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The influence of sex, patella tendon properties and the oral contraceptive pill on markers of exercise-induced muscle damage.

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Declaration

I declare that no material within the current thesis has been submitted for any other academic award. Furthermore, I declare the current thesis complies with the Institutional Code of Practice and Research Degree Regulations. Work from the current thesis that has been published or presented elsewhere is attached in its original format at the end of the thesis.

Signed:

Miss Kirsty-Marie Hicks

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Abstract

Introduction: Exercise-induced muscle damage (EIMD) is an accepted consequence of eccentric exercise. Sex differences in EIMD are attributed to tendon properties, fascicle lengthening and direct hormonal influences, however these remain unreported *in vivo*. Furthermore, the classical definition of eccentric contractions omits the role of the elastic tendon in determining eccentric fascicle lengthening and subsequent EIMD.

Aim: The overall aims of the current thesis were, to investigate the role of the patella tendon during eccentric contractions, to investigate whether muscle and tendon properties are determinants of EIMD and investigate group differences (sex and oral contraceptive (OCP)use) in EIMD.

Materials and method: In brief, *vastus lateralis* (VL) and patella tendon properties were measured in males, females and female OCP users, using a combination of ultrasonography, electromyography and dynamometry. During maximal voluntary eccentric knee extensions ((MVE_{KE}) 12 reps × 6 sets), VL fascicle lengthening and MVE_{KE} torque was recorded every 10° of knee joint angle (20 - 90°). Maximal isometric voluntary knee extensor (MVC_{KE}) torque loss, creatine kinase (CK) and muscle soreness were measured *pre, post, 48, 96 and 168* hours *post damage* as markers of EIMD.

Main findings: Patella tendon properties appear to act as a mechanical buffer on VL fascicle lengthening during MVE_{KE} *in vivo*. Furthermore, due to significantly higher patella tendon stiffness, VL fascicle lengthening was significantly greater in males compared to females. Despite evidencing an attenuating role of the patella tendon on VL fascicle lengthening, patella tendon properties did not correlate with any indirect markers of EIMD, nor did they explain group differences in EIMD. Furthermore, MVE_{KE} torque, MVE_{KE} torque made relative

to estimated total quadriceps anatomical cross-sectional area and VL fascicle lengthening did not correlate with any functional indirect marker of EIMD, nor did they explain group differences in EIMD. Within the current thesis CK was the only indirect marker of EIMD to be significantly different between the groups (males > females < OCP users). Creatine kinase was consistently lower in the groups with lower circulating oestrogen levels. Therefore, it was concluded that the antioxidant and membrane stabilising role of oestrogen might explain the group differences in CK reported in the current thesis.

Conclusion: In agreement with the historical definition, VL fascicles lengthen during MVE_{KE}. Furthermore, it is evident from the current thesis that patella tendon properties determine the magnitude of VL fascicle lengthening during MVE_{KE}, but do not appear to explain the variability or group differences in EIMD.

Abbreviations

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BF	Bicep femoris
Ca^{2+}	Calcium
CK	Creatine kinase
E-C	Excitation- Contraction coupling
EIMD	Exercise-induced muscle damage
EMG	Electromyogram
FSH	Follicle stimulating hormone
HRT	Hormone replacement therapy
ICC	Inter class correlation
LH	Luteinising hormone
MRI	Magnetic resonance imaging
MTU	Muscle tendon unit
MVC	Maximal voluntary contraction
MVC_{KE}	Maximal voluntary knee extension
MVC_{KF}	Maximal voluntary knee flexion
MVE_{KE}	Maximal voluntary eccentric knee extension
OCP	Oral contraceptive pill
PT_{CSA}	Patella tendon cross-sectional area
PT_{L}	Patella tendon length
PT_{MA}	Patella tendon moment arm
Q_{ACSA}	Quadriceps anatomical cross-sectional area
SR	Sarcoplasmic reticulum
TNFα	Tumor necrosis factor-α

VAS	Visual analogue scale
VL	Vastus lateralis
VL_{ACSA}	VL anatomical cross-sectional area
ΔCK_{ABS}	Absolute change in CK (peak CK value - pre CK value)

Figure titles

Table titles

Chapter one: Thesis introduction and literature review

Thesis introduction

Exercise-induced muscle damage (EIMD) is an established consequence of maximal voluntary eccentric contractions (MVE_{KE}). Classically, eccentric contractions are defined as a combination of active and passive forces produced during the lengthening of the muscle tendon unit (MTU). It is apparent however, that based on ultrasonography applied *in vivo* during eccentric phases of gait that this classical definition may not fit the measured movement of the *gastrocnemius* muscle fascicles (Fukunaga et al., 2001). As the use of this method is limited during MVE_{KE}, and is the main measurement outcome of the present thesis, there remains a need to describe the reliability of the ultrasound technique during MVE_{KE} contractions (aim one).

Exercise-induced muscle damage occurs from all forms of muscle contractions, however, eccentric exercise has been reported to result in the most severe EIMD (Newham et al., 1983b; Peñailillo et al., 2013). The measurement of EIMD is achieved through direct and indirect methods. For example using muscle biopsies, Newham et al. (1983b) reported that Z-line disruption, a direct marker of EIMD, was significantly higher following eccentric exercise compared to concentric exercise. Due to the painful procedure and small sampling area associated with muscle biopsies, particularly for repeated measures as is required in the study of EIMD, alternative indirect measurements of EIMD are more frequently used (Clarkson & Hubal, 2002). In agreement with direct measure of EIMD, indirect markers of EIMD: isometric torque loss, muscle soreness and intramuscular proteins (specifically, creatine kinase (CK)) are reported to be significantly higher following eccentric exercise compared to concentric exercise (Poprzecki et al., 2004; Clarkson et al., 1986; Willoughby et al., 2003).

The initial cause of EIMD remains inconclusive with the debate centred on the high strain (Lieber & Friden, 1993; Hoffman et al., 2014) or high eccentric forces (Warren et al., 1993a; Chapman et al., 2008; Hody et al., 2013) associated with eccentric contractions.

Regarding high strains, the argument is conflicting due to literature being predominately evidenced by *in vitro* and *in situ* studies (Warren et al., 1993a; Lieber & Friden, 1993), where strain values are exaggerated compared to *in vivo* and based on the estimation of total MTU behaviour rather than direct measurements of the fascicles (Butterfield, 2010). Using ultrasound technique (Fukashiro et al., 1995), studies have been able to investigate fascicle and tendon behaviour independently during contractions (Fukunaga et al., 1997; Reeves & Narici, 2003; Guilhem et al., 2011). The development of the ultrasound technique has discovered that fascicles lengthen independent of the tendon during MTU lengthening (Fukunaga et al., 2001). Furthermore, using a muscle model Lichtwark and Wilson (2007) have reported a complicated interaction between tendon properties and fascicles where by the degree of fascicle lengthening and efficiency (denoted as energy cost / work done) of the movement being performed depends on the tendon stiffness. These findings support the idea of the tendon acting as a mechanical buffer on fascicle lengthening during eccentric contractions (Roberts & Azizi, 2010; Roberts & Konow, 2013; Reeves & Narici, 2003; Hoffman et al., 2014), however these assumptions have yet to be supported experimentally during MVE_{KE} *in vivo* (aim two). The interaction between fascicle lengthening and tendon properties raises questions regarding the role and behaviour of the tendon on fascicle lengthening during MVE_{KE} (aim two).

Although it is commonly accepted that eccentric exercise causes the most severe level of EIMD, large individual variability exists in the EIMD (Marginson et al., 2005; Chen et al., 2011; Wolf et al., 2012). For example, peak CK responses range from to 100 U/L to < 2000 U/L (Saka et al., 2009; Sewright et al., 2008; Hyldahl et al., 2011). In order to understand the large variability in EIMD, determinants of EIMD need to be established. Fascicle strain (isolated rabbit muscle (Lieber & Friden, 1993)), eccentric force (*in vitro* rat muscle (Warren et al., 1993a)), the architectural properties of the muscle (*in vivo* human upper and lower limb (Chen et al., 2011)), and circulating oestrogen levels (*in vivo* quadriceps (Carter et al., 2001)) have all been suggested to act as determinants of EIMD. These variables however

have yet to be fully investigated *in vivo*, as determinants of muscle damage in the knee extensors (aim three). Furthermore, with the new insight on the interaction between tendon properties and fascicle behaviour during eccentric contractions (Lichtwark & Wilson, 2007; Spanjaard et al., 2007; Fukunaga et al., 2001), the tendon has been suggested to explain group differences in EIMD (Marginson et al., 2005; McHugh et al., 1999); however, the role of the tendon on EIMD has yet to be evidenced experimentally (aim three).

Large variability in EIMD has been evidenced between groups, specifically males and females (Joyce et al., 2014; Wolf et al., 2012). Within animal studies, males are reported to have significantly higher levels of EIMD compared to females (Clarkson & Hubal, 2002; Komulainen et al., 1999), however within humans a sex difference remains unconfirmed (Stupka et al., 2000; Sewright et al., 2008). Historically, the lower EIMD in females has been attributed to the antioxidant role of the female sex hormone oestrogen (Tiidus, 2005; Kendall & Eston, 2002). Alternatively, oestrogen has been reported to have an effect on tendon properties (Hansen et al., 2009b), specifically tendon stiffness (Kubo et al., 2003). Kubo et al. (2003) attributed significantly higher tendon stiffness in males to higher circulating levels of oestrogen in females. With insights on the interaction between tendon properties and fascicle behaviour during eccentric contractions (Lichtwark & Wilson, 2007), it remains in question whether the sex differences in EIMD could be attributed to higher tendon stiffness in males compared to females (aim four).

As mentioned previously the antioxidant role of oestrogen has been used to explain sex differences in EIMD (Tiidus, 2005; Kendall & Eston, 2002), however to directly measure the role of oestrogen on EIMD, hysterectomies (in animals) and synthetic hormones (e.g. the oral contraceptive pill (OCP)) have been used to investigate the role of oestrogen on EIMD (Thompson et al., 1997; Savage & Clarkson, 2002; Joyce et al., 2014). The OCP alters the hypothalamic-pituitary-ovarian feedback loop, thus inhibiting the peak in oestrogen levels during the ovulatory phase (Elliott-Sale et al., 2013; Van Heusden & Fauser, 2002; Nudemberg et al., 1973). Thus, women who take the OCP have significantly lower levels of

oestrogen throughout their menstrual cycle compared to non-users (Bryant et al., 2008). Despite maximising the difference in oestrogen levels through the use of the OCP, the role of oestrogen on EIMD remains inconclusive (Joyce et al., 2014; Savage & Clarkson, 2002). Interestingly, Bryant et al. (2008) reported, that lower levels of oestrogen in OCP users resulted in a decrease in tendon strain compared to non-users. Therefore it remains in question whether, due to the antioxidant role of oestrogen or due to a stiffening of the tendon and therefore a loss of the hypothesised "buffering" role of the tendon, OCP users are more susceptible to EIMD during eccentric contractions (aim five).

Literature review

1.1. Muscle contractions

Eccentric and concentric contractions are used during daily activities and sporting movements, however their contractile behaviour differs (Katz, 1939; Reeves & Narici, 2003). For example, by classical definition during an eccentric contraction the external load is larger than the force production of the muscle, therefore the muscle lengthens under contraction (Katz, 1939). Whereas during a concentric contraction, the external load is lower than the force production of the muscle, thus the muscle shortens under contraction (Katz, 1939). Although the force production during concentric and isometric contractions can be explained by the sliding filament theory (Huxley, 1957), it is unable to fully explain force production during eccentric contractions (Huxley, 1980; Herzog, 2014). Recently, to try and explain the fundamental contractile properties of eccentric contractions unsolved by the sliding filament theory, Herzog (2014) proposed that the sliding filament theory is a three, rather than a two filament model, with the addition of titin to actin and myosin filaments. It is proposed that titin may play a role during eccentric contractions by altering its stiffness in an activation (calcium) dependent manner (Herzog, 2014). However the proposed modification of the sliding filament theory is in its infancy, and needs to be investigated further to fully describe the contractile properties of eccentric contractions (Herzog, 2014).

Eccentric contractions differ to concentric contractions for a couple of reasons. Firstly, eccentric contractions produce significantly higher forces compared to concentric contractions. The higher forces produced during eccentric contractions can be evidenced using the force-velocity relationship. Initially the force-velocity relationship was proposed by Fenn and Marsh (1935) however, the relationship has evolved over time (Hill, 1938; Edman et al., 1997). The force-velocity relationship is now accepted as two distinct curvatures (Figure 1. 1.) (Edman et al., 1997). *In vivo* the force-velocity relationship is largely calculated using torque-angular speed, however, the in-series compliance and fascicle mechanics

perplex the direct comparison between torque-angular speed and the force-velocity relationship at the fascicles (De Brito Fontana et al., 2014). Secondly, energy requirements are considerably lower during eccentric compared to concentric contractions (Abbott et al., 1952; Peñailillo et al., 2013). Lower energy costs during eccentric contractions have been attributed to several mechanisms, including the contribution of titin (Herzog, 2014), the dissipation of energy rather than the generation of energy (Ryschon et al., 1997), and / or weakly bound myosin-actin states breaking rather than requiring ATP hydrolysis to detach the myosin heads as reported with concentric contractions (Huxley, 1957; Ryschon et al., 1997). The latter may be associated with the greater levels of EIMD reported following eccentric contractions compared to concentric contractions, however there is no systematic evidence to support this hypothesis (Ryschon et al., 1997; Huxley, 1957). Therefore, although the mechanisms remain unclear, it is accepted that eccentric contractions produce significantly higher levels of force at a lower metabolic cost compared to concentric contractions (Peñailillo et al., 2013; Abbott et al., 1952).

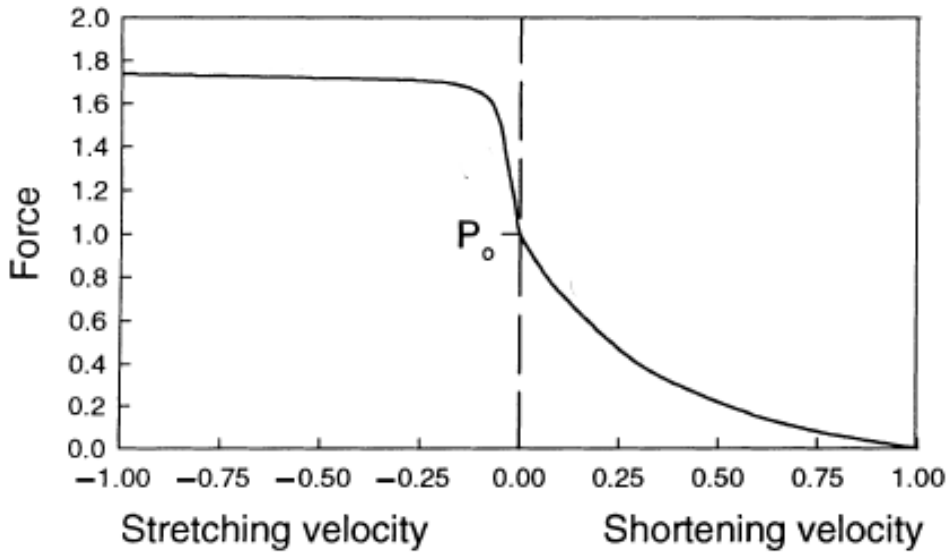

Figure 1. 1. Force-velocity relationship of a skeletal muscle. P_o represented 100% maximal voluntary isometric torque (Allen, 2001).

A consequence of eccentric exercise is significantly higher levels of EIMD compared to concentric exercise (Peñailillo et al., 2013; Willoughby et al., 2003). For example, Peñailillo et al. (2013) found that decreases in isometric torque, counter movement jump and squat jump height and increase in muscle soreness were significantly higher following eccentric compared to concentric cycling bouts. The greater levels of EIMD associated with eccentric contractions have been attributed to several mechanisms however, they predominately focus on two contractile properties of eccentric contractions 1) high eccentric forces and 2) high levels of strain (Peñailillo et al., 2013; Lieber & Friden, 1993; Warren et al., 1993a; Hody et al., 2013; Chapman et al., 2008). Although determinants of EIMD have been proposed (see below), it still remains to be established whether one of these mechanisms per se or a combination of the two can be used to explain eccentric EIMD in vivo.

1.2. Model of exercise-induced muscle damage

Armstrong (1990) proposed a four-stage model to describe the pathology of EIMD: 1) initial stage, 2) loss of calcium (Ca^{2+}) homeostasis / autogenetic phase, 3) phagocytic stage and 4) regenerative stage (Armstrong, 1990; Armstrong et al., 1991). The first three stages of Armstrong (1990) model will be covered below in regards to the mechanisms involved, however the regenerative phase (fourth stage) will not be discussed.

1.2.1. Stage one: Initial stage

Following eccentric exercise the initial stage of EIMD remains controversial. The debate regarding the initial cause of EIMD focuses on two mechanical factors, either structural damage to the muscle sarcomeres or damage to the excitation-contraction coupling (E-C) process (Proske & Morgan, 2001; Armstrong et al., 1991).

1.2.1.1. Initial stage: The popping sarcomere theory

The mechanical theory of EIMD has stemmed from the lengthening characteristics of eccentric contractions, and can be applied to the length-tension relationship described by Gordon et al. (1966). The length-tension relationship reports that peak tension occurs between sarcomere lengths of 2.00 μm and 2.20 μm (Morgan, 1990; Gordon et al., 1966). If sarcomeres exceed 2.20 μm then they are stretched onto the descending limb of the length-tension relationship until there is no actin-myosin interaction (Figure 1. 2, (Morgan, 1990; Gordon et al., 1966)). The popping sarcomere theory, proposed by Morgan (1990), suggests that EIMD occurs when sarcomeres are extended onto the descending limb of the length-tension relationship. The popping sarcomere theory is based on the concept of 'systematic sarcomere lengthening' from the weakest to the strongest sarcomeres during active lengthening. Initially, the weakest sarcomeres lengthen to accommodate the increase in myofibril length during eccentric contractions. Consequently, the weakest sarcomeres surpass their yield point on the descending limb of the length-tension relationship and lengthen beyond the point of no myofilament overlap. During subsequent contractions, a number of sarcomeres fail to re-interdigitate, placing neighbouring sarcomeres under greater lengthening tension. Consequently, the next weakest sarcomeres surpass their yielding point, increasing the area of disrupted sarcomeres (Morgan, 1990). Morgan (1990) suggested that this process continues during subsequent active stretch, resulting in large areas of muscle damage.

Although the principle of the popping sarcomere theory is appealing, its integrity has recently been questioned (Telley et al., 2006; Panchangam & Herzog, 2011). For example, following the attainment of maximal isometric force in rabbit psoas myofibrils, Telley et al. (2006) passively stretched the myofibrils 15-20% beyond resting length. Despite reporting non-uniform lengthening of sarcomeres, Telley et al. (2006) reported no evidence of sarcomeres lengthening beyond the point where no myofilament overlap occurred,

subsequently rejecting the popping sarcomere theory. The severity of the damage protocol used by Telley et al. (2006) however, is questioned due to the use of a activation solution (pCa 4.5) and uncertainty as to whether sarcomeres extended onto the descending limb of the length-tension relationship at all, which is necessary for investigating the popping sarcomere theory (Morgan & Proske, 2006). The aforementioned limitations may explain why Telley et al. (2006) did not see sarcomeres stretched to the point of no myofilament overlap (Morgan & Proske, 2006).

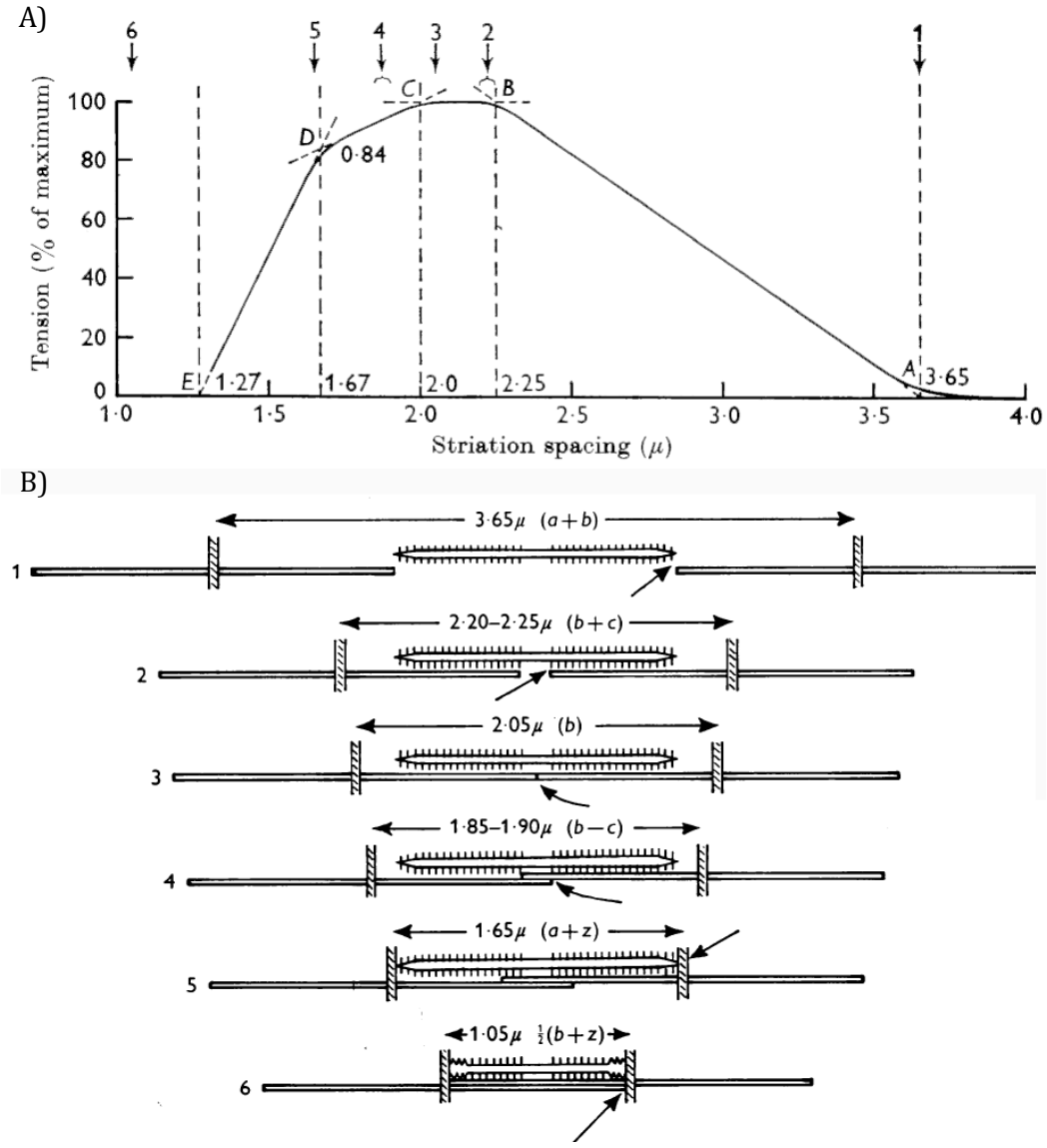

Figure 1. 2: A) Schematic diagram of the length-tension relationship. B) Schematic diagram of myosin and actin overlap at different sarcomere lengths. The numbers in graph 2A) and 2B)

correspond to demonstrate the myosin and actin overlap at each stage of the length-tension relationship (Gordon et al., 1966).

More recently in agreement with Morgan (1990), during active lengthening of isolated rabbit *psoas* myofibrils, Panchangam and Herzog (2011) reported that several *psoas* myofibrils were unable to re-interdigitate following active lengthening (sarcomere length > 4.0 μm). However, Panchangam and Herzog (2011) still reported torque loss in myofibrils where no overstretched sarcomeres were reported, thus Panchangam and Herzog (2011) concluded that structural damage to myofibrils and the popping sarcomere theory *per se* do not fully explain EIMD.

1.2.1.2. Is the popping sarcomere applicable in vivo?

The popping sarcomere theory described as the initial stage of EIMD is predominately evidenced *in situ* and *in vitro*, however due to different muscle preparations, activation techniques and the magnitudes of fascicle lengthening used, the application to *in vivo* remains unclear. It is suggested that *in vivo* the tendon alters the degree of fascicle lengthening, resulting in the muscle fibres to contract predominately on and around the plateau region (optimal region) of the length-tension relationship (Austin et al., 2010; Hoffman et al., 2014; Butterfield & Herzog, 2005). For example recently, Hoffman et al. (2014) investigated *gastrocnemius* fascicle lengthening, the length-tension relationship and EIMD during *in vivo* backwards walking. Hoffman et al. (2014) reported that an 18% increase in *gastrocnemius* fascicle length resulted in several fascicles extending onto the descending limb of the length-tension relationship but predominantly the fascicles contracted onto the ascending and plateau region. Therefore, the conclusion that the magnitude of *gastrocnemius* fascicle lengthening and maximal isometric voluntary contraction (MVC) torque loss were not significantly associated, and that a loss in MVC torque was reported despite fascicles predominantly contracting on the plateau region is inconsistent with the popping sarcomere

theory. From the findings of Hoffman et al. (2014) study however, it is impossible to discount whether weaker individual sarcomeres have lengthened onto the descending limb of the length-tension relationship, as result of heterozygous lengthening (Palmer et al., 2011), which would support the mechanism reported by the popping sarcomere theory.

Although the mechanics of the popping sarcomere theory are plausible *in vivo*, potentially due to the role of the tendon, it appears that the magnitude of fascicle lengthening investigated *in vivo* is considerably smaller than those used *in vitro* and *in situ* (e.g. 125% *in situ* (Lieber & Friden, 1993) and 18% *in vivo* gait (Hoffman et al., 2014)) (Butterfield, 2010). Therefore although *in vitro* and *in situ* studies are necessary to understand the popping sarcomere theory and strain induced muscle damage, it may be difficult to directly extrapolate and apply these findings *in vivo*.

The popping sarcomere theory is the preferable mechanism for the initial phase of EIMD but due to the complex role of tendon and fascicle lengthening during eccentric EIMD the data *in vivo* is not over whelming (Hoffman et al., 2014; Butterfield, 2010).

1.2.1.3. Initial stage: Excitation-Contraction coupling

Alternative to the popping sarcomere theory, the initial stage of EIMD has been attributed to failure of the E-C process (Ingalls et al., 1998; Warren et al., 1993b). The E-C process refers to an electric stimulus, typically an action potential that results in a mechanical response (see Costantin (1976) for a review). In brief the E-C process encompasses the following sequence. An action potential travels down to the neuromuscular junction, where acetylcholine is released into the synaptic cleft. The release of acetylcholine into the synaptic cleft alters the electrical impulse to a chemical stimulus, causing the opening of ion channels in the fibre membrane which depolarises and spreads the impulse to the transverse or T-tubules. Dihydropyridine receptors detect the depolarisation and trigger the opening of ryanodine receptors, resulting in an efflux of Ca^{2+} from the sarcoplasmic reticulum (SR) into the cytoplasmic concentration. Calcium finally interacts with troponin-C to initiate the muscle

contraction. Although damage to the E-C process could occur at any stage of the aforementioned sequence, it has predominately been attributed to a loss of sarcolemma integrity, shearing of the SR and / or t-tubles.

To investigate whether damage occurs as a result of disruption to the E-C process, exogenous exposure to caffeine has been used (Warren et al., 1993b). Caffeine facilitates activation of fibres by bypassing E-C process and triggers Ca^{2+} release from the SR (Lüttgau & Oetliker, 1968). For example, in isolated mouse soleus muscle, Warren et al. (1993b) reported large differences in force loss following 20 eccentric contractions compared to 20 isometric contractions (43% vs. 4%, respectively). When the muscle fibres were exposed to 50 mM of caffeine post the 20 eccentric contractions, peak isometric tetanic force post damage was significantly increased by 118% (Warren et al., 1993b). The 118% restoration of force loss through exposure to exogenous caffeine suggests that EIMD occurs during the E-C process and not to structural properties of the muscle (Warren et al., 1993b). Following a review of the literature, Warren et al. (2001) suggested that ≥ 75% of tension lost during the first 32 hours post EIMD, can be ascribed to failure of the E-C process. Although these findings suggest the majority of damage is initiated through failure at the E-C process, it also insinuates that 25% of damage must be attributed to other mechanisms. Nevertheless, if failure during the E-C process is the primary event of EIMD it fails to explain, 1) why damage is prevalent on the descending limb of the length-tension relationship and 2) why a rightward shift in the length-tension relationship is found following EIMD (Proske & Morgan, 2001; Katz, 1939). Whereas the mechanisms associated with the popping sarcomere provides evidence to answer these question.

It remains unexplained why eccentric damage would 'target' the E-C process first, then cause structural damage (Proske & Morgan, 2001). Through the strain and high force associated with eccentric contractions, it is plausible that damage initially occurs through the popping sarcomere theory (Proske & Morgan, 2001; Morgan, 1990), with damage to the E-C

process occurring almost immediately after or even concurrently to structural damage (Allen, 2001).

1.2.2. Stage two: Loss of Ca^{2+} homeostasis / autogenetic phase

1.2.2.1. Loss of Ca^{2+} homeostasis

Although it remains unclear whether EIMD is a resultant of initial damage to the structural integrity, E-C process or both, in the time frame of events post EIMD a subsequent loss of Ca^{2+} homeostasis is reported (Armstrong et al., 1991).

As discussed previously, during the final stages of the E-C process Ca^{2+} is released out of the sarcoplasmic reticulum (Berchtold et al., 2000), increasing Cystolic Ca^{2+} levels 100 times higher than resting Ca^{2+} levels (50 nM). Through Ca^{2+} channels and structural changes of the dihydropyridine receptor, Ca^{2+} is released into the muscle where Ca^{2+} binds to troponin on the thin filament to initiate a contraction (Catterall, 1991). Thus, Cytosolic free Ca^{2+} concentration is essential to muscle function as it exerts control over the initiation, time course and force of all muscle contractions (Catterall, 1991).

At rest, to maintain Ca^{2+} within the physiological range, ATPase pumps transport Ca^{2+} across the cell membrane (Gissel, 2006; Berchtold et al., 2000). However, due to the large Ca^{2+} gradient differences between extracellular (1 mM Ca^{2+} (Gissel, 2006)) and intracellular Ca^{2+} levels (50 nM (Berchtold et al., 2000)), if the sarcolemma is damaged during EIMD, there is a rapid influx of Ca^{2+}. Following eccentric contractions Ca^{2+} can enter the intracellular membrane through mechanical tears and / or through the opening of stretch-activated channels, resulting in a disruption to Ca^{2+} homeostasis (Zhang et al., 2012; Guharay & Sachs, 1984). A loss of Ca^{2+} homeostasis occurs when Ca^{2+} levels exceed the physiological resting values (~50 nM) and the muscle's transport systems are unable to buffer the Ca^{2+} influx (Berchtold et al., 2000; Armstrong, 1990). Once Ca^{2+} homeostasis is lost for a sufficient period of time, or elevated in specific components of the fibres, various Ca^{2+} activated degradative

mechanisms are activated, e.g. calpain, which initiates the start of the autogenetic phase (Gissel, 2006; Morgan, 1990).

1.2.2.2. Autogenetic phase

Once Ca^{2+} homeostasis is lost, the remaining sequences associated with the damaging process is irreversible (Armstrong et al., 1991; Verburg et al., 2005). The loss of Ca^{2+} homeostasis activates proteases and protolytic enzymes (Armstrong, 1990). For example, the influx Ca^{2+} post EIMD, activates the Ca^{2+} dependent protease, calpain, which has been suggested to explain the uncoupling of the E-C process post EIMD (Verburg et al., 2005).

There are three types of calpain, μ-calpain, m-calpain and calpain-3 (Goll et al., 2003). The isoform of calpain that is activated however is dependent on the severity of the change in Ca^{2+} homeostasis, with low levels of Ca^{2+} (μM) activating μ-calpain, whereas m-calpain is activated with higher levels of Ca^{2+} (mM). Calpain-3 is slightly different as it is sensitive to Ca^{2+} at submicromolar levels and is specific to the skeletal muscle (Belcastro et al., 1998; Allen et al., 2005). When Ca^{2+} homeostasis is lost, calpain mediates the proteolysis of titin (Huff-Lonergan et al., 1996; Lim et al., 2004). Titin, a large elastic protein which anchors the M line to the Z line, provides structural integrity of the myofibril (Verburg et al., 2005) and transmits force to the sarcolemma (Zhang et al., 2008). The role of calpain on EIMD has been evidenced through the use of calpain inhibitors such as leuptin (Verburg et al., 2005). Following exposure to 40 μM of Ca^{2+} solution Verburg et al. (2005) reported that passive force in toad fibres was reduced by 69% however, when exposed to 1 mM of leuptin force was only reduced by 15%. Furthermore, Zhang et al. (2008) concluded, although they reported force loss to be significantly reduced when exposed to 100 μM leuptin (44% to 31% torque loss), following exposure to leuptin however there was still a decrease in force reported. Although the findings of Zhang et al. (2008) and Verburg et al. (2005) suggest that EIMD is caused by loss of Ca^{2+} homeostasis, the influx of Ca^{2+} and the activation of calpain, it is apparent that it does not fully explain the force loss post EIMD. Although the loss of Ca^{2+}

homeostasis has been shown to activate Ca^{2+} dependent protease, which result in the proteolysis of structural proteins like titin, the proteolysis of such proteins has also been reported through eccentric strain (Verburg et al., 2005). For example, Verburg et al. (2005) reported that titin was most susceptible to proteolysis when toad fibres were stretched to twice the length of concentric contractions, suggesting mechanical strain also plays a role in proteolysis of structural proteins.

1.2.3. Stage three: Phagocytic phase

The phagocytic stage, also known as the inflammatory response, starts within one hour post EIMD (Figure 1. 3, (MacIntyre et al., 2001)). Neutrophils, macrophages and T-lymphocytes migrate through the circulation to the injured site via chemotaxis. Neutrophils are the first to infiltrate the cell and they contribute to the phagocytic phase in two ways, 1) they have a phagocytic function which degrades cellular debris and activates proteolysis and 2) neutrophils magnify the damage response through the releases of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor-α (TNFα). The release of pro-inflammatory cytokines may cause further damage to surrounding tissue (Tidball, 1995; MacIntyre et al., 2001), for example, when neutrophil infiltration was blocked the magnitude of muscle damage (denoted by cell membrane permeability) was reduced by 38% (Kyriakides et al., 1999).

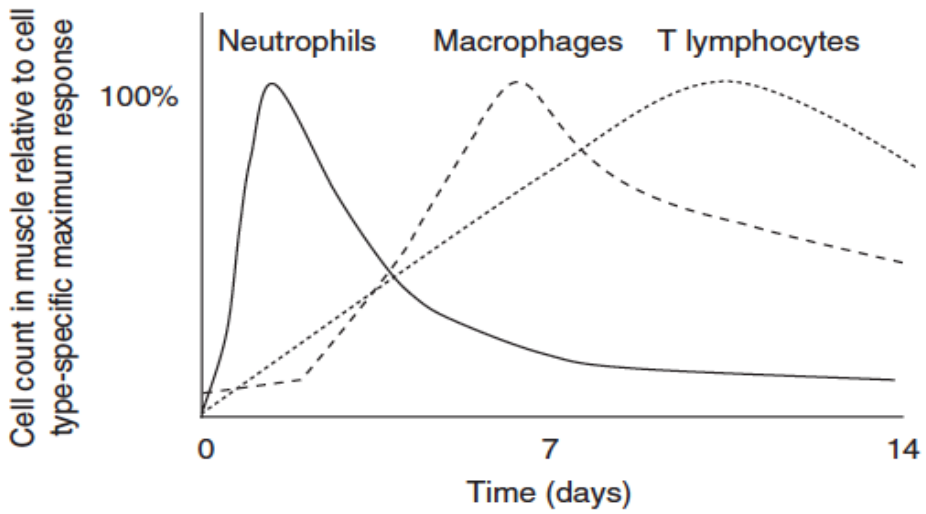

Figure 1. 3: The average time scale of peripheral immune cells following muscle damage. The extent of the immune response depends on the severity of the muscle damage (Smith et al., 2008).

In addition to the invasion of neutrophils, the invasions of phagocytic macrophages also play a major role in the breakdown of cellular debris (Smith et al., 2008). Unlike neutrophils, macrophages have different subtypes which invade the damaged area at different times, thus suggesting multiple functions of macrophages (Smith et al., 2008). In addition to the phagocytic role, macrophages have been suggested to promote muscle regeneration, specifically through the chemotaxis of satellite cells (Chazaud et al., 2003). It is clear that the inflammatory response is an almost immediate process which is essential to the damage process, however the primary role of neutrophils and macrophages remains inexact with a discourse between further injury or repair (Tidball, 2005). Although Armstrong (1990) describes the model of EIMD as a sequence of events, it is more than likely that the four stages over lap. It is essential to understand the fundamental mechanisms of EIMD to comprehend how they manifest through markers of EIMD. The current thesis has discussed a narrow explanation of the model of EIMD therefore caution must be taken when interpreting the basic schematic summary of EIMD presented in Figure 1. 4.

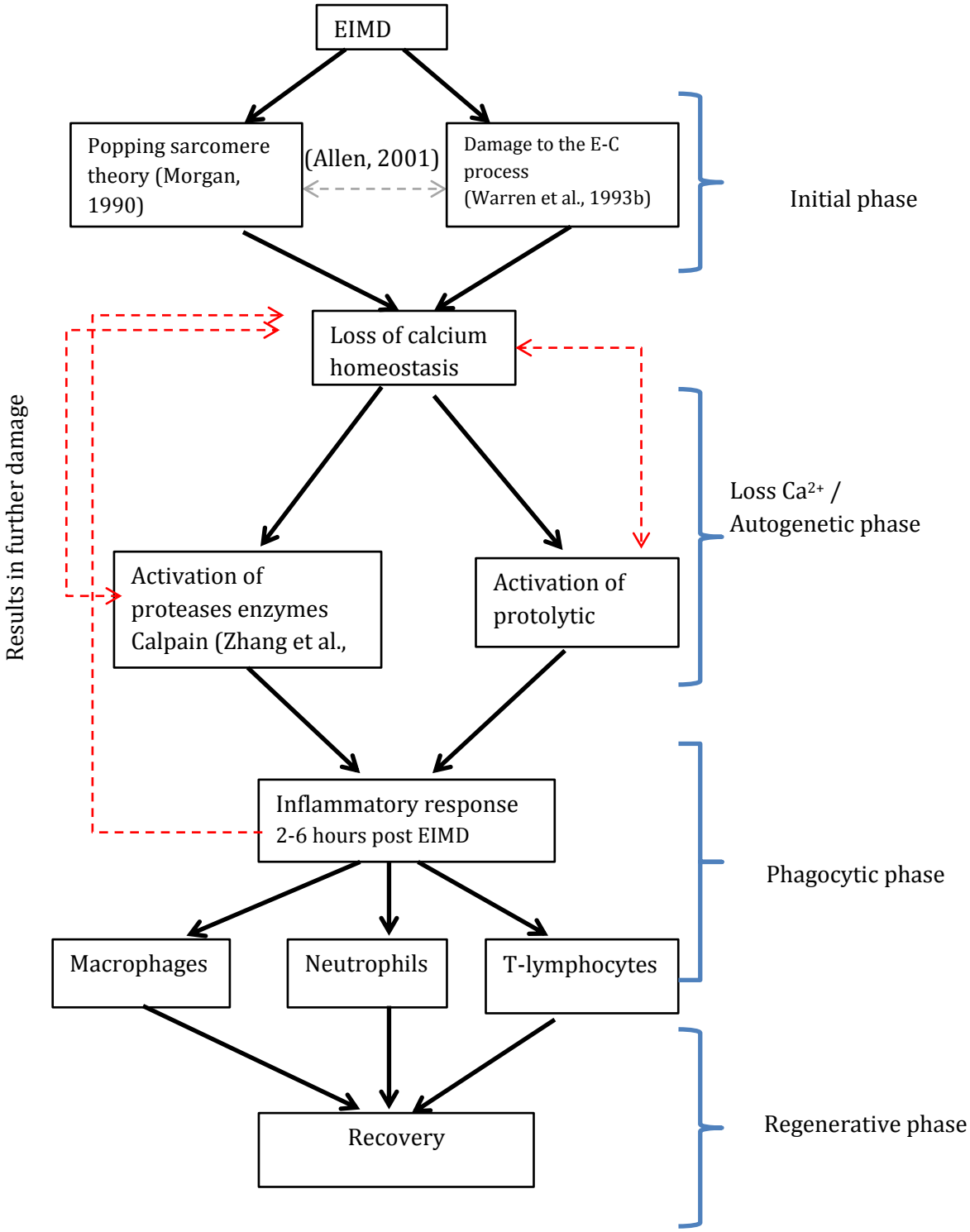

Figure 1. 4: Schematic diagram of the four stage model of exercise-induced muscle damage (EIMD) proposed by Armstrong (1990). Red lines indicate stages that may result in further damage.

1.3. Markers of exercise-induced muscle damage

Although the fundamental process of EIMD is predominately reported at subcellular levels, the magnitude of EIMD can manifest itself through direct and indirect markers of EIMD (Warren et al., 1999). The severity of EIMD is evidenced directly by using muscle biopsies or indirectly through muscle swelling, muscle proteins in the blood (e.g. CK), reduction in MVC isometric torque production and muscle soreness (Warren et al., 1999). Indirect methods are commonly used in the literature due to direct methods being invasive, only providing a small sample and requiring a technique which may exacerbate the amount of EIMD (Roth et al., 2000; Staron et al., 1992). Each marker of EIMD has a unique time frame of appearance and clearance following EIMD; therefore in order to understand the determinants of EIMD it is essential to understand the temporal characteristics of these measurable markers.

1.3.1. Structural damage: Muscle biopsies

To investigate the structural damage following EIMD, studies have used histological and ultrastructure analysis of muscle biopsies (typical muscle sample size 10-50 mg) (Friden et al., 1981). Within skeletal muscle, sarcomeres are enclosed by two Z-lines at the end of each sarcomere, which are organised in a square lattice (an example of square lattice can be seen in Figure 1. 6. However, following EIMD, broadening, smearing and total disruption of the Z-lines has been reported (Figure 1. 6 (Takekura et al., 2001; Friden & Ekblom, 1983)). For example, Friden and Ekblom (1983) was one of the first groups to investigate muscle biopsies post EIMD in humans. Muscle damage was evidenced through streaming, broadening and disruption of the Z-lines, 1, 72 and 144 hours post backwards cycling (Friden & Ekblom, 1983). Initially, Z-line disruption was reported on every third muscle fibre, then between 72 - 144 hours, Z-line disruption was reduced to every tenth fibre (Friden & Ekblom, 1983). Thus structural damage is accepted to peak immediately post EIMD and slowly regress to baseline (Takekura et al., 2001; Friden & Ekblom, 1983). Although it is evident that structural damage

does occur post EIMD, it remains unclear whether it is a result of damage to the E-C process (e.g. through an activation of calpain) or due to the mechanical strain placed on the fibres (Zhang et al., 2008).

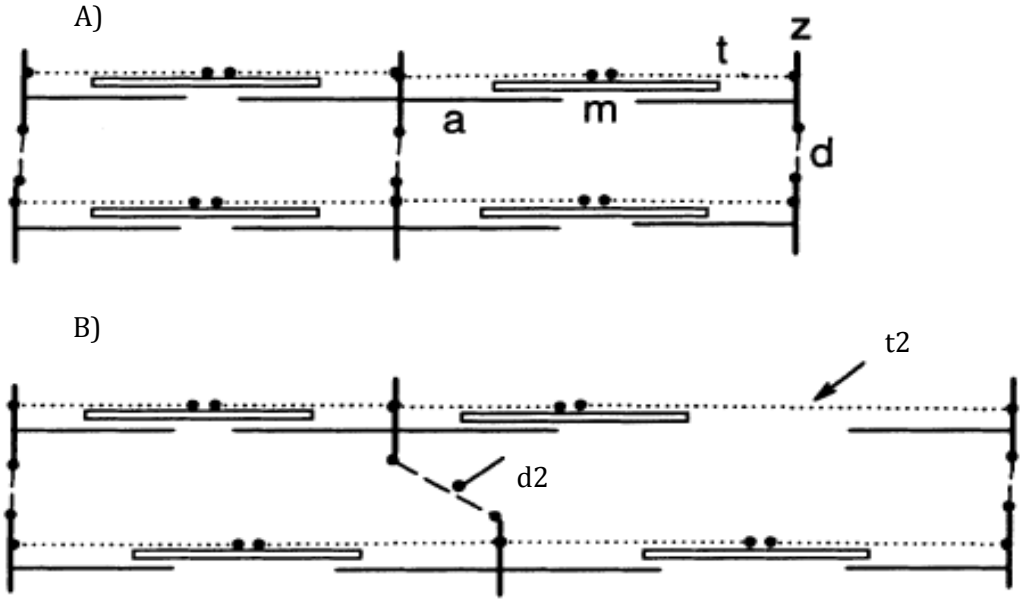

Figure 1. 5: Schematic diagram of sarcomeres during A) resting and B) under contraction. a: thin filaments, m: thick filaments, z: Z-lines, d: desmin and t: titin. The dotted line resemble titin anchoring the Z-line to the thick filament, t2 represents titin lengthening during contraction. The dashed lines represent desmin linking adjacent Z-lines, d2 represents desmin under strain during contraction (adapted from Allen (2001)).

Z-line streaming has been associated with the loss of two cytoskeletal proteins, desmin and titin (Barash et al., 2002; Zhang et al., 2008; Lieber et al., 1996). Desmin and titin are involved in the transverse and longitudinal filament structure, respectively (Luther, 2009; Horowits & Podolsky, 1987; Tskhovrebova & Trinick, 2002; Paulin & Li, 2004). Firstly, desmin anchors each Z-line to adjacent Z-lines and costameres found on the surface membrane, therefore any disruption to desmin can be expected to be evidenced at the Z-line (Magaudda et al., 2004).

For example, Lieber et al. (1996) reported ~23% loss in desmin staining 24 hours post eccentric exercise of the extensor digitorum longus. In an earlier study Lieber et al. (1994) reported a loss in desmin staining 24 hours post EIMD in rabbit extensor digitorum longus, before any increase in extracellular matrix protein fibronectin was reported. Therefore, a loss in desmin staining despite no change in fibronectin indicates that fascicle strain and not cellular disruption is responsible for the initial loss of desmin during eccentric EIMD (Lieber et al., 1994). Secondly, titin anchors the Z-line to the myosin filaments, which provides stability and plays an essential role in re-interdigitating sarcomeres following EIMD (Horowits & Podolsky, 1987; Tskhovrebova & Trinick, 2002). Following EIMD, a loss of irregular labelling of titin has been associated with Z-line disruption in muscle biopsies (Trappe et al., 2002). For example, following eccentric exercise in the lower limb Trappe et al. (2002) reported a 30% reduction in titin 24 hours post EIMD. The association between a loss of titin and EIMD could be explained by the popping sarcomere theory (Morgan, 1990). Whereby damage to titin during fascicle lengthening prevents sarcomeres from regaining normal shape, thus increasing the strain on surrounding sarcomeres (Figure 1. 5 (Trappe et al., 2002)). Furthermore, damage to titin, may reduce muscle stability and increases fibre compliance (Herzog, 2014), which may also explain the rightward shift in the length-tension relationship following EIMD (Allen, 2001).

It is clear that the use of muscle biopsies to measure structural damage and cytoskeletal protein response is the gold standard for measuring EIMD (Warren et al., 1999). The uncomfortable procedure and the potential of accentuating muscle damage through the sampling method has resulted in alternative markers of EIMD being used (Warren et al., 1999).

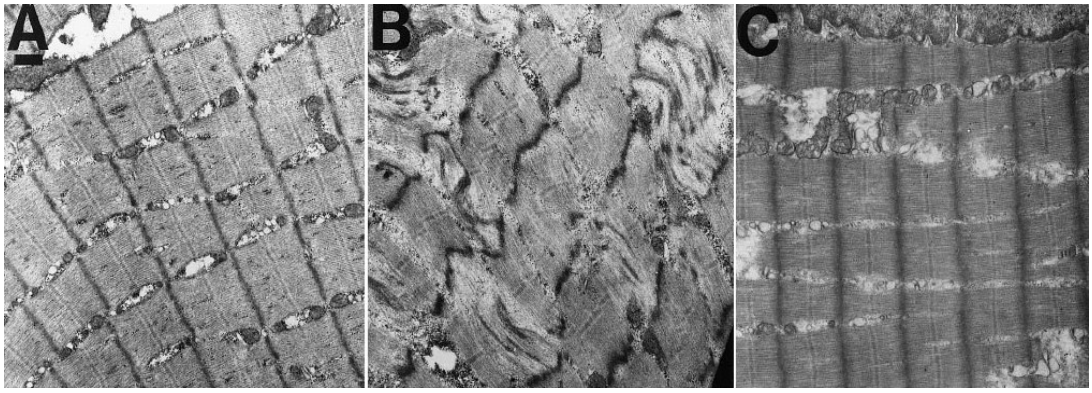

Figure 1. 6: Longitudinal muscle biopsies of the human *vastus lateralis* (VL) before A) 48 hours B) and 168 hours C) post exercise-induced muscle damage (EIMD) (Hortobágyi et al., 1998).

1.3.2. Loss of torque

Post EIMD loss of isometric torque or isometric force is valued as one of the most reliable *indirect* markers of EIMD (Warren et al., 1999). Although conceptually different, the calculation of force reflects the inclusion of an external moment arm in the position of the force transducer during measurement of MVCs, whereas torque does not. Within the context of this literature review the functional marker of strength measured during maximal isometric contractions will be referred to as MVC torque and MVC force loss interchangeably. To ensure the measurement of MVC torque loss is reliable and valid however, several points need to be taken into consideration. Firstly, although, torque is directly proportional to force it is essential for measurements of torque to be performed at the same joint angle for a valid comparison between and within participants to be made (Byrne et al., 2001; Warren et al., 1999). Secondly, torque is velocity dependent therefore to control for the confounding factors of shortening and lengthening velocities, isometric contractions should be used to measure MVC torque loss post EIMD.

Maximal voluntary torque loss is reported immediately post EIMD with a gradual return to *pre-damage* within 7-14 days (Brown et al., 1997; Clarkson et al., 2005; Byrne et al.,

2001); however, in extreme cases, MVC torque loss has taken 47 days to fully recover (Sayers & Clarkson, 2001). The extended duration of MVC torque loss (> 1 hour) post EIMD enables torque loss to be attributed to muscle damage and not fatigue (Walsh et al., 2004). Post EIMD studies have reported peak MVC torque loss to range between 10-70% of *pre-damage* values, however the severity of torque loss depends on the type and intensity of the eccentric exercise (Brown et al., 1997). For example, downhill running results in MVC torque losses ranging from 10-30% (Eston et al., 1996), whereas isolated eccentric extensions of the elbow flexors report torque losses > 60% (Sayers & Clarkson, 2001).

In addition to torque loss post EIMD being a result of damage to the aforementioned contractile elements of the muscle (peripheral damage), MVC torque loss may be attributed to the reduction in neural drive from the brain to the contracting muscle (central mechanisms) (Hubal et al., 2007). For example, a reduction in neural drive can occur through a decrease in the motor neuron firing rate (Hubal et al., 2007; Sayers et al., 2003). Recently however, Hubal et al. (2007) reported similar neural functions (Electromyogram (EMG) and central muscle action potential) between high and low responders despite large variability in MVC torque loss post damage (MVC torque loss 49% and 23% respectively). Therefore, in agreement with recent work (Sayers et al., 2003), Hubal et al. (2007) concluded that torque loss is a result of difference in peripheral rather than neural mechanisms. The mechanisms of damage at the contractile muscle however, remain divided between the popping sarcomere theory and damage to the E-C process.

