NOTTINGHAM TRENT UNIVERSITY

The Effect of β-alanine Supplementation on Neuromuscular Performance

by

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(née Stannard)

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Copyright statement

Abstract

Carnosine (β-alanyl-$_{L}$-histidine), a histidine containing dipeptide, is one of the most abundant small-molecular compounds in human skeletal muscle. Supplementation with the rate limiting amino acid, β-alanine, has resulted in significant improvements to high-intensity exercise performance. The role of carnosine as an intracellular pH buffer is undisputable, yet other physiological roles have been proposed, including the potential influence of increased carnosine content on regulation of skeletal muscle calcium (Ca^{2+}) kinetics. The movement of Ca^{2+} is vital during both skeletal muscle contraction and relaxation phases. The overall aim of this thesis was to investigate the effect of β-alanine supplementation on voluntary and electrically evoked contractile properties of *in-vivo* human skeletal muscle. To examine this research question, there were several aims of this thesis, initially to examine the effect of β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and skeletal muscle contractility in both fresh and fatigued human skeletal muscle in young (Studies 1 and 2; Chapters 4 and 5) and older (Study 3; Chapter 6) adults. The distribution of the carnosine molecule across subcellular fractions within rat skeletal muscle tissue was explored, as well as the impact of increased carnosine availability on ATPase activity, a measure associated with skeletal muscle relaxation, estimated by Pi generation. In young adults, 28-days of β-alanine supplementation did not significantly influence voluntary and evoked force responses, or the force-frequency relationship, the *in-vivo* analogue of the force-Ca^{2+} relationship, in either fresh (Studies 1 and 2; Chapters 4 and 5) or fatigued (Study 2; Chapter 5) skeletal muscle. Furthermore, older adults experiencing pre-existing declines in skeletal muscle function due to ageing, demonstrated no beneficial effect of 28-days β-alanine supplementation on voluntary or electrically evoked skeletal muscle contractions (Study 3; Chapter 6). In young adults, there was, however, a significant decline in skeletal muscle half-relaxation time (HRT) during electrically evoked octet contractions, resting and potentiated twitches (Studies 1 and 2; Chapters 4 and 5). Two possible steps influence skeletal muscle relaxation speed include the Ca^{2+} removal from the myoplasm and Ca^{2+} dissociation from troponin followed by cross-bridge detachment. Based on the *in-vivo* research, it was proposed that there was a direct or indirect mechanism associated with activity of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, the proposed rate-limiting step of muscle relaxation. *In-vitro* analysis of ATPase activity demonstrated that SERCA activity was unaffected by increased carnosine concentrations, although there was a significant increase in overall ATPase activity (Study 4; Chapter 7). The results in this thesis showed that β-alanine supplementation was effective in improving skeletal muscle HRT in young adults, although not in healthy older adults. The exact mechanism associated with the *in-vivo* decline in skeletal muscle HRT remains unclear, yet raising the availability of carnosine *in-vitro*, does increase overall ATPase activity, although not Ca^{2+}-dependent or SERCA activity.

Publications

Journal articles (Appendix 2)

Jones, R.L., Barnett, C.T., Davidson, J., Maritza, B., Fraser, W.D., Harris, R.C., & Sale, C. (2017). β-alanine supplementation improves *in-vivo* fresh and fatigued muscle relaxation speed. *European Journal of Applied Physiology*, 1-13. DOI: 10.1007/s00421-017-3569-1

Hannah, R., **Stannard, R.L.,** Minshull, C., Artioli, G.G., Harris, R.C., Sale, C. (2015). β-alanine Supplementation Enhances Human Skeletal Muscle Relaxation Speed but Not Force Production Capacity. *Journal of Applied Physiology*. 118 (5), 604-612. DOI: 10.1152/japplphysiol.00991.2014

Poster presentations

Stannard, R.L., Hannah, R., Minshull, C., Artioli, G.G., Harris, R.C., Sale, C. (2015). β-alanine Supplementation Enhances Human Skeletal Muscle Relaxation Speed but Not Force Production Capacity. In Proceedings of the 62nd America College of Sport Medicine (ACSM) Annual Meeting, San Diego, USA.

Stannard, R.L., Hannah, R., Minshull, C., Artioli, G.G., Harris, R.C., Sale, C. (2015). β-alanine Supplementation Enhances Human Skeletal Muscle Relaxation Speed but Not Force Production Capacity. Science and Technology Annual Research (STAR) conference, Nottingham Trent University.

Oral presentations

Stannard, R.L., Barnett, C.T., Davidson, J., Maritza, B., Fraser, W.D., Harris, RC., & Sale, C. (2016). β-alanine supplementation improves *in-vivo* fresh and fatigued muscle relaxation speed. In Proceedings of the 21st Annual European College of Sport Science Annual Conference, Vienna, Austria.

Stannard, R.L. (2015). Effects of β-alanine supplementation on human skeletal muscle contractile properties and voluntary muscle performance. STAR conference, Nottingham Trent University.

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Abbreviations

n_g^2	Generalised eta squared
n_p^2	Partial eta squared
^{1}H-MRS	Proton magnetic resonance spectroscopy
1RM	One-repetition maximum
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
Ca^{2+}	Calcium
CCT110%	Cycling capacity test at 110% Powermax
CNDP1	Serum carnosinase
CNDP2	Tissue carnosinase
CV	Coefficient of variation
DHPR	Dihydropyridine receptor
DM	Dry muscle
EGTA	Ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetra acetic acid
EMD	Electromechanical delay
EMG	Surface electromyography
ES	Effect size
h	Hour
H^+	Hydrogen cations
HRT	Half-relaxation time
ICC	Interclass correlation
IKET	Isometric knee extensor endurance task
M wave	Muscle action potential
mA	Milliamps
Min	Minutes
M_{max}	Maximum muscle action potential
MVIC	Maximal voluntary isometric contraction
MVIF	Maximal voluntary isometric force
NAI	Natural Alternatives International
PBS	Phosphate buffered saline
PEPT1	Proton-coupled oligopeptide transporter
Pi	Inorganic phosphate

PMCA	Plasma Membrane Ca^{2+}-ATPase
PWC_{FT}	Physical working capacity at neuromuscular fatigue threshold test
RFD	Rate of force development
RMS	Root mean square
RyR(s)	Ryanodine receptor(s)
S	Seconds
SD	One standard deviation
SDS-PAGE	Sodium dodecyl sulphate –polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SPCA	Secretory Pathway Ca^{2+}/Mn^{2+}-ATPases
SR	Sarcoplasmic reticulum
Tau-T	Taurine transporter
TPT	Time-to-peak tension
TTBS	Tween 20 in 1 X PBS
TTE	Time-to-exhaustion
TTF	Time to task failure
V	Voltage
Y	Years

Chapter 1: General introduction

1.1 Introduction

Carnosine, is a histidine containing dipeptide, found in high-concentrations in the skeletal muscle of vertebrates and non-vertebrates. Following ingestion from the diet, carnosine is broken down into its two constituent amino acids, β-alanine and L-histidine (Sadikali *et al.,* 1975). Upon entering the skeletal muscle, carnosine is formed by the bonding of β-alanine and L-histidine, in a reaction catalysed by carnosine synthase (Harding & Margolis, 1976; Horinishi, Grillo & Margolis, 1978). The availability of β-alanine is the rate limiting factor of skeletal muscle carnosine synthesis (for a brief review, see Harris *et al.,* 2012). Supplementation with β-alanine is regarded as the most beneficial means of increasing muscle carnosine content (Asatoor *et al.,* 1970). In young adults, daily doses of β-alanine have been shown to raise skeletal muscle carnosine content in a dose-dependent manner, with 40 to 80% increases evident over a 4 to 10-week duration, almost without exception, on an individual-by-individual basis (Harris *et al.,* 2006; Hill *et al.,* 2007; Baguet *et al.,* 2010; Sale *et al.,* 2013). Increasing skeletal muscle carnosine content, with β-alanine supplementation, has been shown to be ergogenically beneficial to high-intensity exercise performance, as highlighted by several reviews (see Artioli *et al.,* 2010; Sale *et al.,* 2010; 2013). Two meta-analyses have supported the positive effect of β-alanine supplementation on high-intensity exercise performance (Hobson *et al.,* 2012; Saunders *et al.,* 2017). Tasks lasting between 60 and 240 s have gained the largest beneficial effect, specifically when the tasks include open ended exercise bouts, such as time to exhaustion tests (TTE) (Hobson *et al.,* 2012; Saunders *et al.,* 2017). Supplementation with daily doses of β-alanine in older adults (60-80 y) have also shown to significantly increase skeletal muscle carnosine content, alongside improvements in physical exercise capacity (Stout *et al.,* 2008; del Favero *et al.,* 2012; McCormack *et al.,* 2013; Glenn *et al.,* 2016). That said, β-alanine supplementation has been shown to not significantly benefit measures of muscle function or quality of life (del Favero *et al.,* 2012; Glenn *et al.,* 2016). Due to the small number of investigations examining the impact of β-alanine supplementation in this population, the impact of increased skeletal muscle carnosine content on skeletal muscle function remains undetermined.

Carnosine was first described as an intracellular pH buffer (Bate-Smith, 1938; Deutsch & Eggleton, 1938), due to the imidazole ring of carnosine having a pKa of 6.83, making carnosine a suitable intracellular buffer over the whole exercise induced intramuscular pH transit-range (Bate-Smith *et al.,* 1938; Harris *et al.,* 2006). The role of carnosine as a pH buffer, explains some of the ergogenic effects of β-alanine supplementation on exercise performance and capacity (Hobson *et al.,* 2012; Saunders *et al.,* 2017). This does not, however, exclude the possibility that carnosine may have other physiological roles. One such role, is the potential influence of carnosine on regulating Ca^{2+} kinetics within the skeletal muscle (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.,* 2011; Everaert *et al.,* 2013). *In-vitro* research has suggested that

increased skeletal muscle carnosine content may impact the amount of Ca^{2+} released during skeletal muscle contraction and/or the sensitivity of contractile properties to Ca^{2+} (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.*, 2011; Everaert *et al.*, 2013). Carnosine may interact with the Ca^{2+}-channel itself (Batrukova & Rubstov, 1997), through the existence of saturable binding site(s) on the Ca^{2+}-channel. *In-vitro* research indicated that increasing skeletal muscle carnosine content, via β-alanine supplementation, could alter the Ca^{2+} kinetics properties of the muscle. It remains unclear, however, if this proposed *in-vitro* mechanism is apparent during *in-vivo* skeletal muscle contractions. Any improvements in skeletal muscle function following increased skeletal muscle carnosine content, via β-alanine supplementation, would possibly benefit overall skeletal muscle performance.

The overall aim of this programme of work was to investigate the effect of β-alanine supplementation on voluntary and electrically evoked contractile properties of *in-vivo* human skeletal muscle. The impact of increased skeletal muscle carnosine content, via 28-days of β-alanine supplementation, on *in-vivo* neuromuscular performance, in young and older healthy adults was examined. *In-vitro* research was undertaken to examine the potential effect of increased carnosine concentrations on SERCA activity, a mechanism possibly associated with the improvements in skeletal muscle relaxation reported *in-vivo.*

1.2 Overview

Brief outlines of the experimental chapters reported within this thesis are as follows; the research reported within study 1 (Chapter 4) examined the effect of 28-days β-alanine supplementation on the intrinsic contractile properties of human skeletal muscle *in-vivo*, as well as on voluntary muscle function in young (18 - 30 y) healthy males. Neuromuscular performance was examined using measures such as the force-frequency relationship, pre- and post- β-alanine supplementation, providing an *in-vivo* analogue of the force-Ca^{2+} relationship (Balnave & Allen, 1996).

Study 2 (Chapter 5) examined the effect of β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and muscle contractility in both fresh (rested conditions) and fatigued human skeletal muscle. These data in young adults provide support to the previous research (Study 1; Chapter 4), whilst also examining the influence of increased skeletal muscle carnosine content on neuromuscular function following the completion of an isometric knee extensor endurance task (IKET).

Following studies 1 and 2 (Chapter 4 and 5) conducted in young adults, study 3 (Chapter 6) investigated the effect of β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and muscle contractility in both fresh and fatigued human skeletal muscle in older (60 - 80 y) healthy adults. These data extend the currently limited β-alanine supplementation research in this population, investigating the potential impact of increased carnosine content on skeletal muscle function in older adults.

The *in-vivo* data within the thesis explored the influence of 28-days β-alanine supplementation on neuromuscular function in young and older adults, under fresh and fatigued skeletal muscle conditions. Study 4 (Chapter 7) isolated the microsomal fraction of rat skeletal muscle tissue and examined the impact of increased carnosine concentrations on ATPase activity, a factor related to skeletal muscle relaxation. These data also included analysis of the distribution of the carnosine molecule, and its constituents, β-alanine and L-histidine within the nucleic, mitochondrial, cytosolic and microsomal fractions of the skeletal muscle cell. The *in-vitro* findings reported within study 4 (Chapter 7) are supported by the development of several methodological processes, as reported in appendix 1.

Chapter 2: Review of literature

2.1 Carnosine

Carnosine, a dipeptide consisting of β-alanine and L-histidine (Krimberg, 1906; 1908) characterised by three ionisable groups: the carboxyl group (pKa 2.76), the amino group of the β-alanine residue (pKa 9.32), and the nitrogen of the imidazole ring (pKa 6.83; Vistoli *et al.*, 2012). Carnosine was first isolated by Gulewitsch and Amiradzhibi (1900) in the early 1900s from Liebigs meat extract, and is now known to be one of the most abundant small-molecular compounds in human skeletal muscle (Fig. 2.1). In addition to its high content within in human skeletal muscle, over the last 100 years' carnosine has been identified in numerous other animal species (Boldyrev, 2013), most prominently in those involved in athletic performance, including horses, greyhounds, and camels (Harris *et al.*, 1990; Dunnett & Harris, 1997). It has been suggested that animal species where a periodical demand from anaerobic exercise, or tolerance to imposed hypoxic condition elevates muscle carnosine content (Abe, 2000; Harris *et al.*, 1990). Cetaceans' mammals have some of the largest reported skeletal muscle carnosine contents (Suyama *et al.*, 1977), theoretically due to experiencing prolonged periods of hypoxia when diving. The concentration of skeletal muscle carnosine of vertebrates varies according to the species from 0.6 mM in mice, up to 10 and 30 mM in humans and horses (Crush, 1970).

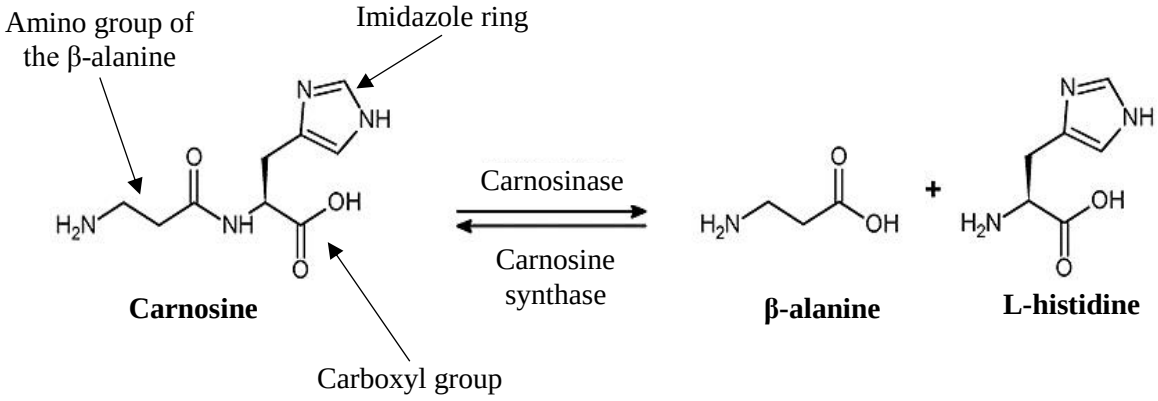

Fig. 2.1: Molecular structure of carnosine, β-alanine and L-histidine

2.1.1 Carnosine transport into human skeletal muscle

In humans, carnosine enters the small intestine following dietary intake, where it is transported across the cellular membrane by a peptide transporter (PEPT1; Fig. 2.2). PEPT1 is characterised as a high-capacity, low-affinity transporter and plays a major nutritive role in the intestinal absorption of peptides. The presence of carnosinase, an enzyme that hydrolyses carnosine, in the enterocytes of humans (Sadikali *et al.*, 1975) suggests that carnosine is broken down into its constituent amino acids (β-alanine and L-histidine) via tissue/jejunal carnosinase enzyme (CNDP2) prior to entering the blood stream. Nonetheless, due to the low activity of CNDP2 in the jejunum

(Sadikali *et al.*, 1975), parts of the ingested carnosine reach the blood stream via peptide transporters (Fig. 2.2). Upon entering the blood stream, the remaining carnosine is rapidly hydrolysed due to the presence of the highly active serum carnosinase enzyme (CNDP1) (Asatoor *et al.*, 1970; Perry *et al.*, 1967). Small amounts (<14% of ingested carnosine) of intact carnosine have, however, been shown in urine in the 5 h following carnosine ingestion (Block *et al.*, 1965; Perry *et al.*, 1967; Gardner *et al.*, 1991). β-alanine and L-histidine enter the blood stream from the enterocytes, via amino acid transporters, ready for transportation into the skeletal muscle by a Taurine transporter (Tau-t; Fig. 2.2). Upon entering the skeletal muscle, β-alanine and L-histidine are re-synthesised into carnosine by the enzyme carnosine synthase (Harding & Margolis, 1976; Horinishi, Grillo & Margolis, 1978; Fig. 2.1). Carnosine synthetase is a member of the adenosine triphosphate (ATP)-grasp family of ATPases, which catalyses ATP, β-alanine and L-histidine into carnosine, phosphate and adenosine diphosphate (ADP). The process of skeletal muscle carnosine synthesis is limited by the availability of β-alanine from the diet (for a brief review see Harris *et al.*, 2012).

