of NKA content adaptation from endurance training occurs in the first 1-2 weeks of training; although it should be noted that these studies all contained a very high volume of endurance training for untrained populations. In recreationally trained male runners that underwent intensified training additional to their current training, a 13.3% increase in $[^3]$Houabain binding site content was reported after 6 weeks (Madsen et al., 1994). Similarly, in 20 male and female junior elite cross country skiers, five months of intensified training caused a 16% increase in $[^3]$Houabain binding site content (Evertsen et al., 1997). This establishes that training can increase NKA even in trained/elite populations and is not a phenomenon only in untrained participants who are unaccustomed to exercise. However, others did not report increased
NKA content after training in trained participants (Aughey et al., 2007), suggesting that substantial changes in either the type of training or volume of training must be made to increase NKA content in trained populations.

The effect of exercise training on NKA content is not limited to traditional endurance exercise. Intensified sprint training on a cycle ergometer caused a 16% increase in \[^3\text{H}]\text{oouabain binding sites}\) in untrained male participants (McKenna et al., 1993). Lower limb resistance training three times per week for 12 weeks caused a 16% increase in \[^3\text{H}]\text{oouabain binding site content}\) in untrained males (Green et al., 1999a), whilst 1-3 training sessions per week of intensified eccentric resistance training for 12 weeks caused a 15% increase in \[^3\text{H}]\text{oouabain binding site content}\) in well trained alpine skiers (Medbø et al., 2001). Increased NKA content after chronic exercise training is also associated with an increased maximal K\(^+\) stimulated 3-O-MFPase activity (Fraser et al., 2002; Murphy et al., 2007), although chronic exercise training can improve skeletal muscle max 3-O-MFPase activity by \(\sim 5.6\%\) without any change in \[^3\text{H}]\text{oouabain binding site content}\), or NKA isoform content changes (Aughey et al., 2007). Increased phospholemman phosphorylation may explain such changes in NKA activity, as phospholemman phosphorylation is increased after training (Thomassen et al., 2010). Hence, both increased NKA activity and content are potential mechanisms which could contribute to the decreased fatiguability seen after chronic exercise training (McKenna et al., 2008).

In agreement with exercise studies using \[^3\text{H}]\text{oouabain binding site content}\), NKA \(\alpha_1\), \(\alpha_2\) and \(\beta_1\) isoform relative abundance are increased after chronic exercise. Two weeks of intensified training increased the NKA \(\alpha_2\) isoform by 15% in elite male soccer players
compared to players who underwent no training (Thomassen et al., 2010). Six to nine weeks of high intensity, but lower volume training increased NKA α₂ isoform abundance by 68% and significantly decreased venous plasma [K⁺] during repeated sprints (Bangsbo et al., 2009). The α₁ isoform was also increased in untrained participants after 10 consecutive d of cycling at 72% of VO₂ peak for one hour; with a 49% increase in NKA α₂ isoform abundance, 113% increase in α₁ isoform abundance and 29% increase in β₁ abundance (Benziane et al., 2011). Four weeks of sprint training in trained runners resulted in a 29% increase in α₁ isoform abundance, with concomitant lowered plasma [K⁺] during repeated high intensity running (Iaia et al., 2008). Conversely, 3 weeks of high intensity intervals in trained cyclists caused no change in NKA isoform abundance or in [³H]ouabain binding site content (Aughey et al., 2007). However, there were only seven training sessions in the 21 day period in conjunction with the cyclists original training regime, thus the additional training may not have been of sufficient additional volume or intensity in highly trained athletes to induce changes in skeletal muscle NKA content, despite increases in performance and 3-O-MFPase activity (Aughey et al., 2007).

2.3.12.1 Adaptations to phospholemman with training

Adaptations to the NKA accessory protein phospholemman (PLM) (Crambert et al., 2002; Crambert et al., 2003; Rasmussen et al., 2008) have only recently been investigated in response to acute and chronic physical training. In rats, a 200-350% increase in sarcolemma PLM was reported, concomitant with at 67% increase in NKA activity (Vmax) (Rasmussen et al., 2008). Further, five d of swim training also increased PLM ser68 phosphorylation in rats (Galuska et al., 2009). In humans, acute cycling exercise caused an increased...
phosphorylation of PLM by 30-75% depending on the phosphorylation site (Ser63 or Ser68) and was accompanied by increased PKC phosphorylation, an upstream activator of PLM (Benziane et al., 2011). In trained populations, two weeks of intensified training in male soccer players caused a 27% increase in PLM phosphorylation on the Ser68 site, which correlated weakly with increased physical performance (Thomassen et al., 2010). Conversely, there was no change in PLM phosphorylation at either Ser63 or Ser68, at rest or after training comprising 10 consecutive d of cycling training for 1 hour at ~72% \( \dot{V}O_2 \) peak in untrained participants (Benziane et al., 2011). This discrepancy is surprising and these divergent findings suggest more research is required to assess the change in phosphorylated and total PLM after training.

### 2.3.12.2 Significance and mechanisms of increased NKA content after exercise training

The increased NKA content after chronic exercise may be an important adaptation to physical training. A higher \([^{3}\text{H}]\text{ouabain binding site content was correlated } \dot{V}O_2 \text{ peak (Fraser et al., 2002; Murphy et al., 2007), inversely related to fatigability (Fraser et al., 2002) and correlated with NKA activity measured by 3-O-MFPase activity (Fraser et al., 2002; Murphy et al., 2007). Further, several studies report a concurrent increase in NKA with exercise performance specific to the training modality (McKenna et al., 1993; Evertsen et al., 1997; Green et al., 1999a; Medbø et al., 2001; Bangsbo et al., 2009; Thomassen et al., 2010). These increases in muscle NKA content and activity after training in some cases also occur along with decreased plasma [K⁺] in response to exercise (Tibes et al., 1976; McKenna et al., 1993; Iaia et al., 2008; Bangsbo et al., 2009). Combined with in vitro data showing increased fatigability in isolated muscle preparations at high, but physiologically relevant
concentrations of $[K^+]$, increased NKA content after chronic exercise training is proposed to be a key adaptation to allow maintenance of muscle excitability during intense repeated muscle contractions (Clausen, 2003; Clausen, 2008a; McKenna et al., 2008).

The mechanism of how muscle NKA content is increased with chronic exercise is incompletely understood. Chronic transient increases in intracellular $Na^+$ have been implicated as a possible cause of increased NKA content seen after exercise (Wolitzky et al., 1986; Brodie et al., 1990; Ladka et al., 2000). Myocytes harvested from chick embryo leg muscle treated with veratridine, which allows for increased $Na^+$ influx via the voltage gated $Na^+$ channels caused a 60-100% increase in NKA measured via labelled monoclonal antibodies over a 36 hour time period; this was reversible when Tetrodotoxin (TTX), a neurotoxin which blocks the $Na^+$ channels was administered (Wolitzky et al., 1986). Further, prolonged increased intracellular $Ca^{2+}$ caused no change in NKA content, while only a small increase was seen in NKA at a non-physiologically low extracellular $K^+$ (0.5 mmol/L) (Wolitzky et al., 1986). These findings were confirmed in rat myotubes where TTX concomitantly decreased intracellular $Na^+$ and $[^3H]ouabain$ binding sites, which was reversible when TTX was washed from the solution and veratradine stimulated a 50-75% increase in NKA and 40-70% increase in NKA activity using $^{86}Rb$ uptake (Brodie et al., 1990). Veratradine treatment caused an 88% increase in the NKA $\alpha_2$ isoform abundance, with no change in $\alpha_1$ isoform content using muscle cell line cultured from mouse; using the same muscle line TTX caused a 58% decrease in NKA $\alpha_2$ isoform abundance (Ladka et al., 2000).

In conclusion, chronic exercise training in humans of various modalities causes increased skeletal muscle NKA content (Green et al., 1993; McKenna et al., 1993; Evertsen et al.,
1997; Green et al., 1999a), which is correlated with increased 3-O-MFPase activity (Fraser et al., 2002; Murphy et al., 2007), increased VO\textsubscript{2} peak and decrease fatiguability (Fraser et al., 2002) and decreased plasma [K\textsuperscript{+}] during exercise (McKenna et al., 1993; Iaia et al., 2008; Bangsbo et al., 2009). The above findings suggest that skeletal muscle NKA content is an important adaptation in various types of exercise and a potential contributing limiting factor in skeletal muscle performance.

2.3.13 Physical inactivity and K\textsuperscript{+} regulation

Research investigating direct adaptations to systemic and peripheral [K\textsuperscript{+}] regulation after inactivity in humans is sparse and highly speculative. Potassium regulation is mediated by multiple factors on both a short (<6 hours) and long term basis. An early descriptive study suggested increased venous plasma [K\textsuperscript{+}] at the submaximal and fatiguing exercise after 20 d bed rest (Saltin et al., 1968), but the low sample size (5 participants) with some missing data for [K\textsuperscript{+}] did not allow for statistical analysis and any definitive conclusions. Bed rest also increases urinary excretion of K\textsuperscript{+} due to changes in hydrostatic pressure (Grenon et al., 2004; Grenon et al., 2005), where fluid shifts from lower limbs are directed into vascular compartments of the upper body, triggering central carotid, aortic and cardiac receptors to increase fluid and sodium excretion (Pavy-Le Traon et al., 2007). The increased K\textsuperscript{+} excretion seen during bed rest does not manifest in lowered [K\textsuperscript{+}], however, likely due to the concurrent decline in plasma volume (Saltin et al., 1968; Grenon et al., 2004; Grenon et al., 2005). The lack of change in plasma [K\textsuperscript{+}] during resting conditions in bed rest studies is not surprising considering the large store of K\textsuperscript{+} available in skeletal muscle, which contains over 96% of K\textsuperscript{+} in humans (Kjeldsen, 2010). Despite this, there may be negative implications of
increased urinary K⁺ excretion with bed rest; in young healthy participants increased urinary K⁺ excretion which the authors suspected as being a cause in increased incidence in microvolt T wave alternans, a risk factor in cardiac dysrhythmias (Grenon et al., 2005). However, with no alteration in plasma [K⁺], there was no causative evidence linking alterations in resting K⁺ regulation during bed rest to the observed cardiac effects in that study (Grenon et al., 2005).

2.3.14 Inactivity and NKA

Inactivity has been repeatedly demonstrated to reduce muscle NKA in animals. This was first investigated when 28 d of denervation caused a 22% and 13% decrease in the soleus and EDL NKA content respectively using [³H]ouabain binding site content analysis (Clausen et al., 1982). However, while denervation does cause a vast decrease in muscle activity, severing nerve connection and thus all electrical activity to the muscle has dramatic effects on thousands of neurotrophic factors, blood flow and resting membrane potential (Jones et al., 2001; Shields, 2002). Even in prolonged full limb immobilisation there is still EMG activity noted in the muscle at rest (Fudema et al., 1961; Duchateau et al., 1990). Hence, denervation does not replicate inactivity or muscle disuse. A later study confirmed that inactivity alone decreased NKA content in rodents, when hindlimb immobilisation in rats caused a 20-30% decrease [³H]ouabain binding sites in hindlimb muscles (Kjeldsen et al., 1986). Leivseth et al. (1992) used a novel system of partial immobilisation in guinea pigs in order to investigate the time course of [³H]ouabain binding site content change during inactivity and found a 25% and 23% decrease after 14 and 21 d respectively in [³H]ouabain binding site content in the gastrocnemius (Leivseth et al., 1992). After 28 d, NKA content
unexpectedly returned to levels similar to baseline, likely due to greater use of the partially immobilised limb as the inactivity system allowed for weight bearing (Leivseth et al., 1992). In female sheep, nine weeks of immobilisation decreased skeletal muscle NKA content by 38% in the immobilised leg and a 22% in the control leg (Jebens et al., 1995). The validity of the decreased ambulatory activity was supported by a 30% decrease in citrate synthase activity in both limbs (Jebens et al., 1995). Re-ambulation of the sheep returned \[^{[3]H}\]ouabain binding site content to baseline levels, demonstrating reversibility of NKA content with physical activity. Surprisingly, after immobilisation resting muscle \([K^+]_i\) was ~40% higher, although this is likely because the sheep were in a hypokalemic state before commencing the study (Jebens et al., 1995).

Despite the consistent findings in the various animal studies investigating the effect of inactivity on NKA, no studies have investigated NKA content after controlled inactivity in humans. However, three studies have investigated NKA content in human skeletal muscle after participants were inactive due to prior injury (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012), Figure 2.8. Hence, the severity and length of the injury is variable among participants and confounding effects from the injury itself or medication cannot be excluded to affect skeletal muscle NKA. Leivseth et al. (1994) obtained deltoid muscle from participants with unilateral shoulder impingement syndrome, who typically experience pain in the affected shoulder when raised above 60° and found a 26.6% decrease in \[^{[3]H}\]ouabain binding site content in the muscle from the injured shoulder. In addition, there was an 18% and 25% decrease in Type II and Type I fibre cross sectional area, respectively, compared to the healthy deltoid muscle (Leivseth et al., 1994). While this research confirms the
fundamental concept that inactivity and injury combined causes a decrease in NKA content in humans, the research provides no insight into the timeline, magnitude or functional effects of the maladaptations to skeletal muscle NKA content. In paraplegics, a 33.8% decline in vastus lateralis muscle NKA content was seen compared to the deltoid (Ditor et al., 2004); whether a muscle biopsy from the deltoid would have an altered content of NKA compared to the vastus lateralis due to its different muscle fibre composition and prior exercise training is unknown. However, the value of 188 pmol g⁻¹ wet wt⁻¹ [³H]ouabain binding sites obtained by Ditor et al., (2004) was considerably lower (35-58%) than healthy sedentary individuals reported in multiple studies from the same laboratory (Green et al., 1993; Green et al., 1999a).
Figure 2.8: Mean change or difference in human skeletal muscle $[^3H]$ouabain binding site content after various training (TRN) modalities ranging from 10 d–12 weeks duration and injuries. All muscle sampled from vastus lateralis except in the shoulder impingement research (filled bars). Data from: (Green et al., 1993; McKenna et al., 1993; Leivseth et al., 1994; Green et al., 1999a; Ditor et al., 2004; Boon et al., 2012)

In patients with chronic cervical spinal injury, a 45% decrease in $[^3H]$ouabain binding site content was reported, as well as 75%, 52% and 38% decreases in the NKA $\alpha_1$, $\alpha_2$ and $\beta_1$ isoforms, respectively, compared to healthy controls (Boon et al., 2012). In patients who were monitored post-complete cervical injury, 80% and 53% decreases in NKA $\alpha_1$ and $\alpha_2$ isoforms, respectively, were reported 12 months post injury, with the vast majority of the decline noted within the first 3 months of injury (Boon et al., 2012). The $[^3H]$ouabain binding site content was not reported 1, 3 or 12 months post injury in this group; this would
have provided insight into the timeline of functional NKA decline with spinal injury, which is still unclear in humans. In contrast, patients with partial cervical spinal injury, who were able to ambulate and perform daily activities had no change in NKA isoform abundance up to 12 months after injury (Boon et al., 2012). Additionally, complete cervical injury caused a 52% decrease in total PLM content after chronic complete cervical spinal injury compared to controls. However, no change in phosphorylation state at Ser63 or Ser68 was found, suggesting that total PLM decreased due to a concomitant decrease in muscle NKA. No changes were noted in total or phosphorylated PLM in the incomplete cervical spinal injury patients (Boon et al., 2012). These findings suggest that while total PLM may be decreased by injury or inactivity, there is no evidence suggesting injury decreases the phosphorylation of PLM (Boon et al., 2012), which indicates that PLM may not be a key maladaptation to inactivity, as opposed to the drastic reduction exhibited in NKA content after long-term injury (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012).

In the Lievseth and Reikerås (1994), Ditor et al., (2004) and Boon et al., (2012) studies, the sample sizes were small, with the studies having 6-7 participants in each injury or control group. Additionally, the magnitude of the injury eliciting inactivity in each study could not be controlled or normalised between participants, hence these studies demonstrate that combined injury and inactivity causes decreased NKA content. Considering the substantial body of research demonstrating increased NKA content with physical activity (Green et al., 1993; McKenna et al., 1993; Madsen et al., 1994; Green et al., 1999a; Medbø et al., 2001; Clausen, 2003), combined with animal immobilisation studies (Leivseth et al., 1992; Jebens et al., 1995) and human injury studies (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012).
it is likely that inactivity decreases NKA content. However, a controlled methodology of eliciting inactivity without accompanying injury in humans is required to confirm such a hypothesis. Hence Chapter 5 investigates the role of controlled unilateral inactivity and subsequent resistance training on NKA content and isoform abundance in skeletal muscle of humans.

### 2.3.14.1 Mechanisms and considerations of NKA content decrease

The cellular mechanisms underlying the potential adaptations seen in NKA content after inactivity are not understood. Dietary K$^+$ depletion causes a decrease in muscle K$^+$, concomitant with a decrease in NKA content (Clausen et al., 1982). The hypothesis of decreased potassium content within the muscle during inactivity causing decreased NKA pumps has been investigated indirectly in several animal studies (Clausen et al., 1983; Leivseth et al., 1992; Jebens et al., 1995). Interestingly, Leivseth et al., (1992), Jebens et al., (1995) and Clausen et al., (1983) all showed an increased muscle K$^+$ content after disuse in both rats and sheep, suggesting that lowered intracellular [K$^+$] is unlikely to mediate reduced NKA content after inactivity. In exercise training, increased NKA content has been suggested to be due to chronic increases in skeletal muscle [Na$^+$]; (Wolitzky et al., 1986; Brodie et al., 1990); hence inactivity could lead to decreased NKA content via a decreased frequency of transient [Na$^+$]; accumulation, although this requires investigation.

Differing rates of atrophy between fibre types during inactivity could also affect NKA content. If NKA content is higher in a specific fibre type, such as Type II fibres (Clausen, 2003; Fowles et al., 2004; Thomassen et al., 2013) preferential atrophy and changes in fibre type distribution could play a fundamental role in the magnitude of skeletal muscle NKA
decline. Bed rest for 84 d caused almost double the magnitude of atrophy in Type I fibres compared to Type II fibres and a ~30% increase in hybrid fibres in the soleus, suggesting a shift towards increased Type II muscle fibres in humans (Rudnick et al., 2004; Gallagher et al., 2005). In addition, 42 d of bed rest caused significant atrophy only in Type I fibres with no significant decrease noted in Type II fibres or changes in fibre type distribution (Berg et al., 1997a). Shorter durations of inactivity in humans, however, have not been associated with changes in fibre type distribution or preferential fibre type atrophy (Hather et al., 1992; Hortobagyi et al., 2004). Hence, changes in fibre type distribution and fibre-specific atrophy are unlikely to have any contribution in potentially decreased muscle NKA after short term inactivity, but may have some role in long term inactivity. A final consideration for reduced muscle NKA content after inactivity is the potential concomitant effect of muscle atrophy on NKA content. Increased sarcolemmal surface area compared to intracellular space, as found in smaller muscle fibres, correlate with $[^3]H$ouabain binding site content in porcine muscle (Harrison et al., 1994). Hence, a sample of smaller muscle fibres may give a higher NKA content than larger fibres (Harrison et al., 1994), suggesting that the atrophy induced by inactivity could conceal what is an actual decrease in absolute NKA content.

2.4 Conclusions

Severe inactivity which occurs during injury and illness, such as osteoarthritis and ACL injury, causes substantial impairment of muscle function (Narici et al., 2011). The biomolecular causes of this impairment to skeletal muscle function after inactivity is incompletely understood, although one likely maladaptation is a reduction in the content of
skeletal muscle NKA. The NKA regulates cellular $[\text{Na}^+]$ and $[\text{K}^+]$ during muscle contractions across the t-tubular and sarcolemmal membranes and thus plays an important role in muscle excitability (Clausen, 2003; McKenna et al., 2008). Hence, any decrease in skeletal muscle NKA content after physical inactivity or disuse can potentially impair muscle fatiguability and function.

2.5 Aims

This thesis investigated the effects of chronic osteoarthritis, voluntary short-term unilateral inactivity and acute ACL injury on various markers of muscle function, skeletal muscle NKA content and NKA isoform relative abundance ($\alpha_{1-3}$, $\beta_{1-3}$). In addition, the broader whole-body effects of unilateral inactivity model (ULLS) were investigated.

Study One

This study aimed to investigate the effects of osteoarthritis on skeletal muscle strength, NKA content, NKA isoform protein relative abundance and gene expression, comparing osteoarthritis participants to age-and BMI-matched healthy controls. A second aim of the study was to investigate the association between age and skeletal muscle $[^3\text{H}]$ouabain binding site content in both the osteoarthritis and control groups.

Study Two (Part I)

Study Two investigated the functional adaptations in skeletal muscle mass, strength, power and postural sway after 23 days of unilateral lower limb suspension (ULLS) and four weeks of subsequent resistance training in both the unloaded and weight-bearing legs of healthy young adults.
**Study Two (Part II)**

The second part of Study 2 investigated the effects of 23 days of ULLS and four weeks of subsequent resistance training in the same participants from Part I on exercise time to fatigue, plasma $[K^+]$ and $[\text{Lac}^-]$, physiological indices of performance during incremental and fatiguing one-legged exercise, skeletal muscle NKA content and isoform relative abundance in both the unloaded and weight-bearing legs.

**Study Three**

Study Three investigated the difference in subjective knee function, knee extensor strength, cross sectional area, postural sway, skeletal muscle NKA content and relative isoform protein abundance in young participants with acute ACL injury, compared to both the non-injured leg and age and BMI-matched controls.

**2.6 Hypotheses**

The specific hypotheses tested were:

**Study One**

1. Patients with knee osteoarthritis would have reduced knee extensor strength and self-reported physical activity compared to controls.

2. Patients with knee osteoarthritis will exhibit lower skeletal muscle $[^3H]\text{ouabain}$ binding site content, NKA $\alpha_2$ relative protein abundance and gene expression than healthy controls.
3. Skeletal muscle $[^{3}H]$ouabain binding site content would be inversely associated with age independent of knee osteoarthritis.

**Study Two (Part I)**

1. Short-term unilateral lower limb suspension (ULLS) will cause declines in each of muscle strength, mass, vertical jump height and postural sway in the unloaded leg compared to baseline.

2. These changes after 23 days of ULLS will be reversible following four weeks of resistance training.

**Study Two (Part II)**

1. Twenty three days of ULLS will reduce time to fatigue, increase heart rate and perceived exertion during one-legged cycling during exercise.

2. After ULLS, skeletal muscle $[^{3}H]$ouabain binding site content and NKA α$_2$ abundance will be reduced in the unloaded leg compared to baseline and the weight-bearing leg.

3. Venous plasma [K$^+$] during exercise will be higher during one-legged exercise in the unloaded leg compared to baseline and the weight-bearing leg.

4. All changes after ULLS will be reversible after four weeks of resistance training.
Study Three

In the injured leg of the ACL group:

1. The injured leg will have reduced knee extensor strength, cross sectional area and increased postural sway compared to the non-injured leg and controls.

2. Skeletal muscle [$^3$H]ouabain binding site content and $\alpha_2$ NKA isoform relative abundance will be lower compared to the non-injured leg and controls.
CHAPTER 3: THE EFFECTS OF OSTEOARTHRITIS AND AGE ON SKELETAL MUSCLE STRENGTH, Na\(^+\),K\(^+\)-ATPase CONTENT, GENE AND ISOFORM EXPRESSION

3.1 Introduction

Osteoarthritis is a common joint disorder prevalent in aged populations (Fejer et al., 2012), characterised by restricted movement (Brooks, 2002) and disability (Yelin, 2003; Buckwalter et al., 2004; Lawrence et al., 2008). Aging, independent from chronic diseases, causes a marked decrease in muscle strength, size and exercise performance (Deschenes, 2004; Narici et al., 2010). Sarcopenia, or age-related muscle atrophy, accounts for the majority of the loss in strength with aging, but aging and disease may also alter the contractility of muscle through mechanisms independent of muscle size (Frontera et al., 2000; Goodpaster et al., 2006). Aging and knee OA are both implicated in causing decreased muscle strength, muscle size and lower functional capacity seen in many older-adults (Yelin, 2003; Deschenes, 2004; Lawrence et al., 2008; Narici et al., 2010).

Physical inactivity has a higher incidence in both osteoarthritis and aging populations (Deschenes, 2004; Bohannon, 2007; Lawrence et al., 2008; Naal et al., 2010) and causes vast maladaptation in skeletal muscle which may mediate decreased muscle strength and performance (Narici et al., 2011). This might conceivably include the ubiquitously expressed sodium-potassium transporter, Na\(^+\),K\(^+\)-ATP-pump (NKA). The NKA has a vital role in skeletal muscle excitability and function via the regulation of Na\(^+\) and K\(^+\) gradients across sarcolemmal and t-tubular membranes (Clausen, 2003). NKA is a heterodimer which
consists of a catalytic α isoform and a regulatory β isoform together with a regulatory FXYD1 protein (phospholemman). In human skeletal muscle three α isoforms (α1-3) and three β isoforms (β1-3) are expressed, although the α4 mRNA was also detected at very low levels (Blanco et al., 1998; Murphy et al., 2004; Nordsborg et al., 2005; Petersen et al., 2005). The dominant α isoform in skeletal muscle is the α2 (Hansen, 2001), although the relative abundance of α isoforms in human skeletal muscle is not known. Further, a recent study suggests that the NKA α2 isoform plays a specific role in exercise; rats with muscle-specific α2 knockout exhibited no difference in ion concentrations or membrane potential at rest, but had substantial decrements in muscle strength and treadmill running performance (Radzyukevich et al., 2013). Importantly, muscle NKA content is decreased with physical inactivity and up-regulated by exercise training (Kjeldsen et al., 1986; Green et al., 1993; McKenna et al., 1993; Leivseth et al., 1994; Jebens et al., 1995; Green et al., 1999a; Clausen, 2003; Boon et al., 2012; Edge et al., 2013). Physical inactivity may cause up to 26-50% reduction in NKA content after shoulder (Leivseth et al., 1994) and spinal injury (Ditor et al., 2004; Boon et al., 2012), suggesting that lowered physical activity in people with osteoarthritis may also have an adverse effect on skeletal muscle NKA content.

As the incidence of osteoarthritis is highest amongst elderly populations (Lawrence et al., 2008), the possible concomitant effects of aging and osteoarthritis on skeletal muscle NKA should be considered. We recently reported a 24% lower NKA α2 isoform abundance and increased β3 abundance in old than in young adults, but without any difference in muscle NKA content (McKenna et al., 2012). Other research in humans however has not demonstrated similar findings. In asymptomatic participants, no correlation was found
between age and ouabain binding site content in participants aged 25-80 years old (Nørgaard et al., 1984), or significant difference in [³H]ouabain binding between young and mature aged humans (Klitgaard et al., 1989; McKenna et al., 2012). It is possible that decrements in muscle [³H]ouabain binding are apparent at advanced ages.

This study investigated the effects of osteoarthritis and aging on skeletal muscle strength, NKA content, isoform protein abundance and gene expression, comparing these in osteoarthritis to matched controls; this study then further investigated the relationship between age and NKA content in subgroups of these older adults. It was hypothesized that osteoarthritis patients would have reduced muscle function, lower [³H]ouabain binding site content, NKA α₂ protein relative abundance and physical activity than controls, and that [³H]ouabain binding site content would also be inversely associated with age.