As previously discussed the popping sarcomere theory describes the process of damage when fibres are stretched beyond the point of no myosin overlap (Morgan, 1990), which according to the length-tension relationship, results in loss of torque generation. Furthermore, sarcomere disruption can cause damage to contractile proteins which may further impede the muscles ability to contract (Herzog, 2014). Following subsequent contractions, the loss in torque through the popping sarcomere theory can be evidenced by a rightward shift in the length-tension relationship (Jakeman & Eston, 2013; Hoffman et al.,

2014). A rightward shift in the length-tension relationship and greater torque loss at shorter muscle lengths represents an increase in fascicle length required to produce optimal torque, and lower myofilament cross bridge formation at the original optimal tension length (Jakeman & Eston, 2013; Byrne et al., 2001). Alternatively another well supported theory to explain MVC torque loss post EIMD is damage to the E-C process (Warren et al., 1993b). Damage to the E-C involves shearing of the t-tubules and SR. As discussed previously, evidence that caffeine reduces the magnitude of torque loss post EIMD, suggests that damage does occur to the E-C process (Warren et al., 1993b). Furthermore, the association between damage to the E-C process and torque loss has been evidenced through the reduction in both (JP1 and JP2) junctophilin proteins following eccentric contractions but not concentric contractions (Corona et al., 2010). Junctophilin proteins anchor the t-tubule membrane to the SR membrane, and are associated with Ca^{2+} release channels. Corona et al. (2010) reported a significant correlation between junctophilin proteins content post EIMD and loss in isometric torque post EIMD following eccentric exercise. Further research however, is required to understand which initial cause of EIMD can be associated with the prolonged torque loss post EIMD (Hyldahl & Hubal, 2014).

Alternative methods (electrical stimulation) can be used to measure torque loss post EIMD to ensure that participants are contracting maximally, a caveat associated with MVC, however electrical stimulation can alter the recruitment pattern of muscle fibres reported *in vivo* (Crameri et al., 2007). Therefore, although the primary mechanisms for MVC torque loss remains unconfirmed, it is deemed and accepted the most representative *indirect* marker of muscle function and EIMD (Warren et al., 1999).

1.3.3. Intracellular proteins: Creatine kinase

Post EIMD due to structural damage to the sarcomeres or the E-C process; there is an increase in the detection of intracellular proteins within the blood. Myoglobin, troponin and

myosin heavy chains are several proteins which have been used as markers of EIMD, however, possibly due to its large response post EIMD, CK is the most investigated intracellular protein within EIMD research (Baird et al., 2012; Clarkson & Hubal, 2002; Sewright et al., 2008; Stupka et al., 2000). Creatine kinase has three tissue-specific isoenzymes, CK-BB (found in the brain), CK-MB (found in cardiac muscle) and the muscle specific CK, (CK-MM) (Baird et al., 2012; Dawson & Fine, 1967; Eppenberger et al., 1964). As the current thesis is focusing on the skeletal muscle, 'CK' will refer to the muscle specific isoform unless otherwise stated. Within the muscle 5-10% of CK is bound to the M-line (Hornemann et al., 2003), therefore post EIMD CK within the blood, represents structural damage and an accompanying loss of muscle membrane permeability.

Similar to MVC torque loss, the severity and timeframe of the CK response is dependent on the type of exercise. Downhill running results in the quickest, but lowest CK response, with peak CK attained within 24 hours and CK values ranging from 100-900 U/L (Sorichter et al., 2001; Malm et al., 2004); whereas with isolated unilateral, maximal eccentric contractions, CK peaks between 3-6 days, with peak CK values between 100-1000 U/L (Hyldahl et al., 2011; Stupka et al., 2001). The greatest increase in CK however, is reported following upper body exercise and bilateral eccentric exercise, where CK peaks 4-6 days post EIMD with CK >2000 U/L (Sewright et al., 2008; Saka et al., 2009; Clarkson et al., 1992). However, caution needs to be taken when measuring average CK within and between groups as the CK can demonstrate large within participant variability (Hyldahl et al., 2011). The determinants of this large variability in the CK response has yet to be determined (Warren et al., 1993a; Chrismas et al., 2014).

The large variability with CK has resulted in scepticism regarding CK as a structural measure of EIMD (Baird et al., 2012; Friden & Lieber, 2001; Komulainen et al., 1995; Chrismas et al., 2014). For example, an increase in CK despite no damage reported at the sarcolemma or the Z-line (Costa et al., 2009; Yu et al., 2002), and, a lack of correlation between Z-line disruption (Stupka et al., 2000) and torque loss (Friden & Lieber, 2001; Lieber

et al., 1994) with CK, questions CK as a structural marker of EIMD. Regardless, an influx in CK does represent an extracellular expression of an intracellular protein, and is widely used as a marker of EIMD (Warren et al., 1999). It is not possible to decipher however, whether an increase in CK represents a change in cell membrane permeability or the magnitude of structural damage. Therefore, CK data should be considered as a quantitative measure of EIMD with the caveat in mind that any increase may be due to either a loss of membrane stability or disruption to the contractile proteins.

1.3.4. Muscle soreness

Muscle soreness otherwise known as 'delayed onset muscle soreness' (DOMS) is a sensation of pain or discomfort following exercise (Armstrong, 1984). Muscle soreness is measured using a visual analogue scales (VAS), which typically ranges from 0 (no soreness at all) to 10 (very, very sore). Although the measure of muscle soreness using the VAS scale is subjective, it is a reliable (inter class correlation (ICC) 0.95 – 0.98) method of measuring muscle soreness (Bijur et al., 2001). The validity of muscle soreness however remains in question, Nosaka et al. (2002) reported significant correlations, albeit weak, between muscle soreness and peak CK ($r = 0.23$) or MVC torque loss ($r = -0.27$). The negative correlation between muscle soreness and MVC torque loss however, is opposite to what physiologically would be expected (i.e. greater muscle soreness = greater MVC torque loss), therefore raising further questions regarding the validity of muscle soreness as an indicator of EIMD (Nosaka et al., 2002). Using VAS and measuring soreness during extension, rather than flexion, improves the correlation with other markers of EIMD, although the correlations remain weak (Nosaka et al., 2002). Despite poor validity with other markers of EIMD, muscle soreness consistently peaks 24-48 hours post EIMD and subsides 7-10 days post EIMD (Cleak & Eston, 1992; Howell et al., 1993; Clarkson et al., 1992; Savage & Clarkson, 2002). Similar to other markers of EIMD, the severity of muscle soreness depends on the type of eccentric exercise, for

example downhill running causes lower muscle soreness scores compared to dynamometer based, single joint muscle contractions (Clarkson & Hubal, 2002).

Although muscle soreness may be seen as the most familiar marker of EIMD with participants, the aetiology of muscle soreness remains unclear. Armstrong (1984) proposed a four-stage model to describe the aetiology of muscle soreness. The proposed stages are, 1) Damage to structural proteins. 2) Loss of Ca^{2+} homeostasis and the activation of proteolytic enzymes. 3) Invasion of monocytes and macrophages. 4) Activation and sensitisation of nociceptors. When initially proposed, Armstrong (1984) model of muscle soreness was mainly hypothetical, however it is now proposed that these four stages work in concert. Recently there has been evidence to suggest that noxious substances released following EIMD, contribute to the sensation of muscle soreness. By altering the administration time of antibodies, i.e. before or after eccentric exercise, the timeline of noxious substances can be calculated (Nie et al., 2009; Murase et al., 2010). For example, Murase et al. (2010) found that hyperalgesia (muscle soreness) was completely prevented in mice, when a bradykinin antibody was administered 30 minutes prior to eccentric exercise. Interestingly, when the bradykinin antibody was administered post EIMD no effect on muscle soreness was reported (Murase et al., 2010). Therefore Murase et al. (2010) concluded bradykinin is involved in the primary onset of muscle soreness but does not explain a delayed response in peak muscle soreness post EIMD. Additionally, the importance of nerve growth hormone and muscle soreness was evidenced when Murase et al. (2010) administered an antibody for the nerve growth hormone post EIMD which completely attenuated muscle soreness. Further evidence put forward to support the role of the nerve growth hormone on muscle soreness is the fact that the up regulation of nerve growth hormone post EIMD is 12 hours and can last up to 48 hours, which coincides with the time response of muscle soreness (Murase et al., 2010). Murase et al. (2010) concluded that bradykinin initiates muscle soreness whereas nerve growth hormone maintains muscle soreness post EIMD.

It is apparent that eccentric exercise causes structural and metabolic damage, which results in disruption to, contractile properties, structural integrity and muscle function. It is evident however, that the response to EIMD varies between groups and individuals. In order to discuss and understand these group differences, it is necessary to understand the determinants of EIMD.

1.4. Determinants of exercise-induced muscle damage

The previous section has described the typical response of direct (Z-line smearing) and indirect (loss of force, CK release and muscle soreness) markers of EIMD. The timeframe and response to EIMD using direct and indirect markers of EIMD is well investigated within the literature however, an understanding for the variability in the response remains unclear. For example, post EIMD participants have been categorised into high and low responders for MVC torque loss (Hubal et al., 2007) and the CK response (Brancaccio et al., 2007), furthermore, group differences have been reported between children and adults (Marginson et al., 2005), and males and females (Joyce et al., 2014). The next sections of the literature review will discuss some of the suggested determinants of EIMD.

1.4.1. High strain versus high torque

During eccentric contractions the muscle produces high torques whilst lengthening, however it remains unclear which, if any, of these characteristics are the main determinant of EIMD. By independently manipulating the level of strain and torque during eccentric contractions authors have investigated the effects on markers of EIMD.

1.4.1.1. Fascicle strain

Fascicle strain, denoted by fascicle lengthening, has been described as a determinant of EIMD *in situ* (Lieber & Friden, 1993; Talbot & Morgan, 1998). For example, using isolated tibialis anterior muscle in rabbits, Lieber and Friden (1993) categorised eccentric contractions into

two groups, either high strain or low strain (125% and 112.5% respectively). Following 900 eccentric stretches Lieber and Friden (1993) found significantly higher force loss when the tibialis anterior was stretched 125% compared to 112.5% of optimal muscle length. Thus, Lieber and Friden (1993) concluded that strain was the main determinant of EIMD. Alternatively, *in vitro* Warren et al. (1993a) investigated the role of fascicle strain (110, 120 and 130% of muscle length at maximal isometric tetanic tension) during five eccentric contractions of rat soleus muscle. Warren et al. (1993a) concluded that there was no association between fascicle strain and markers of EIMD between the different strain levels. The discrepancies between the two studies may be attributed to a couple of reasons; firstly to the volume of eccentric contractions used within the two studies was substantially different (900 versus five, respectively). Secondly, due to fascicle lengths not being measured directly in either study, and estimated as a percentage of MTU lengthening, the true fascicle lengthening may have been under or overestimated in the studies. Although, the theory of fascicle strain being associated with EIMD fits well with the mechanisms proposed by the popping sarcomere theory (Morgan, 1990), evidence supporting strain as a determinant of EIMD remains inconclusive.

As described previously, the role of strain has been investigated *in vitro* and *in situ* however, it is difficult to extrapolate and apply these findings *in vivo* (Butterfield, 2010). Firstly, *in vitro* and *in situ* preparations typically involve the removal of or a reduction in the contribution of the in series compliance of the MTU. *In vivo* the tendon has been reported to play an essential role in muscle contractions and has been suggested to alter fascicle lengthening (Lichtwark & Wilson, 2007; Finni et al., 2001), peak fascicle torques (Roberts & Azizi, 2010) and potentially attenuate EIMD (Roberts & Konow, 2013; Joyce et al., 2014; McHugh et al., 1999). Therefore, *in vivo* MTU behaviour is not directly replicated by *in situ* and *in vitro* studies (Butterfield, 2010). Secondly, fascicle strains used *in situ* and *in vitro* are considerably larger than those reported *in vivo*. For example *in situ* studies have investigated fascicle strains of 125% whereas *in vivo* during backwards walking peak fascicle lengthening

has been reported to be as small as 18% of *gastrocnemius* fascicle length at peak isometric MVC. Finally as mentioned previously, due to either the role of the tendon, the reduced fascicle lengthening or an interaction between the two, the popping sarcomere theory may not fully explain EIMD *in vivo*. For example, Hoffman et al. (2014) are the first group to investigate whether *gastrocnemius* fascicle lengthening during a backwards walking protocol was a determinant of EIMD *in vivo*. Hoffman et al. (2014) reported that several fascicles did lengthen onto the descending limb of the length-tension relationship, however due to the Achilles tendon accounting for ~90% of the MTU lengthening, the majority of fascicles contracted onto the ascending and plateau region of the length-tension relationship. The 23% reduction in MVC torque loss could not be explained by fascicle lengthening, thus Hoffman et al. (2014) suggested that, due to the role of the tendon the popping sarcomere theory may not explain EIMD *in vivo*. It cannot be discounted however, that the loss in torque may be attributed to the lengthening of sarcomeres being heterozygous along a fibre (Palmer et al., 2011). As a result of heterozygous lengthening, it remains possible that individual sarcomeres may have been extended onto the descending limb of the length-tension relationship thus adhering to the mechanisms of the popping sarcomere theory.

Compared to other types of eccentric exercise, backwards walking results in the low levels of EIMD, thus a more vigorous damaging protocol is required *in vivo* to establish whether, 1) fascicle strains used *in vitro* and *in situ* are comparable *in vivo* and, 2) whether greater fascicle strain *in vivo*, denoted by fascicle lengthening, is a determinant of EIMD (Hoffman et al., 2014).

1.4.1.2. High torque

It is well accepted that eccentric contractions can produce high torques (Katz, 1939), it remains unclear however, whether eccentric torques can explain differences in EIMD. For example, by delaying the timing of stretch (200 ms and 0 ms) in rabbit tibialis anterior, Lieber and Friden (1993) investigated the effect of high and low forces (respectively) on

muscle damage. Following 900 eccentric stretches, Lieber and Friden (1993) reported no significant differences in markers of EIMD between high and low eccentric forces. As mentioned previously, Lieber and Friden (1993) reduced the compliance of the MTU by applying the forces directly to the muscle. Therefore, due to the mediating role of the tendon on peak force and peak torque (Roberts & Azizi, 2010; Roberts & Konow, 2013), it is difficult to apply Lieber and Friden (1993) findings *in vivo*. In agreement with Lieber and Friden (1993) however, Chapman et al. (2008) was the first group to investigate whether *in vivo* eccentric torque correlated with any markers of EIMD in the elbow flexors. In agreement with previous work (Talbot & Morgan, 1998; Lieber & Friden, 1993), Chapman et al. (2008) reported no correlation between markers of EIMD (MVC torque loss or CK) and eccentric torque (averaged over 10 sets of 6 maximal eccentric elbow contractions) *in vivo*. The findings of Chapman et al. (2008) appear to disagree with those reported by Warren et al. (1993a). Warren et al. (1993a) manipulated force levels and investigated forces 100%, 125% and 150% of peak isometric force. Warren et al. (1993a) found a significant linear relationship between eccentric torque and torque loss post EIMD, however this linear relationship was lost when eccentric torque was below ~113% of isometric torque. It is concluded that the yield strength of a muscle fibre is above 113% of maximal isometric torque (Warren et al., 1993a; Hasselman et al., 1995) in animals. Interestingly, when Chapman et al. (2008) expressed the eccentric torque as a percentage of *pre-damage* isometric torque, eccentric torque was 94% of *pre-damage* isometric torque. Therefore, when eccentric torque is made relative to isometric torque, the findings of Chapman et al. (2008) do support those reported by Warren et al. (1993a). It remains in question however, if eccentric torque *in vivo* can go beyond the suggested yield threshold, and if so, is eccentric torque is a determinant of EIMD?

1.4.2. Tendon as a determinant of exercise-induced muscle damage

Previously the compliance of the MTU has been *suggested* to explain group differences in EIMD (McHugh et al., 1999; Marginson et al., 2005); however, it remains to be evidenced experimentally. Due to the tendon's ability to engage energy at initial impact and therefore reduce or delay the energy being absorbed by the muscle, the tendon has commonly been referred to as a mechanical buffer (Roberts & Azizi, 2010; Griffiths, 1991; Reeves & Narici, 2003). Although, due to its ability to alter the storage and timing of energy release, the term 'capacitor' has been suggested as a more accurate description of the tendon (Roberts & Konow, 2013). However, because the term is more consistent with the passive absorption of energy during eccentric contractions (rather than rebound plyometric contractions), within the current thesis the tendon will be referred to as a 'buffer'. Although the tendon has been suggested to act as a mechanical buffer during eccentric contractions (Roberts & Azizi, 2010; Griffiths, 1991; Reeves & Narici, 2003), the role of the tendon in association with EIMD has yet to be established *in vivo*.

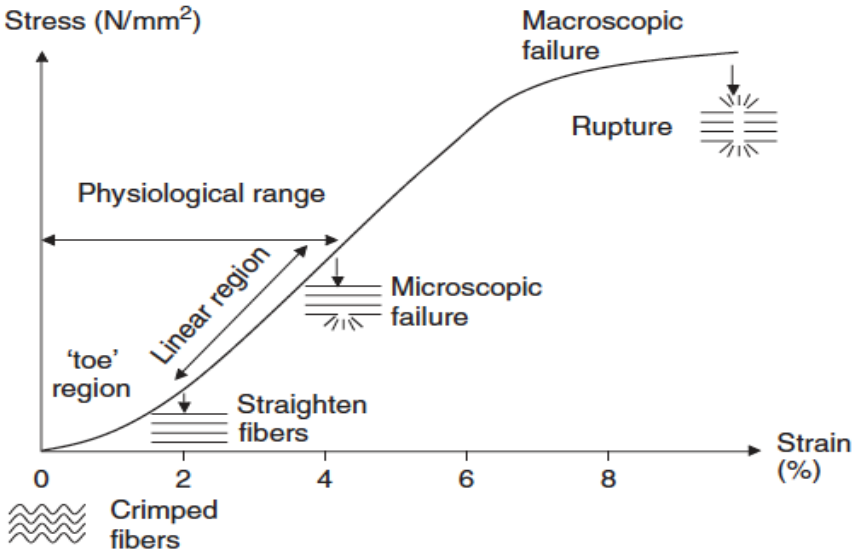

Figure 1. 7: Schematic example of a stress-strain curve demonstrating the mechanical properties of tendon (Wang, 2006).

1.4.2.1. Measuring tendon properties

Collagen, predominantly type 1 collagen, forms 65-85% of the tendon, with 1-2% elastin (O'Brien, 1997). Within the tendon collagen fibrils cross over each other creating plaits and spirals to form a collagen fibre, which is the basic structure of a tendon (Jozsa et al., 1991). Under light microscope, the collagen has a wavy configuration, otherwise known as the crimped region, which can explain the toe region of the stress-strain relationship (Figure 1. 7). When the tendon is stretched, due to the straightening of the collagen, the crimped region is lost, however if the stretch remains below 4% the crimped region will be regained upon relaxation (Rigby et al., 1959). Previously it was anticipated that if the stretch exceeds 8% the tendon would rupture, however *in vitro* it has been established that strains of >14% can be obtained without rupture in chicken tendons (Devkota & Weinhold, 2003) and human patella tendons (Johnson et al., 1994). The strength of the tendon is indicated by the collagen content and the size of the tendon. The tendon's tensile strength can be calculated, independent of cross-sectional area, by dividing tendon stress (tendon force divided by tendon cross-sectional area) by tendon strain (ratio of tendon lengthening to tendon length) and is expressed as Young's modulus (Maganaris & Paul, 1999). Although the higher the Young's modulus the 'stiffer' the tendon, this equation does not calculate tendon stiffness. Tendon stiffness is calculated as tendon force divided by change in tendon length. Unlike Young's modulus however, tendon stiffness does not take into consideration tendon size or length.

Previously the viscoelastic properties of the tendon have been measured indirectly by either the 'α method' (Morgan, 1977) or the spindle null-point' method (Rack & Westbury, 1984). However, with the introduction of real time imaging techniques, Fukashiro et al. (1995) used ultrasound to directly measure tendon properties *in vivo*. During a ramped isometric dorsiflexion contraction, Fukashiro et al. (1995) used the ultrasound to record the movement of the tibialis anterior tendon. Further research has established that total tendon strain is under estimated by 38-45% if tibial movement is not account for (Onambélé et al.,

2007; Hansen et al., 2006). Therefore to measure total tendon elongation, patella tendon elongation at the distal and proximal end of the tendon needs to be recorded (Onambélé et al., 2007; Hansen et al., 2006). Additional measurements, including tendon moment arm, antagonist co-activation, tendon cross-sectional area, and tendon length, are required to calculate tendon force, tendon compliance and Young's modulus (Maganaris & Paul, 1999). What is more, the introduction of imaging technique has allowed for an *in vivo* understanding of tendon compliance and how it interacts in series with the muscle during functional movements (Fukunaga et al., 2001), and more specifically to this thesis how it may impact on eccentric contractions and the associated EIMD.

1.4.2.2. Tendon as a mechanical buffer

The role of the tendon is multifaceted and depends on what movement is being carried out. For example, during counter-movement jumps, the tendon has been reported to slowly absorb energy and then rapidly release energy (Aerts, 1998; Kawakami et al., 2002), whereas during gait (walking and running) the tendon has been reported to reduce the degree of fascicle lengthening thus reducing metabolic work and improve the metabolic efficiency of gait (Fukunaga et al., 2001; Roberts et al., 1997; Lichtwark & Wilson, 2007).

Historically it has been assumed that fascicles follow similar lengthening patterns to the tendon and the MTU during eccentric contractions, however recent evidence disproves this assumption (Fukunaga et al., 2001). For example, during the eccentric phase of gait and stair decent the *gastrocnemius medialis* fascicles have been reported to contract quasi-isometrically (Fukunaga et al., 2001) and shorten (Spanjaard et al., 2007), respectively despite a lengthening of the MTU. Although the differences in fascicle lengthening may be attributed to the different foot placements required during gait (heel contact) and stair descent (toe contact), both Spanjaard et al. (2007) and Fukunaga et al. (2001) concur that lengthening of the MTU occurs mainly at the tendon during eccentric contractions. Due to the majority of MTU lengthening occurring at the tendon, rather than the fascicles, the tendon

has been suggested to act as a mechanical buffer during eccentric contractions (Reeves & Narici, 2003; Roberts & Azizi, 2010; Roberts & Konow, 2013; Lichtwark & Wilson, 2007). For example, Lichtwark and Wilson (2007) used a three element Hill muscle model to estimate the effect of increasing Achilles tendon stiffness by 0.5, 1, 2, and 4 times above previously measured average Achilles tendon stiffness (Lichtwark & Wilson, 2006). Lichtwark and Wilson (2007) calculated that during the eccentric phase of gait, *gastrocnemius medialis* fascicle lengthening increased with an increase in tendon stiffness, despite no change in total MTU lengthening. Therefore, Lichtwark and Wilson (2007) concluded that the tendon does act as a mechanical buffer on fascicle lengthening. However, the model used by Lichtwark and Wilson (2007) assumed tendon stiffness to be linear despite the previously reported 'toe region' in tendon stress-strain properties (Figure 1. 7), thus this may result in an under estimation of fascicle shortening during eccentric contractions, particularly at low forces. Although the findings of Lichtwark and Wilson (2007) have yet to be established *in vivo*, group differences in fascicle lengthening have been suggested, although not measured, to differences in tendon properties. For example, despite the same MTU lengthening during the eccentric stance phase of gait, Mian et al. (2007) attributed differences in fascicle lengthening to differences in tendon properties between the elderly and young. Mian et al. (2007) explained the lower fascicle lengthening in the elderly compared to the young, to higher tendon stiffness in the young. However, Mian et al. (2007) did not measure tendon stiffness within or between the two groups. Therefore, it remains unknown whether *in vivo* tendon stiffness does act as a mechanical buffer on fascicle lengthening, and explain group differences in fascicle lengthening behaviour.

In addition to the tendon being suggested to act as a mechanical buffer on fascicle lengthening during eccentric contractions, the tendon has also been suggested to mediate the degree of peak force and torque transmitted to the fascicles (Roberts & Azizi, 2010). For example, using turkey gastrocnemius, Roberts and Azizi (2010) found the peak power input during MTU lengthening was significantly lower at the fascicles compared to the MTU.

Furthermore, Roberts and Azizi (2010) found the negative work done by the fascicles during the force rise of muscle lengthening contractions was significantly lower than that of the MTU. Thus, the tendon accounted for the differences in negative work between fascicles and the MTU. Roberts and Azizi (2010) concluded, the tendon attenuates peak power, energy and decouples fascicle lengthening from MTU lengthening, which may serve to protect the muscle from high forces during eccentric contractions (Roberts & Azizi, 2010; Roberts & Konow, 2013).

As discussed previously, it remains unknown whether EIMD can be attributed to the high strain or high stress of eccentric contractions, however it appears that the tendon may act as a mediator of both high stress and high strain (Roberts & Azizi, 2010; Roberts & Konow, 2013). Therefore tendon properties have been suggested to act as a determinant of EIMD (Marginson et al., 2005; Roberts & Azizi, 2010; Roberts & Konow, 2013). Previous studies have attributed group differences in EIMD to the flexibility of the MTU as an entity. For example, Marginson et al. (2005) reported that young boys experienced significantly lower levels of EIMD compared to adult men following repeated maximal plyometric jumps. Marginson et al. (2005) attributed the reduced muscle damage to greater MTU flexibility in the boys compared to men. Furthermore, in an earlier study McHugh et al. (1999), reported that muscle damage in the hamstring was significantly higher in participants with stiffer hamstring MTU compared to those with a more compliant hamstring MTU. Applying the popping sarcomere theory proposed by Morgan (1990), both Marginson et al. (2005) and McHugh et al. (1999) suggested that EIMD may be lower in compliant participants due to a reduction in fascicle strain. However, neither McHugh et al. (1999) or Marginson et al. (2005) measured tendon stiffness, thus it remains unknown whether the tendon can act as a determinant or explain group differences in EIMD.

1.4.3. Oestrogen as a determinant of exercise-induced muscle damage

1.4.3.1. Oestrogen as an antioxidant

Before discussing oestrogen as a determinant of EIMD, an understanding of oestrogen structure and its antioxidant properties needs to be established. Oestrogen is a group of 18-carbon steroids, which are mainly secreted from the female ovaries, with smaller quantities secreted by the adrenal glands (Kendall & Eston, 2002). Despite oestrogen being predominately a female sex hormone, small quantities, albeit significantly lower than females, are secreted from male testes and adrenal glands (Velle, 1966). Oestrogen is an umbrella term for estradiol-17b, estrone and estriol. Although all three types of oestrogen are structurally similar, estradiol-17b is most commonly investigated because it is the most abundant form of oestrogen (Kendall & Eston, 2002). Oestrogen has three properties that have been associated with attenuating EIMD (Figure 1. 8). Firstly, oestrogen has been reported to have a high antioxidant capacity (Tiidus, 1995; Ayres et al., 1998; Sugioka et al., 1987). Oestrogen displays a hydroxyl group on their phenolic ring in a similar configuration as vitamin E, another well-known antioxidant (Tiidus et al., 2001; Traber & Stevens, 2011). Although it remains unclear how oestrogen limits lipid peroxidation, it is suggested that oestrogen donates the hydrogen atom from its a-phenolic ring to lipid peroxy radicals (Sugioka et al., 1987; Ayres et al., 1998; Badeau et al., 2005). The donation of the hydrogen atom prevents the hydroxyl radical attacking the polyunsaturated fatty acids in the cell membranes and subsequently maintaining cell membrane stability (Kendall & Eston, 2002). Secondly, oestrogen has been reported to decrease membrane fluidity and in turn increases membrane stability (Wiseman et al., 1993). Oestrogen is lipophilic therefore, it intercalates into the bilayer of the cell membrane. It is this interaction between oestrogen and membrane phospholipids which is suggested to decreases cell membrane fluidity and increase the stability of the membrane (Wiseman et al., 1993). Wiseman et al. (1993) reported a positive association between oestrogen's antioxidant ability and decreased membrane fluidity. Wiseman et al. (1993) concluded that the hydrophobic rings of oestrogen may interact with

the poly-unsaturated residues within the phospholipid layer, thus decreasing membrane fluidity and stabilising the membrane against peroxidation. Thirdly, oestrogen has been reported to have a gene regulatory effect (Ghisletti et al., 2005). Oestrogen has been found to inhibit nuclear factor kappa beta intracellular transport (central mediator of the immediate inflammatory response), thus preventing inflammatory gene transcription (Ghisletti et al., 2005). By reducing the inflammatory process (Silvestri et al., 2003), oestrogen may allay further damage by attenuating the infiltration of neutrophils and cytokines (MacNeil et al., 2011). Although the primary role of oestrogen as an antioxidant remains unclear, it is clear that through the several mechanisms descried above and presented in Figure 1. 8, levels of oestrogen may act to attenuate EIMD.

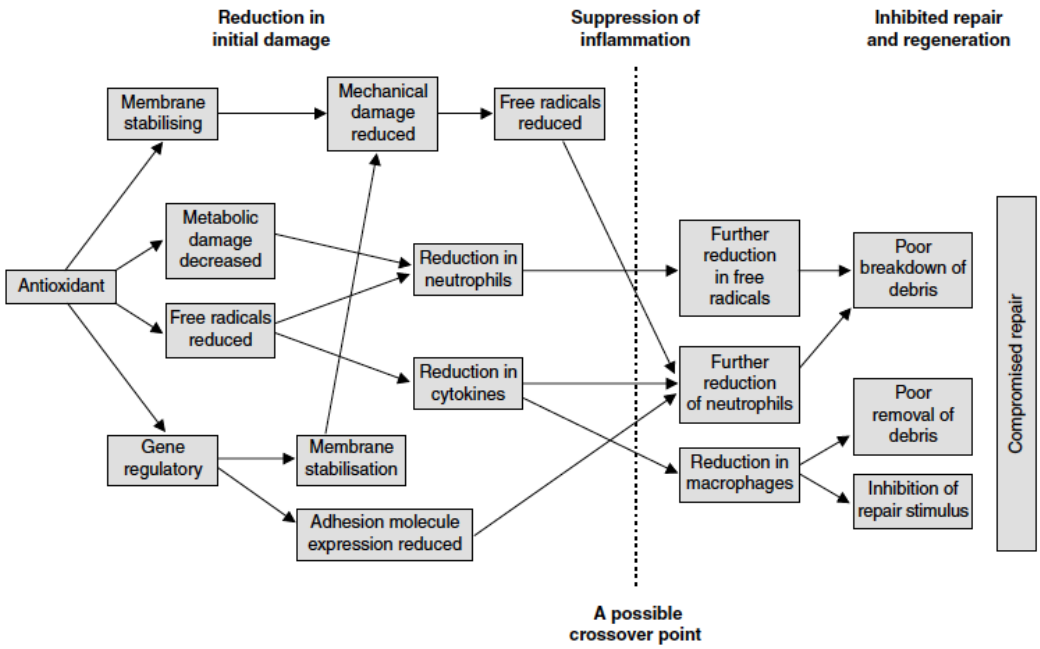

Figure 1. 8: The interaction between oestrogen and exercise-induced muscle damage (EIMD) (Kendall & Eston, 2002).

1.4.3.2. Muscle damage and oestrogen

To establish whether oestrogen may attenuate EIMD, oestrogen levels have previously been manipulated either experimentally or through group comparisons. For example, Bär et al. (1988) reported that ovariectomised female rats demonstrate the same CK response as male rats however, the CK response in both males and ovariectomised females can be diminished following oestrogen supplements prior to exercise. Bär et al. (1988) concluded that oestrogen attenuates the leakage of muscle proteins by stabilising cell membrane permeability. Interestingly the level of protection has been reported to depend on what age the ovariectomy is performed (Amelink et al., 1988). For example, female rats that were ovariectomised before reaching sexual maturity resulted in greater damage compared to female rats ovariectomised after reaching sexual maturity (Amelink et al., 1988). This indicates that in addition to an acute protective mechanisms (Bär et al., 1988), oestrogen may also provide a long term protective mechanism to EIMD (Amelink et al., 1988). It may be speculated that the acute role may be attribute to the antioxidant and membrane stabilising properties of oestrogen, whereas the long term may reflect an additional protective mechanism through the role of oestrogen on the tendon, however there is no systematic evidence to support this.

Contrasting the data showing an influence of oestrogen on EIMD, Moran et al. (2007) reported oestrogen supplementation did not attenuate EIMD. Ovariectomized mice were separated into three groups one group consumed 0.18 mg of oestrogen for either 30 or 60 days, whereas the other groups received either a placebo or no treatment. Following 10 eccentric contractions of the *soleus in vitro*, Moran et al. (2007) reported no significant difference in MVC isometric force loss or membrane damage (assessed by Lactate Dehydrogenase levels) following EIMD regardless of oestrogen levels. Moran et al. (2007) concluded oestrogen supplementation did not alter markers of EIMD. Furthermore the lack of difference between the loss of membrane damage, contradicts the theory of oestrogen acting

as a membrane stabiliser. It must be noted that Moran et al. (2007) only investigated the acute damage response for 30 minutes post EIMD, therefore the protective effect of oestrogen may have been missed.

The methods used to manipulate oestrogen levels within animal studies (ovariectomizes) are difficult to replicate in humans. Alternatively *in vivo* oestrogen can be manipulated in several ways, 1) natural fluctuations of oestrogen throughout the menstrual cycle, 2) damage comparisons between males and females, and 3) the use of synthetic hormones (hormone replacement therapy (HRT) or the OCP). The following section will discuss the role of the OCP on EIMD.

1.4.3.3. The oral contraceptive pill

Within females, oestrogen is heavily involved with the menstrual cycle. The menstrual cycle (from first menses to the next menses) last 28 days and occurs repeatedly from menarche (median age 13 years, (Whincup et al., 2001)) and ceases post menopause (aged ~51 years (Rosner & Colditz, 2011)). The menstrual cycle can be divided into three phases, the follicular phase, ovulation and the luteal phase (Maki et al., 2002). Oestrogen levels fluctuate throughout the menstrual cycle: Low levels of oestrogen are reported in the mid follicular phase (~175 pg/ml) oestrogen then peaks *pre*-ovulation (day 14 ± 1, ~510 pg/ml), and slowly decreases throughout the luteal phase (day 14-28, mid luteal ~336 pg/ml) (Eiling et al., 2007). The natural fluctuations in oestrogen can be manipulated using synthetic hormones, specifically the OCP (Bryant et al., 2008) or reverted using HRT (Dieli-Conwright et al., 2009).

According to the National Survey for Family Growth, the OCP is the leading form of hormone contraception, with 10.7 million women using the OCP (Mosher & Jones, 2010). The OCP is a combination of synthetic oestrogen and progesterone hormones, and is taken either continuously (taken for the whole 28 day cycle) or cyclical (21 days pill, seven days pill free), the cyclical method however has been associated with ovarian rebound activity (Schlaff et al.,

2004). Ovarian rebound activity is where oestrogen levels peak after the seven day free period (Schlaff et al., 2004). Regardless of the type of OCP being used it has the same main effect, of attenuating oestrogen levels throughout the menstrual cycle (Schlaff et al., 2004). During a normal cycle, the hypothalamus secretes Gonadotropins-releasing hormone, which in turn stimulates the release of follicle stimulating hormone (FSH) and luteinising hormone (LH) (Silberstein & Merriam, 2000). Through negative feedback, the OCP alters the hypothalamic-pituitary-ovarian feedback loop subsequently reducing the secretion of FSH and LH and preventing follicular development (ovulation) (for a review see (Rubinstein et al., 1978; Van Heusden & Fauser, 2002). Within OCP users oestrogen levels increase twofold during the menstrual cycle and then plateau, whereas within non-users oestrogen levels increase fourfold and fluctuate (Yeung et al., 2012; Endrikat et al., 2002). Thus OCP users have significantly lower levels of endogenous oestrogen and progesterone levels compared to non-users (Bryant et al., 2008; Fleischman et al., 2010; Kuhl et al., 1985). Therefore, if oestrogen does attenuate EIMD, a difference in EIMD between non-users and OCP users may be expected.

Although within animal studies, the role of oestrogen on EIMD has been evidenced through ovariectomies, the role of oestrogen on EIMD is not as compelling when using the OCP within human studies. For example, no difference in EIMD between OCP users and non-users has been concluded following eccentric stepping (Thompson et al., 1997) and eccentric contractions of the elbow flexors (Savage & Clarkson, 2002; Sewright et al., 2008). Alternatively, following eccentric knee extensions Joyce et al. (2014) reported the CK response and the rightward shift in optimal knee angle post EIMD to be significantly greater in female OCP users compared to female non-users. The discrepancies within human studies may be attributed to several reasons, 1) different measurement days, specifically with markers of EIMD not being measured at their peak (i.e. Joyce et al. (2014) measured CK 24 hours post EIMD, rather than 96 hours). 2) Different markers of EIMD being investigated, for example MVC torque loss was not measured by Joyce et al. (2014). 3) Due to the different

damage responses in upper and lower limbs (Chen et al., 2011; Saka et al., 2009), it is difficult to compare the role of the OCP and oestrogen when comparing studies using different muscle groups.

Future studies are required to establish whether 1) oestrogen levels are a determinant of EIMD and 2) whether women taking the OCP are more susceptible to EIMD.

1.5. Group differences in exercise-induced muscle damage

The following section will discuss group differences in EIMD and potential mechanisms that may explain the reported group differences.

1.5.1. Sex differences

A large variability in markers of muscle damage following eccentric contractions is accepted however, it still remains unclear whether a sex difference in EIMD exists. Within animals, males are reported to have higher values of markers for EIMD compared to females (Clarkson & Hubal, 2002; Komulainen et al., 1999) however; within human studies it remains inconclusive. Human studies have evidenced (Borsa & Sauers, 2000; Sewright et al., 2008; Wolf et al., 2012; Joyce et al., 2014) or refuted (Rinard et al., 2000; Stupka et al., 2000; Dannecker et al., 2012) sex differences in EIMD.

Following eccentric exercise in the lower limbs (36 unilateral leg press and 108 unilateral knee extensions), Stupka et al. (2000) investigated sex differences in EIMD. Using muscle biopsies of the VL, Stupka et al. (2000) reported no sex differences in Z-line disruption or the serum CK response post EIMD. Interestingly, the inflammatory response (presented as changes in bcl-2 cell count) was significantly higher in males compared to females. Stupka et al. (2000) attributed the higher inflammatory response in males, to oestrogen acting as a membrane stabiliser in females. The findings of no differences in the CK response and Z-line disruption may only appear to contradict the conclusion of oestrogen acting as a membrane stabiliser because they were not measured at their peak. Stupka et al.

(2000) measured muscle biopsies at 48 hours post EIMD and CK at 48 and 144 hours post EIMD, whereas Z-line disruption and CK are reported to peak immediately (Takekura et al., 2001; Friden & Ekblom, 1983), and 72-96 hours (Saka et al., 2009; Clarkson et al., 1992; Stupka et al., 2001) post eccentric EIMD respectively. Recently however, Joyce et al. (2014) investigated sex differences in EIMD following 240 eccentric knee extension, unilaterally, on both legs (480 contractions in total). Joyce et al. (2014) reported CK to be significantly higher in males compared to females, despite measuring peak CK at the same time as Stupka et al. (2000) (48 hours). Regardless of using the same muscle groups, the differences in the volume (Machado et al., 2012) of eccentric knee extensions (140 versus 480 respectively) and the different methods used (leg press machine versus isokinetic dynameters respectively) may explain the discrepancies between Stupka et al. (2000) and Joyce et al. (2014) respectively. Other studies have reported peak CK following eccentric exercise in the elbow flexors to be significantly higher in males compared to females (Sewright et al., 2008). It is difficult to compare these findings to the aforementioned studies due to damage in upper limbs being significantly higher compared to the lower limb (Chen et al., 2011; Saka et al., 2009). Furthermore, due to the sensitivity and variability of CK as a marker of EIMD, different rest intervals may potentially explain the discrepancies regarding the CK responses (for a review see Koch et al., (2014)).

Although CK is a marker of EIMD, as mentioned previously loss of isometric MVC torque is deemed the best *indirect* marker of EIMD (Warren et al., 1999) however, investigations regarding sex differences in MVC torque loss is sparse. Furthermore, the limited work that has investigated a sex difference in torque loss has predominately been in the elbow flexors, where a sex difference has been rejected (Rinard et al., 2000; Sayers & Clarkson, 2001) or evidenced (Sewright et al., 2008). Sewright et al. (2008) reported MVC torque loss in the elbow flexors to be significantly greater in females compared to males immediately post EIMD; however, they found no significant difference at any other time point. Therefore, although Sewright et al. (2008) findings could suggest that EIMD is higher in

females, the sex difference in MVC torque loss was only found immediately post EIMD which is a timeframe more representative of fatigue rather than EIMD (Walsh et al., 2004).

It remains unclear, especially in the lower limbs, whether a sex difference in EIMD does exist, however, it is clear that the markers of EIMD used, the measurement day post EIMD and the type of eccentric exercise undertaken, may confound the conclusion. Markers of EIMD need to be measured at peak time frames, with set rest intervals to establish a confident conclusion as to whether a sex difference does exist.

1.5.2. Alternative explanations

Typically the sex differences in EIMD, specifically the CK response, has been attributed to oestrogen's antioxidant and membrane stabilising properties (Joyce et al., 2014), however Wolf et al. (2012) controlled for the menstrual cycle by testing women in the early follicular phase of their menstrual cycle, where oestrogen levels were not significantly different between males and females. Despite similar oestrogen levels, Wolf et al. (2012) reported the CK response to be significantly higher in males compared to females. Highlighting that sex differences in EIMD, may not be attributed to the direct role of oestrogen *per se*.

In addition to oestrogens antioxidant properties, oestrogen has also been reported to alter tendon properties (Hansen et al., 2009b), specifically tendon stiffness (Kubo et al., 2003). Although oestrogen levels fluctuate naturally during the menstrual cycle, no differences in tendon stiffness has been reported at different phases of the menstrual cycle (Burgess et al., 2010). Nevertheless the attenuation in oestrogen is only acute throughout the menstrual cycle and may therefore not be severe enough to see a difference in tendon stiffness. However, the interaction between oestrogen and tendon properties has been evidenced through sex differences, where significantly lower tendon stiffness in males has been attributed to significantly higher levels of oestrogen in females (Kubo et al., 2003). Within OCP users however, despite lower oestrogen levels compared to non-users, the

findings remain contentious with arguments supporting (Bryant et al., 2008) or contesting (Hansen et al., 2013) the role of oestrogen on tendon properties. Therefore, it remains unknown if the OCP alters tendon properties in females.

As described previously, the tendon may act as a mechanical buffer and explain group differences in EIMD in males versus females, and OCP users versus non-users (e.g. Joyce et al. (2014)). Furthermore, an understanding of oestrogen, tendon properties and EIMD may provide further information regarding the risk of injury in females during their menstrual cycle (Lefevre et al., 2013). Therefore, tendon properties in OCP users and non-users need to be investigated to establish whether the tendon acts as a determinant of EIMD, alongside oestrogen.

1.6. Aims

In light of the aforementioned research areas, the five aims of the current thesis are as follows:

1. The aim of chapter two is to investigate the reliability of the measurement techniques used throughout the thesis, specifically the ultrasound to measure fascicle lengthening during MVE_{KE}.

2. The aim of chapter three is to establish whether patella tendon stiffness alters VL fascicle lengthening during eccentric contractions in males and females.

3. The aim of chapter four is to establish whether muscle and tendon properties are associated with EIMD in vivo.

4. The aim of chapter five is twofold: Firstly to establish whether there is a sex difference in EIMD. Secondly, dependent on the outcome of the first aim, to establish whether tendon stiffness may explain the potential sex difference in EIMD.

5. The aim of chapter six is twofold: Firstly to determine whether tendon properties differ between OCP users and non-users and secondly, to establish whether muscle damage is significantly different between OCP users and non-users.

To answer the five aims of the current thesis, the majority of the method will be replicated throughout the thesis. Where required the method has been altered to make it specific to the additional variables being measured within each chapter.

Chapter two: The reliability of using ultrasound to measure patella tendon and muscle properties at rest and during MVE_{KE}.

2.1. Introduction

Historically, knowledge of human muscle tendon unit (MTU) morphology and elastic properties has been based on cadaveric data (Wickiewicz et al., 1983; Friederich & Brand, 1990; Makihara et al., 2006). When using cadavers however, the architectural and structural muscle and tendon properties are typically altered through the age of the deceased, the preservatives used and the fixation techniques adopted (typically fixed in an elongated position) (Narici, 1999; Cutts, 1988). The introduction of imaging techniques has enabled tendon and muscle properties to be measured non-invasively *in vivo*, either at rest or during contraction (Kawakami et al., 1993; Fukunaga et al., 1997; Fukashiro et al., 1995).

Two types of imaging techniques are commonly used to non-invasively measure muscle architecture *in vivo*, nuclear magnetic resonance imaging (MRI) and B-mode ultrasound (Narici, 1999). Although by using MRI several muscles can be scanned simultaneously, the scans are typically performed at rest and require multiple scans (Cutts, 1988). The requirement to stay still in a semi-enclosed/enclosed 'tunnel' during MRI scans, has resulted in MRI being impractical, specifically in a clinical setting (i.e. stroke patients) (Singer et al., 2004). Furthermore, MRI scans are required to be performed in a fixed plane, whereas the ultrasound technique enables muscle and tendon properties to be measured during contraction (Fukunaga et al., 1997; Fukunaga et al., 2001). Measuring muscle and tendon properties during contraction provides further insight on their independent contribution to total MTU behaviour (Fukunaga et al., 1997; Fukashiro et al., 1995; Reeves & Narici, 2003). Therefore, ultrasound is adopted when assessing the *in vivo* properties of the muscle under isometric and dynamic conditions.

The reliability of imaging techniques is crucial when carrying out longitudinal analysis of muscle properties involving repeated measurements. For example, the importance of precise measurements is evidenced when measuring changes in muscle architecture following a 12 week training intervention e.g. 1.57 cm (19.4%) increase in resting fascicle length (Baroni et al., 2013). High reliability has been reported when using ultrasound to

measure patella tendon cross-sectional area at rest (interclass correlation (ICC), 0.96) and patella tendon lengthening under contraction (ICC 0.91) (Onambélé et al., 2007). Furthermore, a recent review has investigated the reliability of using ultrasound to measure fascicle length and pennation angle in a variety of muscle groups (Kwah et al., 2013). Within the review, Kwah et al. (2013) collated moderate–to-high ICCs for repeated measures of *vastus lateralis* (VL) fascicle length (0.62-0.99) and VL pennation angle (0.51-1.00), however, contractile state was not specified. When measuring muscle architecture, ultrasound reliability may be affected by part of the fascicle extending out of the ultrasound's field of view. Thus to calculate the missing portion of the fascicle length, the linear extrapolation method is used to estimate total fascicle length (Figure 2. 6). During isometric contractions of the tibialis anterior, a 2.4% error has been associated with the linear extrapolation method (Reeves & Narici, 2003). Furthermore, potentially due to the longer fascicle length in the VL compared to the tibialis anterior (~11.3 cm and ~5.4 cm respectively), a 2-7% error has been reported when using the extrapolation in the VL at a knee angle of 120° (Finni et al., 2001). Recently, when comparing ultrasound to cadaveric measurements, the linear extrapolation method has been concluded as a valid technique for measuring VL fascicle length at rest (ICC = 0.853) (Ando et al., 2014). During eccentric contractions however, unlike isometric contractions where the MTU length remains constant, the MTU undergoes lengthening (Fukunaga et al., 2001; Guilhem et al., 2011). To account for the change in MTU during maximal eccentric knee extensions (MVE_{KE}) either the tendon, fascicles or both are required to lengthen (Fukunaga et al., 2001; Lichtwark & Wilson, 2007). Therefore, although it remains to be determined *in vivo*, by nature of the changing joint angle during eccentric contractions, it can be assumed a greater proportion of the fascicle extends out of the ultrasound field compared to isometric contractions. Furthermore, due to the rapid change in MTU length during MVE_{KE} it may be difficult to prevent small probe movements, which may contribute to lower reliability during eccentric movements. Klimstra et al. (2007) reported small rotations

of the ultrasound probe altered measurements of tibialis anterior architecture (fascicle length and pennation angle) during maximal dorsi-flexion contractions. For example, rotating the ultrasound probe -5° longitudinal and sagittal-frontal (from a neutral position 14° longitudinal rotation and 4° sagittal-frontal rotation), resulted in a 12% (1.9°) change in pennation angle (Klimstra et al., 2007). Therefore, it is essential to investigate whether the reliability of ultrasound to measure muscle architecture during isometric contractions can be extended to MVE_{KE}.

To the author's knowledge Guilhem et al. (2011) is the only study to report the reliability of ultrasound during MVE_{KE}. Guilhem et al. (2011) reported moderate ICCs for VL fascicle length, pennation angle and muscle thickness (0.82, 0.75 and 0.93 respectively). However, Guilhem et al. (2011) only used ICC, which is only deemed appropriate for reliability when combined with Bland-Altman plots (Rankin & Stokes, 1998; Atkinson & Nevill, 1998). Furthermore, Guilhem et al. (2011) did not measure the degree of probe movement during the $MVE_{KE.}$ Thus, the aim of this chapter is to investigate between session reliability of using ultrasound to measure patella tendon properties and muscle properties, specifically during MVE_{KE}.

2.2. Materials and method

2.2.1. Subjects

A total of 13 participants (22.1 ± 1.1 years of age, with a mass and stature of 68.0 ± 5.5 kg and 175 ± 8 cm, respectively) signed written informed consent to participate in the current study. All participants self-reported no lower limb injuries. All procedures complied with the Declaration of Helsinki (World Health Association, 2013) and ethical approval was obtained through the local ethics committee.

2.2.2. Testing protocol

Participants were asked to visit the laboratory on two different occasions (day one and day two). The two testing sessions were separated by 48 hours, to represent the time differences between *pre-damage* and the *damage session* in the subsequent chapters. Day one and two consisted of morphological measures of the patella tendon (tendon size and stiffness) and VL (muscle architecture, muscle length and VL anatomical cross-sectional area (VL_{ACSA})), and 12 reps MVE_{KE}. The order of measurements in day two mirrored day one, and was performed at the same time of day to control for the effect of time-of-day variation on torque and tendon properties (Pearson & Onambele, 2006; Martin et al., 1999).

All tests were carried out in the non-dominant leg. The non-dominant leg was defined as the leg that provided stability during movements, e.g. kicking. Participants were seated in an isokinetic dynamometer (Cybex Norm, Cybex International, NY, USA), with a hip angle of 85° (Figure 2. 1). To reduce any extraneous movement during maximal efforts participants were secured in a seated position using inextensible straps around the shoulders and hips. The isokinetic dynamometer axis of rotation was visually aligned with the knee joint's centre of rotation. The setup of the dynamometer chair on day one (including lever arm length, distance from the chair to dynamometer, seat height and back angle) was recorded to ensure precise replication of participant posture in the following session (i.e. day two).

Figure 2. 1: Example of participant seated in the isokinetic dynamometer with a hip angle of 85°.