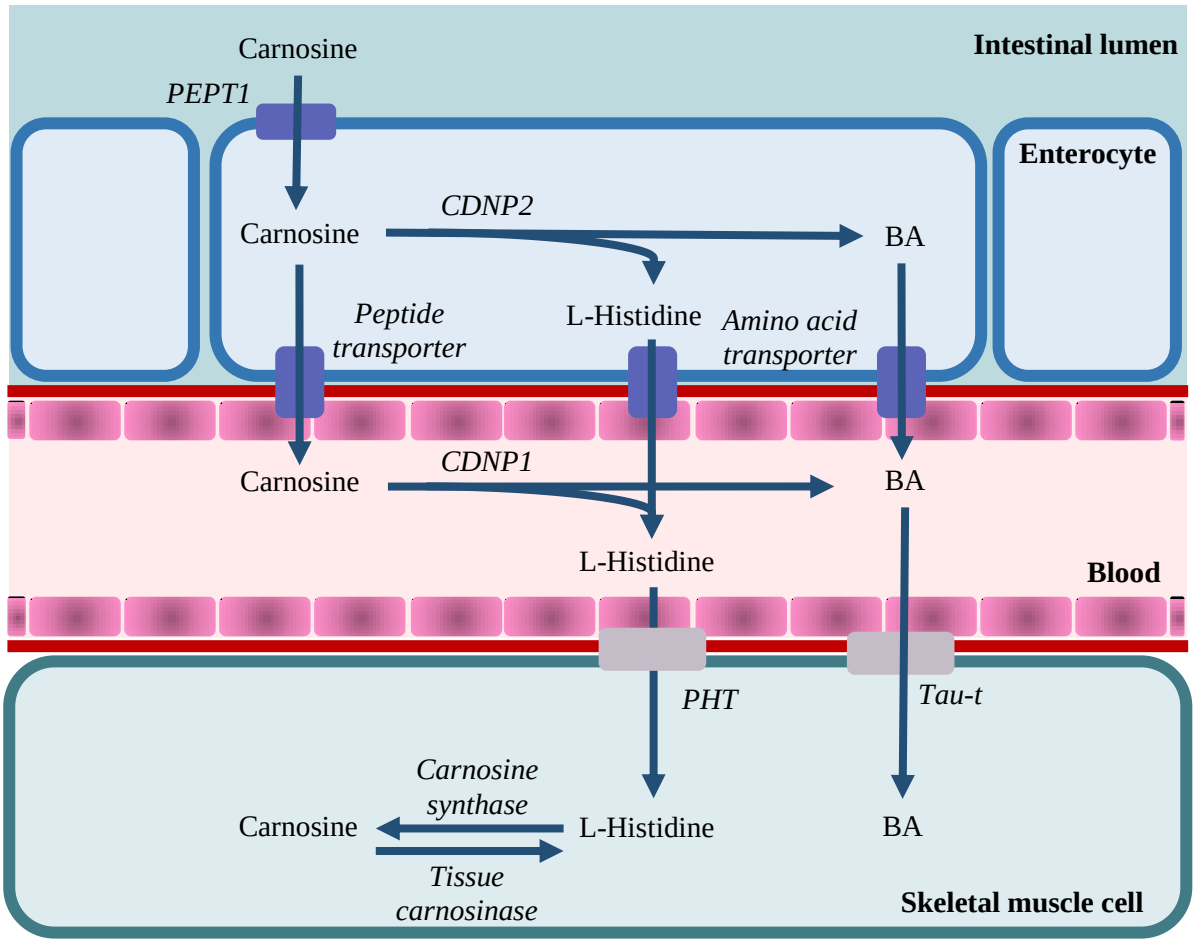

Fig. 2.2: A schematic illustration of the pathways of intestinal absorption of carnosine, β-alanine (BA) and L-histidine in humans. Highlighting potential roles of carnosine in skeletal muscles cells. CNDP2: Jejunal carnosinase enzyme; CNDP1: Serum carnosinase enzyme. PEPT1: Peptide transporter 1. PHT: Peptide histidine transporter. Tau-t: Taurine transporter (Adapted from Boldyrev, 2013).

2.1.2 Determinants of skeletal muscle carnosine content

2.1.2.1 Diet

In humans, carnosine can be ingested directly through several dietary sources, including red and white meats (*e.g.*, beef and poultry) and fish (*e.g.*, salmon) (Abe, 2000). Neither dairy nor vegetable food products contain L-histidine containing dipeptides or β-alanine, except for the very small presence of β-alanine in vegetable oils, at ~100 to 1,000 times lower than those in meat (Sanchez-Hernandez, Marina & Crego, 2011). In an omnivorous Western diet, due to the ingestion of animal products, the average daily intake of β-alanine has been calculated at ~330 $mg \cdot day^{-1}$ (Everaert *et al.*, 2010). This estimate is likely to be highly variable given that the cooking method is a critical factor in maintaining meat carnosine content, with some cooking procedures resulting in a complete carnosine loss (Harris *et al.*, 2012). Individuals who do not consume animal products (vegans or vegetarians) rely solely on endogenous sources of β-alanine, with ~20% lower muscle carnosine content compared to omnivores counterparts (Everaert *et al.*, 2010). Long term vegetarianism (>8 y) has been associated with declined carnosine content in the *m. soleus* (-17%; P = 0.05), *m. gastrocnemius* (-26%; P = 0.01) and *m. tibialis anterior* (20%; P = 0.07), when compared to omnivorous. Short term dietary restriction of β-alanine (5 weeks) through transiently switching omnivores onto vegetarian diets, reported a 9% reduction in *m. soleus* carnosine concentrations. Highlighting the highly sensitive and dependant nature of skeletal muscle carnosine concentrations upon the availability of dietary β-alanine in humans.

2.1.2.2 Muscle fibre type

Carnosine is evident in both slow (Type I) and fast (Type II) skeletal muscle fibres, with greater content in Type II fibres compared to Type I (Dutka *et al.*, 2012; Stellingwerff *et al.*, 2011; Harris *et al.*, 2012). Within the same human skeletal muscle (*m. vastus lateralis*) carnosine content can vary widely, with Type I muscle fibres around half the carnosine content of those in Type II muscle fibres (Type I: 10.5 $mmol \cdot kg^{-1}$ dry muscle (DM); Type II: 23.2 $mmol \cdot kg^{-1}$ DM; Harris *et al.*, 1998). A similar distribution of muscle carnosine concentration between fibre type in the human *m. vastus lateralis* was replicated by Hill *et al.*, (2007), with Type II fibres displaying 1.7 times greater than those reported in Type I fibres (Type I; 17.8 $mmol \cdot kg^{-1}$ DM, Type II; 29.6 $mmol \cdot kg^{-1}$ DM). Larger carnosine content in Type II muscle fibres are also been shown in numerous other species, with content reaching 4 – 5 times those in Type I fibres (Dunnett & Harris, 1995; Dunnett *et al.*, 1997). β-alanine supplementation over a 10-week period does, however, increase muscle fibre carnosine content in both Type I and II muscle fibres to a similar extent (16.5 $mmol \cdot kg^{-1}$, and 17.0 $mmol \cdot kg^{-1}$), thereby maintaining the muscle carnosine content ratio between muscle fibre types.

2.1.2.3 Sex

In humans, males have a ~21% greater skeletal muscle carnosine content (21.3 ± 4.2 $mmol \cdot kg^{-1}$) than those reported in females (17.5 ± 4.8 $mmol \cdot kg^{-1}$; Mannion *et al.,* 1992; Everaert *et al.,* 2011). These sex-related differences are dependent on muscle fibre type, with males displaying a 30% increase in carnosine content in skeletal muscles that are predominantly Type I fibres (*m. soleus* and *m. gastrocnemius*; Everaert *et al.,* 2011). The magnitude is increased to 82% in skeletal muscles predominantly Type II fibres, such as the *m. tibialis anterior* (Everaert *et al.,* 2011). The differences between sexes may also be associated with a significantly higher plasma carnosinase activity in females ($P < 0.001$). Nonetheless, during the period of skeletal muscle carnosine loading, sex and body mass variances have only a minimal effect on the absolute increases in skeletal muscle carnosine content (Stegen *et al.,* 2014).

2.1.2.4 Training

Compared to untrained individuals, skeletal muscle carnosine content are significantly higher in elite athletes (Parkhouse *et al.,* 1985; Tallon *et al.,* 2005), specifically in competitive bodybuilders where carnosine contents were twice that of those in their age-matched untrained counterparts (Tallon *et al.,* 2005). These contents are some of the largest human skeletal muscle carnosine content reported (Tallon *et al.,* 2005), possibly due to these individuals experiencing prolonged repetitive exposure to low muscle pH, alterations to their diet, ingestion of dietary supplements, and/or the use of anabolic steroids (Tallon *et al.,* 2005). Exercise training may also be responsible for these increased skeletal muscle carnosine concentration (Suzuki *et al.,* 2004), yet there are limited exercise intervention studies to support this theory (Kendrick *et al.,* 2008; Baguet *et al.,* 2011). Research combining 10 weeks of β-alanine supplementation (6.4 $g \cdot day^{-1}$) and resistance training reported increased skeletal muscle carnosine content only following β-alanine supplementation (+12.81 ± 7.97 $mmol \cdot kg^{-1}$ DM; Kendrick *et al.,* 2008). Furthermore, no change in skeletal muscle carnosine content was evident within the placebo group, even after the completion of the resistance training programme (Kendrick *et al.,* 2008). Nonetheless, resistance trained individuals are likely to partake in extended periods of training, over several months and years, rather than participating in short training programmes. It could be speculated that the current resistance training protocols were not of sufficient duration to illicit increased muscle carnosine contents. It could also be hypothesised that elite athletes have higher skeletal muscle carnosine contents compared to untrained individuals' due to increased muscle mass, specifically more Type II muscle fibres where larger carnosine content are reported (see section 2.1.2.2; page 8).

2.1.2.5 Ageing

The research examining the impact of ageing on skeletal muscle carnosine content is limited, with no longitudinal research to support the negative correlation evident in cross-sectional investigations (Peñafiel *et al.,* 2004; Tallon *et al.,* 2007; Everaert *et al.,* 2011; Fig. 2.3). Current research reports large reductions in skeletal muscle carnosine content (~53%) in Type II muscle fibres of older participants, compared to younger adult counterparts (Tallon *et al.,* 2007). Cross-sectional data from the m. soleus of individuals aged 19 - 47 y demonstrated a negative correlation between age and skeletal muscle carnosine content, with carnosine content declining at a rate of 1.2% per year (Everaert *et al.,* 2011; Fig. 2.3). These data, however, are largely focused on individuals under the age of 30 y, with no data in adults over the age of 60 y, limiting the interpretation of these analyses. The negative correlation between carnosine concentrations and ageing, reported previously (Everaert *et al.,* 2011), is weak (r= -0.26). As such, to fully understand this relationship additional confirmation data is required, specifically in older adults. Furthermore, the current data is undertaken in Type I muscle fibres (Everaert *et al.,* 2013), Type I muscle fibres are known to contain around half the carnosine content of those in Type II muscle fibres (Harris *et al.,* 1998; Section 2.1.2.2), to support the reported decline in carnosine concentrations with ageing, and understand this relationship, analysis of both Type I and Type II muscle fibres would be essential The exact reasons for the decline in skeletal muscle carnosine content remains unclear (Peñafiel *et al.,* 2004; Tallon *et al.,* 2007; Everaert *et al.,* 2011). Although numerous potential mechanisms have been proposed, including a decline in physical activity, the amount of meat consumed by older individuals (Harris *et al.,* 2006), an overall reduction in muscle mass, and/or lower levels of circulating free testosterone (Peñafiel *et al.,* 2004).

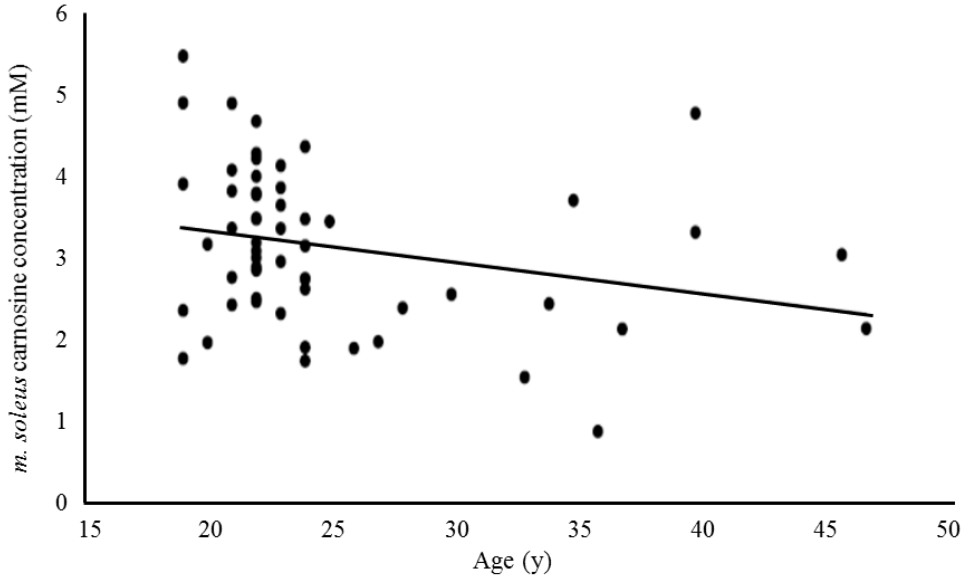

Fig. 2.3: Skeletal muscle carnosine content of the *m. soleus* in male and female adult human omnivores ($P < 0.05$, $r = -0.26$, $n = 58$). Redrawn from Everaert *et al.,* (2011).

2.1.3 Increasing muscle carnosine content

Increasing the availability of free β-alanine, either via dietary or supplemental means, results in significantly increased in skeletal muscle carnosine content (Harris *et al.,* 2006; Hill *et al.,* 2007; Derave *et al.,* 2007; Baguet *et al.,* 2009; Baguet *et al.,* 2010). In humans, carnosine does not remain under tight homeostatic control, unlike other intracellular buffers (Gollnick *et al.,* 1991), therefore, the ability to significantly alter carnosine content making it highly valuable. Supplementation with β-alanine is regarded as the most beneficial means of increasing muscle carnosine content, above that of supplementation with carnosine (Asatoor *et al.,* 1970), potentially due to the mechanism by which carnosine is transported and formed within the skeletal muscle (Perry *et al.,* 1967; Asatoor *et al.,* 1970). The relationship between β-alanine supplementation and skeletal muscle carnosine content occurs in a dose dependent manner, with increases in carnosine content of between 40 and 80%, dependent upon β-alanine supplemental dose (between 3.2 and 6.4 $g \cdot d^{-1}$) and duration (between 4 - 10 weeks; Harris *et al.,* 2006; Derave *et al.,* 2007; Hill *et al.,* 2007; Baguet *et al.,* 2010; Sale *et al.,* 2013; Saunders *et al.,* 2017; Fig. 2.4). Harris and colleagues (2006) were the first to report this relationship, with human skeletal muscle carnosine content increasing by ~65% following 28-days of β-alanine supplementation (6.4 $g \cdot d^{-1}$). Parallel increases in the *m. vastus lateralis* carnosine content of 60% are reported by Hill *et al.,* (2007) following a similar 28-day β-alanine supplementation protocol (6.4 $g \cdot d^{-1}$). The supplementation period was extended for an additional six weeks, where skeletal muscle carnosine content continued to increase (~20%),

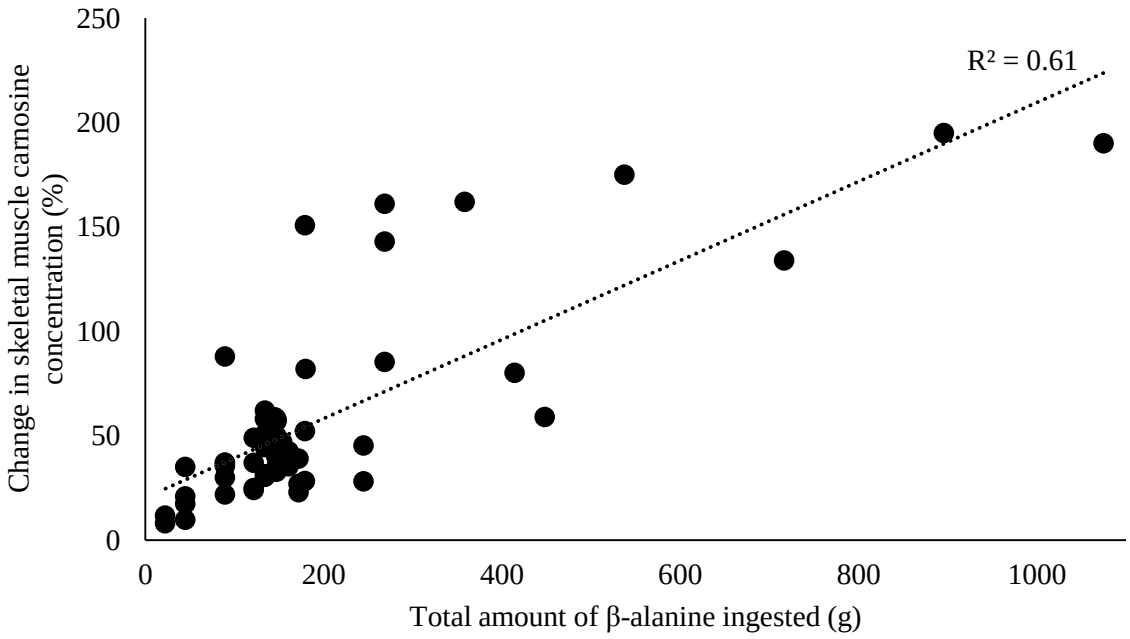

Fig. 2.4: Percentage increase in skeletal muscle carnosine concentration following β-alanine supplementation, data from muscle biopsy or proton magnetic resonance spectroscopy (^{1}H-MRS) analysis. Data from Harris *et al.,* (2006); Derave *et al.,* (2007); Hill *et al.,* (2007); Kendrick *et al.,* (2008; 2009); Baguet *et al.,* (2010); Stellingwerff *et al.,* (2011); del Favero *et al.,* (2012); Bex *et al.,* (2013); Sale *et al.,* (2013); Chung *et al.,* (2014); Gross *et al.,* (2014); Stegen *et al.,* (2014); Danaher *et al.,* (2014); Saunders *et al.,* (2016).