3.2 Methods

3.2.1 Participants

Nineteen older adults with diagnosed knee osteoarthritis (OA; 9 males and 10 females; age: 69.9 ± 6.5 yrs; height: 166.2 cm ± 8.3 cm; mass: 82.4 kg ± 11.8 kg; BMI: 29.8 ± 3.83, mean ± SD) and 17 asymptomatic healthy controls matched for age and BMI (CON; 9 males and 8 females; age 66.8±6.4 yrs, height: 168.6 cm ± 7.6 cm; mass: 82.7 kg ± 16.9 kg; BMI: 28.9 ± 4.7) provided written, informed consent to participate in the research. The OA patients were recruited when scheduled for knee replacement surgery. Patients were excluded if they were not capable of walking 45 m continuously without a walking aid, or had any uncontrolled systemic disease, neurological or orthopaedic condition affecting their ability
to walk. The CON participants were asymptomatic with no knee pain and were free from knee injury. The OA participants received numerous medications, including diuretics (n = 3), antidiabetic agents (n = 2), anticholesterol agents (n = 3), beta-blockers (n = 3), angiotensin II receptor antagonists (n = 5), calcium-channel blockers (n = 2), glucosamine (n = 6), angiotensin converting enzyme inhibitors (n = 6) and aspirin (n = 3). Nine OA participants were taking anti-inflammatory medication, which was discontinued one week prior to the scheduled surgery where muscle was sampled. CON participants receiving medications included anti-diabetic (n=2), anti-cholesterol (n=2), beta-blockers (n=3), angiotensin II receptor antagonist (n=2), platelet inhibitor (n=1), ACE Inhibitor (n=3) and aspirin (n =2). The study was approved by the Victoria University, La Trobe University and Warringal Private Hospital Human Research Ethics Committees.

3.2.2 Physical activity questionnaire

The Incidental and Planned Activity Questionnaire for older people was used to assess the physical activity level of the participants (Delbaere et al., 2010). This includes 10 questions that estimates the physical activity during the previous week and covers the frequency and duration of both planned activity (planned exercise and walks) and incidental activities (casual day-to-day activities). Frequency and duration scores were multiplied to create a total duration for incidental and planned activity as well as an overall total score. Total activity time was summed across all components (hr.wk⁻¹). Physical activity was recorded in each of the OA but only in a smaller cohort of the CON participants due to some participants not being able to attend the testing session (n = 7).
3.2.3 Muscle strength measurement

Maximal isometric voluntary torque was measured as described previously (Levinger et al., 2011a). In brief, a non-extendable strain gauge was attached to the participant’s leg at approximately 10cm above the ankle from a hard surfaced tall chair using a webbing strap with a Velcro fastener. The hip and knee joints were each kept at 90° angle, but no strapping was applied. The distance from the knee joint to the strap around the ankle was measured with a tape measure and used to calculate torque (Nm). The participant exerted maximal force with verbal feedback from the researcher against the strap for 3 s during each of three trials, with the largest peak torque recorded and used for analysis. Maximal knee extensor muscle strength was obtained in 19 OA and in a smaller cohort of 7 CON participants due to some participants not being able to attend the testing session.

3.3.4 Muscle sampling and analysis

Approximately one week after the strength and physical activity assessment, a vastus lateralis muscle sample from the OA group was obtained during elective knee replacement surgery as described previously (Levinger et al., 2011a; Levinger et al., 2011b). In brief, the sample was taken ~5 cm proximal to the suprapatellar pouch after the skin was incised and prior to knee joint capsule incision to ensure no trauma to the muscle or the joint at that time. The CON participants had a single resting vastus lateralis muscle biopsy under local anaesthesia from the middle third of the thigh. In detail, after injection of a local anaesthetic into the skin and fascia (1% Xylocaine), a small incision was made and a muscle sample was taken (~100-200 mg) from the mid-thigh using a Bergström biopsy needle.
Muscle samples were blotted to remove excess blood, immediately frozen in liquid nitrogen and stored at -80°C until analyses.

3.2.5 Muscle [3H]ouabain binding site content

Twenty milligrams of muscle sample was used in the [3H]ouabain binding content analysis, as previously described (Nørgaard et al., 1984; Petersen et al., 2005). In brief, each sample was washed for 2 x 10 min at 37°C in vanadate buffer (250 mM sucrose, 10 mM Tris-HCl, 3 mM MgSO₄, 1 mM NaVO₄; pH 7.3). Muscle samples were then incubated for 2 h at 37°C in vanadate buffer with the addition of [3H]ouabain (2.0 Ci/ml and 10⁻⁶ M, PerkinElmer, Boston, MA). The muscle was then placed in ice-cold vanadate solution for 4 x 30 min to remove any unbound [3H]ouabain. Muscle samples were blotted on filter paper and weighed before being soaked in 500 µl of 5% trichloroacetic acid and 0.1 mM ouabain for ~20 h. Following this, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard, PerkinElmer, Boston, MA) were added before liquid scintillation counting of [3H]ouabain. The [3H]ouabain binding site content was calculated on the basis of the sample wet weight and specific activity of the incubation buffer and samples and expressed as pmol.g ww⁻¹. The final [3H]ouabain binding site content was then calculated as previously described (Nørgaard et al., 1984; Petersen et al., 2005; McKenna et al., 2012). Due to small muscle biopsy samples in some participants, [3H]ouabain binding site content was analysed in 12 OA and 8 CON participants. Data on the NKA in the CON group have been reported previously in comparison to young adults (McKenna et al., 2012).
3.2.6 RNA extraction and RT-PCR

Total cellular RNA was extracted using a modification of the phenol-chloroform extraction and isopropanol precipitation protocol, using the TOTALLY RNA Kit (Applied Biosystems, Foster City, CA), as described previously (Trenerry et al., 2007). RNA quality and concentration was determined using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). First-strand cDNA was generated from 1.0 g total RNA using high-capacity RNA-to-cDNA kit (Applied Biosystems). RT-PCR was performed using the Applied Biosystems 7500 Real Time PCR machine (Applied Biosystems) and PCR was performed in duplicate with reaction volumes of 14 µl, containing Power SYBR Green (Applied Biosystems), forward and reverse primers and cDNA template (1.25 ng/µl). Data was analysed using a comparative critical threshold (Ct) method described previously (McKenna et al., 2012). The efficacy of TBP (NM_003194) as an endogenous control was examined using the equation $2^{-\Delta\Delta Ct}$. No changes in the expression of this gene was observed (data not shown), so it was considered an appropriate endogenous control for this study. NKA primers was used as previously described (Murphy et al., 2004). Muscle NKA gene expression analyses was performed on samples from 19 OA patients and 17 CON participants (except α3 where n = 16 for CON group).

3.2.7 Western blotting

In brief, 40 mg of frozen muscle samples was used for NKA immunoblot analyses as previously described (Murphy et al., 2004; McKenna et al., 2012). Muscle proteins were extracted in ice-cold buffer containing 20 mM Tris pH 7.8 (Bio-Rad Laboratories, Hercules, CA), 137 mM NaCl, 2.7 mM KCl (Merck, Kilsyth, Australia), 1 mM MgCl2, 5 mM Na$_4$H$_2$P$_2$, 10
mM NaF, 1 % Triton X-100, 10 % Glycerol (Ajax Finechem, Australia), 0.5 mM Na$_4$VO$_3$, 1 µg.mL$^{-1}$ Leupeptin, 1 µg.mL$^{-1}$ Aprotinin, 200 mM PMSF, 1 mM DTT, 1 mM Benzamidine. All reagents were analytical grade (Sigma-Aldrich, St Louis, MI), unless otherwise specified. Samples were homogenised (1 : 37.5 dilution) for 2 x 20 s, using a tissue homogeniser (TH220, Omni International, Kennesaw, GA). Homogenates were rotated for 60 min at 4°C and protein concentration of the homogenates was determined using a commercially-available kit (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA).

Muscle sample analyses did not include any membrane isolation steps, to maximize recovery of NKA enzymes. Aliquots of the muscle homogenate were mixed with Laemmli sample buffer and proteins were separated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (6 – 20 % gradient gels), for 2 h at 80 mA and overnight at 16 mA, in a standard vertical electrophoresis unit (SE 600 Chroma®, Hoefer Inc, San Francisco, CA). For the analysis of protein abundance of the α$_1$, α$_3$, β$_2$ and β$_3$ NKA isoforms, 60 µg of total protein per sample were loaded in each gel, while 20 µg was loaded for the α$_2$ and β$_1$ isoforms. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) for 3 h at 320 mA using a semi-dry blotter (TE70X, Hoefer). Membranes were blocked in TBST buffer (10 mM Tris, 100 mM NaCl, 0.02 % Tween-20) containing 7.5 % non-fat milk, for 1 h at room temperature. After being washed (3 x 10 min in TBST), membranes were incubated with the appropriate primary antibody overnight at 4°C. Primary antibodies were diluted in TBS buffer containing 0.1 % NaN$_3$ and 0.1 % albumin bovine serum. All membranes were incubated with the same amount of dilution buffer. Membranes were ponceau stained to confirm complete transfer.
To determine NKA protein abundance, membranes were incubated with antibodies for NKA α₁ (monoclonal α6F, developed by D. Fambrough, obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA), NKA α₂ (polyclonal anti-HERED, kindly donated by T. Pressley, Texas Tech University, USA), NKA α₃ (monoclonal, Thermo Scientific, Rockford, IL, # MA3-915), NKA β₁ (monoclonal, Thermo Scientific # MA3-930), NKA β₂ (monoclonal, Becton Dickinson Bioscience, San Jose, CA, # 610915) and NKA β₃ (monoclonal, Becton Dickinson # 610993), as earlier described (Murphy et al., 2004). The abundance of all proteins was normalised for loading control with GAPDH (Santa Cruz Biotechnology # FL-335). Following incubation with the primary antibodies, membranes were washed in TBST buffer (3 x 10 min) and incubated with the appropriate anti-rabbit (PerkinElmer # NEF812001EA) or anti-mouse (PerkinElmer # NEF822001EA) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were diluted 1:5000 in TBST buffer containing 5% non-fat milk. After washing the membranes in TBST, immunoreactive proteins were detected using chemiluminescence reagents (ImmobilonTM HRP Substrate, Millipore, Billerica, MA) and quantified by densitometric scanning (VersaDocTM Imaging System, BioRad). NKA protein abundance analyses were performed in 19 OA and 13 CON participants.

### 3.2.8 Statistical analyses

All data are reported as mean ± standard deviation (SD). Independent t-tests and effect size (ES) with 90% confidence intervals (CI) were used to calculate differences between groups in [³H]ouabain binding site content, isoform protein abundance and mRNA expression. Spearman correlation coefficient was used to examine correlations between [³H]ouabain
binding site content, muscle strength, total physical exercise and age in the OA and CON groups separately and then sub-analysis performed with pooled data from both groups where no significant differences were found between the OA and CON groups. Additional sub-analysis of the difference with age and [³H]ouabain binding site content was performed using a t-test, with the median age (68.5 years) of the sample used to divide the participants into two groups: 55-68 (mean ± SD, n =12) years and 69-81 (mean ± SD, n = 8) years old. Log-transformation was performed on data which was not normally distributed to reduce bias. Statistical significance was accepted at P<0.05. Magnitudes of change using Cohen’s effect size was classified as defined by Batterham and Hopkins (2006); small, 0.2-0.6; moderate, 0.6-1.2; large, 1.2-2.0; and very large, 2.0-4.0 (Hopkins, 2007). Effects with less certainty (magnitude of <75%) was classified as no meaningful difference (Batterham et al., 2006; Hopkins, 2007). Independent t-tests and correlations were calculated using SPSS version 20 (SPSS Inc., Champaign, IL) and effect size calculated via a custom spreadsheet (Hopkins, 2007).

3.3 Results

3.3.1 Knee extensor muscle strength and physical activity levels

Voluntary maximal knee extensor torque was lower in OA than in CON whether peak torque expressed in absolute units (-40.8%, 84.69 ± 38.2 vs. 143.1 ± 55 Nm respectively, p = 0.005, ES 1.16±0.83) or standardised for body weight (-33.7%, 1.03 ± 0.44 vs. 1.55 ± 0.44 Nm.kg⁻¹ respectively, p = 0.013, ES 1.13 ± 0.77). Whilst OA tended to perform less planned physical activity than CON (50%; 5.8 ±6.2 vs. 11.6 ±11 hr.wk⁻¹, respectively, p = 0.068, ES 0.61 ± 0.86), they performed more incidental exercise (79%; 37.6 ± 18.4 vs. 21 ± 10.5 hr.wk⁻¹, p =
0.035, ES 1.07 ± 0.65) and there was a tendency for greater total exercise time in OA than in CON (33.1%; 43.4 ±19.4 vs. 32.6 ± 10.2 hr.wk⁻¹, respectively, p = 0.083, ES 0.67 ± 0.64).

3.3.2 [³H]Ouabain binding site content

The muscle [³H]ouabain binding site content did not differ between OA and CON (357± 98 versus 352± 76pmol.g wet weight⁻¹, respectively, p = 0.908, ES 0.05±0.75).

3.3.3 NKA isoform gene expression

There was a tendency for lower α₁ and β₂ mRNA expression in OA than in CON, but there were no differences between OA and CON for α₂, α₃, β₁, or β₃ gene expression (p > 0.34; Table 3.1).
Table 3.1: Skeletal muscle NKA α and β isoform gene expression in patients with osteoarthritis (OA) and age, sex and BMI matched controls (CON); n =17 CON (except α₃ where n = 16) and for n = 19 OA (except β₂ where n = 18).

<table>
<thead>
<tr>
<th>NKA isoform mRNA (a.u)</th>
<th>OA</th>
<th>CON</th>
<th>ES</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁</td>
<td>5.11 ± 3.2</td>
<td>6.53 ± 3.14</td>
<td>0.62 ± 0.55‡</td>
<td>0.06</td>
</tr>
<tr>
<td>α₂</td>
<td>37.51 ± 25.35</td>
<td>33.95 ± 28.69</td>
<td>0.01 ± 0.55</td>
<td>0.99</td>
</tr>
<tr>
<td>α₃</td>
<td>0.02 ± 0.03</td>
<td>0.02 ± 0.01</td>
<td>0.32 ± 0.54</td>
<td>0.34</td>
</tr>
<tr>
<td>β₁</td>
<td>20.66 ± 16.37</td>
<td>19.69 ± 15.96</td>
<td>0.02 ± 0.55</td>
<td>0.95</td>
</tr>
<tr>
<td>β₂</td>
<td>1.87 ± 1.12</td>
<td>2.33 ± 1.24</td>
<td>0.47 ± 0.56†</td>
<td>0.15</td>
</tr>
<tr>
<td>β₃</td>
<td>0.68 ± 0.48</td>
<td>0.56 ± 0.31</td>
<td>0.18 ± 0.54</td>
<td>0.57</td>
</tr>
</tbody>
</table>

‡Small Effect Size, †Moderate Effect Size. Values are means ± SD in arbitrary units.

3.3.4 Muscle NKA isoform protein abundance

A higher protein abundance was found in OA than CON for the NKA α₂ (34.1%, p = 0.006, ES 1.09± 0.56) and α₃ isoforms (105%, p = 0.016, ES 0.93±0.57, Figure 3.1). There was also a tendency for higher NKA β₃ abundance in OA than in CON (18%, p= 0.06, ES 0.59±0.54, Figure 3.1).
**Figure 3.1:** NKA (A) α and (B) β isoform relative protein abundance in OA and CON. Values are means ± SD in arbitrary units (a.u); n =19 OA and n = 13 CON. * p < 0.05; OA greater
than CON, † Small Effect Size, ‡ Moderate Effect size. Magnitudes of difference were classified as substantial when there was a >75% likelihood of the effect being meaningful.

**Figure 3.2**: Relationship between skeletal muscle \[^{3}\text{H}]\text{ouabain}\) binding site content and age in older adults. OA: Filled circles, CON empty triangles. Unbroken regression line represents pooled data, which is the \(r\) and \(p\)-value listed on the graph, dashed line represents OA regression and dotted line represents CON regression.

### 3.3.5 Age, strength, physical activity, sex and NKA interrelationships

No significant correlations were found between muscle strength (in absolute units or when standardised for bodyweight) and age in either OA or CON, or between total physical activity and age in either group. There was a significant inverse correlation between age and \[^{3}\text{H}]\text{ouabain}\) binding site content within the OA group \((r = -0.63, p = 0.030; n =12)\), but
not within the CON group \( (r = -0.41, p = 0.317; n = 8) \). No difference was found in 
\[^3H\]ouabain binding or isoform content between sexes, either within the pooled data or
within either the OA or CON groups \( (p>0.05) \). Further sub-analysis with data pooled from
both groups revealed a significant inverse correlation between age and muscle \[^3H\]ouabain
binding site content \( (r = -0.47, p = 0.038; n = 20, \text{ Figure 3.2}) \). Additionally, when the
participants were divided according to the median age, the older group \( (69-81 \text{ yrs}) \) had a
25.5\% lower \[^3H\]ouabain binding site content than the younger \( (55-68 \text{ yrs}) \) group \( (294.3 \pm 74 \text{ vs } 395.5 \pm 73 \text{ pmol/g wet weight}^{-1}, \text{ respectively, } p = 0.007, \text{ ES } 1.31\pm0.76) \).

3.4 Discussion

This is the first study to investigate possible differences in skeletal muscle NKA content,
isof orm protein abundance and gene expression between people with osteoarthritis and
age- and BMI-matched controls. This thesis reports two important findings with respect to
the muscle total NKA content as measured by the \[^3H\]ouabain binding site content. Firstly
there was no difference in NKA content between OA and CON, secondly we demonstrate
that the NKA content was 25.5\% lower in those participants whose age exceeded the
pooled group median age than in those below \( (68.5 \text{ yrs}) \). This suggests that advanced age is
adversely associated to muscle NKA content independent of the osteoarthritis disease
state. Further, we found that patients with osteoarthritis exhibited greater muscle NKA \( \alpha_2 \)
and \( \alpha_3 \) protein abundance than controls, together with greater physical activity, suggesting
there is a complex regulation of muscle NKA in these patients.
3.4.1 Osteoarthritis, physical activity and skeletal muscle NKA

Osteoarthritis is a prevalent joint disease in aged populations and is reported to cause joint movement and physical activity restriction in many sufferers (Dillon et al., 2006; Michael et al., 2010). The knee OA group demonstrated markedly reduced knee extensor muscle strength; although it is important to note that pain during the testing could have contributed to this finding. Further, the strength testing was performed only at one knee angle (90°), and the protocol used was not appropriate to measure the rate of force development from the knee extensors. Future research could build on these limitations and integrate muscle electromyography measurements from the quadriceps and hamstrings muscle groups to assess voluntary activation, as well as assessment of twitch potentiated force. However, such extra parameters must remain practical to apply to participants in a clinical setting, which can be logistically difficult.

The current study cohort remained capable of performing low intensity physical activity including walking and were quite physically active. It is well established that the muscle [³H]ouabain binding site content increases with various training types, including resistance training, submaximal endurance exercise and sprint training with increases of 10-20% typical in young adults (Green et al., 1993; McKenna et al., 1993; Green et al., 1999a). Previous studies reported that OA participants may have a lower level of physical activity compared to asymptomatic controls (Naal et al., 2010; Lee et al., 2012; Veenhof et al., 2012) and on this basis we hypothesized that OA would also exhibit a reduced muscle NKA content as the vastus lateralis is substantially affected by physical inactivity (Hackney et al., 2011). However, a surprising finding was that the OA participants in the present study
reported significantly higher incidental activity and tended to have higher reported total physical activity. This is inconsistent with the lack of difference in $[^3]H$ouabain binding site content between OA and controls. The reason why these patients with OA had higher levels of incidental physical activity than controls is not clear, but may result from the recommendations given to patients prior to undergoing knee replacement surgery, specifically to remain active to assist with the post-knee replacement surgery recovery. The participants did not, however, participate in any formal training or physiotherapy prior to surgery. It is unclear why the greater physical activity in the OA group did not result in increased ouabain binding, considering that long term swim, running and resistance trained older males had 30-40% higher $[^3]H$ouabain binding site content compared to age-matched sedentary controls (Klitgaard et al., 1989). It is possible that the OA group were inactive for a long period before becoming physically active in the short-term before surgery, potentially explaining why there was no difference in NKA content, although long term physical activity habits were not recorded in this study to substantiate this premise. The lack of direct control regarding exercise mode, duration and intensity between the OA and CON groups and age categories is a limitation of this study. Hence, the long-term physical activity of the participants may have contributed to the research findings; hence future studies should document this carefully.

3.4.2 Age and skeletal muscle NKA

The effects of age on skeletal muscle NKA content in humans are controversial. Here we report for the first time a significant inverse relationship between age and $[^3]H$ouabain binding content in human skeletal muscle; this was evident both in people with
osteoarthritis and the entire older adult cohort. This contrasts an earlier finding of a lack of association between age and skeletal muscle \[^{3}H\]ouabain binding site content in 20 participants ranging from 25-80 years old (Nørgaard et al., 1984). To strengthen this finding, when pooled participants were separated according to the median age (68.5yr), the older group aged 69-81 years had a 25% lower \[^{3}H\]ouabain binding site content than the mature age participants aged 55-68 years. Two previous studies investigating young versus older participants (mean age ~25-28 vs. 64-68 years) reported no difference in \[^{3}H\]ouabain binding site content (Klitgaard et al., 1989; McKenna et al., 2012). However, Klitgaard and Clausen (1989) reported a (non-significant) 14% lower \[^{3}H\]ouabain binding in six older participants compared to young controls. Our results suggest that an age related decline in muscle \[^{3}H\]ouabain binding site content may only be apparent in elderly individuals over 70 years old. This would thus explain the previously reported absence of statistically significant results in NKA content between young mature-aged adults (Klitgaard et al., 1989; McKenna et al., 2012).

Older participants in this study typically took multiple forms of medications, which is typical for participants in this age bracket (Qato et al., 2008). This occurred in both the OA and CON groups, where medications taken included beta-blockers, non-steroidal anti-inflammatories, anti-hypertension medication and cholesterol-reducing medications; approximately 50% took more than one prescription medication. While cholesterol-lowering medication can have side effects on skeletal muscle including myositis (Armitage, 2007), it is unclear whether these individual or multiple medications may have affected muscle NKA content or isoform abundance. However, because participants from both
groups were taking similar medications, it is unlikely that medication use alone had any substantial contribution to the results of this study.

3.4.3 Divergence between skeletal muscle \( \alpha_2 \) and \([^3]H\)ouabain binding site content after osteoarthritis

A surprising finding was the greater muscle NKA \( \alpha_2 \) isoform relative abundance in OA than in CON, which is counter to the lack of difference in \([^3]H\)ouabain binding site content between the groups. It is however, consistent with the greater physical activity in this cohort of OA and with numerous other reports of increased NKA \( \alpha_2 \) with exercise training (Bangsbo et al., 2009; Thomassen et al., 2010). We noted previously a disassociation between \([^3]H\)ouabain binding site content and NKA isoform abundance, where there was decreased \( \alpha_2 \) protein abundance with no difference in the \([^3]H\)ouabain binding site content in older adults (2012). Divergent results between \( \alpha_2 \) abundance and \([^3]H\)ouabain binding site content have also been previously reported in aged rat muscle (Sun et al., 1999). The reasons for an increase in \( \alpha_2 \) in OA despite no change in ouabain binding content remain unclear. Studies in rodents (Clausen et al., 1982; Bundgaard et al., 2002; Fowles et al., 2002) and recently in humans using single muscle fibres (Thomassen et al., 2013) report that Type II muscle fibres have a higher abundance than Type I of NKA \( \alpha_2 \), the dominant \( \alpha \) isoform in skeletal muscle. Hence, any specific loss or atrophy of Type II fibres in OA could change NKA isoform abundance. However, lower type II cross sectional area and selective type II muscle fibre atrophy suggestive of disuse in 78 OA patients (Fink et al., 2007) would suggest a lower, not higher, \( \alpha_2 \) abundance in OA muscle. Hence, fibre type differences were unlikely to explain our finding of reduced \( \alpha_2 \) abundance diverging NKA content and \( \alpha_2 \) isoform
abundance results. Small differences in sample size due to limited muscle samples in some participants are unlikely to have influenced the $[^3]$Houabain binding site content results, as the $[^3]$Houabain binding site content was only 1% difference in OA, with a negligible effect size of 0.05. Given that $\alpha_2$ western blot analyses were performed on muscle from 19 OA and 13 CON with statistical power of 0.77, a limited sample size seems unlikely.

The most likely explanation for the divergent NKA results is methodological differences used to detect muscle NKA via the $[^3]$Houabain binding technique for total muscle NKA determination, and western blot analyses for the relative abundance of NKA isoforms. Western blot analysis involves quantification of NKA isoforms from a standardised total protein concentration from a muscle homogenate, while the $[^3]$Houabain binding technique detects functional NKA content in larger intact portions (~3-5mg) of muscle. While our OA group reported higher physical activity, potentially due to the upcoming surgery, osteoarthritis populations generally have reduced physical activity (Naal et al., 2010) and muscle characteristics typical of disuse (Fink et al., 2007). Disuse lowers myofibrillar density of skeletal muscle (Larsson et al., 1996b). Hence, if the OA group had a higher $\alpha_2$ isoform abundance, but decreased myofibrillar density due to long-term inactivity, this may manifest in an unchanged $[^3]$Houabain binding site content as seen in this study. This would imply that the functional NKA content in a given muscle volume is actually the same in OA. Because we have no measure of myofibrillar density or long term physical activity, this is speculative, but remains the most likely explanation for the divergent results of $\alpha_2$ abundance and $[^3]$Houabain binding site content reported in our study.
3.5 Conclusions

This investigation into skeletal muscle NKA in people with knee osteoarthritis reveals firstly that there was no difference in total muscle NKA content despite greatly reduced muscle strength. This suggests that appropriate physical activity might maintain muscle NKA even in populations with functional joint restriction. Secondly we show for the first time an inverse association between skeletal muscle NKA content and age in elderly participants. This has broader implications of a reduced NKA content in older individuals in both healthy and clinical population, including contributing to reduced exercise tolerance in older populations.
CHAPTER 4: SHORT-TERM UNILATERAL LIMB UNLOADING IMPAIRS MUSCLE SIZE, STRENGTH, POWER AND POSTURAL SWAY

4.1 Introduction

Physical inactivity causes major maladaptations in skeletal muscle morphology, power and strength (Narici et al., 2011). The prevalence of lower limb injury is considerable; almost 3 million people per year in the USA suffer a sports related lower limb injury, 464,000 of which include fractures likely requiring immobilisation or unilateral inactivity (Conn et al., 2003). Unilateral lower limb suspension (ULLS) can mimic the disuse which occurs in lower limb injury; three weeks of ULLS induces localised detrimental effects on muscle cross sectional area (CSA) and strength of the knee extensors and the plantar flexors (Hackney et al., 2011). After 3-4 weeks of ULLS, a 7-10% reduction in knee extensor CSA has been reported (Berg et al., 1991; Schulze et al., 2002; Clark et al., 2007; de Boer et al., 2007a) and a 21-42% decrease in knee extensor maximal voluntary torque (Dudley et al., 1992b; Ploutz-Snyder et al., 1995; Schulze et al., 2002; Clark et al., 2007; de Boer et al., 2007a). Such substantial decline in muscle strength may adversely affect the capacity to perform activities of daily living and reduce leisure activities (Kell et al., 2001). The ULLS protocol could cause a detraining effect via the promotion of a sedentary lifestyle. Hence, muscle strength, CSA and postural sway may also be impaired in the weight-bearing or “unaffected” limb after ULLS, although the few studies which have investigated knee extensor muscle endurance or size in the weight-bearing leg after prolonged ULLS found no differences (Hather et al., 1992; Berg et al., 1993). Elucidation of the effects on both the
unloaded and weight-bearing leg after ULLS will provide more complete and clinically relevant information which will be applicable to populations requiring the use of crutches.

Functional changes after ULLS are not limited to muscular strength and size. Previous research suggests that balance, measured via postural sway, is impaired after as little as 14 d of bed rest (Kouzaki et al., 2007; Sarabon et al., 2013). This is an important finding as decreased balance has been associated with increased joint injury risk in several sports (Hrysomallis, 2007), and increased falls history in the elderly (Muir et al., 2013). However, it is unknown whether such maladaptations to postural sway occur in a localised and less severe model of disuse and persist after cessation of inactivity. Muscle power is another less investigated, yet important, measure after ULLS. Vertical jump height was reduced after short-term ULLS by 20% in two-legged vertical jump and by 26% in one-legged vertical jump height of the unloaded leg, while no change was found on the weight-bearing leg (Horstman et al., 2012). However, the recovery of vertical jump height and muscle power after ULLS has not been investigated.