2.2.3. Vastus lateralis resting architecture

Vastus lateralis resting architecture (fascicle length, pennation angle and muscle thickness) was measured using a real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy). Prior to the measurement of resting VL muscle architecture, participants laid supine for 20 minutes to allow for fluid distribution to equilibrate (Berg et al., 1993). With the participant laid supine and their non-dominant leg fully extended (knee angle 0°), the distal and proximal insertions sites of the VL were identified using an ultrasound probe (7.5 MHz linear array probe, 38 mm wide). For muscle properties, the ideal ultrasound probe frequency range is between 7.0-12.0 MHz, however there is a trade-off between higher frequency / precision and lower depths (Gibbon, 1996). Within the current chapter a probe frequency of 7.5 MHz allowed depths of up to 8 cm with an average resolution of 0.20 mm to be used (Gibbon, 1996). At 50% of VL length the ultrasound probe was positioned in the mid-sagittal plane. A hypo-allergenic ultrasound gel (Parker, Park Laboratories Inc., Fairfield) was used to enhance coupling between the skin and ultrasound probe. To ensure there was no movement artefact included in the assessment of muscle architecture, an echo-absorptive marker was fixed on the skin to provide a visual baseline of the internal structures.

Measurements of fascicle length, pennation angle and muscle thickness were measured offline using digitising software (ImageJ 1.45, National Institutes of Health, USA). Fascicle length was measured from the visible insertion of the fibre into either the deep or superficial aponeurosis. Where the fascicle extended longer than the ultrasound image, linear continuation of the fascicle and aponeurosis was assumed (Figure 2. 6). A 2-7% error has been associated with measuring VL fascicle length at 120° knee angle (Finni et al., 2001). Recently, when comparing ultrasound to cadaveric measurements', the linear extrapolation method has been concluded as a valid technique for measuring VL fascicle length (ICC =

0.853) (Ando et al., 2014). The ultrasound can only scan in 2D despite fascicle rotation forming a 3D structure; therefore great care was taken to orientate the probe as precisely as possible with the fascicle. To reduce error associated with the estimation of fascicle length, an average of three fascicles across the image was taken. Pennation angle was determined as the angle created from the visible insertion of the fascicle into the deep aponeurosis (Figure 2. 6). An average of three pennation angles was taken across the image. VL muscle thickness was determined as the vertical distance between the deep and superficial aponeurosis, an average was taken at three points across the width of the probe (Figure 2. 6).

2.2.4. Vastus lateralis anatomical cross-sectional area

Vastus lateralis anatomical cross-sectional area (VL_{ACSA}) was measured using a real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy). With the participant laid supine and their non-dominant leg fully extended (knee angle 0°), the distal and proximal insertions sites of the VL were identified using an ultrasound probe (7.5 MHz linear array probe, 38 mm wide). At 50% of VL muscle length echo-absorptive markers were placed in parallel, at intervals of 30 mm, from the lateral to the medial edge of the VL muscle. The ultrasound probe was held perpendicular to the VL muscle in the axial plane. The ultrasound probe was moved steadily over the echo-absorptive markers from the lateral to the medial edge of the muscle (Figure 2. 2). Constant, light pressure was placed on the muscle during scanning to avoid compression of the muscle (Esformes et al., 2002). The images were recorded in real time at 25 frames per second (Adobe Premier pro Version 6, Adobe Systems Software, Ireland). Using capturing software (Adobe Premier Elements, version 10), individual images were acquired at each 30 mm interval. Shadows cast by the echo-absorptive markers allowed the images to be aligned by the outline of the muscle, thus forming the entire VL_{ACSA} in a single image (Adobe Photoshop Elements, version 10, (Figure 2. 2)). Digitising software (ImageJ 1.45, National Institutes of Health, USA) was used to measure VL_{ACSA}. This method of calculating VL_{ACSA} has previously been accepted as reliable and valid

when compared to MRI, with a reported interclass correlations between 0.997 - 0.999 and 0.998 and 0.999 respectively (Reeves et al., 2004).

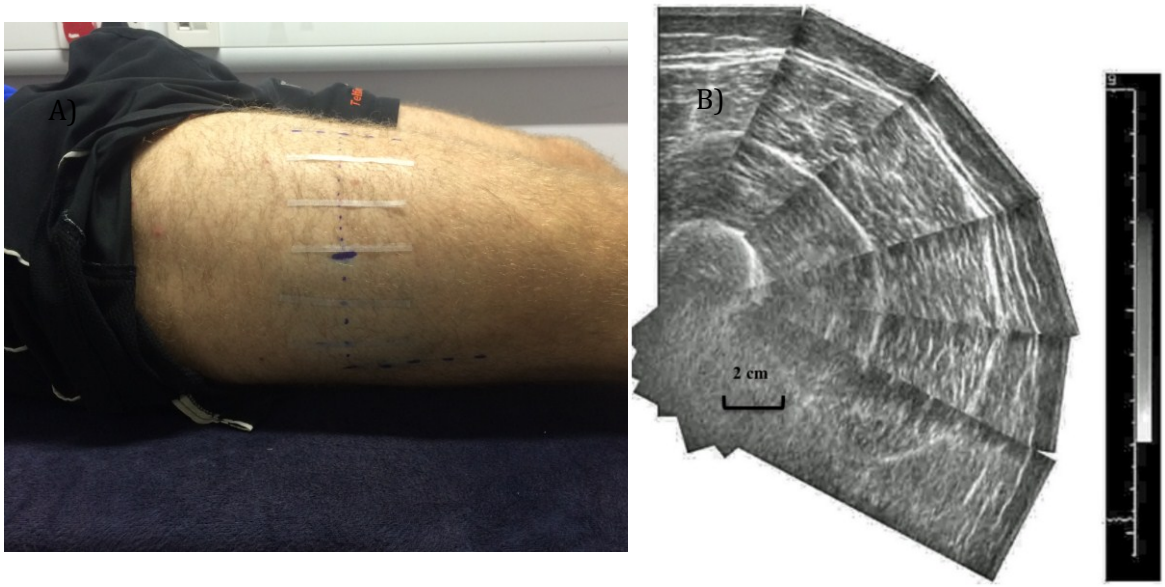

Figure 2. 2: A) Example of echo-absorptive markers at 50% of VL length B) Example figure of VL_{ACSA} in one male participant using ultrasound.

2.2.5. Patella tendon length and cross-sectional area

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure patella tendon cross-sectional area (PT_{CSA}) and patella tendon length (PT_L) at a fixed 90° knee angle. The distance between the apex of the patella and the tibial tuberosity, marked using sagittal ultrasound images, was taken as PT_L. To measure PT_{CSA} the ultrasound probe was placed in the transverse plane and images were captured at 25%, 50%, and 75% of PT_L (Figure 2. 3). The images were later analysed offline using ImageJ (1.45, National Institutes of Health, USA (Figure 2. 3), with the mean of all three images being used for further analysis (O'Brien et al., 2010).

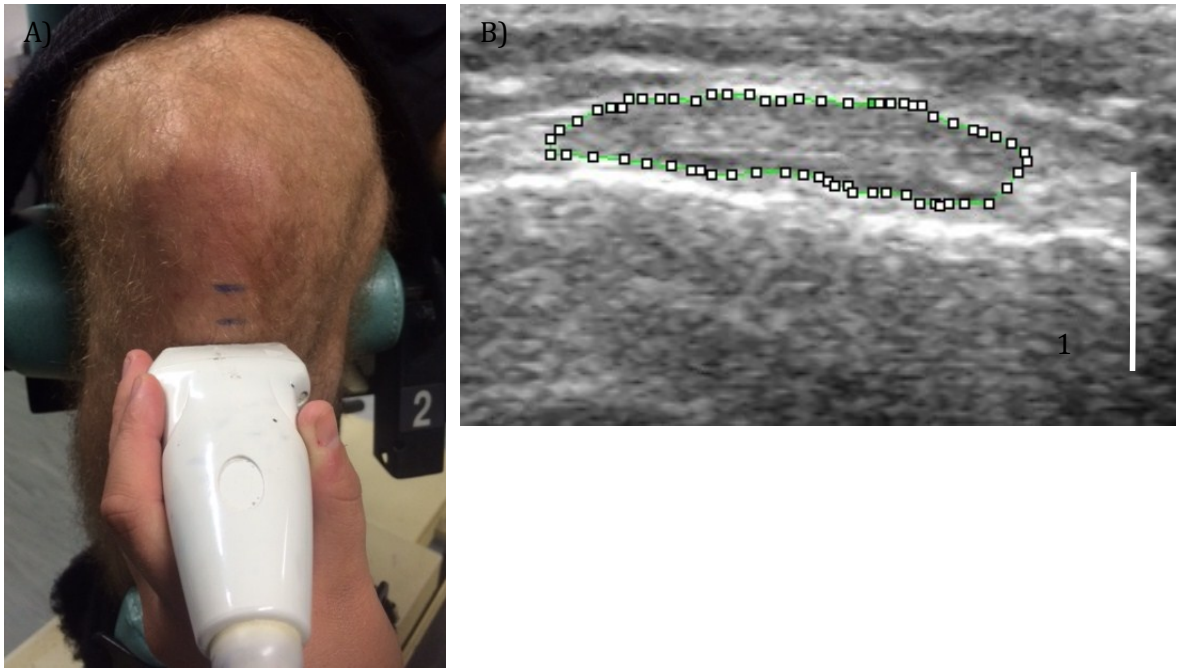

Figure 2. 3: A) Example of the ultrasound probe placed in the transverse plane at 50% of patella tendon length. B) Example figure of PT_{CSA} at 50% of patella tendon length. Area measured using ImageJ (1.45, National Institutes of Health, USA).

2.2.6. Patella tendon elongation

The participants were seated in the isokinetic dynamometer, with the knee angle fixed at 90°, and were instructed to perform a ramped, isometric MVC_{KE} lasting ~5-6 seconds. Ramped MVC_{KE} torque and displacement of the patella tendon were synchronised using a voltage (10-V) square wave signal generator. Patella tendon displacement was measured over two MVC_{KE}, once with the ultrasound probe positioned over the distal edge of the patella and on the second contraction over the tibial tuberosity (Onambélé et al., 2007). Total tendon displacement was computed from the composite of proximal and distal patella motions (see below). Torque was presented on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter and subsequently analysed with the accompanying software (Acknowledge, Biopac Systems, Santa Barbara, CA). To create an external marker on the

ultrasound images, an echo-absorptive marker was placed on the skin. Using the marker to calculate displacement, the distance of the marker (shadow) from an anatomical reference point at the beginning of the contraction, to the position of the shadow at the end of the contraction was calculated (Figure 2. 4). Images were captured at ~10% intervals of ramped MVC_{KE} torque (Onambélé et al., 2007). Total patella tendon displacement was calculated as displacement at the apex of the patellar plus the displacement at the tibial tuberosity (Onambélé et al., 2007).

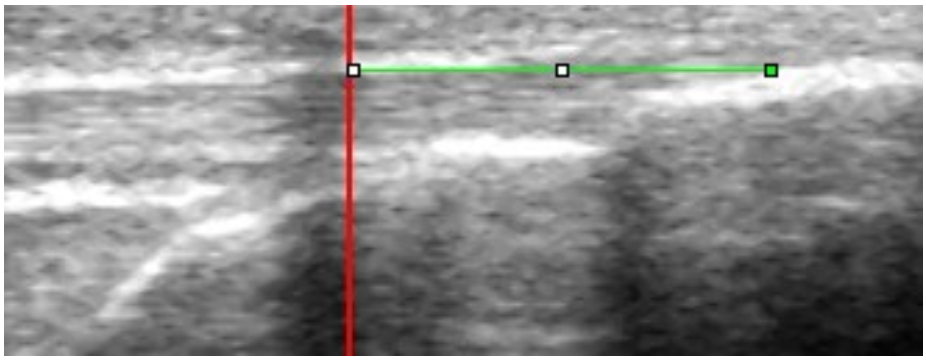

Figure 2. 4: Example of measuring distal patella tendon lengthening using ultrasound. Red line emphasise the shadow cast by the echo-absorptive marker on the skin.

Patella tendon moment arm was measured at 90° knee angle (full extension = 0°) in the sagittal plane (Figure 2. 5), from a single energy (frame 23.3 cm × 13.7cm) DEXA scan (Hologic Discovery, Vertec Scientific Ltd, UK). Using OsiriX (DICOM viewer, ver. 4.0, Pixemo, Switzerland), PT_{MA} length was determined as the perpendicular distance from the centre of the patella tendon to the estimated tibio–femoral contact point ((Baltzopoulos, 1995; Erskine et al., 2014) Figure 2. 5). Compared to the MRI, the DEXA has been reported a reliable and valid technique when measuring moment arm length (Erskine et al., 2014)

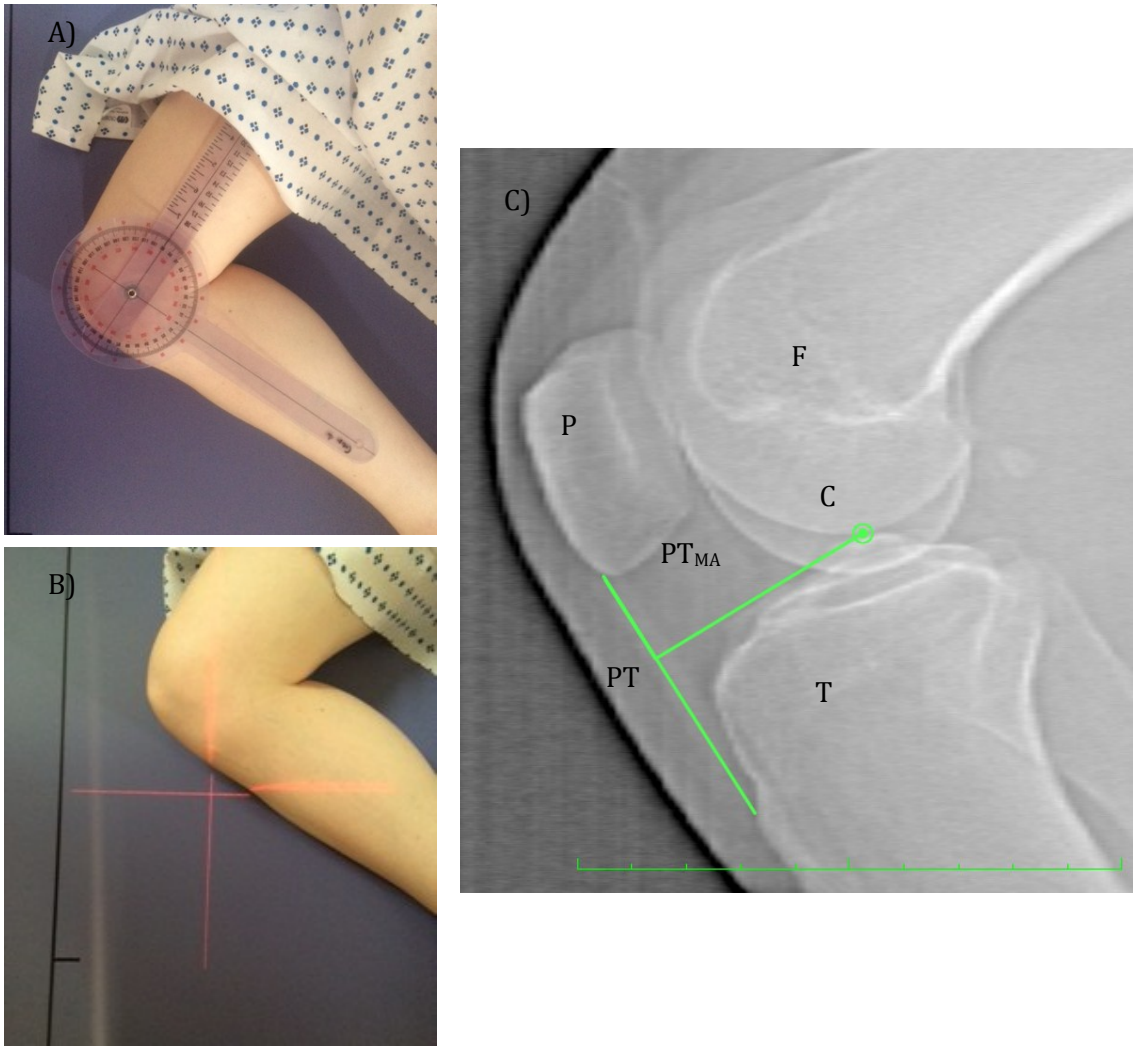

Figure 2. 5: A) Representative image of a participant positioned on the DEXA bed with the knee set at 90° B) field of view correctly identified using laser beam C) single energy image to measure patella tendon moment arm (PT_{MA}). P: Patella, F: Femur, T: Tibia, PT: Patella tendon and C: Tibio–femoral contact point.

2.2.7. 'Damaging' eccentric exercise

Prior to eccentric exercise, a warm-up of 10 isokinetic knee extensions and knee flexions were carried out at $60°{\cdot}s^{-1}$, ensuring a progressive increase in effort (with the last contraction being maximal). A maximal effort contraction performed during the warm up has been

reported to reduce systematic error in repeated eccentric contractions (Almosnino et al., 2012). For the eccentric exercise, the knee extension range of motion was set at 20 – 90° (0° = full extension). Participants were asked to perform 12 MVE_{KE} repetitions. The eccentric phase of the contractions was performed at an isokinetic angular velocity of $30°·s^{-1}$ (Jamurtas et al., 2005). The concentric phase was performed sub-maximally at an angular velocity of $60°·s^{-1}$ to minimise fatigue and enhance eccentric damage (Chapman et al., 2006). The combination of visual and verbal feedback has been reported to increase maximal efforts when compared to no feedback or just verbal encouragement (Campenella et al., 2000). Therefore visual feedback and verbal encouragement was continuously provided throughout the protocol. Torque was presented, in real time, on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter (Biopac Systems, Santa Barbara, CA). Torque measurements were later analysed offline with the accompanying software (Acknowledge, version 3.9.2). Peak MVE_{KE} torque was determined as the highest torque out of the 12 repetitions.

2.2.8. Change in vastus lateralis fascicle length during the eccentric protocol

To measure VL fascicle length during MVE_{KE}, the ultrasound probe was fixed at 50% of VL muscle length in the mid-sagittal plane of the non-dominant leg. During the 12 MVE_{KE} contractions, ultrasound images were recorded onto a PC, in real time, at 25 frames per second (Adobe Premier pro Version 6, Adobe Systems Software, Ireland). An externally generated square wave signal was used to synchronise the ultrasound images with the torque acquisition system. Three MVE_{KE} contractions were chosen from the set of 12 repetitions for architectural analysis. Using frame capture software (Adobe Premier Elements, version 10), an ultrasound image (frame corresponding to every 10° of knee angle, ranging from 20 - 90°) was acquired for offline analysis. To ensure there was no movement artefact included in the measurement of fascicle length, an echo-absorptive marker was fixed on the skin to provide a visual reference point for the internal structures. If movement of the reference line (relative

to its baseline position on the ultrasound screen gridline) was observed, the contraction was discarded and another repetition was chosen for analysis. Using an external marker to calculate change in muscle architecture is a common procedure, however it assumes that the marker, skin and ultrasound do not move during the contraction. Using the same ultrasound method to measure displacement of the *gastrocnemius medialis* MTU under passive stretch, Morse et al. (2008) reported the ultrasound probe translated distally by 1.8 ± 0.2 mm. However, the movement of the ultrasound probe, marker or skin has not been measured during MVE_{KE} (see below).

Using digitising software (ImageJ 1.45, National Institutes of Health, USA), VL fascicle length was analysed offline at every 10°. Fascicle length was measured from the visible insertion of the fibre into either the deep and superficial aponeurosis (Reeves & Narici, 2003). Where the fascicle extended longer than the ultrasound image (frame width 3.50 cm and height 4.15 cm), linear continuation of the fascicle and aponeurosis was assumed (Figure 2. 6). To reduce error associated with the estimation of VL fascicle length, an average of three fascicles across the image was taken (Guilhem et al., 2011). Change in fascicle length during eccentric contractions was measured at 10° increments throughout the MVE_{KE}. Using the ultrasound to track lengthening of the muscle tendon junction, Morse (2011) has reported a mean error of 0.06 mm, with a high coefficient correlation (r = 0.999). Morse (2011) therefore evidences that ultrasound is reliable when measuring small increases in architecture, i.e. fascicle lengthening. Absolute fascicle length is presented as fascicle length from 20° to 90°, change in fascicle length is then made relative to fascicle length measured at a knee angle of 20°. Due to the sparse data regarding the reliability of absolute and relative fascicle lengthening during MVE_{KE}, a total of 13 participants were used rather than 8 used in the other measures of tendon and muscle architecture.

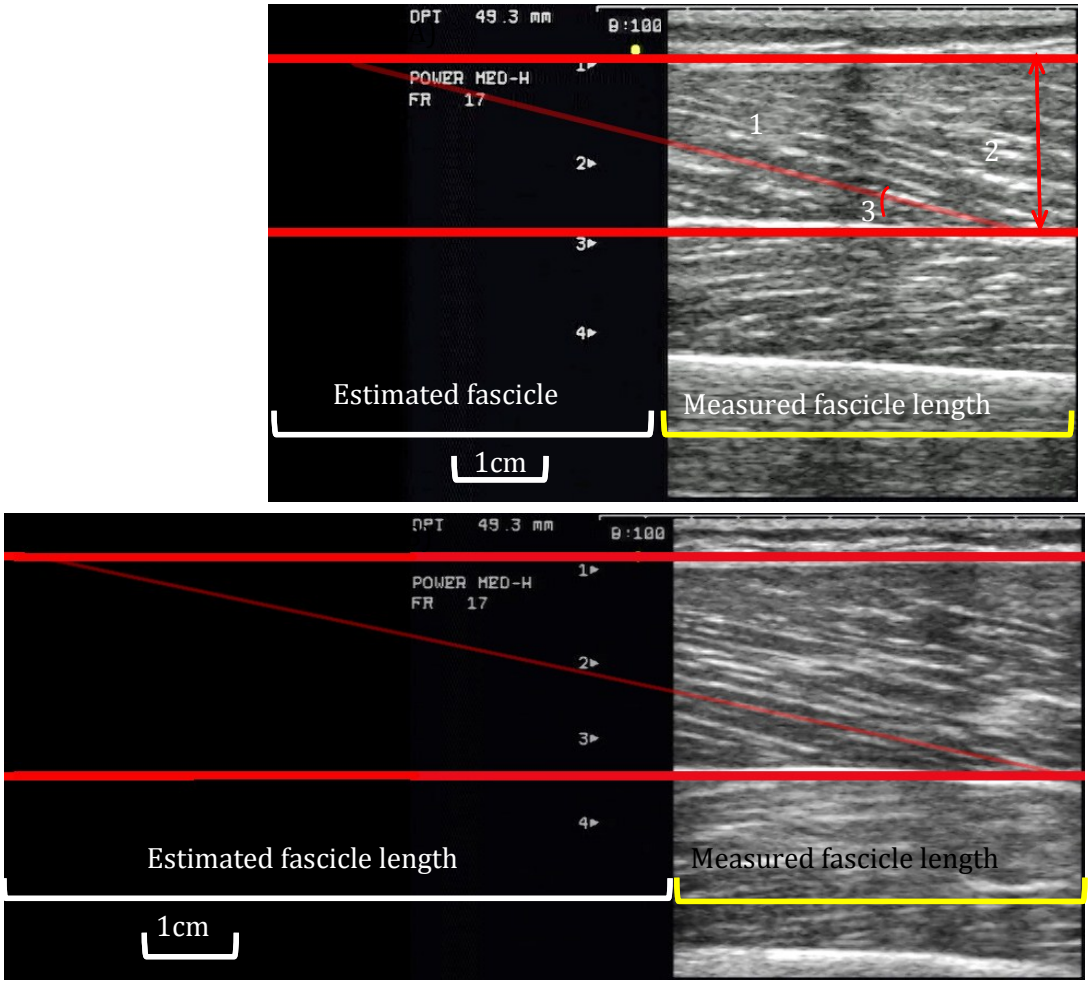

Figure 2. 6: Example of the linear extrapolation method used to calculated total vastus lateralis (VL) fascicle length A) at rest (full leg extension) B) at 90° knee angle during maximal voluntary eccentric knee extensions (MVE_{KE}). At rest and under MVE_{KE} estimated fascicle length was 48% and 67% (respectively) of total fascicle length. 1: Total fascicle length, 2: Muscle thickness, 3: VL pennation angle.

2.2.9. Ultrasound probe movement

As mentioned previously, the ultrasound method used to measure fascicle lengthening during MVE_{KE} relies on the assumption that the ultrasound probe does not move. Although ultrasound probe movement has been measured during stretch of the *gastrocnemius medialis*

(Morse et al., 2008), ultrasound probe movement has yet to be measured during $MVE_{KE.}$ Thus to measure whether the ultrasound probe moved whilst measuring fascicle lengthening during MVE_{KE}, an external video camera (Casio EX-F1, 60 frames per second) fixed to a three-legged tripod, was aligned in parallel with the non-dominant leg. At the distal end of the ultrasound probe, echo absorptive tape was placed on the skin as an external marker. During the 12 MVE_{KE}, images were captured at 20° knee angle (0° = full extension) and 90° knee angle, as indicated by the isokinetic chair. Using digitising software (ImageJ 1.45, National Institutes of Health, USA) probe movement (forward and backwards) was measured offline. Movement of the probe was determined as the change in distance from the distal edge of the probe to the external marker on the skin, from 20° to 90° knee angle.

2.2.10. Statistics

The difference between the mean values of both testing days was plotted on a scatter plot to assess the presence of heteroscedasticity. The corresponding association was assessed using Pearson's product moment correlation. All data was homoscedastic therefore log transformation was not required. To ensure the data were parametric, the Shapiro-Wilk and Levene's tests were utilized to assess the normality and variance of the data, respectively. These statistical assumptions were not breached. Inter-reliability of tendon and muscle properties between day one and day two, were analysed with a one-way random effects ICC model. The coefficient of variation, independent T-test and 95% limits of agreement (LoA) were reported to assess the agreement between day one and two for tendon and muscle properties (Atkinson & Nevill, 1998). All data is presented as mean ± standard deviations.

2.3. Results

2.3.1. Vastus lateralis muscle architecture

There was significant ICC between day one and day two measurements of all muscle architecture measures (Table 1. 2). Coefficient of variations remained below 6% for all architectural measures at rest (Table 1. 2). There was no significant systematic bias between day one and day two for resting muscle architecture: VL fascicle length (5.13 ± 1.00 cm, 5.12 ± 0.99 cm, respectively, p = 0.99 (*n* = 8)), muscle thickness (3.60 ± 0.53 cm, 3.57 ± 0.45 cm, respectively, p = 0.93, (*n* = 8)), pennation angle (15.9 ± 3.0°, 15.8 ± 3.0°, respectively, p = 0.40, (*n* = 8)), VL_{ACSA} (22.4 ± 2.7 cm^2, 22.4 ± 2.7 cm^2, respectively, p = 0.98, (*n* = 8)) and VL length (30.0 ± 2.0 cm, 30 ± 2.0 cm, respectively, p = 1.00, (*n* = 8)). The ICC, coefficient of variance, and 95% absolute limits of agreements are shown in Table 1. 2. Figure 2. 7 shows Bland-Altman plots for resting muscle architecture using 95% confidence interval levels.

2.3.2. Patella tendon properties

There was significant ICC between day one and day two measurements of all tendon properties measures (Table 1. 1.). There was no significant systematic bias between day one and day two for resting tendon measures: PT_L (5.02 ± 0.04 cm, 5.04 ± 0.05 cm, respectively, p = 0.19, (*n* = 8)), PT_{CSA} (85.0 ± 13.2 mm^2, 84.0 ± 12.9 mm^2, respectively, p = 0.18, (*n* = 8)), PT_{MA} (4.30 ± 0.30 cm, 4.32 ± 0.31 cm, p = 0.459, (*n* = 8)) and relative PT elongation (5.55 ± 1.26 cm, 5.88 ± 1.32 cm, respectively, p = 0.73, (*n* = 8)). The ICC, coefficient of variance and 95% absolute limits of agreements are shown in Table 1. 1. Figure 2. 8 shows Bland-Altman plots for patella tendon properties using 95% confidence interval levels.

Table 1. 1. The reliability of B-mode ultrasound when measuring patella tendon properties at rest and during MVC_{KE}.

	ICC	CV (%)	LoA (95%)
PT length (mm)	0.58	0.69	0.02 ± 0.09
PT_{CSA} (cm^2)	0.99	3.50	0.01 ± 0.04
PT elongation (cm)	0.97	5.98	0.17 ± 0.67
PT_{MA} (cm)	0.97	0.45	0.02 ± 0.04

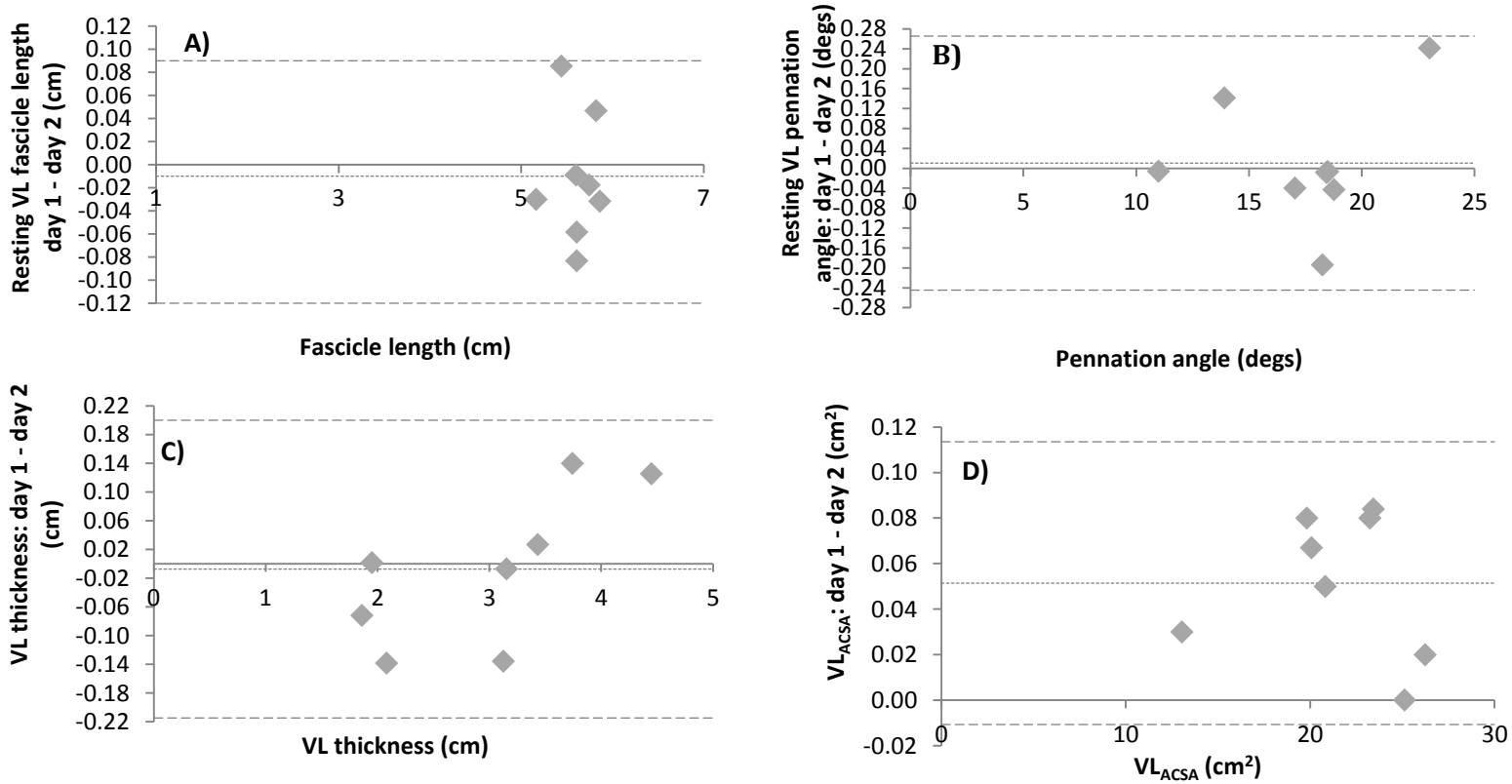

Figure 2. 7: Bland Altman plots identifying the bias (small dashed line) and the limits of agreement (large dashed line, ± 1.96) between day one and day two for the measurements of A) VL resting fascicle length, B) VL resting pennation angle, C) VL resting thickness and D) VL anatomical cross-sectional area.

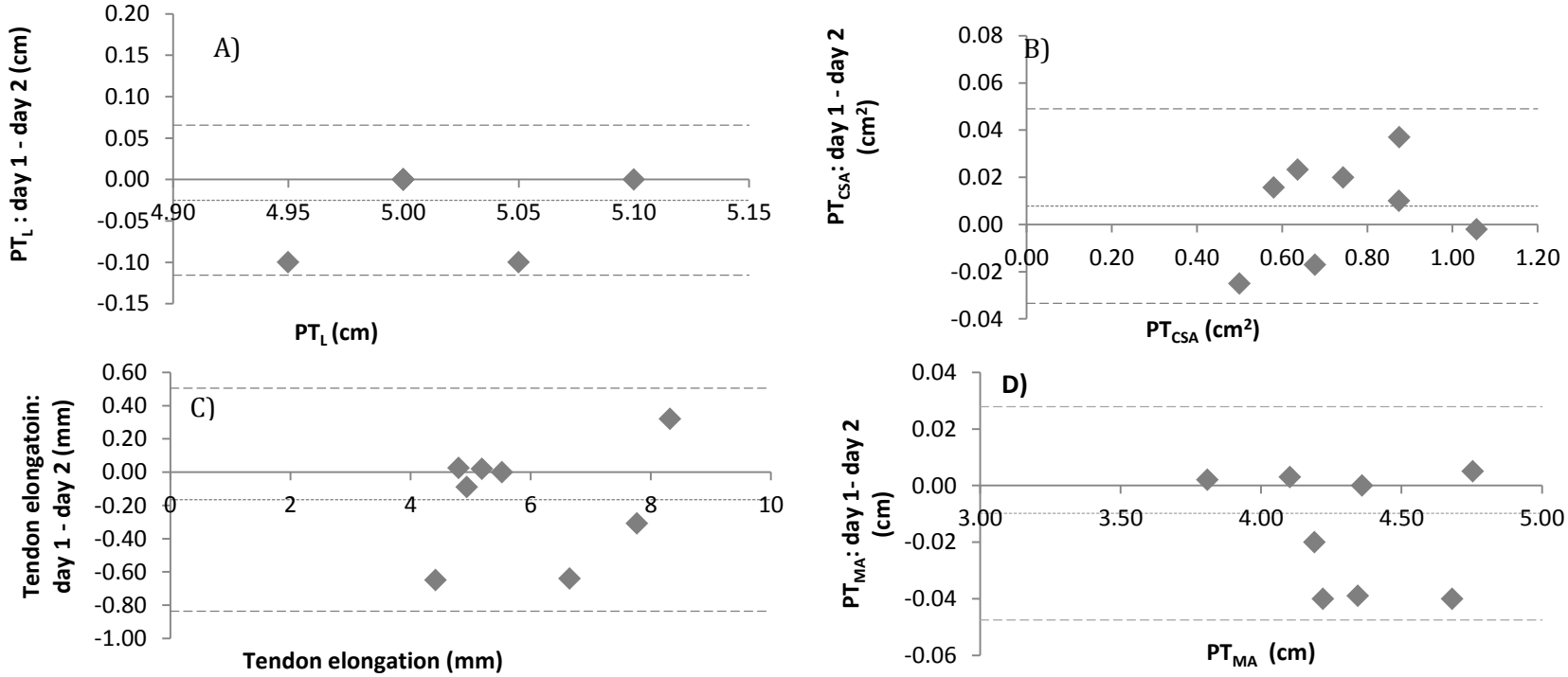

Figure 2. 8: Bland Altman plots identifying the bias (small dashed line) and the limits of agreement (large dashed line, ± 1.96) between day one and day two for the measurements of A) PT_L: Patella tendon length, B) PT_{CSA}: Patella tendon cross-sectional area, C) Patella tendon elongation during maximal isometric knee extension and D) PT_{MA}: Patella moment arm at 90° knee angle.

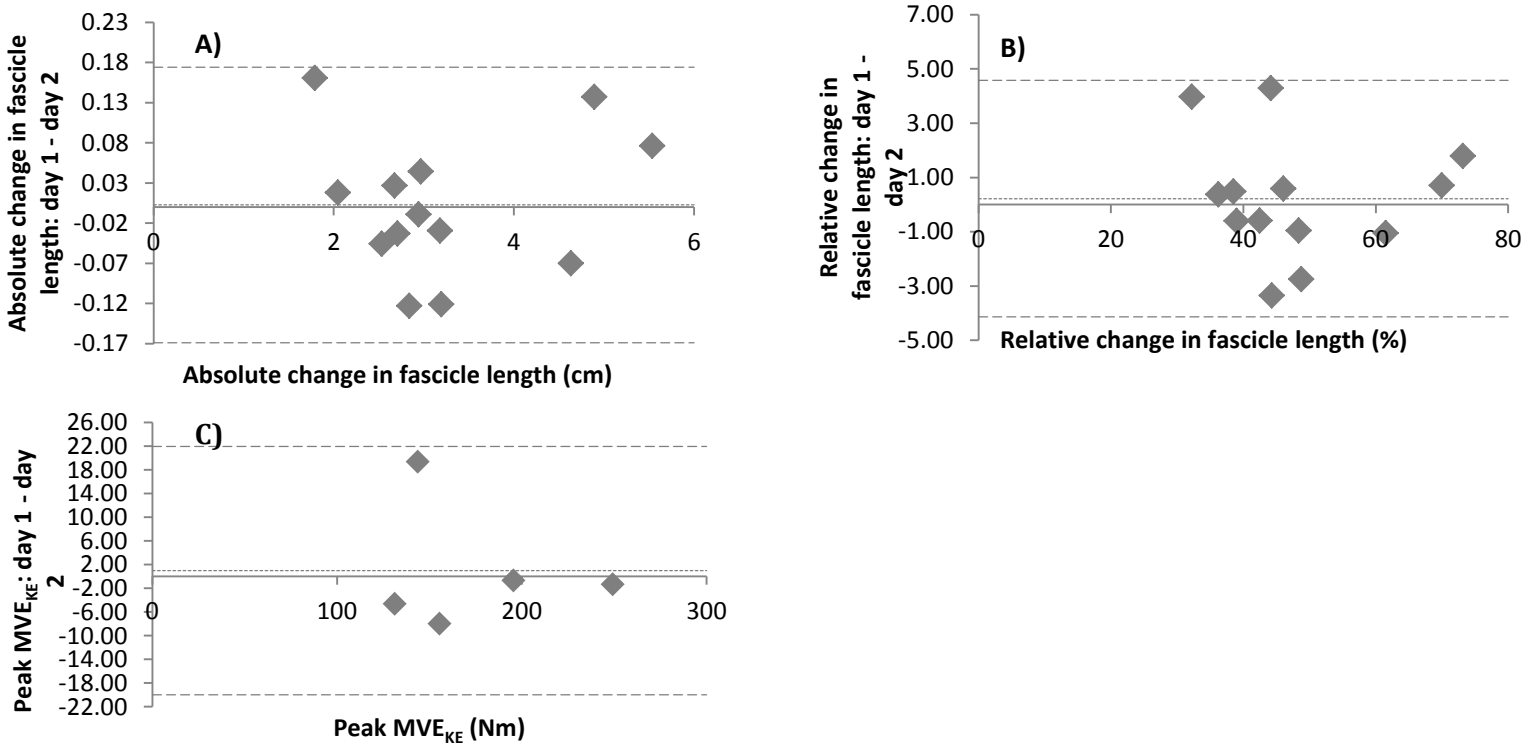

Figure 2. 9: Bland Altman plots identifying the bias (small dashed line) and the limits of agreement (large dashed line, ± 1.96) between day one and day two for the measurements of A) Absolute change in VL fascicle length from 20° to 90° knee angle (0°= full extension) B) Relative change in VL fascicle length, and C) Peak MVE_{KE}: Maximal voluntary eccentric knee extension.

2.3.3. Vastus lateralis properties during eccentric contractions

There was significant ICC between day one and day two measurements of all muscle architecture measures (Table 1. 2). There was no significant systematic bias between day one and day two for absolute fascicle lengthening (2.47 ± 1.15 cm, 2.46 ± 0.71 cm, respectively, p = 0.46, (*n* = 13)), relative fascicle lengthening (40.5 ± 3.9%, 40.4 ± 3.85%, respectively, p = 0.492, (*n* = 13)), and MVE_{KE} (176 ± 47 Nm, 175 ± 49 Nm, respectively, p = 0.488, (*n* = 8)). The ICC, coefficient of variance and 95% absolute limits of agreements are shown in Table 1. 2. Figure 2. 9 shows the Bland-Altman plots for fascicle lengthening and MVE_{KE} using 95% confidence interval levels.

Table 1. 2. The reliability of B-mode ultrasound when measuring muscle properties at rest and during MVE_{KE}.

	ICC	CV (%)	LoA (95%)	
Resting fascicle length (cm)	0.99	1.12	0.01	± 0.11
Resting pennation angle (°)	0.99	0.68	0.01	± 0.26
Muscle thickness (cm)	0.99	3.76	0.01	± 0.21
VL_{ACSA} (cm^2)	1.00	0.33	0.05	± 0.06
VL length (cm)	1.00	0.00	0.00	± 0.00
MVE_{KE} (Nm)	0.99	5.38	0.95	± 21.0
Absolute Fascicle lengthening (cm)	0.99	2.95	0.00	± 0.17
Relative fascicle lengthening (%)	0.99	4.77	0.22	± 4.36

2.3.4. Ultrasound probe movement

The movement of the ultrasound probe when measuring fascicle lengthening from 20° knee angle to 90° knee angle during MVE_{KE} was 0.02 ± 0.05 cm proximally. Therefore, ultrasound

probe movement in the current chapter represents a 0.6% error in change in fascicle length (average change in fascicle length 3.2 cm) during MVE_{KE}.

2.4. Discussion

The main aim of the current chapter was to determine whether B-mode ultrasound is a reliable imaging technique for repeated measures of muscle and tendon properties at rest and under isometric and MVE_{KE} contractions. Three main findings merit highlighting: 1) ultrasound is a reliable method for measuring PT_L and PT_{CSA} at rest and patella tendon lengthening under maximal isometric contraction, 2) ultrasound is a reliable method for measuring resting VL architecture (fascicle length, pennation angle and muscle thickness) and 3) ultrasound is a reliable method for measuring fascicle lengthening during MVE_{KE}.

The measurement of Young's modulus is dependent on the accurate measurement of PT_{CSA} and PT_L. It is therefore essential to ensure resting tendon measurements are accurate to prevent errors in subsequent calculations of Young's modulus. In agreement with previous literature (Kubo et al., 2003), the current chapter reported ultrasound to be a reliable technique when measuring tendon properties, specifically PT_{CSA} and PT_L. Kubo et al. (2003) reported a high correlation coefficient of 0.97 between two separate measurements of PT_{CSA}. Although a correlation coefficient of 0.97 is high (Taylor, 1990), correlation coefficients can be affected by large variability and do not account for systematic bias (Bates et al., 1996; Bland & Altman, 1996; Atkinson & Nevill, 1998). To account for systematic bias, ICCs are commonly used in place of correlation coefficients in reliability analysis (Atkinson & Nevill, 1998). In agreement with the current chapter, Onambélé et al. (2007) reported high ICCs for patella tendon elongation, PT_{CSA} and isometric patella tendon knee extension torque (0.91, 0.96 and 0.99 respectively). Although ICCs are an acceptable measure of reliability, the output could be skewed by sample heterogeneity (Bland & Altman, 1990). It is therefore

recommended that ICCs should not be used as a sole indicator of reliability and should be supported by the use of Bland-Altman plots (Bland & Altman, 1990; Atkinson & Nevill, 1998). The triangulation of ICC, coefficient of variance and limits of agreement reported within the current chapter indicates high reliability when using ultrasound to measure patella tendon properties at rest and under maximal contraction.

Following exercise training programmes adaptations in resting muscle architecture are used to measure the training response, for example Baroni et al. (2013) reported VL fascicle length to increase by 1.57 cm (19.4%) following 12 weeks of eccentric training. Thus, high reliability of imaging techniques is crucial to avoid under / over estimation of changes to resting muscle architecture (Klimstra et al., 2007). In agreement with previous research the current chapter can conclude that ultrasound is a highly reliable technique for measuring resting VL muscle architecture (De Boer et al., 2008; Alegre et al., 2006). In agreement with the ICCs reported in the current chapter, Alegre et al. (2006) reported ICCs of 0.95, 0.96 and 0.96 for resting VL fascicle length, muscle thickness and pennation angle respectively. It remains unknown however, if the high ICCs presented by the current chapter and by Alegre et al. (2006) are skewed by the large variability (a known limitation of ICCs (Bland & Altman, 1990)) associated with resting muscle architecture (Stebbings et al., 2013; Weir, 2005). By using a triangulation of methods, ICC, coefficient of variance and Bland-Altman plots, the current chapter demonstrates high agreement and low bias. Thus the current chapter can confirm that the ultrasound is a reliable measurement for resting VL muscle architecture, including fascicle length, pennation angle and muscle thickness.

In addition to resting muscle architecture, the introduction of ultrasound has enabled fascicle behaviour to be directly measured during contraction (Fukunaga et al., 1997). Due to a small field of view (typically 38 mm wide) on the ultrasound probe, extrapolation and partial estimation of the fascicles is required to calculate total fascicle length. Within the VL, a 2-7% error is associated with the linear extrapolation method when used to calculate fascicle length at a knee angle of 120° (Finni et al., 2003a). However, under maximal isometric

contraction, a 2.4% error has been associated with estimating fascicle length in the tibialis anterior (Reeves et al., 2003). The greater error within the VL may be attributed to the greater fascicle length (~11.3 cm) in the VL (Finni et al., 2003a) compared to the *tibialis anterior* (~5.4 cm) (Reeves et al., 2003), thus a greater portion of the fascicle may have to be estimated within the VL. For example within the current chapter, at rest a VL fascicle length of 6.91 cm required 48% of the fascicle length to be estimated, whereas during MVE_{KE} a VL fascicle length of 10.4 cm at 90° knee angle required 66.7% of the fascicle to be estimated (Figure 2. 6). Furthermore, greater error reported from the VL may be attributed to, unlike during isometric contractions and at rest, fascicles lengthening during eccentric contractions (Reeves & Narici, 2003). Although the validity of the linear extrapolation method has been unreported in the VL during MVE_{KE}, the fascicle length we report is similar to those reported by Finni et al. (2003a), thus similar measurements of error (2-7%) can be assumed. The current chapter confirms that during eccentric contractions the ultrasound can be used as a reliable imaging technique, despite greater estimation of fascicle length, to directly calculate fascicle lengthening during MVE_{KE}. Our findings concur with Guilhem et al. (2011) who reported ICCs of 0.93-0.95, 0.82-0.88, and 0.75-0.87 for muscle thickness, fascicle angle and fascicle length, respectively in the VL, during MVE_{KE}. Unlike Guilhem et al. (2011), to account for systematic error, the current chapter combined ICCs and Bland-Altman plots. Supported by a triangulation of reliability statistics and an extremely low bias (0.0025 cm), the current chapter can therefore conclude that ultrasound is a reliable method of measuring fascicle length *in vivo* during MVE_{KE}.

During eccentric contractions the muscle lengthens rapidly under high torque, therefore it may be difficult to maintain probe placement. Morse et al. (2008) reported the ultrasound probe translated distally by 1.8 ± 0.2 mm during stretch of the *gastrocnemius medialis*, however probe movement during MVE_{KE} has yet to be investigated. The current chapter however, can confirm that the probe movement when measuring fascicle lengthening during MVE_{KE} was negligible, accounting for 0.6% measurement error in change in fascicle

length during MVE_{KE}. Thus, providing further confidence that the measurements can be attributed to fascicle lengthening and not probe movement.

When measuring maximal voluntary contractions an assumption is made that the participants are contracting maximally. The adherence to the instructions to push maximally is crucial when investigating MVE_{KE} and exercise-induced muscle damage. Introducing a maximal effort within the warm up has been reported to increase the probability of the participants to contract maximal in repeated measures of isometric torque (Almosnino et al., 2012). Although high ICCs (0.96) have been reported when using the isokinetic dynamometer to measure MVE_{KE} torque at $60°·s^{-1}$ (Impellizzeri et al., 2008), the low bias and CVs within the current chapter can conclude high reliability when MVE_{KE} are performed at a slower speed ($30°·s^{-1}$).

2.5. Conclusion

The current chapter can conclude that the ultrasound is a highly reliable technique to measure resting architecture in both tendon and muscle. Therefore, the current chapter supports the use of ultrasound as an alternative method for measuring muscle and tendon morphology when an MRI is impractical, e.g. within stroke patients (Singer et al., 2004). Furthermore, during MVE_{KE} the ultrasound is a highly reliable technique to measure change in muscle architecture, specifically VL fascicle lengthening, with negligible probe movement. Due to the repeated use of the same techniques and methods throughout this thesis, the findings of this chapter provide confidence that the results within the subsequent chapter of the current thesis are true and not confounded by measurement error.

Chapter three: Sex differences in fascicle lengthening during eccentric contractions: the role of the patella tendon stiffness.

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3.1. Abstract

Aim: Tendons have been suggested to attenuate fascicle lengthening during eccentric contractions however there is no *in vivo* evidence to support this hypothesis. Therefore, the aim of this chapter was to determine whether patella tendon stiffness modulates *vastus lateralis* fascicle lengthening during eccentric contractions in males and females. *Materials and method:* V*astus lateralis* and patella tendon properties were measured in males and females owing to previously reported intrinsic sex differences in tendon properties. During maximal voluntary eccentric knee extensions, *vastus lateralis* fascicle lengthening and torque was recorded at every 10° (range of motion 20-90°). *Results:* A significant correlation between maximal patella tendon stiffness and change in fascicle length ($r = 0.476$, $p = 0.023$) was observed. Similarly, there was a significant correlation between maximal patella tendon Young's modulus and change in *vastus lateralis* fascicle length ($r = 0.470$, $p = 0.049$). As expected, patella tendon stiffness and Young's modulus were significantly higher in males compared to females ($p \leq 0.05$). Interestingly, change in *vastus lateralis* fascicle length during the eccentric contractions was significantly greater in males compared to females ($p \leq 0.05$). Based on patella tendon moment arm (PT_{MA}) measurements, *vastus lateralis* muscle-tendon unit elongation was estimated to be significantly greater in males compared to females (5.24 cm and 4.84 cm, respectively) *Conclusion:* The significant difference in fascicle lengthening during eccentric contractions may be partly explained by the significantly higher PT_{MA}, patella tendon stiffness and Young's modulus found in males compared to females. The current chapter provides *in vivo* evidence to support the hypothesis that the tendon acts as a 'mechanical buffer' during eccentric contractions.