increasing baseline skeletal muscle carnosine content by ~80% (Hill *et al.*, 2007). These data have been extended by numerous investigations, consistently reporting increased skeletal muscle carnosine contents following β-alanine ingestion (Derave *et al.*, 2007; Kendrick *et al.*, 2008; Baguet *et al.*, 2010; Stellingwerff *et al.*, 2011; del Favero *et al.*, 2012; Bex *et al.*, 2014; Chung *et al.*, 2014; Gross *et al.*, 2014; Stegen *et al.*, 2014; Danaher *et al.*, 2014; Saunders *et al.*, 2017; Fig. 2.4). Increased skeletal muscle carnosine content, following β-alanine supplementation, was examined using either muscle biopsies, or proton magnetic resonance spectroscopy (^{1}H-MRS) (Harris *et al.*, 2006; Hill *et al.*, 2007; Derave *et al.*, 2007; Kendrick *et al.*, 2008; Baguet *et al.*, 2010; Stellingwerff *et al.*, 2011; del Favero *et al.*, 2012; Bex *et al.*, 2014; Chung *et al.*, 2014; Gross *et al.*, 2014; Stegen *et al.*, 2014; Danaher *et al.*, 2014). To date, only one participant has displayed no increase in carnosine content following β-alanine ingestion (Harris *et al.*, 2006). The longest duration of β-alanine supplementation to assess skeletal muscle carnosine concentration was undertaken by Saunders *et al.*, (2017), with individuals completing a total of 24-weeks of β-alanine supplementation (5.2 g·d^{-1}). Skeletal muscle carnosine content significantly increased from baseline at all assessed time points during the 24-week β-alanine supplementation protocol (all $P < 0.001$; Week 4: +11 ± 7 mmol·kg^{-1}DM, Week 8: +14 ± 8 mmol·kg^{-1}DM, Week 12: +17 ± 9 mmol·kg^{-1}DM, Week 16: +18 ± 8 mmol·kg^{-1}DM, Week 20: +21 ± 8 mmol·kg^{-1}DM, Week 24: +20 ± 8 mmol·kg^{-1}DM). The current body of β-alanine supplementation research has largely been conducted in younger adult males (18 – 30 y; see reviews Sale *et al.*, 2010; 2013; Hobson *et al.*, 2012; Saunders *et al.*, 2017), and although these data suggest a dose-dependent relationship there remains limited understanding of the impact of β-alanine supplementation on skeletal muscle carnosine contents in older adults.

The impact of β-alanine supplementation on skeletal muscle carnosine content in older individuals (60 – 80 y) has recently been investigated (del Favero *et al.*, 2012). Skeletal muscle carnosine content of the *m. gastrocnemius*, assessed via ^{1}H-MRS measurements in older adults significantly increased (β-alanine: + 85%, PLA: +7%; $P = 0.004$) following 12-weeks of β-alanine supplementation (3.2 g·d^{-1}; total dose; ~269 g). The ingestion of β-alanine at 3.2 g·d^{-1} is currently the largest daily dose reported in older adults (del Favero *et al.*, 2012; McCormack *et al.*, 2013; Glenn *et al.*, 2016), half that currently implemented in similar investigations in younger adults (~6.4 g·d^{-1}; see review Sale *et al.*, 2010; 2013). Ingestion of β-alanine at the lower daily dose of 3.2 g·d^{-1} is sufficient in significantly raising skeletal muscle carnosine content, with an 85% increase evident following 12-weeks of supplementation (total dose: 269 g; del Favero *et al.*, 2012). The smaller daily dosing strategies are largely undertaken due to the associated declines in muscle mass experienced by older individuals, and the potential risk of experiencing the negative side effect of β-alanine supplementation, paraesthesia. Currently there has only been one documented case of older individuals experiencing feelings of paraesthesia with β-alanine supplementation at 3.2 g·d^{-1}

(Glenn *et al.*, 2016). There was, however, no change between research findings with and without this individual's data (Glenn *et al.*, 2016). Based on the present literature, supplementation with β-alanine at a daily dose of 3.2 $g \cdot d^{-1}$ is suitable for adults aged 60 to 80 y, without the risk of paraesthesia, maintaining the double-blind status of research investigations. Currently, it remains unknown if older adults can tolerate daily doses of β-alanine above 3.2 $g \cdot d^{-1}$, without experiencing the negative side effect of paraesthesia.

Short term (4-weeks) β-alanine supplementation has been widely undertaken to raise skeletal muscle carnosine content (Derave *et al.*, 2007; Kendrick *et al.*, 2008; Baguet *et al.*, 2010; Stellingwerff *et al.*, 2011; del Favero *et al.*, 2012; Bex *et al.*, 2014; Chung *et al.*, 2014; Gross *et al.*, 2014; Stegen *et al.*, 2014; Danaher *et al.*, 2014). Recently long-term (24 weeks) β-alanine supplementation protocols have been undertaken showing that skeletal muscle carnosine content may not increase continuously until maximal in all individuals, with skeletal muscle carnosine content declining at certain times points, despite maintaining ingestion (Saunders *et al.*, 2017). Interestingly, a fifth of the participants (5 out of 25) reported their highest carnosine content at the 24-week time-point, suggesting that for these individuals further increases in skeletal muscle carnosine content would have been evident if β-alanine supplementation was maintained.

Based on the current short term (~ 28-days) β-alanine supplementation data, maintenance of increased muscle carnosine concentration is achievable with a much smaller concentration of β-

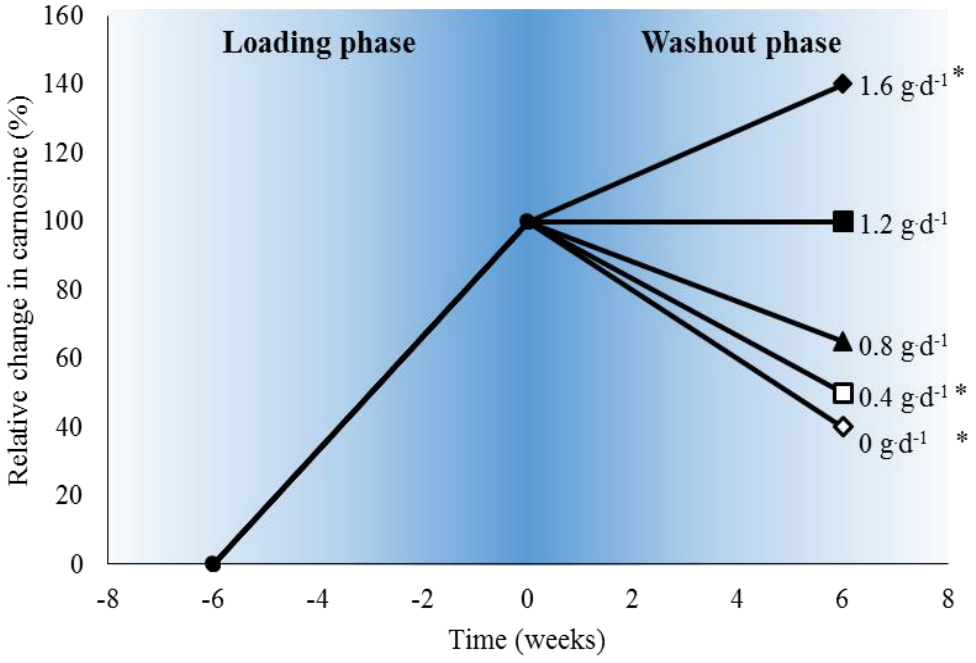

Fig. 2.5: The relative change in muscle carnosine concentrations after six weeks of loading (3.2 $g \cdot d^{-1}$), proceeded by five different maintenance phases. *P < 0.05 from post-loading levels. Redrawn using data from Stegan *et al.*, (2014), Stellingwerff *et al.*, (2012) and Baguet *et al.*, (2009).

alanine (1.2 $g \cdot d^{-1}$; Stegen *et al.,* 2014; Fig. 2.5). Upon discontinuation of β-alanine supplementation, carnosine synthesis returns to normal, resulting in a 2 to 4% decline in skeletal muscle carnosine content per week (Baguet *et al.,* 2009); a process referred to as carnosine washout (Baguet *et al.,* 2009; Stellingwerff *et al.,* 2012; Stegen *et al.,* 2014; Fig. 2.5). Based on current literature, an individual with increased muscle carnosine content of greater than 55% following β-alanine supplementation would require a washout period of up to 15 weeks. To ensure that participants are all starting investigations at a normal skeletal muscle carnosine content, researchers must confirm that all individuals refrain from β-alanine supplementation for a minimum of almost four months before participating. This significant period required for skeletal muscle carnosine washout demonstrates the impracticality of implementing cross-over research designs when implementing β-alanine supplementation protocols.

2.1.4 Side effects of β-alanine supplementation

Ingestion of β-alanine in powder form at a single dose of > 10 $mg \cdot kg^{-1}$ BM rapidly increases blood plasma content, associated with symptoms of paraesthesia, which can continue for > 60 min (Harris *et al.,* 2006; Fig. 2.6). Paraesthesia is a neuropathic pain, resulting in the experience of a prickly sensation affecting areas of the face, neck, shoulders, chest and buttocks (Harris *et al.,* 2012; Decombaz *et al.,* 2012). To reduce the incidence of paraesthesia, a sustained release formulation was developed by Natural Alternatives International (NAI; Carnosyn, San Marcos, California, USA). The sustained release formulation imposes a physical restriction on the

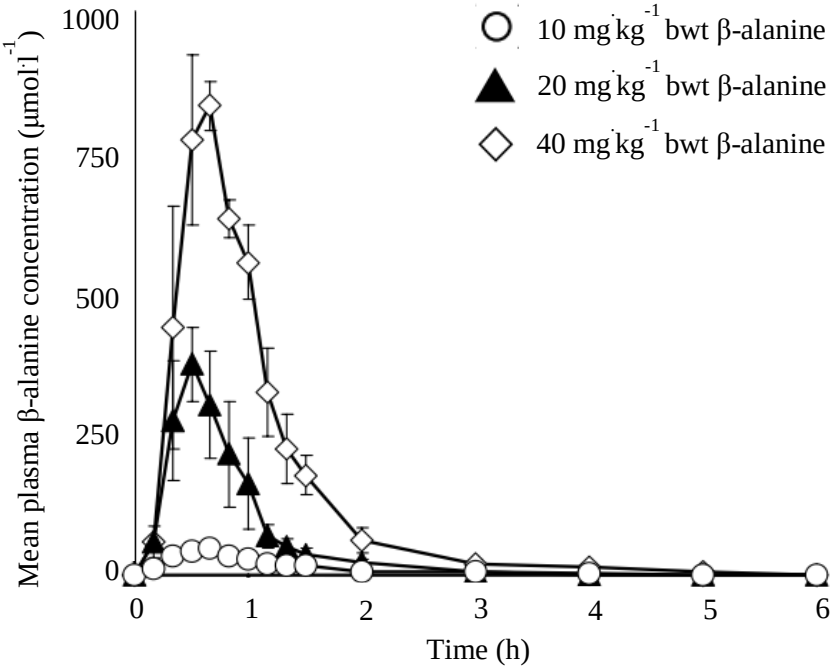

Fig. 2.6: Mean plasma β-alanine concentration following ingestion of 10, 20 and 40 $mg \cdot kg^{-1}$ bwt β-alanine. For clarity SE of the means is shown only for measurements following 20 and 40 $mg \cdot kg^{-1}$ bwt β-alanine. Redrawn from Harris *et al.,* (2006).

amount of β-alanine released into the gut, slowing the release of β-alanine into the blood stream (~6 h), and reducing the peak plasma concentration from a single dose (Decombaz *et al.*, 2012). Due to the reduced symptoms of paraesthesia, even at larger single doses of β-alanine, ingestion of sustained release tablets is now more commonplace (Sale *et al.*, 2011; Saunders *et al.*, 2012). Participants appear to be unable to differentiate between the β-alanine and matched placebo tablets in this sustained release form (Decombaz *et al.*, 2012). Apart from paraesthesia, no other side effects of β-alanine have been reported (Harris *et al.*, 2006).

2.2 β-alanine supplementation and exercise performance

In the last 10 years, there has been significant research examining the influence of increased skeletal muscle carnosine content on exercise performance (see meta-analyses Hobson *et al.*, 2012; Saunders *et al.*, 2017). Due to the undeniable role of carnosine as an intracellular pH buffer (see section 2.3; see reviews Sale *et al.*, 2010; 2013), exercise performances that are likely to experience the generation of large quantities of H^+, and thus disruption to acid-base balance, have been significantly improved following β-alanine supplementation (Hill *et al.*, 2007; Sale *et al.*, 2011). The current review will specifically focus on the literature surrounding β-alanine supplementation to increase skeletal carnosine concentrations and neuromuscular performance, in line with the aim of the current body of work.

2.2.1 Younger individuals

Stout *et al.*, (2006) investigated the effect of 28-days of β-alanine and creatine monohydrate supplementation on the onset of neuromuscular fatigue as assessed by the physical working capacity at neuromuscular fatigue threshold test (PWC_{FT}). In untrained males, β-alanine supplementation at 1.6 $g \cdot d^{-1}$ for 28-days, delayed the onset of neuromuscular fatigue during the PWC_{FT}, with cycling performance times increasing by ~17%. A similar 14% increase in ventilatory threshold was shown in physically active women following 28-days of β-alanine supplementation; 3.2 $g \cdot d^{-1}$ in week one, increasing to 6.4 $g \cdot d^{-1}$ in weeks two to four. It is likely that these reported improvements in performance are associated with the increase in skeletal muscle carnosine content, thereby a greater capacity to buffer H^+ during high-intensity exercise. To explore this mechanism in an isolated condition, Ponte and colleagues (2006) examined the effect of β-alanine supplementation (6.4 $g \cdot d^{-1}$; total dose of 179 g) on an IKET at 45 – 50% maximal voluntary isometric force (MVIF). Twenty-eight days of β-alanine supplementation improved IKET performance by 10 - 15%, equating to around 8-s (Ponte *et al.*, 2006). Sale *et al.*, (2012) reported a similar increase in IKET hold times, around 10 s (13%), following 28-days of β-alanine supplementation at 6.4 $g \cdot d^{-1}$ (total dose: 179.2 g). The IKET hold time were within 3 - 4 s of the expected hold duration of 78 s (Ahlborg *et al.*, 1972), as predicted by the Rohmert equation (Rohmert, 1960). These hold times indicated that participants completed the IKET at the hold

target of 45% MVIF. Research examining the influence of β-alanine supplementation on IKET hold time in trained sprinters, however, showed no significant improvement following 28-days of supplementation (4.8 $g \cdot d^{-1}$; total dose: 134.4 g; Derave *et al.,* 2007). The duration of the IKET hold times were markedly above those expected at a hold intensity of 45% MVIF (~78 s; Ahlborg *et al.,* 1972). Furthermore, based on the hold times reported, it has been speculated that the trained sprinters were completing the IKET at ~25% MVIF (Sale *et al.,* 2012); much lower than instructed (Derave *et al.,* 2007). Completion of the IKET at this intensity may have restricted skeletal muscle blood flow, yet complete occlusion of the skeletal muscle would have been unlikely (Sale *et al.,* 2012). As such, this hold intensity would have allowed maintenance of H^+ transport from the skeletal muscle, prolonging the duration of the task.

A recent meta-analysis by Hobson *et al.,* (2012) demonstrated that supplementation with β-alanine for 28-days, at 6.4 $g \cdot d^{-1}$, significantly improved exercise measures by ~3% when compared to the placebo condition. A significant overall ES of 0.18 (95% CI 0.08 to 0.28) following β-alanine supplementation was reported, based on 40 individual studies, employing 65 different exercise protocols, totalling 70 exercise measures, in 1461 participants. The majority of these data however, were undertaken in recreationally active individuals, rather than elite athletes, who may not experience the same levels of ergogenic effect (Hobson *et al.,* 2012). Disparity between investigations may be associated with variations in methodological design, such as, exercise intensity and duration, supplementation dosage, publication bias, training status, and lack of familiarisation (Hobson *et al.,* 2012).