Despite the major impairment in muscle function caused by ULLS, the recovery of voluntary strength after cessation of ULLS is surprisingly rapid. One half of the decrement in knee extensor strength after 42 d of ULLS was recovered after only four d of re-ambulation (Berg et al., 1991), whilst after three weeks of ULLS, the decrements in muscle cross sectional area, fibre size, myosin content and strength were all reversed by three weeks of resistance retraining (Campbell et al., 2013). Conversely, muscle peak power after 90 d of bed rest did not return until 140 d of re-ambulation (Rittweger et al., 2007). Despite the notable decrements to vertical jump height and potential decrement to postural sway (Kouzaki et
al., 2007; Horstman et al., 2012) after inactivity, the rate of recovery of either of these factors is not known and may provide important information for the rehabilitation of patients from lower limb injury, especially to return to sporting and leisure activity.

This study investigated the functional maladaptations in skeletal muscle size, strength, power and postural sway induced by 23 d of ULLS and their recovery following four weeks of resistance training in both the unloaded and weight-bearing limbs in healthy young humans. It is hypothesized that short-term unloading will cause a substantial reduction in muscle strength, size, vertical jump height and postural sway only in the unloaded leg, which will be reversible after four weeks of resistance training.

4.2 Methods

4.2.1 Participants

Six sedentary healthy adults (4 males, 2 females; age: 22.4± 2.1 yrs; mass: 71.3 ± 14.3 kg; height: 175 ± 11.7 cm; BMI: 23.2 ± 3.6 kg.m⁻², VO₂ peak 45.5 ± 5.8 ml.kg⁻¹.min⁻¹; mean ± SD) provided written informed consent and participated in the research. Exclusion criteria included recent history of lower limb injury, family or personal history of deep vein thrombosis and any other chronic disease contraindications to maximal exercise, strength testing, balance testing, or muscle biopsies. The study was approved by the Victoria University Human Research Ethics Committee, and was part of a larger study also investigating changes to skeletal muscle NKA content and time to fatigue and [K⁺] regulation during exercise after ULLS and resistance training, as presented in Chapter 5.
4.2.2 General design and testing overview

All participants underwent 23 consecutive days of ULLS to induce isolated unloading of the left leg, followed by four weeks of resistance training that included 12 lower body-based resistance training sessions (Figure 4.1). The testing battery was performed over three days on three separate days and completed at baseline, after the ULLS period and after the 4 week resistance training period. The first day in order of testing, included measurement of postural sway, vertical jump height and maximal knee extensor strength testing. The second day consisted of body composition testing (Dual-energy X-ray absorptiometry; DXA scan), whilst on the third day, resting muscle samples were taken and exercise testing completed as described in Chapter 5. The testing was completed over these three days to ensure complete recovery before the dynamic exercise testing. The muscle function testing completed after ULLS was performed 48 hr before the biopsy; hence these muscle function results reflect the effects of only 21 days of ULLS whereas muscle samples reflect 23 days of ULLS.
4.2.3 Inactivity protocol: Unilateral Lower Limb Suspension

Participants completed ULLS with the left leg unloaded using a modified ULLS protocol described previously (Tesch et al., 2004). In brief, the participant ambulated with the aid of forearm crutches and a 10 cm extended-sole shoe on the right leg, causing the left leg to become unloaded while the participant completed daily tasks and occupational requirements. There was no strapping or bracing of the unloaded leg, thus the 10 cm-extended sole shoe allowed the leg to hang freely during ambulatory activity. The left leg was chosen to be unloaded to allow participants to continue to drive an automatic transmission car and thus to minimise the impact on their daily lives. Participants were instructed to continue to perform all daily tasks, but without any weight bearing on the left leg and to avoid any exercise. To encourage compliance to the ULLS protocol, participants kept a daily diary and were contacted daily by either telephone or a meeting. Additionally,
Calf skin temperature was measured two to three times per week throughout the 23 d of ULLS to demonstrate compliance to the ULLS protocol via evidence of reduced skin temperature on the unloaded leg (Ploutz-Snyder et al., 1995; Tesch et al., 2004). As ULLS may increase the risk of superficial and deep vein thrombosis (DVT) (Bleeker et al., 2004), participants wore a compression stocking on the unloaded leg, performed self-massage, passive joint movement and consumed a 100 mg aspirin on a daily basis throughout the ULLS period to decrease the risk of DVT. Additionally, a venous blood sample was taken twice per week during ULLS and analysed for plasma d-dimer at an independent clinical pathology lab (Melbourne Pathology) using an automated quantitative test which analyses fibrin monomers (STA Liatest D-DI, USA). Finally, participants were contacted daily to check for any signs of possible thrombosis including redness, swelling and pain (Rittweger et al., 2006). Throughout ULLS, no participant recorded D-dimer levels greater than the reference range of 500 µg/L and no participant acquired, or presented symptoms of a superficial or deep vein thrombosis.

4.2.4 Resistance training

Three to four days after the completion of ULLS, participants commenced a lower-body based progressive resistance training program, comprising three sessions per week for four weeks (12 sessions total). The program comprised three to four sets of each the following exercises: one-legged press, one-legged knee extension, one-legged hamstring curls and one-legged calf-raises. The sets were performed at 65-70% of 1 repetition maximum (1RM), with 8-12 repetitions per set; 1RM testing was conducted similar to as previously described (Levinger et al., 2009). To induce progressive overload, weight was increased by 5-10%.
when the participant could complete three sets of 12 repetitions of an exercise. Rest between sets was 60 s and a warm up comprised two submaximal sets before the start of each exercise. Participants had 48 hr rest between each resistance training session. To compensate for the likely greater loss in muscle mass and strength in the unloaded leg, the first four training sessions were only performed on the unloaded leg and thereafter both legs were trained.

4.2.5 Body composition, muscle fibre cross sectional area and fibre typing

4.2.5.1 DXA scans
Dual x-ray absorptiometry scans (DXA, Hologic Discovery W, MA, USA) were performed at baseline, day 22 of ULLS and after 4 weeks resistance training. Scans were performed on the same machine and by an experienced clinical technician throughout the study. The total body mass, lean mass and fat mass of the whole body and thighs were determined.

4.2.5.2 Muscle biopsies
After 20 minutes of supine rest, a local anaesthetic was injected into the skin and fascia of the middle third of the vastus lateralis muscle (1% Xylocaine). A small incision was made; and a muscle sample was taken (~100-200mg) using a Bergström biopsy needle as described by our group previously (Murphy et al., 2006). Muscle samples were blotted to remove excess blood, immediately frozen in liquid nitrogen and stored at -80°C until analyses. An additional piece of muscle was mounted in OCT gel (OCT gel, ProSciTech, Australia) and frozen in pre-cooled isopentane (Sigma Aldrich, MO, USA) for later immunofluorescent analysis of fibre type and cross sectional area.
4.2.5.3 Muscle fibre type and cross sectional area analysis

Serial tissue sections of approximately 5 µm thickness were cut at -20°C (HM 550 cryostat, Thermo Fisher Scientific, Australia) and thaw-mounted onto glass microscope slides (Starfrost, ProSciTech, Australia). Slides were stored at -80°C until analysed. Tissue sections were removed from -80°C storage and left to air dry for 30 min at room temperature before being fixed in 4% (wt/vol) paraformaldehyde (Sigma-Aldrich, Australia), permeated with 5% (vol/vol) Triton X-100 (Sigma-Aldrich, Australia) and blocked in 3% (wt/vol) bovine serum albumin (Sigma-Aldrich, Australia) for 30 min at room temperature in a humid chamber. Sections were rinsed four times with phosphate buffered saline (PBS) in between each stage. Primary antibodies, diluted in 3% BSA in PBS, were applied and left to incubate in a humid chamber overnight at 4°C. The sections were washed four times in PBS. Secondary antibodies, diluted in 3% BSA in PBS, were applied and incubated for 2 hr in a humid chamber at room temperature in the dark. Sections were washed four times and then incubated with the nuclear stain bis-benzimide (Hoechst 33285, Sigma-Aldrich, Australia), then mounted in PBS, coverslips applied and sealed.

Fibre-typing of the cross-sections was performed using the monoclonal anti-myosin antibody A4.480 specific to Type I myosin (developed by H. Blau and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa, Department of Biological Sciences, IA, USA) and anti-laminin antibody produced in rabbit (L9393, Sigma, MO, USA). All unstained fibres were classified as myosin Type II. Secondary antibody-coupled fluorophores used were Alexa Fluor® 488 goat anti-
mouse A21042 and Alexa Fluor® 594 donkey anti-rabbit A21207 (Invitrogen, USA). For negative controls and the assessment of background fluorescence, the primary antibody was omitted and in all cases the fluorescence signal was removed.

Immunostained sections were visualised with an Olympus BX51 fluorescent microscope and digital images were captured using a DP72 colour CCD camera and Cell*F software (Olympus Corporation, Japan). Wavelength specific filters were used to visualise the Alex Fluor 488 and 594 fluorophores, respectively. Captured images were analysed using ImageJ Software (NIH, Bethesda, MD). Using the laminin stained membranes for reference the individual muscle fibres were selected using the polygonal tool and a cross-sectional area was calculated in µm² for each fibre. Only well cross-sectioned fibres with clear borders were measured, all fibres on the edge of the field of view were also excluded. The number of fibres counted per sample was 153± 64 for fibre typing and 124 ± 52 for fibre CSA.

4.2.6 Functional tests

4.2.6.1 Maximal voluntary strength

Peak maximal voluntary torque of the knee extensors was measured using an isokinetic dynamometer (Cybex II Lumex, NY, USA), whilst in a seated position with hips fixed at 90° at the following speeds: 0 (isometric; 60° knee extension), 60, 120, 180, 240, 300 and 360 °.s⁻¹. Participants completed a familiarisation session prior to baseline testing. The testing order of the weight-bearing and unloaded legs was counterbalanced between participants, but remained identical for each participant’s testing sessions. Each set comprised three warm up repetitions and then three consecutive maximal efforts at that velocity, each set was
separated by one minute rest. Participants were verbally encouraged to give a maximal effort during each repetition and the highest peak torque achieved during each speed of contraction was recorded as the maximum torque for that cadence.

4.2.6.2 Vertical jump height

Vertical jump height (VJH) was estimated from flight time (Bosco et al., 1983) using a force platform (400 Series Performance Force Plate; Fitness Technology, Adelaide, Australia) with data sampled at 200 Hz and interfaced with a personal computer (Ballistic software, Innervations, Australia). Flight time was estimated from the equation: \( h = g \cdot \frac{ft^2}{8} \); where \( h \) is the jump height in m; \( g \) is gravity acceleration [9.81 m \( \cdot \) s\(^{-2}\)]; \( ft \) is the flight, time in s (Bosco et al., 1983). VJH was measured for three separate tests comprising two-legged and single-leg jumps on the weight-bearing and unloaded legs. Participants were familiarised with the jumping protocol several days before baseline with a minute rest between sets (de Ruiter et al., 2007); participants performed five counter-movement two-legged jumps with 20 s rest between each jump. This was followed by four, one-legged jumps on each of the weight-bearing and unloaded legs. Jump data were discarded if landing occurred more than 2 cm posterior or anterior to the starting position. The one-legged jumps were performed with the contra-lateral leg remaining in front of the jumping leg. The depth of knee flexion was self-selected in both the two and one legged jumps, with hands held behind the back to minimise countermovement from the upper-body. The order of the weight-bearing and unloaded leg testing was counterbalanced between participants, but was identical in each participant’s testing sessions. Data presented is the combined average of the three highest estimated jump heights.
4.2.6.3 Postural sway

Balance was assessed via the anterior-posterior standard deviation (APSD) of the centre of pressure (Suomi et al., 2000; Qiu et al., 2012). The APSD was chosen as a measure of postural sway as the muscle groups involved, such as the knee extensors and plantar flexors, are commonly affected by inactivity (Adams et al., 2003; Hackney et al., 2011; Narici et al., 2011) furthermore anterior-posterior postural was increased in two-legged quiet stance after bed rest (Kouzaki et al., 2007). Postural sway was measured on a force platform (400 Series Performance Force Plate; Fitness Technology, Adelaide, Australia) with a data sampling rate of 200 Hz interfaced with a personal computer with software for analysis of postural sway (InnerBalance software, Innervations, Australia). The participants were familiarised to the protocol before baseline testing. Postural sway APSD were measured under quiet conditions during two-legged stance, on the weight-bearing and unloaded leg alone with both eyes open and closed. The two-legged testing was performed three times, with each trial 30 s in duration; feet were kept at hip width apart and this distance was standardised between trials. The one-legged tests were performed four times, each of 10 s duration. Hands were kept behind the participants back during all conditions and the tests were performed in a quiet environment. The order of the weight-bearing and unloaded leg testing was counterbalanced between participants, but remained in the same order in each participant’s testing sessions. Data presented is the average of all APSD trials for each test. Coefficient of variance at baseline was 20.9% for two-legged stance, 17.1% for the single-legged eyes open stance and 13.2% for the single legged eyes closed condition.
4.2.7 Statistical analysis

All data are reported as mean ± standard deviation (SD). Log-transformation was performed on data which were not normally distributed to reduce bias. Statistical significance was accepted at $P<0.05$. One way repeated measures ANOVA was used to analyse changes in body composition, maximal knee extensor torque and vertical jump height on both weight-bearing and unloaded legs, with after ULLS and resistance training, with Tukey’s LSD used for post-hoc comparison. Correlations were analysed using Pearson’s product-moment correlation coefficient. Effect size with 90% confidence intervals (ES ±90% CI) used to compare magnitudes of effect between and within legs, to account that the weight-bearing leg may have still been affected during ULLS. Magnitudes of change using Cohen’s effect size was classified as; trivial <0.2; small, 0.2-0.6; moderate, 0.6-1.2; large, 1.2-2.0; and very large, 2.0-4.0 (Batterham et al., 2006; Hopkins, 2007). Effects with less certainty (magnitude of <75%) was classified as no meaningful difference (Batterham et al., 2006; Hopkins, 2007). One way repeated measures ANOVA, mixed linear models were calculated using SPSS version 20 (SPSS Inc., Champaign, IL) and effect size calculated via a custom spreadsheet (Hopkins, 2007).

4.3 Results

4.3.1 Compliance to ULLS protocol and resistance training

Participants verbally reported full compliance to the protocol and calf skin temperature was 1.4°C lower on the unloaded leg than in the weight-bearing leg (27.8 ± 0.9 vs 29.2 ± 1.1°C; $p = 0.001$). Participants completed 92% of the resistance training sessions (66 out of 72 sessions, n = 6).
4.3.2 Body composition and muscle fibre cross sectional area

Total body mass, lean body mass and body fat percentage did not change after ULLS, or after resistance training (p > 0.4; Table 4.1). Thigh mass decreased after ULLS in the unloaded leg by 4.43% (p = 0.047, ES: 0.15±0.12) and was then increased by 5.90% above post-ULLS following training (p = 0.01, ES 0.19 ± 0.07) reaching levels not significantly different to from baseline. In the weight-bearing leg, no significant changes were found in the thigh mass between any of the time points (Table 4.2). After ULLS, there was a trivial effect size between the unloaded and weight-bearing thigh mass (ES: 0.12±0.1).

Table 4.1: Body mass, lean mass (muscle and bone) and body fat percentage at baseline, after 21 d unilateral lower limb suspension (ULLS) and after 4 weeks resistance training.

<table>
<thead>
<tr>
<th>Body composition</th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (kg)</td>
<td>70.6 ± 14.5</td>
<td>70.3 ± 14.8</td>
<td>71.0 ± 14.8</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>50.8 ± 12.6</td>
<td>50.2 ± 12.7</td>
<td>51.0 ± 12.9</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>24.8 ± 6.4</td>
<td>25.3 ± 6.4</td>
<td>25.1 ± 6.4</td>
</tr>
</tbody>
</table>

Values are mean ± SD, n = 6.
Table 4.2: Total thigh mass at baseline, post-ULLS and post training.

<table>
<thead>
<tr>
<th>Thigh Mass (kg)</th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unloaded leg</td>
<td>6.59 ± 1.52</td>
<td>6.31 ± 1.61 *</td>
<td>6.68 ± 1.61 #</td>
</tr>
<tr>
<td>Weight-bearing leg</td>
<td>6.65 ± 1.51</td>
<td>6.60 ± 1.67</td>
<td>6.79 ± 1.69</td>
</tr>
</tbody>
</table>

ES ± 90% CI between legs 0.03 ± 0.07 0.15 ± 0.1 ^ 0.06 ± 0.07

* significantly lower than baseline (p <0.05), # significantly higher than post-ULLS. ^ trivial effect size (>75% certainty). Values are Mean± SD, n = 6.

After ULLS and resistance training there were no significant changes in muscle CSA. However, combined muscle fibre CSA tended to decrease by 20.1% in the unloaded leg after ULLS, with a small effect size (p = 0.057, ES: 0.54 ± 0.37) and there was a trend for a 22.7% decrease with a moderate effect size in Type II fibre CSA after ULLS (p = 0.11. ES: 0.63±0.4; Table 4.3) and a trend with a small effect size in Type I fibre CSA after ULLS (p = 0.22, ES: 0.42 ± 33). In addition, there were small ES in combined, Type I and Type II fibres after ULLS in the weight-bearing leg, but no meaningful ES were found between legs (Table 4.3). No changes were found in Type I fibre distribution after ULLS or after resistance training in either the unloaded or weight-bearing leg, or any difference between legs (Table 4.4).
Table 4.3: Muscle fibre cross sectional area at baseline, post-ULLS and post-training.

<table>
<thead>
<tr>
<th>Muscle Fibre CSA (µm)</th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unloaded leg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>4009.1 ± 1631.6</td>
<td>3328.1 ± 1052.7†</td>
<td>3693.1± 1130.8</td>
</tr>
<tr>
<td>Type II</td>
<td>4710.0 ± 1618.4</td>
<td>3636.6 ± 1219.7†</td>
<td>4415 ± 1136.37</td>
</tr>
<tr>
<td>Combined</td>
<td>4359.7 ± 1598.9</td>
<td>3482.4 ± 1201.2†</td>
<td>4054.2 ± 1101.6</td>
</tr>
<tr>
<td><strong>Weight-bearing leg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>4074.3 ± 961.6</td>
<td>3371.6 ± 953.4†</td>
<td>3873.1 ± 1019.9</td>
</tr>
<tr>
<td>Type II</td>
<td>4695.8 ± 1162.5</td>
<td>3860.4 ± 1201.1†</td>
<td>4375.4 ± 723.5</td>
</tr>
<tr>
<td>Combined</td>
<td>4385.1 ± 1009.7</td>
<td>3616.0 ± 1017.8†</td>
<td>4124.3 ± 806.9</td>
</tr>
<tr>
<td><strong>ES± 90% CI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>0.04±0.5</td>
<td>0.03±0.75</td>
<td>0.14 ±0.89</td>
</tr>
<tr>
<td>Between legs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II</td>
<td>0.01±0.36</td>
<td>0.16±0.16</td>
<td>0.03±0.59</td>
</tr>
<tr>
<td>Combined</td>
<td>0.01±0.25</td>
<td>0.1±0.73</td>
<td>0.06±0.72</td>
</tr>
</tbody>
</table>

† small effect size. Values are Mean ± SD, n = 6.
Table 4.4: Muscle fibre type I distribution at baseline, post-ULLS and post-training.

<table>
<thead>
<tr>
<th>Type I fibre distribution (%)</th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unloaded</td>
<td>54.1±7.4</td>
<td>54.4±9.5</td>
<td>51.1±4.3</td>
</tr>
<tr>
<td>Weight-bearing</td>
<td>53.3±6.8</td>
<td>50.5±6.2</td>
<td>51.7±7.4</td>
</tr>
<tr>
<td>ES±90% CI between legs</td>
<td>0.07±0.32</td>
<td>0.40±0.56</td>
<td>0.18±0.91</td>
</tr>
</tbody>
</table>

Values are Mean ± SD, n = 6.

4.3.3 Functional testing

4.3.3.1 Maximal knee extensor torque

After 21 d ULLS maximum knee extensor torque in the unloaded leg, decreased compared to baseline by 24.4% at 0 °.s\(^{-1}\) (p = 0.003, ES: 0.67±0.25) and by 22.6% at 60°.s\(^{-1}\) (p = 0.002, ES: 0.59±0.2) but with no change when measured at faster contraction velocities (Figure 4.2). After resistance training, torque increased above post ULLS levels by 34.2% at 0 °.s\(^{-1}\) (p = 0.004, ES: 0.72±0.29) and 22.3% at 60 °.s\(^{-1}\) (p = 0.004, ES: 0.45±0.18) to levels not statistically different from baseline (Figure 4.2).

In the weight-bearing leg after ULLS, isometric maximal knee extensor torque tended to decrease (13.7%, p = 0.057, ES: 0.33±0.27) compared to baseline, decreased by 14.5% at 60 °.s\(^{-1}\) (p = 0.015, ES: 0.38±0.21) and by 9.2% at 120 °.s\(^{-1}\) (p=0.001, ES: 0.22±0.10; Figure 4.2). Compared to post-ULLS, resistance-training caused a 27% increase in isometric torque (p=0.010, ES: 0.56±0.22), a 14.1% increase at 60 °.s\(^{-1}\) (p=0.001, ES: 0.31±0.08) and a 13.3% increase at 120 °.s\(^{-1}\) (p = 0.004, ES: 0.29±0.11) to levels not statistically different than
baseline (Figure 4.2). No other significant changes or meaningful effect sizes were found. There was no meaningful effect size between the unloaded and weight-bearing legs at any of baseline, post-ULLS and post-training.

**Figure 4.2**: Maximal voluntary torque during isokinetic dynamometry measured at 0° - 360°.s⁻¹ on the unloaded (A) and weight-bearing leg (B). Black squares represent baseline, empty inverted triangle represent post-ULLS and solid circles represent post-training. * lower than baseline (p <0.05). † Small effect size from baseline, ‡ Moderate effect size from baseline. Values are mean ± SD, n = 6.

### 4.3.3.2 Vertical jump height

Vertical jump height decreased by 16.2% after ULLS (p = 0.041, ES: 0.46±0.3) and increased after training by 13.6% to levels similar to baseline (p = 0.004, ES: 0.33±0.11, Table 4.5). There was a small effect size between the unloaded and weight-bearing leg after ULLS. No
significant changes or meaningful effect sizes were found on the weight-bearing or for two-legged jump height, Table 4.5.

**Table 4.5:** Two-legged, unloaded single-leg and weight-bearing single-leg VJH as calculated from flight time at baseline, post-inactivity and post-training.

<table>
<thead>
<tr>
<th>Vertical Jump height (cm)</th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-legged</td>
<td>28.3± 9.6</td>
<td>27.5 ± 8.5</td>
<td>28.5 ± 9.3</td>
</tr>
<tr>
<td>Unloaded leg</td>
<td>14.9 ± 5.1</td>
<td>12.5 ± 4.2</td>
<td>14.2 ± 4.8 *†</td>
</tr>
<tr>
<td>Weight-bearing leg</td>
<td>14.5 ± 5.2</td>
<td>14.3 ± 5.3</td>
<td>15.6 ± 5.4</td>
</tr>
<tr>
<td>ES ± 90% CI Between legs</td>
<td>0.04±0.12</td>
<td>0.38±0.28</td>
<td>^ 0.26±0.2</td>
</tr>
</tbody>
</table>

* lower than baseline (p <0.05), # greater than post-ULLS, ^ meaningful effect size (>75% certainty), † small effect size from baseline. Values are Mean± SD, n = 6.

**4.3.3.3 Postural sway**

After ULLS, APSD increased by ~2.2 fold in the two-legged eyes closed condition (p=0.021, ES: 1.5±0.91) and for the unloaded leg increased during both the eyes open (34%; p = 0.015, ES: 1.0±0.55,) and closed conditions (32%; p = 0.002,ES: 1.66±0.55, Table 4.6). After training, APSD decreased 71% in comparison to post-ULLS in the two-legged eyes closed condition (p =0.044, ES: 1.15±0.87) and on the unloaded leg in the eyes closed condition (26% decrease, p = 0.046,ES: 1.43±1.09). Post-training APSD was not different to baseline in any condition. Post-ULLS there was a small effect size between legs with eyes closed APSD.
and a moderate effect with the eyes open condition (Table 4.6). There was no change in APSD post ULLS or post-training in the weight-bearing leg.

Table 4.6: Anterior-posterior standard deviation (cm) of the centre of pressureduring two-legged and one-legged stances with eyes open and closed, measured at baseline, post-ULLS and post-training.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Two-legged stance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes open</td>
<td>0.185 ± 0.056</td>
<td>0.409 ± 0.193</td>
<td>0.237 ± 0.129</td>
</tr>
<tr>
<td>Eyes closed</td>
<td>0.205 ± 0.047</td>
<td>0.448 ± 0.186 *§ 0.261 ± 0.094 #</td>
<td></td>
</tr>
<tr>
<td><strong>Unloaded leg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes open</td>
<td>0.488 ± 0.169</td>
<td>0.654 ± 0.105 *‡ 0.567 ± 0.122</td>
<td></td>
</tr>
<tr>
<td>Eyes closed</td>
<td>1.084 ± 0.200</td>
<td>1.430 ± 0.146 *§ 1.132 ± 0.310 #</td>
<td></td>
</tr>
<tr>
<td><strong>Weight-bearing leg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes open</td>
<td>0.566 ± 0.151</td>
<td>0.562 ± 0.079</td>
<td>0.554 ± 0.232</td>
</tr>
<tr>
<td>Eyes closed</td>
<td>1.178 ± 0.145</td>
<td>1.278 ± 0.266</td>
<td>1.276 ± 0.256</td>
</tr>
<tr>
<td><strong>ES ± 90% CI between legs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes open</td>
<td>0.41±0.43 ^ 0.84±0.33 ^ 0.06±0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes closed</td>
<td>0.45±0.59 0.59±0.46 ^ 0.43±0.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* greater than baseline p <0.05, # different from post-ULLS (p <0.05), ^ meaningful effect size (>75% certainty). ‡ moderate effect size from baseline, § large effect size from baseline.

Values are mean ± SD, n = 6.
4.3.3.4 Correlations

No significant correlations were found between the percentage change in torque at any speed, muscle CSA, muscle fibre CSA, postural sway, or vertical jump height on either leg after ULLS or post training (p >0.05).

4.4 Discussion

This study investigated the broad changes to muscle function in both the unloaded and weight-bearing legs after 23 d ULLS and four weeks resistance training and found impaired muscle strength, mass, vertical jump height and postural sway in the unloaded leg after ULLS, which was restored after resistance training. Unexpectedly, there was also a decrease in strength in the weight bearing leg which was also restored after training. The broad impairment of muscle function after ULLS, which was not solely localised to the unloaded leg, has clinical implications for the rehabilitation of patients with lower limb injuries requiring the use of crutches.

4.4.1 Muscle strength and mass decreased after 21 d ULLS

This thesis found a 4.4% reduction in muscle mass and a 24% decline in isometric knee extensor strength in the unloaded limb after three weeks ULLS. The decline in knee extensor strength after ULLS was substantial, and the discrepancy between the loss of strength and loss of muscle mass after ULLS demonstrates that muscle mass or CSA alone likely represents a minor contribution to impairment of muscle function, a finding consistent with previous ULLS studies (Berg et al., 1991; Dudley et al., 1992b; Ploutz-Snyder et al., 1995; Schulze et al., 2002; Clark et al., 2006a; Clark et al., 2007; de Boer et al., 2007a). Factors which could be impaired after ULLS leading to decreased knee extensor torque
include reduced neural drive and impaired spinal reflex response (Kawakami et al., 2001; Deschenes et al., 2002; Clark et al., 2006a; de Boer et al., 2007a; Seynnes et al., 2009), changes to muscle pennation angle, reduced tendon stiffness and increased fascia length (de Boer et al., 2007a). In addition, isolated skeletal muscle fibres from the vastus lateralis and soleus show reduction in peak force normalised for CSA after bed rest (Larsson et al., 1996a; Widrick et al., 1997), suggesting intrinsic factors within the muscle are also impaired after inactivity. The factors within skeletal muscle that could lead to impaired muscle strength and fatiguability after inactivity include impaired sarcoplasmic reticulum functioning (Thom et al., 2001), decreased mitochondrial function (Abadi et al., 2009) and potentially muscle NKA content, as investigated in Chapter 5.