3.2. Introduction

Historically eccentric contractions are considered and often defined as "muscle lengthening" (e.g. (Lindstedt et al., 2001)). However, with the advent of the sonomicrometry method pioneered by Griffiths (1987) and B-mode ultrasonography (Fukashiro et al., 1995), the quantification of muscle lengthening during eccentric contractions has been directly investigated. Although the findings from a small sub-population within chapter two suggest that *vastus lateralis* (VL) fascicles lengthen during knee extension, previous eccentric work has concluded different fascicle behaviour. During the stance phase in a freely walking cat, Griffiths (1991) found that muscle fascicles were shortening despite an increase in muscle tendon unit (MTU) length. In agreement with Griffiths (1991), Spanjaard et al. (2007) reported that during the eccentric phase of stair descent, the *gastrocnemius medialis* fascicles shortened despite a lengthening of the MTU. In contrast, Fukunaga et al. (2001) reported *gastrocnemius medialis* fascicles to contract quasi-isometrically during the stance phase of human walking, despite significant lengthening of the MTU. Although some of the discrepancy in fascicle lengthening can be attributed to differences in, joint range of motion and gait patterns during flat walking versus stair decent (Spanjaard et al., 2007), both Spanjaard et al. (2007) and Fukunaga et al. (2001) reported that the lengthening of the MTU during the eccentric phase, occurs predominately at the tendon rather than at the muscle fascicles.

It is widely accepted that tendons are not inextensible and are in fact a viscoelastic tissue that deforms under loading (Magnusson et al., 2008). The degree of deformation (elongation) depends on the level of loading and intrinsic stiffness of the tendon (Maganaris & Paul, 1999). Non-invasive techniques such as B-mode ultrasonography allow the in-series muscle and tendon behaviour to be measured independently, at rest and during locomotion (Fukunaga et al., 1996; Cronin & Finni, 2013; Fukashiro et al., 1995). Understanding the interaction between the tendon and muscle is essential to fully understand the contribution of each to everyday tasks such as postural balance (Onambele et al., 2006). The shortening or

quasi-isometric behaviour of fascicles during eccentric contractions has previously been attributed to the tendon lengthening under load (Spanjaard et al., 2007; Fukunaga et al., 2001; Reeves & Narici, 2003). The greater proportion of MTU lengthening occurring at the tendon, reduces the metabolic cost of muscle contractions (Roberts & Scales, 2002) and economy of walking is enhanced (Fukunaga et al., 2001). The impact of tendon properties on fascicular elongation during eccentric loading is further demonstrated through the role that tendon stiffness plays in explaining group differences in fascicular elongation (Mian et al., 2007). The *gastrocnemius medialis* fascicles during the eccentric stance phase of gait, in the elderly and young, are indeed reported to behave either quasi-isometrically (in the old) or to lengthen (in the young) (Mian et al., 2007). With these differences in fascicle behaviour being attributed to tendon stiffness decreasing with age (Onambele et al., 2006) thus attenuating fascicle lengthening in the elderly. To explain the interaction between tendon stiffness and fascicle displacement under loading, Lichtwark et al. (2007) used a three element Hill muscle model. Using the Achilles tendon and the *gastrocnemius medialis* fascicle lengthening during the eccentric stance phase of walking they predicted increasing Achilles tendon stiffness increased the required amount of fascicle lengthening (Lichtwark & Wilson, 2007).

The interaction between the tendon and muscle fascicles have also been reported within the VL during eccentric tasks, such as drop jumps and countermovement jumps (2001). Finni et al., (2001) reported the magnitude of VL fascicle lengthening during the eccentric phase was smaller in the drop jump compared to the countermovement jump. Due to similar MTU lengthening, they suggested the difference of fascicle lengthening could be attributed to the greater tendon elongation required in the drop jump. These findings suggest a modulating role of the patella tendon on the degree of VL fascicle lengthening, which is dependent on the movement being performed (Ishikawa et al., 2003). Thus, the above supports the hypothesis that the tendon may act as a 'mechanical buffer' (Reeves & Narici, 2003; Roberts & Azizi, 2010).

The role of the tendon in attenuating fascicle loading during eccentric contractions is particularly relevant when trying to address the previously reported sex differences in response to eccentric contractions (Sewright et al., 2008). It is well established that males have higher tendon stiffness than females (Kubo et al., 2003; Onambélé et al., 2007), which is associated with a direct impact of raised oestrogen levels on tendon properties (Bryant et al., 2008). For example, Onambélé et al. (2007) reported patella tendon stiffness to be 57% higher in males compared to females. Even when corrected for tendon length and patella tendon cross-sectional area (PT_{CSA} (Young's modulus)), males' tendon stiffness remained 12% higher than females'. It is these sex differences in tendon properties that have previously been alluded to as the mechanism by which adult males experience greater levels of muscle damage following eccentric exercise compared to boys (Marginson et al., 2005). Although a lengthening of VL fascicles during eccentric knee extensions has been shown within chapter two and by Guilhem et al. (2011), to the authors' knowledge, it is not known whether a sex difference in the elongation of muscle fascicles during eccentric contractions exists.

Therefore, the aim of the present chapter was to determine whether patella tendon stiffness alters VL fascicle lengthening during eccentric contractions in males and females. In view of the evidence that males have stiffer tendons than females, it is hypothesised that fascicular lengthening during eccentric contractions would be greater in males compared to females.

3.3. Materials and methods

3.3.1. Subjects

Nine males (22 ± 2 years of age, 71.9 ± 13.1 kg and 173 ± 8 cm) and nine females (21 ± 2 years of age, 62.2 ± 6.9 kg and 165 ± 7 cm) volunteered to participate in this study. All participants self-reported as being recreationally active (undertaking no more than 1 hour of

"moderate" physical activity per week) and not undertaking any structured training regime. Due to the effects of the oral contraceptive pill on tendon properties (Bryant et al., 2008; Hansen et al., 2009b), females had no history of oral contraceptive pill (OCP) use. Ethical approval was obtained through the Department of Exercise and Sport Science, Manchester Metropolitan University and all participants signed informed consent prior to taking part in the chapter. All procedures complied with the Declaration of Helsinki (World Health Association, 2013). Exclusion criteria were established through participant questionnaire prior to testing. Exclusion criteria included an occupation or lifestyle that required regular heavy lifting or carrying, resistance trained in the last six months, the use of dietary supplements (e.g. Vitamin E), known muscle disorder, and any musculoskeletal injury in the last three months. Further exclusion criteria for female participants included, irregular menstrual cycles (where regular cycles were defined as 24-36 days (Cole et al., 2009; Landgren et al., 1980)), previous use of any other forms of hormone based contraception, and been pregnant or breast fed in the year preceding their application to participate in the present study.

3.3.2. Testing protocol

Participants were asked to visit the laboratory on two occasions, separated by two days; the first session consisted of anthropometric measures (stature and mass), VL anatomical cross-sectional area (VL_{ACSA}), patella tendon moment arm (PT_{MA}), and dynamometer familiarisation. Stature and mass were measured using a wall mounted stadiometer (Harpenden stadiometer, Holtain Crymych, UK) and digital scales (Seca model 873, Seca, Germany) respectively. Familiarisation included two practice MVCs, at two knee joint angles (90 seconds rest). The second laboratory visit consisted of patella tendon stiffness measurements followed by measurement of VL fascicle length during eccentric contractions. All testing was carried out on the non-dominant leg. The non-dominant leg was defined as the leg that provided stability during movements, e.g. kicking. During the tendon properties and eccentric

test protocols, participants were secured (i.e. inextensible straps around the shoulders and hips to reduce any extraneous movement during maximal efforts) in a seated position to an isokinetic dynamometer (Cybex Norm, Cybex International, NY, USA), with a hip angle of 85°. During *pre-damage* the isokinetic dynamometer settings (chair position, distance from the dynamometer, dynamometer arm length) and anatomical zero (leg full extended, knee angle 0°) were recorded to ensure repeatability in the following sessions.

3.3.3. Vastus lateralis anatomical cross-sectional area

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure VL_{ACSA}. Participants laid supine with their leg full extended (knee angle 0°), an ultrasound probe (7.5 MHz linear array probe, 38 mm wide) was used to identify the distal and proximal insertion sites of the VL. At 50% of VL muscle length echo-absorptive markers were placed in parallel, at intervals of 30 mm, from the medial to the lateral edge of the VL muscle. The ultrasound probe was placed perpendicular to the VL muscle in the axial plane. The ultrasound probe was moved slowly across the echo-absorptive markers from the medial to the lateral edge of the muscle. Consistent minimal pressure was placed on the muscle during scanning to avoid compression of the muscle. The images were recorded in real time onto a PC at 25 frames per second (Adobe Premier pro Version 6). At each 30 mm interval, individual images were acquired using capturing software (Adobe Premier Elements, version 10). The shadows cast by the echo-absorptive markers allowed the images to be aligned by the contour of the muscle and the entire VL_{ACSA} to be recreated in a single image (Adobe Photoshop Elements, version 10). The VL_{ACSA} was then measured using digitising software (ImageJ 1.45, National Institutes of Health, USA). This method for calculating VL_{ACSA} has previously been accepted as reliable and valid when compared to MRI, with a reported interclass correlation between 0.998 and 0.999, for reliability and validity respectively (Reeves et al., 2004). Furthermore, chapter two confirms high reliability and low day-to-day measurement error for measuring VL_{ACSA} using the present ultrasound technique.

Within males a VL_{ACSA} of 24.3 cm^2 has previously reported to contribute to ~32% of total quadriceps anatomical cross-sectional area ((Q_{ACSA}) Q_{ACSA} = 74.9 cm^2 (Tsakoniti et al., 2008)). Whereas in females, a VL_{ACSA} of ~21 cm^2 has been reported to contribute to 38% of Q_{ACSA} (Q_{ACSA} = ~55 cm^2 (Grosset & Onambele-Pearson, 2008)). Therefore, assuming all things equal, and the contribution of VL_{ACSA} remains constant within males and females, Q_{ACSA} within males and females was estimated by dividing VL_{ACSA} by 32% and 38% respectively.

3.3.4. Patella tendon length and cross-sectional area

Patella tendon cross-sectional area and patella tendon length (PT_L) were measured, seated in the isokinetic chair with the knee fixed at 90° knee angle. The distance between the tibial tuberosity and the apex of the patella, marked using sagittal ultrasound images, was taken as PT_L. To measure PT_{CSA} the ultrasound probe was placed in the transverse plane and images were captured at 25%, 50%, and 75% of PT_L. The images were subsequently analysed offline using (ImageJ 1.45, National Institutes of Health, USA). A mean of the three images was taken as PT_{CSA} (O'Brien et al., 2010).

3.3.5. Patella tendon moment arm

In order to calculate patella tendon force PT_{MA} was measured at a knee joint angle of 90° (full extension = 0°) in the sagittal plane, from a single energy (frame 23.3 cm x 13.7 cm) DEXA scan (Hologic Discovery, Vertec Scientific Ltd, UK). A goniometer was used to ensure the knee angle was 90°. Using OsiriX, (DICOM viewer, ver. 4.0, Pixemo, Switzerland) the perpendicular distance from the center of the patella tendon to the tibio–femoral contact point was determined as the PT_{MA} length (Baltzopoulos, 1995). Compared to the MRI, the DEXA has been reported a reliable and valid technique when measuring moment arm length (Erskine et al., 2014). Furthermore, chapter two confirms the reliability of DEXA when measuring PT_{MA}.

3.3.6. Force- elongation relationship / patella tendon stiffness

With the knee angle fixed at 90° in the isokinetic dynamometer the participants were instructed to perform a ramped maximal voluntary knee extension (MVC_{KE}) lasting ~6 seconds. Concomitantly, ramped MVC_{KE} torque and displacement of the patella tendon were recorded. Patella tendon displacement was measured over two MVC_{KE}, once with the ultrasound probe positioned over the tibial tuberosity and on the second contraction over the apex of the patella. This is consistent with Onambélé et al. (2007) as a means of obtaining total patella tendon displacement. Torque was displayed on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter (Biopac Systems, Santa Barbara, CA) and subsequently analysed with the accompanying software (Acknowledge, version 3.9.2). Patella tendon displacement was measured by positioning the ultrasound probe (7.5 MHz linear array probe, 38 mm wide) in the axial-plane on the tibial tuberosity (or the apex of the patella during alternate contractions). An echo-absorptive marker was placed on the skin to cast a shadow on the ultrasound and act as an external marker. The distance of the shadow from an internal marker at the beginning of the contraction, to the position of the shadow at the end of the contraction, allows patella tendon excursion to be measured. Ramped MVC_{KE} torque and displacement of the patella tendon were synchronised using a 10-V square wave, signal generator. Images were captured at ~10% intervals of ramped MVC_{KE} torque (Onambélé et al., 2007). Total patella tendon displacement was calculated as displacement at the tibial tuberosity plus the displacement at the apex of the patella (Onambélé et al., 2007). Patella tendon forces were calculated as: (MVC_{KE} torque + antagonist co-activation torque) / PT_{MA}. The measurement of antagonist co-activation torque is described below. The force – elongation curve stemming from data at every 10% MVC_{KE} was then fitted with a second-order polynomial function, forced through zero (Onambélé et al., 2007), and the tangential slope at discreet sections of the curve, relative to MVC_{KE}, was computed by differentiating the curve at each point of interest (i.e. every 10% force intervals). To account for between subject

differences in maximal force production, the slope of the tangential line, corresponding to the MVC_{KE} of the weakest participant (regardless of sex), was computed for each subject. This therefore standardised the comparison of tendon stiffness and Young's modulus at an absolute load, hereafter referred to as standardised force patella tendon stiffness and Young's modulus.

3.3.7. Co-activation

Electromyogram amplitude of the *bicep femoris* (BF) was measured to determine co-activation during the ramped MVC_{KE}. Guided by an axial-plane ultrasound of the BF, two bipolar electrodes (Ambu, Neuroline 720, Malaysia) were placed in the mid-sagittal line at 75% of BF muscle length (distal end = 0%). A reference electrode (Ambu, blue sensor, Malaysia) was placed on the lateral tibia condyle. The electrodes were placed in a bipolar configuration with a constant inter-electrode distance of 20 mm (Hermens et al., 2000). Preceding electrode placement; the skin was shaved, abraded and cleansed with an alcohol wipe to reduce skin impedance below 5000 Ω (Hermens et al., 2000). The participants were asked to contract and relax to ensure correct electrode placement. The raw EMG signal was amplified (×2000) and filtered (through low and high pass filters; i.e. 10 and 500 Hz respectively) with the sampling frequency set at 2000 Hz. Ramped MVC_{KE} torque and BF EMG were recorded in real time and synchronised using a (10-V) square wave signal generator. In the isokinetic dynamometer, participants were asked to perform two maximal voluntary knee flexions (MVC_{KF}) at 90° knee angle. Participants were instructed to perform MVC_{KF} as quickly and as forcefully as possible against the dynamometer's lever arm. The participants were instructed to stop contracting once a two second plateau had been attained (as observed on the dynamometer screen display). The integral of the root mean square of the BF EMG signal, was calculated 500 ms either side of instantaneous MVC_{KF} maximal torque from the contraction corresponding to the highest MVC_{KF} torque. Prior to contraction the baseline signal noise was calculated as the integral root mean square over 1s and removed from the

measured EMG during MVC_{KF} and MVC_{KE}. The absolute integral of the BF EMG was taken over 250 ms at every 10% of ramped MVC_{KE} torque. Co-activation torque was calculated as, (BF EMG during ramped MVC_{KE} / BF EMG during MVC_{KF}) × peak MVC_{KF} torque (Onambélé et al., 2007). This equation assumes that the BF is representative of the entire hamstring (Carolan & Cafarelli, 1992), and that a linear relationship exists between BF EMG and MVC_{KF} torque (Lippold, 1952).

3.3.8. Patella tendon stress / strain relationship

Patella tendon stress was calculated as, patella tendon force / PT_{CSA}. Tendon strain was calculated as a ratio of total patella tendon displacement and PT_L.

3.3.9. Patella tendon Young's modulus

Young's modulus was calculated as: patella tendon stiffness × (PT_L (mm) / PT_{CSA} (mm^2)) at MVC_{KE}. Furthermore, Young's modulus was calculated with patella tendon stiffness standardised to the MVC_{KE} force of the weakest participant.

3.3.10. Eccentric exercise

A warm-up of 10 isokinetic knee extensions and knee flexions, ensuring a progressive increase in effort (with the last contraction being maximal), was carried out prior to eccentric exercise. For the eccentric exercise, the knee extensor range of motion was set at 20 – 90° (0° = full extension). Participants were asked to perform 1 set of 12 maximal voluntary eccentric knee extensions (MVE_{KE}) repetitions. During the eccentric phase (knee angle 20 to 90°), contractions were performed at an isokinetic angular velocity of $30°·s^{-1}$ (Saka et al., 2009). The concentric phase was performed sub-maximally at an angular velocity of $60°·s^{-1}$ to minimise fatigue and enhance eccentric damage (Chapman et al., 2006). Visual and verbal encouragement was continuously provided throughout the eccentric exercise (Campenella et

al., 2000). MVE_{KE} torque was recorded throughout each MVE_{KE} and displayed via the torque acquisition system.

3.3.11. Change in vastus lateralis fascicle length during the eccentric protocol

To measure VL fascicle length during MVE_{KE}, the ultrasound probe was fixed at 50% of VL muscle length in the mid-sagittal plane of the non-dominant leg. A hypo-allergenic ultrasound gel (Parker, Park Laboratories Inc., Fairfield) was used to enhance coupling between skin and the ultrasound probe.

During the 12 MVE_{KE} contractions, ultrasound images were recorded onto a PC, in real time, at 25 frames per second (Adobe Premier pro Version 6). An externally generated square wave pulse was used to synchronise the ultrasound images with the torque acquisition system. Three MVE_{KE} contractions were randomly chosen from the 12 repetitions for architectural analysis. Using frame capture software (Adobe Premier Elements, version 10), an ultrasound image (frame corresponding to every 10° of knee angle, ranging from 20-90°) was acquired for offline analysis. To ensure there was no movement artefact included in the assessment of fascicle length, an echo-absorptive marker was fixed on the skin to provide a visual baseline of the internal structures. If movement of the probe was observed relative to the VL, the contraction was discarded and another MVE_{KE} contraction was chosen for analysis.

Using digitising software (ImageJ 1.45, National Institutes of Health, USA), VL fascicle length was analysed offline at every 10° of knee joint angle. Fascicle length was measured from the visible insertion of the fibre into either the deep or superficial aponeurosis (Reeves & Narici, 2003). The fascicle was only measured if it remained visible across the entire ultrasound image. Where the fascicle extended beyond the ultrasound image (frame width 3.50 cm and height 4.15 cm), linear continuation of the fascicle and aponeurosis was assumed. Using ultrasound a 2-7% error has been associated with assuming linear continuation to calculate VL fascicle length at 120° knee angle (Finni et al., 2003a). Recently,

when comparing ultrasound to cadaveric measurements', the linear extrapolation method has been concluded as a valid technique for measuring VL fascicle length at rest (ICC = 0.853) (Ando et al., 2014). Additionally, within chapter two the current thesis reports high reliability and low day-to-day measurement error when measuring VL fascicle length during MVE_{KE}. To reduce error associated with the estimation of VL fascicle length, an average of three fascicles across the image was taken (Guilhem et al., 2011). Change in fascicle length was calculated at each angle by subtracting the fascicle length reported at knee angle 20°. Change in fascicle length at each angle was made relative to fascicle length at 20°.

3.3.12. Vastus lateralis excursion

In order to estimate the total VL MTU elongation, the tendon excursion method was adopted (Spoor et al., 1990); whereby the change in knee angle (70°, 1.22 rad) was multiplied by measured PT_{MA} at 90° knee angle.

3.3.13. Statistics

All statistical analyses were carried out using the statistical software package SPSS (v.19, Chicago, IL) for Windows and Microsoft Excel. To ensure the data were parametric, the Shapiro-Wilk and Levene's tests were utilized to assess the normality and variance of the data. These statistical assumptions were not breached. For sex differences in anthropometric measures, VL fascicle length and patella tendon properties, Independent T-tests were used. A 2 × 8 mixed design ANOVA (between factors: sex (2 levels), and knee angle (8 levels)) was used for MVE_{KE} torque, MVE_{KE} torque corrected for Q_{ACSA}, and change in VL fascicle length. Wherever the assumption of sphericity was violated, the Greenhouse-Geisser correction was used. When a significant group effect was found, post-hoc Independent T-tests (planned contrast) with LSD correction were carried out. Pearson's correlations were used to investigate whether significant associations were present at 90° between 1) MVE_{KE} corrected for Q_{ACSA} and change in fascicle length 2) patella tendon stiffness, at MVC_{KE} and change in

fascicle length and, 3) Young's modulus, at MVC_{KE}, and change in fascicle length. Significance was set at $p \leq 0.05$. Data are presented as mean ± standard deviation.

3.4. Results

3.4.1. Anthropometric measurements

There were no significant difference in age (males aged 22 ± 2 years, females aged 21 ± 2 years, $p = 0.449$) and mass (males 71.9 ± 13.1 kg, females 62.2 ± 6.9 kg, $p = 0.066$) in males compared with females. Males were significantly taller than the females (173 ± 8 cm and 165 ± 7 cm, respectively, $p = 0.045$).

3.4.2. Vastus lateralis anatomical cross-sectional area

VL_{ACSA} was significantly higher in males compared to females (23.4 ± 2.9 cm^2 and 19.5 ± 3.8 cm^2 respectively, $p = 0.030$). Estimated Q_{ACSA} was significantly higher in males compared to females (73.0 ± 9.2 cm^2 and 51.4 ± 10.1 cm^2 respectively, $p = 0.0002$).

3.4.3. Patella tendon properties

PT_L (males 5.73 ± 0.50 cm, females 5.16 ± 0.55 cm, $p = 0.034$), PT_{CSA} (males 0.81 ± 0.16 cm^2, females 0.62 ± 0.14 cm^2, $p = 0.016$) and PT_{MA} (males 4.30 ± 0.27 cm, females 4.0 ± 0.29 cm, $p = 0.023$) were significantly greater in males compared to females. Ramped MVC_{KE} torque was significantly higher in males compared to females at every 10% MVC_{KE} torque ($p \leq 0.01$). Patella tendon force was significantly higher in males compared to females at every 10% MVC_{KE} torque (($p \leq 0.05$), Table 3. 1.). Patella tendon elongation was not significantly different between males and females at any 10% MVC_{KE} torque (Table 3. 1). Patella tendon stiffness, at MVC, was significantly higher in males compared to females (≥ 50% MVC_{KE} $p \leq 0.05$, < 50% MVC_{KE} $p \leq 0.01$, (Table 3. 1)). The greater patella tendon stiffness in males was reflected in a relative shift to the left (compared to the females' trend line) in the patella tendon force-elongation curve (Table 3.1).

Table 3. 1: Structural and mechanical patella tendon properties in males and females, *in vivo*.

Sex	MVC_{KE} (%)	Tendon force (N)			Elongation (mm)			Stiffness (N·mm-1)		
Females	10	317	±	47	1.81	±	0.82	256	±	59
	20	633	±	93	3.01	±	0.95	334	±	84
	30	951	±	139	3.97	±	0.88	397	±	106
	40	1267	±	185	4.55	±	1.03	450	±	127
	50	1583	±	229	5.46	±	1.05	498	±	144
	60	1899	±	274	6.07	±	1.25	541	±	160
	70	2217	±	319	6.46	±	1.35	581	±	174
	80	2535	±	365	7.14	±	1.15	619	±	188
	90	2855	±	412	7.64	±	1.25	655	±	201
	100	3177	±	460	8.06	±	1.51	689	±	214
Males	10	411 *	±	94	1.90	±	1.09	443	±	264
	20	821 *	±	187	3.05	±	1.34	568*	±	271
	30	1233*	±	281	4.08	±	1.66	668*	±	285
	40	1646*	±	376	4.89	±	1.50	754*	±	301
	50	2061*	±	472	5.56	±	1.69	829*	±	315
	60	2475*	±	567	5.98	±	1.75	901**	±	333
	70	2888*	±	660	6.36	±	1.75	966**	±	349
	80	3302*	±	756	6.63	±	1.84	1027**	±	365
	90	3725*	±	858	7.00	±	2.12	1085**	±	381
	100	4136*	±	945	7.25	±	2.18	1139**	±	395

Note: MVC_{KE} (%): ramped isometric maximal voluntary knee extension. Data is presented as means and standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$.

Young's modulus at MVC_{KE} was significantly higher (p = 0.014) in males (811 ± 263 MPa) compared to females (582 ± 186 MPa). To account for the significantly higher MVC_{KE} torque in males, a patella tendon force of 2330N (corresponding to the highest MVC_{KE} torque of the weakest participant (independent of sex)) was used to calculate standardised force level patella tendon stiffness and Young's modulus. Standardised force patella tendon stiffness (males 880 ± 293 $N \cdot mm^{-1}$, females 553 ± 179 $N \cdot mm^{-1}$, p = 0.013) and standardised force Young's modulus (males 632 ± 213 MPa, females 498 ± 137 MPa, p = 0.033) remained significantly higher in males compared to females.

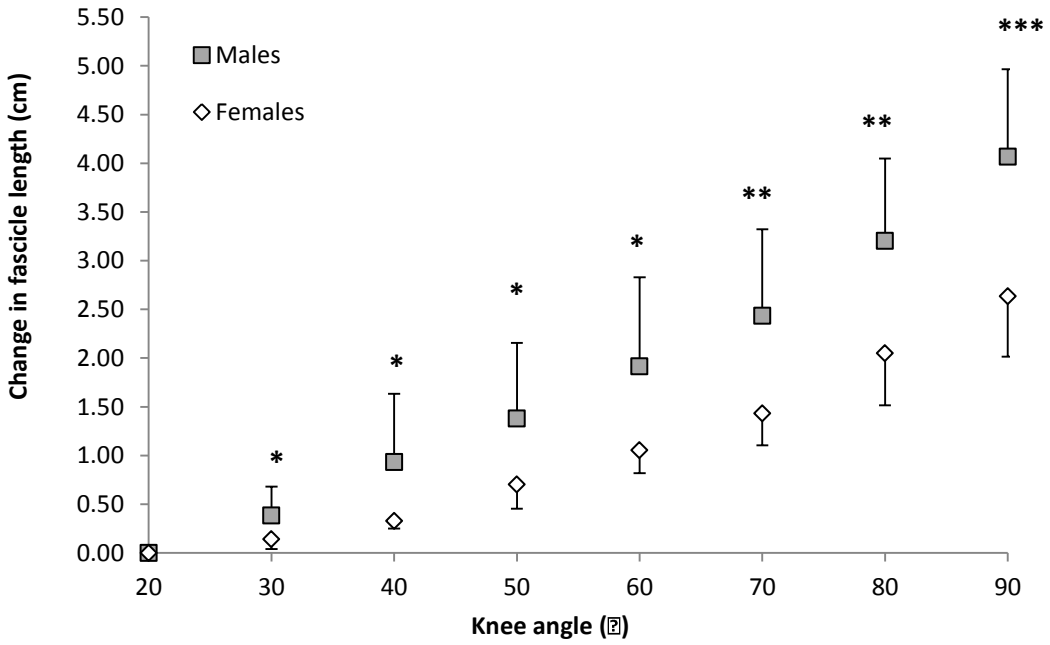

Figure 3. 1: Change in VL fascicle length from 20°, at each knee angle (20-90°) during eccentric exercise in males and females. Data is reported as mean and standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.4.4. Change in vastus lateralis fascicle length during eccentric exercise

A two-way mixed repeated measures ANOVA for change in VL fascicle length, reported a significant main effect of angle ($p \leq 0.001$) and a significant group effect of sex ($p = 0.004$), with a significant interaction effect ($p = 0.001$). A planned contrast showed at 30 - 90°, change in fascicle length was significantly higher in males compared to females ($p \leq 0.05$, Figure 3. 1). When the change in fascicle length was made relative to fascicle length at 20° knee angle, male's fascicles increased by 57.6 ± 10.9% and females increased by 39.6 ± 9.8% at 90° knee angle. Thus, relative fascicle length increased significantly further in males compared to females ($p = 0.002$).

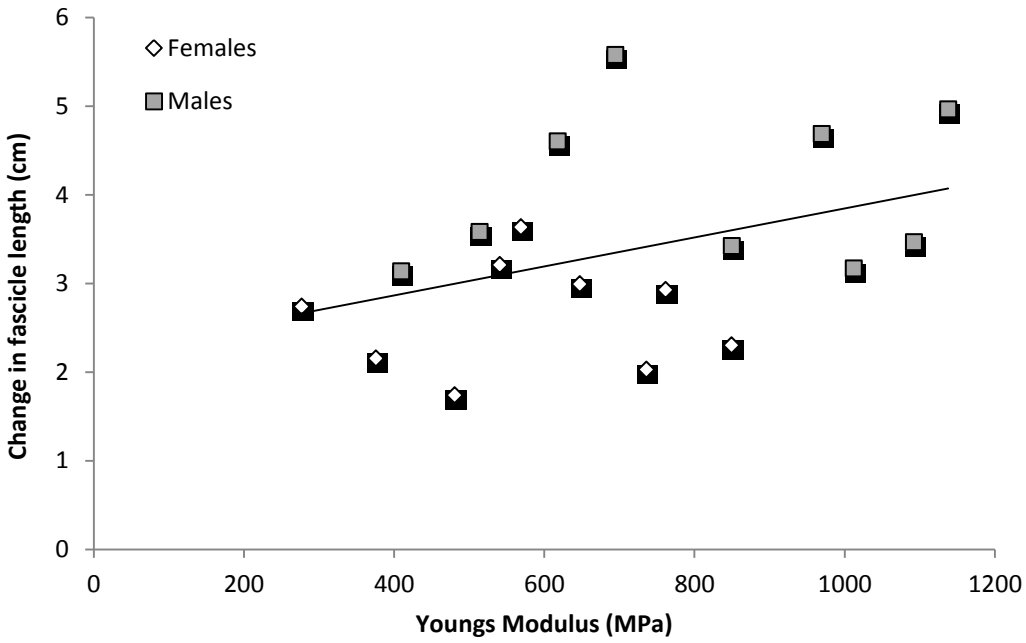

Figure 3. 2: Correlation between patella tendon Young's modulus and change in *vastus lateralis* fascicle length. Note: R^2 value is for the entire sample.

3.4.5. Maximal voluntary eccentric knee extensor torque

MVE_{KE} torque showed a significant main effect of angle ($p \leq 0.001$) and a significant group effect of sex ($p = 0.004$), with a significant interaction effect ($p = 0.020$). A planned contrast demonstrated that MVE_{KE} torque was significantly higher in males compared to females at 60° (males 148 ± 33.2 Nm, females 101 ± 30.4 Nm, $p = 0.007$), 70° (males 160 ± 38.9 Nm, females 99.2 ± 29.1 Nm, $p = 0.002$) and 80° (males 145 ± 33.8 Nm, females 88.9 ± 28.8 Nm, $p = 0.002$). Independent T-test showed that peak MVE_{KE} torque was significantly higher in males compared to females (161 ± 0.67 Nm, 113 ± 1.36 Nm, respectively, $p = 0.015$).

When MVE_{KE} torque was made relative to Q_{ACSA} (i.e. MVE_{KE} torque / Q_{ACSA}) a significant main effect of knee angle ($p = 0.0004$) was observed but no effect of sex ($p = 0.979$), or an interaction effect was reported ($p = 0.367$). No significant difference in peak MVE_{KE} made relative to Q_{ACSA} was reported between males and females (2.24 ± Nm/cm^2, 2.25 ± 0.74 Nm/cm^2, respectively, $p = 0.969$).

3.4.6. Vastus lateralis excursion

Based on the tendon moment arm excursion, the estimated increase in VL MTU length from 20-90° was significantly greater in the males compared to the females (5.25 ± 0.33 cm, 4.84 ± 0.35 cm, respectively, $p = 0.023$).

3.4.7. Correlation

Pearson's correlations were conducted at 90° knee angle, as the change in fascicle length was greatest at this angle. There was no correlation between change in fascicle length and peak MVE_{KE} ($r = 0.336$, $p = 0.173$) or peak MVE_{KE} / Q_{ACSA} ($r = 0.049$, $p = 0.848$). A significant, although weak, correlation between patella tendon stiffness at MVC_{KE} and change in fascicle length was reported ($r = 0.476$, $p = 0.023$). A significant correlation between patella tendon

Young's modulus at MVC_{KE} and change in fascicle lengthening was reported (r = 0.508, p = 0.016, Figure 3. 2).

3.5. Discussion

The aim of the present chapter was to determine whether patella tendon stiffness alters VL fascicle lengthening during MVE_{KE} in males and females. There were three main findings, 1) VL fascicles lengthen during MVE_{KE}, 2) in agreement with our hypothesis, the change in VL fascicle length and relative change in fascicle length during eccentric contractions was significantly greater in males compared to females, 3) a significantly longer PT_{MA}, higher tendon stiffness and Young's modulus in males compared to females, partially accounts for increased fascicle lengthening under loading.

Consistent with the classical definition of eccentric contractions and the findings of Guilhem et al., (2011) the current chapter reported a lengthening of VL fascicles during MVE_{KE}. A lengthening of VL fascicles has previously been reported during eccentric movements (Finni et al., 2001), with greater lengthening during a low intensity countermovement jump compared to a drop jump where the fascicle lengthening was minimal. As a result of similar MTU lengthening between the two jumps, Finni et al. (2001), attributed the difference in fascicle lengthening to the greater elongation of the tendon and higher EMG activity found in the drop jump. The difference in fascicle behaviour was further explained by Ishikawa et al., (2003) who reported VL fascicle lengthening to reduce, and patella tendon elongation to increase when the rebound intensity of the drop jump was increased. These findings suggest the interaction between the fascicles and tendon during the eccentric phase allows for the fascicle to operate on the most 'efficient' part of the force–lengthening relationship, for greater rebound intensities (Ishikawa et al., 2003). Therefore although the previous literature and the current chapter report a lengthening of VL fascicles during eccentric movements, it should also be clear that the magnitude of lengthening is

regulated by the amount of tendon elongation, the type of eccentric task and intensity of the task itself (Ishikawa et al., 2003; Finni et al., 2001).

In the current chapter, change in VL fascicle length was found to be significantly higher in males compared to females during MVE_{KE}. The significant difference remained when change in fascicle length was made relative to fascicle length at 20°. To the best of the authors' knowledge, sex differences in fascicle behaviour during eccentric contractions have yet to be reported. The observed sex difference in fascicle behaviour during eccentric contractions can be partially attributed to the observed difference in patella tendon compliance. Although sex differences remain unreported, the role of the tendon and group differences in fascicle lengthening during eccentric phases of gait have recently been considered (Mian et al., 2007). For example, Mian et al. (2007) suggested that the group differences in the fascicle behaviour of young and elderly participants might be a result of reduced tendon stiffness in the elderly; however this was not confirmed experimentally in their study.

The idea that the tendon acts as a mechanical buffer and attenuates fascicle lengthening however is not novel (Fukunaga et al., 2001; Spanjaard et al., 2007; Reeves & Narici, 2003). Litchwark et al. (2007) used a three element Hill muscle model to predict the effect of different levels of tendon stiffness on the behaviour of fascicle lengthening during gait. They predicted that increasing Achilles tendon stiffness, increased the required amount of *gastrocnemius medialis* fascicle lengthening during the stance phase of gait (Lichtwark & Wilson, 2007). Thus further supporting the hypothesis that the tendon may modulate eccentric fascicle lengthening (Reeves & Narici, 2003; Roberts & Azizi, 2010). The accuracy of the model remains in question however, as Litchwark et al. (2007), did not take into account fibre pennation angle and used a linear model for tendon stiffness despite tendon being known to have a non-linear force-elongation relationship (Maganaris & Paul, 2002). The current chapter however, provides *in vivo* evidence to demonstrate the association between patella tendon properties and VL fascicle lengthening. In agreement with previous literature

(Onambélé et al., 2007; Kubo et al., 2003), the current chapter reported significantly higher patella tendon stiffness and Young's modulus, at MVC_{KE}, in males compared to females. This significant difference remained when patella tendon stiffness and Young's modulus was calculated at a standardized absolute force level of 2330N. Significant correlations were found between 1) Patella tendon stiffness when calculated at MVC_{KE} and change in VL fascicle length and 2) Young's modulus, when calculated at MVC_{KE} and change in VL fascicle length. Thus, greater fascicle lengthening in males can be partially attributed to, the significantly higher patella tendon stiffness and Young's modulus in males. Although the attenuating role of the tendon on fascicle behaviour has been alluded to previously, the current chapter provides *in vivo* evidence to support previous models (Lichtwark & Wilson, 2007) and hypotheses (Reeves & Narici, 2003; Spanjaard et al., 2007; Fukunaga et al., 2001).

In addition to the tendon one of the other contributory factors to greater fascicle elongation in males could be the greater MVE_{KE} torque. MVE_{KE} torque was significantly higher at 60, 70 and 80° in males compared to females. Furthermore, peak MVE_{KE} torque was significantly higher in males compared to females. Males however, were estimated to have significantly larger Q_{ACSA} compared to females. When peak MVE_{KE} torque was corrected for Q_{ACSA} (stress), there was no significant difference between males and females. Torque was therefore 'distributed evenly' over Q_{ACSA} in both males and females. Furthermore, a significant correlation between MVE_{KE} torque and change in fascicle length was not observed in the current chapter. Therefore, although the current chapter cannot completely dismiss the contribution of greater eccentric torque on fascicle lengthening, the findings conclude no difference in relative MVE_{KE} torque, or any correlation with change in VL fascicle length.

During isokinetic contractions the lengthening of the MTU through a range of motion is affected by the joint kinetics specifically, the joint moment arm (Spoor et al., 1990; Visser et al., 1990; Herbert et al., 2002). During knee extension, the most influential moment arm, is the PT_{MA} (Erskine et al., 2014). Males had a significantly longer PT_{MA} than females thus, in order to establish whether there was a significant contribution of the PT_{MA} to the fascicle

elongation MTU elongation was estimated based on the tendon excursion method (Spoor et al., 1990). Although this method estimates the entire quadriceps MTU elongation, the current chapter estimated a significantly greater MTU elongation in males compared to females (5.24 cm and 4.84 cm, respectively). Therefore, the difference in PT_{MA} contributes to the difference in fascicle lengthening. Based on measured fascicle lengthening of 4.07 cm in males and 2.63 cm in females, the current chapter calculated that 89% and 54% (males and females respectively) of the estimated total change in MTU length, from 20-90°, could be attributed to an increase in the fascicle length. It is therefore likely that the remaining portion of the increase in MTU length (11% and 46% in males and females, respectively), which is not attributed to this increase in fascicle length, may be due to the in series elastic component. Highlighting that although PT_{MA} may contribute to VL fascicle lengthening, the sex differences in VL fascicle lengthening can be attributed to the sex differences in patella tendon stiffness.

Although the current chapter has provided evidence to support the role of patella tendon stiffness on VL fascicle lengthening during MVE_{KE}, it is acknowledged that the elastic component also incorporates the aponeurosis. It is however unclear how the distinct contributions of the tendon vs. the aponeurosis may interplay since the relative mechanical properties of the aponeurosis are indeterminate with reports of significantly higher (Maganaris & Paul, 2000), significantly lower (Magnusson et al., 2003) or similar (Arampatzis et al., 2005) strain values to the tendon. It should be noted that the apparent variability within these cited studies could be attributed to different participant activity levels, the specific anatomical MTU investigated and the method used to determine MTU lengthening. Therefore, a limitation of the current chapter is that the mechanical properties of the VL aponeurosis alongside those of the tendon cannot be reported; hence the current chapter cannot determine the contribution of the VL aponeurosis to VL fascicle lengthening behaviour. However, it remains that a longer VL fascicle elongation in males compared to females is attributable to a larger PT_{MA}, and an increase in stiffness of the in series elastic components, that of the patella tendon in particular.

3.6. Conclusion

In conclusion the current chapter demonstrates that during eccentric contractions, VL fascicles lengthen significantly further in males compared to females. The significant difference in lengthening may be partly attributed to the significantly longer PT_{MA}, higher patella tendon stiffness and Young's modulus found in males compared to females. The current chapter provides *in vivo* evidence to support the hypothesis that the tendon acts as a mechanical buffer during eccentric contractions. Future research needs to investigate the contribution of the aponeurosis to the lengthening of the fascicles during eccentric contractions.

Chapter four: Fascicle lengthening and maximal voluntary eccentric torque are not associated with indirect markers of EIMD.

Chapter four was presented at the 19th annual Congress of the European College of Sport Science – Amsterdam 2014. The abstract was entered into the Young Investigators Award and published in the congress Book of Abstracts.

4.1. Abstract

Aim: The aim of the current chapter was to establish whether patella tendon properties, eccentric torque and *vastus lateralis* fascicle lengthening are associated with the magnitude of indirect markers of exercise-induced muscle damage (EIMD). *Materials and method:* 16 recreationally active males performed maximal voluntary eccentric knee extensions (12 reps x 6 sets), during which *vastus lateralis* fascicle lengthening and eccentric torque were recorded every 10° (range of motion 20-90°). Maximal isometric torque loss, Creatine Kinase activity (CK) and muscle soreness were measured *pre-damage, post damage, 48, 96 and 168* hours post damage as markers of EIMD. *Results:* Change in CK (peak CK value - *pre* CK value (33)) significantly correlated with relative VL fascicle lengthening ((from 20° to 90° knee angle), r = 0.534, p = 0.017). Neither ΔCK_{ABS} to peak nor loss in maximal isometric knee extensor torque, correlated with any other indirect marker of EIMD. *Conclusion:* The current chapter concludes that VL fascicle lengthening and eccentric torque are not associated with functional indirect markers of EIMD.

4.2. Introduction

Unaccustomed eccentric exercise is accepted as the greatest cause of exercise-induced muscle damage (EIMD), however there is considerable variability in the range of EIMD reported within the literature (see Clarkson and Hubal (2002) and Hyldahl and Hubal (2014) for a review). The cause of differences in EIMD have been attributed to the level of fascicle strain experienced within the muscle (isolated rabbit muscle) (Lieber & Friden, 1993), the level of eccentric force the muscle is exposed to during eccentric contractions (*in vitro* rat muscle) (Warren et al., 1993a), the architectural properties of the muscle (*in vivo* human upper and lower limb)(Chen et al., 2011), the elastic properties of the tendon (although not confirmed experimentally) (Marginson et al., 2005; McHugh et al., 1999) and circulating oestrogen levels (*in vivo* human quadriceps)(Carter et al., 2001). While these determinants have mainly been identified in various animal studies (Lieber & Friden, 1993; Warren et al., 1993a), they may not be directly applicable to *in vivo* human studies.

Although EIMD has previously been associated with the high torque and fascicle strain produced during eccentric contractions in animal studies (Lieber & Friden, 1993; Warren et al., 1993a), within human studies experimental evidence investigating the association between fascicle lengthening (Hoffman et al., 2014) and eccentric torques (Hody et al., 2013; Chapman et al., 2008) with EIMD remains limited. For example, Hoffman et al. (2014) reported no significant correlation between maximal *gastrocnemius* fascicle lengthening and plantar flexion MVC torque loss following a damaging bout of backwards walking. However, during backwards walking the magnitude of fascicle lengthening is small (~18% (Hoffman et al., 2014)) compared to fascicle lengthening in chapter 3 (~58%). Therefore it remains unknown if *in vivo*, using a more severe damaging protocol and investigating a greater magnitude of fascicle lengthening, whether fascicle lengthening may be associated with indirect markers of EIMD.

Recently, studies have alluded to the properties of the tendon being associated with EIMD (Roberts & Azizi, 2010; Roberts & Konow, 2013; McHugh et al., 1999; Hoffman et al., 2014). For example Hoffman et al. (2014) concluded that no relationship between fascicle lengthening and EIMD may have been reported due to the Achilles tendon accounting for ~90% of total muscle tendon unit (MTU) lengthening. Within chapter three it was reported that the patella tendon mediates VL fascicle lengthening during eccentric contractions, thus supporting the hypothesis that the tendon may prevent sarcomeres extending onto the descending limb of the length-tension relationship (Hoffman et al., 2014). Furthermore, the tendon has also been suggested to reduce EIMD by mediating peak force, peak torque and fascicle lengthening velocity (Roberts & Azizi, 2010; Roberts & Konow, 2013). However, the association between tendon properties and indirect markers of EIMD remain speculative and has not been evidenced experimentally *in vivo*.

In addition to the potential association between tendon properties and EIMD, muscle architecture has also been proposed to have an association with EIMD (Chen et al., 2011; Roberts & Konow, 2013). Chen et al. (2011) reported EIMD to be greatest in the upper limbs (specifically elbow flexors), with the lower limbs (specifically knee extensors) exhibiting the least damage. Chen et al. (2011) attributed the differences in EIMD to relatively larger pennation angles, muscle length and fibre orientation in the knee extensors compared to the elbow flexors. Although muscle architecture has been alluded to as a determinant of EIMD by Chen et al. (2011), to the current author's knowledge it has yet to be investigated experimentally.

Eccentric torque, fascicle lengthening, tendon and muscle properties have all been associated with EIMD, particularly in animal studies (Lieber & Friden, 1993; Warren et al., 1993a), however animal studies may not be directly comparable to *in vivo* human studies for a couple of reason. Firstly, strain values used in animal studies (Lieber & Friden, 1993) are considerably larger than those reported *in* vivo (Hoffman et al., 2014) (125% versus 18%, respectively) (Butterfield, 2010). Secondly, protein metabolism and caspase pathways (i.e.

caspase 8 and 10 regarding cell death and lymphocyte activation) are significantly different between animal and human studies (for a review please refer to Sakamaki and Satou (2009) and Phillips et al. (2009)). Therefore measuring and establishing variables associated with EIMD directly *in vivo* is essential to further our knowledge in the events leading to EIMD and to further understand group differences (and / or within group variability) in EIMD. Therefore the aim of this chapter was to establish whether patella tendon properties, eccentric torque and VL fascicle lengthening are associated with indirect markers of EIMD, including creatine kinase (CK) and maximal voluntary isometric knee extension (MVC_{KE}) torque loss.

4.3. Materials and method

4.3.1. Subjects

16 males (21.1 ± 1.6 years of age, 72.0 ± 7.5 kg and 176 ± 6 cm) signed written informed consent to participate in this study. All participants self-reported as being recreationally active (undertaking no more than 1 hour of "moderate" physical activity per week) and did not take part in any structured resistance training. All experimental procedures complied with the Declaration of Helsinki (World Health Association, 2013) and ethical approval was obtained through the local Ethics Committee. Exclusion criteria were, any resistance training in the last six months, occupation or lifestyle that required regular heavy lifting or carrying, any known muscle disorder, the use of dietary supplements (i.e. vitamin E), and any musculoskeletal injury in the last three months. All inclusion and exclusion criteria were determined via a questionnaire.

4.3.2. Testing protocol

Once selected, participants were asked to visit the laboratory on five different occasions over nine days. The sessions were as follows: 1) *pre-damage* 2) *damage* (48 hours post *pre-damage*), 3) *48 hours post damage*, 4) *96 hour post damage,* and 5) *168 hour post damage*. *Pre-*

damage assessments consisted of stature and mass (anthropometric measures), patella tendon moment arm (PT_{MA}), 5-6ml blood sample, dynamometer familiarisation (within *pre-damage*), morphological measures of the patella tendon (tendon size and stiffness), VL (muscle size and architecture), and MVC_{KE} torque measurements at six knee angles (60, 65, 70, 75, 80 and 90° (0° = full extension). Participants were tested at six knee angles to obtain optimal knee angle for MVC_{KE} torque. Participants only performed two practice MVCs, at two knee joint angles during the familiarisation session. Stature and mass were measured using a wall mounted stadiometer (Harpenden, Holtain Crymych, UK) and digital scales (Seca model 873, Seca, Germany) respectively. The *damage* session consisted of eccentric exercise, 5-6 mL venous blood sample and MVC_{KE} torque measurements. *48, 96 and 168 hours session* consisted of 5-6 mL blood sample and MVC_{KE} torque measurements at all six knee angles (60, 65, 70, 75, 80 and 90°).

All tests were carried out in the self-reported, non-dominant leg, defined as the leg that provided stability during movements. Participants were seated in an isokinetic dynamometer (Cybex Norm, Cybex International, NY, USA), with a hip angle of 85° (0° = supine). To reduce any extraneous movement during maximal efforts, participants were secured in a seated position using inextensible straps around the shoulders and hips. The isokinetic dynamometer axis of rotation was visually aligned with the estimated knee joint's centre of rotation. During *pre-damage* testing the isokinetic dynamometer settings (chair position, distance from the dynamometer, dynamometer arm length) and anatomical zero (leg full extended, knee angle 0°) were recorded to ensure repeatability in the following sessions.

4.3.3. Vastus lateralis anatomical cross-sectional area

Vastus lateralis anatomical cross-sectional area (VL_{ACSA}) was measured at 50% of VL length using a real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy). With the participant laid supine and their non-dominant leg fully extended (knee angle = 0°), the distal and proximal insertions sites of the VL were identified using an ultrasound probe (7.5

MHz linear array probe, 38 mm wide). At 50% of VL muscle length echo-absorptive markers were placed in vertically in parallel, at intervals of 30 mm, from the lateral to the medial edge of the VL muscle. The ultrasound probe was held perpendicular to the VL muscle in the axial plane. The ultrasound probe was moved steadily over the echo-absorptive markers from the lateral to the medial edge of the muscle. Constant, light pressure was placed on the muscle during scanning to avoid compression of the underlying soft tissue. The images were recorded in real time at 25 frames per second (Adobe Premier pro Version 6, Adobe Systems Software, Ireland). Using video editing software (Adobe Premier Elements, version 10), still images were acquired at each 30 mm interval. The shadows cast by the echo-absorptive markers allowed the neighbouring still images to be aligned, thus reconstructing the entire VL_{ACSA} in a single image (Adobe Photoshop Elements, version 10). Digitising software (ImageJ 1.45, National Institutes of Health, USA) was used to measure VL_{ACSA}. This method of calculating VL_{ACSA} has previously been accepted as reliable and valid when compared to magnetic resonance imaging (MRI), with a reported interclass correlation (ICC) between 0.998 and 0.999 (Reeves et al., 2004). Furthermore within chapter two of the current thesis, high reliability and low day-to-day measurement error was reported when using this method to calculate VL_{ACSA}.

Using MRI, a VL_{ACSA} of 24.3 cm^2 has previously reported to contribute to ~32.4% of total quadriceps anatomical cross-sectional area ((Q_{ACSA} = 74.9 cm^2 (Tsakoniti et al., 2008)). Therefore, assuming the contribution of VL_{ACSA} remains constant within males, Q_{ACSA} within the current chapter was estimated using the following equation: (measured VL_{ACSA} / 0.324) × 100.

4.3.4. Maximal voluntary isometric knee extensor torque measurements

At six different knee angles (60, 65, 70, 75, 80 and 90° (full extension = 0°)) participants were instructed to perform two rapid MVC_{KE} lasting ~two seconds at the plateau with 90 seconds rest between contractions. Torque was presented, in real time, on a Macintosh G4 computer

(Apple Computer, Cupertino, CA, USA), via an A/D converter (Biopac Systems, Santa Barbara, CA). Torque measurements were later analysed offline with the accompanying software (Acknowledge, version 3.9.2). The highest torque produced at each angle was taken as MVC_{KE} peak torque. The angle where the highest MVC_{KE} torque was produced during the *pre-damage* session was recorded as optimal knee angle in every subsequent session. Optimal knee angle torque was used to calculate MVC_{KE} torque loss in the subsequent four sessions. To calculate loss of MVC_{KE} torque following eccentric exercise, MVC_{KE} torque measurements were repeated 60 minutes post eccentric exercise (to reduce any fatigue effect (Walsh et al., 2004)) and *48 hours, 96 hours* and *168 hours* (recovery) post eccentric exercise.