There is currently a large body of evidence to support the ergogenic effect of increased skeletal muscle carnosine content, via β-alanine supplementation, on high-intensity exercise performance. Nonetheless, there remains limited support for the beneficial effect of β-alanine supplementation on maximal strength performance measures (Hoffman *et al.,* 2006; Kendrick *et al.,* 2008). One of the few studies to explore this was undertaken in physically active males, supplemented with either placebo, creatine, or creatine and β-alanine in combination with a resistance training programme, over a 10-week period (3.2 $g \cdot d^{-1}$; total dose: 224 g; Hoffman *et al.,* 2006). Resistance training significantly improved free weight squat one-repetition maximum (1RM), and individuals supplemented with either creatine or creatine plus β-alanine improved to a greater extent, that those in the placebo group ($P < 0.05$; $ES > 0.89$). Bench press performance increased two-fold greater in the creatine and β-alanine group, and two and a half-fold greater with creatine supplementation, compared to the placebo group. Creatine supplementation increased strength performance, although there was no additional benefit to co-ingestion of creatine and β-alanine supplementation. The effect of 10 weeks resistance training and β-alanine (6.4 $g \cdot day^{-1}$) demonstrated no beneficial effect on whole-body strength (box squat, bench press and dead lift)

and isokinetic dynamometer strength, even though skeletal muscle carnosine content increased by 13 ± 8 $mmol \cdot kg^{-1}$ DM (Kendrick *et al.,* 2008). These types of strength activities are not limited by the buffering capacity of the skeletal muscle; therefore, it seems expected that β-alanine supplementation would not result in improved muscular strength.

2.2.2 Older individuals

The current literature examining the impact of β-alanine supplementation, on high-intensity exercise performance and muscular strength have been predominately conducted in young healthy adults (see meta-analyses Hobson *et al.,* 2012; Saunders *et al.,* 2017). Emerging, although limited research has shown similar improvements in exercise performance measures following β-alanine supplementation in older adults (Stout *et al.,* 2008; del Favero *et al.,* 2012; McCormack *et al.,*2013; Glenn *et al.,* 2015; 2016; Table 2.1). Older individuals experience declined cross-sectional area of Type II muscle fibres (Verdijk *et al.,* 2010), loss of both Type I and II muscle fibres (Lexell, 1995; Doherty 2003; Tallon *et al.,* 2007), as well as reductions in skeletal muscle carnosine content (Stuerenburg & Kunse, 1999; Tallon *et al.,* 2007). Older adults, may therefore, experience declines in their ability to complete anaerobic activity, during which reduced intracellular pH limits performance (Stout *et al.,* 2008). The amalgamation of these factors increases the sense of frailty in older adults, associated with impairments in balance, gait speed, and an increased risk of falls (Madureira *et al.,* 2010). As such, it is imperative to find techniques to maintain and even improve physical function in older individuals. Supplementation with β-alanine over 12-weeks (3.2 $g \cdot d^{-1}$; total dose 269 g) in older adults, has been shown to significantly increase skeletal muscle carnosine content (85%; del Favero *et al.,* 2012). Furthermore, these increases in skeletal muscle carnosine content were positively correlated with improved TTE performance, in both the constant-load submaximal ($r = 0.62$; $P = 0.01$) and incremental tests ($r = 0.48$; $P = 0.02$; del Favero *et al.,* 2012). Supplementation with β-alanine in older adults has also been associated with delayed onset of neuromuscular fatigue (Stout *et al.,* 2008). Ninety days of β-alanine supplementation significantly improved PWC_{FT} (28.6%) in older adults, with no change to the placebo group performances. Interestingly, the improvements in exercise performance, were two-fold greater than those previously reported in young healthy adult males (younger; 12 – 15% vs. older; 29%; Stout *et al.,* 2006). There were however, clear differences in supplemental dosing strategies. Whilst the older participants ingested a lower daily dose of β-alanine, compared to the younger counterparts (older; 2.4 $g \cdot d^{-1}$; young; 6.4 $g \cdot d^{-1}$), supplementation was provided over an additional 62 days. Therefore, total dose ingested by the older adults was around 30 g more (younger; ~179 g, older; 216 g). Ingestion of shorter 28-day β-alanine supplementation periods at 3.2 $g \cdot d^{-1}$ have also been shown to improve older female physical function (Glenn *et al.,* 2015; 2016). Female masters athletes following 28-days of β-alanine supplementation, exhibited improved TTE (23%) performance and total work completed (21%) during a cycling performance at 120% aerobic capacity (Glenn *et al.,* 2015). These data are in-line with the improvements in exercise performance lasting between 60 and 240 s reported in young healthy adults (see meta-analysis, Hobson *et al.,* 2012).

Although several investigations have shown improved exercise performance following β-alanine supplementation (Stout *et al.,* 2008; del Favero *et al.,* 2012; Glenn *et al.,* 2015), measures

associated with skeletal muscle function (timed-stand and timed-up-and-go, hand-grip strength), and quality of life parameters (del Favero *et al.*, 2012; Glenn *et al.*, 2016) have remained unaffected. The lack of change in quality of life and skeletal muscle function variables, may be due to the healthy and physically active status of these older individuals. Alterations to functional and health related measures may be more apparent in older individuals whom have already experienced declines in physical function, activity and quality of life, such the frail elderly.

The current research highlights the efficiency of β-alanine supplementation in raising skeletal muscle carnosine content in older individuals, as well as the potential beneficial effect of supplementation on improving exercise performance. Furthermore, these data suggest that older individuals may experience a greater beneficial effect following increased skeletal muscle carnosine content in comparison to younger adult counterparts. It can also be proposed that the positive effects of β-alanine supplementation may be to an even greater degree in older adults whom already have declined muscle function.

Table 2.1: Overview of current research examining the effect of β-alanine supplementation on older adult's physical performance measures.

Participants				Supplementation				Methodology
Author (year)	**Supplementation groups (n)**	**Age (y)**	**Gender**	**Dose ($g.d^{-1}$)**	**Duration (days)**	**Total Intake (g)**	**Placebo**	**Performance Measure**
Glenn *et al.*, (2016)	β-alanine (11) Placebo (11)	54 ± 2 53 ± 1	F	3.2	28	89.6	Dextrose	Isokinetic flexor and extensor strength, isometric grip strength.
Glenn *et al.*, (2015)	β-alanine (11) Placebo (11)	54 ± 2 53 ± 1	F	3.2	28	89.6	Dextrose	Time to exhaustion, total work completed
McCormack *et al.*, (2013)	ONS (16) ONS+ β-alanine 800 (15) ONS+ β-alanine 1200 (13)	71 ± 7 71 ± 6 72 ± 6	5F/11M 10F/5M 7F/6M	1.6 or 3.2	84	134.4 or 268.8	N/A	$PWC_{FT,}$ hand dynamometer, 30 s sit to stand.
del Favero *et al.*, (2012)	β-alanine (12) Placebo (6)	65 ± 4 64 ± 7	Mixed (8F/10M)	3.2	84	268.8	Maltodextrin	PWC_{FT}, timed stands, timed up and go.
Stout *et al.*, (2008)	β-alanine (12) Placebo (14)	72 ± 10 73 ± 11	Mixed (9M/17F)	2.4	90	216	Cellulose	$PWC_{FT.}$

F: Females. M: Males. PWC_{FT}: Physical working capacity at neuromuscular fatigue threshold test. ONS: Oral nutritional supplement

2.3 Potential roles of carnosine in the skeletal muscle

Although carnosine is evident within excitable tissues, including the cardiac muscle and neuronal tissue, based on the objectives of the present thesis, this section will discuss the potential roles of carnosine within the skeletal muscle (Fig. 2.7). Furthermore, it has been suggested that carnosine may not display the same functions in different tissues, and may even fulfil several functions within one tissue (Boldyrev *et al.*, 2013).

2.3.1 Proton buffer

Carnosine was first described as an intracellular pH buffer in 1938 by two independent research groups (Bate-Smith, 1938; Deutsch & Eggleton, 1938), following the isolation of the molecule in the early 1900s (Gulewitsch & Amiradzhibi, 1900). Research by Severin *et al.*, (1953), showed that, in the presence of 10 mM carnosine, there was a greater accumulation of lactate within the isolated frog nerve and muscle without limiting muscle performance. In the absence of carnosine, lactate accumulation occurred, and resulted in rapid muscle fatigue and acidosis. Due to its molecular structure, the role of carnosine as an intracellular pH buffer is undisputable (Bate-Smith *et al.*, 1938; Sale *et al.*, 2010; 2013; Fig. 2.7), furthermore with the imidazole ring of carnosine having a pKa of 6.83, carnosine is a suitable intracellular buffer over the whole exercise induced intramuscular pH transit-range (Bate-Smith *et al.*, 1938; Harris *et al.*, 2006). In untrained human individuals, carnosine can neutralise between 2 and 10 mmol $H^{+}\cdot kg^{-1}$dm as intramuscular pH declines, as a minimum estimate this can add at least 7 to 10% to total intramuscular buffering capacity (Bate-Smith, 1938; Mannion *et al.*, 1992; Cairns, 2006). These analyses, however, were conducted in skeletal muscle samples approaching rigour, likely resulting in an underestimation of the true contribution carnosine has to total muscle buffering capacity. The ability of carnosine to buffer H^{+} is dependent on both animal species and muscle fibre type (Bate-Smith, 1938; Abe, 2000). The improvements in high-intensity exercise performance following β-alanine supplementation, specifically at durations of 60 to 240 s where H^{+} accumulation is evident (for a review see Hobson *et al.*, 2012) are associated with the role of carnosine as an intracellular pH buffer (see review; Sale *et al.*, 2010; 2013), and therefore increased buffering capacity (Hill *et al.*, 2007; Sale *et al.*, 2011; Fig. 2.7). Although the role of carnosine as an intracellular pH buffer is undisputable, this does not exclude the possibility of other physiological roles.

2.3.2 Calcium kinetics

Raising skeletal muscle carnosine content is suggested to influence the regulation of Ca^{2+} kinetics within the sarcoplasmic reticulum (SR) of the skeletal muscle, through increased Ca^{2+} release during skeletal muscle contraction and/or improved sensitivity of contractile properties to Ca^{2} (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.*, 2011; Everaert *et al.*, 2013). The SR has three key functions regarding Ca^{2+} movement; 1) storage of Ca^{2+} pending excitation (Tripathy *et al.*, 1995); 2) Ca^{2+} release through Ca^{2+} specific channel proteins (Ryanodine Receptor; RyR); and 3) Ca^{2+} reuptake initiating muscle relaxation via Ca^{2+}-dependent ATPases. The carnosine molecule can bind to both Ca^{2+} and H^{+}, potentially acting to serve as a cytoplasmic regulator of Ca^{2+} and H^{+} coupling (Baran, 2000). *In-vitro* research on rabbit hind leg muscles showed that raising carnosine concentrations decreased the rate of Ca^{2+} accumulation within the muscle, implying that carnosine influenced the amount of Ca^{2+} released during muscle contraction (Batrukova & Rubtsov, 1997). The addition of L-histidine and β-alanine, assessed separately and together, reported no activation of the Ca^{2+} channels (Rubstov, 2001), indicating that the whole carnosine molecule is required to alter Ca^{2+} release. There is also emerging evidence to suggest that carnosine may influence the sensitivity of the contractile apparatus to Ca^{2+} (Lamont & Miller, 1992; Dutka & Lamb, 2004). In rat *m. extensor digitorum longus* fibres, increased muscle carnosine concentrations improved Ca^{2+} sensitivity of the contractile apparatus in a concentration-dependent manner (Dutka & Lamb, 2004). Similar improvements in contractile properties Ca^{2+} sensitivity where shown in Type I and Type II fibres of skinned human *m. vastus lateralis* fibres following the addition of 8 and 16 mM cytoplasmic carnosine concentrations, with little or no change to either the Hill coefficient or maximum Ca^{2+}-activated force (Dutka *et al.*, 2012). Improving the sensitivity of the contractile apparatus to Ca^{2+} would potentially allow the same amount Ca^{2+} to be released, whilst either increasing force production, or increasing the duration a force is produced (Dutka & Lamb 2004). Currently, it remains unclear if these responses to increased skeletal muscle carnosine content alters the amount of Ca^{2+} released, and/or the sensitivity of the contractile properties to Ca^{2+} (Dutka *et al.*, 2012). Nonetheless, these data highlight the potential of increasing skeletal muscle carnosine content to alleviate the decline in exercise performance, through a mechanism of improved Ca^{2+} kinetics.

In-vivo research examining the effect of increased carnosine content, through supplementation of mice drinking water with 1.2% β-alanine, over eight weeks, reported significantly higher carnosine concentrations in the *m. extensor digitorum longus* (+156%, $P < 0.001$) and the *m. tibialis anterior* (+160%, $P < 0.001$) compared with control mice (Everaert *et al.*, 2013). Furthermore, analysis of submaximal *m. quadriceps femoris* stimulation at a range of frequencies (1 – 100 Hz) was undertaken in the mice to examine the force-frequency relationship, a correspondent of the *in-vivo* force-Ca^{2+} concentration relationship (Balnave & Allen, 1996;

MacIntosh & Willis, 2000). The amount of relative force produced in the *m. extensor digitorum longus* of the mice supplemented with β-alanine, increased by 10 – 31%, inducing a leftward shift of the force–frequency relationship (Everaert *et al.,* 2013). The alterations to the force-frequency relationship implied that Ca^{2+} kinetics was improved following increased carnosine concentrations, via β-alanine supplementation. These data highlight the possibility that increasing carnosine concentrations, via β-alanine supplementation, may alter *in-vivo* human contractile properties, and thus muscle performance by a Ca^{2+} related mechanism. The *in-vitro* nature of the current literature should be considered, although the saturable binding sites of carnosine on the Ca^{2+} release channels in skeletal muscle highlights the potential for carnosine to alter the Ca^{2+} channel itself (Batrukova & Rubtsov, 1997). It remains unclear if the association between skeletal muscle carnosine content and Ca^{2+} kinetics *in-vitro*, is apparent within a human *in-vivo* scenario.

2.3.3 Antioxidant

Carnosine is suggested to provide protection against exercise-induced production of reactive oxidant species (ROS) in skeletal muscle (Kohen *et al.,* 1988; Boldyrev *et al.,* 1992; Boldyrev, 2007; Fig. 2.7). Under normal physiological conditions ROS carry out important regulatory functions in the organism (Halliwell & Gutteridge, 2007; Valko *et al.,* 2007), yet when an increased generation of free radicals and/or reduced physiological activity of antioxidant defences against free radicals is evident (Poljsak *et al.,* 2013), oxidative stress occurs. There are numerous causes associated with increased ROS production, including exposure to microbial infections, extensive exercise, or pollutants/toxins such as cigarette smoke, alcohol, ultra-violet radiation and pesticides (Poljsak *et al.,* 2013). Increased levels of oxidative stress can negatively impact deoxyribonucleic acid, proteins and lipids, increasing the risk of cardiovascular disease, cancer, autism and other diseases (Lu *et al.,* 2010). The skeletal muscle is relatively resistant to exercise-induce oxidative damage, however, exposure to intense and/or prolonged muscular activity can negatively influence performance (Clarkson & Hubal, 2002). Antioxidants are substances that are existing at low concentrations, in comparison to an oxidisable substrate, and significantly delay or prevent the oxidation of that substrate (Halliwell & Gutteridge, 1999). Ingestion of antioxidants has been shown to prevent harmful effects of exercise-induced oxidative stress, accelerate recovery of muscle function and improve exercise performance (see review, Peternelj & Coombes, 2011). Carnosine is suggested to meet almost all the requirements for an ideal antioxidant; it is synthesised and contained in human skeletal muscle and nervous tissues, it is easily absorbed in the digestive tract, it can penetrate through the blood-brain barrier, and has membrane-stabilising actions (Prokopieva *et al.,* 2016). The antioxidant activity of carnosine is supposedly multifunctional, with the ability to display a buffering effect, interact in the initial step of oxidation, decrease the amount of preformed peroxides and react with some secondary products (Kansci *et al.*, 1997).