Contrary to one of the hypotheses, this thesis found a 9 and 14% reduction in knee extensor peak torque in the weight bearing leg at 60 and 120 °.s\(^{-1}\) respectively after three weeks ULLS; which is more than half of the decrement in peak torque found in the unloaded leg. This decline could reflect a sedentary lifestyle or detraining effect promoted by the ULLS protocol; although physical activity of the weight-bearing leg was not quantified in this or previous research which has investigated the decline in muscle CSA in both the weight-bearing and unloaded legs (Berg et al., 1991; de Boer et al., 2007a). Participants in this study were able to drive an automatic car, which is contrary to other research using immobilisation which did not allow participants to drive (Suetta et al., 2009), and thus required participants to use public transport. Hence, it is plausible that the participants in our study adopted a more sedentary lifestyle than that of participants in previous ULLS studies. This demonstrates the importance of greater monitoring of physical activity in
future ULLS studies. While untrained populations are typically resistant to detraining within a short time-frame (Houston et al., 1983), detraining effects have been noted in trained populations. For example, dancers who ceased training for 8 weeks had a 12% decline in knee extension force (Dahlström et al., 1987). Hence, the restriction which ULLS places on overall incidental activity may induce a detraining effect in as little as three weeks that is not related to a reduction in muscle mass, an important clinical consideration for patients with lower limb injury.

4.4.2 Postural sway is impaired after ULLS

This thesis investigated postural sway after ULLS and found increased postural sway only in the unloaded limb that was reversible with four weeks of resistance training. This increase in postural sway after three weeks of ULLS is important considering that poor balance is associated with increased sporting injury and falls in the elderly (Hrysomallis, 2007; Muir et al., 2013). Knee extensor strength or mass were not correlated with the impairment of postural sway, similar to a bed rest finding (Kouzaki et al., 2007). In addition, postural sway was impaired only in the unloaded leg, suggesting localised peripheral factors were impaired, rather than a “whole-body” reduction in balance. The underlying mechanisms for the impairment of postural sway after inactivity are not clear; although it is likely due to undescribed impairment of sensorimotor control, including impairment of proprioceptive organs in muscle such as the golgi tendon organ (Fitts et al., 2001). However, the restoration of postural sway after four weeks of resistance training indicates that the impairment caused by inactivity is rapidly reversible in young healthy populations.
4.4.3 ULLS impairs vertical jump height in the unloaded leg

A 16% decrease in VJH was found only in the unloaded leg, with no changes in the two-legged or weight bearing leg VJH. Previous research reported an association between VJH and maximal knee extensor torque after ULLS (Horstman et al., 2012) using video analysis of VJH from a half-squat position, but found no relationship previous to the ULLS intervention. However, this thesis found no correlations between knee extensor torque of the unloaded leg at any contraction speed and VJH. Further, there was no change in VJH in the weight-bearing leg despite a 9-14% decrease in knee extensor torque. Hence, the results suggest that ULLS can reduce VJH via maladaptations localised to the unloaded leg, including decreased movement co-ordination impaired corticospinal excitability (Clark et al., 2006a; Clark et al., 2007) and changes in plantar flexion strength (Hackney et al., 2011).

4.4.4 Resistance training restores muscle strength, mass and VJH after ULLS

Measures impaired by ULLS, including knee extensor strength, mass, power and postural sway were all restored to levels similar to baseline after four weeks of resistance training. This full restoration in muscle function is remarkable after 4 weeks considering the 24% decrement in knee extensor strength, 4.4% decrease in thigh muscle mass and 16% reduction in VJH after ULLS. Similar recovery in muscle CSA was found seven weeks after 42 d of ULLS (Hather et al., 1992) and impairment of muscle pennation angle and tendon stiffness caused by three week ULLS were reversed within three weeks of resistance training (Campbell et al., 2013). While re-ambulation itself may be a powerful stimulus after ULLS, the addition of resistance training after inactivity is superior to re-ambulation alone in reversing inactivity-induced strength loss; isometric and eccentric knee extensors strength
loss after short-term immobilisation was reversed to a greater extent with resistance training compared to participants who performed no structured training (Hortobagyi et al., 2000). Hence, it is apparent that even short-term resistance training of similar length to the inactivity period rapidly reverses the broad impairments on muscle function and morphology after ULLS, perhaps due to the reversal of impairments to central drive and corticospinal excitability caused by ULLS (Deschenes et al., 2002; Clark et al., 2007; de Boer et al., 2007a; Deschenes et al., 2008; Seynnes et al., 2009) and changes in muscle architecture, including tendon stiffness, pennation angle and fascicle length (de Boer et al., 2007a; Campbell et al., 2013).

4.4.5 Limitations

This research had some limitations, including a relatively small sample size due to the difficulty of recruiting participants for ULLS studies. This was demonstrated with the lack of significant change in muscle fibre CSA despite a 20.2% decrease and small effect size after ULLS ($p = 0.057$, ES: $0.54 \pm 0.37$). This small sample size also likely made between-leg comparisons underpowered, although use of effect size did provide limited insight. Regardless, the study was powered sufficiently to detect changes in muscle strength, mass, power and postural sway; the key outcomes of this chapter. This study had both male and female participants, an important factor considering females may undergo greater strength loss in response to unloading (Deschenes et al., 2012). However, this was not able to be assessed in this study due to the small sample size. Finally, it is important to note that this is one of the few studies to investigate the weight-bearing leg comprehensively with the ULLS model, an important consideration for injured populations.
4.5 Conclusions

In conclusion, this study found that a short period of ULLS reduced each of muscle strength, VJH, thigh muscle mass and postural sway in the unloaded leg and also caused reduced strength in the weight-bearing leg. Importantly, four weeks of resistance training restored these impairments to muscle function. The findings suggest that impairments to muscle function after ULLS may not be localised only to the unloaded leg, which suggests potential benefit of ensuring rehabilitation in the weight-bearing, as well as the injured leg.
CHAPTER 5: THE EFFECTS OF SHORT-TERM UNLOADING AND RESISTANCE TRAINING ON EXERCISE PERFORMANCE, K⁺ REGULATION, MUSCLE Na⁺,K⁺-ATPase CONTENT AND ISOFORM ABUNDANCE IN HUMANS

5.1 Introduction

Physical inactivity causes substantial decrements in skeletal muscle strength, whole muscle cross sectional area (CSA) and endurance, which adversely impact quality of life (Kell et al., 2001; Narici et al., 2011). Decreased muscle CSA, changes in muscle pennation angle, fascicle length, decreased central drive and impaired neuromuscular responses all have a prominent role in the decline in muscle strength and endurance after inactivity (Berg et al., 1993; Clark et al., 2006a; de Boer et al., 2007a; Narici et al., 2011). However, after bed rest, isolated muscle fibres exhibit up to 40% reduction in peak force normalised for CSA (Larsson et al., 1996a; Widrick et al., 1997), implying that intramuscular factors could also be impaired after inactivity.

One potential maladaptation in skeletal muscle is in the ubiquitously expressed Na⁺,K⁺-ATPase (NKA) protein. In skeletal muscle NKA has a vital role in muscle excitability via the maintenance of [Na⁺] and [K⁺] gradients across sarcolemmal and t-tubular membranes (Clausen, 2003). A decreased NKA content, and/or activity has the potential to impair muscle excitability and contractility (McKenna et al., 2008). In human skeletal muscle, the NKA is a heterodimer comprises a catalytic α subunit with three isoforms (α₁-₃), a regulatory β subunit with three isoforms (β₁-₃) and a regulatory FXYD1 (phospholemman) protein (Blanco et al., 1998; Murphy et al., 2004; Nordsborg et al., 2005; Petersen et al., 2005;
Thomassen et al., 2010). The role of each individual isoform in skeletal muscle is not well understood, although the NKA α2 isoform represents approximately 75-80% of the α subunits in rat EDL (Hansen, 2001) and is vital for muscle function and fatigue resistance (Radzyukevich et al., 2013). Mice with complete NKA α2 knockout in skeletal muscle were unable to run on a treadmill at speeds over 4 m.s\(^{-1}\), compared to control mice which could reach up to 26 m.s\(^{-1}\), and exhibited substantial impairment of in vitro muscle strength and fatiguability, despite compensatory increases in the NKA α1 isoform (Radzyukevich et al., 2013). In humans skeletal muscle, NKA α2 relative abundance increased after only 10-14 d of endurance or sprint training (Thomassen et al., 2010; Benziane et al., 2011) and lowered by approximately 50% in spinal injury (Boon et al., 2012). A lowered skeletal muscle NKA content mediated by inactivity could contribute to the impairment of muscle function in injured and inactive populations.

In human skeletal muscle, NKA content, as measured using \(^{3}\text{H}\)ouabain binding site content, which assess total functional content of NKA in skeletal muscle (Nørgaard et al., 1984), is increased by 10-20% with resistance, endurance and sprint exercise training (Kjeldsen et al., 1986; Green et al., 1993; McKenna et al., 1993; Green et al., 1999a; Clausen, 2003; Edge et al., 2013). Conversely, injury which promotes inactivity lowers skeletal muscle NKA content by 25-58% (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012) and an 18-27% lower muscle NKA content is reported in chronic diseases associated with reduced physical inactivity, such as Type II diabetes mellitus and McArdle’s syndrome (Haller et al., 1998; Djurhuus et al., 2001). Patients with complete cervical spine injury had an 80% and 53% decrease in NKA α1 and α2 isoform protein abundance, respectively, at 12
months post-injury; with most of the decline in the first three months (Boon et al., 2012). In contrast, no change in NKA isoform abundance was seen in patients with less severe spinal injury that allowed for gait and basic mobility (Boon et al., 2012). Patients with shoulder impingement injury for a minimum of seven months had 26% lower NKA content in the deltoid muscle compared to the non-injured shoulder (Leivseth et al., 1994), suggesting that inactivity was a major factor underlying lower muscle NKA content. However, the use of injured and chronically ill populations to investigate inactivity effects on skeletal muscle NKA presents several issues. Injured populations do not have a standardised severity and duration of injury and concomitant effects of the injury itself, and possibly medications, are likely to reduce muscle function (Shields, 2002). Further, the potential reversibility of skeletal muscle NKA content after disuse is difficult to monitor with injured populations. Whilst muscle NKA content after inactivity has not been tested in humans, a 20-38% reduction in NKA content was exhibited in response to limb casting in other mammals (Kjeldsen et al., 1986; Leivseth et al., 1992; Jebens et al., 1995).

No study has investigated the effects of controlled inactivity, such as bed rest, immobilisation or unilateral lower limb suspension (ULLS) on skeletal muscle NKA content or NKA isoform abundance in humans. Hence, the magnitude, time-course, relation to exercise performance and reversibility of potential impairment to skeletal muscle NKA after muscle inactivity are not known. This study therefore investigated the effects of unilateral lower limb suspension (ULLS) and subsequent resistance training on skeletal muscle NKA content, NKA isoform (α₁-3, β₁-2) relative abundance, exercise performance as well as selected physiological variables during incremental one-legged exercise in humans. It was
hypothesized that in the unloaded leg after ULLS there would be decrements in muscle NKA content and $\alpha_2$ abundance, as well as reduced time to fatigue and increased venous plasma $[K^+]$ during cycling exercise after ULLS compared to both baseline and the weight-bearing leg, which will be reversible with four weeks of resistance training.

5.2 Methods

5.2.1 Participants

Six sedentary healthy adults (4 males, 2 females; age: $22.4\pm 2.1$ yrs; mass: $71.3 \pm 14.3$ kg; height: $175 \pm 11.7$ cm; BMI: $23.2 \pm 3.6$ kg.m$^{-2}$, $\dot{V}O_2$ peak $45.5 \pm 5.8$ ml.kg$^{-1}$.min$^{-1}$; mean $\pm$ SD) provided written informed consent and participated in the research. Exclusion criteria included recent history of lower limb injury, family or personal history of deep vein thrombosis and any other chronic disease contraindications to maximal endurance exercise, strength testing, balance testing, or muscle biopsies. The study was approved by the Victoria University Human Research Ethics Committee, and was part of a larger study investigating changes to muscle function after ULLS and resistance training as presented in Chapter 4.

5.2.2 General design and testing overview

All participants underwent 23 consecutive days of unilateral lower limb suspension (ULLS) to induce isolated unloading of the left leg, followed by four weeks of resistance training, which included 12 lower-body based resistance training sessions, with testing performed at baseline, after 23 d of ULLS and after 4 wks of resistance training. One week prior to testing, participants completed an incremental two-legged cycling test with measurement of
expired gases for determination of $\dot{V}O_2$ peak and subsequent calculation of one-legged cycling workrates, which were piloted at least 3 d before testing. The testing day comprised vastus lateralis muscle biopsies at rest in both legs, followed by one-legged cycling exercise of the unloaded and weight-bearing legs. Participants rested for two hours in a supine position between the testing of the unloaded and weight-bearing legs. The unloaded leg was always tested first to ensure no order effects were present from previous testing bout for that leg. Prior to and during the one-legged cycling exercise and in recovery, antecubital venous blood was sampled, while heart rate, $\dot{V}O_2$ and rating of perceived exertion were measured throughout the exercise period. Participants abstained from exercise, caffeine and alcohol for 48 hr before all testing sessions.

5.2.3 Inactivity protocol

ULLS was performed for 23 d as detailed (Chapter 4.2.3). In brief, the participant ambulated with the aid of forearm crutches and a 10 cm extended-sole shoe worn on the right foot, causing the left leg to swing freely but to be functionally unloaded, while the participant completed normal daily tasks. Participants verbally reported compliance to the protocol and skin temperature was on average 1.4 °C lower on the unloaded leg compared to the weight-bearing leg (Chapter 4).

5.2.4 Resistance training

Approximately 3-4 d after the completion of ULLS, participants commenced a lower-body based resistance training program three times per week for four weeks (12 sessions total) as detailed (Chapter 4.2.4).
5.2.5 One legged-cycling testing

5.2.5.1 VO₂ peak testing and one-legged cycling intensities

A two-legged incremental cycling test was initially performed to obtain peak oxygen consumption (VO₂ peak) and comprised four minutes at 40, 80 and 120 W for males, and 30, 60 and 90 W for females, followed by a 20 W increase every minute until volitional fatigue. The participant breathed through a 3-way rebreathing valve and expired gas volume was measured using a turbine flowmeter (KL Engineering, Sunnyvale, CA); mixed expired O₂ and CO₂ fractions were analysed by rapidly responding gas analysers (Applied Electrochemistry S-3A O₂ and CD-3A CO₂, USA), interfaced with Turbofit software (Vacumetrics, USA). Analysers were calibrated with known gas concentrations (BOC, Australia). The Turbine flowmeter was calibrated using a 3 L Hans Rudolph syringe. The one-legged VO₂ peak was estimated as 74% of two-legged VO₂ peak (Pernow et al., 1971; Pilegaard et al., 2002). The one-legged cycling workrates were linearly derived from the relationship between VO₂ and power output to represent 45, 65 and 85% VO₂ peak-1leg and piloted 48-hr afterwards with the same workrate used for each leg. The VO₂ at the end of the 45, 65 and 85% of estimated VO₂ peak-1leg exercise bouts corresponded to (mean ± SD): 32.1 ± 3.6, 44.8 ± 5.5, 52.5 ± 4.1 and 75 ± 4.7% of two-legged VO₂ peak.

5.2.5.2 One-legged cycling protocol

Participants performed an incremental one-legged cycling protocol followed by a time to fatigue test, with the protocol first conducted on the unloaded (left) leg, followed by 120 min of supine rest and then repeated on the weight-bearing (right) leg. The protocol consisted of two consecutive four-min bouts of cycling on an electronically-braked cycle
(Lode, Netherlands) ergometer at 45% and 65% of the estimated one legged \( \dot{V}O_2 \text{peak}_{\text{1leg}} \), followed by one min rest and one min cycling at 85% of one leg \( \dot{V}O_2 \text{peak} \), a 5-min rest followed by cycling at 85% \( \dot{V}O_2 \text{peak}_{\text{1leg}} \) until volitional fatigue, defined as when the cadence could not be maintained above 60 RPM. The protocol was completed by \( n = 6 \) at baseline and post-ULLS and \( n = 5 \) for post-training.

### 5.2.5.3 Heart rate and perceived exertion

Heart rate (HR) was measured throughout the trial via 12-lead ECG (Mortara, USA) with the HR averaged over the final 10 s of each exercise intensity. Rating of perceived exertion (RPE) was measured using a 20 point scale of perceived exertion (Borg, 1982) at the completion of the 45, 65 and 85% \( \dot{V}O_2 \text{peak}_{\text{1leg}} \) cycling bouts.

### 5.2.5.4 Blood sampling and analysis

A 20-gauge catheter was inserted into an antecubital vein, attached to a 30 cm extension tube, covered by a waterproof patch (Tegaderm) and kept patent via injection of sterile isotonic saline. The participant rested supine for 20 min before a resting blood sample was obtained, followed by a muscle biopsy on each of the unloaded and weight-bearing legs. Following the biopsy, the participant was seated on the cycle ergometer for five min before a second resting blood sample was obtained immediately pre-exercise (0 min). Blood samples were taken during exercise at the end of each workrate, prior to commencement of the 1 minute 85% cycling bout and immediately prior to the commencement of the final bout at 85% of \( \dot{V}O_2 \text{peak}_{\text{1leg}} \). During the time to fatigue test, a sample was taken every 30 s and immediately prior to fatigue. In recovery, blood was sampled at 1, 2, 5, 10, 20 and 30 min post-exercise. Approximately 3.5 mL of blood was sampled on each occasion into two
syringes; approximately 1.5 ml was collected in a plain syringe, with 1 mL transferred to an Eppendorf tube and analysed in duplicate for [Hb] and Hct using automated analysers (Sysmex, K-800, Kobe, Japan) and the remaining 0.5 mL of blood was transferred into a separate Eppendorf tube and analysed for blood [Lac] (2300 Stat Plus, YSI, USA). Two mL of blood was sampled in a separate heparin-coated syringe and analysed immediately for plasma [K⁺] (Rapid point 405, Siemens Medical Solutions and Diagnostics, NY, USA).

5.2.6 Muscle biopsies
A single muscle biopsy was taken from the middle third of the vastus lateralis muscle at rest on each leg, as described in Chapter 4.2.5.2. After injection of a local anaesthetic into the skin and fascia (1% Xylocaine), a small incision was made and a muscle sample was taken (∼100-200 mg) using a Bergström biopsy needle. Muscle samples were blotted to remove excess blood, immediately frozen in liquid nitrogen and stored at -80°C until analyses.

5.2.7 [³H]ouabain binding site content
Analysis of skeletal muscle NKA content was determined by the [³H]ouabain binding site content assay as described previously (Chapter 3.2.5).

5.2.8 Western blotting
NKA isoform relative protein abundance was measured using western blotting, but using a different running, transfer and protein normalisation protocol to that described in chapter 3.2.7, due to upgrades in laboratory equipment. Approximately 10-20 mg of frozen muscle sample was used for NKA immunoblot analyses. Muscle proteins were extracted in ice-cold buffer containing 20 mM Tris pH 7.8 (Bio-Rad Laboratories, Hercules, CA), 137 mM NaCl, 2.7
mM KCl (Merck, Kilsyth, Australia), 1 mM MgCl₂, 5 mM Na₃O₂P₂, 10 mM NaF, 1 % Triton X-100, 10 % Glycerol (Ajax Finechem, Australia), 0.5 mM Na₄VO₃, 1 µg.mL⁻¹ Leupeptin, 1 µg.mL⁻¹ Aprotinin, 200 mM PMSF, 1 mM DTT and 1 mM Benzamidine. All reagents were analytical grade (Sigma-Aldrich, St Louis, MI). Samples were homogenised (1 : 37.5 dilution) for 2 x 20 s, using a tissue homogeniser (TH220, Omni International, Kennesaw, GA). Homogenates were rotated for 60 min at 4°C and protein concentration of the homogenates was determined using a commercially-available kit (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA).

Muscle NKA isoform analyses did not include any membrane isolation steps, to maximise recovery of NKA enzymes (Murphy et al., 2004). Aliquots of the muscle homogenate were mixed with Laemmli sample buffer and proteins were separated with pre-made 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (10%, Criterion TGX, Bio-Rad, Bio-Rad Laboratories, Hercules, CA %), for 45 min at 200 mA. For the analysis of protein abundance of the α₁, α₂, α₃, β₁ and β₂ NKA isoforms, 10 µg of total protein per sample were loaded in each gel, while 15 µg was loaded for the α₁ isoform. The β₃ NKA isoform was attempted at several total protein concentrations, but could not be detected. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (TurboTransfer pack, Bio-Rad) for 7 m at 320 mA using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in TBST buffer (10 mM Tris, 100 mM NaCl, 0.02 % Tween-20) containing 7.5 % non-fat milk, for 1 h at room temperature. After being washed (4 x 8 min in TBST), membranes were incubated with the appropriate primary antibody overnight at 4°C. Primary antibodies were diluted in TBS buffer containing 0.1 % NaN₃ and 0.1 % albumin
bovine serum. All membranes were incubated with the same amount of dilution buffer. Membranes were ponceau stained to confirm complete transfer.

To determine NKA protein abundance, membranes were incubated with antibodies for NKA α₁ (monoclonal α6F, developed by D. Fambrough, obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA), NKA α₂ (polyclonal anti-HERED, kindly donated by T. Pressley, Texas Tech University, USA), NKA α₃ (monoclonal, Thermo Scientific, Rockford, IL, # MA3-915), NKA β₁ (monoclonal, Thermo Scientific # MA3-930), NKA β₂ (monoclonal, Becton Dickinson Bioscience, San Jose, Ca, # 610915) using a protocol similar to as described (Murphy et al., 2004). Following incubation with the primary antibodies, membranes were washed in TBST buffer (4 x8 min) and incubated with the appropriate anti-rabbit (PerkinElmer # NEF812001EA) or anti-mouse (PerkinElmer # NEF822001EA) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were diluted 1:20,000, except α₁ which was diluted 1:5000. After washing the membranes in TBST, immunoreactive proteins were detected using chemiluminescence reagents (Bio-Rad) and quantified by densitometric scanning (VersaDocTM Imaging System, Bio-Rad). The abundance of NKA isoforms was normalised to total protein content of the lane as assessed using a coomassie stain (50% methanol, 50% H₂O and 0.1% Brilliant Blue R-250 powder) on the membrane (Welinder et al., 2011).

5.2.9 Statistical analysis

All data are reported as mean ± standard deviation (SD). Log-transformation was performed on data which was not normally distributed to reduce bias. A mixed linear model was used
for the time to fatigue test on the weight-bearing and unloaded leg individually, whilst $[^3]$Houabain binding content, NKA isoform abundance RPE, heart rate, lactate, [K$^+$], [Lac$^-$] and VO$_2$ were analysed with both the weight-bearing and unloaded legs within the same analysis. Statistical significance was accepted at P<0.05. Magnitudes of change using Cohen’s effect size was performed on strength and time fatigue testing, $[^3]$Houabain binding and NKA isoform abundance. Effect size was calculated via a custom spreadsheet and classified as: small, 0.2-0.6; moderate, 0.6-1.2; large, 1.2-2.0; and very large, 2.0-4.0 (Batterham et al., 2006; Hopkins, 2007). Effects with less certainty (magnitude of <75%) were classified as no meaningful difference (Batterham et al., 2006; Hopkins, 2007). Two way repeated measures ANOVA and mixed linear models were calculated using SPSS version 20 (SPSS Inc., Champaign, IL).

5.3 Results

5.3.1 One-legged cycling testing

5.3.1.1 Time to fatigue

Time to fatigue at 85% of estimated VO$_2$ peak-1leg decreased in the unloaded leg by 22.5% after ULLS (p = 0.041, ES: 1.19±0.67) and increased by 31% after resistance training compared to post-ULLS (ES: 1.11±0.85, p = 0.004) not different from baseline (Figure 5.1). No significant changes or meaningful effect sizes were seen in the weight-bearing leg after ULLS or resistance training (Figure 5.1).
Figure 5.1: Time to fatigue at 85% $\dot{V}O_2 \text{peak-1leg}$ at baseline, post-ULLS and post-training. Filled bars represent the unloaded leg; unfilled bars represent the weight-bearing leg. Percentage of $\dot{V}O_2 \text{ peak-1 leg}$ were determined from values gained prior to ULLS. * less than baseline, $p <0.05$. ‡ moderate effect size from baseline. Values are mean ± SD. $n = 6$ for baseline and post-ULLS, $n = 5$ for post-training.

5.3.1.2 Heart rate and $\dot{V}O_2$ during exercise

After ULLS, HR during exercise increased compared to baseline in both the unloaded and weight-bearing legs, at 45% $\dot{V}O_2 \text{ peak-1leg}$ (unloaded leg: 5.6 %, weight-bearing leg: 7.7%, $p = 0.005$) and 85% of $\dot{V}O_2 \text{ peak-1leg}$ (unloaded leg: 10.2 %, weight-bearing leg: 11%, $p = 0.001$). Post-training, HR compared to post ULLS decreased and did not differ significantly to baseline in either leg ($p>0.05$), nor was HR different between legs at any time-point (Figure 5.2). No change was seen in the pulmonary $\dot{V}O_2$ during exercise at any workrate in either the weight-bearing or unloaded leg after ULLS or training (Data not shown; Appendix I).
**Figure 5.2**: Heart rate (bpm) during one-legged cycling exercise conducted at baseline, post-ULLS and post-training, in the unloaded (A) and weight-bearing (B) legs. The 45% and 65% bouts were 4 min in duration and the first 85% bout was 1 minute in duration. TTF (time to fatigue) was completed until participant reached volitional fatigue. Percentage of VO$_2$ peak -1 leg were determined from values gained prior to ULLS. Filled bars represent baseline, hollow bars represent post-ULLS and hatched bars represent post-training. * greater than baseline, p<0.05. Values are mean ± SD. n = 6 for Baseline and Post ULLS and n = 5 for Post-training.
5.3.1.3 RPE during exercise

The RPE during exercise was increased at 65% $\dot{V}O_2_{peak-1 \text{ leg}}$ (unloaded leg: 15.9 %, weight-bearing leg: 9.2 %, $p = 0.001$) and at 85% $\dot{V}O_2_{peak-1 \text{ leg}}$ (unloaded leg: 17.1 %, weight-bearing leg: 9.6%, $p = 0.001$) after ULLS compared to baseline (Figure 5.3). After resistance training, RPE decreased compared to post ULLS and was not significantly different to baseline in either leg after training ($p>0.05$), Figure 5.3.
Figure 5.3: Rating of perceived exertion (a.u.) at the completion of different one-legged cycling intensities conducted at baseline, post-ULLS and post-training, in the unloaded (A) and weight-bearing (B) legs. The 45% and 65% bouts were 4 min in duration and the first 85% bout was 1 minute in duration. TTF (time to fatigue) was completed until participant reached volitional fatigue. Percentage of VO2_peak -1 leg were determined from values gained prior to ULLS. Filled bars represent baseline, hollow bars represent post-ULLS and hatched bars represent post-training. * greater than baseline, p<0.05. Values are mean ± SD. n = 6 for Baseline and Post ULLS and n = 5 for Post-training.
5.3.1.4 Plasma [K⁺] during exercise

Plasma [K⁺] increased during exercise and decreased in recovery in each of the conditions (p<0.001) but were no differences between legs or any interaction effect between leg and time after ULLS and resistance training (Figures 5.4).