4.3.5. Patella tendon length and cross-sectional area

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure patella tendon cross-sectional area (PT_{CSA}) and patella tendon length (PT_L) at a fixed 90° knee angle. The distance between the apex of the patella and the tibial tuberosity, marked using sagittal ultrasound images, was taken as PT_L. To measure PT_{CSA} the ultrasound probe was placed in the transverse plane and images were captured at 25%, 50%, and 75% of PT_L. The images were later analysed offline using (ImageJ 1.45, National Institutes of Health, USA). A mean of all three images were taken for PT_{CSA} (O'Brien et al., 2010)

4.3.6. Patella tendon stiffness

The participants were seated on the isokinetic dynamometer, with the knee angle fixed at 90°, and were instructed to perform a ramped, isometric MVC_{KE} lasting ~5-6 seconds. Ramped MVC_{KE} torque and displacement of the patella tendon were synchronised using a 10-V square wave signal generator. Patella tendon displacement was measured over two MVC_{KE}, once with the ultrasound probe positioned over the distal edge of the patella and on the second contraction over the tibial tuberosity (Onambélé et al., 2007), so that total displacement would be computed from the composite of proximal and distal patella motions

(see below). Torque was presented on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter and subsequently analysed with the accompanying software (Acknowledge, Biopac Systems, Santa Barbara, CA). To create an external marker on the ultrasound images, an echo-absorptive marker was placed on the skin. Using the marker to calculate displacement of the tendon, the distance of the marker (shadow) from an anatomical reference point at the beginning of the contraction, to the position of the shadow at the end of the contraction was calculated. A 10-V square wave signal generator was used to synchronise the ultrasound images with the torque acquisition system. Images were captured at ~10% intervals of ramped MVC_{KE} torque (Onambélé et al., 2007). Total patella tendon displacement was calculated as displacement at the apex of the patellar plus the displacement at the tibial tuberosity (Onambélé et al., 2007). Patella tendon forces were calculated as: (MVC_{KE} torque + antagonist co-activation torque) / PT_{MA}.

Patella tendon moment arm was measured at 90° (full extension = 0°) in the sagittal plane, from a single-energy X-ray absorptiometry scan (frame 23.3 cm x 13.7cm) Hologic Discovery, Vertec Scientific Ltd, UK). Using OsiriX (DICOM viewer, ver. 4.0, Pixemo, Switzerland), patella tendon moment arm length was determined as the perpendicular distance from the centre of the patella tendon to the estimated tibio–femoral contact point. Single-energy X-ray absorptiometry scans have been compared to MRI measures, demonstrating consistent reliability and validity against this standard (Erskine et al., 2014).

To compute tendon forces, the calculation of antagonist co-activation torque is described below. The patella tendon force - elongation curve stemming from data at every 10% MVC_{KE} was then fitted with a second-order polynomial function forced through zero (Onambélé et al., 2007). The tangential slope at discreet sections of the curve, relative to MVC_{KE} force, was computed by differentiating the curve at every 10% force intervals. In addition, to standardise the comparison of tendon stiffness and Young's modulus at an absolute load, the slope of the tangential line, corresponding to the MVC_{KE} force of the weakest participant (2972 Nm), was computed for each subject.

4.3.7. Co-activation

To determine co-activation during the ramped MVC_{KE}, Electromyogram (EMG) of the *bicep femoris* (BF) was recorded. Guided by an axial-plane ultrasound of the BF, two bipolar electrodes (Ambu, Neuroline 720, Denmark) were placed in the mid-sagittal line at 25% of BF muscle length (distal end = 0%). A reference electrode (Ambu, Blue Sensor, Denmark) was placed on the lateral tibial condyle. The electrodes were placed in a bipolar configuration with a constant inter-electrode distance of 20 mm (Hermens et al., 2000). Prior to electrode placement; the skin was shaved, gently abraded and cleansed with an alcohol wipe to reduce skin impedance below 5000 Ω (Hermens et al., 2000). To minimise cross talk, and ensure a mid-sagittal placement of electrodes, ultrasonography was used to identify the medial and lateral aspects of the BF muscle. The raw EMG signal was amplified (× 2000) and filtered (through low and high pass filters of 10 and 500 Hz respectively) with the sampling frequency set at 2000 Hz. Ramped MVC_{KE} torque and BF EMG were recorded in real time and synchronised using an external square wave signal generator. Participants performed two maximal voluntary isometric knee flexions (MVC_{KF}) at 90° knee angle. They were instructed to perform MVC_{KF} rapidly and as forcefully as possible against the dynamometer's lever arm. The participants were instructed to relax once a two second plateau had been attained (as observed on the dynamometer screen display). The root mean square of the integral of the raw BF EMG signal, was calculated 500 ms either side of instantaneous MVC_{KF} maximal torque from the contraction corresponding to the highest MVC_{KF} torque. Prior to contraction the baseline signal noise was calculated as the integral root mean square over 1s and removed from the measured EMG during MVC_{KF} and MVC_{KE}. At every 10% of ramped MVC_{KE} torque the absolute integral of the BF EMG was taken over 250 ms. Co-activation torque was calculated as, (BF EMG during ramped MVC_{KE} / BF EMG during MVC_{KF}) × peak MVC_{KF} torque (Onambélé et al., 2007). This equation assumes that the BF is representative of the entire

hamstring (Carolan & Cafarelli, 1992) and that a linear relationship exists between BF EMG and MVC_{KF} torque (Lippold, 1952).

4.3.8. Patella tendon stress / strain relationship

Patella tendon strain was calculated as a ratio of total patella tendon displacement to PT_L. Patella tendon stress was calculated as: patella tendon force (N) / PT_{CSA} (mm^2).

4.3.9. Patella tendon Young's modulus

Young's modulus was calculated as: Patella tendon stiffness × (PT_L (mm) / PT_{CSA} (mm^2)).

4.3.10. 'Damaging' eccentric exercise

Prior to eccentric exercise, a warm-up of 10 isokinetic knee extensions and knee flexions were carried out, ensuring a progressive increase in effort (with the last contraction being maximal). For the eccentric exercise, the knee extension range of motion was set at 20 – 90° (0° = full extension), and the Participants performed six sets of 12 MVE_{KE} repetitions. The eccentric phase of the contractions was performed at an isokinetic angular velocity of $30°·s^{-1}$ (Jamurtas et al., 2005). The concentric phase was performed sub-maximally at an angular velocity of $60°·s^{-1}$ to minimise fatigue and enhance eccentric damage (Chapman et al., 2006). Two minutes rest was provided between each set. Participants remained seated throughout the entire exercise protocol, including rest periods. Visual feedback and verbal encouragement was continuously provided throughout the protocol (Campenella et al., 2000). Average peak MVE_{KE} torque was calculated as an average of peak MVE_{KE} across six sets.

4.3.11. Change in vastus lateralis fascicle length during the eccentric protocol

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure VL fascicle length during MVE_{KE}. The ultrasound probe (7.5 MHz linear array probe) was fixed at 50% of VL muscle length in the mid-sagittal plane of the non-dominant leg. VL fascicle length was determined as the linear distance along the fascicle as it ran from the deep to the superficial aponeurosis. Pennation angle was determined as the angle created from the visible insertion of the fascicle into the deep aponeurosis. A hypo-allergenic ultrasound gel (Parker, Park Laboratories Inc., Fairfield) was used to enhance acoustic coupling between the skin and the ultrasound probe.

During the first set (out of six) of MVE_{KE} contractions, ultrasound images were recorded onto a PC, in real time, at 25 frames per second (Adobe Premier pro Version 6). An externally generated square wave signal was used to synchronise the ultrasound images with the torque acquisition system. Three MVE_{KE} contractions were chosen at random from the first set of 12 repetitions for architectural analysis. Using frame capture software (Adobe Premier Elements, version 10), an ultrasound image (frame corresponding to every 10° of knee angle, ranging from 20 - 90°) was acquired for offline analysis. To ensure there was no movement artefact included in the measurement of fascicle length, an echo-absorptive marker was fixed on the skin to provide a visual reference point for the internal structures. If movement of the reference line was observed, the contraction was discarded and another repetition was chosen for analysis.

Using digitising software (ImageJ 1.45, National Institutes of Health, USA), VL fascicle length was analysed offline at every 10° knee angle (range 20 - 90°, 0° = full extension) throughout the MVE_{KE}. Where the fascicle extended longer than the ultrasound image (frame width 3.50 cm and height 4.15 cm), linear continuation of the fascicle and aponeurosis was assumed. Within the VL, a 2-7% error is associated with the linear extrapolation method when used to calculate VL fascicle length (e.g. measured at 11.3 cm) at a knee angle of 120°

(Finni et al., 2003a). Recently, when comparing ultrasound to cadaveric measurements', the linear extrapolation method has been concluded as a valid technique for measuring VL fascicle length at rest (ICC = 0.853) (Ando et al., 2014). Within chapter two, high reliability and low day-to-day measurement error has been demonstrated using this method to measure VL fascicle length during $MVE_{KE.}$ Although the validity of the linear extrapolation method has been unreported in the VL during MVE_{KE}, the fascicle length reported is similar to those reported by Finni et al. (2003a), thus similar measurements of error (2-7%) can be assumed. In order to reduce any error associated with the estimation of VL fascicle length, an average of three fascicles across the image was taken. Change in fascicle length is presented as fascicle length at a knee angle of 90° made relative to fascicle length measured at a knee angle of 20°; hereafter termed "relative fascicle lengthening" and reported as a percentage change from starting length at 20°. Pennation angle was measured and is presented at a knee angle of 90°. An average of three pennation angles was taken across a single image.

4.3.12. Blood samples

Venous blood samples were taken to measure CK levels. A 21-gauge needle was inserted into the antecubical vein of the forearm, using a 10 mL syringe. 5-6 mL of blood was drawn into a serum collection tube. The sample was left on a crushed ice bed for 60 minutes. The sample was then centrifuged at 4500 rpm at 0°C for 10 minutes. Using a 200 – 1000 µl pipette (Eppendorf), the resulting serum sample was separated into three aliquots (~500 µl each) and stored in eppendorfs at -20°C until CK analysis was performed. Creatine kinase levels were measured using a standard colorimetry procedure, measuring at optical density 340 nm (BioTek ELx800 96 well Microplate Reader) and immediately analysed (Gen5, version 2.0). Individual samples were run in either duplicate or quadruplets using an EnzyChromTM CK Assay Kit (BioAssay Systems, Hayward, CA, sensitivity 5 U/L, manufacturer reported intra-assay variability ≤ 5%), reading enzyme activity after 20 minutes (25 minutes if necessary) and 40 minutes of exposure to the assay mix. An average of two – four readings was taken. CK

activity is reported in absolute values and absolute change from *pre-damage* values ((ΔCK_{ABS}) i.e. the peak CK value - the *pre* CK values).

4.3.13. Statistics

Statistical analysis was carried out using the statistical software package SPSS (v.19, Chicago, IL) for Windows and Microsoft Excel. To check for parametricity, the Levene's and Shapiro-Wilk tests were used to assess the variance and normality of the data, respectively. These assumptions were not violated. A one-way repeated measures ANOVA (time, 5 levels) was used for absolute CK and MVC_{KE} torque loss. Wherever the assumption of sphericity was violated, the Greenhouse-Geisser correction was applied. Pairwise comparison, with Boneforroni correction was used to investigate if there was any significant difference to *pre-damage*. Pearson linear correlations were used to determine whether ΔCK_{ABS} to peak correlated with muscle properties, tendon properties, fascicle lengthening, MVE_{KE} torque or MVE_{KE} torque / Q_{ACSA}. Pearson linear correlations were also used to determine whether MVC_{KE} torque loss was associated with, muscle properties, tendon properties, VL fascicle lengthening, MVE_{KE} torque or MVE_{KE} torque / Q_{ACSA}. Data is presented as mean ± standard deviation. Statistical significance was accepted at $p \leq 0.05$.

4.4. Results

4.4.1. Muscle and patella tendon measurements

Muscle architecture and patella tendon properties, assessed at the '*pre-damage* phase', are presented in Table 4.1.

4.4.2. Patella tendon properties

To account for varying ramped MVC_{KE} torques, the patella tendon force corresponding to the highest MVC_{KE} torque of the weakest participant (2972 N) was used to calculate standardised

force level patella tendon stiffness (1213 ± 436 $N \cdot mm^{-1}$) and Young's modulus (1030 ± 591 MPa).

4.4.3. Torque during maximal voluntary eccentric knee extensions

During MVE_{KE}, average peak (over six sets) MVE_{KE} torque was 255 ± 51 Nm. To account for varying muscle size, MVE_{KE} torque was normalised to the estimated Q_{ACSA} (3.28 ± 0.69 Nm/cm^2). Peak MVE_{KE} torque was 96% (± 15%) of '*pre-damage*' MVC_{KE} torque.

4.4.4. Vastus lateralis fascicle lengthening during maximal voluntary eccentric knee extensions

Absolute change in VL fascicle length from 20° knee angle (7.06 ± 0.43 cm) to 90° knee angle during MVE_{KE} contractions (11.3 ± 0.20 cm) was 4.20 ± 0.82 cm. Absolute change in VL fascicle length was made relative to fascicle length at knee angle of 20° denoting a 59.4 ± 12.0% increase in relative fascicle length.

Table 4. 1. *Vastus lateralis* and patella tendon properties.

Variable	Mean ± SD
Pennation angle (°)	12.1 ± 1.6
VL muscle length (cm)	32.8 ± 2.1
VL_{ACSA} (cm^2)	25.3 ± 4.2
Q_{ACSA} (cm^2)	79.0 ± 13.1
Tendon length (mm)	57.8 ± 6.0
Tendon CSA (mm^2)	78.1 ± 26.1
Ramped MVC_{KE} (Nm)	196 ± 37
Maximal tendon elongation (mm)	6.56 ± 2.12
Patella tendon strain (%)	11.5 ± 4.10
Patella tendon stress (MPa)	58.0 ± 17.3
PT_{MA} (cm)	4.33 ± 0.34
Maximal tendon force (N)	4590 ± 847
Maximal tendon stiffness ($N \cdot mm^{-1}$)	1450 ± 554
Maximal Young's modulus (MPa)	1247 ± 808

Note: Patella tendon stiffness and Young's modulus are calculated at ramped 100% maximal voluntary isometric knee extension (MVC_{KE}). Pennation angle is presented at 90° knee angle during maximal voluntary eccentric knee extension (MVE_{KE}). VL: *Vastus lateralis*. VL_{ACSA}: V*astus lateralis* anatomical cross-sectional area. Q_{ACSA}: estimated quadriceps anatomical cross-sectional area. PT_{MA}: Patella tendon moment arm. Data is presented as means ± standard deviation.

4.4.5. Creatine kinase levels

One-way repeated measures ANOVA reported a main effect of time ($p = 0.005$) on absolute CK values. Pairwise comparison, with Bonferroni correction, reported CK to significantly increase from *pre* to 96 hours ($p = 0.014$) but was not significantly different at any other time

point compared with *pre-damage* (Figure 4. 1). Relative to *pre-damage* (136 ± 114 U/L), ΔCK_{ABS} to peak (883 ± 667 U/L) increased by 855% (± 779%).

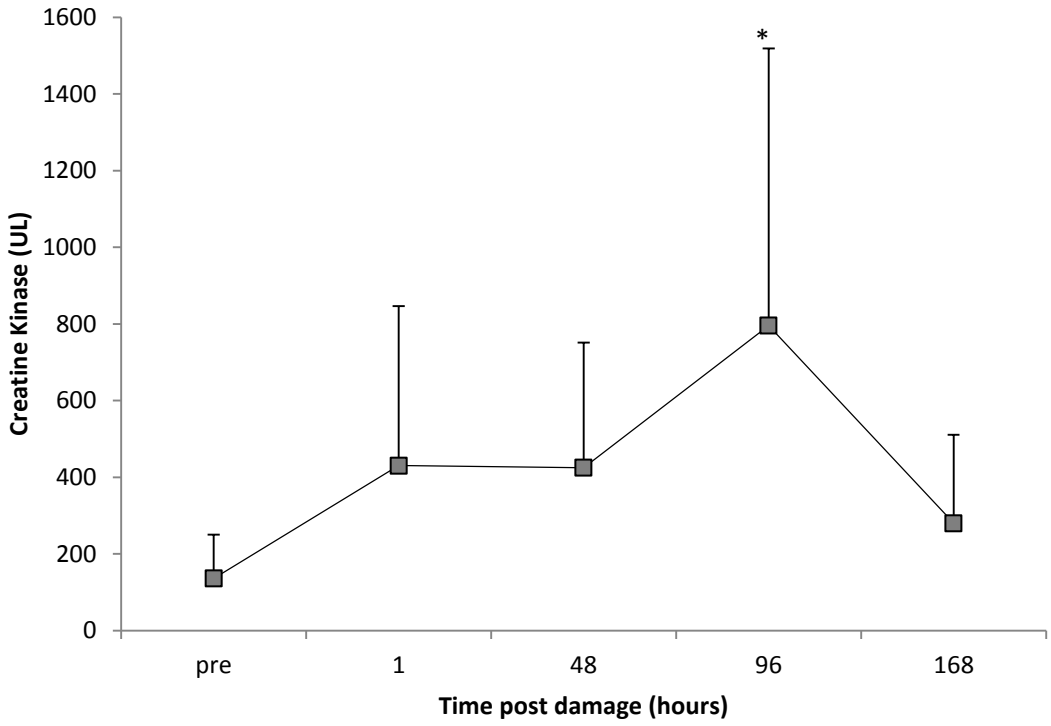

Figure 4. 1: Absolute creatine kinase (CK) response following exercise-induced muscle damage (EIMD). Data is presented as means ± standard deviation. * Significantly higher than *pre* $p \leq 0.05$.

4.4.6. Maximal voluntary isometric knee extensor torque loss

One-way repeated measures ANOVA reported a main effect of time ($p = 0.0004$) on torque loss. Pairwise comparison, with Bonferroni correction, reported torque to significantly decrease from *pre* to, 1 ($p = 0.0004$) and 48 hours ($p = 0.004$) post EIMD but was not significantly different at any other time point(Figure 4.2).

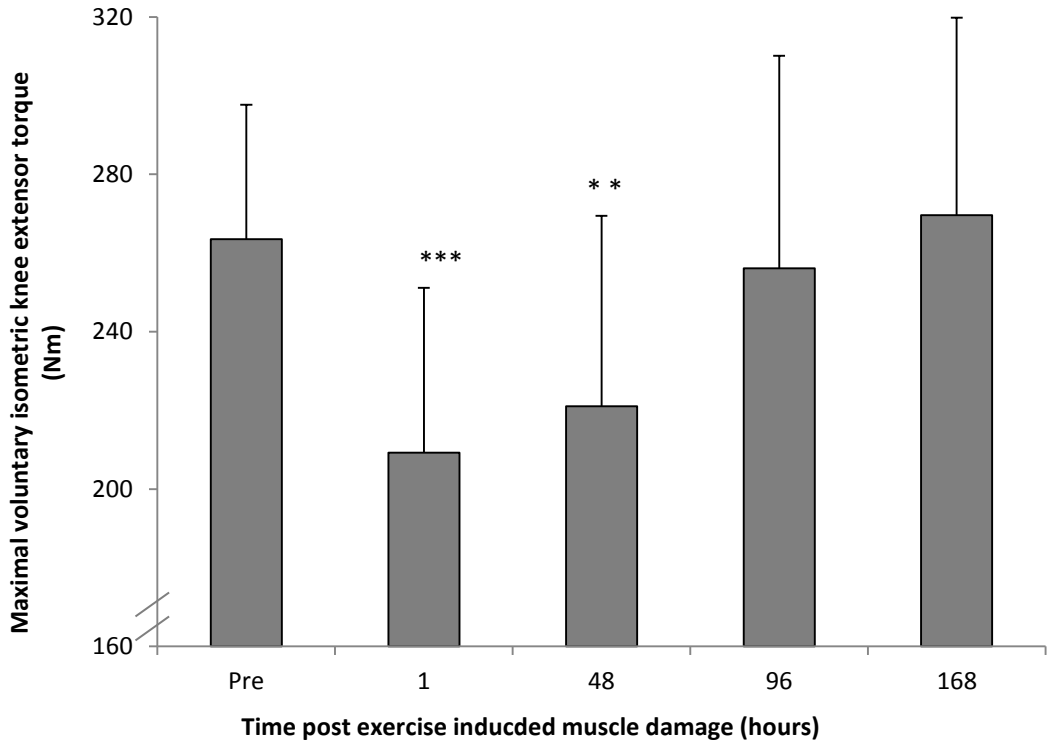

Figure 4. 2: Maximal voluntary isometric knee extension (MVC_{KE}) torque following exercise-induced muscle damage (EIMD). Data is presented as means ± standard deviation. Significantly lower than *pre*, ** $p \leq 0.01$ *** $p \leq 0.001$.

4.4.7. Correlations between markers of exercise-induced muscle damage

Pearson correlations between muscle and tendon properties and ΔCK_{ABS} to peak and MVC_{KE} torque loss are presented in Table 4. 2.

There was no significant correlation between ΔCK_{ABS} to peak and, pennation angle (at 90° knee angle during MVE_{KE}), resting VL length, Q_{ACSA}, relative tendon stiffness or relative Young's modulus ($p \geq 0.05$). ΔCK_{ABS} to peak did significantly correlate with average peak MVE_{KE} torque ($p = 0.027$) and relative VL fascicle lengthening (Figure 4. 3, $p = 0.017$). However, when peak MVE_{KE} was made relative to Q_{ACSA} the significant correlation was lost ($p \geq 0.05$).

There was no significant correlation between MVC_{KE} torque loss and, pennation angle (at 90° knee angle during MVE_{KE}), Q_{ACSA}, relative tendon stiffness, relative Young's modulus, average peak MVE_{KE} torque, MVE_{KE} torque made relative to Q_{ACSA} or relative fascicle lengthening ($p \geq 0.05$).

Table 4. 2. Pearson correlations between markers of exercise-induced muscle damage and *vastus lateralis* and patella tendon properties.

		Pennation angle (°)	VL muscle length (cm)	Q_{ACSA} (cm^2)	Relative tendon stiffness ($N \cdot mm^{-1}$)	Relative Young's modulus (MPa)	MVE_{KE} (Nm)	MVE_{KE} Q_{ACSA} ($Nm \cdot cm^2$)	Relative change in FL (%)
ΔCK_{ABS} to peak	R	-0.195	0.276	0.360	0.131	0.073	0.500 *	0.208	0.534 *
	Slope	-81.9	89.1	18.6	0.201	0.123	6.58	197.6	29.8
MVC_{KE} torque loss	R	0.202	-0.060	0.164	-0.221	-0.146	-0.201	-0.334	0.180
	Slope	1.62	-0.371	0.16	-0.007	-0.005	-0.051	-7.012	0.192

Note: Pennation angle is presented at 90° knee angle. VL: *Vastus lateralis*. $Q_{ACSA:}$ quadriceps anatomical cross-sectional area. MVE_{KE}: Maximal voluntary eccentric knee extension torque, presented as the peak torque averaged over six sets. MVE_{KE} / VL_{ACSA}: Maximal voluntary eccentric knee extension torque made relative to *vastus lateralis* anatomical cross-sectional area. Relative change in FL: VL fascicle length at a knee angle of 90° made relative to VL fascicle length measured at a knee angle of 20°. *Slope* represents the gradient of the line, where $y = mx + b$. * = $p \leq 0.05$.

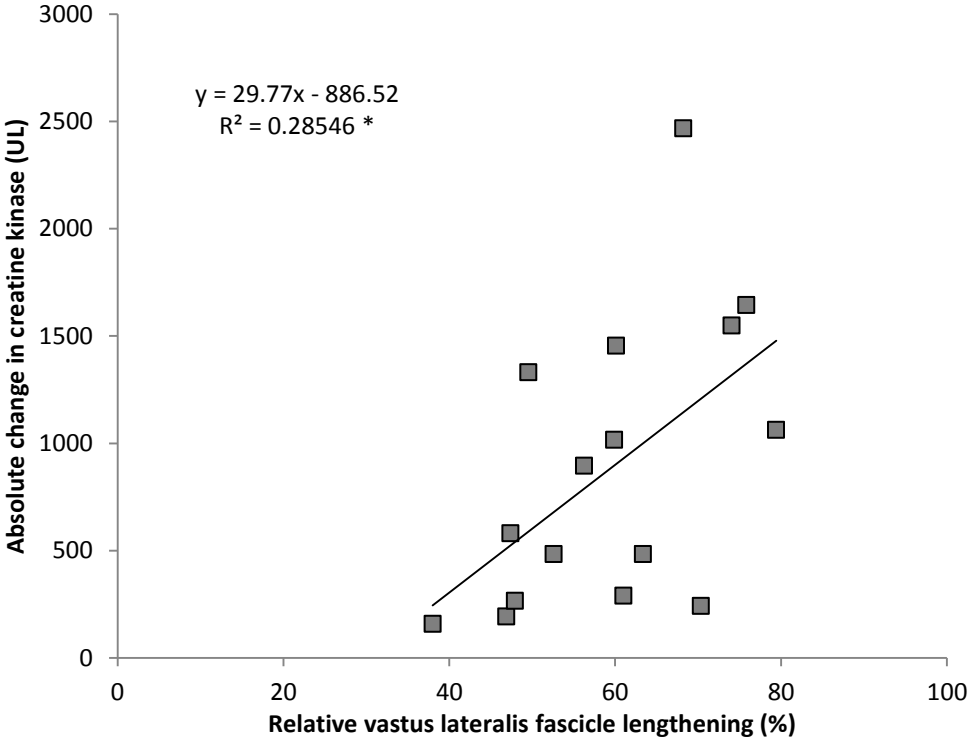

Figure 4. 3: Pearson's correlation between relative *vastus lateralis* (VL) fascicle lengthening and absolute change in creatine kinase (CK). Relative VL fascicle lengthening was calculated as: fascicle length at a knee angle of 90° made relative to fascicle length measured at a knee angle of 20° during maximal eccentric knee extension. Change in CK was calculated as: the peak CK value - the *pre* CK values (ΔCK_{ABS}). * $p \leq 0.05$. absolute

4.5. Discussion

The aim of the current chapter was to establish whether patella tendon properties, eccentric torque and VL fascicle lengthening during a damaging eccentric knee extension protocol, were associated with indirect markers of EIMD. The current paper reports three main findings. 1) Patella tendon stiffness does not correlate with ΔCK_{ABS} to peak or MVC_{KE} torque loss. 2) Relative VL fascicle lengthening and MVE_{KE} torque significantly correlated with ΔCK_{ABS}

to peak. And 3) MVE_{KE} torque and MVE_{KE} torque made relative to Q_{ACSA} do not correlate with ΔCK_{ABS} to peak or MVC_{KE} torque loss.

In addition to the patella tendon attenuating VL fascicle lengthening within the VL in chapter three, the tendon has previously been reported to reduce peak forces and torques during eccentric contractions (Roberts & Azizi, 2010), which have all been hypothesised to be determinants of EIMD. Therefore, tendon properties have been suggested to act as a mechanical buffer during lengthening contractions (Roberts & Azizi, 2010; Fukunaga et al., 2001), and potentially explain group differences in EIMD (McHugh et al., 1999; Marginson et al., 2005). The current chapter provides evidence for the first time that there is no direct association between patella tendon stiffness and ΔCK_{ABS} to peak or peak MVC_{KE} torque loss following EIMD. Although the current chapter reports no direct association between patella tendon stiffness and indirect markers of EIMD, it cannot account for the role of the aponeurosis or an indirect effect of the tendon on markers of EIMD during MVE_{KE}.

Within the current chapter relative VL fascicle lengthening was not associated with peak MVC_{KE} torque loss. These findings agreed with the *in vivo* data conveyed by Hoffman et al. (2014), who reported that during an eccentric walking protocol there was no association between maximal medial *gastrocnemius* fascicle lengthening and planter flexion MVC torque loss. Although within the current chapter, the contribution of tendon to total MTU lengthening during MVE_{KE} remains unknown, Hoffman et al. (2014) suggested that fascicle lengthening was not associated with MVC torque loss due to the Achilles tendon accounting for ~90% of total MTU lengthening. Despite incomparable differences between the Achilles and patella tendon properties (specifically stiffness), based on a similar concept to the findings of Hoffman et al. (2014), the observation of no relationship between VL fascicle lengthening and MVE_{KE} torque loss may be attributed to the role of the patella tendon / aponeurosis mitigating VL fascicle length change during MVE_{KE}. This remains to be confirmed

in the VL. The findings of no significant correlation between fascicle lengthening and MVC_{KE} torque loss may be miss-interpreted to contradict the popping sarcomere theory (Morgan, 1990). Due to heterozygous lengthening of sarcomeres however, the current chapter cannot discount the possibility of sarcomeres lengthening onto the descending limb of the length-tension relationship and increasing EIMD as proposed by the popping sarcomere theory (Morgan, 1990). Furthermore, the current chapter cannot account for the contribution of fascicle lengthening at the distal and proximal region of the VL or within the other three quadriceps muscles. Further investigation is required to conclude whether sarcomere lengthening and fascicle behaviour in the total quadriceps muscle or within other regions of the VL are associated with EIMD in the knee extensors.

Despite no significant correlation with MVC_{KE} torque loss, the current chapter did find a significant correlation between VL fascicle lengthening and ΔCK_{ABS} to peak. The current chapter is the first to investigate and report a correlation *in vivo*, however these findings do contradict previous *in vitro* findings (Warren et al., 1993a). *In vitro,* Warren et al. (1993a) reported no correlation between the magnitude of fascicle lengthening in rat soleus muscle and CK following EIMD. The differences between *in vitro* animal studies and the current chapter could be attributed to several reasons including; Firstly, the use of electrical stimulation applied directly to the muscle *in vitro* (Warren et al., 1993a). Electrical stimulation applied directly to the muscle alters the contractile behaviour, making it difficult to compare to voluntary contractions (Crameri et al., 2007). Secondly, *in vitro* studies use large fascicle lengthening to investigate fascicle strain on EIMD, which may not be attainable *in vivo* (Butterfield, 2010). Regardless, the discrepancies between fascicle lengthening being correlated with ΔCK_{ABS} to peak, and not MVC_{KE} torque loss make the association between fascicle lengthening and EIMD perplexing. Although MVC_{KE} torque loss is accepted as marker of contractile muscle function, there remains uncertainty concerning the validity of ΔCK_{ABS} to peak

as a marker of EIMD (Friden & Lieber, 2001; Komulainen et al., 1995). The release of CK represents EIMD through a loss of membrane permeability. A loss of membrane permeability allows for CK to leak into the interstitial fluid however, the influx includes the release and the clearance of CK in the serum (Bijsterbosch et al., 1985). Although an increase in CK may confirm EIMD has occurred, the magnitude of the CK influx does not necessarily quantify the severity of the EIMD (Friden & Lieber, 2001). Therefore, within the current chapter, an increase in fascicle lengthening and ΔCK_{ABS} to peak may denote the severity of the loss of membrane stability rather than the severity of damage to the contractile proteins.

Previously, significantly higher torques produced during eccentric contractions compared to any other type of contraction has been associated with EIMD (Armstrong et al., 1991). The current chapter however, can report when made relative to Q_{ACSA}, peak MVE_{KE} torque is not associated with MVC_{KE} torque loss or ΔCK_{ABS} to peak. The findings of the current chapter support previous *in vivo* work, which has concluded no association between total eccentric work, and CK (Chapman et al., 2008; Hody et al., 2013) or MVC torque loss (Chapman et al., 2008). It would appear however our findings are at odds with Warren et al. (1993a) who reported peak eccentric torque to be significantly correlated with MVC torque loss in rat muscle. However, Warren et al. (1993a) reported that the significant correlation was lost when eccentric torque was below 113% of *pre-damage* isometric torque. Within the current chapter and the work of Chapman et al. (2008), MVC torque was 96% and 94% (respectively) of *pre-damage* MVC torque, therefore upon closer inspection, the current findings are consistent with those reported by Warren et al. (1993a). The discrepancies regarding peak MVE_{KE} torque values within the current studies and in other *in vivo* studies, with those reported by Warren et al. (1993a) represent an inherent limitation to the comparisons made between *in vitro* versus *in vivo* comparisons of EIMD. Therefore, although the current thesis cannot confirm experimentally that participants were contracting

maximally, our findings represent EIMD resulting from maximal eccentric efforts *in vivo*. Therefore future research needs to investigate whether voluntary eccentric torque *in vivo*, surpassing contractile yield strength (estimated above 113% of isometric MVC (Warren et al., 1993a)), is associated with EIMD.

Muscle architecture has previously been suggested to explain variations in EIMD between muscle groups. Indeed previous authors (Chen et al., 2011), hypothesised that the smaller pennation of the *biceps* could be a determinant of the greater CK following EIMD than in the VL. Within the current chapter however, pennation angle was not associated with ΔCK_{ABS} to peak or MVC_{KE} torque loss. It is possible that pennate fibres result in a lower level of force transmission from the eccentric load to the muscle. However, based on the current data, between the lowest to highest pennation angles measured in the current participant groups (~5°), the change in mean MVE_{KE} torque (272 Nm) transmitted along the muscle fascicles would be ~6 Nm. Clearly this is not a sufficient difference in stress to expect any significant differences in EIMD. Therefore within the VL pennation angle during EIMD is not a determinant of EIMD however pennation angle may contribute to differences in EIMD between two muscle groups that have larger differences in pennation angle (Chen et al., 2011).

4.6. Conclusion

In conclusion the current chapter has established no association between patella tendon properties, MVE_{KE} torque made relative to Q_{ACSA}, VL fascicle lengthening and pennation angle, with EIMD based on MVC_{KE} torque loss or ΔCK_{ABS} to peak. The current chapter reports significant correlation between VL fascicle lengthening and ΔCK_{ABS} to peak which may reflect the magnitude of EIMD or indicate a loss membrane permeability has occurred however, due to fascicle lengthening having no association with any other marker of EIMD, it is likely the

significant correlation represent the latter. Future research needs to investigate whether the same findings would be reported if higher *in vivo* MVE_{KE} torques were measured.

Chapter five: Sex differences in muscle damage following maximal eccentric knee extensions

5.1. Abstract

Aim: To investigate whether there is a sex difference in exercise-induced muscle damage (EIMD). *Materials and method: Vastus lateralis* (VL) and patella tendon properties were measured in males and females using a combination of ultrasonography, electromyography and dynamometry. During maximal voluntary eccentric knee extensions ((MVE_{KE}) 12 reps × 6 sets), VL fascicle lengthening and MVE_{KE} torque was recorded every 10° of knee joint angle (20-90°). Maximal voluntary isometric knee extension (MVC_{KE}) torque loss, creatine kinase (CK) and muscle soreness were measured *pre, post, 48, 96 and 168* hours post damage as markers of EIMD. *Results:* Patella tendon stiffness and VL fascicle lengthening were significantly higher in males compared to females ($p \leq 0.05$). There was no sex difference in MVC_{KE} torque loss and muscle soreness post EIMD ($p \geq 0.05$). Post EIMD CK was higher in males compared to females ($p \leq 0.05$) and remained so when MVE_{KE} torque relative to estimated quadriceps anatomical cross-sectional area was taken as a covariate ($p \leq 0.05$). *Conclusion:* Based on MVC_{KE} torque loss, there is no sex difference in EIMD. The higher CK in males could not be explained by differences in MVE_{KE} torque, VL fascicle lengthening or patella tendon stiffness. The comparable MVC_{KE} torque loss and rightward shift in optimal knee angle could reflect the fact that EIMD was similar between sexes. It is proposed that the role of oestrogen in stabilising the cell membrane may have contributed to lower CK post EIMD in females rather than higher EIMD in males.

5.2. Introduction

It is well established that unaccustomed eccentric exercise causes exercise-induced muscle damage (EIMD). Although there remains no conclusion on the initial events leading to EIMD (direct damage to sarcomeres, or the Excitation-Contraction (E-C) coupling process); the high stress and strain of eccentric contractions are associated with subsequent increases in markers of EIMD (Warren et al., 1993a; Lieber & Friden, 1993). The degree of EIMD has been quantified using both direct (e.g. muscle biopsies) and indirect techniques (Warren et al., 1999; Clarkson & Hubal, 2002). Due to the invasive procedure and the small sampling area of muscle biopsies, indirect techniques are more common (Clarkson & Hubal, 2002). Reduction in maximal isometric torque following EIMD has been reported as the most valid indirect marker of EIMD (Warren et al., 1999). Change in range of motion, muscle soreness, and the detection of intramuscular enzymes (e.g. Creatine kinase (CK)) in venous blood however, are frequently reported as indirect markers of EIMD (Warren et al., 1999; Savage & Clarkson, 2002; Sayers & Clarkson, 2001; Sewright et al., 2008).

Within animals, EIMD is reported to be significantly higher in males compared to females (Komulainen et al., 1999). Within human research however, the evidence is not as compelling; for example, Stupka et al. (2000) were among the first to measure human sex differences in EIMD. Following maximal leg press and leg extensions, Stupka et al. (2000) reported similar amounts of Z-line streaming and CK between males and females. In contrast, following EIMD in the elbow flexors, Sewright et al. (2008) found males to have significantly higher levels of CK compared to their female counterparts. The different muscle architecture and tendon properties in the elbow flexors compared to the knee extensors may partly explain the aforementioned discrepancies (Chen et al., 2011). It remains unknown however whether the architectural and structural properties of the muscle-tendon unit (MTU) may play a role in determining sex differences in EIMD.

Previous literature has alluded to the tendon potentially acting as a mechanical buffer during eccentric contractions (Roberts & Azizi, 2010; Roberts & Konow, 2013; Reeves & Narici, 2003). Using Hill's three element muscle model, Lichtwark and Wilson (2007) found during the stance phase of walking, increasing Achilles tendon stiffness augmented fascicle lengthening despite total MTU lengthening remaining constant. In agreement, chapter four attributed greater *vastus lateralis* (VL) fascicle lengthening during eccentric contractions in males, to a significantly higher patella tendon Young's modulus compared to females. Thus the males experienced significantly higher levels of VL fascicle lengthening, which, as a determinant of EIMD *in vitro* (albeit in animals (Lieber & Friden, 1993; Talbot & Morgan, 1998)), may explain higher levels of EIMD previously reported in males (Sewright et al., 2008; Joyce et al., 2014). However, any link between fascicle lengthening and EIMD *in vivo* remains to be confirmed experimentally and there are inherent limitations of extrapolating findings from animal studies (Butterfield, 2010).

Although no data exists to attribute lower EIMD in females to sex differences in MTU properties, the attenuated EIMD in females has previously been attributed to the protective properties of oestrogen (Joyce et al., 2014). An anti-inflammatory action through high antioxidant capacity, suppression of nuclear factor-kappa beta, and a direct membrane stabilising property of oestrogen, have been suggested to contribute to the suppression of EIMD (Kendall & Eston, 2002). Through manipulation of oestrogen levels, e.g. using the oral contraceptive pill (OCP (Savage & Clarkson, 2002)), hormone replacement therapy (HRT) (Dieli-Conwright et al., 2009) and *pre* vs. *post* menopause comparisons (Buckley-Bleiler et al., 1989), both a protective (Dieli-Conwright et al., 2009; Savage & Clarkson, 2002) or no effect (Buckley-Bleiler et al., 1989; Sewright et al., 2008; Savage & Clarkson, 2002) of oestrogen on EIMD has been reported. Due to the inconsistent nature of these findings, it is unclear whether oestrogen can account directly for sex differences in EIMD. Alternatively, oestrogen

may indirectly reduce levels of EIMD through altering tendon properties. For instance, lower levels of oestrogen have been associated with lower tendon strain in OCP users compared to non-users (Bryant et al., 2008). Previously, differences in tendon stiffness have been suggested to explain group differences in EIMD (McHugh et al., 1999; Marginson et al., 2005) however, to date, there is no systematic research supporting this hypothesis.

Sex differences in EIMD remain equivocal. Furthermore, a physiological explanation for the potential differences has yet to be reported. Therefore, the aims of the current chapter were twofold: firstly, to establish whether there is a sex difference in EIMD, when difference in muscle size is accounted for; and secondly, dependent on the outcome of the first aim, to establish whether tendon stiffness may explain the potential sex difference in EIMD.

5.3. Materials and methods

5.3.1. Subjects

11 males (21.1 ± 1.6 years of age, 72.0 ± 7.5 kg and 176 ± 6 cm) and 11 females (21.4 ± 1.6 years of age, 63.0 ± 5.8 kg and 165 ± 8 cm) signed written informed consent to participate. All participants self-reported as being recreationally active (undertaking no more than 1 hour of "moderate" physical activity per week) and did not take part in any structured resistance training. None of the female participants had ever used any form of oestrogen-based contraception. All women reported regular menstrual cycles, documenting an average cycle length of 28 ± 1 days (Cole et al., 2009). Females were tested on the 14th day of their menstrual cycle (self-reported) to measure oestrogen levels at ovulation (Brown, 1955; Cole et al., 2009). All procedures complied with the Declaration of Helsinki and ethical approval was obtained through the local ethics committee (World Health Association, 2013). Exclusion criteria included any resistance training in the last six months, occupation or lifestyle that required regular heavy lifting or carrying, any known muscle disorder, the use of dietary supplements (i.e. vitamin E), and any musculoskeletal injury in the last three months. Further exclusion criteria for female participants included, irregular menstrual cycles (where regular cycles were defined as 24-35 days (Cole et al., 2009; Landgren et al., 1980)) in the last 12 months, and pregnant in the year preceding inclusion in the current chapter. All inclusion and exclusion criteria were determined through participant questionnaire prior to inclusion within this chapter.

5.3.2. Testing protocol

Participants attended the laboratory on five different occasions over nine days. The sessions were as follows: 1) *pre-damage* 2) *damage* (48 hours post *pre-damage*), 3) *48 hours*, 4) *96 hours,* and 5) *168 hours* post *damage*. *Pre-damage* assessments consisted of stature and mass

(anthropometric measures), patella tendon moment arm (PT_{MA}), 5-6ml blood sample, dynamometer familiarisation (within *pre-damage*), morphological measures of the patella tendon (tendon size and stiffness) and isometric maximal voluntary knee extension (MVC_{KE}) torque measurements at six knee angles (60, 65, 70, 75, 80 and 90° (0° = full extension). Participants were tested at six knee angles to obtain optimal knee angle for MVC_{KE} torque. Participants performed two practice isometric MVCs, at two knee joint angles during the familiarisation session (within *pre-damage*). Stature and mass were measured using a wall mounted stadiometer (Harpenden, Holtain Crymych, UK) and digital scales (Seca model 873, Seca, Germany) respectively. The *damage* session consisted of maximal voluntary eccentric knee contractions (MVE_{KE}), 5-6 mL venous blood sample, rating of muscle soreness and MVC_{KE} torque measurements. *48, 96 and 168 hours session* consisted of 5-6 mL blood sample, rating of muscle soreness, and MVC_{KE} torque measurements at all six knee angles (60, 65, 70, 75, 80 and 90°).

All tests were carried out in the non-dominant leg. The non-dominant leg was defined as the leg that provided stability during movements, e.g. kicking. Participants were seated in an isokinetic dynamometer (Cybex Norm, Cybex International, NY, USA), with a hip angle of 85° (0° = supine). To reduce any extraneous movement during maximal efforts participants were secured in a seated position using inextensible straps around the shoulders and hips. The isokinetic dynamometer axis of rotation was visually aligned with the knee joint's centre of rotation. During *pre-damage* the isokinetic dynamometer settings (chair position, distance from the dynamometer, dynamometer arm length) and anatomical zero (leg full extended, knee angle 0°) were recorded to ensure repeatability in the following sessions.

5.3.3. Vastus lateralis anatomical cross-sectional area

Vastus lateralis anatomical cross-sectional area (VL_{ACSA}) was measured using a real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy). With the participant laid supine and their non-dominant leg fully extended (knee angle 0°), the distal and proximal insertions sites of the VL were identified using an ultrasound probe (7.5 MHz linear array probe, 38 mm wide). At 50% of VL muscle length echo-absorptive markers were placed in parallel, at intervals of 30 mm, from the lateral to the medial edge of the VL muscle. The ultrasound probe was held perpendicular to the VL muscle in the axial plane. The ultrasound probe was moved steadily over the echo-absorptive markers from the lateral to the medial edge of the muscle. Constant, light pressure was placed on the muscle during scanning to avoid compression of the muscle. The images were recorded in real time at 25 frames per second (Adobe Premier pro Version 6, Adobe Systems Software, Ireland). Using capturing software (Adobe Premier Elements, version 10), individual images were acquired at each 30 mm interval. Shadows cast by the echo-absorptive markers allowed the images to be aligned by the outline of the muscle, thus forming the entire VL_{ACSA} in a single image (Adobe Photoshop Elements, version 10). Digitising software (ImageJ 1.45, National Institutes of Health, USA) was used to measure VL_{ACSA}. This method of calculating VL_{ACSA} has previously been accepted as reliable and valid when compared to magnetic resonance imaging (MRI), with a reported interclass correlation between 0.998 and 0.999 (Reeves et al., 2004). Within chapter two, high reliability and low day-to-day measurement error was reported when using this method to measure VL_{ACSA}.

Within males a VL_{ACSA} of 24.3 cm^2 has previously reported to contribute to ~32% of total quadriceps anatomical cross-sectional area ((Q_{ACSA}) Q_{ACSA} = 74.9 cm^2 (Tsakoniti et al., 2008)). Where in females, a VL_{ACSA} of ~21 cm^2 has been reported to contribute to 38% of Q_{ACSA} (Q_{ACSA} = ~ 55 cm^2 (Grosset & Onambele-Pearson, 2008)). Therefore, assuming the

contribution of VL_{ACSA} remains constant within males and females, Q_{ACSA} within males and females was estimated by dividing VL_{ACSA} by 32% and 38% respectively.

5.3.4. Maximal voluntary isometric knee extension torque measurements

At six different knee angles (60, 65, 70, 75, 80 and 90° (full extension = 0°)) participants were instructed to perform two MVC_{KE} lasting ~two seconds at the plateau with 90 seconds rest between contractions. Torque was presented, in real time, on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter (Biopac Systems, Santa Barbara, CA). Torque measurements were later analysed offline with the accompanying software (Acknowledge, version 3.9.2). The highest torque produced at each angle was taken as MVC_{KE} peak torque. The angle where the highest MVC_{KE} torque was produced during the *pre-damage* was recorded as optimal knee angle. Optimal knee angle was used to calculate MVC_{KE} torque loss in the subsequent sessions. To calculate loss of MVC_{KE} torque following eccentric exercise, within the *damage* session MVC_{KE} torque measurements were repeated 60 minutes post eccentric exercise (to reduce any fatigue effect (Walsh et al., 2004)) and *48 hours, 96 hours* and *168 hours* (recovery) post eccentric exercise.

5.3.5. Patella tendon length and cross-sectional area

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure patella tendon cross-sectional area (PT_{CSA}) and patella tendon length (PT_L) at a fixed 90° knee angle. The distance between the apex of the patella and the tibial tuberosity, marked using sagittal ultrasound images, was taken as PT_L. To measure PT_{CSA} the ultrasound probe was placed in the transverse plane and images were captured at 25%, 50%, and 75% of PT_L. The images were later analysed offline using ImageJ (1.45, National Institutes of Health, USA). A mean of all three images were taken for PT_{CSA} (O'Brien et al., 2010).

5.3.6. Patella tendon stiffness

The participants were seated in the isokinetic dynamometer, with the knee angle fixed at 90°, and were instructed to perform a ramped, isometric MVC_{KE} lasting ~5-6 seconds. Ramped MVC_{KE} torque and displacement of the patella tendon were synchronised using a 10-V square wave signal generator. Patella tendon displacement was measured over two MVC_{KE}, once with the ultrasound probe positioned over the distal edge of the patella and on the second contraction over the tibial tuberosity (Onambélé et al., 2007), so that total displacement would be computed from the composite of proximal and distal patella motions (see below). Torque was presented on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter and subsequently analysed with the accompanying software (Acknowledge, Biopac Systems, Santa Barbara, CA). To create an external marker on the ultrasound images, an echo-absorptive marker was placed on the skin. Using the marker to calculate displacement of the tendon, the distance of the marker (shadow) from an anatomical reference point at the beginning of the contraction, to the position of the shadow at the end of the contraction was calculated. A 10-V square wave signal generator was used to synchronise the ultrasound images with the torque acquisition system. Images were captured at ~10% intervals of ramped MVC_{KE} torque (Onambélé et al., 2007). Total patella tendon displacement was calculated as displacement at the apex of the patellar plus the displacement at the tibial tuberosity (Onambélé et al., 2007). Patella tendon forces were calculated as: (MVC_{KE} torque + antagonist co-activation torque) / PT_{MA}.

Patella tendon moment arm was measured at 90° (full extension = 0°) in the sagittal plane, from a single-energy X-ray absorptiometry scan (frame 23.3 cm x 13.7 cm) Hologic Discovery, Vertec Scientific Ltd, UK). Using OsiriX (DICOM viewer, ver. 4.0, Pixemo, Switzerland), patella tendon moment arm length was determined as the perpendicular distance from the centre of the patella tendon to the tibio–femoral contact point. Single-

energy X-ray absorptiometry scans have been compared to MRI measures, demonstrating consistent reliability and validity against this standard (Erskine et al., 2014).

To compute tendon forces, the calculation of antagonist co-activation torque is described below. The force – patella tendon elongation curve stemming from data at every 10% MVC_{KE} was then fitted with a second-order polynomial function forced through zero (Onambélé et al., 2007). The tangential slope at discreet sections of the curve, relative to MVC_{KE} force, was computed by differentiating the curve at every 10% force intervals. In addition, to standardise the comparison of tendon stiffness at an absolute load, the slope of the tangential line, corresponding to the MVC_{KE} force of the weakest participant, was computed for each subject.

5.3.7. Co-activation

To determine co-activation during the ramped MVC_{KE}, Electromyogram (EMG) of the *bicep femoris* (BF) was measured. Guided by an axial-plane ultrasound of the *bicep femoris*, two bipolar electrodes (Ambu, Neuroline 720, Denmark) were placed in the mid-sagittal line at 25% of BF muscle length (distal end = 0%). A reference electrode (Ambu, Blue Sensor, Denmark) was placed on the lateral tibial condyle. The electrodes were placed in a bipolar configuration with a constant inter-electrode distance of 20 mm (Hermens et al., 2000). Prior to electrode placement; the skin was shaved, gently abraded and cleansed with an alcohol wipe to reduce skin impedance below 5000 Ω (Hermens et al., 2000). To minimise cross talk, and ensure a mid-sagittal placement of electrodes, ultrasonography was used to identify the medial and lateral aspects of the BF muscle. The raw EMG signal was amplified (×2000) and filtered (through low and high pass filters of 10 and 500 Hz respectively) with the sampling frequency set at 2000 Hz. Ramped MVC_{KE} torque and BF EMG were recorded in real time and synchronised using an external square wave signal generator. Participants performed two maximal voluntary isometric knee flexions (MVC_{KF}) at 90° knee angle. They were instructed

to perform MVC_{KF} rapidly and as forcefully as possible against the dynamometer's lever arm. The participants were instructed to relax once a two second plateau had been attained (as observed on the dynamometer screen display). The integral of the root mean square of the BF EMG signal, was calculated 500 ms either side of instantaneous MVC_{KF} maximal torque from the contraction corresponding to the highest MVC_{KF} torque. Prior to contraction the baseline signal noise was calculated as the integral root mean square over 1s and removed from the measured EMG during MVC_{KF} and MVC_{KE}. At every 10% of ramped MVC_{KE} torque the absolute integral of the BF EMG was taken over 250 ms. Co-activation torque was calculated as, (BF EMG during ramped MVC_{KE} / BF EMG during MVC_{KF}) × peak MVC_{KF} torque at 90° knee angle (Onambélé et al., 2007). This equation assumes that the BF is representative of the entire hamstring (Carolan & Cafarelli, 1992) and that a linear relationship exists between BF EMG and MVC_{KF} torque (Lippold, 1952).