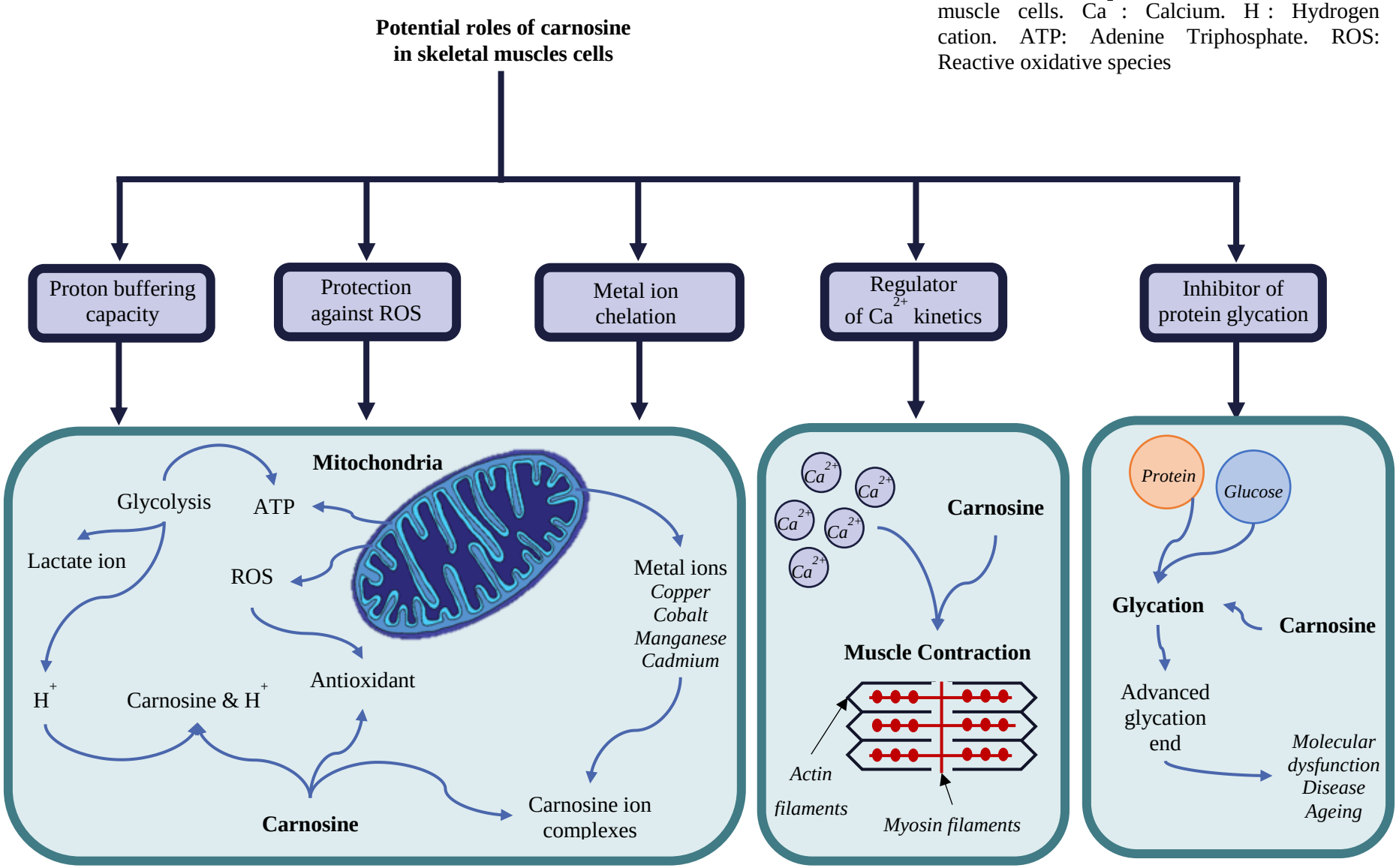

Fig. 2.7: Potential roles of carnosine in skeletal muscle cells. Ca^{2+}: Calcium. H^{+}: Hydrogen cation. ATP: Adenine Triphosphate. ROS: Reactive oxidative species

2.3.4 Metal ion chelation

Another assumed role of carnosine in skeletal muscle tissue is as a physiological metal ion chelator, forming complexes with bivalent metals including ions of copper, cobalt, manganese, and cadmium (Brown & Antholine, 1979; Vladimirov, 1996). Of these ions, copper (Cu^{2+}) and zinc complexes have been the most examined due to their biological relevance. These molecules are able to neutralise free radicals by accepting the unpaired election, inhibiting the oxidation of other molecules (Steinbacher & Eckl, 2015). Dobbie and Kermack (1955) was the first to suggest a complex formation between carnosine and Cu^{2+}, although the exact structure of this molecule has been debated (Baran *et al.,* 2000). In human calf muscle, the presence of carnosine has been shown to chelate copper when examined *in-vivo* through ^{1}H-MRS (Schröder *et al.,* 2008). Although carnosine at pH 7.84 ± 0.18, displayed a much lower binding constant with Cu^{2+} ions (1.1 M^{-1}) compared with that of L-histidine (71 M^{-1}; Velez *et al.,* 2008). There is currently very limited research within this area, with most of the studies examining this potential mechanism conducted *in-vitro*, implemented during un-physiological conditions.

2.3.5 Inhibitor of protein glycation

When elevated glucose levels are evident, formation of covalent adducts with plasma proteins occurs, through a non-enzymatic process known as glycation. Protein glycation reactions leading to advanced glycation end products; thought to be a critical factor in ageing, neurodegeneration, diabetes and its related complications (Kikuchi *et al.,* 2003; Miller *et al.,* 2003; Chen *et al.,* 2004; Davydov *et al.,* 2004; Ahmed, 2005). A large majority of these secondary complications from diabetes are associated with protein glycation and oxidation (Brownlee, 2001; Ahmed, 2005). Glycation of proteins interferes with their normal functions by disrupting molecular conformation, altering enzymatic activity, and interfering with receptor functioning (Singh *et al.,* 2014). The carnosine molecule may provide protection of proteins against glycation, by either acting as a sacrificial peptide (Hipkiss *et al.,* 1995), or though prevention of the formation of protein-protein cross-links via reactions with protein-carbonyl groups (Hipkiss *et al.,* 2001; Fig. 2.7). Evaluation of plasma carnosine levels in diabetic rats demonstrated significantly lower concentrations when compared to those of normal animals (Nagai *et al.,* 2003). The authors proposed that carnosine provides a regulatory effect on the blood glucose levels within the rats. In-line with the current proposed mechanism, Ahmed (2005) indicated that carnosine has the properties required to act as a putative glycation inhibitor, including carnosine's role as a metal ion chelator (Browan & Antholine, 1979; Vladimirov, 1996; Baran, 2000) and anti-oxidant (Kohen *et al.,* 1988; Boldyrev *et al.,* 1993; Boldyrev, 2007).

2.3.6 Conclusion

Based on the existing body of literature, the carnosine molecule may perform several roles within the skeletal muscle (Fig. 2.7), albeit based on varying levels of scientific support. The role of carnosine as an intracellular pH buffer is undisputable, although it is possible that some of the associated improvements in exercise performance are due to the other potential mechanisms of carnosine, which are currently less well studied. The current programme of work will focus predominately on the suggested influence of increased carnosine content on regulating Ca^{2+} kinetics within the SR, and the potential influence of carnosine on the amount of Ca^{2+} released during skeletal muscle contractions and/or the sensitivity of the contractile properties to Ca^{2+}.

2.4 Calcium kinetics within skeletal muscle

Given the previous body of literature, and the potential association between carnosine content and Ca^{2+} kinetics within the skeletal muscle, the following section will give a brief overview on the role of Ca^{2+} within the skeletal muscle, specifically regarding skeletal muscle activation and relaxation.

2.4.1 Calcium kinetics during skeletal muscle activation and relaxation

The movement of Ca^{2+} within the skeletal muscle is vital due to the involvement of Ca^{2+} during both the contraction and relaxation phases. To activate a skeletal muscle contraction, an impulse (action potential) transfers from the nerve into the skeletal muscle, described as neuromuscular transmission, this action potential propagates change across the surface membrane (Allen *et al.*, 2008; Fig. 2.8). Depolarisation of the motor end plate occurs, directing the action potential down the T-tubules into the skeletal muscle, and the SR. Depolarisation allows for cross-talk between the voltage sensitive dihydropyridine receptor (DHPR), located on the T-tubule, and the RyR located in the SR. RYRs are the Ca^{2+} release channels of the SR, and have a high

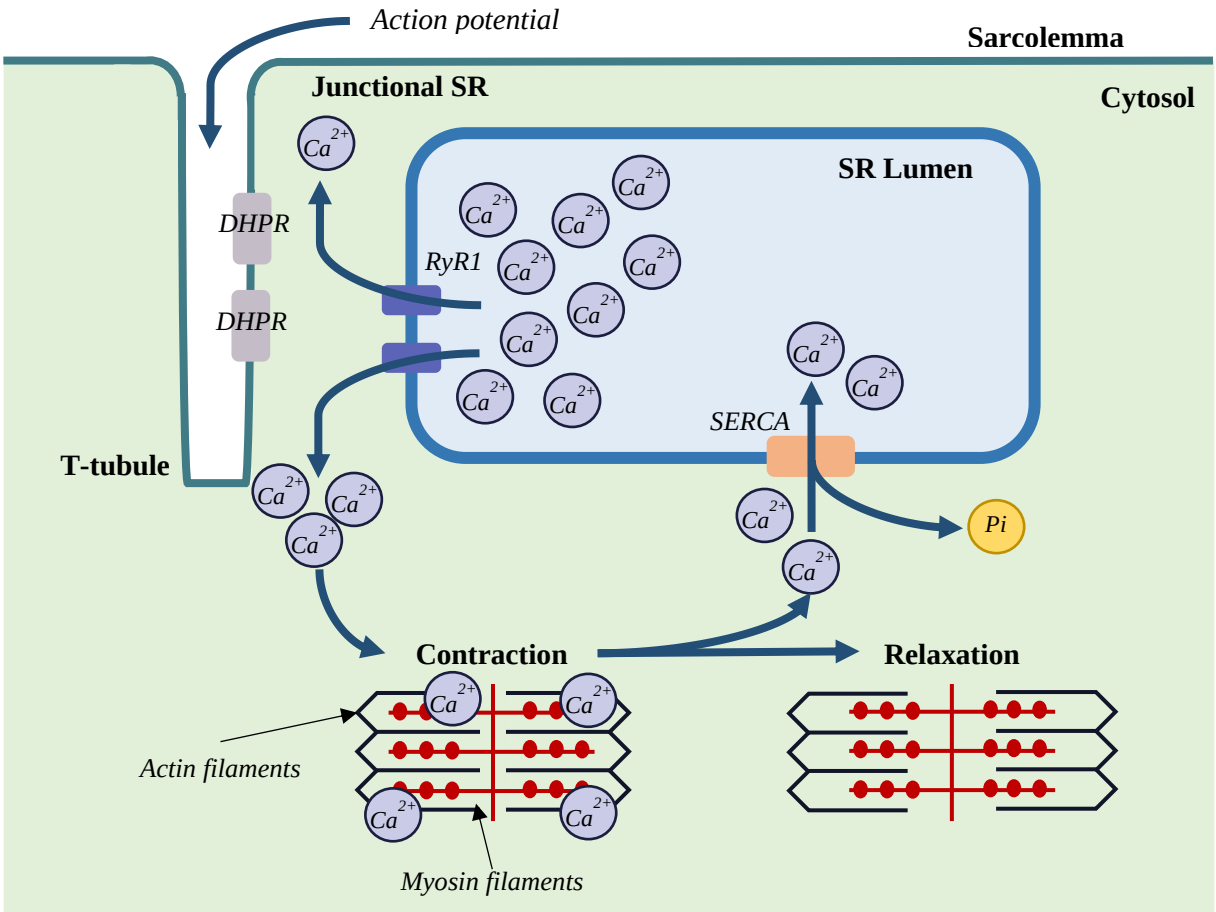

Fig. 2.8: Schematic representation of skeletal muscle excitation, contraction and relaxation. DHPR: Dihydropyridine Receptor; RyR: Ryanodine Receptor; Ca^{2+}: Calcium: SERCA; Sarcoplasmic endoplasmic reticulum calcium ATPase. Pi: Inorganic phosphate

permeability to Ca^{2+} (Fig. 2.8). When opened, the RYRs permit a rapid efflux of Ca^{2+} from the SR lumen to the myofibrils, driven by the large luminal-cytoplasmic concentration gradient. The junctional membrane complex between the plasma membrane and the SR is an important structural foundation for crosstalk between the cell surface and intracellular ionic channels (Pozzan *et al.*, 1994; Berridge, 1998). In a relaxed muscle state, tropomyosin obstructs the attachment site for the myosin cross bridge, held in place by troponin complexes, thus preventing skeletal muscle contraction. Troponin has three subunits within the skeletal muscle; Ca^{2+}-binding protein troponin C (TnC), the inhibitory troponin I (TnI) and tropomyosin-binding troponin T (TnT) (Farah & Reinach, 1995). Upon release of Ca^{2+} by the RyRs into the SR, the Ca^{2+} binds to the TnC exposing a patch of hydrophobic residues located in the N-terminal domain of TnC, allowing interaction of TnC with both TnI and Tnt (Gomes *et al.*, 2002). These interactions promote alterations to the shape and position of the troponin, freeing the tropomyosin filament (Jurkat-Rott & Lehmann-Horn, 2005). The process of sarcoplasmic Ca^{2+} release can be directly and indirectly inhibited by H^{+} cations during skeletal muscle contraction (Laver, Eager, Taoube & Lamb, 2000; Laver, O'Neill & Lamb, 2004). Myosin and actin filaments can connect through the movement of the myosin globular heads, forming a cross-bridge between the two proteins. ATP is broken down within the skeletal muscle, resulting in the release of energy, allowing the myosin globular heads to twist and move into position. The actin filament is pulled inwards, shortening and thus contracting the skeletal muscle. ATP is then required to break the bond between the actin filament and the myosin globular head, allowing the myosin globular head to move along the action chain, allowing continuation of the shortening and relaxation of the muscle. The speed of skeletal muscle relaxation can be impacted by the rate of: (1) dissociation of Ca^{2+} from troponin (Little *et al.*, 2011); (2) the rate of translocation of Ca^{2+} to near the site of entry into the SR (Muntener *et al.*, 1995); (3) re-uptake of Ca^{2+} into the SR by Ca^{2+} pumps (Nogueira *et al.*, 2013) and cross-bridge detachment (Allen *et al.*, 2008). Research conducted by Westerblad and Allen (1993), in fatigued mouse muscle fibres, suggested that the slowing of muscle relaxation, apparent under fatigued conditions, was a reflection of slowed cross-bridge kinetics, rather than a reduction in the rate of Ca^{2+} decline at the end of the stimulation train. Yet, when these data were repeated in Xenopus muscle fibres, the slowing of muscle relaxation was associated with a combination of altered cross-bridge kinetics and impaired Ca^{2+} kinetics, rather than just slowed cross-bridge kinetics. Whilst there is an adequate supply of both ATP and Ca^{2+}, the contraction of the skeletal muscle will continue. If the action potential is halted, thus no longer permitting the release of Ca^{2+} into the muscle, the contraction is stopped. Additionally, if there is a reduction in available ATP to make or break the myosin and actin filament connection, in circumstances such as muscle fatigue, the myosin globular head will remain bound to the actin filament, preventing muscle contraction, even if the action potential stimuli and Ca^{2+} levels are sufficient.

2.4.2 Assessment of calcium kinetics *in-vivo*

There are several measures that can be implemented *in-vivo* to monitor skeletal muscle Ca^{2+} kinetics, alterations to these variables are indicative of adaptations to either the sensitivity of the contractile properties to Ca^{2+} and/or the amount of Ca^{2+} released from the SR lumen to the myofibrils. The *in-vivo* force-frequency relationship is the association between skeletal muscle force output and electrically evoked stimulation frequency, this relationship is associate with intracellular skeletal muscle Ca^{2+} levels (Batrukova & Rubstov 1997). Alterations to the force-frequency relationship could be influenced by adjusted Ca^{2+} kinetics and/or sensitivity (Jones, 1981). Research examining the intracellular Ca^{2+} in mammalian fibres, noted that for a given stimulation frequency there was a reduced Ca^{2+} in the fatigued fibre, although there was no evidence of altered intracellular buffering of Ca^{2+} (Westerblad *et al.,* 1993). These data suggested that the alterations to the force-frequency relationship were due to a reduction in Ca^{2+} release from the SR, rather than decreased Ca^{2+} binding. Interpretation of Ca^{2+} kinetics within the skeletal muscle based solely on the force-frequency relationship should be avoided, as the assessment of the force-frequency relationship can be influenced by the potentiation of the muscle (Jones, 1996), potentially altering muscle responses and thus any findings based solely on these assumptions. It should also be noted that there may be a compensatory mechanism occurring within the skeletal muscle, which would be overlooked by solely assessing the force-frequency relationship, as such, reductions in SR Ca^{2+} release may be obscured by an associated increase in Ca^{2+} sensitivity of the myofibrils, consequential the same skeletal muscle force output will be produced.

The activation of a single action potential, via a 1 Hz electrical impulse, described as a twitch contraction, will result in a quick contraction of the skeletal muscle, generating a small force response (Enoka, 2008). Generation of a twitch contraction is delivered in a voluntary passive state, which is described as a resting or un-potentiated twitch, or shortly following a voluntary muscle contraction, referred to as a potentiated twitch (Rassier & MacIntosh, 2000; Hodgson *et al.,* 2005; Ebben, 2006; Fukutani *et al.,* 2013). Potentiated twitches generally generate larger forces than resting twitches, with possible increases of up to 100% (Kopman *et al.,* 2001). The increases in force response are due to the phosphorylation of the myosin regulatory light chain, leading to a larger number of attached cross brides at a given Ca^{2+} concentration (Sweeney *et al.,* 1993; Rassier & MacIntosh, 2000; Kamm & Stull, 2011). Both resting and potentiated twitch contractions are typically characterised by a few key variables including, peak force, time-to-peak tension (TPT), the interval between the onset of tension and the peak value, and the time it takes for the force to decline to one half of its peak value, half relaxation time (HRT) (Enoka, 2008; MacIntosh, Gardiner & McComas, 2005). There are numerous parameters used to describe skeletal muscle relaxation, although HRT is the most frequently used index because it considers the whole relaxation process (Gillis, 1985). Alterations to evoked twitch contraction time course variables

(TPT and HRT) are proposed to reflect changes in Ca^{2+} kinetics (Enoka, 2008). TPT is known to be significantly influenced by skeletal muscle fatigue, with reported increases during both electrically evoked resting and potentiated twitches (Klitgaard *et al.,* 1989), potentially a reflection of declined efficiency of the SR to release Ca^{2+}. Alongside prolonged TPT, skeletal muscle HRT significantly increases with the ageing process (Edstrom & Larsson, 1987; Kent-Braun & Ng, 1999), with an increase of 30 ms in HRT in older individuals compared to younger counterparts (130 ± 9 ms older vs 107 ± 7 young, P = 0.05; Kent-Braun & Ng, 1999). The negative impact of ageing on skeletal muscle HRT was later supported by McPhee *et al.,* (2014), with men having faster relaxation than women (~12 ms), and younger adults faster than older (~19 ms). This slowing of skeletal muscle relaxation is potentially due to a reduced rate of dissociation of cross-bridges following the removal of the activating Ca^{2+} back into the SR (Edwards, Hill & Jones, 1975; Cady *et al.*, 1989) or a reduced rate of Ca^{2+} pumping by the SR (Dawson, Gadian & Wilkie, 1980). Skeletal muscle HRT is also known to be influenced by other physiological factors including localised muscle fatigue (Jewell & Wilkie, 1960; Edwards, Hill & Jones, 1972, 1975; Bigland-Ritchie *et al.*, 1983). During muscle exhaustion, the *m. quadricep femoris* displays increased HRT, associated with decreased Ca^{2+} uptake of ~58% of baseline measures (Gollnick *et al.,* 1991).