There was also no difference in the unloaded leg in peak plasma [K⁺] (p = 0.519, Baseline: 5.38± 0.47, Post ULLS: 5.25 ± 0.47, post-training: 5.22± 0.37 mmol/L) or Δ plasma [K⁺]/work ratio (p= 0.1, Baseline: 58.5 ± 39.2, Post ULLS: 77.9 ± 43.5, post-training: 45.7 ±16.5 nmol.L J⁻¹) or in the weight bearing leg in peak plasma [K⁺] (p = 0.542, Baseline: 5.21± 0.22, Post ULLS: 5.11 ± 0.26, post-training: 5.36 ± 0.46 mmol/L) or Δ plasma [K⁺]/work ratio (p= 0.12, Baseline: 51.1 ± 18.1, Post ULLS: 59.4 ± 23.8, post-training: 35.8 ± 4.9 nmol.L J⁻¹).
Figure 5.4: Plasma [K⁺] during incremental one-legged exercise workloads time to fatigue and recovery on the unloaded (A) and weight-bearing (B) legs. Black squares represent baseline, hollow inverted triangles represent post-ULLS and hollow circles represent post-training. Values are expressed as mean ± SD. n =6, except post-training where n = 5.
5.3.1.5 Plasma [Lac] during exercise

Blood [Lac] increased during exercise and decreased in recovery in each of the conditions (p<0.001), but there were no differences between legs or any interaction effect between leg and time after unloading and retraining (Figure 5.4).
**Figure 5.5**: Plasma [Lac'] during incremental one-legged exercise workloads, time to fatigue and recovery on the unloaded (A) and weight-bearing (B) leg. Black squares represent baseline, hollow inverted triangles represent post-ULLS and hollow circles represent post-training. Values are expressed as mean ± SD. n = 6, except post-training where n = 5.
5.3.2 Muscle NKA content and isoform abundance

5.3.2.1 \[^{3}H\]ouabain binding site content

There was no change in the muscle \[^{3}H\]ouabain binding site content, or meaningful effect sizes after ULLS or post-training in either the unloaded or weight-bearing leg (Figure 5.6).

![Figure 5.6: Skeletal muscle \[^{3}H\]ouabain binding site content in the unloaded and weight bearing legs measured at baseline, after 23 d ULLS and 4 wk resistance training. Values are expressed as mean ± SD, n = 6.]

5.3.2.2 NKA isoform abundance

No significant differences were found in NKA α\(_1\), α\(_2\), α\(_3\), β\(_1\) or β\(_2\) isoform protein abundance after ULLS or after training in either the weight-bearing or unloaded leg, or between legs (p>0.07). However, in the unloaded leg there was a trend for increased NKA β\(_2\) abundance
with a small effect size ($p = 0.074$, ES: $0.83 \pm 0.83$) after inactivity. Post training there was a trend with a small effect size for increased NKA $\alpha_3$ ($p=0.16$, ES: $0.58 \pm 0.63$) isoform relative abundance after training compared to post-ULLS (Table 5.1 and Table 5.2).

**Table 5.1**: Skeletal muscle NKA $\alpha$ and $\beta$ isoform protein relative abundance in the unloaded and weight bearing legs at baseline, after 23 d ULLS and following 4 wk resistance training.

<table>
<thead>
<tr>
<th>NKA isoform</th>
<th>Leg</th>
<th>Baseline</th>
<th>Post-ULLS</th>
<th>Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$</td>
<td>Unloaded</td>
<td>21.4 ± 14.3</td>
<td>26.9 ± 15.9</td>
<td>24.4 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>20.7 ± 17.9</td>
<td>18.3 ± 14.0</td>
<td>21.1 ± 13.0</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>Unloaded</td>
<td>12.6 ± 10.4</td>
<td>12.7 ± 6.1</td>
<td>15.5 ± 11.1</td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>12.5 ± 8.5</td>
<td>11.6 ± 5.3</td>
<td>16.9 ± 11.4</td>
</tr>
<tr>
<td>$\alpha_3$</td>
<td>Unloaded</td>
<td>6.9 ± 4.1</td>
<td>8.3 ± 8.1</td>
<td>3.9 ± 3.4 †</td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>3.5 ± 2.9</td>
<td>4.8 ± 3.3</td>
<td>4.3 ± 4.6</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>Unloaded</td>
<td>23.7 ± 14.5</td>
<td>23.5 ± 15.6</td>
<td>27.8 ± 24.5</td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>13.3 ± 4.0</td>
<td>16.6 ± 8.8</td>
<td>17.1 ± 6.7</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Unloaded</td>
<td>8.4 ± 3.6</td>
<td>19.0 ± 14.7 ‡</td>
<td>15.2 ± 8.7 †</td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>9.9 ± 5.2</td>
<td>11.7 ± 9.8</td>
<td>10.1 ± 7.5</td>
</tr>
</tbody>
</table>

† small effect size from baseline, ‡ moderate effect size from baseline. Values in arbitrary units (a.u.). Values are means ± SD, n = 6.
Table 5.2: Effect size comparison of skeletal muscle NKA content ([^3]H)ouabain binding site content), NKA α and β isoform protein abundance in the unloaded and weight bearing legs between baseline vs post ULLS, post-ULLS vs post-training and post-training vs baseline.

<table>
<thead>
<tr>
<th>NKA measure</th>
<th>Leg</th>
<th>Baseline vs.</th>
<th>Post ULLS</th>
<th>Post-ULLS vs Post-training</th>
<th>Post-training vs Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>[^3]H)ouabain</td>
<td>Unloaded</td>
<td>0.12 ± 0.25</td>
<td>0.04 ± 0.15</td>
<td>0.16 ± 0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>0.07 ± 0.22</td>
<td>0.06 ± 0.47</td>
<td>0.14 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>α1</td>
<td>Unloaded</td>
<td>0.31 ± 0.45</td>
<td>0.14 ± 0.47</td>
<td>0.17 ± 0.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>0.13 ± 0.29</td>
<td>0.15 ± 0.56</td>
<td>0.02 ± 0.74</td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td>Unloaded</td>
<td>0.19 ± 0.58</td>
<td>0.60 ± 1.73</td>
<td>0.79 ± 1.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>0.18 ± 0.29</td>
<td>0.31 ± 0.71</td>
<td>0.49 ± 0.89</td>
<td></td>
</tr>
<tr>
<td>α3</td>
<td>Unloaded</td>
<td>0.18 ± 1.02</td>
<td>0.58 ± 0.63 †</td>
<td>0.40 ± 0.56 †</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>0.35 ± 0.87</td>
<td>0.14 ± 0.88</td>
<td>0.20 ± 0.59</td>
<td></td>
</tr>
<tr>
<td>β1</td>
<td>Unloaded</td>
<td>0.05 ± 0.51</td>
<td>0.54 ± 1.6</td>
<td>0.59 ± 1.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>0.41 ± 0.75</td>
<td>0.06 ± 1.29</td>
<td>0.47 ± 0.68 †</td>
<td></td>
</tr>
<tr>
<td>β2</td>
<td>Unloaded</td>
<td>0.83 ± 0.83 ‡</td>
<td>0.30 ± 0.63</td>
<td>0.54 ± 0.45 †</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight-bearing</td>
<td>0.23 ± 0.57</td>
<td>0.22 ± 0.33</td>
<td>0.01 ± 0.39</td>
<td></td>
</tr>
</tbody>
</table>

† small effect size from baseline, ‡moderate effect size from baseline. ES classified as meaningful if >75% certainty. Values in arbitrary units (a.u.). Values are means ± SD, n = 6.
5.4 Discussion

This was the first study to investigate the effects of a controlled period of unloading in healthy humans on skeletal muscle NKA content, NKA isoform abundance and plasma $[K^+]$ during and following exercise. The results are surprising in that there were no changes in either the NKA content or any of the individual NKA isoforms after ULLS, despite clear evidence of functional impairment with decreased muscle strength, power (Chapter 4) and reduction in time to fatigue during intense exercise with increased heart rate and rating of perceived exertion. This suggests that the decrements in muscle function and performance after short-term disuse or inactivity is not accompanied by a reduction in NKA content or NKA isoform abundance in skeletal muscle.

5.4.1 The effect of ULLS and skeletal muscle NKA content and isoform abundance

An intriguing finding was the lack of difference in NKA content, as measured by $[^3H]$ouabain binding site content, after 23 d ULLS. This is in contrast to several studies using joint- or spinal-injured populations in humans (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012) and inactivity studies using animals (Kjeldsen et al., 1986; Jebens et al., 1995). It is likely that 23 d of ULLS was an insufficient stimulus to reduce muscle NKA content in healthy young humans; as previous research investigated patients whom had been injured for many months or even years (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012). However, spinal injury promotes a broad range of maladaptations to skeletal muscle, including cell death, myelin inhibition and glial scarring independent of inactivity, which impairs skeletal muscle function (Jones et al., 2001; Shields, 2002) and as such is not appropriate to study the effects of inactivity or disuse. While short timeframes of inactivity,
under 30 d, was used in rats and guinea pigs previously (Kjeldsen et al., 1986; Leivseth et al., 1992), the brief lifespan and decreased metabolic stability in rodents during inactivity makes comparisons to humans problematic (Rennie et al., 2010). In addition, the low effect size and lack of change after ULLS in [\(^3\)H]ouabain binding content in this thesis (2.3%, ES: 0.12 ± 0.26) indicate that the study was unlikely to be statistically underpowered.

A second explanation for the lack of change in skeletal muscle NKA content or relative isoform abundance after 23 d of ULLS, relates to the ULLS method itself. ULLS allows the unloaded leg to hang without any immobilisation or restriction to the unloaded leg; hence the unloaded leg may continue to undergo passive movements and contractions. Patients with partial spinal injury that were capable of walking and basic mobility had unchanged NKA isoform abundance, despite great restriction on muscle function (Boon et al., 2012). This suggests that partial or reduced physical activity for only a short time-frame may not be substantial enough to decrease NKA content in skeletal muscle. While ULLS is capable of inducing decreased muscle CSA, strength and endurance as seen in Chapter 4 and previous ULLS studies (Hackney et al., 2011; Narici et al., 2011), our results suggest that skeletal muscle NKA content may be resilient to decrement after short term-inactivity. Hence a more restrictive inactivity method, such as limb casting, may be required to induce a decrease in skeletal muscle NKA content. The resilience of NKA content in response to short-term inactivity is surprising considering how rapidly NKA content can be increased with training; for example, 3 d of consecutive endurance training increased skeletal muscle NKA content by 9% in untrained participants (Green et al., 2004).
Another possible explanation for the lack of change in NKA content after ULLS in this thesis was the concomitant effects of muscle atrophy. In porcine skeletal muscle, a greater sarcolemmal muscle membrane area, as occurs in smaller fibres, compared to intracellular area was associated with greater $[^3]$Houabain binding site content (Harrison et al., 1994). If true in human skeletal muscle, this suggests that the atrophy caused by ULLS may in fact compensate for an absolute decrease in NKA content, due to an increased proportion of sarcolemma to intracellular area of the muscle fibre. However, there is no evidence to properly support or refute a higher sarcolemmal NKA concentration in humans, thus, this possibility requires further investigation.

This research was part of a larger study investigating other functional effects of ULLS, and included vertical jump and knee extensor strength testing (less than 45 minutes duration) two days prior to the muscle biopsies. This was highly unlikely, however, to affect either the muscle NKA content or isoform abundance measures, as a single bout of highly fatiguing exercise was not sufficient to increase muscle NKA content or isoform protein abundance (Murphy et al., 2004). Further, the earliest an increase in muscle NKA content has been reported is after 9 consecutive bouts of very high intensity exercise in 10 hours (Green et al., 2007a). Hence, it is unlikely that the minimal-volume strength and jump testing changed muscle NKA content or isoform abundance in this study.

The underlying mechanisms which could cause decreased NKA content in skeletal muscle in response to disuse are not understood. Whilst transient increases in intracellular $[\text{Na}^+]$ are suspected (Brodie et al., 1990), although not proven, to upregulate NKA content in muscle in response to training, even less is known about the potential mechanisms which could
cause a decrease in muscle NKA content after injury and inactivity. Whilst it is possible that reduced muscle NKA could be due to an overall decline in the frequency of intracellular \([\text{Na}^+]\) fluxes, due to reduced muscle contraction, other underlying molecular mechanisms could exist. Inactivity produces vast maladaptations to muscle molecular signalling, including increased transcription of ubiquitin ligases associated with atrophy such as FoxO, MuRF-1 and Atrogin-1 (Bodine et al., 2001; Sandri et al., 2004; Abadi et al., 2009; Bunn et al., 2011). The upregulation of ubiquitin ligases and other as yet undescribed signalling pathways in skeletal muscle disuse, or changes in corticospinal excitability (Deschenes et al., 2002; Clark et al., 2007; de Boer et al., 2007a; Deschenes et al., 2008; Seynnes et al., 2009) via reduced release of CGRP from nerve endings (Clausen, 2003) may have some direct or indirect role with inactivity-induced reduction in muscle NKA (Leivseth et al., 1994; Ditor et al., 2004; Boon et al., 2012) which requires future investigation.

Consistent with the unchanged \([\text{³H}]\text{ouabain}\) binding site content findings, no difference was found in the isoform relative abundance in any of the NKA \(\alpha\) (\(\alpha_{1-3}\)) isoforms or \(\beta\) isoforms (\(\beta_{1-2}\)) in response to either ULLS or subsequent resistance training; although the trend (\(p = 0.07\)) and moderate effect size for increased \(\beta_2\) after ULLS is intriguing, but difficult to interpret considering the lack of knowledge regarding the specific function of the isoform. The minimal effect sizes in NKA \(\alpha_2\) (-0.1% decrease, 0.19 ± 0.57) and NKA \(\beta_1\) (2.2% increase, ES:0.05 ± 0.51) indicate there was no difference in what is likely to be the most common NKA isoforms in human muscle after ULLS or four weeks of subsequent resistance training (Hansen, 2001).
5.4.2 Effects of ULLS on exercise fatigability

An important finding was the 22% shorter time to fatigue during intense one-legged cycling exercise after ULLS which was fully restored after four weeks of resistance training. Conversely, there was no change in venous plasma [K+] during exercise or recovery after ULLS or subsequent resistance training. Taken together with the lack of change in skeletal muscle NKA content and isoform abundance in this thesis, it is unlikely that the decreased time to fatigue was associated with impaired exercise K+ regulation. However, there are two important considerations with this assumption. Venous blood taken from the arm is not an indicator of interstitial [K+] of the active leg musculature; which is likely to influence muscle excitability and possibly muscle fatigue (Clausen, 2003; McKenna et al., 2008). Hence, the lack of change in venous [K+] after ULLS does not reflect the interstitial [K+]. Secondly, NKA activity or the NKA partner protein FXDY1, which is implicated in modulating NKA activity were not investigated (Rasmussen et al., 2008). Reduced NKA activity was proposed have an important role in muscle fatigue (McKenna et al., 2008), although recent evidence suggests more research is required to better investigate the role of NKA activity, exercise and fatigue in human skeletal muscle (Juel et al., 2013). There is only limited evidence of changes in FXDY1 abundance after inactivity; one study found a 17% reduction in phosphorylated FXDY1 in soccer players who ceased all training for two weeks (Thomassen et al., 2010). Hence it is plausible that NKA activity, perhaps via reduced FXDY1 phosphorylation, may have changed after ULLS with no difference in total NKA content or venous [K+].
One of the most unexpected findings of this thesis was the elevated heart rate and perceived exertion in both the unloaded and weight-bearing legs after ULLS. While a decline in one-legged cycling performance and knee extensor endurance has been reported previously after ULLS in the unloaded leg (Berg et al., 1993; Sato et al., 2010), the apparent detraining effect seen in the weight-bearing leg is potent considering untrained populations are usually resistant to short-term detraining (Mujika et al., 2001). As the greater heart rate after ULLS was almost identical in either the weight bearing or unloaded limbs, it is likely that either increased sympathetic activation, decreased parasympathetic drive or a combination of both occurred after ULLS (Hughson et al., 1994). The greater HR may also reflect a decreased stroke volume after ULLS (Coyle et al., 1986; Martin III et al., 1986; Convertino, 2007). However, there was no reduction in time to fatigue on the weight-bearing leg despite increased heart rate and perceived exertion, suggesting that these central cardiovascular and perceptual changes were of limited relevance to exercise performance. Hence, the actual cause of the decreased in time to fatigue performance after ULLS is not known from this research, but previous research with ULLS and limb immobilisation suggest mechanisms could include impairment of muscle pennation angle, tendon stiffness and fascicle length (de Boer et al., 2007a; Kinugasa et al., 2010), which could lead to decreased efficiency of muscle force. Within the muscle, mitochondrial protein activity and size have been found to decrease in response to 10-14 d of immobilisation (Abadi et al., 2009; Nielsen et al., 2010), leading to impaired oxidative capacity of skeletal muscle; finally, sarcoplasmic reticulum uptake rate was reduced after 10
d immobilisation (Thom et al., 2001) suggesting impairment to sarcoplasmic reticulum function in muscle may also contribute to the increased fatigability after inactivity.

5.4.3 The effects of resistance training after ULLS

The lack of change in skeletal muscle NKA content or isoform abundance after four weeks of resistance training was unexpected. While studies report rapid increases in skeletal muscle NKA content after as little as three d of endurance training (Green et al., 2004), the two studies that have reported an increased NKA content after resistance training performed resistance training for at least 12 weeks (Green et al., 1999a; Medbø et al., 2001). Hence, the 4-week resistance training program performed subsequent to ULLS in this thesis may have lacked sufficient volume or intensity to stimulate an increase in skeletal muscle NKA content. It was also anticipated that an increase in NKA content would start from a lower NKA content after ULLS, but this was not the case. However, the decrease in time to fatigue after ULLS was restored after resistance training; suggesting that despite the lack of change in NKA content, a short-term resistance training program is of substantial functional benefit after inactivity. Interestingly, re-ambulation alone can be a potent stimulus to return baseline levels of muscle strength and size after ULLS (Hather et al., 1992), but resistance training is superior to re-ambulation alone in order restore muscle strength after inactivity (Hortobagyi et al., 2000).

5.5 Conclusions

In conclusion, 23 d of unilateral unloading and four weeks of subsequent resistance training caused no changes in skeletal muscle NKA content, NKA isoform abundance ($\alpha_{1-3}$ and $\beta_{1-2}$) or venous plasma [$K^+$] during or following exercise. However, ULLS did cause impairment to
intense exercise time to fatigue, increased heart rate and perceived exertion, which were each reversible with resistance training. Hence, skeletal muscle NKA content was surprisingly resistant to decrement after short-term unloading and down-regulation may require a greater duration or restrictiveness of disuse to occur. This finding demonstrates the complexity and little understood regulation of NKA content in skeletal muscle after inactivity.
CHAPTER 6: THE EFFECTS OF ANTERIOR CRUCIATE LIGAMENT INJURY ON
MUSCLE FUNCTION, MUSCLE Na⁺,K⁺-ATPase CONTENT AND ISOFORM
ABUNDANCE

6.1 Introduction

Rupture of the anterior cruciate ligament (ACL) is a debilitating injury which adversely
impacts knee and lower limb function and may lead to physical inactivity (Miyasaka et al.,
1991; Daniel et al., 1994; Snyder-Mackler et al., 1997). Over 10,000 people in Australia
undergo ACL reconstructive surgery annually, with sporting injuries the most common
cause (Gianotti et al., 2009; Janssen et al., 2012). The debilitating effects of ACL injury
include decreased knee extensor muscle size and strength, decreased knee proprioception
and increased postural sway (Wilk et al., 1994; MacDonald et al., 1996; Ageberg et al.,
2005; Williams et al., 2005b; Makihara et al., 2006; Lee et al., 2009). Together, these
functional decrements greatly impact daily activities and quality of life in ACL-injured
patients (Miyasaka et al., 1991; Frobell et al., 2010).

Muscle disuse induced by ACL injury can impair both muscle morphology and function.
Atrophy of the knee extensor muscle after ACL injury is common, with 4.5-13% reduction in
cross sectional area (CSA) in whole muscle and in vastus lateralis single muscle fibres
compared to the non-injured leg (Lorentzon et al., 1989; Williams et al., 2005a). In addition,
84% of ACL-injured patients have some degree of knee extensor muscle strength loss in
their injured leg compared to their non-injured leg (Wilk et al., 1994). The reported
magnitude of strength loss varies greatly between studies, from 8% to 37.5% lower strength
in the injured leg compared to the non-injured leg (Keays et al., 2001; Makihara et al., 2006). The atrophy and strength loss after ACL injury is critical; ACL patients exhibiting worse knee function, stability and “knee related” quality of life also have considerably lower knee extensor CSA and strength (Williams et al., 2005a; Williams et al., 2005b; Roberts et al., 2007). In addition, ACL injury causes reduced knee proprioception and increased postural sway, suggesting functional and neuromuscular impairments to balance after ACL injury (MacDonald et al., 1996; Ageberg et al., 2005; Lee et al., 2009).

However, no studies have investigated the potential biomolecular mechanisms in skeletal muscle which contribute to these functional deficits after ACL injury. Part of this weakness and muscle loss after ACL injury may be related to inactivity, as disuse on both a localised and whole body-scale produces substantial decrements in muscle strength and size (Adams et al., 2003; Narici et al., 2011) as demonstrated with ULLS in Chapters 4 and 5. One potential maladaptation in skeletal muscle after ACL injury is to the Na⁺,K⁺-ATPase (NKA), a protein vital for the maintenance of muscle membrane excitability and muscle contraction (McKenna et al., 2008). The NKA is a heterodimer protein comprising a catalytic α and a regulatory β subunit, each with three isoforms (α₁-₃ and β₁-₃) detected in human skeletal muscle (Murphy et al., 2004). The α₂ isoform is the dominant NKA α isoform in skeletal muscle (Hansen, 2001) and has an important role in muscle strength and endurance. For example, mice with a muscle-specific α₂ knockout had substantially reduced exercise endurance on a graded treadmill test, where they could not surpass running speeds higher than 4 m.s⁻¹, compared to controls which could reach up to 26 m.s⁻¹ and exhibited impaired in vivo muscle strength (Radzyukevich et al., 2013). The NKA content in skeletal muscle, as
measured by [³H]ouabain binding site content, is increased with acute training (Green et al., 1993; McKenna et al., 1993; Green et al., 1999a; Medbø et al., 2001; Thomassen et al., 2010) and conversely is reduced by 25-58% in chronic injuries which enforce physical inactivity, such as spinal injury (Ditor et al., 2004; Boon et al., 2012) and shoulder impingement syndrome (Leivseth et al., 1994). The reduction in NKA content after injury may be rapid with 80% and 50% reduction in NKA α₁ and α₂, respectively, after the first three months of spinal cord injury (Boon et al., 2012). Hence, acute ACL injury may also depress muscle NKA content through disuse of the injured leg and the possible functional correlates of this are explored.

This study therefore investigated the effects of ACL injury on knee function, knee extensor strength, muscle fibre CSA, postural sway, skeletal muscle NKA content and NKA isoform (α₁-3, β₁-3) relative abundance in otherwise healthy, young adult participants. It was hypothesised that the ACL-injured leg will exhibit reduced knee extensor strength, CSA, NKA content, NKA α₂ isoform abundance and increased postural sway compared to both the non-injured leg and to healthy asymptomatic matched controls.

6.2 Methods

6.2.1 Participants

Six adults with ACL injury that were scheduled for ACL reconstruction (ACL; 4 females, 2 males; age: 25.0 ± 4.9 yrs; body mass: 76.6 ± 5.6 kg; height: 174.2 ± 4.7 cm; BMI: 25.3 ± 2.3 kg.m⁻² mean ± SD) and seven age- and-BMI-matched asymptomatic controls (CON; 5 females, 2 males; age: 23.3 ± 2.0 y.o; mass: 64.0 ± 11.6 kg; height: 170.1 ± 9.1 cm; BMI: 22.0
+ 3.8 kg.m\(^{-2}\)) gave written informed consent and participated in the study. The ACL-injured participants were recruited after confirmation of ACL reconstructive surgery and had been injured 15 ± 17 weeks prior to surgery (mean ± SD, range 5-50 weeks). Exclusion factors for the ACL group included any condition apart from ACL injury which would affect physical activity, a BMI over 30 kg.m\(^{-2}\), pregnancy, age exceeding 35 years and any condition contraindicative for muscle biopsies, strength or balance testing. The control group in addition to the exclusion criteria already described, were matched for age, sex and BMI, and had to be free from any serious lower limb injury in the past three years. The study protocol was approved by the St. Vincents Hospital Human Research Ethics Committee and the Victoria University Human Research Ethics Committee.

6.2.2 General design

The ACL-injured participants were tested on two separate days. The first day comprised knee extensor muscle strength testing, measurement of one- and two-legged postural sway, thigh anthropometry in each of the injured and non-injured legs, as well as completion of a physical activity questionnaire and a subjective knee function evaluation. Approximately seven d later, a *vastus lateralis* muscle biopsy was taken from both the injured and non-injured legs whilst under a general anaesthetic, just prior to commencement of their ACL reconstruction surgery. The control participants underwent the same functional testing and physical activity questionnaire, but did not complete the subjective knee function questionnaire and had a *vastus lateralis* muscle biopsy from a single leg, which was the same corresponding leg as the ACL injury in the ACL injured participant (right or left leg). The dominant leg of the CON participants, which is used with
thigh CSA, maximal torque and postural sway analysis, was designated as the leg the participant used to kick a ball.

6.2.3 Subjective knee function

Subjective knee function was assessed using the International Knee Documentation Committee (IKDC) Subjective Knee Form, which consists of 18 items relating to injury symptoms, knee function and sporting physical activity (Hambly et al., 2010). A greater overall indexed score on the IKDC reflects a higher level of knee functioning and less injury symptoms (Anderson et al., 2006) and is scored by calculating the difference between the raw score and the lowest possible score and then dividing this difference by the range of potential scores, multiplied by 100 (Irrgang et al., 2001).

6.2.4 Physical activity questionnaire

The Incidental and Planned Activity Questionnaire (IPAQ) was used to assess the physical activity level for all participants (Hallal et al., 2004). The questionnaire contains 27 questions that estimate the physical activity during the previous week and covers the frequency and duration of occupation-related, transportation, housework/gardening and leisure-time physical activities. Frequency and duration scores were multiplied to create a total duration for incidental and planned activity, as well as an overall total score. Total activity time was summed across all components (hr.wk\(^{-1}\)).

6.2.5 Thigh cross sectional area

Thigh cross sectional area (CSA) of each leg was measured using a non-invasive anthropometric method as described and validated by Knapik et al. (1996). In brief, this
method involves measurement of thigh circumference at the midpoint of the thigh, which was measured as halfway between the lateral epicondyle and greater trochanter, skin fold assessment and estimate of femur epicondyle width to calculate an estimate of total CSA in cm$^2$. The same experienced investigator performed all measurements. The technique has a standard error of estimate of 10.1 cm$^2$ and correlated highly ($r = 0.97$) with MRI scans of the thigh (Knapik et al., 1996).

6.2.6 Muscle fibre cross sectional area and fibre type

Muscle fibre CSA and fibre typing of the vastus lateralis in both legs was determined using immunofluorescence microscopy, as described in Chapter 4.2.5.3. Muscle fibres counted were $159 \pm 77$ fibres per sample. Due to small muscle samples in two participants, the muscle fibre CSA is presented for four ACL participants and their four matched controls.

6.2.7 Maximal voluntary strength

Knee extensor maximal voluntary strength was measured in each leg using a non-extendable strain gauge, with 90° knee and hip angles, as detailed in Chapter 3.2.3.

6.2.8 Postural sway

Postural sway (anterior-posterior SD; APSD) was used to determine balance, using the same protocol and equipment as detailed in Chapter 4.2.6.3, in two-legged and one-legged stances, with eyes open and closed.

6.2.9 Muscle biopsies

For the ACL participants, a muscle biopsy from the vastus lateralis of both the injured and non-injured legs were taken under a general anaesthetic (propofol) immediately prior to
the ACL reconstructive surgery. In the Control group, a single *vastus lateralis* muscle biopsy was performed under a local anaesthetic (1% Xylocaine, Astra Zeneca, Australia). The biopsy procedure, processing and storage of muscle are as detailed in Chapter 4.2.5.2.