5.3.8. Patella tendon stress / strain relationship

Patella tendon strain was calculated as a ratio of total patella tendon displacement and PT_L Patella tendon stress was calculated as: patella tendon force (N) / PT_{CSA} (mm^2).

5.3.9. Patella tendon Young's modulus

Young's modulus was calculated as, patella tendon stiffness × (PT_L (mm) / PT_{CSA} (mm^2)).

5.3.10. 'Damaging' eccentric exercise

Prior to eccentric exercise, a warm-up of 10 isokinetic knee extensions and knee flexions were carried out through the full test range of motion (20-90°, at $60°{\cdot}s^{-1}$), ensuring a progressive increase in effort (with the last contraction being maximal). For the eccentric exercise, the knee extension range of motion was set at 20 – 90° (0° = full extension). Participants were asked to perform six sets of 12 maximal voluntary eccentric knee

extensions (MVE_{KE}) repetitions, which has previously been reported to induce significant EIMD (Jamurtas et al., 2005). The eccentric phase of the contractions was performed at an angular velocity of 30°·s^{-1} (Jamurtas et al., 2005). The concentric phase was performed sub-maximally at an angular velocity of 60°·s^{-1} to minimise fatigue and enhance eccentric damage (Chapman et al., 2006). Two minutes rest was provided between each set. Participants remained seated in the isokinetic dynamometer throughout the entire exercise protocol, including rest periods. Visual feedback and verbal encouragement was continuously provided throughout the protocol (Campenella et al., 2000). MVE_{KE} torque was recorded throughout each contraction and displayed via the torque acquisition system. For each set, peak MVE_{KE} torque was determined as the highest torque out of the 12 repetitions. Average peak MVE_{KE} torque was calculated as an average of peak MVE_{KE} across six sets.

5.3.11. Change in vastus lateralis fascicle length during the eccentric protocol

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure VL fascicle length during MVE_{KE}. The ultrasound probe (7.5 MHz linear array probe) was fixed at 50% of VL muscle length in the mid-sagittal plane of the non-dominant leg. A hypo-allergenic ultrasound gel (Parker, Park Laboratories Inc., Fairfield) was used to enhance acoustic coupling between the skin and the ultrasound probe.

During the first set (out of six) of MVE_{KE} contractions, ultrasound images were recorded onto a PC, in real time, at 25 frames per second (Adobe Premier pro Version 6). An externally generated square wave signal was used to synchronise the ultrasound images with the torque acquisition system. Three MVE_{KE} contractions were chosen from the first set of 12 repetitions for architectural analysis. Using frame capture software (Adobe Premier Elements, version 10), an ultrasound image (frame corresponding to every 10° of knee angle, ranging from 20 - 90°) was acquired for offline analysis using ImageJ (1.45, National

Institutes of Health, USA). To ensure there was no movement artefact included in the measurement of fascicle length, an echo-absorptive marker was fixed on the skin to provide a visual reference line for the internal structures. If movement of the reference line was observed in a recording, the contraction was discarded and another repetition was chosen for analysis.

Using digitising software (ImageJ 1.45, National Institutes of Health, USA), VL fascicle length was analysed offline at every 10° knee angle (range 20 - 90°, 0° = full extension) throughout the MVE_{KE}. Fascicle length was measured from the visible insertion of the fibre into either the deep and superficial aponeurosis (Reeves & Narici, 2003). Where the fascicle extended longer than the ultrasound image (frame width 3.50 cm and height 4.15 cm), linear continuation of the fascicle and aponeurosis was assumed. Using ultrasound a 2-7% error has been associated with assuming linear continuation to calculate VL fascicle length at 120° knee angle (Finni et al., 2003a). Recently, when comparing ultrasound to cadaveric measurements', the linear extrapolation method has been concluded as a valid technique for measuring VL fascicle length at rest (ICC = 0.853) (Ando et al., 2014). Furthermore, chapter two concludes high reliability and low day-to-day measurement error when measuring VL fascicle lengthening during MVE_{KE}. To reduce error associated with the estimation of VL fascicle length, an average of three fascicles across the image was taken. Change in fascicle length is presented as fascicle length at a knee angle of 90° made relative to fascicle length measured at a knee angle of 20°; hereafter termed "relative fascicle lengthening" and reported as a percentage change from starting length at 20°.

5.3.12. Muscle soreness

Muscle soreness was measured using a visual analogue scale. The visual analogue scale consisted of a 100 mm line, with 0 mm labelled "No pain at all" and 100 mm labelled

"Unbearable pain". Seated in the isokinetic dynamometer the non-dominant leg was passively moved through a full range of motion at $30°\cdot s^{-1}$. Participants were asked to mark a line perpendicular to the visual analogue scale to denote the level of pain they experienced during the passive movement. The visual analogue scale has been reported to be a valid and reliable measure of muscle soreness (inter class correlation ≥ 0.96 (Bijur et al., 2001)).

5.3.13. Blood samples

Venous blood samples were taken to measure CK levels. A 21-gauge needle was inserted into the antecubical vein of the forearm, using a 10 mL syringe. 5-6 mL of blood was drawn into a serum collection tube. The sample was left on a crushed ice bed for 60 minutes. The sample was then centrifuged at 4500 rpm at 0°C for 10 minutes. Using a 200 – 1000 µl pipette (Eppendorf), the resulting serum sample was separated into three aliquots (~500 µl each) and stored in eppendorfs at -20°C until CK analysis was performed. Creatine kinase levels were measured using a standard colorimetry procedure, measuring at optical density 340 nm (BioTek ELx800 96 well Microplate Reader) and immediately analysed (Gen5, version 2.0). Each sample was run in duplicate-quadruplets using an EnzyChromTM CK Assay Kit (BioAssay Systems, Hayward, CA, sensitivity 5 U/L, intra-assay variability ≤ 5% (as reported by the manufacturer)). An average of two – four readings was taken (at 20 minutes, (25 minutes if necessary i.e. enzyme activity above assay's detection upper limit of 300 U/L), or 40 minutes following sample exposure to assay reagents (i.e. Substrate, assay buffer and enzyme mix)). CK activity is reported in absolute and absolute change ((ΔCK_{ABS}) i.e. the peak CK value - the *pre* CK values) terms.

5.3.14. Statistics

Statistical analyses was carried out using the statistical software package SPSS (v.19, Chicago, IL) for Windows and Microsoft Excel. To ensure the data were parametric, the Levene's and

Shapiro-Wilk tests were used to assess the variance and normality of the data. If parametric tests were violated, the equivalent non-parametric tests were used. For sex differences in anthropometric measures, VL_{ACSA}, Q_{ACSA} and patella tendon properties, independent T-tests and Mann-Whitney U tests were used. A 2×5 mixed design ANOVA (between factors: sex (2 levels), and time from EIMD (5 levels) was used for muscle soreness, torque loss and CK levels. Wherever the assumption of sphericity was violated, the Greenhouse-Geisser correction was used. When a significant group effect was found, post-hoc independent T-tests was used (planned contrast) with LSD correction. Since an association was reported between change in CK levels and MVE_{KE} normalised to Q_{ACSA}, an analysis of covariance was used. Significance was set at $p \leq 0.05$. Data are presented as mean ± standard deviation.

5.4. Results

5.4.1. Anthropometric measurements

There was no significant difference in age between males and females ($p = 0.326$). Males had significantly greater mass ($p = 0.009$) and were taller ($p = 0.007$) than females. *Pre-damage* peak MVC_{KE} torque was significantly higher in males compared to females (263 ± 27 Nm, 190 ± 33 Nm, respectively, $p = 0.0004$). Other dimension and functional characteristics of the population are described in Table 5.1.

5.4.2. Vastus lateralis anatomical cross-sectional area

Vastus lateralis anatomical cross-sectional area was significantly higher in males compared to females (24.4 ± 3.9 cm^2 and 20.4 ± 3.4 cm^2 respectively, $p = 0.019$). Estimated Q_{ACSA} was significantly higher in males compared to females (72.8 ± 14.0 cm^2 and 53.9 ± 8.51 cm^2 respectively, $p = 0.0001$).

5.4.3. Patella tendon properties

Patella tendon properties for males and female are presented in Table 5.1. Patella tendon length was 14% longer in males compared to females. Patella tendon cross-sectional area was 39% larger in males. Ramped MVC_{KE} was 36% higher in males compared to females. Patella tendon moment arm length was 12% longer in males compared to females ($p = 0.0007$). Tendon stiffness and Young's modulus at MVC_{KE} was 54% and 35% (respectively) higher in males compared to females. To account for the significantly higher ramped MVC_{KE} torque in males, the patella tendon force corresponding to the highest ramped MVC_{KE} torque of the weakest participant (independent of sex) was used to calculate standardised patella tendon stiffness at a standardised force (2330 N). Standardised patella tendon stiffness (males 998 ± 329 $N \cdot mm^{-1}$, females 542 ± 119 $N \cdot mm^{-1}$, $p = 0.003$) remained significantly higher in males compared to females.

Table 5. 1. Patella tendon properties in males and females.

	Males		Females	
Patella tendon length (mm)	56.4 **	± 5.1	48.3	± 6.0
Patella tendon cross-sectional area (mm^2)	83.9***	± 16.4	54.2	± 16.0
Patella tendon moment arm (cm)	4.41***	± 0.31	3.90	± 0.90
Ramped MVC_{KE} (Nm)	203***	± 23	129	± 26
Patella tendon force (N)	4707***	± 603	3385	± 700
Patella tendon stiffness ($N \cdot mm^{-1}$)	1387***	± 560	610	± 146
Young's modulus (MPa)	948*	± 492	620	± 284

Note: Patella tendon stiffness and Young's modulus calculated at 100% maximal voluntary isometric knee extension (MVC_{KE}), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data is presented as means ± standard deviation.

5.4.4. Torque during maximal voluntary eccentric knee extensions

Average peak MVE_{KE} torque of each set (six in total) was significantly higher in males compared to females ($p \leq 0.05$). Peak MVE_{KE} torque averaged over six sets was significantly higher in males compared to females (255 ± 50.0 Nm and 167 ± 29.0 Nm, respectively, $p = 0.0001$). When peak MVE_{KE} torque was normalised to Q_{ACSA}, there was no significant difference between males and females (3.40 ± 0.78 $Nm \cdot cm^2$ and 3.15 ± 0.66 $Nm \cdot cm^2$, respectively, $p = 0.160$).

Peak MVE_{KE} torque made relative to *pre-damage* MVC_{KE} torque, was not significantly different between males and females (97 ± 17% and 87 ± 12% respectively, $p = 0.139$)

5.4.5. Vastus lateralis fascicle length

Fascicle length at 20° knee joint angle was not significantly different between males and females (6.86 ± 0.31 cm, 6.67 ± 0.75 cm respectively, p = 0.197). The increase in fascicle length from 20° to 90° knee angle during MVE_{KE} was significantly higher in males compared to females (3.84 ± 0.92 cm, 2.82 ± 0.45 cm, respectively, p = 0.007). VL fascicle length at 90° knee angle made relative to fascicle length at knee angle of 20°, was significantly greater in males compared to females (56.0 ± 13.6% and 43.7 ± 10.1%, respectively, p = 0.034).

5.4.6. Change in optimal knee angle

Pre-damage, optimal MVC_{KE} knee angle was not significantly different between males and females (median, 75.0 ± 10.0°, and 75.0 ± 10.0°, respectively, p = 0.458). Post EIMD both males (90.0 ± 5.0°, p = 0.002) and females (median 80.0 ± 5.0°, p = 0.007) demonstrated a significant rightward shift in MVC_{KE} optimal angle. There was no significant difference in the magnitude of the rightward shift in MVC_{KE} optimal angle between males and females (p = 0.099).

5.4.7. Maximal voluntary isometric knee extension torque loss

Maximal voluntary isometric knee extension torque expressed as a percentage of *pre-damage* MVC_{KE} torque is illustrated in **Error! Reference source not found.**. A two way mixed repeated measures ANOVA for MVC_{KE} torque loss reported a significant main effect of time (p = 0.0005), however there was neither a sex effect (p = 0.201) nor a time × sex interaction (p = 0.324). There was no significant difference in peak MVC_{KE} torque loss between males and females (22.5 ± 8.5% and 27.1 ± 13.1% respectively, p = 0.332).

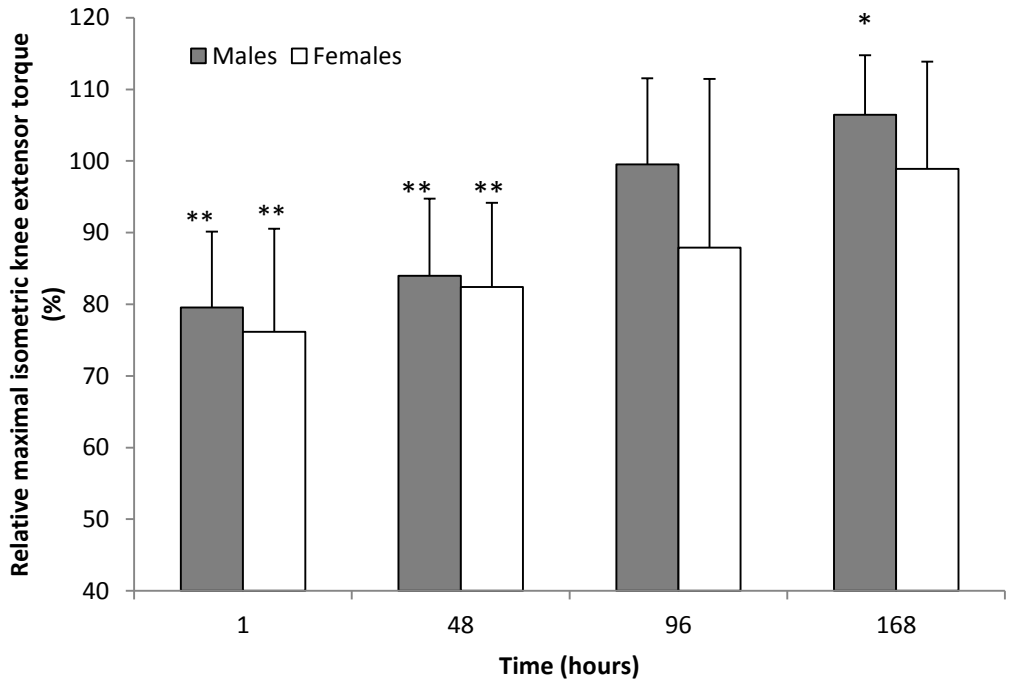

Figure 5. 1: Maximal voluntary isometric knee extension (MVC_{KE}) torque in males and females, expressed as a percentage of *pre-damage* MVC_{KE} torque. Data is presented as means ± standard deviation. Significant difference from *pre* –damage * $p \leq 0.05$, ** $p \leq 0.01$.

5.4.8. Creatine kinase levels

Absolute CK response for males and females is illustrated in Figure 5. 2. Inter-assay variability within the current chapter was ≤ 10%. A two way mixed repeated measures ANOVA for the CK response reported a significant main effect of time ($p = 0.004$), group effect of sex ($p = 0.0004$) and interaction effect ($p = 0.014$). *Pre-damage* CK was not significantly different between males and females (138 ± 140 U/L and 80.8 ± 56.7 U/L, respectively, $p = 0.097$). Absolute peak CK was significantly higher in males compared to females (1607 ± 1247 U/L and 409 ± 421 U/L, respectively, $p = 0.003$). ΔCK_{ABS} to peak was significantly higher in males compared to females (1468 ± 1247 U/L and 329 ± 441 U/L, respectively, $p = 0.005$).

Creatine kinase response made relative to Q_{ACSA} for males and females is illustrated in Figure 5. 2. A two way mixed repeated measures ANOVA for CK response made relative to Q_{ACSA} reported a significant main effect of time ($p = 0.003$) and a group effect of sex ($p = 0.004$), however there was no significant interaction ($p = 0.112$). ΔCK_{ABS} to peak made relative to Q_{ACSA} remained significantly higher in males compared to females (18.4 ± 15.7 U/L·cm^2 and 6.84 ± 10.75 U/L·cm^2, respectively, $p = 0.043$).

A significant correlation was observed between MVE_{KE} torque made relative to Q_{ACSA} and ΔCK_{ABS} to peak ($r = 0.635$, $p \leq 0.001$), it was therefore considered as a covariate of CK. The analysis of covariance revealed a significant effect of sex on ΔCK_{ABS} to peak when controlling for average peak MVE_{KE} torque made relative to Q_{ACSA} as a covariate ($p = 0.0001$, power 0.468).

5.4.9. Creatine kinase correlation

Within males and females there was no significant correlation between ΔCK_{ABS} to peak and relative patella tendon stiffness ($r = -0.260$, $p = 0.220$ and $r = 0.086$, $p = 0.419$ respectively). Similarly, pooling the whole population, there was no correlation between ΔCK_{ABS} to peak and relative patella tendon stiffness ($r = 0.184$, $p = 0.212$).

Within males and females there was no significant correlation between ΔCK_{ABS} to peak and relative change in fascicle length (males $r = 0.037$, $p = 0.457$ and females $r = -0.109$, $p = 0.382$). Again, pooling the whole population, there was no significant correlation between ΔCK_{ABS} to peak and relative change in fascicle length ($r = 0.251$, $p = 0.136$).

5.4.10. Muscle soreness

A two way mixed measures ANOVA for muscle soreness reported a significant main effect of time ($p = 0.0004$); however, there was no significant group difference (sex, $p = 0.395$) nor a significant interaction ($p = 0.759$). For both males and females, peak muscle soreness

occurred 48 hours post EIMD. There was no difference in peak muscle soreness in males and females (4.82 ± 2.23 and 3.75 ± 1.68, respectively, p = 0.134).

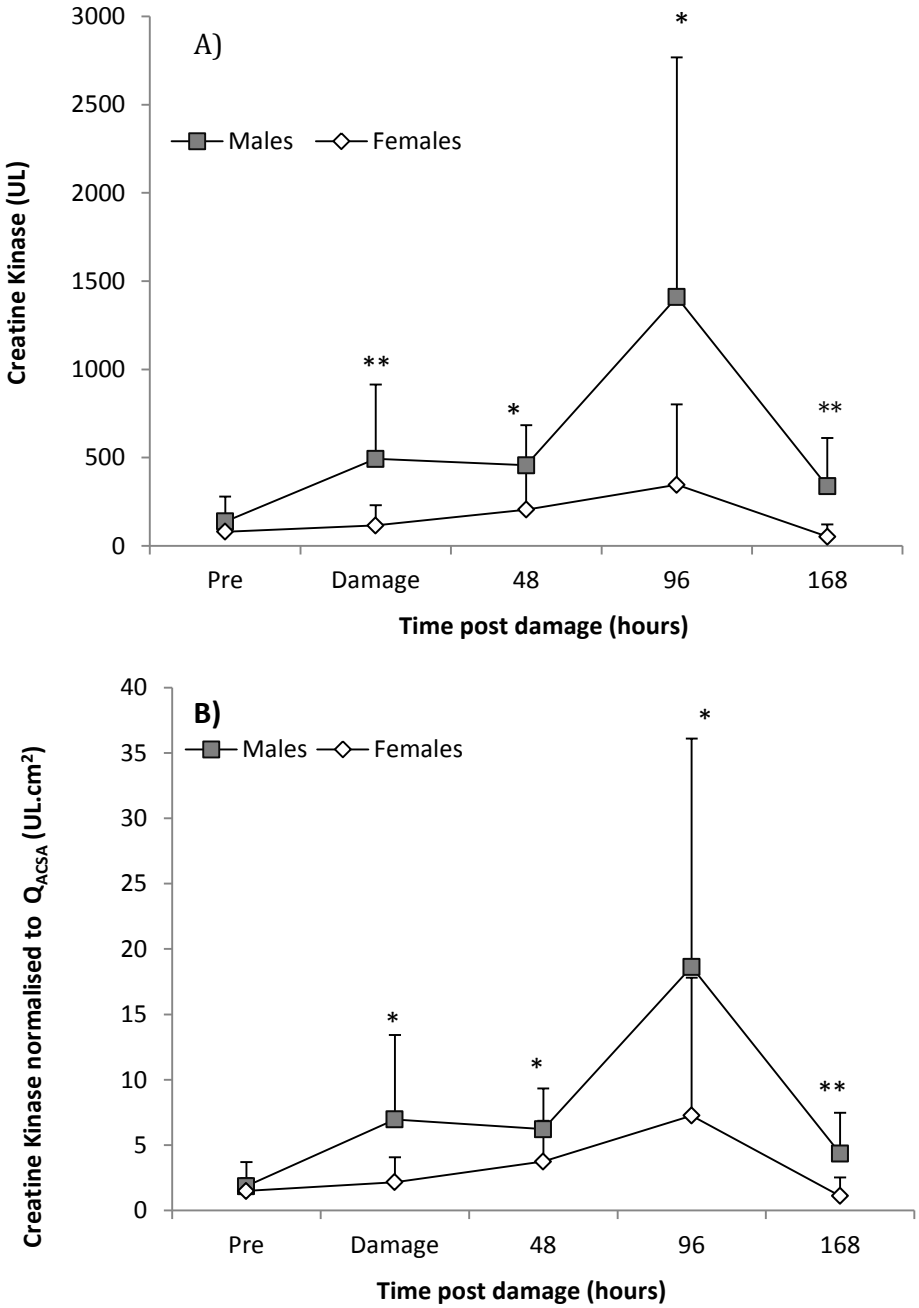

Figure 5. 2: A) Creatine kinase (CK) response following exercise-induced muscle damage (EIMD) in males and females B) CK made relative to *vastus lateralis* anatomical cross-sectional area (VL_{ACSA}). Data is presented as means ± standard deviation. * Males CK

significantly higher than females $p \leq 0.05$, ** Males CK significantly higher than females $p \leq 0.01$.

5.5. Discussion

The aims of the current chapter were twofold, firstly to determine whether there is a sex difference in EIMD; and secondly, dependent on the outcome of the first aim, to establish whether tendon stiffness may explain the potential sex difference in EIMD. The three main findings of the current chapter are as follows: 1) MVC_{KE} torque loss and muscle soreness post EIMD do not differ between males and females, 2) the CK response is significantly higher in males compared to females following EIMD, and remains higher when MVE_{KE} torque relative to Q_{ACSA} is considered, and 3) patella tendon stiffness and VL fascicle lengthening do not explain the sex differences in CK.

In agreement with previous literature (Sayers & Clarkson, 2001; Rinard et al., 2000) the current chapter reports no sex difference in MVC torque loss post EIMD. Through a significant loss in MVC_{KE} torque and a rightward shift in MVC_{KE} optimal knee angle it can be concluded that the protocol used in this chapter was sufficient to cause EIMD. Although there was a trend for males to demonstrate a greater rightward shift in optimal knee angle post EIMD, despite sufficient power ($p = 0.99$) it did not reach a significant difference between males and females. Further supporting the finding of no difference in MVC_{KE} torque loss between males and females. The findings from the current chapter concur with Sayers and Clarkson (2001), despite the current chapter reporting substantially smaller MVC torque loss (23% and 27% versus 54% and 60% in males and females, respectively). The greater MVC torque loss reported by Sayers and Clarkson (2001) can be attributed to the current chapter exercising the lower limb rather than the upper limb, which is consistent with previous data (Chen et al., 2011; Saka et al., 2009). The similar loss of MVC_{KE} torque in both sexes within the current chapter, may be supported by the findings of Stupka et al. (2000) who, using muscle

biopsies of the VL, found no sex difference in structural damage (z-disk streaming) post EIMD. On the other hand, the current chapter contradicts the findings of Sewright et al. (2008) who reported MVC torque loss to be significantly higher in females compared to males immediately following 50 eccentric contractions of the elbow flexors. Interestingly, Sewright et al. (2008) reported no further difference in torque loss post EIMD (12, 72, 96, 168 and 240 hours post EIMD). As no difference in isometric MVC torque loss was reported at any other time point, Sewright et al. (2008) attributed the loss in MVC torque immediately post EIMD to fatigue rather than EIMD. Therefore, when the confounding factor of fatigue was avoided in the current chapter (by measuring MVC_{KE} torque loss 60 minutes after the damage protocol (Walsh et al., 2004)), it can be concluded that a sex difference in MVC_{KE} torque loss post EIMD does not exist in the VL.

Despite no sex difference in MVC_{KE} torque loss, a significant sex difference in the CK response was reported within the current chapter. The CK response in both males and females followed the typical CK response post EIMD, with a peak at 96 hours post EIMD (Newham et al., 1983a). Furthermore, the elevated CK in males in the present chapter is consistent with the CK responses observed following both eccentric contractions in elbow flexors (Sewright et al., 2008) and knee extensors (Wolf et al., 2012; Joyce et al., 2014). Specifically, the current chapter has demonstrated that the acute CK response following EIMD observed by Wolf et al. (2012) and Joyce et al. (2014), persists past 24 and 48 hours (respectively), to remain significantly higher in males 168 hours post EIMD. To account for the larger muscle mass in males within the current chapter, and the potential confounding impact this could have, CK was also presented relative to estimated Q_{ACSA}. Creatine kinase normalised to Q_{ACSA} remained significantly higher in males. Thus suggesting that the greater muscle mass in males does not contribute to the greater CK levels post EIMD. A sex difference in CK is not consistent with Stupka et al. (2000) whom reported no sex difference in CK

following eccentric exercise of the lower limbs. Despite exercising the same muscle groups, the discrepancies between Stupka et al. (2000) and the current chapter may be attributed to Stupka et al. (2000) measuring CK 48 and 144 hours post EIMD, whereas CK is suggested to peak 72-96 hours post EIMD (Newham et al., 1983a). Therefore, the current chapter confirms, regardless of greater muscle size, when CK is measured at its peak, there is a sex difference in the CK response post EIMD.

When MVC_{KE} torque loss is taken as the primary marker of EIMD, the current chapter can conclude that there are no sex differences in EIMD however, if CK is used as the primary marker of EIMD, a sex difference is present. It remains unclear why, despite no difference in MVC_{KE} torque loss, a sex difference in CK has been reported within the current chapter. The higher CK levels following EIMD in males may be attributed to three factors, these are: 1) Significantly higher patella tendon stiffness in males, 2) greater VL fascicle lengthening in males and 3) greater MVE_{KE} torque in males. Firstly, higher circulating oestrogen levels have been reported to alter tendon properties (Hansen et al., 2009b), specifically lowering tendon stiffness in females (Bryant et al., 2008). In agreement with chapter three and previous literature, the current chapter reported males to have significantly higher tendon stiffness compared to females (Onambélé et al., 2007). Previously, differences in tendon properties have been suggested to explain differences in EIMD between compliant and stiff hamstrings however, the role of the tendon on EIMD has yet to be evidenced experimentally (McHugh et al., 1999). Within the current chapter there was no significant correlation between patella tendon stiffness and ΔCK_{ABS} to peak, in males, females or when participants were pooled by sex. Therefore, it is likely that sex differences in patella tendon properties do not explain sex differences in CK and some other factors are contributing to the reported sex difference.

Secondly, the higher CK in males may be attributed to the significantly lower VL fascicle lengthening in females. *In situ* animal the magnitude of fascicle strain, denoted by

fascicle lengthening has been concluded as the main determinant of EIMD (Lieber & Friden, 1993; Talbot & Morgan, 1998), *in vivo* however the role of fascicle lengthening on EIMD remains unclear (Hoffman et al., 2014). Within chapter three and within the current chapter, VL fascicle lengthening was significantly higher in males compared to females during MVE_{KE}. Higher fascicle lengthening in the males may increase the number of fascicles extending onto the descending limb of the length-tension relationship, thus increasing EIMD (Morgan, 1990). The current chapter however, showed no relationship between relative VL fascicle lengthening and ΔCK_{ABS} to peak, suggesting that greater VL fascicle lengthening in males does not explain the sex difference in CK. Fascicle length throughout the VL is heterozygous (Blazevich et al., 2006), however, the current chapter cannot account for fascicle lengthening in other regions of the VL (i.e. the distal or proximal region), nor indeed, the other three muscles that form the quadriceps.

Thirdly, the higher CK in males may be attributed to the lower MVE_{KE} torque in females. *In vitro* animal studies have attributed EIMD to the magnitude of eccentric torque (Warren et al., 1993a), within *in vivo* studies however, the association between eccentric torque and EIMD remains undetermined (Hody et al., 2013; Chapman et al., 2008). Peak MVE_{KE} torque was significantly higher in males compared to females within the current chapter. However, when peak MVE_{KE} torque was normalised to Q_{ACSA} and accounted for as a covariate, CK values remained significantly higher post EIMD in males compared to females. Therefore, although MVE_{KE} made relative to Q_{ACSA} has an interaction with ΔCK_{ABS} to peak (as would be expected given the greater strength and CK of the males), it did not fully account for the sex differences in CK following EIMD.

Within the current chapter the higher CK response in males could not be attributed to sex differences in patella tendon properties, VL fascicle lengthening or MVE_{KE} torque; it is possible therefore, that damage is equivocal between the sexes (evidenced by similar MVC_{KE}

torque loss, rightward shift in optimal angle and muscle soreness), but in females oestrogen may suppress the release of CK. Oestrogen has a high antioxidant capacity in skeletal muscle and enhances cell membrane stability (Wiseman & Quinn, 1994). The lower CK response in females may be attributed to oestrogen maintaining the structural integrity of the cell membrane, thus reducing the leakage of CK into the serum and giving an impression of lower EIMD. Although CK has historically been used as an indirect marker of EIMD (Nosaka & Clarkson, 1996), the validity of CK has been questioned (Friden & Lieber, 2001; Komulainen et al., 1995). In addition to large variability, it remains unclear as to whether CK is a true representation of muscle function and the magnitude of damage post EIMD, but may reflect the membrane permeability to intramuscular proteins (Friden & Lieber, 2001). Therefore, the findings from the current chapter are presented with the caveat that the elevated CK may confirm that a loss of membrane stability and thus EIMD has occurred, rather than the severity of EIMD *per se* (Heled et al., 2007). In support of CK representing a loss of membrane stability, significantly higher levels of CK in males and oral contraceptive users compared to non-users has been attributed to the membrane stabilising role of oestrogen in females (Komulainen et al., 1999; Joyce et al., 2014).

5.6. Conclusion

The current chapter can therefore conclude; that whether a sex difference in EIMD is observed depends on which measure of EIMD is reported. It is anticipated that MVC_{KE} torque loss is the best indirect marker of EIMD (Warren et al., 1999), whereas it remains unclear whether the magnitude of CK is a true indicator of the severity of EIMD (Friden & Lieber, 2001). Within the current chapter significantly higher patella tendon stiffness, VL fascicle lengthening and MVE_{KE} torque in males, could not explain the lower ΔCK_{ABS} to peak in females. Therefore, the lower CK in females may be attributed to oestrogen increasing membrane stability, thus giving the impression of greater EIMD in males (Joyce et al., 2014).

Therefore, with the caveat associated with CK in mind, using MVC_{KE} torque loss as the primary marker of EIMD, the current chapter concludes a sex differences in EIMD does not exist.

Chapter six: Oral contraceptive pill users are not more susceptible to exercise-induced muscle damage compared to non-users.

6.1. Abstract

Aim: Firstly to establish whether oral contraceptive pill (OCP) users are more susceptible to exercise-induced muscle damage (EIMD) compared to non-users, and secondly, whether differences can be attributed, in part, to differences in patella tendon properties. *Materials and method:* 9 female OCP users and 9 female non-users participated on the 14th day of their menstrual cycle. *Vastus lateralis* (VL) and patella tendon properties were assessed using ultrasonography. During maximal voluntary eccentric knee extensions ((MVE_{KE}) 12 reps × 6 sets), VL fascicle lengthening and MVE_{KE} torque were recorded every 10° of knee joint angle (20 - 90°). Oestrogen levels were measured at ovulation (14th day). Maximal isometric knee extension (MVC_{KE}) torque loss, Creatine Kinase (CK) and muscle soreness were measured *pre, post, 48, 96* and *168* hours post *damage* as markers of EIMD. *Results:* As expected oestrogen levels were significantly lower in OCP users compared to non-users ($p \leq 0.05$). Patella tendon stiffness, VL fascicle lengthening and MVE_{KE} torque did not differ between OCP users and non-users ($p \leq 0.05$). Absolute change in CK ((ΔCK_{ABS}) i.e. peak CK value - *pre-damage* CK values) was significantly higher in OCP users compared to non-users. There were no other differences in markers of EIMD. *Conclusion:* The magnitude of EIMD, as determined through MVC_{KE} torque loss and muscle soreness was similar between OCP users and non-users. The significantly higher ΔCK_{ABS} to peak in the OCP users may be attributed to oestrogen increasing membrane stability in non-users and/or a partially protective mechanism against EIMD.

6.2. Introduction

The female sex hormone, oestrogen, has antioxidant properties (Enns et al., 2008; Wiseman et al., 1993; Ayres et al., 1998), which have been suggested to attenuate exercise-induced muscle damage (EIMD) in females compared to males (Sewright et al., 2008; Joyce et al., 2014). Through manipulation of circulating levels, oestrogen has been shown to attenuate EIMD in male and female rats (Amelink et al., 1990; Amelink et al., 1991; Bär et al., 1988). For example, Bär et al. (1988) reported the increase in creatine kinase (CK) post EIMD, in both males and ovariectomised female rats, was supressed by oestrogen supplements prior to EIMD. However, although methods used to manipulate hormone levels within animal studies are very precise (ovariectomy), they are not readily used within humans in the investigation of EIMD. Alternatively, oestrogen levels can be manipulated through the use of synthetic hormones (e.g. hormone replacement therapy (HRT) or the oral contraceptive pill (OCP)). The OCP down regulates oestrogen levels throughout the menstrual cycle by altering the hypothalamic-pituitary-ovarian feedback loop, thus inhibiting the peak in oestrogen at ovulation (Elliott-Sale et al., 2013; Van Heusden & Fauser, 2002). Consequently, women who take the OCP have significantly lower levels of circulating oestrogen compared to non-users throughout the menstrual cycle (Bryant et al., 2008; Fleischman et al., 2010). For example, throughout the menstrual cycle oestrogen levels within non-users increases four fold from baseline (~50 to ~ 200 pg/ml (Yeung et al., 2012)), whereas within OCP users oestrogen increases 2 fold from baseline and then plateaus (mean 66 pg/ml (Endrikat et al., 2002)). Due to the lower levels of oestrogen, it is suggested that OCP users may be more susceptible to EIMD. However, the literature remains inconclusive with authors supporting (Carter et al., 2001; Joyce et al., 2014) or refuting (Thompson et al., 1997; Sewright et al., 2008) this increased EIMD susceptibility hypothesis. Measurement days at different phases of the menstrual cycle and different exercising muscle groups may explain the discrepancies within

previous literature. Furthermore, although the OCP attenuates oestrogen levels, post intake of the OCP and following the seven day pill free period, oestrogen levels temporarily spike during the first 7-10 days of the next cycle (Sneader, 2005; Legro et al., 2008). Consequently, due to this delayed spike in oestrogen, it is crucial to control measurement times and measurement days not only within the non-users but also within the OCP users. Therefore, due to the inconsistencies within data collection, the effect of the OCP and by extension oestrogen on EIMD remains inconclusive in the lower limbs.

In addition to acting as an antioxidant, oestrogen may attenuate EIMD in a couple of other ways. Firstly, it appears oestrogen may attenuate EIMD by mediating the influx of neutrophils post EIMD (MacNeil et al., 2011; Silvestri et al., 2003) and reducing markers of inflammation (Silvestri et al., 2003), thus preventing further EIMD occurring through the secondary damage response. Secondly, oestrogen has been reported to decrease tendon stiffness by increasing tendon fractional synthesis rate (Kubo et al., 2003; Hansen et al., 2009a; Hansen et al., 2009b). For example, greater tendon stiffness in males compared to females, has been attributed to significantly higher levels of oestrogen in females (Kubo et al., 2003). Within OCP users however, despite lower oestrogen levels compared to non-user, it remains unclear, with arguments supporting (Bryant et al., 2008) or contesting (Hansen et al., 2013) differences in tendon properties between OCP users and non-users. Therefore, it remains unknown if the OCP alters tendon properties.

Within chapter five we attributed the significantly lower CK response in the females compared to the males, to the protective role of oestrogen on EIMD. Furthermore, within chapter three it was shown that the patella tendon mitigated fascicle lengthening during MVE_{KE}, with a greater attenuation in fascicle lengthening within males compared to females being attributed to higher patella tendon stiffness in males. Additionally the tendon has been reported to attenuate peak forces and torques during eccentric contractions, thus the tendon

has been suggested to act as a mechanical buffer on EIMD (Roberts & Azizi, 2010; Roberts & Konow, 2013; Hoffman et al., 2014). Consequently, if the OCP does alter tendon stiffness, differences in EIMD could be attributed to oestrogen's antioxidant properties (as hypothesised in chapter five), and / or oestrogen's effects on tendon properties. Therefore, the aim of the current chapter is twofold: Firstly to establish whether OCP users are more susceptible to EIMD compared to non-users and secondly, whether differences can be attributed, in part, to differences in tendon properties.

6.3. Materials and method

6.3.1. Subjects

A total of 18 females were divided into two groups: women taking the OCP (n = 9, 23.4 ± 2.4 years of age, 70.0 ± 9.9 kg and 170 ± 5 cm), and eumenorrheic women who had never taken the OCP ((non-users) n = 9, 21.2 ± 1.5 years of age, 63.2 ± 5.6 kg and 165 ± 9 cm). All participants self-reported as being recreationally active (undertaking no more than 1 hour of "moderate" physical activity per week) and did not take part in any structured resistance training. All procedures complied with the Declaration of Helsinki (World Health Association, 2013) and ethical approval was obtained through the local ethics committee. All women reported regular menstrual cycles, documenting an average cycle length of 28 ± 1 days (Cole et al., 2009). On average the OCP users had been taking an OCP prescription with an ethinyl estradiol dosage between 20-30 μg for 3 ± 1 years. Exclusion criteria included any resistance training in the last six months, occupation or lifestyle that required regular heavy lifting or carrying, any known muscle disorder, the use of dietary supplements (i.e. vitamin E), and any musculoskeletal injury in the last three months. Further exclusion criteria included, previous use of any other forms of hormone based contraception, irregular menstrual cycles (where regular cycles were defined as 24-36 days (Cole et al., 2009; Landgren et al., 1980)) in the last

12 months, and pregnancy in the year preceding inclusion in the present chapter. All inclusion and exclusion criteria were determined through participant questionnaire prior to inclusion within this chapter.

6.3.2. Testing protocol

Once selected, participants were asked to visit the laboratory on five different occasions over nine days. Non-users were tested on the 14th day (self-reported) of the menstrual cycle to measure oestrogen levels at ovulation (Brown, 1955; Cole et al., 2009). To allow for direct comparison, OCP users were also tested on the 14th day of their menstrual cycle. Testing the OCP users on the 14th day of their menstrual cycle ensured the secondary peak in oestrogen witnessed following the seven day pill free period in the OCP users was avoided (Legro et al., 2008). To avoid an acute peak in oestrogen levels the OCP users were not tested within two hours of taking their OCP (Sneader, 2005; Endrikat et al., 2002). The sessions were as follows: 1) *pre-damage* 2) *damage* (48 hours post *pre-damage*), 3) *48 hours*, 4) *96 hours,* and 5) *168 hours*. *Pre-damage* assessments consisted of stature and mass (anthropometric measures), patella tendon moment arm (PT_{MA}), 5-6 ml blood sample, dynamometer familiarisation (within *pre-damage*), morphological measures of the patella tendon (tendon size and stiffness) and isometric maximal voluntary knee extension (MVC_{KE}) torque measurements at six angles (60, 65, 70, 75, 80 and 90° (full extension = 0°)). Participants performed two practice MVCs, at two knee joint angles during the familiarisation session. Stature and mass were measured using a wall mounted stadiometer (Harpenden, Holtain Crymych, UK) and digital scales (Seca model 873, Seca, Germany), respectively. The *damage* session consisted of maximal voluntary eccentric knee contractions (MVE_{KE}), 5-6 mL venous blood sample, rating of muscle soreness and MVC_{KE} torque measurements. *48, 96 and 168 hours session* consisted of 5-6 mL blood sample, rating of muscle soreness, and MVC_{KE} torque measurements at six angles (60, 65, 70, 75, 80 and 90°).

All tests were carried out in the non-dominant leg. The non-dominant leg was defined as the leg that provided stability during movements. Participants were seated in an isokinetic dynamometer (Cybex Norm, Cybex International, NY, USA), with a hip angle of 85°. To reduce any extraneous movement during maximal efforts participants were secured in a seated position using inextensible straps around the shoulders and hips. The isokinetic dynamometer axis of rotation was visually aligned with the knee joint's centre of rotation. During *pre-damage* the isokinetic dynamometer settings (chair position, distance from the dynamometer, dynamometer arm length) and anatomical zero (leg full extended, knee angle 0°) were recorded to ensure repeatability in the following sessions.

6.3.3. Vastus lateralis anatomical cross-sectional area

Vastus lateralis (VL) anatomical cross-sectional area (VL_{ACSA}) was measured using a real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy). With the participant laid supine and their non-dominant leg fully extended (knee angle 0°), the distal and proximal insertions sites of the VL were identified using an ultrasound probe (7.5 MHz linear array probe, 38 mm wide). At 50% of VL muscle length echo-absorptive markers were placed in parallel, at intervals of 30 mm, from the lateral to the medial edge of the VL muscle. The ultrasound probe was held perpendicular to the VL muscle in the axial plane. The ultrasound probe was moved steadily over the echo-absorptive markers from the lateral to the medial edge of the muscle. Constant, light pressure was placed on the muscle during scanning to avoid compression of the underlying soft tissue. The images were recorded in real time at 25 frames per second (Adobe Premier pro Version 6, Adobe Systems Software, Ireland). Using video editing software (Adobe Premier Elements, version 10), still images were acquired at each 30 mm interval. The shadows cast by the echo-absorptive markers allowed the neighbouring still images to be aligned, thus reconstructing the entire VL_{ACSA} in a single image

(Adobe Photoshop Elements, version 10). Digitising software (ImageJ 1.45, National Institutes of Health, USA) was used to measure VL_{ACSA}. This method of calculating VL_{ACSA} has previously been accepted as reliable and valid when compared to magnetic resonance imaging (MRI), with a reported interclass correlation between 0.998 and 0.999 (Reeves et al., 2004). In females, a VL_{ACSA} of ~21 cm^2 has been reported to contribute to 38% of total quadriceps ACSA ((Q_{ACSA}) Q_{ACSA} = ~55 cm^2 (Grosset & Onambele-Pearson, 2008)). Therefore, assuming the contribution of VL_{ACSA} remains constant within females, Q_{ACSA} within female OCP users and non-users was estimated using the following equation: (VL_{ACSA} / 0.38) × 100.

6.3.4. Maximal isometric voluntary knee extension torque measurement

At six different knee angles (60, 65, 70, 75, 80 and 90° (full extension = 0°)) participants were instructed to perform two MVC_{KE} lasting ~two seconds with 90 seconds rest between contractions. Torque was presented, in real time, on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter (Biopac Systems, Santa Barbara, CA). Torque measurements were later analysed offline with the accompanying software (Acknowledge, version 3.9.2). The highest peak torque produced at each angle was taken as MVC_{KE} peak torque. The knee angle where the highest torque was produced was recorded as optimal knee angle. In a randomised order, MVC_{KE} torque measurements were repeated at all six knee angles 60 minutes post eccentric exercise (to reduce any fatigue effect (Walsh et al., 2004)) and *48 hours, 96 hours* and *168 hours* post eccentric exercise. MVC_{KE} torque loss was calculated from optimal knee angle identified at *pre-damage*.

6.3.5. Patella tendon length and cross-sectional area

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure patella tendon cross-sectional area (PT_{CSA}) and patella tendon length (PT_L) at a fixed 90° knee angle. The distance between the apex of the patella and the tibial tuberosity, marked

using sagittal ultrasound images, was taken as PT_L. To measure PT_{CSA} the ultrasound probe was placed in the transverse plane and images were captured at 25%, 50%, and 75% of PT_L. The images were later analysed offline using (ImageJ 1.45, National Institutes of Health, USA). PT_{CSA} is presented as a mean of all three images (O'Brien et al., 2010).

6.3.6. Patella tendon stiffness

The participants were seated in the isokinetic dynamometer, with the knee angle fixed at 90°, and were instructed to perform a ramped, isometric MVC_{KE} lasting ~5-6 seconds. Ramped MVC_{KE} torque and displacement of the patella tendon were synchronised using a 10-V square wave, signal generator. Patella tendon displacement was measured over two MVC_{KE}, once with the ultrasound probe positioned over the distal edge of the patella and on the second contraction over the tibial tuberosity (Onambélé et al., 2007), so that total displacement would be computed from the composite of proximal and distal patella motions (see below). Torque was presented on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter and subsequently analysed with the accompanying software (Acknowledge, Biopac Systems, Santa Barbara, CA). To create an external marker on the ultrasound images, an echo-absorptive marker was placed on the skin. Using the marker to calculate displacement, the distance of the marker (shadow) from an anatomical reference point at the beginning of the contraction, to the position of the shadow at the end of the contraction was calculated. A square wave, signal generator was used to synchronise the ultrasound images with the torque acquisition system. Images were captured at ~10% intervals of ramped MVC_{KE} torque (Onambélé et al., 2007). Total patella tendon displacement was calculated as displacement at the apex of the patellar plus the displacement at the tibial tuberosity (Onambélé et al., 2007). Patella tendon forces were calculated as: (MVC_{KE} torque + antagonist co-activation torque) / PT_{MA}.

Patella tendon moment arm was measured at 90° knee angle (full extension = 0°) in the sagittal plane, from a single energy (frame 23.3 cm x 13.7 cm) DEXA scan (Hologic Discovery, Vertec Scientific Ltd, UK). Using OsiriX (DICOM viewer, ver. 4.0, Pixemo, Switzerland), patella tendon moment arm length was determined as the perpendicular distance from the centre of the patella tendon to the tibio–femoral contact point (Baltzopoulos, 1995). The DEXA has been compared to MRI measures, demonstrating consistent reliability and validity against this standard (Erskine et al., 2014).

For the calculation of tendon force, the calculation of antagonist co-activation torque is described below. The patella tendon force – elongation curve constructed from data analysed at every 10% MVC_{KE}, was then fitted with a second-order polynomial function forced through zero (Onambélé et al., 2007). The tangential slope at discreet sections of the curve, relative to MVC_{KE} force, was computed by differentiating the curve at every 10% patella tendon force intervals. In addition, to standardise the comparison of patella tendon stiffness at an absolute load, the slope of the tangential line, corresponding to the MVC_{KE} force of the weakest participant, was computed for each subject.

6.3.7. Co-activation

In order to compute patella tendon force co-activation was measured. To determine co-activation during the ramped MVC_{KE}, Electromyogram (EMG) of the *bicep femoris* (BF) was measured. Guided by an axial-plane ultrasound of the BF, two bipolar electrodes (Ambu, Neuroline 720, Denmark) were placed in the mid-sagittal line at 25% of BF muscle length (distal end = 0%). A reference electrode (Ambu, Blue Sensor, Denmark) was placed on the lateral tibial condyle. The electrodes were placed in a bipolar configuration with a constant inter-electrode distance of 20 mm (Hermens et al., 2000). Prior to electrode placement; the skin was shaved, gently abraded and cleansed with an alcohol wipe to reduce skin impedance below 5000 Ω (Hermens et al., 2000). To minimise cross talk, and ensure a mid-sagittal placement of electrodes, ultrasonography was used to identify the medial and lateral aspects of the BF muscle. The raw EMG signal was amplified (×2000) and filtered (through low and high pass filters of 10 and 500 Hz respectively) with the sampling frequency set at 2000 Hz. Ramped MVC_{KE} torque and BF EMG were recorded in real time and synchronised using a (10-V) square wave signal generator. Participants performed two maximal voluntary isometric knee flexions (MVC_{KF}) at 90°. They were instructed to perform MVC_{KF} rapidly and as forcefully as possible against the dynamometer's lever arm. The participants were instructed to relax once a two second plateau had been attained (as observed on the dynamometer screen display). The integral of the root mean square of the BF EMG signal, was calculated 500 ms either side of instantaneous MVC_{KF} maximal torque from the contraction corresponding to the highest MVC_{KF} torque. Prior to contraction the baseline signal noise was calculated as the integral RMS over 1s and removed from the measured EMG during MVC_{KF} and MVC_{KE}. At every 10% of ramped MVC_{KE} torque the absolute integral of the BF EMG was taken over 250 ms. Co-activation torque was calculated as, (BF EMG during ramped MVC_{KE} / BF EMG during MVC_{KF}) × MVC_{KF} torque at 90° knee angle (Onambélé et al., 2007). This

equation assumes that the BF is representative of the entire hamstring (Carolan & Cafarelli, 1992), and that a linear relationship exists between BF EMG and MVC_{KF} torque (Lippold, 1952).

6.3.8. Patella tendon stress / strain relationship

Patella tendon strain was calculated as a ratio of total patella tendon displacement and PT_L. Patella tendon stress was calculated as: patella tendon force (N) / PT_{CSA} (mm^2).

6.3.9. Patella tendon Young's modulus

Young's modulus was calculated as: Patella tendon stiffness × (PT_L (mm) / PT_{CSA} (mm^2)).

6.3.10. 'Damaging' eccentric exercise

Prior to eccentric exercise, a warm-up of 10 isokinetic knee extensions and knee flexions were carried out, ensuring a progressive increase in effort (with the last contraction being maximal). For the eccentric exercise, the knee extension range of motion was set at 20 – 90° (0° = full extension). Participants were asked to perform six sets of 12 maximal voluntary eccentric knee extensions (MVE_{KE}) repetitions. The eccentric phase of the contractions was performed at an isokinetic angular velocity of $30°·s^{-1}$ (Jamurtas et al., 2005). The concentric phase was performed sub-maximally at an angular velocity of $60°·s^{-1}$ to minimise fatigue and enhance eccentric damage (Chapman et al., 2006). Two minutes rest was provided between each set. Participants remained seated in the isokinetic dynamometer throughout the entire exercise protocol, including rest periods. Visual feedback and verbal encouragement was continuously provided throughout the protocol. MVE_{KE} torque was recorded throughout each contraction and displayed via the torque acquisition system. For each set, peak MVE_{KE} torque was determined as the highest torque out of the 12 repetitions. Average peak MVE_{KE} torque was calculated as an average of peak MVE_{KE} across six sets.