2.5 Calcium-dependent ATPase

Ca^{2+}-dependent ATPases are members of the P-type ATPase family of ion pumps, and responsible for the ATP dependent active transport of ions across a wide variety of cellular membranes. Ca^{2+} pumps, together with Ca^{2+} release channels, form ubiquitous Ca^{2+} regulatory systems in muscle and non-muscle cells (McMullan *et al.,* 1997). Ca^{2+}-dependent ATPases are cation transporting proteins that utilise the energy of ATP hydrolysis for the transport of Ca^{2+} after a skeletal muscle contraction. Within the human skeletal muscle, Ca^{2+}-dependent ATPases are in either the plasma membrane or in the internal membranes of the sarcoplasmic reticulum (SR), there are three types of enzymes: Plasma Membrane Ca^{2+}-dependent ATPase (PMCA), Secretory Pathway Ca^{2+}/Mn^{2+}-ATPases (SPCA) and SERCA.

2.5.1 Plasma membrane calcium-dependent ATPase

PMCAs are Ca^{2+}-dependent ATPases found on the plasma membrane that catalyse the active transport of Ca^{2+} from the cytoplasm into the extracellular space (Carafoli, 1994), and are critical regulators of intracellular Ca^{2+} concentrations (Jensen *et al.,* 2004). The PMCA was discovered in erythrocytes (Schatzmann, 1966) and shows the same essential membrane topology properties of the SERCA pump. The pump operates with a 1:1 Ca^{2+}/ATP stoichiometry as a Ca^{2+}: H^+ exchanger, yet the matter of $Ca^{2+/}H^+$ stoichiometry is still controversial (Niggli *et al.,* 1982; Hao *et al.,* 1994). Due to the numerous variants and specific distribution of the PMCA, it is purposed that PMCA pump plays a prominent role as signalling molecules, in addition to having a constitutive role as Ca^{2+} housekeeping enzymes (Brini & Carafoil, 2011). Upon skeletal muscle relaxation, Ca^{2+} ions are pumped out of the cytosolic compartment by PMCAs and pumped into intracellular compartments by SERCAs, where Ca^{2+} is stored and released upon necessity (Kochegarov, 2003).

2.5.2 Sarcoplasmic/endoplasmic reticulum calcium-ATPase

The SERCA pump is a high-affinity low-capacity 110-kDa transmembrane protein that transports Ca^{2+} from the cytosolic compartment to the SR lumen (MacLennan, Rice & Green, 1997), maintaining the Ca^{2+} gradient across the membrane of intracellular vesicles of all cells (MacLennan & Kranias, 2003; Rossi & Dirksen, 2006). The SERCA pumps are in the longitudinal SR, found in abundance in cardiac and skeletal muscle cells, and are the primary protein responsible for maintaining the Ca^{2+} gradient across the membrane of intracellular vesicles (MacLennan & Kranias, 2003; Fig 2.8 and Fig. 2.9). The SERCA serves as a dual function, (a) lowering the cytosolic Ca^{2+} content to induce muscle relaxation, and (b) concurrently restoring the SR Ca^{2+} store required for following muscle contractions (MacLennan, 1970; Gommans *et al.,* 2002; Rossi & Dirksen, 2006; Periasamy & Kalyanasundraam, 2007; Bers, 2008). The speed by which muscle relaxation occurs has been associated with the reuptake of Ca^{2+} by the SERCA

pumps (Gafni & Yuh, 1989), the pump is proposed to be the rate-limiting step in muscle relaxation (Gillis, 1985; Dux, 1993). SERCA and PMCA have the highest affinity for Ca^{2+} removal from the cytoplasm, and therefore set resting cytoplasmic Ca^{2+} concentrations (MacLennan, Rice & Green, 1997). The SERCA pump is responsible for removing 80% of the Ca^{2+} within the cytoplasm into the lumen of the SR, generating a 10,000-fold concentration gradient across the SR membrane, and provides a large store of Ca^{2+} inside muscle cells. Resting SERCA activity maintains a normal intracellular lumen of free Ca^{2+} at concentrations of 0.3 and 1.0 mM (Bers, 2001; Ginsburg *et al.*, 1998), which is more than 1000 times greater than free Ca^{2+} concentrations in the extracellular cytoplasm (~20-50 nM; Laver *et al.*, 2007).

The SERCA pump undergoes a series of structural transitions, producing a reversibly phosphorylated state (Kühlbrandt, 2004; Wuytack, Raeymaekers, & Missiaen, 2002), according to the E1-E2 model these transitions include changing the affinity of Ca^{2+}-binding sites from high (E1) to low (E2; Toyoshima & Nomura, 2002; Fig. 2.9). This transition process is initiated by the binding of either Ca^{2+} or ATP to the high-affinity site on the cytoplasmic face of the SERCA pump (Fig. 2.9). The ATP molecule undergoes phosphorylation into ADP and Pi, and resulting in E1P-Ca^{2+} (Fig. 2.9). This intermediate is ADP-sensitive, with the presence of ADP resulting in a backwards reaction occurring, thus releasing the Ca^{2+} and synthesising ATP. The Ca^{2+} binding sites are converted to a low affinity state, reorienting toward the luminal face, releasing the Ca^{2+} and inorganic phosphate (Pi), returning the molecules from the low affinity state (E2), to high affinity (E1; Fig. 2.9). For each ATP molecule hydrolysed, the SERCA pump transports two molecules of Ca^{2+} from the cytoplasm to the lumen of the SR, which takes only a few milliseconds. The re-accumulation of Ca^{2+} through the SERCA pump is directly controlled by the mitochondrial supply of ATP. SERCA activity accounts for 10 - 25% of basal ATP turnover (Clasusen *et al.*, 1991; Simonides *et al.*, 2001), yet this is dependent on the cell type and the physiological state. The transfer of Ca^{2+} into the SR lumen by the SERCA pump is accompanied by a counter-transport of H^{+} out into the cytosolic (Tran *et al.*, 2009), by acting on Ca^{2+} kinetics proteins directly or via other molecules, Ca^{2+} signalling can be inhibited or excited (Swietach *et al.*, 2013). The function of the SERCA pump appears to be rather well adapted to the faster Ca^{2+} replenishment of releasable Ca^{2+} stores and the decline of cytosolic Ca^{2+} transients after cell stimulation (Dode *et al.*, 2005).

2.5.3 Secretory protein ATPases

SPCAs represent a group of ion-motive ATPases consisting of single subunit integral membrane enzymes specifically mediating the ATP-powered transport of either Ca^{2+} or Mn^{2+} from the cytosolic compartment into the Golgi lumen (Wuytack *et al.*, 2002; van Baelen *et al.*, 2004). The importance of SPCAs in suppling sufficient Ca^{2+} and Mn^{2+} to different Golgi compartments for the correct execution of diverse luminal functions was recently documented in clinical, genetic, and

functional investigations (Hu *et al.*, 2000; Sudbrak *et al.*, 2000; Fairclough *et al.*, 2003). Like the SERCA pump, the SPCA pump undergoes a series of structural transitions producing a reversibly phosphorylated state (Fig. 2.9). The SPCA reaction cycle, however, transports only one Ca^{2+} per hydrolysed ATP rather than the two transported by the SERCA pump. The SPCA pump is a high affinity and low turnover rate pump can be expected to render the adjustment of the luminal Ca^{2+} level less dependent on the cytosolic Ca^{2+} concentration, mainly by limiting the fluctuation of pumping activity during cytosolic Ca^{2+} transients (Dode *et al.*, 2005).

2.5.4 Calcium-dependent ATPase and carnosine

Carnosine has been suggested to alter the Ca^{2+} kinetics within the skeletal muscle (Zaloga, Roberts & Nelson, 1996), possibly via the existence of saturable binding site(s) for carnosine on the Ca^{2+}-channel (Batrukova & Rubstov, 1997). The presence of carnosine has been shown to improve isolated rat heart muscle contraction and increase free intracellular Ca^{2+} concentrations (Zaloga, Roberts & Nelson 1996). At a pH of 6.0, where a complete decline in Ca^{2+} release pump activity was evident, the presence of carnosine maintained ~30% of pump activity at the same pH. Although speculative, these data suggest that increased carnosine content might alter Ca^{2+}-channel activity by interacting with the Ca^{2+}-channel itself (Batrukova & Rubstov, 1997). These data, however, are limited by several methodological factors, including but not limited to, the lack of a Ca^{2+} buffer, the overloading of the SR with Ca^{2+} concentrations approximately ten times greater than normal, and the inclusion of non-physiological magnesium concentrations.

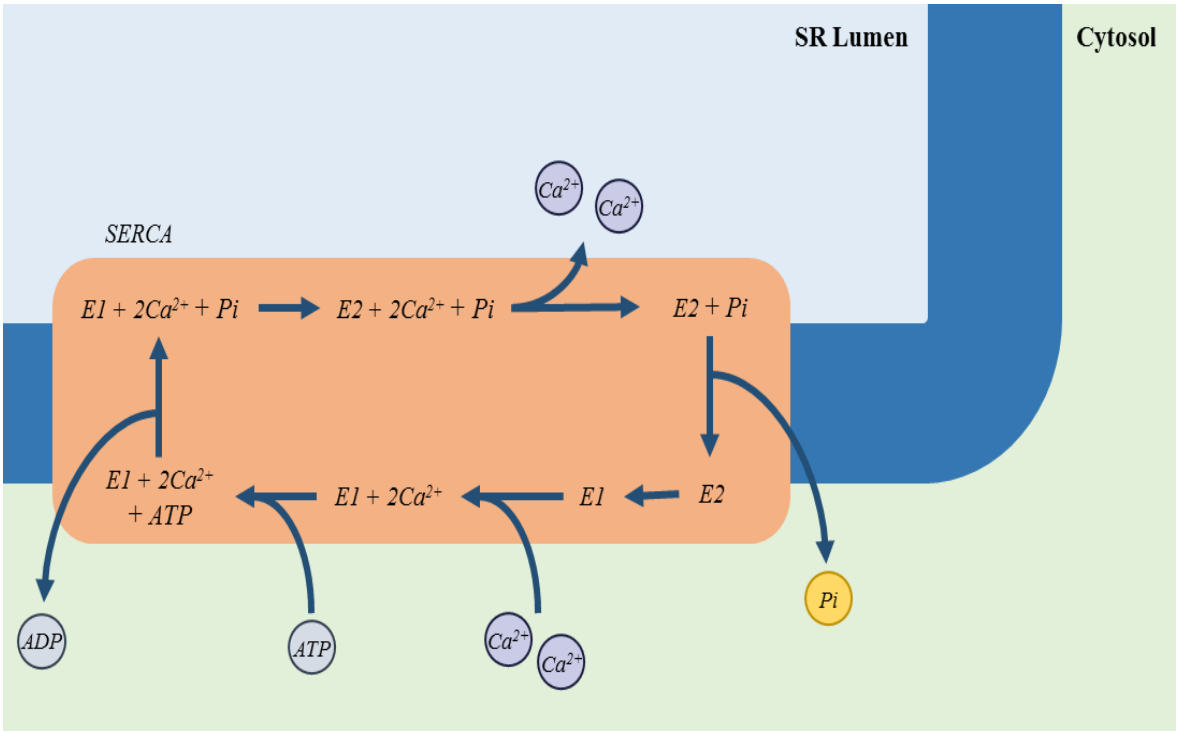

Fig. 2.9: Simplified version of the transition of the sarcoplasmic endoplasmic reticulum calcium ATPase pump during the transportation of calcium (Ca^{2+}) per the E1-E2 model. Redrawn from Toyoshima & Nomura (2002).

2.6 Summary

Since 1938, carnosine, a dipeptide consisting of β-alanine and L-histidine, has been described as an intracellular pH buffer within the skeletal muscle (Bate-Smith, 1938; Deutsch & Eggleton, 1938). In the following years, the role of carnosine as an intracellular buffer has been supported by reported improvements in exercise performance measures, following increased muscle carnosine content, via β-alanine supplementation (see reviews Sale *et al.,* 2010; 2013; Hobson *et al.,* 2012; Saunders *et al.,* 2017. In the last ten years, the role of carnosine in the skeletal muscle has been extended (see Begum *et al.,* 2005; Sale *et al.,* 2010; Fig. 2.7). One such mechanism is the potential influence of increased carnosine content on regulating Ca^{2+} kinetics, through either altered the amount of Ca^{2+} released during skeletal muscle contraction and/or the sensitivity of contractile properties to Ca^{2+} (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.,* 2011; Everaert *et al.,* 2013). The current *in-vitro* data highlights the possibility that increasing carnosine content, via β-alanine supplementation, may in turn, alter *in-vivo* human contractile properties, and thus skeletal muscle performance through a Ca^{2+} kinetics mechanism. Despite the importance, there remains no research examining the impact on increased skeletal muscle carnosine content, via β-alanine supplementation, on *in-vivo* measures associated with *in-vitro* Ca^{2+} kinetics. The current thesis reports on four studies that extend the previously limited body, evaluating the impact of increased carnosine content, via β-alanine supplementation on measures associated with skeletal muscle Ca^{2+} kinetics.

Chapter 3: General methodology

3.1 Introduction

This chapter provides a description of the general methodologies implemented during the studies including within this programme of work (Chapters 4–6). Any specific protocols implemented during an individual investigation are described within the respective chapter. Development of the research specific methodologies, reported with study 4 (Chapter 7), are presented in Appendix 1.

3.2 Ethical approval

All invasive research conducted on human participants required ethical approval, as such, each individual investigation obtained permission from the Nottingham Trent University Invasive Human Ethical Review Committee. All approvals were obtained prior to the commencement of data collection. Research presented in Study 4 (Chapter 7), conducted using rat skeletal muscle tissue did not require institutional ethical approval, however, to comply with the principles of replacement, reduction and refinement, as stated in the Animals (Scientific Procedures) Act 1986 (Amendment Regulations 2012, SI 2012/3039), the research was conducted on tissue samples collected as a by-product from animals sacrificed as part of another research programme. The researcher was not involved in the life or death of the animal, or collection of the tissue.

3.3 Human participants

All human participants were fully informed of any risks and discomforts associated with the research (in verbal and written form) prior to providing written informed consent and completing a health screen. Participants were immediately excluded from the research if they presented with any medical conditions documented in the exclusion criteria, or if these conditions arose during their participation in the study. Participants were excluded if they had ingested β-alanine or carnosine during the previous 6 months, this time frame was due to the long washout period of muscle carnosine (Baguet *et al.,* 2009). None of the participants were vegetarian/vegan, and would have ingested small amounts of β-alanine in their normal diet; typically 50 – 400 $mg \cdot d^{-1}$ (Yeum *et al.,* 2010; Jones 2011; Harris *et al.,* 2012). Participants were excluded from partaking in the current research if they had undertaken a resistance training programme in the previous 6 months. Participants were instructed to avoid strenuous/unaccustomed exercise for 48 h prior to each visit to the laboratory, with alcohol and caffeine prohibited on test days. Participants arrived to each session at least 3 h postprandial. Compliance with these requests was confirmed verbally with each participant prior to each session.

3.3.1 Young human participants

Within this thesis, participants classified as 'young' adults were physically active males aged between 18 - 30 y. Exclusion criteria for these participants were; a) bone disorders in the assessed lower limb, sustained within the previous 2 y, including osteoarthritis, osteoporosis, bone cyst and osteopenia, b) non-arthroscopic joint surgery, or joint replacement, ever, in the assessed limb (knee, hip and ankle), c) lower limb leg injuries including sprains and strains, joint dislocations and fractures at the time of assessment and d) regular knee pain in the assessed limb when performing daily movement tasks.

3.3.2 Older human participants

Participants classified throughout this thesis as 'older' adults were male or female individuals aged between 60 - 80 y, and defined as 'medically stable for exercise studies' by Grieg *et al.,* (1994; Table 3.1). These criteria were designed for safety, ensuring participants were free from diseases that might affect exercise performance (Greig *et al.,* 1994).

Table 3.1: Exclusion criteria, defining 'medically stable' older participants (Greig *et al.,* 1994).

Exclusion criteria:
History of myocardial infarction within the previous 2 y
Cardiac illness: symptoms of aortic stenosis, acute pericarditis, acute myocarditis, aneurysm, severe angina, clinically significant valvular disease, uncontrolled dysrhythmia, claudication, within the previous 10 y
Thrombophlebitis or pulmonary embolus within the previous 2 y
History of cerebrovascular disease
Acute febrile illness within the previous 3 months
Severe airflow obstruction
Uncontrolled metabolic disease (e.g. diabetes, thyroid disease)
Major systemic disease active within the previous 2 y (e.g. cancer, rheumatoid arthritis)
Significant emotional distress, psychotic illness or depression within the previous 2 y
Lower limb arthritis, classified by inability to perform maximal contractions of lower limbs without pain
Lower limb fracture sustained within the previous 2 y
Upper limb fracture sustained within the previous 6 months
Non-arthroscopic lower limb joint surgery within the previous 2 y
Any reason for a loss of mobility for greater than 1 week in the previous 2 months or greater than 2 weeks in the previous 6 months
Resting systolic blood pressure > 200 mmHg or resting diastolic blood pressure > 100 mmHg
Taking beta-blockers or digoxin, or not in sinus rhythm
On daily analgesia

3.4 Supplementation

3.4.1 Supplement disclosure

All β-alanine (Carnosyn™) and placebo (maltodextrin) supplements used as a part of this thesis were provided free of charge from NAI (San Marcos, California, USA), although no additional funding was provided.