6.2.10 $[^3]$Houabain binding site content

Analysis of skeletal muscle $[^3]$Houabain binding site content, to determine muscle NKA content, was performed as described in Chapter 3.2.5.

6.2.11 Western blotting

Individual NKA isoform abundance was measured using the same western blotting methodology as described in Chapter 5.2.8. No signal could be detected for NKA $\beta_3$ and was subsequently not analysed.

6.2.12 Statistical analysis

All data are reported as mean ± standard deviation (SD). Statistical significance was accepted at $P<0.05$. Log-transformation was performed on data which were not normally distributed to reduce bias. Paired t-tests were used to determine differences between the injured and non-injured legs in the ACL participants for muscle fibre CSA, $[^3]$Houabain binding site content, NKA isoform abundance, while independent t-tests were performed to compare the above measures to the CON group. A two-way mixed model ANOVA (leg by group) was used to assess the difference in knee extensor strength, thigh cross sectional area and postural sway between the injured and non-injured legs of the ACL group and compared to CON. Correlations were analysed using Pearson’s product-moment correlation coefficient. Magnitudes of change using Cohen’s effect size was assessed on all variables.
except physical activity and measures between the dominant and non-dominant legs in the CON and was defined as small, 0.2-0.6; moderate, 0.6-1.2; large, 1.2-2.0; and very large, 2.0-4.0 (Batterham et al., 2006; Hopkins, 2007). Effects with less certainty (magnitude of <75%) were classified as no meaningful difference (Batterham et al., 2006; Hopkins, 2007). T-tests and mixed models ANOVA were calculated using SPSS version 20 (SPSS Inc., Champaign, IL) and effect size calculated via a custom spreadsheet (Hopkins, 2007).

6.3 Results

6.3.1 Subjective knee function and physical activity
In ACL, subjective knee function was 53.8 ± 18.7 a.u. (arbitrary units, range 37.9-85 a.u.), which places knee function and symptoms with daily activity in the lowest 5-15th percentile of normative data from the 18-34 year old age group (Anderson et al., 2006). Total physical activity did not differ between ACL and CON (10.7 ± 7.0 vs 12.1± 4.73 hr.wk$^{-1}$, respectively, p = 0.65), nor did leisure/planned activity (ACL: 2.2 ± 2.3 vs CON: 3.7 ± 2.7 hr.wk$^{-1}$, p = 0.29).

6.3.2 Muscle fibre and thigh cross sectional area
In ACL, there were no significant differences in vastus lateralis Type I, Type II, or combined muscle fibre CSA between the injured and non-injured leg (Table 6.1), or between CON group and the injured leg in ACL (n = 4, p>0.22). In addition, there was no difference in the percentage of Type I muscle fibre distribution between legs or groups (p>0.21, Table 6.1). There was a small effect size for the combined muscle fibre CSA between the injured and non-injured legs (ES: 0.41±0.35). Total thigh CSA was 7.1% lower in the injured leg than the
non-injured leg (p = 0.021, ES: 0.61±0.38, Table 6.2). The ACL thigh CSA overall was higher than in CON (p = 0.039).

**Table 6.1**: Muscle fibre cross sectional area and fibre type distribution of the *vastus lateralis* in patients undergoing ACL surgery (ACL) and in the healthy, matched controls (CON).

<table>
<thead>
<tr>
<th>Group</th>
<th>ACL</th>
<th>CON</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injured</td>
<td>Non-injured</td>
</tr>
<tr>
<td><strong>Muscle fibre CSA (µm²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>3719.7 ± 1746.7</td>
<td>4649.4 ± 768.9</td>
</tr>
<tr>
<td>Type II</td>
<td>3752.9 ± 2693.5</td>
<td>4881.3 ± 1376.1</td>
</tr>
<tr>
<td>Combined</td>
<td>3736.2± 2161.3†</td>
<td>4765.1 ± 992.8</td>
</tr>
<tr>
<td><strong>Type I fibre distribution (%)</strong></td>
<td>56.8 ± 12.1</td>
<td>42.0 ± 28.4</td>
</tr>
</tbody>
</table>

† small effect size. Values are Mean ± SD. n = 6 ACL, n = 7 CON.

**6.3.3 Maximal knee extensor torque**

In ACL, the maximal isometric knee extensor torque was 21.2% lower in the injured compared to the non-injured leg (p = 0.021, ES: 0.59±0.38, Table 6.2). Knee extensor isometric torque expressed relative to body mass was similarly 21.7% lower in the injured than the non-injured leg (p = 0.027, ES: 0.7±0.46). Max knee extensor torque relative to thigh CSA also tended to be lower in the injured compared to the non-injured leg (15.5%, p = 0.086, ES: 0.41±0.35, Table 6.2). There were, however, no differences between the dominant and non-dominant legs in CON, or between groups (ACL vs CON) in any of the maximal torque measures (p>0.28).
Table 6.2: Thigh cross sectional area (CSA), maximal isometric torque expressed in absolute units, relative for body mass and thigh CSA, in the ACL and CON groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>ACL</th>
<th></th>
<th>CON</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injured</td>
<td>Non-injured</td>
<td>Dominant</td>
<td>Non-dominant</td>
</tr>
<tr>
<td>Leg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thigh CSA (cm)</td>
<td>150.5 ± 16.2**†</td>
<td>162.0 ± 15.3</td>
<td>127.1 ± 29.3</td>
<td>125.3 ± 26.9</td>
</tr>
<tr>
<td>Maximum torque (Nm)</td>
<td>94.6 ± 35.1*†</td>
<td>120.2 ± 37.8</td>
<td>106.4 ± 28.4</td>
<td>102.7 ± 23.5</td>
</tr>
<tr>
<td>Torque: Body mass (Nm.kg⁻¹)</td>
<td>1.22 ± 0.39*†</td>
<td>1.56 ± 0.42</td>
<td>1.61 ± 0.26</td>
<td>1.56 ± 0.17</td>
</tr>
<tr>
<td>Torque: CSA (Nm.cm⁻²)</td>
<td>0.63 ± 0.24†</td>
<td>0.75 ± 0.23</td>
<td>0.84 ± 0.14</td>
<td>0.82 ± 0.09</td>
</tr>
</tbody>
</table>

* different to non-injured leg (p <0.05; interaction effect between leg and group), † small effect size compared to non-injured leg. Mean ± SD. n = 6 ACL, n = 7 CON.

6.3.4 Postural sway

There was a 43% increase in two-legged APSD with eyes closed in ACL compared to CON (p = 0.04, ES: 1.16±0.96), but no difference with eyes open (p =0.14, ES: 0.74±1.0; Table 6.3).

There were no differences in single-leg APSD between the injured and non-injured legs in ACL with either the eyes open (p= 0.75, ES:0.08±0.97) or eyes closed (p = 0.13, ES: 0.46±0.84; Table 6.3). There was no difference in postural sway between legs in the control group in either condition (p > 0.33), or any overall difference between groups in either the eyes open or closed conditions, although there was a trend for higher overall single-leg postural sway with eyes open in ACL compared to CON ( p = 0.06).
Table 6.3: Two- and one-legged postural sway (APSD) in ACL and CON groups with eyes open and eyes closed.

<table>
<thead>
<tr>
<th></th>
<th>Eyes open</th>
<th>Eyes closed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Two-legged APSD (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACL</td>
<td>0.31 ± 0.18</td>
<td>0.30 ± 0.09*‡</td>
</tr>
<tr>
<td>CON</td>
<td>0.19 ± 0.05</td>
<td>0.21 ± 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Eyes open</th>
<th>Eyes closed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>One-legged APSD (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACL Non-injured</td>
<td>0.59 ± 0.09</td>
<td>1.12 ± 0.19</td>
</tr>
<tr>
<td>ACL Injured</td>
<td>0.65 ± 0.16</td>
<td>1.26 ± 0.26</td>
</tr>
<tr>
<td>Con Dominant</td>
<td>0.51 ± 0.13</td>
<td>1.02 ± 0.09</td>
</tr>
<tr>
<td>Con Non-Dominant</td>
<td>0.49 ± 0.20</td>
<td>1.19 ± 0.45</td>
</tr>
</tbody>
</table>

* higher than CON, p <0.05. ‡ moderate effect size compared to CON. Values are Mean ± SD. n = 6 ACL, n = 7 CON.

6.3.5 Skeletal muscle NKA content and NKA isoform abundance

6.3.5.1 Skeletal muscle [³H]ouabain binding site content

Skeletal muscle [³H]ouabain binding site content in the ACL injured leg was 20.2% lower than in the non-injured leg (p=0.045, ES: 0.8±0.61) and 22.5% lower than CON (p=0.043, ES: 1.19±0.91; Figure 6.1).
Figure 6.1: Vastus lateralis muscle $[^{3}\text{H}]$ouabain binding site content in from the injured and non-injured leg of the ACL group and from the CON group. * less than the non-injured leg, # Less than CON, ‡ moderate effect size compared to non-injured leg. Values are Mean ± SD, ACL, n = 6 for each leg, CON n = 7.

6.3.5.2 Skeletal muscle NKA isoform abundance

The NKA α₂ abundance in the injured leg was 63% lower compared to the non-injured leg in ACL (p = 0.032, ES: 1.11 ± 0.76), but did not differ significantly from CON, although a moderate effect size was found (p = 0.167, ES: 0.77 ± 0.91; Figure 6.3). There was also a non-significant difference, but a moderate effect size in β₁ abundance between the injured and non-injured leg in ACL (p = 0.17, ES: 0.68 ± 0.87; Figure 6.3). There were no differences or meaningful effect sizes in the relative abundance of α₁, α₃ or β₂ isoforms either between the injured and non-injured legs, or in the ACL injured leg compared to the CON group (Figures 6.2 and 6.3).
Figure 6.2: Muscle NKA α₁ (A), α₂ (B) and α₃ (C) relative isoform abundance from the vastus lateralis of the injured and non-injured legs of participants with ACL injury (ACL) and from a single leg of age-and BMI-matched controls (CON). Unfilled bars represent the injured leg from ACL, filled bars represent the non-injured leg from ACL and the hatched bars represent the CON. *less than non-injured leg (p<0.05), ‡ moderate effect size compared to non-injured leg. Values are Mean ± SD, n =6 for each leg ACL, n = 7 CON.
Figure 6.3: Muscle NKA $\beta_1$ (A) and $\beta_2$ (B) relative isoform abundance from the vastus lateralis of the injured and non-injured legs of participants with ACL injury (ACL) and from a single leg of age and BMI-matched controls (CON). Hollow bars represent the injured leg from ACL, filled bars represent the non-injured leg from ACL and the hatched bars represent the CON. ‡ moderate effect size compared to non-injured leg in ACL. Values are Mean ± SD, n =6 for each leg ACL, n = 7 CON.

6.3.6 Correlations

There were no significant correlations between any of the $[^3\text{H}]$ouabain binding site content, NKA $\alpha_2$ abundance, strength, thigh CSA, IPAQ, IKDC scores and duration on injury ($p>0.05$).
6.4 Discussion

This is the first study to demonstrate both a lower skeletal muscle NKA content and NKA $\alpha_2$ relative abundance, in addition to lower muscle CSA and strength, after short-term ACL injury. Hence, ACL injury causes inactivity-induced maladaptations within skeletal muscle, which could impair muscle excitability and fatiguability during exercise in ACL injured patients.

6.4.1 ACL injury reduces NKA content and NKA $\alpha_2$ relative abundance

The first major finding was that ACL injury lowered skeletal muscle NKA content. A decline in NKA content may adversely affect muscle excitability during exercise, which may ultimately contribute to reduced muscle function and fatiguability (Clausen, 2003; McKenna et al., 2008). The 20% lower in $[^3]$Houabain binding site content in the injured leg compared to the non-injured leg in this study is slightly less than the 26% reduction between deltoid muscles in patients with shoulder impingement syndrome (Leivseth et al., 1994), likely due to the two-fold longer mean duration of the shoulder impingement injury (over 8 months). Further, the reduction in muscle NKA content in this study was substantially less than the 34-58% lower NKA content after complete cervical spinal injury (Ditor et al., 2004; Boon et al., 2012). However, as spinal injury causes considerable dysfunction to muscle excitability and disruption of neurotrophic factors within skeletal muscle independent of disuse (Shields, 2002), it is not a valid comparison to investigate inactivity-mediated maladaptations to NKA content. Hence, the greater decline in $[^3]$Houabain binding site content in skeletal muscle after spinal injury likely reflects the combined maladaptive effects of spinal injury along with muscle disuse. As the innervation of skeletal muscle is not
directly impaired by ACL injury, the 20% reduction in $[^3H]ouabain$ binding site content likely represents the direct effects of disuse per se on the injured leg rather than the ACL injury.

A 63% decrease in muscle NKA $\alpha_2$ isoform protein abundance was also found in the injured leg compared to the non-injured leg in ACL, a difference which is three-fold greater than the lower $[^3H]ouabain$ binding site content. There were no differences with ACL injury to the other NKA isoforms ($\alpha_1$, $\alpha_3$, $\beta_1$ or $\beta_2$). This discrepancy between the $[^3H]ouabain$ binding site content and $\alpha_2$ isoform is surprising considering that ~75-80% of NKA $\alpha$ isoforms in rat EDL muscle is the $\alpha_2$ isoform (Hansen, 2001) and is anticipated to be the same in human muscle. The disparity between the $[^3H]ouabain$ binding site content and $\alpha_2$ findings may reflect the semi-quantitative, non-molar and relative nature of western blotting analysis. The lower NKA $\alpha_2$ abundance in the ACL injured leg suggests that the decline in muscle NKA content after injury is likely due to a reduction in the abundance of $\alpha_2$ isoform. A decline in NKA $\alpha_2$ in skeletal muscle has important implications for muscle function and fatiguability. Muscle-specific NKA $\alpha_2$ knockout mice exhibited vastly reduced electrically-stimulated muscle strength and increased in vivo muscle fatigability, and a vast reduction in performance on a graded treadmill test, despite compensatory up-regulation of the NKA $\alpha_1$ isoform (Radzyukevich et al., 2013). In addition, 28% inhibition of NKA $\alpha_2$ abundance in rat skeletal muscle by incubation of $10^{-6}$ M ouabain in vitro caused a 22% decrease in the time to inhibition of muscle force and a concomitant reduction in NKA activity, as measured via $^{86}$Rb uptake (Clausen et al., 1991). The NKA $\alpha_2$ isoform abundance is altered in human skeletal muscle after chronic training and injury. NKA $\alpha_2$ relative abundance increased by 15-68% in response to 10-14 d high intensity and endurance exercise training (Bangsbo et al., 2009;
Thomassen et al., 2010; Benziane et al., 2011) and decreased by 50% after 12 months of spinal injury (Boon et al., 2012). Hence, even a small decrease in NKA α2 relative abundance or total [3H]ouabain binding site content, as seen in this thesis with ACL injury, could impair muscle function and contractility. Future research following ACL injured-patients after improvement of knee function by either surgery or rehabilitation would be useful to investigate the reversibility of the reduced skeletal muscle NKA content, as would further investigation into the association between muscle strength, excitability, fatigability and NKA content after ACL injury. An inevitable weakness of recruiting participants with ACL injury, especially in a small sample, is the lack of standardisation between participants. The duration of injury in this study ranged from 5-50 weeks; hence it is difficult to accurately assess at what duration of injury muscle NKA decreases, another important area for future research.

The underlying mechanism which causes reduced NKA content in skeletal muscle in response to disuse is not understood. Transient chronic increases in muscle intracellular [Na+] increase NKA content in cultured rat muscle (Brodie et al., 1990) and this is suspected, yet untested, to be the mechanism which causes increased NKA content with training (Clausen, 2003). Hence, one potential mechanism behind the decrease in NKA content after ACL injury is a chronic reduction in transient intracellular [Na+] fluxes. Another speculative mechanism is through the biochemical signalling caused by inactivity in muscle; including atrophy-inducing proteins and pathways such as FoxO, Atrogin-1 and MuRF-1 (Bodine et al., 2001). However, research is required to elucidate the cellular cause of decreased NKA content after disuse and injury.
6.4.2 ACL injury reduces joint and muscle function

The impairment of knee extensor strength (-21%) and muscle CSA (-7.1%) in this study is typical of patients with ACL injury, although other studies have reported strength loss as high as 38% and with up to a 14% reduction in CSA of the knee extensors (Lorentzon et al., 1989; Williams et al., 2005a; Makihara et al., 2006). The average IKDC score of 53.8, with a range of 37.8 - 85 suggests that all participants had some level of knee dysfunction due to ACL injury that adversely impacted daily and leisure activities with the injured leg, with participants on average being in the lowest ~5-15% of knee function for their age group (Anderson et al., 2006). However, there were no significant differences in leisure and overall physical activity with ACL compared to CON, likely due to the comparison with a control group. Analysis of pre-injury physical activity levels of patients may provide a greater insight into the effect of ACL injury on physical activity. The reduced weight-bearing of the ACL injured leg likely mediated the atrophy seen after ACL injury; five d of immobilisation caused a 3.5% decrease in quadriceps muscle CSA in humans (Wall et al., 2013), with more substantial decreases in CSA (8-10%) occurring after 2-3 weeks (Narici et al., 2011). However, the strength loss noted in this study could reflect a combination of knee dysfunction and disuse-mediated maladaptations independent of atrophy. This was evident as when normalised for muscle CSA, knee extensor torque still tended (p= 0.086) to be 15% lower in the injured leg compared to the non-injured leg. The reduction of muscle function is of clinical importance; ACL injured patients with lesser atrophy and strength loss have higher knee functioning and knee-related quality of life (Williams et al., 2005b). Hence, the disuse induced by ACL injury may at least partially mediate the impairment of
muscle function and CSA and may further exacerbate the impairment of knee function following ACL injury.

6.4.3 ACL injury and postural sway

Despite the decrease in knee extensor torque, muscle cross sectional area and knee function after ACL injury, indicating the substantial adverse impact of ACL injury, there was no significant decrease in postural sway between the injured and non-injured legs in the ACL group. However, there was a greater two-legged postural sway in ACL compared to the CON. The lack of change in single leg postural sway is in contrast with previous research investigating single-leg postural sway after ACL injury (Ageberg et al., 2005; Lee et al., 2009). This may be because this study used a different postural sway methodology to both previous studies, had a smaller sample size and used a population with more recent ACL injury. The higher postural sway only in two-legged stance with eyes closed in ACL compared to CON suggests some impairment of postural sway not specific to the injured leg, perhaps due to impaired knee proprioception (Barrack et al., 1989; MacDonald et al., 1996; Roberts et al., 2007; Lee et al., 2009); although it is unusual that increased postural sway was not detected in the injured leg alone if this was the case. Hence, the clinical relevance of proprioceptive and postural sway impairments after ACL-injury requires further investigation (Gokeler et al., 2012).

6.5 Conclusion

In conclusion, ACL injury was associated with a reduction in skeletal muscle NKA content and NKA α2 isoform abundance, accompanied by functional impairments in knee extensor strength and thigh cross sectional area. The disuse-induced reduction in NKA content in
skeletal muscle may further contribute to the impairment of muscle function after ACL and other serious knee injuries through increased fatiguability, although this requires further investigation. Clinically, these findings demonstrate the value of exercise and rehabilitation after injury when safely possible in ACL injured patients.
CHAPTER 7: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

7.1 General discussion

7.1.1 ACL injury, but not short-term voluntary inactivity or knee osteoarthritis, reduces skeletal muscle content and α2 relative protein abundance

This thesis investigated skeletal muscle NKA content, NKA isoform abundance and muscle function in each of osteoarthritis, after short-term voluntary inactivity, older age, and after short term ACL injury. In Chapter 3 (OA study) patients with OA did not have reduced NKA content or isoform abundance, similarly in Chapter 5 (ULLS study) there was no difference in NKA content or isoform abundance after 23 d unilateral unloading, whilst in Chapter 6 (ACL study) ACL injury caused a decrease in both NKA content and NKA α2 isoform relative abundance (see Figure 7.1).

A key finding of this thesis was that skeletal muscle NKA content was reduced only in ACL injury. However, in individuals with knee osteoarthritis who were injured for likely a greater duration of time, skeletal muscle [3H]ouabain binding site content was not affected. Despite the physical restriction placed on the ACL patients with gait and daily/leisure tasks, NKA content was only significantly different in the injured leg compared to the controls, while the non-injured leg, which was presumably still somewhat active was not different. This leg-specific maladaptation suggests that the reduction in skeletal muscle NKA content in the ACL patients was mediated by reduced loading on the injured leg and was not due to a reduction in whole-body physical activity. This is consistent with a finding from another
laboratory which found no change in NKA content or isoform abundance in patients with partial spinal injury, contrasting the substantial reductions in NKA content, α₁, α₂, and β₁ isoform relative abundance in patients with complete cervical spinal injury (Boon et al., 2012).

The findings of the ULLS and ACL study taken together could suggest that muscle NKA content was decreased after ACL injury of approximately four months, but not after three weeks of voluntary unilateral inactivity (Figure 7.1). The lack of change after short term-ULLS was surprising, as an early decline in skeletal muscle NKA might be anticipated since muscle NKA can be rapidly increased with as little as 3 d of consecutive exercise training (Green et al., 2004). However, it is important to consider that the physical activity of the ACL injured participants in this study was not directly quantified and they had varied injury durations of between 5-50 weeks. Further, the OA participants would also be likely to have reduced muscle NKA content given the substantial duration and severity of injury. Instead, no difference in NKA content was found in OA. Thus, more research is required to establish the time-course of muscle NKA decline in response to physical inactivity and injury.

It is proposed that the increase in muscle NKA content after training is due to transient chronic increases in intracellular [Na⁺] (Wolitzky et al., 1986; Brodie et al., 1990); but the lack of change in skeletal muscle NKA content with disuse suggests that different mechanisms could cause reduced NKA content after prolonged disuse. The mechanisms underlying the regulation of NKA content in skeletal muscle after inactivity are not
understood, but potentially include signalling pathways including FoxO, Atrogin-1 and MuRF-1 (Bodine et al., 2001) or the altered corticospinal excitability (Deschenes et al., 2002; Clark et al., 2007; de Boer et al., 2007a; Deschenes et al., 2008; Seynnes et al., 2009), potentially via decreased CGRP release into muscle (Clausen, 2003). The exact mechanisms involved are unclear and require more research.
Figure 7.1: Percentage change or difference in vastus lateralis $[^{3}H]$ouabain binding site content (A) and $\alpha_2$ isoform relative abundance (B) in patients with knee osteoarthritis (OA; compared to matched controls; Chapter 3), after 23 d unilateral lower limb suspension (ULLS; pre vs. post ULLS; Chapter 5) and in patients with ACL injury (ACL; compared to non-injured leg; Chapter 6). Error bars represent SD, * significantly difference, <0.05.
This thesis also investigated changes in the relative abundance of the NKA α<sub>1-3</sub> and β<sub>1-2</sub> isoforms (Chapter 3 also included β<sub>3</sub>) after OA, ULLS and ACL injury. Only after ACL injury was there a reduction in NKA α<sub>2</sub> isoform relative abundance, suggesting the reduction in NKA content seen after ACL injury is likely due to a decline in the α<sub>2</sub> isoform (Figure 7.1). There was no compensatory change in the abundance in any of the other NKA α isoforms in Studies 1-3. However, there was an increase in α<sub>2</sub> and α<sub>3</sub> relative abundance in the OA patients despite no difference in [<sup>3</sup>H]ouabain binding site content. This apparent discrepancy may be due to the high physical activity levels of some of the OA participants and the difference in methodologies between NKA immunoblotting, a relative measure of individual NKA isoforms, and [<sup>3</sup>H]ouabain binding site content, which allows for complete quantification of functional NKA heterodimers. However, the exact reason for the discrepancy between NKA α<sub>2</sub> and [<sup>3</sup>H]ouabain binding site content found in patients with OA is not known and requires investigation.

7.1.2 Implications and association of reduced muscle NKA content and muscle function

No research previous to this thesis has investigated skeletal muscle NKA content after a controlled intervention of inactivity, nor investigated the role that a potential decline in skeletal muscle NKA has on muscle function and fatiguability. Surprisingly, despite decline in skeletal muscle strength, mass, power and reduced exercise time to fatigue after ULLS, no change was found in [<sup>3</sup>H]ouabain binding site content, relative protein abundance of NKA α<sub>1-3</sub> or β<sub>1-2</sub> isoforms, or changes in plasma [K<sup>+</sup>] during exercise. This suggests that impaired muscle function after short term-inactivity is unlikely to be due to reduced NKA content in skeletal muscle. Conversely, longer duration or greater severity of inactivity, such as seen
with ACL injury, reduced NKA content and α₂ isoform protein relative abundance in skeletal muscle and was accompanied by a 21% lower maximal isometric torque than the non-injured leg.

While the direct functional effects of decreased NKA content could not be concluded from this investigation, the decline in NKA content and in the relative abundance of NKA α₂ in skeletal muscle after ACL injury are likely to adversely affect muscle function. A reduction of NKA content may reduce the capacity of skeletal muscle to maintain excitability and prevent excessive extracellular accumulation of K⁺ and hence may increase fatiguability during exercise (McKenna et al., 2008). The decrease in muscle NKA content with ACL injury likely reflected the lower NKA α₂ isoform relative abundance. Mice with muscle-specific NKA α₂ knockout had substantial diminishment of maximal running speed on a treadmill and lower in vivo EDL muscle strength and fatiguability (Radzyukevich et al., 2013); furthermore, inhibition of 28% of NKA α₂ isoforms in isolated rat soleus muscle via incubation in 10⁻⁶ mmol/L ouabain impaired electrically stimulated fatigability in vitro by 22% (Clausen et al., 1991). Hence, it is conceivable that the functional impairment seen after ACL injury could partially be due to an inactivity-induced lowering of muscle NKA, likely due to lower α₂ isoform abundance. However, patients with osteoarthritis had increased muscle NKA α₂, despite greatly reduced strength compared to controls. This disassociation between muscle function, muscle NKA content and α₂ relative abundance in humans demonstrates that there is not always a direct relationship between these variables and requires further investigation. Figure 7.2 conceptually describes the effects of inactivity and training on skeletal muscle function, fatiguability and muscle NKA content.
7.1.3 Concomitant effects of atrophy and inactivity on skeletal muscle NKA content

An important consideration with the analysis of $[^3]$Houabain binding site content and NKA relative protein abundance in Chapters 3-6 is the potential concomitant effects of reduced muscle cross sectional area after inactivity. In pig muscle, a greater muscle sarcolemmal membrane area relative to intracellular area was associated with higher $[^3]$Houabain binding site content (Harrison et al., 1994). Hence, a smaller muscle fibre, as seen after inactivity, with a greater sarcolemma to intracellular area ratio, could potentially compensate for any potential decrease in muscle NKA content with inactivity. An important underlying assumption is that the NKA are predominantly found in the sarcolemma, which may not be the case in human muscle. As this thesis had no measurement of muscle
membrane area, as used by Harrison et al., (1994), it was not possible to confirm or reject whether cellular atrophy could compensate for lowered muscle NKA content, or present data normalised to muscle membrane area. Future research should consider the physiological implications of muscle fibre size on NKA content, including measuring muscle membrane area and presenting normalised \(^{[3]}\text{H}\text{ouabain binding site content.}

7.1.4 The effect of injury and inactivity on skeletal muscle strength, morphology and fatiguability

In each of OA, ULLS and ACL injury there was a clear decrease in muscle strength. Whilst in OA and ACL injury these could be partially mediated by the injury itself, muscle disuse was likely to have caused substantial reduction in muscle strength. After 23 d of controlled unilateral inactivity there was a 24% decline in isometric knee extensor MVC, similar to previous research using ULLS (de Boer et al., 2007a) and the 21% decline in knee extensors isometric strength reported between legs in the ACL group, but substantially less than the 40.8% lower isometric knee extensor torque in the OA participants compared to age-matched controls (see Figure 7.3). Reduced strength can impair quality of life and capacity to perform daily activities (Kell et al., 2001). As a result, any reduction via inactivity and/or injury is of clinical importance and promotes the importance of physical activity when possible to prevent inactivity-induced maladaptations to skeletal muscle.
Figure 7.3: Percentage difference or change in isometric knee extensor torque in patients with knee osteoarthritis (OA; compared to matched controls; Chapter 3), after 23 d unilateral lower limb suspension (ULLS; pre vs. post ULLS; Chapter 4) and in patients with ACL injury (ACL; compared to non-injured leg; Chapter 6). Error bars represent SD, * significantly difference, <0.05.
Muscle atrophy is one important factor which could contribute to the reduced strength in OA, after 23 d ULLS and ACL injury (Houston et al., 1983; Maughan et al., 1983). There was a 4.4% reduction in total thigh mass after 23 d of voluntary inactivity in the unloaded leg, with no difference in the weight bearing leg. Whilst in ACL injured patients, using a less-precise anthropometric based method due to logistical constraints, there was a 7% lower thigh cross sectional area in the injured leg compared to the non-injured leg (Chapter 6). This decline in muscle mass has functional significance; ACL injured patients with less thigh atrophy and knee extensor strength decline also have higher subjective knee function (Williams et al., 2005b), suggesting that the maintenance of CSA and strength may reduce the impairment in joint function caused by ACL injury. Further, conditions which induce muscle atrophy have detrimental metabolic effects, including increased reliance on muscle glycolysis and impairment of aerobic metabolism (Stein et al., 2005; Abadi et al., 2009).