6.3.11. Change in vastus lateralis fascicle length during the eccentric protocol

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure VL fascicle length during MVE_{KE}. The ultrasound probe (7.5 MHz linear array probe) was fixed at 50% of VL muscle length in the mid-sagittal plane of the non-dominant leg. A hypo-allergenic ultrasound gel (Parker, Park Laboratories Inc., Fairfield) was used to enhance acoustic coupling between the skin and the ultrasound probe.

During the first set (out of six) of MVE_{KE} contractions, ultrasound images were recorded onto a PC, in real time, at 25 frames per second (Adobe Premier pro Version 6). An externally generated square wave signal was used to synchronise the ultrasound images with the torque acquisition system. Three MVE_{KE} contractions were chosen from the first set of 12 repetitions for architectural analysis. Using frame capture software (Adobe Premier Elements, version 10), an ultrasound image (frame corresponding to every 10° of knee angle, ranging from 20- 90°) was acquired for offline analysis. To ensure there was no movement artefact included in the measurement of fascicle length, an echo-absorptive marker was fixed on the skin to provide a visual reference point for the internal structures. If movement of the reference line was observed, the contraction was discarded and another repetition was chosen for analysis.

Using image analysis software (ImageJ 1.45, National Institutes of Health, USA), VL fascicle length was analysed offline at every 10°. Fascicle length was measured from the visible insertion of the fibre into either the deep and superficial aponeurosis (Reeves & Narici, 2003). Where the fascicle extended longer than the ultrasound image (frame width 3.50 cm and height 4.15 cm), linear continuation of the fascicle and aponeurosis was assumed. Using ultrasound a 2-7% error has been associated with assuming linear continuation to calculate VL fascicle length at 120° knee angle (Finni et al., 2003a). Recently, when comparing ultrasound to cadaveric measurements', the linear extrapolation method

has been concluded as a valid technique for measuring VL fascicle length at rest (interclass correlation (ICC) = 0.853) (Ando et al., 2014). Furthermore, chapter two concludes high reliability and low day-to-day measurement error when measuring VL fascicle lengthening during MVE_{KE}. To reduce error associated with the estimation of VL fascicle length, an average of three fascicles across the image was taken (Guilhem et al., 2011). Fascicle length during eccentric contractions was measured at every 10° knee angle (range 20 - 90°, 0° = full extension) throughout the MVE_{KE}. Change in fascicle length is presented as fascicle length at a knee angle of 90° made relative to fascicle length measured at a knee angle of 20°; hereafter termed "relative fascicle lengthening" and reported as a percentage change from starting length at 20°.

6.3.12. Muscle soreness

Muscle soreness was measured using a visual analogue scale (VAS). The VAS consisted of a 100 mm line, with 0 mm labelled "No pain at all" and 100 mm labelled "Unbearable pain". Seated in the isokinetic dynamometer the leg was passively moved through a full range of motion at $30°\cdot s^{-1}$. Participants were asked to mark a line perpendicular to the VAS to denote the level of pain they experienced during the passive movement. The VAS has been reported to be a valid and reliable measure of muscle soreness (ICC > 0.96 (Bijur et al., 2001)).

6.3.13. Blood samples

Venous blood samples were taken to measure CK and oestradiol levels. A 21-gauge needle was inserted into the antecubical vein of the forearm, using a 10 mL syringe. 5-6 mL of blood was drawn into a serum collection tube. The sample was left on a crushed ice bed for 60 minutes. The sample was then centrifuged at 4500 rpm at 0°C for 10 minutes. Using a 200 – 1000 µl pipette (Eppendorf), the resulting serum sample was separated into three aliquots (~500 µl each) and stored in eppendorfs at -20°C until CK and oestradiol analysis was performed.

Creatine kinase levels were measured using a standard colorimetry procedure, measuring at optical density 340 nm (BioTek ELx800 96 well Microplate Reader) and immediately analysed (Gen5, version 2.0). Each sample was run in duplicate-quadruplets using an EnzyChromTM CK Assay Kit (BioAssay Systems, Hayward, CA, sensitivity 5 U/L, manufacturer intra-assay variability ≤ 5%). An average of two – four readings was taken. CK activity is reported in absolute values and absolute change from *pre-damage* values ((ΔCK_{ABS}) i.e. the peak CK value - the pre CK values).

Oestradiol was measured using a standard enzyme-linked immunosorbent assay (ELISA) procedures (Alpha Diagnostic International, San Antonio, USA). Absorbency was

measured at optical density of 450 nm (BioTek ELx800 96 well Microplate Reader). The minimal oestroadiol detection was ~10 pg/ml, the manufacturers intra-precision and inter-precision was 9.85% and 10.3% respectively. To calculate oestrogen levels, a standard curve was plotted using the six standards against their absorbance. Using the mean absorbance of each sample, the concentration of the sample was read directly from the standard curve.

6.3.14. Statistics

Statistical analyses was carried out using the statistical software package SPSS (v.19, Chicago, IL) for Windows and Microsoft Excel. To ensure the data were parametric, the Levene's and Shapiro-Wilk tests were used to assess the variance and normality of the data. If parametric tests were violated, the equivalent non-parametric tests were used. For group differences in anthropometric measures, oestrogen levels, VL_{ACSA}, Q_{ACSA}, patella tendon properties, ΔCK_{ABS} to peak and change in VL fascicle length independent T-tests were used. A 2×5 mixed design ANOVA (between factors: OCP use (2 levels), and time from EIMD (5 levels)) was used for muscle soreness, MVC_{KE} torque loss and CK levels. Wherever the assumption of sphericity was violated, the Greenhouse-Geisser correction was used. When a significant group effect was found post-hoc independent T-tests were used (planned contrast) with LSD correction. Since an association was reported between age and MVC_{KE} torque loss an analysis of covariance was used. The variables being investigated as a potential determinant of EIMD, or where p was close to being significant, confidence intervals and Cohen *d* were calculated. Significance was set at $p \leq 0.05$. Data are presented as mean ± standard deviation.

6.4. Results

6.4.1. Anthropometric measurements

Oral contraceptive pill users were significantly older by 2.2 years ($p = 0.010$) and of an 11% greater mass ($p = 0.047$) than non-users. There was no significant difference in stature between OCP users and non-users ($p = 0.061$).

6.4.2. Oestrogen levels

As expected, serum oestrogen levels at ovulation were significantly lower in OCP users compared to non-users (209 ± 115 pg/ml and 433 ± 147 pg/ml, respectively, $p = 0.004$).

6.4.3. Vastus lateralis anatomical cross-sectional area

There was no significant difference in VL_{ACSA} between OCP users and non-users (21.9 ± 2.64 cm^2 and 20.1 ± 3.40 cm^2, respectively, $p = 0.224$). There was no significant difference in estimated Q_{ACSA} between OCP users and non-users (57.1 ± 6.88 cm^2 and 52.4 ± 8.86 cm^2 respectively, $p = 0.223$).

Table 6. 1. Patella tendon properties in oral contraceptive pill users and non-users.

	OCP users		Non-users	
Patella tendon length (mm)	52.9	± 4.3	49.0	± 6.0
Patella tendon cross-sectional area (mm^2)	63.2	± 15.1	56.0	± 17.2
Patella tendon moment arm (cm)	4.01	± 0.24	3.96	± 0.28
Ramped MVC_{KE} (Nm)	143	± 30	132	± 27
Patella tendon force (N)	3634	± 717	3430	± 768
Patella tendon stiffness ($N \cdot mm^{-1}$)	872	± 366	665	± 169
Young's modulus (MPa)	761	± 331	642	± 285

Note: Patella tendon stiffness and Young's modulus are presented at 100% ramped MVC_{KE}.

6.4.4. Patella tendon properties

Patella tendon properties for OCP users and non-users are presented in Table 6. 1. Patella tendon length, PT_{CSA} and PT_{MA} were 7%, 11% and 1% (respectively) greater in OCP users compared to non-users ($p \geq 0.05$). Ramped MVC_{KE} torque was 9% higher in OCP users compared to non-users ($p \geq 0.05$). Patella tendon force was 6% higher in OCP users compared to non-users ($p \geq 0.05$). Patella tendon elongation was not significantly different between OCP users and non-users (7.20 ± 1.30 mm, 8.16 ± 0.80 mm, respectively, $p = 0.075$). Tendon stiffness (stress-strain relationship in Figure 6. 1) at MVC_{KE} was 24% higher in OCP users compared to non-users ($p = 0.153$, CI (-502, 88.0), $d = 0.73$). Young's modulus was 16% higher in OCP users compared to non-users ($p = 0.213$, CI (-190, 428), $d = 0.38$). Patella tendon stiffness calculated at a standardised patella tendon force (2330 N) was not significantly different between OCP users and non-users (725 ± 219 $N \cdot mm^{-1}$ and 565 ± 103 $N \cdot mm^{-1}$, respectively ($p = 0.070$, CI (-10.9, 331), $d = 0.94$). Patella tendon Young's modulus calculated at a standardised patella tendon force (2330 N) was not significantly different

between OCP users and non-users (628 ± 219 N·mm^{-1} and 536 ± 190 N·mm^{-1}, respectively (p = 0.175 CI (-111, 298), *d* = 0.453)).

6.4.5. Torque production during maximal voluntary eccentric knee extension

There was no significant difference in average peak MVE_{KE} torque of each set (six in total) between OCP users and non-users ($p \leq 0.05$). There was no significant difference in average peak MVE_{KE} torque (averaged over 6 sets) between OCP users and non-users (182 ± 33 Nm and 167 ± 32 Nm, respectively, p = 0.093). There was no significant difference between OCP users and non-users when average peak MVE_{KE} torque was made relative to Q_{ACSA} (3.19 ± 0.47 Nm·cm^2 and 3.64 ± 0.73 Nm·cm^2, respectively, p = 0.141). Peak MVE_{KE} torque made relative to *pre* MVC_{KE} torque was significantly higher in OCP users compared to non-users (108 ± 16.7% and 85.0 ± 11.9%, respectively, p = 0.002).

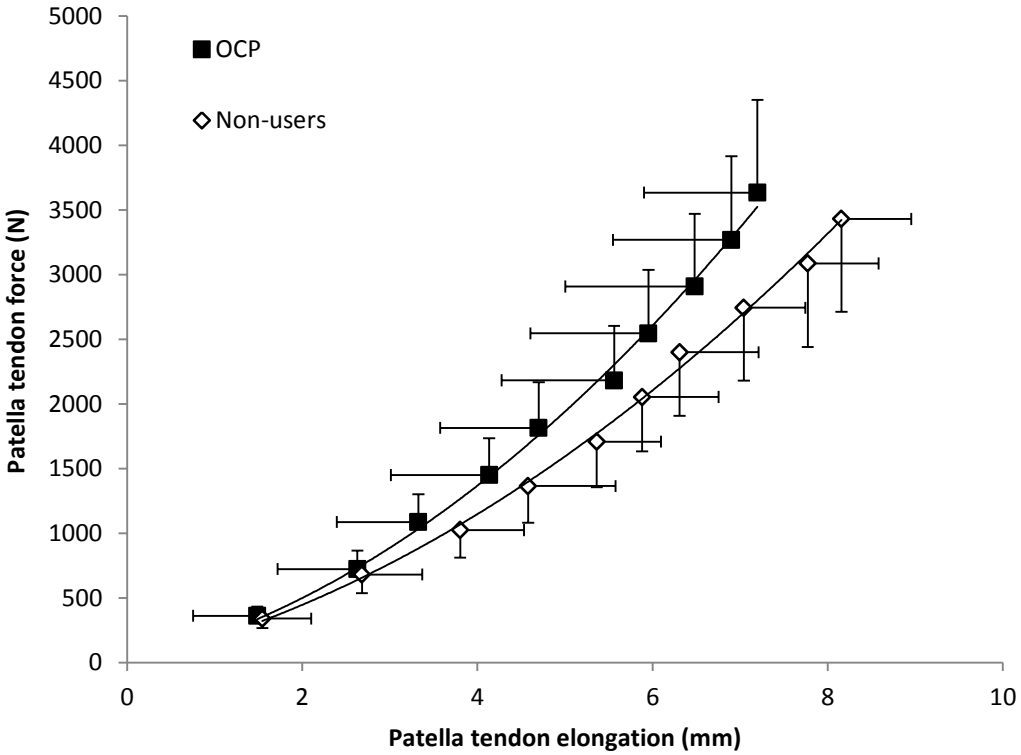

Figure 6. 1: Patella tendon force – elongation relationship, during ramped maximal voluntary isometric contraction, in oral contraceptive pill (OCP) users and non-users. Data is presented as means ± standard deviation.

6.4.6. Vastus lateralis fascicle lengthening

Fascicle length at 20° knee joint angle was not significantly different between OCP users and non-users (7.07 ± 0.42 cm and 6.82 ± 0.61 cm, respectively, p = 0.175). The increase in fascicle length from 20° to 90° knee angle during MVE_{KE} was not significantly different between OCP users and non-users (3.48 ± 0.90 cm and 2.81 ± 0.44 cm, respectively, p = 0.062). VL fascicle length at 90° knee angle made relative to fascicle length at knee angle of 20°, was not significantly different between OCP user and non-users (49 ± 12% and 42 ± 8%, respectively (p = 0.135, CI (-2.60 – 17.6), *d* = 0.74)).

6.4.7. Change in optimal knee angle

Pre-damage, optimal MVC_{KE} knee angle was not significantly different between OCP users and non-user (median 75.0 ± 7.26° and 75.0 ± 7.41°, respectively, p = 0.430). Post EIMD both OCP users (median 80.0 ± 7.07° p = 0.008) and non-users (median 80.0 ± 7.46°, p = 0.026) demonstrated a significant rightward shift in MVC_{KE} optimal knee angle. There was no significant difference in the magnitude of the rightward shift in MVC_{KE} optimal knee angle between OCP users and non-users (p = 0.311).

6.4.8. Maximal voluntary isometric knee extension torque loss

Maximal voluntary isometric knee extension torque expressed as a percentage of *pre-damage* MVC_{KE} torque is illustrated in Figure 6. 2. A two way repeated mixed ANOVA for MVC_{KE} torque loss post EIMD, reported a significant main effect of time (p = 0.0004); however, no group effect (OCP users versus non-users p = 0.257) interaction (times versus group, p = 0.118) were reported. *Pre* MVC_{KE} peak torque was not significantly different between OCP users and non-users (169 ± 24.3 Nm and 194 ± 36.2 Nm, p = 0.055). Peak MVC_{KE} torque loss made relative to *pre-damage* MVC_{KE} torque was not significantly different between OCP users compared to non-users (17 ± 7% and 28 ± 13%, respectively (p = 0.055, CI (-21.5, 10.5), *d* = 0.94)).

A significant correlation was observed between age and MVC_{KE} torque loss (r = 0.58, p = 0.011), age was therefore considered as a covariate of MVC_{KE} torque loss. ANCOVA revealed that no significant difference in MVC_{KE} torque loss between OCP users and non-users remained when age was accounted for as a covariate (p = 0.179).

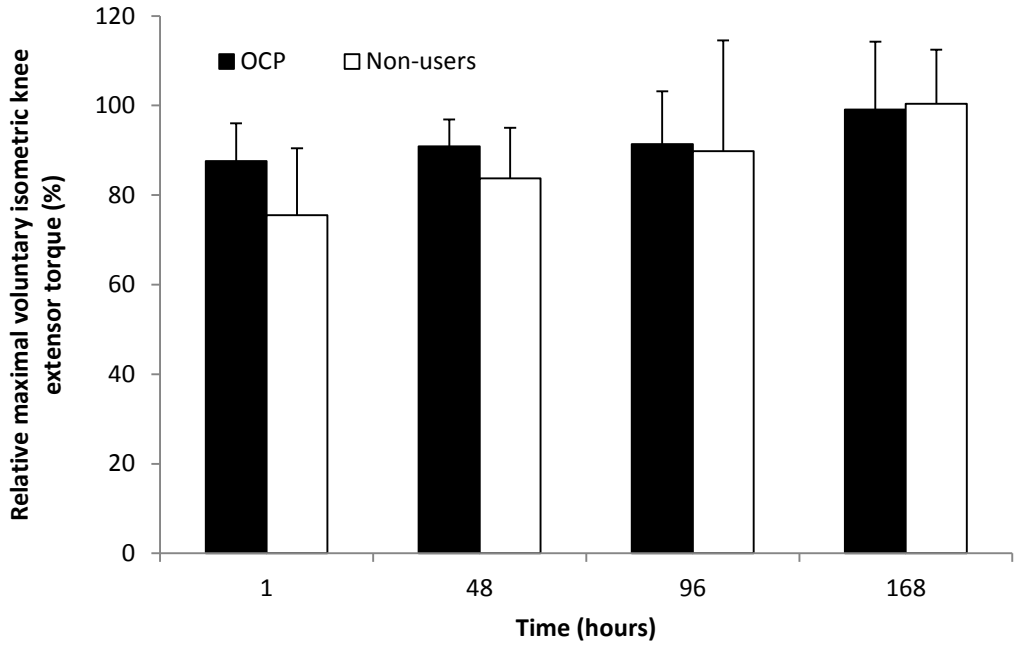

Figure 6. 2: Maximal voluntary isometric knee extensor (MVC_{KE}) torque in oral contraceptive pill (OCP) users and non-users expressed as a percentage of MVC_{KE} torque *pre-damage*. Data is presented as means ± standard deviation.

6.4.9. Creatine kinase levels

A two way repeated mixed ANOVA for absolute CK reported a significant main effect of time ($p = 0.040$) however no significant group (OCP users versus non-users, $p = 0.057$) or interaction (time versus group, $p = 0.576$) effects were reported (Figure 6. 3). Absolute peak CK was significantly higher in OCP users compared to non-users (1086 ± 971 U/L and 453 ± 459 U/L, respectively, $p = 0.023$). ΔCK_{ABS} to peak was significantly higher in OCP users compared to non-users (962 ± 968 U/L and 386 ± 474 U/L, respectively ($p = 0.016$, CI (-186, 1338), $d = 0.76$)).

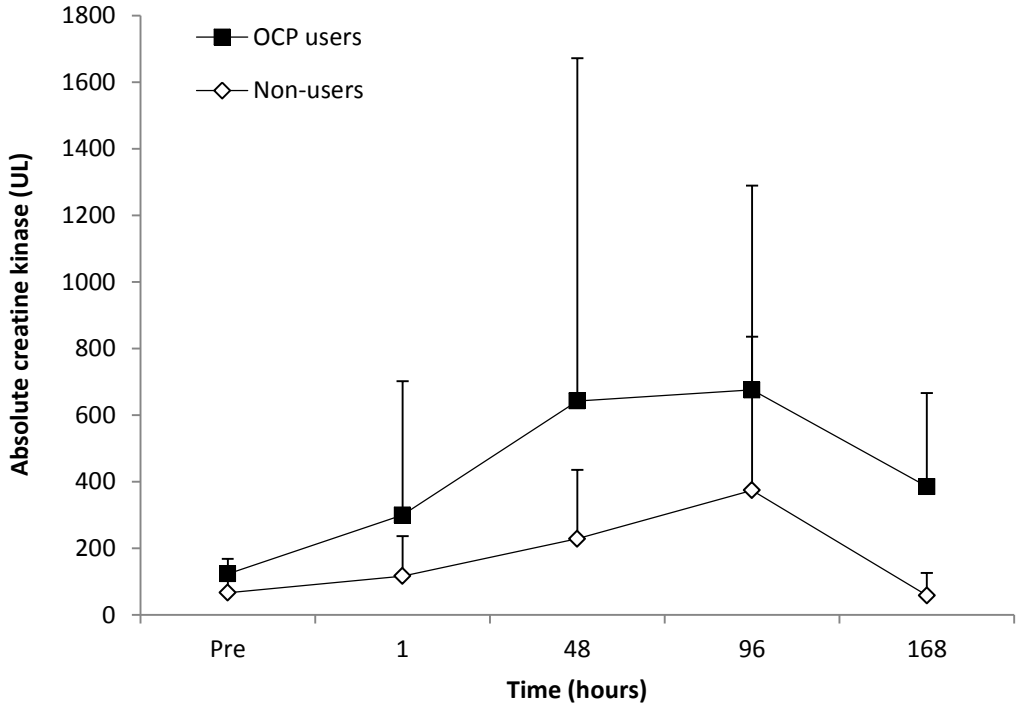

Figure 6. 3: Absolute creatine kinase (CK) response in oral contraceptive pill (OCP) users and non-users following exercise-induced muscle damage (EIMD). Data is presented as means ± standard deviation.

6.4.10. Muscle soreness

A two way mixed measures ANOVA for muscle soreness reported a significant main effect of time ($p = 0.0004$); however, there was no significant group difference (OCP users versus non-users, $p = 0.598$) or an interaction effect ($p = 0.127$). For both OCP users and non-users peak muscle soreness occurred 48 hours post EIMD. There was no significant difference in peak muscle soreness between OCP users and non-users (3.89 ± 2.23 and 4.13 ± 1.27, respectively, $p = 0.358$).

6.5. Discussion

The aim of the current chapter was twofold, firstly to establish whether OCP users are more susceptible to EIMD compared to non-users, and secondly, whether differences can be attributed, in part, to differences in patella tendon properties. The current chapter reports three main findings. 1) Patella tendon properties are not significantly different between OCP users and non-users, 2) MVC_{KE} torque loss and muscle soreness are not different between OCP users and non-users, and 3) ΔCK_{ABS} to peak is significantly higher in OCP users compared to non-users following EIMD.

All participants were tested on the 14th day of their menstrual cycle to measure peak oestrogen levels at ovulation (Brown, 1955). Within the current chapter it can be assumed that the non-users were tested at ovulation as circulating oestrogen levels are comparable with previous research for this phase of the menstrual cycle (510 - 742 pg/ml (Bryant et al., 2008; Eiling et al., 2007)). Circulating oestrogen levels within the OCP users however, are 2.8 times higher than those previously reported (Bryant et al., 2008). The discrepancies with previously reported circulating oestrogen levels may be attributed to the different varieties of the OCP prescriptions used within the current chapter (Elliott-Sale et al., 2013). Furthermore, due to significantly higher mass and similar lean muscle mass (VL_{ACSA}) and stature in OCP users compared to non-users, it may be suggested that fatty mass was higher in OCP users. Therefore the elevated oestrogen levels in the OCP users compared to previous literature may be explained by adipose tissue (Yeung et al., 2012). Regardless, in agreement with previous research OCP users demonstrated a significant down regulation of oestrogen compared to non-users (Bryant et al., 2008), and thus it is reasonable to assume that the suppression of oestrogen remained throughout the menstrual cycle (Bryant et al., 2008).

It has been reported that oestrogen alters tendon properties through an increases in type 1 collagen and tendon fractional synthesis rate (Lee et al., 2004; Hansen et al., 2009a).

Within the current chapter however, despite significantly lower oestrogen levels in OCP users, there was no significant difference in patella tendon stiffness *in vivo* between OCP users and non-users. Although our data shows a trend towards higher stiffness in the OCP users, our findings did not reach significance and contradict those of Bryant et al. (2008) who concluded that exposure to the OCP (≥1 year) decreases Achilles tendon strain properties. The discrepancies with the current chapter may be attributed to the higher oestrogen levels reported in OCP users compared to previous research (Bryant et al., 2008), which may result in OCP users displaying similar oestrogenic effects on the tendon as found in non-users. Furthermore, the discrepancies with the current chapter may also be attributed to Bryant et al. (2008) including the aponeurosis in their calculation of strain. Therefore, due to the divergent strain rates between the aponeurosis and the tendon (Magnusson et al., 2003), it is difficult to directly compare Bryant et al's (2008) findings to the current chapter. Additionally, Bryant et al. (2008) measured Achilles tendon strain and not Achilles tendon stiffness or Young's Modulus. It is also possible that the Achilles and Patella tendons show inconsistent responses to the OCP, potentially due to difference in the number of oestrogen receptor sites, as our findings concur with those of Hansen et al. (2013) who recently reported no significant difference in patella tendon stiffness or Young's modulus between female OCP users and non-users. The current chapter confirms that the OCP does not alter patella tendon stiffness or Young's modulus in recreationally active participants. The conclusion that the OCP has no significant effect on tendon properties, specifically patella tendon stiffness, may discount any potential differences in EIMD between OCP users and non-users being directly attributed to patella tendon properties, and this is reflected in similar VL fascicle lengthening during MVE_{KE}.

Consistent with others, the data from the present chapter demonstrates a greater ΔCK_{ABS} to peak in OCP users compared to non-users (Joyce et al., 2014) but in the presence of

no significant difference in MVC_{KE} torque loss between the groups (Thompson et al., 1997; Sewright et al., 2008). Furthermore, within the current study an insignificant finding remained when group differences in age were taken as a covariate of MVC_{KE} torque loss. The findings of the current chapter however appear to contradict those reported by Savage and Clarkson (2002) who investigated EIMD between OCP users and non-users following 50 eccentric contractions of the elbow flexors. Savage and Clarkson (2002) reported MVC torque loss to be significantly higher 48 and 72 hours post EIMD in OCP users compared to non-users. However, due to no difference in MVC torque loss being reported before 48 hours post EIMD, Savage and Clarkson (2002) concluded that OCP users are not more susceptible to EIMD but have a prolonged recovery compared to non-users. A delayed recovery in OCP users may be attributed to higher oestrogen levels attenuating the infiltration of neutrophils and cytokines (MacNeil et al., 2011; Silvestri et al., 2003) thus reducing secondary damage. However, the mechanisms responsible for this attribute of oestrogen requires further investigation (MacNeil et al., 2011).

Regardless of groups, a loss of MVC_{KE} torque and a rightward shift in optimal MVC_{KE} knee angle within the current chapter supports the conclusion that EIMD was achieved (Jakeman & Eston, 2013). Interestingly a comparable shift in optimal knee angle between OCP users and non-users may support that no difference in structural damage exists between the two groups. The current chapter therefore concludes OCP users are not necessarily more susceptible to MVC_{KE} torque loss compared to non-users.

Through an interaction with cell membrane phospholipids, oestrogen has been reported to decrease cell membrane fluidity thus increasing cell membrane stability. An increase in cell membrane stability may prevent muscle proteins (i.e. CK) leaking into the serum (Wiseman et al., 1993; Carter et al., 2001). Furthermore, by increasing cell membrane stability, oestrogen may attenuate the influx of neutrophils and cytokines post EIMD (MacNeil

et al., 2011; Silvestri et al., 2003), thus mitigating the magnitude of secondary damage occurring in the non-users. The current chapter reported ΔCK_{ABS} to peak to be significantly higher in OCP users (low oestrogen levels) compared to non-users (high oestrogen levels). Thus indicating that either through an increase in cell membrane stability or a reduction in the influx of neutrophils, higher oestrogen levels reduce susceptibility to EIMD. Our findings concur with those reported by Joyce et al. (2014) who reported CK following 240 bilateral knee extensors on both legs (480 knee extensions in total), to be significantly higher in OCP users compared to non-users. Joyce et al. (2014) measured CK 48 hours post EIMD, whereas CK is reported to peak 96 hours post EIMD (Hyatt & Clarkson, 1998), however, the current chapter can confirm ΔCK_{ABS} to peak remains significantly higher in OCP users when CK is measured over 168 hours post EIMD. Contradictory to the current findings previous work has reported no significant differences in CK between OCP users and non-users (Thompson et al., 1997; Savage & Clarkson, 2002; Sewright et al., 2008). For example, Savage and Clarkson (2002) reported no significant difference in the CK response between OCP users and non-users (despite significant differences in MVC torque loss). Due to Savage and Clarkson (2002) investigating the elbow flexors, the CK response was ~30% higher than compared to the current chapter (Chen et al., 2011). Therefore, it may be suggested that oestrogen acts as a membrane stabiliser up to a certain threshold, but beyond that threshold, which may be attained through more severe damage protocols or different muscle groups, the protective role of oestrogen on CK is negligible. This hypothesis requires further investigation.

Furthermore, discrepancies regarding the role of the OCP on EIMD with previous research may be confounded by different measurement days at different phases of the menstrual cycle. For example, Carter et al. (2001) tested OCP users at the mid-luteal phase (22-25 days) and non-users during the mid-follicular phase (6-10 days). The measurement days used by Carter et al. (2001) resulted in OCP users having significantly higher oestrogen

levels compared to non-users, which is opposite to the oestrogen levels reported in the current chapter and throughout the menstrual cycle (Bryant et al., 2008). Therefore, although Carter et al. (2001) concluded that CK is significantly lower in OCP users compared to non-users and appears to contradict the findings of the current chapter, at closer inspection the same conclusion can be made, that the higher oestrogen recorded in the OCP participants in their study, attenuates CK levels.

Within the current chapter patella tendon properties, relative VL fascicle lengthening and MVE_{KE} torque (all previously suggested determinants of EIMD (Lieber & Friden, 1993; Warren et al., 1993a; McHugh et al., 1999) were not significantly different between OCP users and non-users, thus cannot explain the group difference in ΔCK_{ABS} to peak. Oestrogen levels however were significantly lower in OCP users compared to non-users. Previously, post downhill running, Carter et al. (2001) reported a significant, albeit weak, moderate correlation ($r = -0.43$) between total resting oestrogen levels and overall CK. The negative correlation between oestrogen and CK reported by Carter et al. (2001) may be explained by the membrane stabilising role of oestrogen reducing the leakage of CK from the intracellular membrane. Therefore, within the current chapter, the significantly higher ΔCK_{ABS} to peak in OCP users may reflect the membrane stabilising properties of oestrogen preventing the leakage of CK in non-users rather than a lower magnitude of EIMD.

It must be noted that age and mass were significantly different between OCP users and non-users within the current chapter. Although mass did not act, as a covariate on any dependent variable, and when age was accounted for as a covariate it had no effect on the difference in MVC_{KE} torque loss. Mass is especially important to control for as it may have a significant effect on oestrogen levels (Yeung et al., 2012), and may explain why the current chapter reports high oestrogen levels in OCP users. The oestrogen levels within the current chapter however, remain significantly lower than non-users, and within measurable limits of

published data from the same phase of the menstrual cycle (Bryant et al., 2008; Eiling et al., 2007).

6.6. Conclusion

Due to no other differences between suggested determinants of EIMD in OCP users and non-users (tendon properties, VL fascicle lengthening and MVE_{KE} torque), the current chapter concludes that the higher ΔCK_{ABS} to peak in OCP users may be attributed to the significantly higher oestrogen levels in the non-users. However, the group differences in ΔCK_{ABS} to peak may represent differences in cell membrane stability and influx in cytokines rather than differences in functional EIMD. Therefore, due to MVC_{KE} torque loss being indicative of muscle function and the most valid indirect marker of EIMD (Warren et al., 1999), and the caveats associated with CK as a marker of EIMD (Friden & Lieber, 2001), the current chapter concludes that OCP users are not at a greater risk of EIMD compared to non-users. Using muscle biopsies, further research is required to investigate the association between CK and oestrogen.

Chapter seven: Discussion, conclusion and future directions

7.1. Discussion

The main findings of this thesis are summarised in Figure 7. 1. Briefly the data has confirmed the classical model for eccentric contractions whereby *vastus lateralis* (VL) fascicles lengthen during maximal voluntary eccentric knee extensions ((MVE$_{KE}$) chapter three). The patella tendon, although a mediator of VL fascicle lengthening (chapter three), does not appear to be associated with functional markers of exercise-induced muscle damage ((EIMD) chapter four). Specifically in populations where the patella tendon properties differ (chapters five and six), there was no difference in maximal voluntary knee extension (MVC$_{KE}$) torque loss post EIMD (chapter five and six). Furthermore, the significance of oestrogen to EIMD was established through sex differences (chapter five) and oral contraceptive pill (OCP) use (chapter six), and the findings were consistent between chapters regarding the possible role of oestrogen. It was observed that although no difference in EIMD MVC$_{KE}$ torque loss was reported (males = females = OCP females), creatine kinase (CK) values were significantly different (males > females < OCP users). It was concluded in both chapters five and six, that as groups show no difference in MVC$_{KE}$ torque loss but display differences in CK (in both instances the higher oestrogen group had lower CK), it is likely that the contractile disruption from EIMD is similar, but the previously reported role of oestrogen on membrane stability, attenuates the CK release post EIMD. The following discussion outlines the themes that were observed in investigating the *in vivo* determinants of EIMD throughout this thesis.

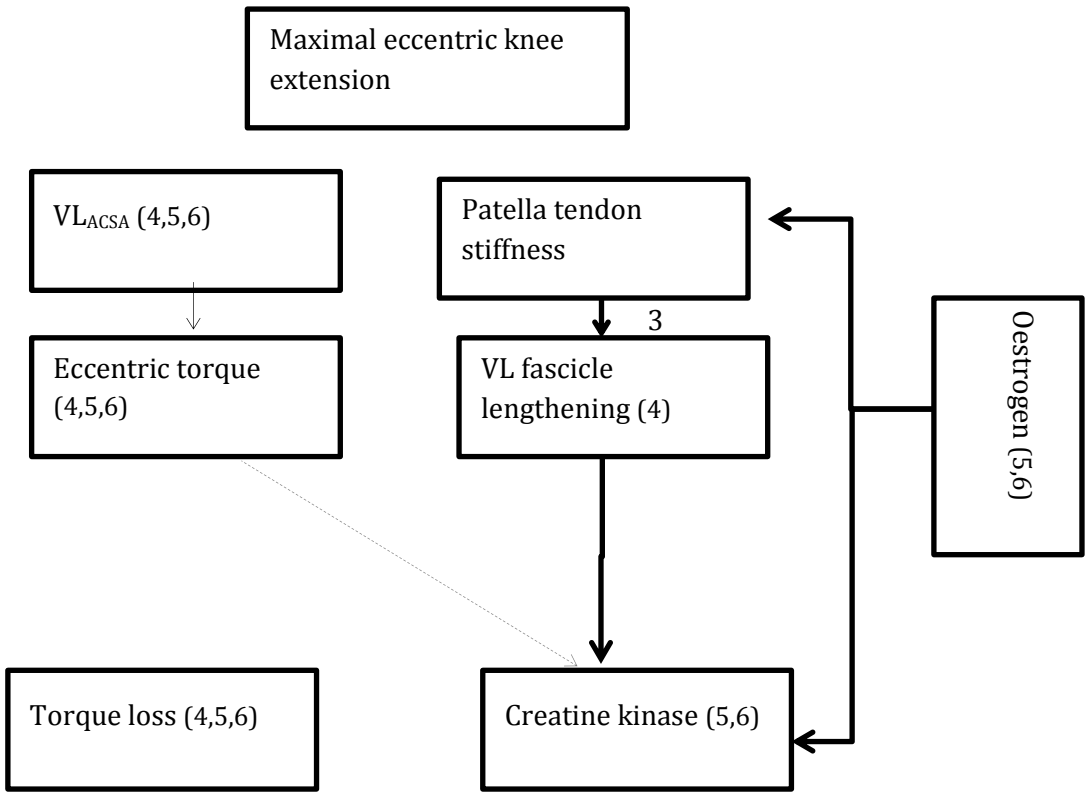

Figure 7.1 The hypothetical and demonstrable connections between the determinants of exercise-induced muscle damage (EIMD) and the measured markers of EIMD within the thesis. The numbers in parenthesis denote the chapter where the findings are presented. The joining arrows show associations within the current thesis. Muscle damage may be dependent on VL fascicle lengthening in males (chapter four). Patella tendon mediates fascicle lengthening but not muscle damage directly (chapter three). Low oestrogen populations have lower creatine kinase (CK) than high oestrogen populations (males > females < oral contraceptive pill (OCP) users (5,6)) but no differences in torque loss (males = females = OCP females (5,6)).

7.2. The role of the patella tendon on exercise-induced muscle damage

Within chapter three it was identified that sex differences in patella tendon stiffness do exist, with males reporting significantly higher tendon stiffness compared to females. Although this has previously been identified (Onambélé et al., 2007), the implication of different tendon properties on fascicle lengthening (the main aim of chapter three) has yet to be established *in vivo*. Lichtwark and Wilson (2007) applied average tendon stiffness reported by Lichtwark and Wilson (2006) to a Hill-type muscle model to estimate the effect of increasing Achilles tendon stiffness by 0.5, 1, 2 and 4 times. Despite the same amount of muscle tendon unit (MTU) lengthening at all stiffness levels, Lichtwark and Wilson (2007) estimated *gastrocnemius* fascicle lengthening to increase with an increase in Achilles tendon stiffness. Due to using a muscle model it is difficult to extrapolate Lichtwark and Wilson (2007) findings *in vivo*. The findings of chapter three however agree with Lichtwark and Wilson (2007) and confirm a significant correlation between patella tendon stiffness and VL fascicle lengthening *in vivo*. Therefore, the findings of chapter three are the first to provide *in vivo* evidence that the magnitude of VL fascicle lengthening is not only dependent on the type of eccentric task and intensity of the task itself (Ishikawa et al., 2003; Finni et al., 2001), but on the mechanical properties of the patella tendon, specifically tendon stiffness and Young's modulus.

Based on the findings of chapter three (that there is an interaction between the patella tendon stiffness and VL fascicle lengthening during eccentric contractions), chapter four investigated the contribution of the patella tendon stiffness to EIMD in a group of males. In addition to attenuating fascicle lengthening, the tendon has been hypothesised to protect the muscle from EIMD and explain group difference in EIMD by altering peak force to the fascicles during eccentric contractions (Roberts & Konow, 2013; Roberts & Azizi, 2010; McHugh et al., 1999). For example, a reduction in the magnitude of fascicle lengthening may

reduce the fascicles extending onto the descending limb of the length-tension relationship, where eccentric EIMD is proposed to occur (Morgan, 1990; Hoffman et al., 2014). However, the data from chapter four showed that within males there was no significant correlation between patella tendon stiffness or Young's modulus with any *indirect* marker of EIMD (ΔCK_{ABS} to peak (peak CK value - the *pre* CK values) or MVC_{KE} torque loss). It cannot be discounted however that within chapter four the patella tendon stiffness range was not large enough to detect a difference (see chapter five). Therefore, contrary to the hypothesis, within the current thesis the mechanical patella tendon properties were not a direct determinant of EIMD in males *in vivo*.

7.2.1. Patella tendon properties and group differences in exercise-induced muscle damage

Although within chapter four, patella tendon properties did not act as a determinant of EIMD in males, group differences in EIMD have been attributed, although not investigated, to differences in tendon properties (McHugh et al., 1999; Marginson et al., 2005). For example, tendon properties have been suggested to explain differences in EIMD between 'stiff' and 'compliant' hamstring MTUs however, McHugh et al. (1999) did not measure tendon properties.

Based on the findings of chapter three, and the potential caveat that the range in tendon stiffness in the male group may not have been sufficient to detect an association, chapters five and six investigated whether group differences in patella tendon properties may explain any group differences in EIMD. Due to oestrogen being known to alter tendon properties (Hansen et al., 2009a), specifically tendon stiffness (Bryant et al., 2008; Kubo et al., 2003), two different groups with high versus low oestrogen levels were investigated, with the hypothesis that patella tendon stiffness would be higher in the low oestrogen groups (males and OCP users). Although within chapter three and chapter five it was found that males have

significantly higher patella tendon stiffness compared to females, a non-significant trend for patella tendon stiffness to be higher in OCP users compared to the non-users was reported in chapter six. Despite oestrogen levels being significantly lower in OCP users compared to non-users, the oestrogen levels in the OCP users were 2.8 times higher than previously reported values (Bryant et al., 2008). Therefore, the unusually high oestrogen levels in the OCP user may explain why in chapter six OCP users displayed similar tendon properties to non-users. Future studies need to control for differences in body composition and OCP prescription being investigated to prevent confounding variables on oestrogen levels in the OCP users (Yeung et al., 2012; Elliott-Sale et al., 2013). Regardless, within chapter five there was no significant difference in MVC_{KE} torque loss or muscle soreness, despite males having significantly higher tendon stiffness compared to females. However, whilst CK was significantly higher in males compared to females, there was no correlation between patella tendon stiffness and ΔCK_{ABS} to peak thus, tendon properties cannot be used to explain differences in ΔCK_{ABS} to peak in chapter five. Chapter six further supported these findings whereby, despite no significant difference in tendon properties a significant difference in ΔCK_{ABS} to peak was still reported.

Therefore, from the findings of chapter four, five and six it would appear that patella tendon properties may not be *directly* associated with EIMD in males nor can they explain group differences in EIMD. A common theme did emerge from chapters five and six, whereby ΔCK_{ABS} to peak was consistently higher in groups with lower oestrogen levels. The common theme suggests that oestrogen may explain differences in ΔCK_{ABS} to peak through its role as a membrane stabiliser and/or preventing the influx of neutrophils thus minimising further damage (MacNeil et al., 2011), rather than its role on tendon properties as hypothesised within the current thesis.

7.3. Oestrogen as a membrane stabiliser

Within the current thesis the groups investigated in chapters five and six (males and females, OCP users and non-users, respectively) were categorised by low and high circulating oestrogen levels. Interestingly the current thesis consistently reports higher ΔCK_{ABS} to peak in the groups with lower circulating oestrogen levels (males and OCP users) despite reporting no significant difference in any other marker of EIMD (MVC_{KE} torque loss or muscle soreness).

Within chapter five a significant correlation with MVE_{KE} torque and ΔCK_{ABS} to peak was reported, when MVE_{KE} torque was accounted for as a covariate, a significant group difference in ΔCK_{ABS} to peak remained. Therefore MVE_{KE} did not fully account for the group difference in ΔCK_{ABS} to peak *per se*. Additionally, patella tendon properties, VL fascicle lengthening or MVE_{KE} torque in chapters five or six could not explain the reported group differences in ΔCK_{ABS} to peak. Interestingly, the significant correlation between VL fascicle lengthening and ΔCK_{ABS} to peak reported in males in chapter four was lost when VL fascicle lengthening was correlated with ΔCK_{ABS} to peak in males, females and even when sex was grouped in chapter five. The loss of association could be due to the variability in CK and the lower participant numbers when the males were sub-grouped in chapter five, however it may also be due to the presence of oestrogen acting as a confounding variable in chapters five and six. Therefore, in agreement with previous work the group differences in ΔCK_{ABS} to peak may be explained by the group differences in oestrogen (Joyce et al., 2014; Carter et al., 2001). Previously Carter et al. (2001) reported a moderate negative correlation ($r = -0.43$) between total oestrogen levels and CK post EIMD. Therefore, the higher ΔCK_{ABS} to peak in males and OCP users within chapter five and six respectively, may be attributed to the higher oestrogen levels in females and female non-users. Oestrogen has been reported to act as an antioxidant, enhance membrane stability, and prevent the influx of neutrophils and cytokines (MacNeil et al., 2011; Wiseman et al., 1993; Wiseman & Quinn, 1994), which individually or collectively

may prevent intracellular proteins, like CK, leaking into the extracellular matrix in females and non-users.

The conclusion that oestrogen may explain group differences in ΔCK_{ABS} to peak in chapters five and six, contributes to the caveat associated with using CK as a marker of EIMD. Although CK has historically been used as an indirect marker of EIMD (Nosaka & Clarkson, 1996; Savage & Clarkson, 2002; Stupka et al., 2000; Sewright et al., 2008), it has been suggested that the magnitude of damage cannot be determined from changes in CK post EIMD *per se* (Friden & Lieber, 2001; Komulainen et al., 1995; Chrismas et al., 2014). The increase in CK may reflect a loss of membrane stability as opposed to the severity of EIMD. The magnitude of CK being indicative of membrane stability rather than the magnitude of structural damage would explain why the current thesis and other studies (Joyce et al., 2014) report ΔCK_{ABS} to peak to be highest in groups with lower oestrogen levels, despite no differences in other markers of EIMD (Friden & Lieber, 2001). The current thesis concludes that ΔCK_{ABS} to peak is presented with the forewarning that although elevated CK may represent a loss of membrane stability and can be seen as an indicator that EIMD has occurred, the inherent increase may not reflect the magnitude of EIMD *per se* (Heled et al., 2007; Friden & Lieber, 2001; Komulainen et al., 1995; Chrismas et al., 2014). Consequently, the current thesis concludes that the severity of EIMD cannot be drawn on the magnitude of CK independently, thus although a group differences in CK were reported consistently, taking other markers of EIMD into consideration it may not be indicative of an actual group difference in EIMD *per se*.

7.4. *In vivo* fascicle lengthening and MVE_{KE} torque.

The group comparisons that the current thesis has identified, are all based on the induction of damage through MVE_{KE}. Eccentric contractions are proposed to have two main characteristics that contribute to the inevitable disruption of the muscle: high force and high strain. The remainder of this discussion outlines the evidence for each of these characteristics that

results in EIMD. Exercise-induced muscle damage will be discussed predominately using MVC_{KE} torque loss and muscle soreness due to the aforementioned issues associated with CK as a marker of EIMD.

7.4.1. The role of maximal voluntary eccentric knee extension torque on exercise-induced muscle damage

Previously *in vitro*, eccentric torque has been identified as the main determinant of EIMD (Warren et al., 1993a). For example, within rat soleus muscle, Warren et al. (1993a) reported force loss to be significantly greater following high eccentric forces compared to low eccentric forces (150% and 125% of maximal isometric tetanic tension, respectively). For several reasons, including the use of maximal electrical stimulation, the reduction of in series MTU compliance (by removing the tendon), and forces being applied directly to the muscle, it is difficult to apply the findings from animal studies to *in vivo* human studies (Crameri et al., 2007). Although work *in vivo* is somewhat limited, studies have reported total or peak MVE_{KE} torque to have no significant association with isometric torque loss in the elbow flexors (Chapman et al., 2008) or with CK in the knee extensors (Hody et al., 2013). The findings of chapter four add to the body of *in vivo* human research supporting that peak MVE_{KE} torque in males does not correlate with MVC_{KE} torque loss post EIMD. To the authors' knowledge chapter four is the first to report no correlation between MVE_{KE} torque and MVC_{KE} torque loss in the lower limbs. The findings in chapter four appear to contradict the classical eccentric stress theory whereby a muscle will fail when an applied tensile strength is greater than its yield strength (Warren et al., 1993a). The yield strength of a muscle fibre is predicted to be above 113% of maximal isometric torque (Warren et al., 1993a; Hasselman et al., 1995) in animals. However, Warren et al. (1993a) reported that when MVC eccentric torque is below 113% of isometric MVC, the association with MVC torque loss is lost. The eccentric torque recorded in chapter four during MVE_{KE} and in previous work during eccentric elbow contractions (Chapman et al., 2008) did not approach the suggested yield strength of muscle fibres (96% and 94% respectively). Therefore upon closer inspection the findings of chapter four agree with the work of Warren et al. (1993a). Although it may be suggested that within chapter four participants

were not contracting maximally, using electrical stimulation to elicit higher eccentric torques may alter the recruitment pattern (Crameri et al., 2007) and potentially mask any protective mechanism of the muscle on EIMD. Therefore, by not using electrical stimulation, the current thesis has reported data using an eccentric torque achieved during voluntary maximal effort (similar to numerous investigations into EIMD). In agreement with Warren et al. (1993a) however the current thesis agrees a potential threshold, whereby beyond this point eccentric torque may become a determinant of EIMD. Although it may be deemed a limitation that the current thesis did not reach this suggested threshold (>113% above MVC_{KE} isometric torque), due to the nature of *in vivo* recruitment and force transmission, our data remains meaningful compared to that of Warren et al. (1993a). Therefore, in males chapter four concludes that MVE_{KE} torque *in vivo* is not associated with indirect markers of EIMD *in vivo*.

7.4.2. Maximal voluntary eccentric knee extension torque and group differences in exercise-induced muscle damage

Within chapter five, peak MVE_{KE} torque averaged over six sets was significantly higher in males compared to females; this is not surprising considering the significantly higher VL_{ACSA} and estimated Q_{ACSA} in males. However, despite significantly higher MVE_{KE} torque in males there was no sex difference in MVC_{KE} torque loss. Therefore, higher MVE_{KE} torque was not associated with a difference in MVC_{KE} torque loss, as mentioned formerly and in agreement with chapter four, no association between eccentric torques and MVC torque loss has previously been reported (Hody et al., 2013; Chapman et al., 2008).

Due to significant sex differences in muscle size within chapter five, MVE_{KE} torque was made relative to estimated Q_{ACSA} (stress). There remained no significant association between MVE_{KE} torque / Q_{ACSA} and MVC_{KE} torque loss. Furthermore, within chapter six there was no significant difference in MVE_{KE} torque or MVE_{KE} torque / Q_{ACSA} between OCP users and non-users nor was there a significant difference in MVC_{KE} torque loss between OCP users and

non-users. Thus, chapter six concurs with chapter five that when corrected for muscle size, MVE_{KE} torque is not associated with MVC_{KE} torque loss.

Despite higher MVE_{KE} torque in males within chapter five, there was no significant difference in MVE_{KE} torque made relative to MVC_{KE} torque *pre-damage* between males and females (96% and 87% respectively). Interestingly, in both males and females MVE_{KE} torque remained below the suggested yield strength of muscle fibres (< 113% albeit in animals (Warren et al., 1993a)). Therefore, comparable MVC_{KE} torque loss between males and females in chapter five may be attributed to a couple of reasons: Firstly, there was no group difference in MVE_{KE} torque made relative to *pre-damage* MVC_{KE} torque; and secondly, MVE_{KE} torque made relative to *pre-damage* MVC_{KE} torque in both males and females was below the suggested yield strength of muscle fibres. Intriguingly, within chapter six despite MVE_{KE} torque made relative to MVC_{KE} being significantly higher in OCP users compared to non-users (108% versus 85% respectively), MVC_{KE} torque loss remained comparable between the two groups. Although OCP users approached the suggested threshold of MVE_{KE} torque, it remained below 113%. Therefore, from the findings of chapter six, the hypothesis that MVE_{KE} torque and group differences in MVE_{KE} torque are negligible until they are above a certain yield threshold remains plausible, but warrants further investigation during voluntary contractions.

It has been reported that the eccentric torque to isometric torque ratio is lower when lengthening contractions are performed at a low compared to a high velocity (Onambele et al., 2004). Therefore, the low MVE_{KE} to MVC_{KE} ratio within chapters four, five and six may be attributed to the low lengthening velocity used within the current thesis ($30°{\cdot}s^{-1}$). The low velocity of the eccentric damage protocol may thus have allowed for active cross-bridge force production thereby adding noise in the data through the involvement of calcium kinetics (i.e. muscle activation level variations) rather than passive torque production from the stretch response *per se*. Further investigation is therefore required to establish the yield strength of

in vivo human muscle and whether the suggested yield strength can be achieved during voluntary contractions of large muscle groups. Ultimately, further research should aim to determine whether voluntary activation level is in fact a determinant of EIMD? A study design to attempt to answer to the above research question would utilise fast lengthening velocities i.e. ≥300 mm/s (so that cross-bridge numbers do not alter during lengthening), so as to separate any impact of activation level from those of stretch.

The findings of chapters five and six therefore suggest that group differences in MVE_{KE} torque do not result in differences in MVC_{KE} torque loss until MVE_{KE} torque surpasses a certain threshold, however the yield threshold for *in vivo* human muscle requires further investigation. Therefore, consistently throughout chapters four, five and six, and in agreement with previous research (Hody et al., 2013; Chapman et al., 2008) the current thesis concludes that peak MVE_{KE} torque, even when made relative to Q_{ACSA}, cannot be used to explain differences in indirect markers of EIMD, specifically MVC_{KE} torque loss.