3.4.2 Supplementation protocol

Participants were supplemented with either sustained-release β-alanine or a matched placebo ingested in the form of 800 mg tablets. Supplement tablets were consumed two at a time (dose of 1.6 g), on three (older individuals; Study 3; Chapter 6) or four occasions (young individuals; Studies 1 and 2; Chapters 4 and 5) per day, across the 28-day period. Older individuals were provided with 134.4 g of either β-alanine or placebo over the 28-day period, younger individuals were provided with around 45 g more, totalling 179.2 g. Supplements were supplied to each participant in identical white tubs, with both the participants and researchers remaining blind to allocations, until data analyses were completed. Participant compliance was assessed across the 28-day period using a supplement log, with participants asked to report any feelings of paraesthesia during this time. Prior to use, β-alanine tablets were tested by the manufacturer and conformed to the label claim for β-alanine content. In addition, β-alanine and placebo supplements were independently tested by HFL Sports Science, UK, to ensure no contamination with steroids or stimulants per International Organization for Standardization 17025 accredited tests.

3.5 Experimental setup

3.5.1 Height and body mass

Upon arrival to the familiarisation session of each study, participant height was measured to the nearest 0.1 cm using a stadiometer (Seca, UK). Body mass was measured while wearing minimal clothing, to the nearest 0.1 kg using calibrated electronic scales (Seca, UK).

3.5.2 Fingertip blood sampling

Fingertip capillary blood sample preparation included wiping with an isopropyl alcohol wipe and puncturing the skin using a spring propelled lancet (Unistik3, Mumford, UK). Fingertip capillary blood lactate measurements at 5 min following exercise provided the best estimate of lower limb blood lactate contractions (Comeau *et al.,* 2011). Sampling involved the collection of 80 µL of whole blood in to a heparin-coated clinitube (Radiometer Ltd, UK); all samples were immediately analysed.

3.5.3 Knee extensor force

The production of voluntary muscular force is determined by a combination of motor unit recruitment and the firing rate of recruited motor units (Watanabe *et al.*, 2012; Hu *et al.,* 2014). A greater number of action potentials will enlarge muscle contraction, increasing force production. The force exerted by each motor unit depends principally on the number of muscle fibres that are innervated by the motor neuron and the rate at which the motor neuron discharges action potentials (Enoka *et al.,* 2012). The evaluation of voluntary muscular force has been widely undertaken to assess physical conditioning, identify muscle weakness (due to ageing or disease), and monitor the influence of external factors such as training and rehabilitation programmes (Dwyer & Davis, 2008).

Participants were seated in a rigid, custom-built isometric dynamometer (Fig. 3.1) with hip and knee joint angles of approximately 95° and 100° (180° = full extension), previously described by Hannah *et al.,* (2012; 2013). Adjustable strapping across the pelvis and shoulders prevented extraneous movement during skeletal muscle contractions. An ankle cuff was attached to the dominant leg of the participant ~2 cm proximal to the medial malleolus and in-line with a uni-linear strain gauge (615, Tedea-Huntleigh, Herzliya, Israel) oriented perpendicular to the tibia. Dynamometer configuration was established during the familiarisation session and replicated thereafter. The force signal was amplified (×1000) in the frequency range of 0 – 500 Hz, and sampled at 2000 Hz using an external A/D converter (1401; CED, Cambridge, UK), interfaced with a computer using Spike 2 software (CED, Cambridge, UK). Force data were low-pass filtered bi-directionally at 450 Hz using a fourth-order zero-lag Butterworth filter prior to analysis. Baseline resting force was subtracted from force recordings to correct for the effects of gravity and

background noise. The force onset was identified manually using visual identification in accordance with previously published methods (Tillin *et al.*, 2010; Hannah *et al.*, 2012).

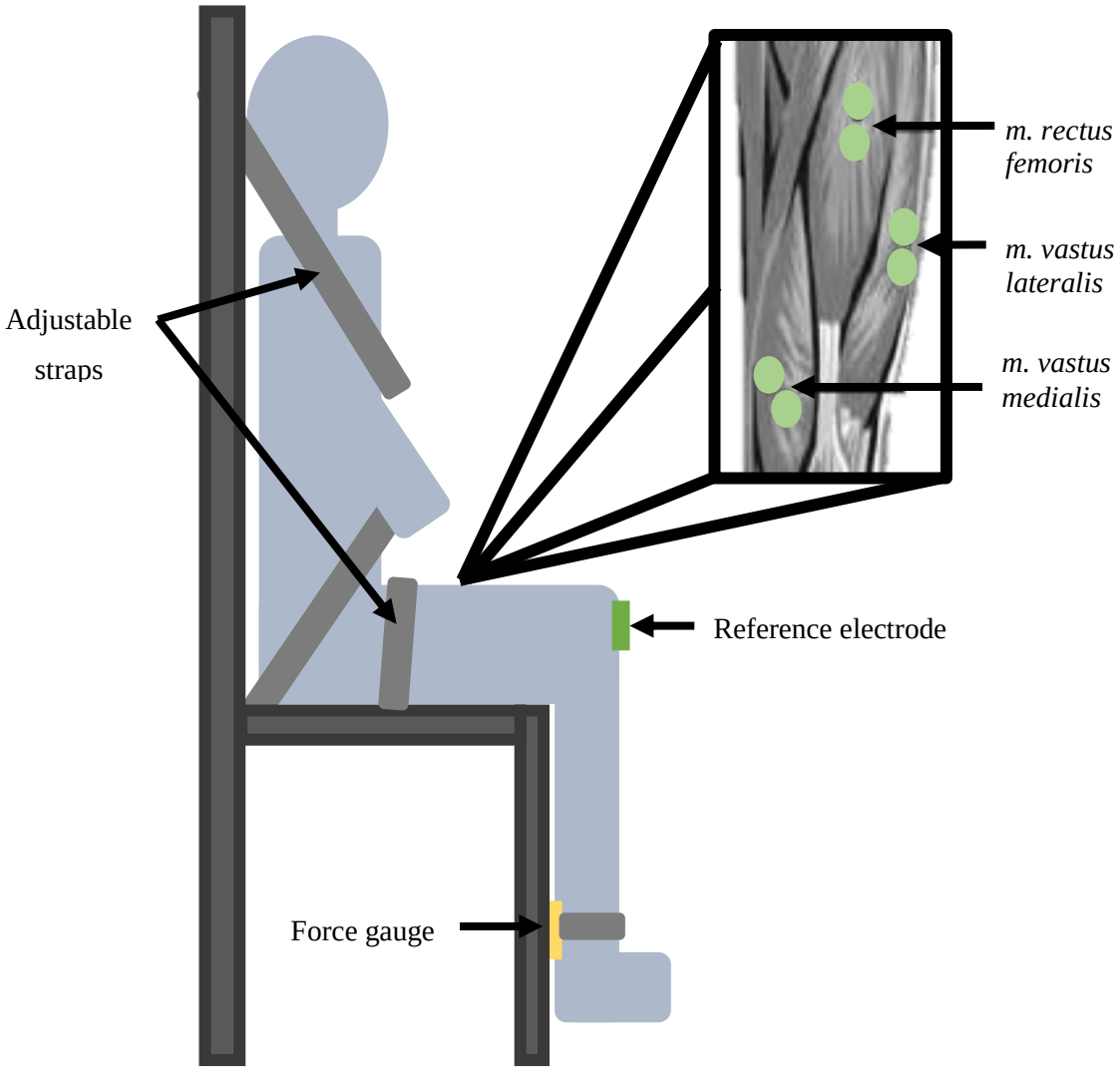

Fig. 3.1: Pictorial representation of the custom-built isometric dynamometer, including adjustable straps, force gauge, and anatomical positions of selected electrode sites: frontal view of the right quadricep. Adapted from Konrad (2006).

3.5.4 Electromyography

Surface electromyography (EMG) provides quantifiable information on the relative contribution of the superficial muscles during muscular activity (Winter, 2005; Esquenazi & Mayer, 2004), with varying amplitude, duration and frequency content (Basmajian & De Luca, 1985). Surface EMG is more suitable for recording neural activation of large muscle groups, including the quadriceps, rather than small or deep muscles, which may require intramuscular EMG (Tucker, 1993). Surface EMG records the action potential travelling across the muscle fibres in response to neural activation (MacIntosh, Gardiner & McComas, 2006), providing information on the relative contribution of the superficial muscles during movement (Basmajian & DeLuca, 1985; Esquenazi & Mayer, 2004). This method provides a non-invasive, easy to access assessment of muscular responses during force generation, movement production and accomplishment of everyday tasks (De Luca, 1997). EMG signals are obtainable during both voluntary and electrically evoked contractions, although during voluntary activation interpretation of EMG signals can become difficult due to inconsistencies in signal formation; EMG activity is normally expressed in millivolts (mV). Surface EMG can be influenced by multiple intracellular and extracellular factors including, electrode placement, signal crosstalk, blood flow, and subcutaneous tissue (De Luca, 1997; Farina *et al.*, 2006). Although, the positive curvilinear relationship between EMG amplitude and force output, supports its implementation to interpret neuromuscular activation. Normalisation of the EMG signal is the process by which the values are expressed as a percentage of the muscle activity during a calibrated test contraction (Lehman & McGill, 1999) typically a MVIC. By normalising EMG signals to a supramaximal muscle action potential (M-wave), the removal of some confounding factors associated with EMG, such as electrode location, subcutaneous fat quantity, and skin impedance, may be reduced (Gandevia 2001). In addition to reducing confounding factors, normalisation of the EMG signal allows comparison between different muscles, across time and between individuals (De Luca, 1997; Knutson *et al.*, 1994; Mathiassen, Winkel & Hagg, 1995).

In the current thesis, surface EMG was recorded from the superficial quadricep muscles (*m. rectus femoris*, *m. vastus medialis* and *m. vastus lateralis*) of the dominant leg, with a reference electrode placed on the patella of the same limb (Fig. 3.1). Bipolar surface electrodes (2.5 cm inter-electrode distance; silver/silver chloride, 95 mm^2 area, Ambu Blue Sensor, Ambu, Ballerup, Denmark) were attached over each assessed muscle at standardised percentages of thigh length, as measured from the knee joint space to the greater trochanter (*m. rectus femoris*, 55%; *m. vastus medialis*, 25% and *m. vastus lateralis*, 45%, see Fig. 3.1). Orientation of the electrode pair were positioned in the direction of the muscle fibre, in accordance with the guidelines set by Konrad (2006). These sites were selected to avoid the innervation zones of each of the assessed muscles (Rainoldi *et al.*, 2004). Preparation of the skin prior to electrode placement was achieved by

removing hair and cleaning of the skin with fine sandpaper and an alcohol pad (Konrad, 2006), attempting to reduce bioelectrical impedance.

EMG signals were pre-amplified by active EMG leads (input impedance 100 MΩ, CMMR > 100 dB, base gain 500, 1st order high pass filter set to 10 Hz; Noraxon, Scottsdale, USA) connected in series to a custom-built junction box and subsequently to the same A/D converter and computer software that enabled synchronisation with the force data. The signals were sampled at 2000 Hz. EMG data were band-pass filtered in both directions between 20 and 450 Hz using a fourth-order zero-lag Butterworth filter prior to analysis.

3.5.5 Electrically stimulation

Electrical stimulation involves the use of low-amplitude electrical pulses to induce an involuntary muscle contraction (Maffiuletti, 2010), through the activation of the motor neuron axons or intramuscular axonal branches (Hultman *et al.*, 1983). Electrical stimulation provides a helpful measure to investigate the physiological properties of the neuromuscular system (Merletti *et al.*, 1992) in healthy and impaired muscles, in both fresh and fatigued muscle conditions (Martin *et al.*, 2004; Wust *et al.*, 2008; Horstman *et al.*, 2008). Electrical stimulation overcomes any central activation failure by bypassing any spinal or voluntary inhibitions (Westing *et al.*, 1990; Fitzgerald *et al.*, 2004), allowing the activation of Type II muscle fibres, even when stimulation is at relatively low intensities (Maffiuletti, 2010).

A constant current variable voltage stimulator (DS7AH, Digitimer Ltd, Welwyn Garden City, UK) was used to assess evoked knee extensor contractile properties. The frequency of electrical stimulation described the number of pulses delivered per s, progressing from 1 Hz upwards. The duration of each pulse is measured in microseconds (μs), with the intensity of the current recorded in milliamps (mA) or voltage (V). As an electrical evoked signal causes the muscle to contract, on M-wave from stimulating motor axons is produced and is used to assess changes in membrane excitability. As the electrical stimulus intensity is increased at rest or during a movement, the M-wave increases until maximum (M_{max}).

3.5.5.1 Femoral nerve stimulation

The cathode (positive electrode) stimulation probe (1 cm diameter, Electro-Medical Supplies Ltd, Wantage, UK; Fig. 3.2) was pressed firmly onto the skin over the femoral nerve high in the femoral triangle. The anode (negative electrode) carbon rubber electrode (7 × 10 cm; Electro-Medical Supplies Ltd, Wantage, UK) was coated with electrode gel and taped to the skin midway between the iliac crest and the greater trochanter (Sidhu *et al.*, 2009). The precise location of the cathode was determined during each testing session as the position that evoked the greatest twitch

response for a submaximal electrical current (typically 30 – 50 mA). Femoral nerve stimulation was implemented for all electrically evoked resting and potentiated twitches, doublet and octet contractions.

3.5.5.2 Percutaneous stimulation

Two carbon rubber electrodes (14 × 10 cm; Electro-Medical Supplies Ltd, Wantage, UK; Fig. 3.2) were coated with electrode gel and placed on the skin directly above the assessed quadricep muscles (*m. rectus femoris*, *m. vastus medialis*, and *m. vastus lateralis*) to induce percutaneous stimulation. Placement of the electrodes was at standardised percentages of thigh length, as measured from the patella to the anterior superior iliac spine. The proximal electrode was placed 20% distal to the anterior superior iliac spine, and the distal electrode placed 10% proximal to the patella. Percutaneous stimulation was implemented to assess the force-frequency relationship, with electrically evoked contractions at a range of frequencies (1, 5, 10, 15, 20, 30, 40, 50, 80 and 100 Hz) to induce submaximal muscle contractions.

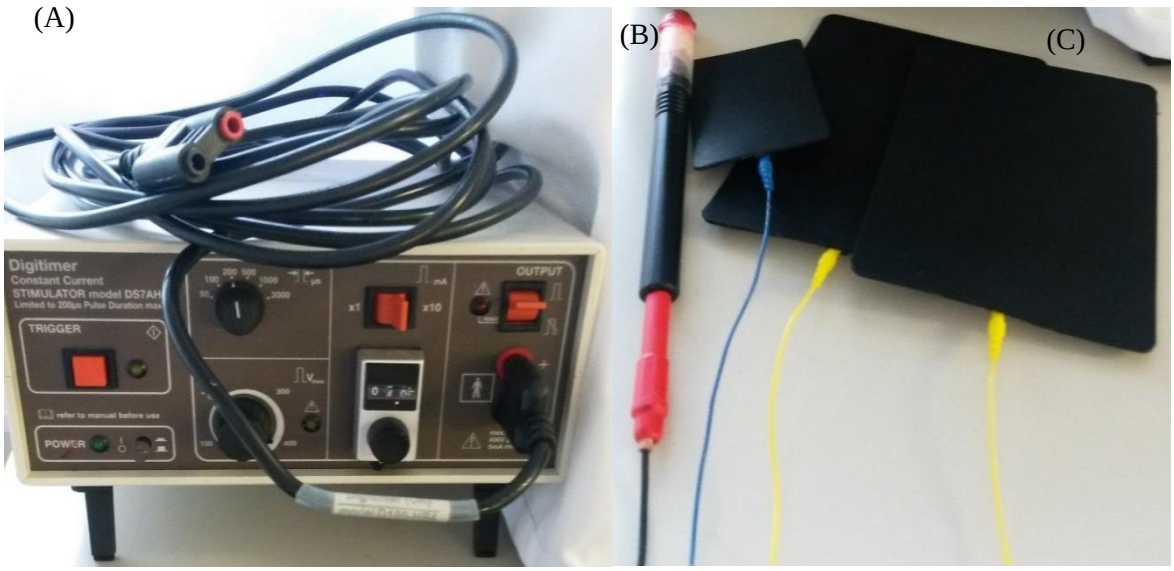

Fig. 3.2: Method of electrical stimulation: (A) a constant current Digitimer DS7AH electrical stimulator; (B) a femoral nerve stimulation probe and carbon rubber electrode; and (C) two carbon rubber electrodes used for percutaneous muscle stimulation.

3.6 Protocol and measurements

Measurements were completed in a specific order, in accordance with a consistent time schedule, with 3 min rest between successive measurements. Force and EMG onsets for all evoked and voluntary contractions were identified using manual visual identification by the same investigator. The method of manual visual identification has been proposed as the 'gold standard' measure to identify signal onset (Pain & Hibbs, 2007; Pulkovski *et al.,* 2008; Tillin *et al.,* 2010; Hannah *et al.,* 2012). Manual identification is considered more valid than the use of automated methods (Tillin *et al.,* 2012), with identification of the signal onsets up to 60 ms earlier than automated methods (Allison, 2003; Pain & Hibbs, 2007).

3.6.1 Maximal voluntary isometric contraction

Voluntary force production by a given muscle, or group of muscles, in each situation is referred to as muscle strength (Knuttgen & Komi, 1992). Muscle strength is specific to both the assessed muscle or muscle groups, and the given situation (Knuttgen & Komi 1992; Logan *et al.*, 2000). The ability to generate a maximal voluntary isometric contraction (MVIC) allows the assessment of an individual's MVIF. MVIF is the difference between baseline force and the greatest peak in force production (Folland & Williams, 2007; Fig. 3.3) and is defined as the peak instantaneous force achieved during a given situation (Wigley & Strauss, 2000) or during a measurement session (Tillin *et al.,* 2010; Hannah *et al.,* 2012). The completion on an MVIC can allow the impact of muscular fatigue to be observed and quantified. Skeletal muscle fatigue is often described as the decline in physical performance, associated with an increase in the real and/or perceived difficulty of a task or exercise (MacIntosh *et al.,* 2005). Muscle fatigue is a decline in MVIF or power, of either a single muscle or muscle group (Millet *et al.,* 2012; Enoka & Duchateau, 2008; Gandevia 2001).