Muscle fatigability was measured after 23 d of ULLS, but not in ACL injury and OA due to logistical and practical limitations of testing these participants. The 22% reduction in one-legged cycling time to fatigue (TTF) after ULLS is unlikely to be due to central cardiovascular changes, including decreased stroke volume, as there was no change in \( \dot{V}O_2 \) after inactivity and TTF was not decreased after ULLS during cycling using the weight-bearing leg. There are numerous factors which could contribute to the impaired muscle strength and fatigability after inactivity, including decreased central drive (Deschenes et al., 2002; Clark et al., 2006a; Deschenes et al., 2008; Narici et al., 2011), impairment of muscle pennation angle and fascicle length (de Boer et al., 2007a) and decreased mitochondrial function, as evidenced by a reduction in mitochondrial proteins, activity and size (Abadi et al., 2009;
Nielsen et al., 2010). After ULLS there was no greater increase in plasma \([K^+]\) during submaximal and fatiguing exercise or slower decline in recovery, which is consistent with the lack of decline in muscle NKA. However, as there was neither measurement of muscle interstitial \([K^+]\), or arterial plasma \([K^+]\) sampled, it is difficult to properly assess the effect of inactivity of \(K^+\) regulation during exercise. Hence, it is possible that higher muscle interstitial \([K^+]\) may still have occurred after ULLS compared to baseline, which could impair muscle excitability and ultimately muscle function (McKenna et al., 2008); but this appears unlikely after short-term inactivity. Whilst not investigated in this thesis, sarcoplasmic reticulum \(Ca^{2+}\) uptake was reduced after 10 d immobilisation (Thom et al., 2001), perhaps due to accumulation of inorganic phosphates (Allen et al., 2008), which could also contribute to impaired muscle fatiguability after inactivity. Whatever the factors responsible for the increased fatiguability after ULLS, the effects on muscle function were reversible with only 4 weeks of resistance training.

7.1.5 The effects of injury and disuse on postural sway

Postural sway, as a measurement of balance, has important implications for the likelihood of injury in competitive sports (Hrysomallis, 2007) and in elderly populations is associated with risk of falls (Muir et al., 2013). Postural sway was impaired after short-term ULLS in the unloaded leg with both eyes open and eyes closed and a trend with two-legged standing \((p = 0.051, \text{Chapter 4})\). In ACL-injured patients, no difference was found between the injured and non-injured leg postural sway, in contrast to previous research (Ageberg et al., 2005; Lee et al., 2009). However, two legged postural sway with eyes closed was 43% greater with ACL injury compared to the matched controls without any impairment of postural sway.
with eyes open, suggesting that unlike short term ULLS, reduced knee proprioception may have a greater role in impairing balance after ACL injury. Hence, in injured populations enforced into inactivity, both disuse and injury itself can impair balance.

The mechanisms behind increased single leg postural sway after inactivity, and increased two-legged postural sway with ACL injury are likely different. ACL injury is marked by reduced knee proprioception (MacDonald et al., 1996; Hassan et al., 2001; Lee et al., 2009); the increased postural sway after ACL injury only with eyes closed implies that impaired postural sway with ACL injury is likely due to reduced knee proprioception. With ULLS, the mechanisms of increased single legged postural sway are less apparent. This thesis found no relationship between knee extensor strength decrease and postural sway, similar to previous studies where maintenance of plantar flexor strength and CSA did not prevent impaired postural sway after 20 d of bed rest (Kouzaki et al., 2007). Taken together this suggests strength loss has little role in the impairment of postural sway after inactivity, and instead this is most likely due to as yet undescribed sensorimotor maladaptations, including impairment of Golgi tendon organs (Fitts et al., 2001).

7.1.6 Training reverses the impairments to muscle strength and power caused by inactivity

One of the most consistent findings of the ULLS study was the potent effect of training after inactivity (Chapters 4 and 5). Four weeks of resistance training after ULLS restored to levels similar to pre inactivity levels each of muscular strength, muscle mass, postural sway, time to fatigue and heart rate during incremental exercise. This is remarkable considering the 22-24% impairment of muscle strength and intense exercise time to fatigue evident after ULLS.
This establishes that at least in healthy young adults, the various maladaptations caused by inactivity on skeletal muscle function and performance are rapidly reversible, consistent with another recent ULLS study of similar duration (Campbell et al., 2013). Ambulation without specific resistance training is also effective; even after six weeks of ULLS, seven weeks of re-ambulation without structured training was sufficient to restore knee extensor strength and muscle size (Berg et al., 1991). However, both eccentric-and concentric-based resistance training enhanced recovery after immobilisation compared to no training (Hortobagyi et al., 2000), supporting an important role for exercise and rehabilitation after injury. The mechanism for this rapid recovery of strength is likely the broad neuromuscular maladaptations seen after ULLS, which are quickly reversible with re-ambulation (Berg et al., 1996). Seven and 14 d of ULLS caused decreased neural drive in both young and older-adults participants (Deschenes et al., 2002; de Boer et al., 2007a; Deschenes et al., 2008) and increased H-reflex (Clark et al., 2007; Seynnes et al., 2009), denoting increased corticospinal excitability. This impairment in neural drive and corticospinal excitability may be rapidly reversible, as the magnitude of strength loss after 6 weeks of ULLS was halved after only 4 d of re-ambulation (Berg et al., 1991), whilst the 13% decrease after 10 d of ULLS was completely reversed after 4 d of re-ambulation (Berg et al., 1996).

The restoration of muscle power (vertical jump height) after inactivity has been sparsely investigated, hence, the mechanisms are not well known. The changes in muscle architecture and tendon stiffness after ULLS (de Boer et al., 2007a), which are returned after 3 weeks of resistance training (Campbell et al., 2013), in combination with restoration of movement co-ordination and impaired corticospinal excitability (Clark et al., 2006a; Clark
et al., 2007) could be responsible for the restoration of vertical jump height after ULLS. In addition, while strength was not associated with vertical jump height in the ULLS study, the restoration in strength may still also have contributed to the restoration of vertical jump height (Wisløff et al., 2004).

7.2 Conclusions

Study One, Chapter 3- Osteoarthritis and aging

i) Skeletal muscle NKA content was not changed in mature age and elderly patients with knee osteoarthritis despite greatly reduced knee extensor muscle strength.

ii) An inverse association was found between skeletal muscle NKA content and age in all participants pooled and in the OA group alone.

Study Two (Part I), Chapter 4-ULLS and muscle function

i) Twenty-three d of ULLS in healthy young participants reduced each of muscle strength, thigh mass and postural sway in the unloaded leg, and also reduced strength in the weight-bearing leg.

ii) Each of these impairments to muscle mass and function after ULLS were restored to baseline levels after four weeks of resistance training.
Study Two (Part II), Chapter 5- ULLS and muscle NKA

i) Twenty-three d of ULLS caused no changes in NKA content, NKA α₁-3 and β₁- isoform abundance, or plasma [K⁺] during exercise and recovery, despite substantial impairment of exercise time to fatigue and associated increased rating of perceived exertion and heart rate.

ii) Resistance training reversed the impairment in one-legged cycling exercise time to fatigue, but did not increase muscle NKA content or change antecubital venous plasma K⁺ dynamics.

Study Three, Chapter 6- ACL injury and muscle NKA

i) Participants with short-term ACL injury had lower knee extensor strength and cross sectional area than the non injured leg and compared to healthy age- and BMI-matched controls.

ii) The injured leg in ACL participants had lower muscle NKA content than the non-injured leg and controls, whilst α₂ isoform relative abundance was lower than the non-injured leg.

7.3 Recommendations for future research

7.3.1 Time-course and severity of inactivity required for reduced NKA content

This thesis found that NKA content was reduced in skeletal muscle after a short-term ACL injury of average duration of 3-4 months (Chapter 6), but not with either knee osteoarthritis (Chapter 3) or after 23 d of voluntary inactivity (Chapter 5). Hence, the time course of
impairment in NKA content after inactivity is not understood. Further, it is possible that in a more debilitating or restrictive form of disuse, such as limb casting (Adams et al., 2003), a decline in muscle NKA could be seen after even short-term disuse. Hence future research should investigate different modalities of voluntary inactivity and injury, as well as the potential time-course of reduced NKA content in skeletal muscle. Additionally, the development of the single fibre western blotting technique (Murphy, 2010) as applied with muscle NKA (Thomassen et al., 2013) could also elucidate whether the decline in NKA with inactivity or injury is greater in one fibre type, or specific to only Type I or Type II muscle fibres. Investigation into the molecular mechanisms underlying the decline in NKA in skeletal muscle is of clinical relevance for the development of pharmacological interventions to minimise the reduction in muscle NKA.

7.3.2 The effect of reduced physical activity on skeletal muscle NKA activity and $K^+$ regulation during exercise

This thesis did not investigate the activity of skeletal muscle NKA after inactivity, exercise or injury. It is possible that NKA activity is impaired after inactivity or injury. The most widely used assay to measure NKA activity in human muscle is the $K^+$-stimulated maximum 3-O-MFPase activity assay, the specificity of which can be shown by its complete inhibition by ouabain (Fraser et al., 1998). However, recent evidence that the 3-O-MFPase activity assay was not accurate for determination of NKA activity after exercise raises doubts about this assay for this purpose (Juel et al., 2013). Hence, the investigation of NKA activity at rest, as well as after acute exercise following inactivity and injury, possibly using the method outlined by Juel et al. (2013) may reveal important information which were not possible
with this research. The level of FXDY1 phosphorylation has an important role in the activation of NKA in skeletal muscle (Rasmussen et al., 2008) which also warrants further investigation using a unilateral or whole-body inactivity method. It should be noted, however, that both previously mentioned methods for determination of NKA activity in humans cannot determine NKA activity during actual exercise. Hence, future studies investigating NKA activity after muscle disuse should still account for total muscle NKA content.

While this thesis emphasized the potential change in skeletal muscle NKA content after injury and inactivity, future research could investigate what functional effect these impairments could have on $K^+$ regulation in the muscle and plasma during exercise and the potential implications for fatigue. Only the one study in healthy young participants (Chapter 5) investigated the adaptations to inactivity to plasma $[K^+]$ during exercise. As this was antecubital venous blood, which allows no quantification for the release and clearance of $K^+$ from active skeletal muscle, or allows assessment of $K^+$ uptake by inactive muscle (Hallen et al., 1994), which is likely important with only limited muscle mass being used in one-legged cycling. Hence, both femoral venous and arterial blood sampling from the exercising leg before and after controlled inactivity, as well as assessment of blood flow would accurately determine $K^+$ release and re-uptake by skeletal muscle and could be applied during exercise and recovery (Hallen et al., 1994). Determination of muscle interstitial $[K^+]$ via microdialysis during rest and exercise after inactivity would also allow for a useful determination of the potential impacts of inactivity on $K^+$ regulation in skeletal muscle (Green et al., 1999b).
7.3.3 Variance in skeletal muscle function in osteoarthritis and ACL injury and association with skeletal muscle NKA content

This thesis investigated relatively homogenous groups of patients with ACL injury and with knee osteoarthritis. Future research could investigate the differing severity within these conditions and explore possible changes to muscle NKA content and activity, to determine whether the degree of the injury/condition could affect the physical activity levels and hence NKA content in skeletal muscle. Indeed, this has been done with spinal injury, where a decrease in NKA content was only found in those with complete, but not partial, cervical spinal injury (Boon et al., 2012). This future work may best be carried out on populations with ACL injury. Some participants have only minimal knee dysfunction even after a complete ACL rupture, and have been termed ‘copers’ (Williams et al., 2005a; Williams et al., 2005b), who have less atrophy and strength impairment compared to non-copers (Williams et al., 2005b). Hence, further work in ACL injury may investigate whether muscle NKA loss is minimised in patients with better knee function, perhaps due to increased knee function and limb use.

7.3.4 Recoverability and age-related effects of skeletal muscle NKA content after injury and osteoarthritis

This thesis investigated the effect of re-ambulation combined with 4 weeks of resistance training after inactivity on muscle function and on skeletal muscle NKA (Chapters 4 and 5). Our findings regarding muscle function after inactivity are broadly consistent with previous research (Berg et al., 1991; Hortobagyi et al., 2000; Campbell et al., 2013) and suggests that the recovery of muscle strength, size and endurance are rapid and can return in a similar
duration to that of the inactivity period. However, as the ULLS period did not cause a decrease in NKA content, the reversibility of potential reductions to skeletal muscle NKA is unknown and warrants future research with longer timeframes of ULLS or other inactivity interventions such as immobilisation. As muscle NKA content can be increased after as little as three consecutive days of endurance training, the restoration of NKA content in skeletal muscle after recovery may be rapid. This could also be investigated during the course of rehabilitation from ACL reconstructive surgery and compared to patients who do not undergo reconstructive surgery after ACL injury, or investigated with many other severe joint injuries.

Finally, older populations may undergo greater impairment in muscle function after physical inactivity, including greater strength decrement at faster speeds of contraction (Deschenes et al., 2008) and on a single muscle fibre level (Hvid et al., 2011). Hence, the role of inactivity in old versus young participants on skeletal muscle NKA content, NKA activity and K⁺ regulation during exercise are all important future areas of research.
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APPENDIX A- CARDIOVASCULAR AND RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the pilot study testing a peripheral muscle stimulation protocol, you are required to complete the following questionnaire which is designed to assess the risk of you having a cardiovascular event occurring during an exhaustive exercise bout.

Name: ____________________________________________    Date: ______________________
Age: ________ years    Weight: ________ kg    Height: _________ cms    Gender:  M  F

Give a brief description of your average activity pattern in the past 2 months:

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

Circle the appropriate response to the following questions.

Are you overweight?  Yes  No  Don't know
Do you smoke?  Yes  No  Social
Are you an asthmatic?  Yes  No  Don't Know
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<th>Question</th>
<th>Yes</th>
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<td>Are you a diabetic?</td>
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<td>Does your family have a history of diabetes?</td>
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<td>Do you have a thyroid disorder?</td>
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<td>Does your family have a history of thyroid disorders?</td>
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<td>Do you have a pituitary disorder?</td>
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<td>Does your family have a history of pituitary disorders?</td>
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<td>Do you have a heart rhythm disturbance?</td>
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<td>Do you have a high blood cholesterol level?</td>
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<td>Do you have elevated blood pressure?</td>
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<td>Are you being treated with diuretics?</td>
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<td>Are you on any other medications?</td>
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List all medications? (including vitamin supplements, antioxidants and oral contraceptives)

___________________________________________________

Do you think you have any medical complaint or any other reason which you know of which you think may prevent you from participating in strenuous exercise?  
If Yes, please elaborate

Have you had any musculoskeletal problems that have required medical treatment (eg, broken bones, joint reconstruction etc)?

If Yes, please provide details (including dates)

Are you currently pregnant or expect to become pregnant during the time in which this experiment is conducted?  

Does your family have a history of premature cardiovascular problems (e.g. heart attack, stroke)?

Don't Know
I, ______________________________, believe that the answers to these questions are true and correct.

Signed: ____________________________  Date:  ________________
MUSCLE BIOPSY, ARTERIAL & VENOUS CATHETERISATION QUESTIONNAIRE:

The effect of ACL injury on skeletal muscle function, contractile proteins and inflammation

NAME: ____________________________________________________________

ADDRESS: ________________________________________________________
__________________________________________________________________
__________________________________________________________________

DATE: _________________________  AGE:   ___________ __ years

1. Have you or your family suffered from any tendency to bleed excessively? (e.g. Haemophilia) or bruise very easily?  
   Yes  No
   Don't Know
   
   If yes, please elaborate

__________________________________________________________________
2. Are you allergic to local anaesthetic?   Yes  No
   Don't Know
   If yes, please elaborate

3. Do you have any skin allergies?   Yes   No
   Don't Know
   If yes, please elaborate

4. Have you any other allergies?   Yes   No
   Don't Know
   If yes, please elaborate

5. Are you currently on any medication?   Yes   No
   If yes, what is the medication?

6. Do you have any other medical problems?   Yes   No
   If yes, please elaborate

7. Have you ever fainted when you had an injection or blood sample taken?   Yes   No
   Don't know
   If yes, please elaborate
8. Have you previously had heparin infused or injected?  
   Yes  No  Don’t know

   If yes, please elaborate
   ______________________________________________________

9. Do you or other members of your family have Raynauds disease, or suffer from very poor circulation in the fingers,

   leading to painful fingers that turn white/blue?  
   Yes  No  Don’t know

   If yes, please elaborate
   ______________________________________________________

To the best of my knowledge, the above questionnaire has been completely accurately and truthfully.

Signature: ________________________________  Date: ______________________
ULLS Risk Factor Questionnaire

In order to be eligible to participate in the study: *The effect of single lower limb unloading on exercise performance, fatigue and potassium regulation in healthy young adults*, you are required to complete the following questionnaire which is designed to assess the risk of Deep vein thrombosis occurring while under Unilateral Lower Limb Suspension.

Name: ____________________________________________    Date: ______________________

Age: ________ years    Weight: ________ kg    Height: ________ cms    Gender:  M    F

Circle the appropriate response to the following questions.

Do you or your family have any history of blood clotting disorders or deep vein thrombosis?

Yes   No   Don't know

Are you on an oral contraceptive?

Yes   No

Have you had any significant lower limb injuries in the past 2 years?

Yes   No

If so, please elaborate:

____________________________________________________________________
____________________________________________________________________
APPENDIX D- EXAMPLE OF ULLS DIARY

Unilateral lower limb suspension Diary

Please complete everyday while on crutches

Please contact Ben Perry on 0414 600 986 if you have any question or problems, or if any of the signs of deep vein thrombosis are present.

<table>
<thead>
<tr>
<th>Name:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Signs</th>
<th>Yes</th>
<th>No</th>
<th>Unsure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Redness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soreness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenderness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swelling</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Daily checklist</th>
<th>Yes</th>
<th>No</th>
<th>Unsure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments?
Did you use the inactive leg today?

- Performed non-weight bearing contractions 2-3 times?
- Worn your compression stocking?
- Performed passive joint movements?
- Had 1 pill of supplied aspirin?
- Consumed alcohol?
- Been contacted by a study investigator?
APPENDIX E - EXAMPLE INFORMATION AND CONSENT FORM

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled: The effect of single lower limb unloading on exercise performance, fatigue and potassium regulation in healthy young adults

This project is being conducted by Prof. Michael McKenna, Dr Suzanne Broadbent, Dr Nigel Stepto, Dr Francois Billaut and Dr Aaron Petersen, Dr. Itamar Levinger and Mr. Ben Perry.

Project explanation

This research is being conducted to assess what effect inactivity, a huge problem in public health, has on various markers of muscle physiology and general health. This research also seeks to investigate whether electrical muscle stimulation in humans can help attenuate some of the effects normally seen with inactivity. This project aims to investigate the effects of 23 days of single lower limb inactivity on exercise performance, muscle physiology and biomedical health markers. This research will also investigate whether electrical muscle stimulation can minimise the effect of inactivity on a single leg.

What will I be asked to do?

We will ask you to fill in several short questionnaires about your family medical history and your exercise habits. You will then be asked to do a familiarisation of the exercise tests, using crutches, and a maximal aerobic test (VO\textsubscript{2 peak}). You will then start the formal exercise tests which span over two visits (total of six visits for exercise testing), one of which will involve blood sampling, muscle samples (from the thigh) and peripheral magnetic stimulation. All muscle samples will be performed by a qualified medical practitioner. This process, called a muscle biopsy, is performed under local anaesthetic, and involves a small sample (about 3-4 grains of rice) of muscle to be taken. After three days rest, you will be asked to do daily activities with the use of crutches, and a raised-sole on one shoe. This will cause one leg to not undertake activity (similar to if you were to injure a knee or ankle and you required crutches). If you agree to the use of crutches and the raised shoe, you will be asked to use crutches for 23 days, in this time you will report in to the exercise physiology laboratory (LCESS building, Victoria University, Footscray park campus) twice per week for blood testing. If you are in the group to receive muscle electrical stimulation, you will be asked to participate five times per week in a training protocol utilising muscle electrical stimulation during the time in crutches. After the 23 days, you will then be asked to complete the same exercise testing, with blood and muscle samples, as completed previously. You will then be asked to undertake a strength training
program for the next four weeks (three times per week). This program is designed to return your
strength power and muscle size to that or exceeding that acquired before participating in the study.
You will then be asked to do the final round of exercise testing (the same tests as previously
completed). A further 3 weeks of resistance training will be offered to you if you wish to keep
training.

During the study, you will also be asked to have a DXA scan (Dual X-ray absorptiometry) on your
lower limbs. This will occur three times during the study, and you will be asked to meet one of the
investigators at Victoria University (Footscray Park) to complete this test. This test will be used to
accurately measure lean muscle mass in the legs. The DXA scans involved in this research study
involves exposure to a very small amount of radiation. As part of everyday living, everyone is
exposed to naturally occurring background radiation and receives a dose of about 2 millisievert
(mSv) each year. The effective dose from this study is about 0.03 mSv. At this dose level, no harmful
effects of radiation have been demonstrated as any effect is too small to measure. The risk is
believed to be minimal. Additionally, your blood samples will be measured for basic metabolic health
markers (Glucose, triglycerides and cholesterol). Weight, height, blood pressure and waist
circumference will also be required throughout the study.

What will I gain from participating?
You will gain a thorough, scientific insight into multiple aspects of your fitness. You will gain a highly
accurate measure of your muscle and fat mass. You will receive free training with supervision at
Victoria rehabilitation clinic for up to seven weeks, and will be shown various resistance exercises
for the lower limbs. Additionally, you will be payed an honorarium of $400 at the completion of your
participation, and will gain the knowledge of your muscle fibre distribution; something which can
only be attained from studies such as these. Finally, you would be contributing to knowledge in an
area which is becoming more prevalent, the effects of inactivity in humans.

How will the information I give be used?
The information which will be gained from the research will be used for analysis of various muscle
markers of muscle excitability and fatigue (Na+/K+ pump activity, content and expression) and how
they are altered after inactivity, and whether electrical stimulation alters this. Additionally, single
fibre characteristics will also be determined. Strength and muscle cross sectional area data will be
used to assess whether the ULLS protocol was effective, and if electrical stimulation causes a
functional adaptation during inactivity. Your samples and data will be stored under alphanumeric
codes (i.e. without your name or personal details) and only the researchers will be able to connect
the samples to you. The data that will be collected during the study will be used/published in peer-
reviewed journals and conference presentations. No personal details will be revealed without your
written consent.
What are the potential risks of participating in this project?

The maximal cycle ergometer incremental exercise test and one legged incremental cycling test involve the risk of sudden death due to myocardial infarct, vasovagal episodes, muscle soreness and stiffness. Risks associated with venous catheterisation include discomfort, bruising and infection (for example pus, tenderness and/or redness). Risks associated with muscle biopsy include discomfort, pain, bruising, bleeding, soreness, localised altered sensation of skin reduced /absent /tingle /hypersensitive) and infection. Risks associated with being on crutches for 23 days include increased risk of deep vein thrombosis, decreased mobility and independence, increased risk of falls due to the use of crutches, decreased muscle strength, atrophy, muscle cramping and muscle soreness. A regular blood sample will be taken while on crutches to test for a pre-clinical marker of deep vein thrombosis (DVT). Symptoms of DVT include redness, swelling and pain in the affected leg. Aspirin (100 mg) will be supplied to you for daily consumption as a preventative measure against DVT. ULLS does increase the risk of DVT or a superficial vein thrombosis, to an incidence of ~1-2%. In the incident of a possible DVT or superficial vein thrombosis forming, you will immediately be referred to a medical doctor for a Doppler ultrasound, with any costs for treatment being covered by funding from the research. You should continue to check for the symptoms of DVT (redness swelling and/or pain in the leg) daily after the ULLS protocol for up to one month. Thereafter, continue to regularly check for the signs and symptoms of DVT for an additional two months after the ULLS protocol. If any signs and symptoms of DVT are noted, immediately seek medical consultation and contact the researchers. Psychological risks from the ULLS protocol include frustration and anxiety from lack of mobility, and frustration from being unable to undertake exercise for the 23 day period.

Inclusion criteria:

- Between the age of 18-35 years of age.
- In good health.

Exclusion criteria:

- Using oral contraceptives.
- A family history of DVT
- Blood clotting problems/disorder
- Any allergies to aspirin
- Obesity.

How will this project be conducted?

As a potential participant you will be initially screened for cardiovascular risk factors and any health issues of relevance to the study. If you are deemed healthy and at low risk of any adverse events you will be ask to complete multiple testing sessions as listed below. The study involves multiple visits to VU, Footscray Park campus over a total 9-12 week period. The schematic on the next page demonstrates the required involvement in the study. This research is completely voluntary; you are free to withdraw from the study at anytime without resentment or ramifications from any of the investigators. However, withdrawal after more than a week of ULLS will leave you with a slightly weaker leg. The testing of DVT will still be offered to you after the inactivity period even if you withdraw at anytime during ULLS.
Visit 1 (~60min): Body Composition, Aerobic fitness and Familiarisation to crutches.

During this session you will undertake some basic measures of body composition including determination of height, weight and waist circumference. Immediately following these measures you will then undertake an aerobic fitness ($\text{VO}_2^{\text{peak}}$) test by performing an incremental exercise test on a cycle ergometer. After recovery, you will be asked to briefly (~5 minutes) ambulate on crutches with an extended show. This is to let you know how it will feel if you have never been on crutches before.

Visit 2 (~2h): Familiarisation Day 1

This will occur approximately one week after your $\text{VO}_2^{\text{peak}}$ test. During this session you will perform familiarisations of the exercise tests to be performed. These include proprioception (balance) tests, vertical jump and quadriceps strength test. Data will not be collected; this session is to ensure you are comfortable and competent with the exercise testing. You will be given substantial breaks between each test to ensure there is no fatigue before the exercise begins.

Visit 3 (~3h): Familiarisation Day 2

This visit will occur approximately 1-3 days after your last visit. Like the last visit, this session is for your familiarisation to the exercise testing. This session will include the one legged incremental cycling protocol (on both legs), with a ~2 hour break between each leg tested. You are asked to wear comfortable exercise gear in all exercise testing.