7.4.3. The role of vastus lateralis fascicle lengthening during maximal voluntary eccentric knee extensions

The findings from chapter three support the classical description of an eccentric contraction, whereby VL fascicles were reported to lengthen during MVE_{KE}. Within animal studies the magnitude of fascicle strain, denoted as fascicle lengthening, has previously been concluded as the main determinant of EIMD (Lieber & Friden, 1993; Talbot & Morgan, 1998). Theoretically, the association between fascicle lengthening and EIMD can be explained by the popping sarcomere theory (Morgan, 1990). Morgan (1990) reported muscle damage to occur when the weakest sarcomeres lengthen onto the descending limb of the length-tension relationship thus, increasing the strain on surrounding sarcomeres. Morgan (1990) suggested that during subsequent contractions the sarcomeres continue to lengthen onto the descending limb, progressively from the weakest to the strongest sarcomere, therefore

increasing the area of EIMD. However, it is difficult to extrapolate findings from *in vitro* and *in situ* animal studies for several reasons. Firstly, electrical stimulation is generally used to lengthen the muscle. During electrical stimulation the recruitment of muscle fibres does not follow the traditional recruitment pattern described by Henneman et al (Henneman et al., 1965), furthermore fibres lengthen in a uniformed manner whereas during voluntary contractions fibres contract in a non-uniformed manner (Gregory & Bickel, 2005). Therefore, using electrical stimulation may prevent the potential protective mechanisms of heterozygous sarcomere lengthening and may not be representative of the contractile behaviour or physiological response to EIMD *in vivo* (Crameri et al., 2007). Secondly, to be able to investigate the association between strain (denoted by fascicle lengthening) and EIMD, *in situ* and *in vitro* studies exaggerate strain values (i.e. 125% of fascicle length at peak MVC (Lieber & Friden, 1993)) (Butterfield, 2010) and remove the tendon from the MTU. The strain values used *in situ* and *in vitro* are considerable larger than those attained *in vivo* (i.e. 18% of fascicle length at peak MVC (Hoffman et al., 2014)) and even to those reported within chapter three of the current thesis (58%). Furthermore, by removing the tendon, *in situ* and *in vitro* studies do not account for the potentially attenuating role of the tendon on fascicle lengthening reported in chapter three and estimated in previous muscle models (Lichtwark & Wilson, 2007). Therefore, although *in situ* and *in vitro* studies are essential to contribute to the understanding of EIMD, due to the complicated mechanical properties of the MTU *in vivo*, findings from these studies they are not necessarily replicable or directly applicable *in vivo* (Butterfield, 2010).

The association between fascicle lengthening and EIMD *in vivo* is limited, with the recent work of Hoffman et al. (2014) being the first to directly investigate the association *in vivo*. Hoffman et al. (2014) reported no association between MVC plantar flexion torque loss and *gastrocnemius* fascicle lengthening during backwards walking. Hoffman et al. (2014)

suggested that *in vivo* the Achilles tendon accounts for ~90% of MTU lengthening thus minimising the magnitude of *gastrocnemius* fascicle lengthening and thus the extent of EIMD. However, the degree of fascicle lengthening investigated by Hoffman et al. (2014) was considerably smaller than the magnitude of VL fascicle lengthening measured during MVE_{KE} in chapter three (~58%). However, despite a larger magnitude of fascicle lengthening compared to the work of Hoffman et al. (2014) (42.0 mm and 10.7 mm respectively), chapter four concurs that VL fascicle lengthening was not associated with MVC_{KE} torque loss. Although the findings of no association between fascicle lengthening and MVC_{KE} torque loss appear to contradict the popping sarcomere theory (Morgan, 1990), chapter four or the work of Hoffman et al. (2014), cannot account for heterozygous individual sarcomere lengthening (Palmer et al., 2011). Thus individual sarcomeres may have extended onto the descending limb of the length-tension relationship but through an increase in fascicle compliance or through a lateral transmission of force (Bloch & Gonzalez-Serratos, 2003; Butterfield, 2010) the true magnitude of MVC torque loss may be masked. Although the current thesis cannot confirm this theory, a significant rightward shift in optimal knee angle in chapter four, five and six strengthens the hypothesis that optimal fascicle length may have increased during and post MVE_{KE}. Therefore, the findings of chapter four conclude that VL fascicle lengthening in males is not associated with markers of EIMD in males.

7.4.4. Fascicle lengthening and group differences in exercise-induced muscle damage

The main finding of chapter three concludes that during MVE_{KE} the magnitude of VL fascicle lengthening is significantly higher in males compared to females. In agreement with chapter three, chapter five reported fascicle lengthening to be significantly higher in males compared to females, however no significant sex difference in MVC_{KE} torque loss or muscle soreness was reported within chapter five. Furthermore, within chapter six, although there was a trend for VL fascicle lengthening to be higher in OCP users, there was no significant difference

in MVC_{KE} torque loss or muscle soreness between OCP users and non-users (potentially due to no differences in patella tendon stiffness). As previously mentioned the current chapters cannot account for potential differences in individual sarcomere lengthening or disruption. However, no significant group difference in the magnitude of the rightward shift in the optimal MVC_{KE} knee angle within chapters five or six may indicate no significant differences in sarcomere disruption. Therefore, chapters four, five and six conclude that VL fascicle lengthening is not associated with indirect markers of EIMD (Figure 7. 1).

7.5. Conclusion

The findings of the current thesis are summarised in Figure 7. 1. Within the current thesis a significant positive association between tendon stiffness and VL fascicle lengthening has been reported for the first time in vivo. Furthermore, as a result of higher patella tendons stiffness, VL fascicle lengthening was significantly higher in males compared to females. When the association with EIMD was investigated within the current thesis however, neither fascicle lengthening or tendon properties were reported to have a direct effect on the magnitude of EIMD. Moreover, higher tendon stiffness and greater magnitude of fascicle lengthening did not cause greater EIMD in males compared to females. It may be concluded that although tendon properties do not directly affect the magnitude of EIMD, it may be a contributing factor during the eccentric contraction. For example, the greater contribution of tendon lengthening during MVE_{KE} may alter the degree of fascicle lengthening thus in-directly attenuating EIMD (Hoffman et al., 2014). However, this requires further investigation.

It appears when using voluntary MVE_{KE} contractions *in vivo* eccentric torques investigated may not surpass fibre yield threshold (>113% (Warren et al., 1993a)). Therefore, the current thesis concludes that MVE_{KE} torques below 108% of MVC_{KE} torques are not associated with, nor do they result in, group difference in MVC_{KE} torque loss. However, further investigation is required to establish whether voluntary MVE_{KE} torques higher than

the suggested yield strength of muscle fibres (> 113% (Warren et al., 1993a)) can be achieved during voluntary eccentric damaging protocols, and if so, is eccentric torque then associated with EIMD.

Consistently within the current thesis ΔCK_{ABS} to peak was highest in groups with lower oestrogen levels (males > females < OCP users), despite no differences in any other markers of EIMD. The lower CK response in the presence of oestrogen may be attributed to oestrogen helping to maintain membrane stability, thus reducing the influx of CK into the serum. Although an influx of CK represents a loss of membrane stability and therefore a marker of EIMD, the severity of the CK influx may not relate to the magnitude of EIMD *per se* (Heled et al., 2007; Friden & Lieber, 2001; Komulainen et al., 1995). Therefore the current thesis concludes that CK is attenuated in groups with higher circulating oestrogen. It is also apparent from the current thesis that different groups seem to demonstrate different profiles in response to EIMD.

7.6. Future directions

Whilst investigating the aims of the current thesis, several areas for future research have been highlighted. The following section will briefly discuss several ideas for further research.

Recently speculation regarding the association between titin and eccentric contractions has increased however, scientific evidence remains in its infancy (Herzog, 2014). The current thesis has investigated the role of patella tendon stiffness on VL fascicle lengthening, and whether tendon stiffness or fascicle lengthening are determinants of EIMD. The current thesis however, could not account for differences in structural properties of the sarcomeres, for example titin stiffness. Titin stiffness has recently been suggested to alter titin force, degree of sarcomere lengthening and stability of the descending limb of the length-tension relationship during eccentric contraction (Herzog, 2014). Although, further evidence is required to support the role of titin, Herzog (2014) hypothesised that titin stiffness may play a significant role in protecting the muscle from EIMD during eccentric contractions. Furthermore, the elastic properties of the muscle depends on the titin isoform expressed (Wang et al., 1991). Therefore, although currently based on speculation, it should be investigated whether sex or muscle group differences in titin stiffness exist, and if so, do different titin isoforms explain variability in EIMD?

Throughout the current thesis, to investigate an association with EIMD in the knee extensors, VL muscle properties (fascicle lengthening, VL_{ACSA}, pennation angle and thickness) were measured at 50% of VL length. Although, during walking, Lichtwark et al. (2007) concluded that fascicle length change at 50% of the *gastrocnemius* muscle allowed for an approximation of fascicle lengthening across the whole muscle, it remains unknown if this generalisation is applicable in the VL during MVE_{KE}. Within the VL, fascicle length, pennation angle and muscle thickness are heterozygous along the muscle (Blazevich et al., 2006), hence magnitudes of strain may vary across the muscle (Finni et al., 2003b). Therefore, due to

assuming homozygous muscle properties from 50% of VL muscle length, the current thesis may have underestimated the association of muscle properties, specifically fascicle lengthening, with EIMD. Consequently future research should investigate the role of muscle properties at the proximal and distal regions of the muscle on EIMD.

It is reported that EIMD is significantly lower in the lower limbs compared to the upper limbs (Saka et al., 2009; Chen et al., 2011). These differences have been previously attributed to the daily exposure of eccentric contractions being higher in the lower limbs compared to the upper limbs (Chen et al., 2011). Furthermore, in light of the current thesis, greater EIMD in the upper limbs compared to the lower limbs may be attributed to differences in muscle architecture and tendon properties (Chen et al., 2011). For example, due to parallel muscle fibres in the *bicep femoris*, the magnitude of fascicle lengthening may be significantly greater than the degree of fascicle lengthening reported in the VL. Although within the current thesis, muscle and tendon properties were not concluded as determinates of EIMD in the lower limbs, it is required to establish whether the same conclusion can be drawn in the upper limbs. Secondly, differences in muscle and tendon properties may explain differences in EIMD between muscle groups, especially when the difference in the magnitude of EIMD investigated is significantly greater than the group differences reported in the current thesis.

Appendix: Publications and conferences

A.1. Gender differences in fascicle lengthening during eccentric contractions: the role of the patella tendon stiffness.

Chapter three was published in the following format, in Acta Physiologica 2014.

K. M. Hicks[1], G. L. Onambele-Pearson[1], K. Winwood[1] and C. I. Morse[1]

K M Hicks carried out data collection, analysis and manuscript preparation

G. L. Onambele-Pearson contributed to study design, data collection and manuscript preparation

K. Winwood contributed to study design and manuscript preparation

C. I. Morse contributed to study design, data collection and manuscript preparation

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Running title: Patella tendon stiffness and fascicle lengthening

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Abstract

Aim: Elastic tendons have been suggested to attenuate fascicle lengthening during eccentric contractions however there is no *in vivo* evidence to support this hypothesis. Therefore, the aim of this study was to determine whether patella tendon stiffness modulates *Vastus Lateralis* fascicle lengthening during eccentric contractions in males and females. *Method: Vastus Lateralis* and patella tendon properties were measured in males and females owing to previously reported intrinsic gender differences in tendon properties. During maximal voluntary eccentric knee extensions, *Vastus Lateralis* fascicle lengthening and torque was recorded at every 10° (range of motion 20-90°). *Results:* A significant correlation between maximal patella tendon stiffness and change in fascicle length ($r=0.476$, $p=0.023$) was observed. Similarly, there was a significant correlation between maximal Young's modulus and change in fascicle length ($r=0.470$, $p=0.049$). As expected, patella tendon stiffness and Young's modulus were significantly higher in males compared to females ($p<0.05$). Interestingly, change in *Vastus Lateralis* fascicle length during the eccentric contractions was significantly greater in males compared to females ($p<0.05$). Based on patella tendon moment arm measurements, *Vastus Lateralis* muscle-tendon unit elongation was estimated to be significantly greater in males compared to females (5.24cm and 4.84cm respectively) *Conclusion:* The significant difference in fascicle lengthening during eccentric contractions may be partly explained by the significantly higher patella tendon moment arm, patella tendon stiffness and Young's modulus found in males compared to females. The current study provides *in vivo* evidence to support the hypothesis that the tendon acts as a 'mechanical buffer' during eccentric contractions.

Key words: Fascicle lengthening, tendon stiffness, eccentric exercise, and gender

Introduction

Historically eccentric contractions are considered and often defined as “muscle lengthening” (e.g. (Lindstedt et al., 2001)). However, with the advent of the sonomicrometry method pioneered by Griffiths (1987) and B-mode ultrasonography (Fukashiro et al., 1995), the quantification of muscle lengthening during eccentric contractions has been directly investigated. During the stance phase in a freely walking cat, Griffiths (1991) found that muscle fascicles were shortening despite an increase in muscle tendon unit (MTU) length. In agreement with Griffiths (1991), Spanjaard et al. (2007) reported that during the eccentric phase of stair descent, the *gastrocnemius medialis* fascicles shortened despite a lengthening of the MTU. In contrast, Fukunaga et al. (2001) reported *gastrocnemius medialis* fascicles to contract quasi-isometrically during the stance phase of human walking, despite significant lengthening of the MTU. Although some of the discrepancy in fascicle lengthening can be attributed to differences in gait patterns during flat walking versus stair decent (Spanjaard et al., 2007), both Spanjaard et al. (2007) and Fukunaga et al. (2001) suggested that the lengthening of the MTU during the eccentric phase, occurs predominately at the tendon rather than at the muscle fascicles.

It is widely accepted that tendons are not inextensible and are in fact a viscoelastic tissue that deforms under loading (Magnusson et al., 2008). The degree of deformation (elongation) depends on the level of loading and intrinsic stiffness of the tendon (Maganaris & Paul, 1999). Non-invasive techniques such as B-mode ultrasonography allows the in-series muscle and tendon behaviour to be measured independently, at rest and during locomotion (Fukunaga et al., 1996; Cronin & Finni, 2013). Understanding the interaction between the tendon and muscle is essential to fully understand the contribution of each to everyday tasks such as postural balance (Onambele et al., 2006) and acute stretching (Morse et al., 2008). The shortening or quasi-isometric behaviour of fascicles during eccentric contractions has

previously been attributed to the tendon lengthening under load (Spanjaard et al., 2007; Fukunaga et al., 2001; Reeves & Narici, 2003). The greater proportion of MTU lengthening occurring at the tendon, reduces the metabolic cost of muscle contractions (Roberts & Scales, 2002) and economy of walking is enhanced (Fukunaga et al., 2001). The impact of tendon properties on fascicular elongation during eccentric loading is further demonstrated through the role that tendon stiffness plays in explaining group differences in fascicular elongation (Mian et al., 2007). The *gastrocnemius medialis* fascicles during the eccentric stance phase of gait, in the elderly and young, are indeed reported to behave either quasi-isometrically (in the old) or to lengthen (in the young) (Mian et al., 2007). With these differences in fascicle behaviour being attributed to tendon stiffness decreasing with age (Onambele et al., 2006) thus attenuating fascicle lengthening in the elderly. To explain the interaction between tendon stiffness and fascicle displacement under loading, Lichtwark et al. (2007) used a three element Hill muscle model. Using the Achilles tendon and the *gastrocnemius medialis* fascicle lengthening during the eccentric stance phase of walking they predicted increasing Achilles tendon stiffness increased the required amount of fascicle lengthening (Lichtwark & Wilson, 2007).

The interaction between the tendon and muscle fascicles have also reported within the *vastus lateralis* (VL) during eccentric tasks, such as drop jumps and countermovement jumps (2001). Finni et al., (2001) reported the magnitude of VL fascicle lengthening during the eccentric phase was smaller in the drop jump compared to the countermovement jump. Due to similar MTU lengthening, they suggested the difference of fascicle lengthening could be attributed to the greater tendon elongation required in the drop jump. These findings suggest a modulating role of the patella tendon on the degree of VL fascicle lengthening, which is dependent on the movement being performed (Ishikawa et al., 2003). Thus, the above

supports the hypothesis that the tendon may act as a 'mechanical buffer' (Reeves & Narici, 2003; Roberts & Azizi, 2010).

The role of the tendon in attenuating fascicular loading during eccentric contractions is particularly relevant when trying to address the gender differences in response to eccentric contractions (Sewright et al., 2008). It is well established that males have higher tendon stiffness than females, which is associated with a direct impact of raised oestrogen levels on tendon properties (Bryant et al., 2008). For example, Onambélé et al. (2007) reported patella tendon stiffness to be 57% higher in males compared to females. Even when corrected for tendon length and patella tendon cross-sectional area (PT_{CSA}(Young's modulus)), males' tendon stiffness remained 12% higher than females'. It is these gender differences in tendon properties that have previously been alluded to as the mechanism by which males experience greater levels of muscle damage following eccentric exercise compared to boys (Marginson et al., 2005). Although a lengthening of VL fascicles during eccentric knee extensions has been shown (Guilhem et al., 2011), to the authors' knowledge, it is not known whether a gender difference in the elongation of muscle fascicles during eccentric contractions exists.

Therefore, the aim of the present study was to determine whether patella tendon stiffness alters VL fascicle lengthening during eccentric contractions in males and females. In view of the evidence that males have stiffer tendons than females, it is hypothesised that fascicular lengthening during eccentric contractions would be greater in males compared to females.

Materials and methods

Subjects

Nine males (22 ± 2 years of age, 71.9 ± 13.1 kg and 173 ± 8 cm) and nine females (21 ± 2 years of age, 62.2 ± 6.9 kg and 165 ± 7 cm) volunteered to participate in this study. All participants self-reported as being recreationally active (undertaking no more than 1 hour of "moderate" physical activity per week) and not undertaking any structured training regime. Due to the effects of the oral contraceptive pill on tendon properties (Bryant et al., 2008), females had no history of oral contraceptive pill use. Ethical approval was obtained through the Department of Exercise and Sport Science, Manchester Metropolitan University and all participants signed informed consent prior to taking part in the study. All procedures complied with the Declaration of Helsinki. Exclusion criteria were established through participant questionnaire prior to testing. Exclusion criteria included an occupation or lifestyle that required regular heavy lifting or carrying, resistance trained in the last six months, the use of dietary supplements (e.g. vitamin E), known muscle disorder, and any musculoskeletal injury in the last three months. Further exclusion criteria for female participants included, irregular menstrual cycles (24-35 days) in the last 12 months, previous use of any other forms of hormone based contraception, and been pregnant or breast fed in the year preceding their application to participate in the present study.

Testing protocol

Participants were asked to visit the laboratory on two occasions, separated by two days; the first session consisted of anthropometric measures (stature and mass), VL anatomical cross-sectional area (VL_{ACSA}), patella tendon moment arm (PT_{MA}), and dynamometer familiarisation. Stature and mass were measured using a wall mounted stadiometer (Harpenden stadiometer, Holtain Crymych, UK) and digital scales (Seca model 873, Seca, Germany) respectively. Familiarisation included two practice MVCs, at two knee joint angles (90

seconds rest). The second laboratory visit consisted of patella tendon stiffness measurements followed by measurement of VL fascicle length during eccentric contractions. All testing was carried out on the non-dominant leg. The non-dominant leg was defined as the leg that provided stability during movements, e.g. kicking. During the tendon properties and eccentric test protocols, participants were secured (i.e. inextensible straps around the shoulders and hips to reduce any extraneous movement during maximal efforts) in a seated position to an isokinetic dynamometer (Cybex Norm, Cybex International, NY, USA), with a hip angle of 85°. The dynamometer axis of rotation was visually aligned with the knee joint's centre of rotation.

VL anatomical cross-sectional area

A real-time B-mode ultrasound (AU5 Harmonic, Esaote Biomedica, Genoa, Italy) was used to measure VL_{ACSA}. Participants laid supine with their leg full extended (knee angle 0°), an ultrasound probe (7.5 MHz linear array probe, 38 mm wide) was used to identify the distal and proximal insertion sites of the VL. At 50% of VL muscle length echo-absorptive markers were placed in parallel, at intervals of 30 mm, from the medial to the lateral edge of the VL muscle. The ultrasound probe was placed perpendicular to the VL muscle in the axial plane. The ultrasound probe was moved slowly across the echo-absorptive markers from the medial to the lateral edge of the muscle. Consistent minimal pressure was placed on the muscle during scanning to avoid compression of the muscle. The images were recorded in real time onto a PC at 25 frames per second (Adobe Premier pro Version 6). At each 30 mm interval, individual images were acquired using capturing software (Adobe Premier Elements, version 10). The shadows casted by the echo-absorptive markers allowed the images to be aligned by the contour of the muscle and the entire VL_{ACSA} to be recreated in a single image (Adobe Photoshop Elements, version 10). The VL_{ACSA} was then measured using digitising software

(ImageJ 1.45, National Institutes of Health, USA). This method for calculating VL_{ACSA} has previously been accepted as reliable and valid when compared to MRI, with a reported interclass correlation between 0.998 and 0.999, for reliability and validity respectively (Reeves et al., 2004). Furthermore, chapter demonstrates high reliability for measuring VL_{ACSA} using the ultrasound.

Tendon length and cross-sectional area

Patella tendon cross-sectional area and patella tendon length (PT_L) were measured with the knee fixed at 90° angle. The distance between the tibial tuberosity and the apex of the patella, marked using sagittal ultrasound images, was taken as PT_L. To measure PT_{CSA} the ultrasound probe was placed in the transverse plane and images were captured at 25%, 50%, and 75% of PT_L. The images were subsequently analysed offline using (ImageJ 1.45, National Institutes of Health, USA). A mean of the three images was taken as PT_{CSA} (O'Brien et al., 2010).

Patella tendon moment arm

In order to calculate patella tendon force PT_{MA} was measured at a knee joint angle of 90° (full extension = 0°) in the sagittal plane, from a single energy (frame 23.3 cm x 13.7cm) DEXA scan (Hologic Discovery, Vertec Scientific Ltd, UK). A goniometer was used to ensure the knee angle was 90°. Using OsiriX, (DICOM viewer, ver. 4.0, Pixemo, Switzerland) the perpendicular distance from the center of the patella tendon to the tibio–femoral contact point was determined as the PT_{MA} length (Baltzopoulos, 1995). Compared to the MRI, the DEXA has been reported a reliable and valid technique when measuring moment arm length (Erskine et al., 2014).

Force- elongation relationship / patella tendon stiffness

With the knee angle fixed at 90° in the isokinetic dynamometer the participants were instructed to perform a ramped maximal voluntary knee extension (MVC_{KE}) lasting ~6 seconds. Concomitantly, ramped MVC_{KE} torque and displacement of the patella tendon were recorded. Patella tendon displacement was measured over two MVC_{KE}, once with the ultrasound probe positioned over the tibial tuberosity and on the second contraction over the apex of the patella. This is consistent with Onambélé et al. (2007) as a means of obtaining total patella tendon displacement. Torque was displayed on a Macintosh G4 computer (Apple Computer, Cupertino, CA, USA), via an A/D converter (Biopac Systems, Santa Barbara, CA) and subsequently analysed with the accompanying software (Acknowledge, version 3.9.2). Patella tendon displacement was measured by positioning the ultrasound probe (7.5 MHz linear array probe, 38 mm wide) in the axial-plane on the tibial tuberosity (or the apex of the patella during alternate contractions). An echo-absorptive marker was placed on the skin to cast a shadow on the ultrasound and act as an external marker. The distance of the shadow from an internal marker at the beginning of the contraction, to the position of the shadow at the end of the contraction, allows excursion to be measured. An externally generated square wave pulse was used to synchronise the ultrasound images with the torque acquisition system. Images were captured at ~10% intervals of ramped MVC_{KE} torque (Onambélé et al., 2007). Total patella tendon displacement was calculated as displacement at the tibial tuberosity plus the displacement at the apex of the patella (Onambélé et al., 2007). Patella tendon forces were calculated as: (MVC_{KE} torque + antagonist co-activation torque) / PT_{MA}. The measurement of antagonist co-activation torque is described below. The force – elongation curve stemming from data at every 10% MVC_{KE} was then fitted with a second-order polynomial function, forced through zero (Onambélé et al., 2007), and the tangential

slope at discreet sections of the curve, relative to MVC_{KE}, was computed by differentiating the curve at each point of interest (i.e. every 10% force intervals). To account for between subject differences in maximal force production, the slope of the tangential line, corresponding to the MVC_{KE} of the weakest participant (regardless of gender), was computed for each subject. This therefore standardised the comparison of tendon stiffness at an absolute load.

Co-activation

Electromyogram amplitude of the bicep femoris (BF) was measured to determine co-activation during the ramped MVC_{KE}. Guided by an axial-plane ultrasound of the BF, two bipolar electrodes (Ambu, Neuroline 720, Malaysia) were placed in the mid-sagittal line at 75% of BF muscle length (distal end = 0%). A reference electrode (Ambu, blue sensor, Malaysia) was placed on the lateral tibia condyle. The electrodes were placed in a bipolar configuration with a constant inter-electrode distance of 20mm (Hermens et al., 2000). Preceding electrode placement; the skin was shaved, abraded and cleansed with an alcohol wipe to reduce skin impedance below 5000 Ω (Hermens et al., 2000). The participants were asked to contract and relax to ensure correct electrode placement. The raw EMG signal was amplified (×2000) and filtered (through low and high pass filters; i.e. 10 and 500 Hz respectively) with the sampling frequency set at 2000 Hz. Ramped MVC_{KE} torque and BF EMG were recorded in real time and synchronised using an externally generated square wave pulse. In the isokinetic dynamometer, participants were asked to perform two maximal voluntary knee flexions (MVC_{KF}) at 90° knee angle. Participants were instructed to perform MVC_{KF} as quickly and as forcefully as possible against the dynamometer's lever arm. The participants were instructed to stop contracting once a two second plateau had been attained (as observed on the dynamometer screen display). The integral of the root mean square of

the BF EMG signal, was calculated 500 ms either side of instantaneous MVC_{KF} maximal torque from the contraction corresponding to the highest MVC_{KF} torque. Prior to contraction the baseline signal noise was calculated as the integral root mean square over 1s and removed from the measured EMG during MVC_{KF} and MVC_{KE}. The absolute integral of the BF EMG was taken over 250 ms at every 10% of ramped MVC_{KE} torque. Co-activation torque was calculated as, (BF EMG during ramped MVC_{KE} / BF EMG during MVC_{KF}) × peak MVC_{KF} torque (Onambélé et al., 2007). An assumption that BF EMG is linear with MVC_{KF} was made (Lippold, 1952)

Stress / strain relationship

Patella tendon stress was calculated as, patella tendon force / PT_{CSA}. Tendon strain was calculated as a ratio of total patella tendon displacement and PT_L.

Young's modulus

Young's modulus was calculated as: patella tendon stiffness × (PT_L (mm) / PT_{CSA} (mm^2)) at MVC. Furthermore, young's modulus was also calculated with patella tendon stiffness standardised to the MVC_{KE} force of the weakest participant.

Eccentric exercise

A warm-up of 10 isokinetic knee extensions and knee flexions, ensuring a progressive increase in effort (with the last contraction being maximal), was carried out prior to eccentric exercise. For the eccentric exercise, the knee extensor range of motion was set at 20 – 90° (0°

= full extension). Participants were asked to perform 1 set of 12 maximal voluntary eccentric knee extensions (MVE_{KE}) repetitions. During the eccentric phase (knee angle 20 to 90°), contractions were performed at an isokentic angular velocity of $30°·s^{-1}$ (Saka et al., 2009). The concentric phase was performed sub-maximally at an angular velocity of $60°·s^{-1}$ to minimise fatigue and enhance eccentric damage (Chapman et al., 2006). Visual and verbal encouragement was continuously provided throughout the eccentric exercise (Campenella et al., 2000). MVE_{KE} torque was recorded throughout each MVE_{KE} and displayed via the torque acquisition system.

Change in VL fascicle length during the eccentric protocol

To measure VL fascicle length during MVE_{KE}, the ultrasound probe was fixed at 50% of VL muscle length in the mid-sagittal plane of the non-dominant leg. A hypo-allergenic ultrasound gel (Parker, Park Laboratories Inc., Fairfield) was used to enhance coupling between skin and the ultrasound probe.

During the 12 MVE_{KE} contractions, ultrasound images were recorded onto a PC, in real time, at 25 frames per second (Adobe Premier pro Version 6). An externally generated square wave pulse was used to synchronise the ultrasound images with the torque acquisition system. Three MVE_{KE} contractions were randomly chosen from the 12 repetitions for architectural analysis. Using frame capture software (Adobe Premier Elements, version 10), an ultrasound image (frame corresponding to every 10° of knee angle, ranging from 20- 90°) was acquired for offline analysis. To ensure there was no movement artefact included in the assessment fascicle length, an echo-absorptive marker was fixed on the skin to provide a visual baseline of the internal structures. If movement of the probe was observed relative to

the VL, the contraction was discarded and another MVE_{KE} contraction was chosen for analysis.

Using digitising software (ImageJ 1.45, National Institutes of Health, USA), VL fascicle length was analysed offline at every 10°. Fascicle length was measured from the visible insertion of the fibre into either the deep or superficial aponeurosis (Reeves & Narici, 2003). The fascicle was only measured if it remained visible across the entire ultrasound image. Where the fascicle extended beyond the ultrasound image (frame width 3.50 cm and height 4.15 cm), linear continuation of the fascicle and aponeurosis was assumed. Using ultrasound a 2-7% error has been associated with assuming linear continuation to calculate VL fascicle length at 120° knee angle (Finni et al., 2003a). To reduce error associated with the estimation of VL fascicle length, an average of three fascicles across the image was taken (Guilhem et al., 2011). Change in fascicle length was calculated at each angle by subtracting the fascicle length reported at knee angle 20°. Change in fascicle length at each angle was made relative to fascicle length at 20°. To normalise change in fascicle length for PT_{MA} length, change in fascicle length from 20-90° was divided by PT_{MA} length at 90° knee angle.

VL excursion

In order to estimate the total VL MTU elongation, the tendon excursion method was adopted (Spoor et al., 1990); whereby the change in knee angle (70°, 1.22 rad) was multipliedpoo by measured PT_{MA} at 90° knee angle.

Statistics

All statistical analyses were carried out using the statistical software package SPSS (v.19, Chicago, IL) for Windows and Microsoft Excel. To ensure the data were parametric, the Shapiro-Wilk and Levene's tests were utilized to assess the normality and variance of the

data. These statistical assumptions were not breached. For gender differences in anthropometric measures, VL fascicle length and patella tendon properties, Independent T-tests were used. A 2×8 mixed design ANOVA (between factors: gender (2 levels), and knee angle (8 levels)) was used for MVE_{KE} torque, MVE_{KE} torque corrected for VL_{ACSA}, and change in VL fascicle length. Wherever the assumption of sphericity was violated, the Greenhouse-Geisser correction was used. When a significant group effect was found, post-hoc Independent T-tests (planned contrast) with LSD correction were carried out. Pearson's correlations were used to investigate whether significant associations were present at 90° between 1) MVE_{KE} corrected for VL_{ACSA} and change in fascicle length 2) patella tendon stiffness, at MVC_{KE} and change in fascicle length and, 3) Young's modulus, at MVC_{KE}, and change in fascicle length. Significance was set at $p \leq 0.05$. Data are presented as mean ± standard deviation.

Results

Anthropometric measurements

There were no significant difference in age (males aged 22 ± 2 years, females aged 21 ± 2 years, p= 0.449) and mass (males 71.9 ± 13.1 kg, females 62.2 ± 6.9 kg, p= 0.066) in males compared with females. Males were significantly taller than the females (173 ± 8 cm and 165 ± 7 cm, respectively, p=0.045).

Tendon Properties

PT_L (males 5.73 ± 0.50 cm, females 5.16 ± 0.55 cm, p= 0.034), PT_{CSA} (males 0.81 ± 0.16 cm^2, females 0.62 ± 0.14 cm^2, p =0.016) and PT_{MA} (males 4.30 ± 0.27 cm, females 4.0 ± 0.29 cm, p=0.023) were significantly greater in males compared to females. Ramped MVC_{KE} torque was significantly higher in males compared to females at every 10% MVC_{KE} torque ($p < 0.01$).

Patella tendon force was significantly higher in males compared to females at every 10% MVC_{KE} torque ((p<0.05), Table 1.). Patella tendon elongation was not significantly different between males and females at any 10% MVC_{KE} torque (Table 1). Patella tendon stiffness, at MVC, was significantly higher in males compared to females (≥ 50% MVC_{KE} p<0.05, < 50% MVC_{KE} p< 0.01, (Table 1)). The greater patella tendon stiffness in males was reflected in a relative shift to the left (compared to the females' trend line) in the force-elongation curve.

<INSERT TABLE 1>

Young's modulus at MVC_{KE} was significantly higher (p= 0.014) in males (8131 ± 2447 MPa) compared to females (5800 ± 1543 MPa). To account for the significantly higher MVC_{KE} torque in males, a patella tendon force of 2330N (corresponding to the highest MVC_{KE} torque of the weakest participant (independent of sex)) was used to calculate standardised force level patella tendon stiffness and Young's modulus. Standardised force patella tendon stiffness (males 880 ± 293 $N \cdot mm^{-1}$, females 553 ± 179 $N \cdot mm^{-1}$, p=0.013) and standardised force Young's modulus (males 6343 ± 2012 MPa, females 4701 ± 1482 MPa, p=0.033) remained significantly higher in males compared to females.

<INSERT FIGURE 1>

Change in VL fascicle length during MVE_{KE}

A two-way mixed repeated measures ANOVA for change in VL fascicle length, reported a significant main effect of angle (p< 0.001) and a significant group effect of gender (p= 0.004), with a significant interaction effect (p= 0.001). A planned contrast showed at 30 - 90°, change in fascicle length was significantly higher in males compared to females (p< 0.05, Figure 1). When this change in fascicle length was made relative to fascicle length at 20°, male's fascicles

increased by 57.6 ± 10.9 % and females increased by 39.6 ± 9.8 % at 90°. Thus, relative fascicle length increased significantly further in males compared to females (p= 0.002)

When change in fascicle length during eccentric contraction was corrected for PT_{MA} length, change in fascicle length remained significantly higher in males (0.96 ± 0.26 cm) compared to females (0.66 ± 0.14 cm, p= 0.008).

<INSERT FIGURE 2>

MVE_{KE} torque showed a significant main effect of angle (p< 0.001) and a significant group effect of gender (p=0.004), with a significant interaction effect (p= 0.020). A planned contrast demonstrated that MVE_{KE} torque was significantly higher in males compared to females at 60° (males 148 ± 33.2 Nm, females 101 ± 30.4 Nm, p= 0.007), 70° (males 160 ± 38.9 Nm, females 99.2 ± 29.1 Nm, p= 0.002) and 80° (males 145 ± 33.8 Nm, females 88.9 ± 28.8 Nm, p=0.002). Peak MVE_{KE} torque was found at 60° for females (101 ± 30.4 Nm) and 70° for males (160 ± 38.9 Nm). Independent T-test showed that peak MVE_{KE} torque in males was significantly higher than in females (p< 0.003).

When MVE_{KE} torque was normalised for VL_{ACSA} (i.e. MVE_{KE} torque / VL_{ACSA}) a significant main effect of angle (p< 0.001) was observed but no effect of gender (p=0.162), with a significant interaction (p= 0.047).

VL excursion

Based on the tendon moment arm excursion, the estimated increase in VL MTU length from 20-90° was significantly greater in the males compared to the females (5.25 ± 0.33 cm, 4.84 ± 0.35 cm, respectively, p =0.023).

Correlation

Pearson's correlations were conducted at 90° (knee angle), as the change in fascicle length was greatest at this angle. There was no correlation between MVE_{KE} corrected for VL_{ACSA} and change in fascicle length ($r=0.010$, $r^2=1.03$ %, $p=0.485$). A significant, although weak, correlation between patella tendon stiffness at MVC and change in fascicle length ($r=0.476$, $r^2=23\%$) was reported ($p=0.023$). A significant correlation between patella tendon Young's modulus at MVC and change in fascicle lengthening ($r=0.508$, $r^2=26\%$) was reported ($p=0.016$) (Figure 2.).

Discussion

The aim of the present study was to determine whether patella tendon stiffness alters VL fascicle lengthening during MVE_{KE} in males and females. There were three main findings, 1) VL fascicles lengthen during MVE_{KE}, 2) in agreement with our hypothesis, the change in VL fascicle length and relative change in fascicle length during eccentric contractions was significantly greater in males compared to females, 3) a significantly longer PT_{MA}, higher tendon stiffness and Young's modulus in males compared to females, partially accounts for increased fascicle lengthening under loading.

Consistent with the classical definition of eccentric contractions and the findings of Guilhem et al., (2011) the current study reported a lengthening of VL fascicles during MVE_{KE}. A lengthening of VL fascicles has previously been reported during eccentric movements (Finni et al., 2001), with greater lengthening during a low intensity countermovement jump compared to a drop jump where the fascicle lengthening was minimal. As a result of similar MTU lengthening between the two jumps, Finni et al. (2001), attributed the difference in fascicle lengthening to the greater elongation of the tendon and higher EMG activity found in the drop jump. The difference in fascicle behaviour was further explained by Ishikawa et al.,

(2003) who reported VL fascicle lengthening to reduce, and patella tendon elongation to increase when the rebound intensity of the drop jump was increased. These findings suggest the interaction between the fascicles and tendon during the eccentric phase allows for the fascicle to operate on the most 'efficient' part of the force–lengthening relationship, for greater rebound intensities (Ishikawa et al., 2003). Therefore although the previous literature and the current study report a lengthening of VL fascicles during eccentric movements, it should also be clear that the magnitude of lengthening is regulated by the amount of tendon elongation, the type of eccentric task and intensity of the task itself (Ishikawa et al., 2003; Finni et al., 2001).

In the current study, change in VL fascicle length was found to be significantly higher in males compared to females during MVE_{KE}. The significant difference remained when change in fascicle length was made relative to fascicle length at 20°. To the best of the authors' knowledge, gender differences in fascicle behaviour during eccentric contractions have yet to be reported. The observed gender difference in fascicle behaviour during eccentric contractions can be partially attributed to the observed difference in patella tendon compliance. Although gender differences remain unreported, the role of the tendon and group differences in fascicle lengthening during eccentric phases of gait have recently been considered (Mian et al., 2007). For example, Mian et al. (2007) suggested that the group differences in the fascicle behaviour of young and elderly participants might be a result of reduced tendon stiffness in the elderly; however this was not confirmed experimentally in their study.

The idea that the tendon acts as a mechanical buffer and attenuates fascicle lengthening however is not novel (Fukunaga et al., 2001; Spanjaard et al., 2007; Reeves & Narici, 2003). Litchwark et al. (2007) used a three element Hill muscle model to predict the effect of different levels of tendon stiffness on the behaviour of fascicle lengthening during gait. They

predicted that increasing Achilles tendon stiffness, increased the required amount of *gastrocnemius medialis* fascicle lengthening during the stance phase of gait (Lichtwark & Wilson, 2007). Thus further supporting the hypothesis that the tendon may modulate fascicle lengthening (Reeves & Narici, 2003; Roberts & Azizi, 2010). The accuracy of the model remains in question however, as Litchwark et al. (2007), did not take into account fibre pennation angle and used a linear model for tendon stiffness despite tendon being known to have a non-linear force-elongation relationship (Maganaris & Paul, 2002). The current study however, provides *in vivo* evidence to demonstrate the association between tendon properties and fascicle lengthening. In agreement with previous literature (Onambélé et al., 2007; Kubo et al., 2003), the current study reported significantly higher tendon stiffness and Young's modulus, at MVC_{KE}, in males compared to females. This significant difference remained when patella tendon stiffness and Young's modulus was calculated at a standardized absolute force level of 2330N. Significant correlations were found between 1) Tendon stiffness when calculated at MVC_{KE} and change in fascicle length and 2) Young's modulus, when calculated at MVC_{KE} and change in fascicle length. Thus, greater fascicle lengthening in males can be partially attributed to, the significantly higher patella tendon stiffness and Young's modulus in males. Although the attenuating role of the tendon on fascicle behaviour has been alluded to previously, the current study provides *in vivo* evidence to support previous models (Lichtwark & Wilson, 2007) and hypotheses (Reeves & Narici, 2003; Spanjaard et al., 2007; Fukunaga et al., 2001).

In addition to the tendon one of the other contributory factors to greater fascicle elongation in males could be the greater MVE_{KE} torque. MVE_{KE} torque was significantly higher at 60, 70 and 80° in males compared to females. Furthermore, peak MVE_{KE} torque was significantly higher in males compared to females. Males however, were reported to have significantly larger VL_{ACSA} compared to females. When MVE_{KE} torque was corrected for VL_{ACSA} (stress),

there was no significant difference between males and females. Torque was therefore 'distributed evenly' over VL_{ACSA} in both males and females. Furthermore, a significant correlation between MVE_{KE} torque and change in fascicle length was not observed in the current study. Therefore, although we cannot completely dismiss the contribution of greater torque on fascicle lengthening, we observed no difference in relative MVE_{KE} torque, or any correlation with change in fascicle length.

Males had a significantly longer PT_{MA} than females thus, in order to establish whether there was a significant contribution of the PT_{MA} to the fascicle elongation MTU elongation was estimated based on the tendon excursion method (Spoor et al., 1990). Although this method estimates the entire quadriceps MTU elongation, we estimated a significantly greater MTU elongation in males compared to females (5.24 cm and 4.84 cm respectively). Therefore the difference in PT_{MA} contributes to the difference in fascicle lengthening. Based on measured fascicle lengthening of 4.07 cm in males and 2.63 cm in females, we estimated that 89% and 54% (males and females respectively) of the estimated total change in MTU length, from 20-90°, could be attributed to an increase in the fascicle length. It is therefore likely that the remaining portion of the increase in MTU length (11% and 46% in males and females, respectively), which is not attributed to this increase in fascicle length, may be due to the in series elastic component. As a way of determining the contribution of the PT_{MA} and patella tendon stiffness on VL fascicle lengthening, change in VL fascicle length was normalised for PT_{MA} length. When corrected for PT_{MA} length, change in VL fascicle length remained significantly higher in the males compared to females. Highlighting that although PT_{MA} does contribute to VL fascicle lengthening, the gender differences in VL fascicle lengthening can be attributed to the gender differences in patella tendon stiffness. Although the current study has provided evidence to support the role of patella tendon stiffness on VL fascicle lengthening during MVE_{KE}, it is acknowledged that the elastic component also incorporates

the aponeurosis. It is however unclear how the distinct contributions of the tendon vs. the aponeurosis may interplay since the relative mechanical properties of the aponeurosis are indeterminate with reports of significantly higher (Maganaris & Paul, 2000), significantly lower (Magnusson et al., 2003) or similar (Arampatzis et al., 2005) strain values to the tendon. It should be noted that the apparent variability within these cited studies could be attributed to different participant activity levels, the specific anatomical MTU investigated and the method used to determine MTU lengthening. Therefore, a limitation of the current study is that we are unable to provide a measure of the mechanical properties of the VL aponeurosis alongside those of the tendon; hence we cannot determine the contribution of the VL aponeurosis to fascicle lengthening behaviour. However, it remains that a longer fascicle elongation in males compared to females is attributable to a larger moment arm length, and an increase in stiffness of the in series elastic components, that of the patella tendon in particular.

In conclusion the current study demonstrates during eccentric contractions, VL fascicles lengthen significantly further in males compared to females. The significant difference in lengthening may be partly attributed to the significantly longer PT_{MA}, higher patella tendon stiffness and Young's modulus found in males compared to females. The current study provides *in vivo* evidence to support the hypothesis that the tendon acts as a mechanical buffer during eccentric contractions. Future research needs to investigate the contribution of the aponeurosis to the lengthening of the fascicles during eccentric contractions.

Acknowledgments

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Conflict of interest

The authors declare that no conflict of interest exists.

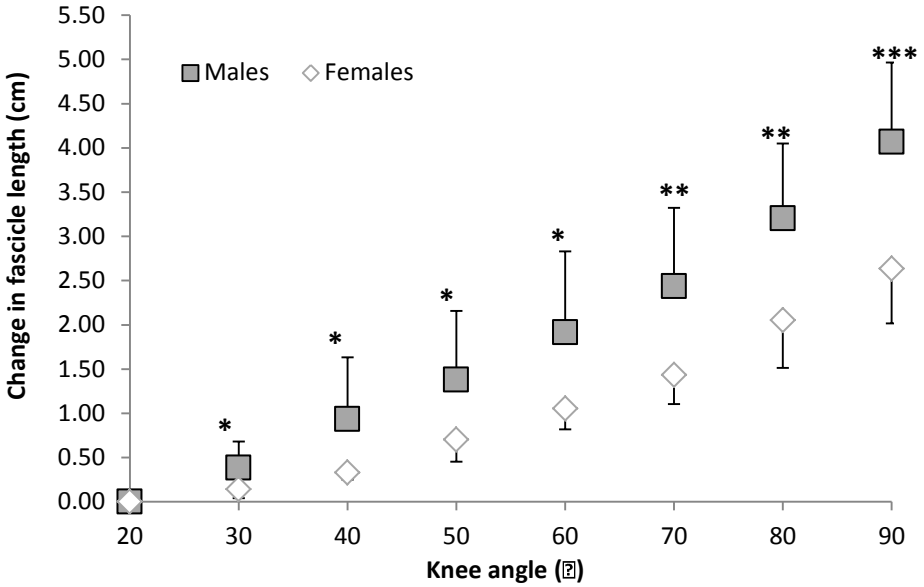

Figure I: Change in VL fascicle length from 20°, at each knee angle (20-90°) during eccentric exercise in males and females. Data is reported as mean and standard deviations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

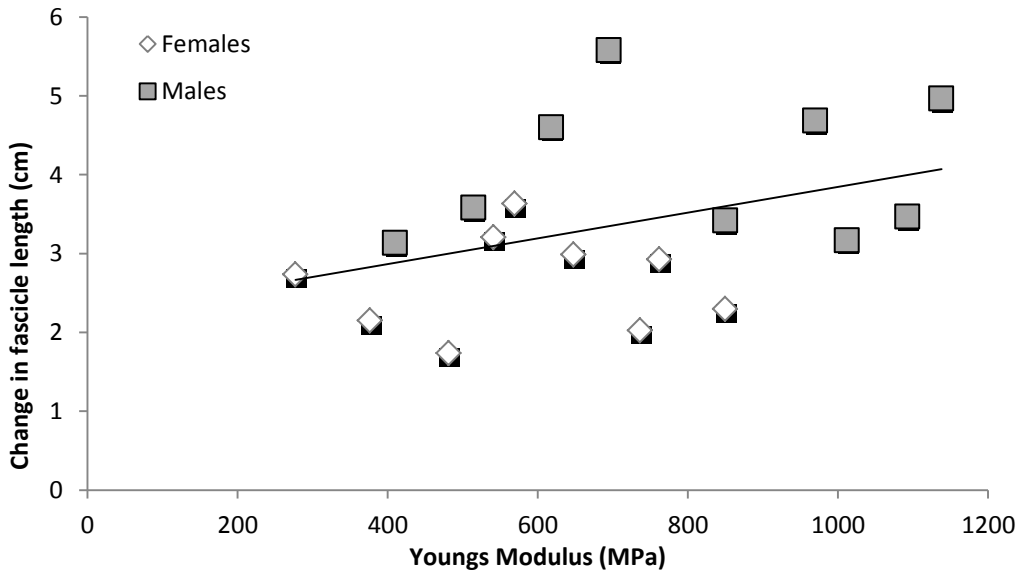

Figure II: Correlation between patella tendon Young's modulus and change in VL fascicle length. Note: R^2 value is for the entire sample.

Table 1. Structural and mechanical patella tendon properties in males and females, *in vivo*.

Gender	MVC_{KE} (%)	Tendon force (N)			Elongation (mm)			Stiffness (N·mm-1)		
Females	10	317	±	47	1.81	±	0.82	256	±	59
	20	633	±	93	3.01	±	0.95	334	±	84
	30	951	±	139	3.97	±	0.88	397	±	106
	40	1267	±	185	4.55	±	1.03	450	±	127
	50	1583	±	229	5.46	±	1.05	498	±	144
	60	1899	±	274	6.07	±	1.25	541	±	160
	70	2217	±	319	6.46	±	1.35	581	±	174
	80	2535	±	365	7.14	±	1.15	619	±	188
	90	2855	±	412	7.64	±	1.25	655	±	201
	100	3177	±	460	8.06	±	1.51	689	±	214
Males	10	411 *	±	94	1.90	±	1.09	443	±	264
	20	821 *	±	187	3.05	±	1.34	568*	±	271
	30	1233*	±	281	4.08	±	1.66	668*	±	285
	40	1646*	±	376	4.89	±	1.50	754*	±	301
	50	2061*	±	472	5.56	±	1.69	829*	±	315
	60	2475*	±	567	5.98	±	1.75	901**	±	333
	70	2888*	±	660	6.36	±	1.75	966**	±	349
	80	3302*	±	756	6.63	±	1.84	1027**	±	365
	90	3725*	±	858	7.00	±	2.12	1085**	±	381
	100	4136*	±	945	7.25	±	2.18	1139**	±	395

Note: MVC_{KE} (%), ramped isometric maximal voluntary knee extension. Data is presented as means and standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$

A.2. Eccentric strain determines exercise-induced muscle damage

Chapter four was presented at the 19th annual Congress of the European College of Sport Science – Amsterdam 2014. The abstract was entered into the Young Investigators Award and published in the congress Book of Abstracts.

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Introduction

Fascicle strain, eccentric torque, muscle and tendon properties, have all been suggested to be determinants of exercise-induced muscle damage (EIMD) yet these remain to be assessed *in vivo* (Butterfield, 2010). Therefore, the aim of the present study was to establish the *in vivo* muscle and tendon determinants of EIMD.

Method

Vastus Lateralis (VL) structural properties (anatomical cross-sectional area (ACSA), architecture), and Patella Tendon (PT) properties (CSA, length) were recorded using B-mode ultrasonography in 16 recreationally active males (22±2 years). PT displacement during Isometric knee extensions was used to determine PT stiffness, and Young's modulus. Six sets of 12 isokinetic maximal eccentric knee extension contractions (knee angle 20-90degs, 30degs/s) were performed. During the first 3 reps, VL fascicle length (strain) was recorded using ultrasonography. During the eccentric phase, torque and strain were recorded at every 10deg knee angle. Markers of EIMD (creatine kinase (CK) [from 6mL venous blood sample] and isometric MVC torque [to calculate torque loss]) were taken pre, 0, 48, 96 and 168 hours post EIMD. Pearson's correlations were used to establish any associations between markers of EIMD and muscle-tendon properties.

Results

Tendon stiffness, normalised for tendon force, did not correlate with change in CK (r=0.131, p=0.314) or isometric torque loss (r=-0.221, p=0.205). There was a significant correlation between change in CK and strain (r=0.534, p=0.017). Eccentric torque, made relative to VL ACSA (stress), did not correlate with markers of EIMD.

Discussion

The current study can report that the tendon does not directly attenuate markers of EIMD. However, tendon may reduce markers of EIMD indirectly through attenuating strain (Hicks et al., 2013). Our data showed change in CK to be associated with VL muscle strain *in vivo*, thus supporting the *in vitro* findings of Lieber and Friden (1993). Despite eccentric contractions generating high torques, when made relative to muscle size, tendon stress was not associated MVC torque loss. In conclusion, *in vivo*, strain is a determinant of EIMD, whereas eccentric stress and the viscoelastic tendon properties are not.

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