Visit 4 (<1h): DXA scan

You will attend the Western Hospital (Footscray), or Sunshine Hospital, St. Albans with one of the investigators for a DXA scan. This whole body scan calculates muscle, bone and fat proportions. The scan uses very low levels of ionising radiation; it utilises less than 1/10 of the radiation emitted from a chest x-ray.
Visit 5 (~2.5h): Baseline testing Day 1

This testing will occur approximately 3-7 days after your familiarisation. This testing day will consist of the following exercise tests: Vertical jump, quadriceps strength and lower limb proprioception. This is the same tests which were performed on visit 2. You will be given appropriate rest between each test to ensure that there is no fatigue at the beginning of each test.

Visit 6 (~4h): Baseline testing Day 2 (One legged cycling protocol)

This exercise testing session will occur the day after the last visit. This is the same exercise test as familiarised as on familiarisation day 2, but this test will also involve blood sampling from venous cannulation, magnetic stimulation (causes muscle contraction), Electromyographic recording of muscle activity and muscle biopsies from the thigh. Additionally, a mask will be fitted to measure expire gases as was used in the first visit (VO₂ peak). A total of two muscle biopsies will be taken: one on each leg at rest

Visit 7-13 (<1h): Unilateral Lower Limb Suspension (ULLS), blood tests and familiarisation.

Three days after the second day of baseline testing, you will be issued with crutches and a customised shoe which will be required to be used for the next 23 days. You will be familiarised with ambulating on crutches, and it will be ensured the equipment supplied is adjusted correctly for your height. You will also be given a compression garment for the inactive leg, and a small pressure pad for the insert of the shoe. The pressure gauge will only monitor the amount of weight placed on the non-weight bearing leg; it cannot retain any information on the destination of the unit. Additionally, if you are selected to receive electrical stimulation, you will be familiarised with the training protocol.

Throughout the 23 days on crutches and the raised sole shoe, you will be required to attend the exercise physiology laboratory twice per week (five times if assigned to electrical stimulation training group) for a single blood sample via venepuncture. This blood sample will test whether there is an increased risk of DVT development. The first and final blood samples will also be used for fasting glucose, triglycerides and cholesterol. Additionally, this is an opportunity for you to discuss any problems/issues you are having with the crutches/study in person during this time. You will also be supplied with the contact details of one of the investigators throughout the study if any issues arise.

Additionally, you may be randomly selected to receive electrical muscle stimulation five times per week while under ULLS. This electrical stimulation training is brief (<30 minutes), and muscle stimulation intensity is kept at a safe and painless level attained during the familiarisation before the ULLS period.

Visit 14 (<1h): DXA scan

Same as previously described DXA scan. Will occur one day prior to the end of ambulating on crutches (day 22).

Visit 15 (~2.5h): Post ULLS testing day 1

This testing day will occur on day 21 of being on crutches. This will be the same tests as the first day of baseline testing. You will undertake the vertical jump, quadriceps strength and proprioception tests.
Visit 14 (~4h): Post ULLS testing day 2
This will occur the day after the last testing session. This will be the same exercise testing protocol (with blood samples and muscle biopsies) as used on baseline testing day 2; the one legged incremental cycling test is completed on both legs. The only difference in this exercise test compared to the previous test is that there are two additional muscle biopsies taken, each after exercise on the cycling leg.

Visit 15-27 (<45 min): Strength training program at Victoria University
3-5 days after the exercise testing, you will begin a strength training program at the Victoria University rehabilitation clinic (Footscray Park) under the supervision of a Postgraduate student in exercise rehabilitation. The program will last for four weeks, with three training programs per week (total 12 sessions). The training will focus primarily on the lower limb affected while you were on crutches. The sessions will last 30-45 minutes, and will be held at a time convenient for your schedule.

Visit 28 (~1.5h): Post resistance training testing Day 1
After four weeks of strength training, you will be asked to complete the final bout of exercise testing. The schedule will be the same as previously described with day 1 consisting of the vertical jump test, quadriceps strength and proprioception testing.

Visit 29 (~4h): Post resistance training testing Day 2
Two days after the previous testing session, you will be required to complete the one legged incremental cycling test using the same protocol, with blood samples and muscle biopsies, as previously described. This test is identical to the first occasion (before ULLS) the one-legged cycling test was completed, there is a total of two muscle biopsies at rest; one on each leg.

Visit 30 (<1 h): DXA scan
Same as previously described DXA scan.

Who is conducting the study?
The study is conducted by the School of Sport and Exercise Science, Victoria University

Main Investigators:

Prof. Michael J. Mckenna, Telephone number 9919 4499, Mobile 0488 475 735,
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Email: nigel.stepto@vu.edu.au

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Email: Aaron Petersen@vu.edu.au

Dr. Francois Billaut Telephone number 9919 9451 Mobile
Email: Francois.Billaut@vu.edu.au
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Email : itamar.levinger@vu.edu.au

Mr. Ben Perry  Telephone number  9919 4207 Mobile 0414600986,
Email: ben.perry@live.vu.edu.au

Any queries about your participation in this project may be directed to the Principal Researcher listed above.

If you have any queries or complaints about the way you have been treated, you may contact the Secretary, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 phone (03) 9919 4781.
CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study investigating *The effect of single lower limb unloading on exercise performance, fatigue and potassium regulation in healthy young adults*

INVESTIGATORS:
Professor Michael McKenna
Dr. Aaron Petersen
Dr. Nigel Stepto
Dr. Suzanne Broadbent
Dr. Francois Billaut
Professor. Graham Lamb
Dr. Robyn Murphy
Dr. Ceric Lamboley
Mr Ben Perry

AIMS OF THE STUDY – This research aims to investigate the effect of single limb inactivity on exercise performance, basic health markers and muscle physiology.

SCREENING- Before entry into the study, participants will be screened for any health factors which may cause adverse effects during exercise testing, blood sampling and the inactivity protocol. This will be done via self questionnaire. If any health factors are found which will increase the participant’s risk, the participant will be unable to be involved in the study.

PARTICIPANT INVOLVEMENT AND OVERVIEW OF TESTING - Participants will be requested to attend the Exercise Physiology Laboratory at Victoria University, Footscray Park Campus (LCESS building) multiple times over a ~10 week period. Within the 10 weeks will be exercise familiarisation and testing, 23 days of single limb inactivity (via crutches and a raised-sole shoe), blood samples, muscle samples, strength training and further exercise tests. Additionally, you may be selected to receive an electrical stimulation protocol during single leg inactivity.
EXERCISE TESTING PROCEDURES – Participants will be asked to undertake 4 separate exercise tests: lower limb proprioception (balance), vertical jump, quadriceps strength and single leg cycling. These tests will be required to be done three times each during the 10 week period, as well as familiarisation. A maximal aerobic test (VO$_2$ peak) will also be required. Additionally, 4-7 weeks (3 times per week) of lower limb strength training will also be utilised after the lower limb inactivity.

INACTIVITY PROTOCOL- The inactivity protocol used in this study is called “unilateral lower limb suspension” (ULLS). The protocol involves walking with the aid of crutches and a raised-sole shoe at all times through the day. This causes one limb to not bear any weight during daily activity. After familiarisation and baseline exercise testing, you will be asked to do the ULLS protocol for 23 continuous days. Because this protocol increases the risk of deep vein thrombosis (like air travel), you will be supplied with a compression garment, shown passive ankle and knee movements and will come in for regular blood testing, and supplied a daily dose of aspirin (100mg). These measures will both minimise the risk of a blood clot occurring, and provide very early detection if at risk. There are psychological risks with this protocol, including frustration and anxiety from the decreased mobility, and frustration from being unable to undertake exercise. Other physical risks of the inactivity also include increased risk of falling, cramping and decreased mobility.

STRENGTH TRAINING – After the inactivity protocol, participants will be required to attend the exercise rehabilitation clinic located at Victoria University (Footscray Park campus, building L, level 3) for four weeks. There will be 3 sessions per week, lasting approximately 30-45 minutes each. You will be under the supervision of a Victoria University postgraduate student (Exercise rehabilitation) in these session

ELECTRICAL STIMULATION DURING INACTIVITY PROTOCOL- Participants will be assigned to receive either electrical stimulation during single limb inactivity, or no intervention. If selected for the electrical stimulation protocol, participants will attend the exercise physiology laboratory five times per week (overlapping with blood sampling days) for the electrical stimulation training program. Electrical stimulation causes contraction of the muscles on which electrodes are placed over (the quadriceps). The isometric contractions will occur at 60° knee flexion, with the leg fixed. The protocol lasts 16 minutes. Like the magnetic stimulation there may be some mild discomfort with this protocol, but familiarisation with the device will ensure the power used is comfortable for the participant.
MUSCLE BIOPSIES - The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of molecular transporters involved in muscle excitation. During the three experimental trials (the one-legged cycling test), muscle biopsies will be taken from the thigh muscle of participants, at rest, in between bouts of one-legged cycling, and immediately post-exercise. Two to four muscle biopsies will be taken on each visit, giving an overall total of eight biopsies. During the procedure you will feel pressure and this will be quite uncomfortable and you may also experience some pain, but this will last for only about 1-2 seconds. Muscle biopsies are routinely carried out in our laboratory by experienced medical practitioners, with no serious adverse effects.

VENOUS CATHETERISATION & BLOOD SAMPLES VIA VENEPUNCTURE – During the single leg cycling endurance test (3 times throughout the study) blood samples will be taken from the forearm via venous catheterisation. Venous catheterisation is slightly uncomfortable, with minimal possibility of bruising and infection. Additionally, during the inactivity protocol you will be required to attend the exercise physiology lab twice per week for a blood sample via venepuncture from the forearm. Like the venous catheterisation, the blood sample is slightly uncomfortable with a minor risk of bruising and infection.

DXA scans – Dual X-Ray absorptiometry is used in this research. This scan accurately determines lean body and fat mass of the lower limbs. The DXA scans used in this research study involves exposure to a very small amount of radiation. As part of everyday living everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisievert (mSv) each year. The effective dose from this study is about 0.03 mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be minimal.

CERTIFICATION BY SUBJECT

I, _____________________________________________________________(participants name) of _____________________________________________________________(suburb)
certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study:

The effect of single lower limb unloading on exercise performance, fatigue and potassium regulation in healthy young adults being conducted at Victoria University by: Prof. Michael McKenna, Dr. Aaron Petersen, Dr Francois Billaut, Dr. Nigel Stepto, Prof. Graham Lamb, Dr. Robyn Murphy, Dr. Cedric Lamboley, Dr. Itamar Levinger and Mr. Ben Perry
I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

_______________________________ (Investigators Name)

and that I freely consent to participation involving the below mentioned procedures:

Screening.

VO$_{2peak}$ testing

Muscle Biopsies

Exercise testing (one-legged cycling, proprioception, vertical jump and quadriceps strength)

Inactivity protocol (ULLS)

Strength training protocol

Electrical stimulation

Venous catheterisation and blood sampling

Magnetic stimulation

Electromyography

DXA scans

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed:_____________________________________________________________
Date: ___________________________(dd/mm/yyyy)

Any queries about your participation in this project may be directed to the researcher

Ben Perry 9919 4207 or 0414 600 986

Prof. Michael J. Mckenna, 9919 4499 or 0488 475 735,

If you have any queries or complaints about the way you have been treated, you may contact the Secretary, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 phone (03) 9919 4781
APPENDIX F- INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (IPAQ)

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.
Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an International Physical Activity Prevalence Study is in progress. For further information see the IPAQ website.

More Information

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

☐ Yes

☐ No  →  Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.
2. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ days per week

☐ No vigorous job-related physical activity → Skip to question 4

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?

_____ hours per day

_____ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.

_____ days per week

☐ No moderate job-related physical activity → Skip to question 6

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

_____ hours per day

_____ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.
7. How much time did you usually spend on one of those days walking as part of your work?

______ hours per day

______ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

______ days per week

☐ No traveling in a motor vehicle → Skip to question 10

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

______ hours per day

______ minutes per day
Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

    _____ days per week

    ☐ No bicycling from place to place  →  **Skip to question 12**

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

    _____ hours per day

    _____ minutes per day

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

    _____ days per week

    ☐ No walking from place to place  →  **Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY**

13. How much time did you usually spend on one of those days **walking** from place to place?

    _____ hours per day

    _____ minutes per day
PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?

_____ days per week

☐ No vigorous activity in garden or yard → Skip to question 16

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?

_____ days per week

☐ No moderate activity in garden or yard → Skip to question 18

17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?
18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

_____ days per week

[ ] No moderate activity inside home

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

_____ hours per day

_____ minutes per day

**PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY**

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?

_____ days per week

[ ] No walking in leisure time

Skip to question 22
21. How much time did you usually spend on one of those days walking in your leisure time?

_____ hours per day

_____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

_____ days per week

☐ No vigorous activity in leisure time  

Skip to question 24

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?

_____ hours per day

_____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

_____ days per week

☐ No moderate activity in leisure time  

Skip to PART 5: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?

_____ hours per day
PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?

______ hours per day
______ minutes per day

27. During the last 7 days, how much time did you usually spend sitting on a weekend day?

______ hours per day
______ minutes per day

This is the end of the questionnaire, thank you for participating.
APPENDIX G- INTERNATIONAL KNEE DOCUMENTATION COMMITTEE (IKDC)

FORM: SUBJECTIVE KNEE FUNCTION SECTION

2000 IKDC SUBJECTIVE KNEE EVALUATION FORM

Your Full Name__________________________

Today's Date: __/__/______ Date of Injury: __/__/______

SYMPTOMS*:
*Grade symptoms at the highest activity level at which you think you could function without significant symptoms, even if you are not actually performing activities at this level.

1. What is the highest level of activity that you can perform without significant knee pain?
   - Very strenuous activities like jumping or pivoting as in basketball or soccer
   - Strenuous activities like heavy physical work, skiing or tennis
   - Moderate activities like moderate physical work, running or jogging
   - Light activities like walking, housework or yard work
   - Unable to perform any of the above activities due to knee pain

2. During the past 4 weeks, or since your injury, how often have you had pain?

   Never □ □ □ □ □ □ □ □ □ □ □ Constant

3. If you have pain, how severe is it?

   No pain □ □ □ □ □ □ □ □ □ □ □ Worst pain imaginable

4. During the past 4 weeks, or since your injury, how stiff or swollen was your knee?

   - Not at all
   - Mildly
   - Moderately
   - Very
   - Extremely

5. What is the highest level of activity you can perform without significant swelling in your knee?

   - Very strenuous activities like jumping or pivoting as in basketball or soccer
   - Strenuous activities like heavy physical work, skiing or tennis
   - Moderate activities like moderate physical work, running or jogging
   - Light activities like walking, housework, or yard work
   - Unable to perform any of the above activities due to knee swelling

6. During the past 4 weeks, or since your injury, did your knee lock or catch?

   - Yes □ □ No

7. What is the highest level of activity you can perform without significant giving way in your knee?

   - Very strenuous activities like jumping or pivoting as in basketball or soccer
   - Strenuous activities like heavy physical work, skiing or tennis
   - Moderate activities like moderate physical work, running or jogging
   - Light activities like walking, housework or yard work
   - Unable to perform any of the above activities due to giving way of the knee
SPONSORS ACTIVITIES:

8. What is the highest level of activity you can participate in on a regular basis?

☐ Very strenuous activities like jumping or pivoting as in basketball or soccer
☐ Strenuous activities like heavy physical work, skiing or tennis
☐ Moderate activities like moderate physical work, running or jogging
☐ Light activities like walking, housework or yard work
☐ Unable to perform any of the above activities due to injury

9. How does your knee affect your ability to:

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<tr>
<th></th>
<th>Not difficult at all</th>
<th>Minimally difficult</th>
<th>Moderately Difficult</th>
<th>Extremely difficult</th>
<th>Unable to do</th>
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<td>Go up stairs</td>
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<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td>b.</td>
<td>Go down stairs</td>
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<td>□</td>
<td>□</td>
<td>□</td>
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<td>c.</td>
<td>Kneel on the front of your knee</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td>d.</td>
<td>Squat</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td>e.</td>
<td>Sit with your knee bent</td>
<td>□</td>
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<tr>
<td>f.</td>
<td>Rise from a chair</td>
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<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td>g.</td>
<td>Run straight ahead</td>
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<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td>h.</td>
<td>Jump and land on your involved leg</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td>i.</td>
<td>Stop and start quickly</td>
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</table>

FUNCTION:

10. How would you rate the function of your knee on a scale of 0 to 10 with 10 being normal, excellent function and 0 being the inability to perform any of your usual daily activities which may include sports?

FUNCTION PRIOR TO YOUR KNEE INJURY:

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<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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CURRENT FUNCTION OF YOUR KNEE:

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<th>8</th>
<th>9</th>
<th>10</th>
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APPENDIX H- DXA SCAN BONE DENSITY RESULTS (CHAPTERS 4 & 5)

**Table Appendix H:** Descriptive Bone mineral density data at baseline, following 23 d ULLS (post-ULLS) and following 4 weeks resistance training (Post-training).

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<tr>
<th></th>
<th>Thoracic spine</th>
<th>Lumbar spine</th>
<th>Pelvis</th>
<th>Left leg</th>
<th>Right leg</th>
<th>Subtotal</th>
<th>Total</th>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>0.849 ± 0.082</td>
<td>1.095 ± 0.147</td>
<td>1.187 ± 0.127</td>
<td>1.242 ± 0.186</td>
<td>1.234 ± 0.194</td>
<td>1.016 ± 0.134</td>
<td>1.118 ± 0.103</td>
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<tr>
<td><strong>Post-ULLS</strong></td>
<td>0.774 ± 0.110</td>
<td>1.084 ± 0.138</td>
<td>1.181 ± 0.130</td>
<td>1.230 ± 0.156</td>
<td>1.225 ± 0.146</td>
<td>1.007 ± 0.125</td>
<td>1.111 ± 0.088</td>
</tr>
<tr>
<td><strong>Post-Training</strong></td>
<td>0.841 ± 0.124</td>
<td>1.086 ± 0.137</td>
<td>1.180 ± 0.114</td>
<td>1.231 ± 0.151</td>
<td>1.239 ± 0.157</td>
<td>1.014 ± 0.122</td>
<td>1.117 ± 0.088</td>
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</table>

Values are Mean±SD.
Figure Appendix I: VO₂ during different one-legged cycling workrate intensities (percentage of one-legged VO₂ peak) on the unloaded (A) and weight-bearing (B) leg at Baseline, after 23 d ULLS and after 4 weeks resistance training. The 45% and 65% bouts were 4 minutes in duration and the first 85% bout was 1 minute in duration. TTF (Time to fatigue) was completed until participant reached volitional fatigue. Filled bars represent baseline, white bars represent post-inactivity and hatched bars represent post-training. Values are mean ± SD. n = 6 for Baseline and Post ULLS and n = 5 for Post-training.
APPENDIX J- INDIVIDUAL DATA CHAPTER 3

Table Appendix J (i) : Individual participant characteristics from osteoarthritis (OA) and controls (CON); Chapter 3.

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<th>Age (yrs)</th>
<th>BMI (kg.m(^{-2}))</th>
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<td>CON</td>
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<td>73</td>
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Table Appendix J (ii): Individual strength and physical activity data from osteoarthritis (OA) and control participants (CON); Chapter 3.

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Table Appendix J (iii): Individual α and β NKA isoform expression data from Osteoarthritis (OA) and control participants (CON); Chapter 3.

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| SD    | 3.14  | 28.69 | 0.01  | 15.96  | 1.24   | 0.31   |
Table Appendix J (iv): Individual NKA content (³Houabain binding site content) and NKA isoform relative abundance from Osteoarthritis (OA) and control participants (CON); Chapter 3.

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Table Appendix K (i): Individual data for age, BMI and $\text{VO}_2$ peak at baseline; individual data for thigh mass at baseline, after 23 d ULLS and 4 wks resistance training; Chapter 4.

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Table Appendix K (iv)- Individual participant vertical jump height at baseline, after 23 d ULLS and 4 weeks resistance training; Chapter 4.

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Table Appendix K (v)- Individual participant postural sway (APSD) with both eyes open and closed testing, at baseline, after 23 d ULLS and 4 weeks resistance training; Chapter 4.

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APPENDIX L- INDIVIDUAL DATA CHAPTER 5

Table Appendix L (i)- Individual participant time to fatigue (TTF) at baseline, after 23 d ULLS and 4 weeks resistance training; Chapter 5.

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Table Appendix L (ii)- Individual participant exercise venous plasma [K⁺] mmol/L at baseline, after 23 d ULLS and 4 weeks resistance training;

Chapter 5.

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<th>Baseline</th>
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| Post-ULLS   |           |                 |          |          |
| Plasma [K⁺]  |           |                 |          |          |
| Rest         | 0         | 1               | 2        | 3        |
| 3.92         | 4.21      | 4.07            | 4.27     | 4.139    |
| 3.41         | 3.73      | 3.91            | 3.88     | 3.71     |
| 3.56         | 3.94      | 3.99            | 4.09     | 3.96     |
| 4.12         | 4.37      | 4.73            | 4.77     | 4.49     |
| 3.85         | 4.06      | 4.02            | 4.39     | 4.36     |
| 3.63         | 4.12      | 4.12            | 4.6     | 4.27     |
| Mean         | 3.748333  | 4.072           | 4.345    | 4.177    |
| SD           | 0.261489  | 0.221           | 0.339    | 0.308    |

<p>| Post-training |           |                 |          |          |
| Plasma [K⁺]    |           |                 |          |          |
| 4.01          |           |                 |          |          |
| 3.74          | 4.06      | 4.43            | 4.42     | 4.43     |
| 3.53          | 3.96      | 4.15            | 4.04     | 4.05     |
| 4.09          | 4.26      | 4.55            | 4.79     | 4.44     |
| 3.96          | 4.13      | 4.14            | 4.34     | 4.34     |
| 3.64          | 4.15      | 4.24            | 4.48     | 4.48     |
| Mean          | 3.823333  | 4.112           | 4.274    | 4.432    |
| SD            | 0.224091  | 0.111           | 0.167    | 0.243    |</p>
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**Post-ULLS**

|             | SD                 | 0.169    | 0.163    | 0.135    | 0.184    | 0.141    | 0.263    | 0.175    | 0.168    | 0.209    | 0.209    | 0.167    | 0.165    | 0.172    | 0.22     |          | 0.231    | 0.329    | 0.294    | 0.243    | 0.165    | 0.193    |

**Post-Training**

|             | SD                 | 0.081    | 0.19     | 0.286    | 0.231    | 0.192    | 0.127    | 0.127    | 0.211    | 0.199    | 0.324    | 0.345    | 0.282    | 0.515    |          | 0.235    | 0.23     | 0.214    | 0.149    | 0.193    | 0.218    |
Table Appendix L (iii)- Individual participant exercise peak venous plasma [K⁺] mmol/L and ∆[K⁺]/Work nmol. J⁻¹ at baseline, after 23 d ULLS and 4 weeks resistance training;

Chapter 5.

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Table Appendix L (iv)- Individual participant muscle $[^3]$Houabain binding site content at baseline, after 23 d ULLS and 4 weeks resistance training; Chapter 5.

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Table Appendix L (v): Individual participant muscle NKA α isoform relative abundance at baseline, after 23 d ULLS and 4 weeks resistance training; Chapter 5.

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**Table Appendix L (vi)**- Individual participant muscle NKA β isoform relative abundance at baseline, after 23 d ULLS and 4 weeks resistance training; Chapter 5.

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APPENDIX M- INDIVIDUAL DATA CHAPTER 6

Table Appendix M (i)- Individual participant characteristics and physical activity levels in the ACL injured group (ACL) and controls (CON); Chapter 6.

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<tr>
<th></th>
<th>Age yrs</th>
<th>BMI kg.m(^2)</th>
<th>Injury length wks</th>
<th>IKDC score; a.u.</th>
<th>Leisure activity hrs.wk(^{-1})</th>
<th>Total activity hrs.wk(^{-1})</th>
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<td>3.7</td>
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<td>3.8</td>
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<td>2.7</td>
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**Table Appendix M (ii)-** Individual participant muscle strength, normalised strength and CSA in the ACL injured group (ACL) and controls (CON);

Chapter 6.

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<tr>
<th></th>
<th>Thigh CSA</th>
<th>Strength</th>
<th>Strength: BW</th>
<th>Strength : CSA</th>
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<td>cm</td>
<td>Nm</td>
<td>Nm.kg⁻¹</td>
<td>Nm.cm⁻²</td>
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<td>Injured</td>
<td>Non-injured</td>
<td>Injured</td>
<td>Non-injured</td>
</tr>
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<td>99</td>
<td>1.33</td>
</tr>
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<td>120.18</td>
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<td>130</td>
<td>1.76</td>
</tr>
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<td>105</td>
<td>1.77</td>
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<td>76</td>
<td>62</td>
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<td>127.11</td>
<td>102.71</td>
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Table Appendix M (iii)- Individual participant muscle CSA and type I fibre distribution in the ACL injured group (ACL) and controls.

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<td>Type II µm²</td>
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<td>Controls</td>
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<td>4935</td>
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<td>6324</td>
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<td>Combined µm</td>
<td>Type I distribution</td>
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<td>ACL</td>
<td>%</td>
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Table Appendix M (iv)- Individual participant postural sway (APSD) in the ACL injured group (ACL) and controls (CON) in one and two legs;

Chapter 6.

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<th>1 leg APSD</th>
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<th></th>
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<td>Open</td>
<td>Injured</td>
<td>Closed</td>
<td>Injured</td>
</tr>
<tr>
<td></td>
<td>cm</td>
<td></td>
<td>Non-injured</td>
<td></td>
<td>Non-injured</td>
<td></td>
</tr>
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<td>0.962</td>
<td>0.656</td>
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<td>0.641</td>
<td>0.674</td>
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<td>0.968</td>
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<td>0.578</td>
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<td>0.429</td>
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<td>0.650</td>
<td>0.594</td>
<td>1.269</td>
<td>1.116</td>
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<td>0.087</td>
<td>0.166</td>
<td>0.090</td>
<td>0.264</td>
<td>0.191</td>
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</tbody>
</table>

|        | Eyes open  | Closed      | Non-dominant| Dominant | Non-dominant| Dominant |
| CON    | 0.176      | 0.190       | 0.151      | 0.730    | 0.950       | 0.980    |
|        | 0.109      | 0.119       | 0.382      | 0.401    | 0.841       | 0.969    |
|        | 0.220      | 0.245       | 0.474      | 0.478    | 0.854       | 1.112    |
|        | 0.236      | 0.231       | 0.515      | 0.411    | 1.265       | 0.991    |
|        | 0.161      | 0.256       | 0.567      | 0.446    | 2.126       | 1.103    |
|        | 0.280      | 0.270       | 0.820      | 0.650    | 1.210       | 1.060    |
|        | 0.183      | 0.175       | 0.522      | 0.421    | 1.064       | 0.907    |
| Mean   | 0.195      | 0.212       | 0.490      | 0.505    | 1.187       | 1.017    |
| SD     | 0.056      | 0.054       | 0.201      | 0.131    | 0.445       | 0.076    |
Table Appendix M (v)- Individual participant muscle $[^{3}\text{H}]$ouabain binding site content in the ACL injured group (ACL) and controls (CON); Chapter 6.

<table>
<thead>
<tr>
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<th>Controls</th>
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<td>413</td>
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<td>441</td>
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<td></td>
<td>276</td>
<td>252</td>
<td>255</td>
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<td>251</td>
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<td></td>
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Table Appendix M (vi)- Individual participant muscle NKA α isoform relative abundance in the ACL injured group (ACL) and controls (CON);

Chapter 6.

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<th>NKA α₃</th>
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<td>a.u.</td>
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<td>Injured</td>
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Table Appendix M (V) - Individual participant muscle NKA β isoform relative abundance in the ACL injured group (ACL) and controls (CON); Chapter 6.

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<td>Controls</td>
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<td>6.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Mean | 12.4  | 8.1   | 10.9   | 17.7   | 19.6   | 23.3   |         |         |         |         |         |
| SD   | 6.2   | 4.3   | 5.3    | 7.6    | 14.5   | 12.1   |         |         |         |         |         |
APPENDIX N: STRAIN GAUGE AND SETUP USED IN CHAPTERS 3 AND 6

The below is the setup for the strain gauge device and a tall chair as used to assess strength at 90° knee angle.