3.6.1.1 Protocol

To elicit a voluntary maximal contraction, participants were instructed to kick out their dominant leg "as hard as possible" for a 2 - 3 s duration. In total, four separate MVIC were undertaken with greater than 60 s rest between each attempt, this rest period was double that reported in previous research (≥30 s; Folland *et al.,* 2013; 2014; Hannah *et al.,* 2014), ensuring participants remained rested throughout. During each MVIC, participants received strong and consistent verbal encouragement reiterating the initial instructions, together with on-screen feedback of the force signal and a marker of their maximum force during that session displayed on-screen. Following the competition of each MVIC two potentiated twitch contractions were elicited.

3.6.1.2 Assessed variables

MVIF was recorded during each maximal contraction, with the RMS (Equation 3.1; Basmaijian & De Luca, 1985) of the EMG signal for each muscle (*m. rectus femoris*, *m. vastus medialis*, and *m. vastus lateralis*) calculated over a 500 ms epoch surrounding MVIF (250 ms either side). The RMS value was normalised to the corresponding M_{max} (Folland & Williams, 2007; Buckthorpe *et al.*, 2012), before being averaged across all three sites to calculate a mean quadriceps value.

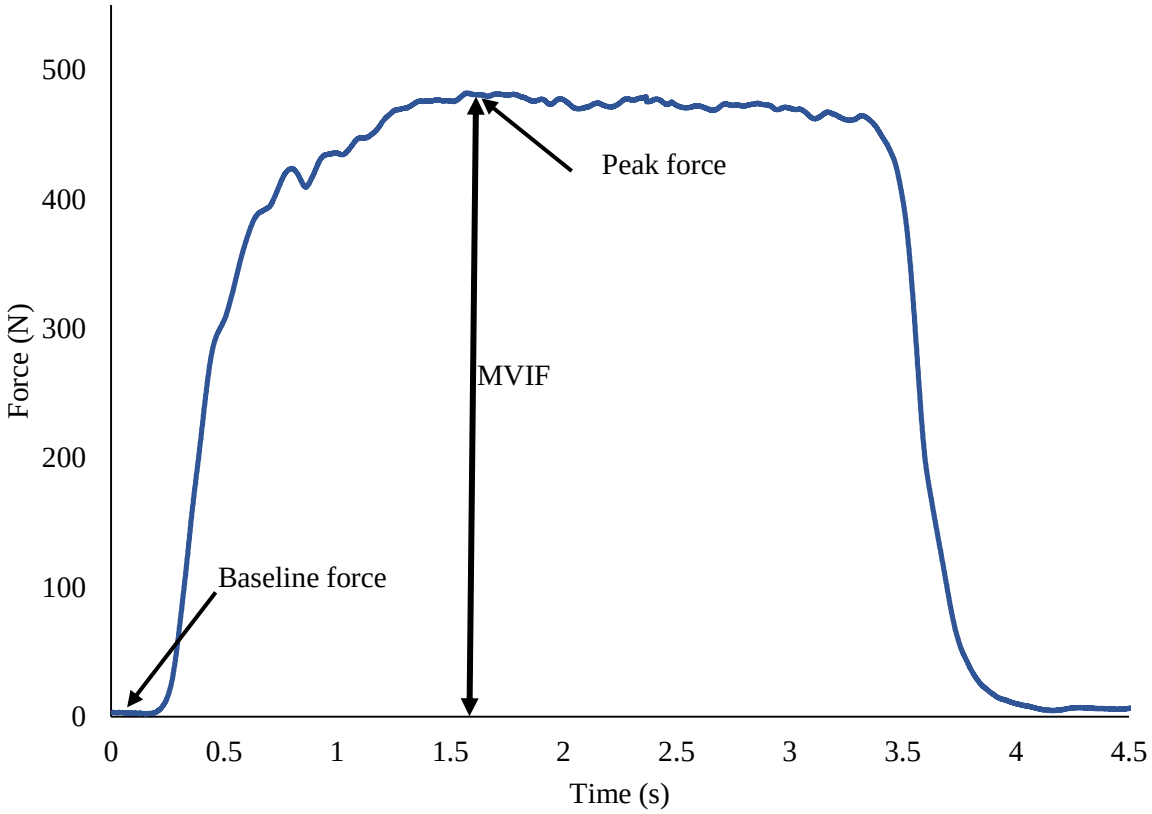

Fig. 3.3: Representative records from a single participant displaying the force response of the knee extensors during a maximal isometric voluntary contraction of the knee extensors. Maximal isometric voluntary force (MVIF) is depicted as the difference between baseline force and the greatest peak in produced force.

3.6.2 Explosive voluntary contractions

The ability to generate force rapidly or as 'fast and hard' as possible from rest, is often described as production of explosive force (Logan *et al.*, 2000). The production of the muscle force generation over a period (ΔForce/ΔTime) is also described as rate of force development (RFD; Aagaard *et al.*, 2002; de Ruiter *et al.*, 2004; Minshull *et al.*, 2007). The RFD at specific time points from the onset of the contraction (Tillin *et al.*, 2010) or across a time (Aagaard *et al.*, 2002) can be used to quantify explosive force (Fig. 3.4). The RFD is an important descriptor of functional performance (Aagaard *et al.*, 2002; de Ruiter *et al.*, 2004), a key factor in numerous populations, including athletes (Luhtanen & Komi, 1979; Kuitunen, Komi, & Kyrolainen, 2002), clinical populations (Suetta *et al.*, 2004) and the elderly (Hakkinen *et al.*, 1996). RFD is considered more important than the production of a MVIC (Tillin *et al.*, 2012; Aagaard *et al.*, 2002; Anderson *et al.*, 2010), due to the many sporting movements occurring within a shorter time than it take to reach maximal force (Hernández-Davó & Sabido, 2014). There is however, a moderate-to-strong relationship between RFD and MVIC, especially as time increases beyond 200 ms from force onset (Driss *et al.*, 2002; Anderson *et al.*, 2006). The use of 50 ms time windows from the onset of force production to provide clearer understanding of the determinants of RFD (Tillin *et al.*, 2010) with

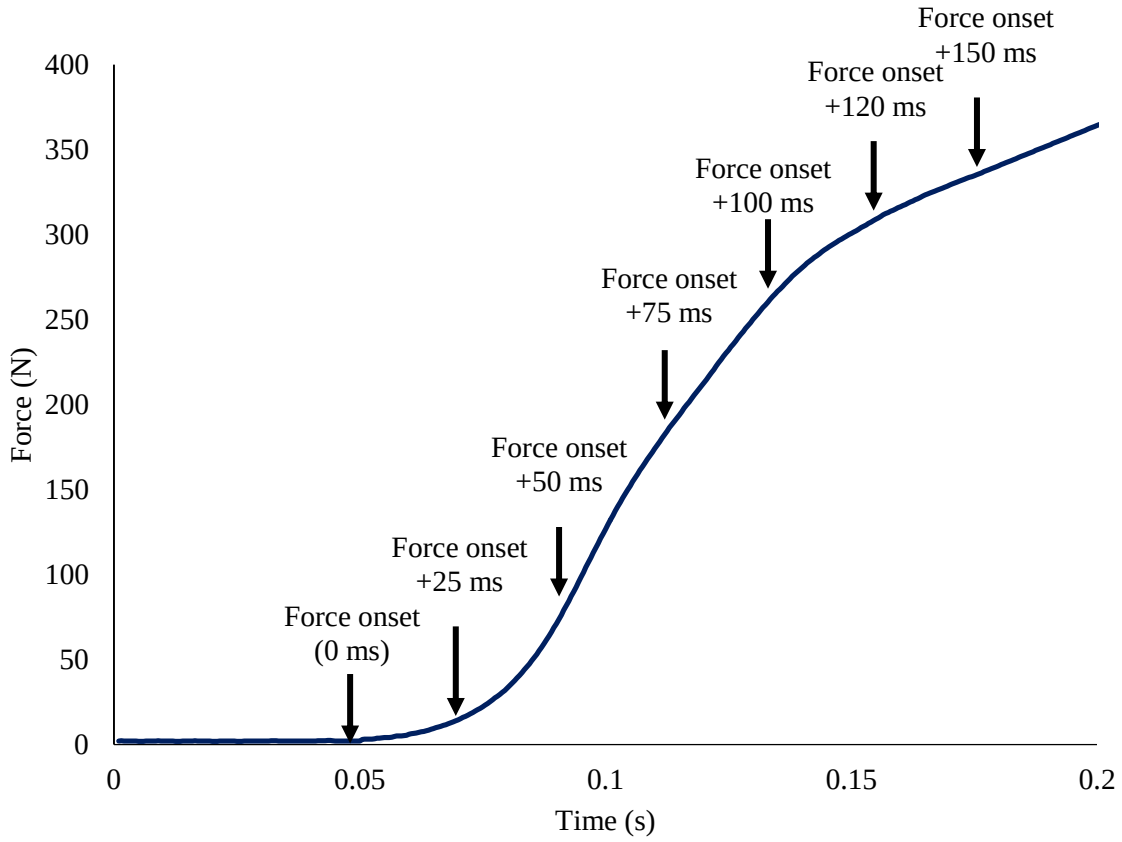

Fig. 3.4: Representative records from a single participant displaying force responses of the knee extensors during an explosive contraction of the knee extensors. Specific time points are depicted every 25 ms following the onset of force (0 ms), to quantify explosive force and rate of force development.

shorter time window of 25 ms being used in more recent research (Hannah *et al.*, 2012).

3.6.2.1 Protocol

Explosive contractions were generated by the participants contracting voluntarily "as fast and as hard as possible" on ten separate occasions. The emphasis was on the speed of voluntary contraction, yet participants were expected to achieve greater than 80% of MVIF. Contractions were performed in accordance with previously published protocols (Tillin *et al.*, 2010; Hannah *et al.*, 2012). Each contraction was held for around one second, with around 20 seconds between each measurement. Feedback was provided using on-screen cursors; one displaying the peak RFD recorded during the session, and the other displaying an 80% MVIC target level. A sensitive scale of resting force level was also displayed on-screen providing feedback on any countermovement or pre-tension (Fig. 3.5).

3.6.2.2 Assessed variables

The three explosive voluntary contractions with the greatest RFD, displaying no prior countermovement or pretension, with peak force ≥80% MVIF were used for analysis, criteria previously described by Hannah *et al.*, (2012; 2014). Force and EMG measurements were averaged across the three selected contractions. Force and RFD measures (absolute and normalised to MVIF) were recorded at 25, 50, 75, 100, 125 and 150 ms from force onset. The RMS of the EMG signal from each muscle (*m. rectus femoris*, *m. vastus medialis*, and *m. vastus lateralis*) was measured over three consecutive 50 ms time periods (*i.e.*, 0 - 50, 50 - 100 and 100 - 150 ms) from the EMG onset of the first activated agonist muscle. The RMS value (Equation 3.1) of each assessed quadricep muscle was normalized to M_{max} and averaged to provide a mean quadriceps value.

$$\mathrm{RMS}\{\mathrm{m(t)}\} = \left(\frac{1}{\mathrm{T}}\int_{\mathrm{t}}^{\mathrm{t+T}} \mathrm{m}^2\,(\mathrm{t})\mathrm{t}\right)^{\frac{1}{2}}$$

Equation 3.1: Root mean square (RMS) equation where t is the time at the start of sampling, T is the time window and m is the amplitude of the surface electromyography signal.

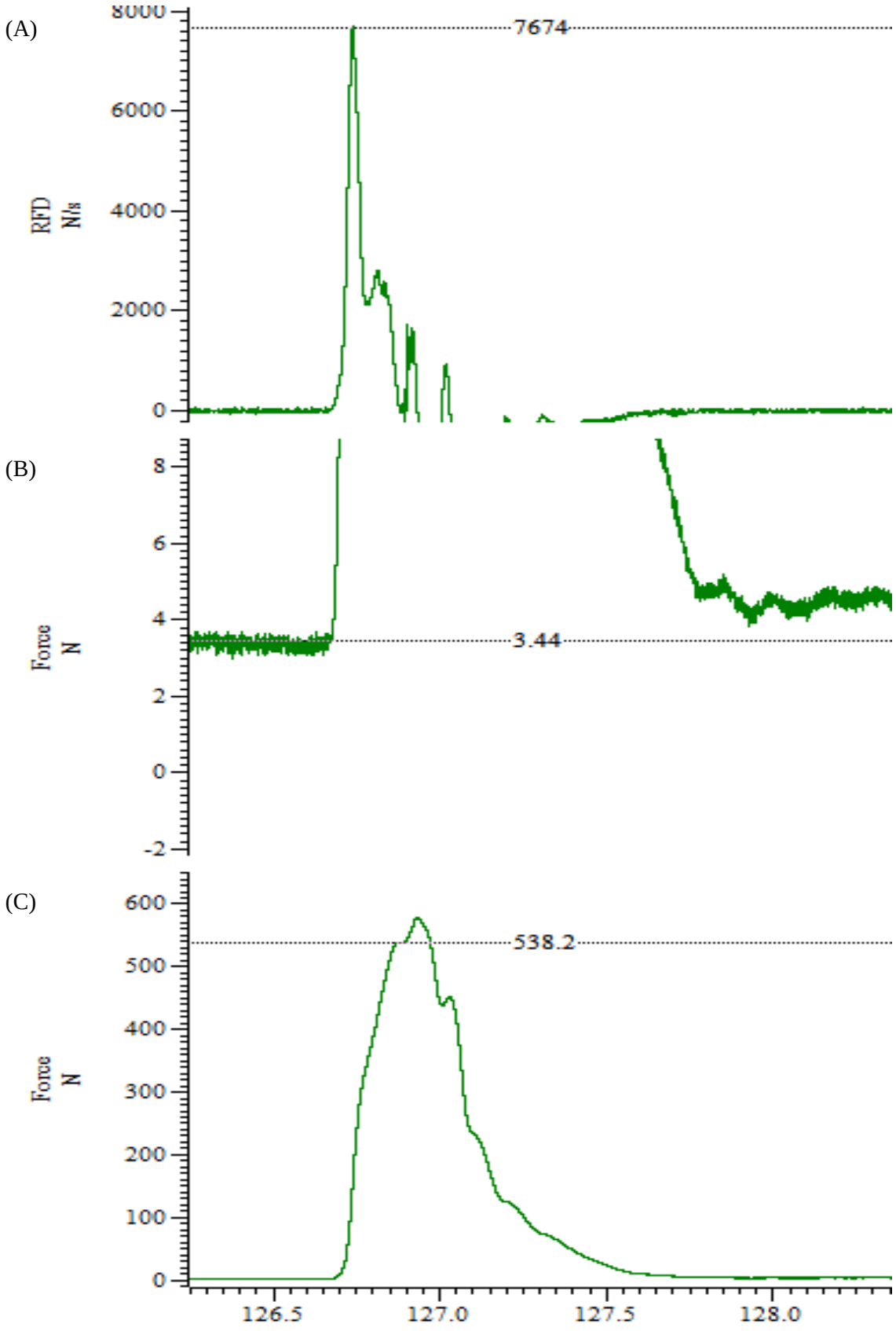

Fig. 3.5: Representative records from a single participant displaying force responses of the knee extensors collected during an explosive contraction; (A) Channel displaying rate of force development, with a horizontal cursor to depict maximum rate of force development; (B) A sensitive scale of resting force level; and (C) Force channel displaying horizontal cursor displayed 80% of maximal isometric voluntary contraction target level.

3.6.3 Sustained isometric knee extensor endurance test

At 45% MVIF, the blood flow to the active muscle becomes occluded by the increased intramuscular pressure (de Ruiter *et al.*, 2007), obstructing circulation to the contracting muscle with minimal efflux on lactate and H^+ (Ahlborg *et al.*, 1972). The active muscle becomes a closed system, solely dependent upon anaerobic energy provision, with physiochemical buffers providing the only defence against local changes in pH (Sale *et al.*, 2012). In 1960, the Rohmert equation was formulated, following the discovery of a non-linear relationship between an individual's ability to maintain a static force and the percentage of MVIF (Rohmert, 1960; Bloswick & Ellis, 1974). It was predicted, based on the following equation (Equation 3.2), that a constant isometric contraction of the knee extensors would fail to maintain 45% MVIF after approximately 78 s (Ahlborg *et al.*, 1972).

$$T(\mathrm{s}) = -90 + \left(\frac{126}{P}\right) - \left(\frac{36}{P^2}\right) + \left(\frac{6}{P^3}\right)$$

Equation 3.2: The Rohmert equation (Rohmert, 1960), where *P* is the decimal percentage of maximal isometric voluntary force, *T* is the time window in seconds (s).

3.6.3.1 Protocol

Participants were instructed to contract their dominant leg and hold this isometric contraction at 45% MVIF. Individual targets were visually displayed on-screen in the form of a horizontal cursor, with participants asked to complete the IKET as close as possible to the target force. Two additional horizontal cursors were displayed to indicate 5% above and below the target force (Fig. 3.6). Verbal feedback was provided throughout the contraction, including "too high", "too low" or "on the line". The start of the IKET was defined as when the force response reached greater than 40% MVIF for more than one second (Sale *et al.*, 2010), and ended when force fell below 40% MVIF (5% drop from target) for more than three seconds, despite strong verbal encouragement (Nordez *et al.*, 2009; Nam *et al.*, 2013; McPhee *et al.*, 2014).

3.6.3.2 Assessed variables

The Rohmert equation (Equation 3.2) was used to calculate, 1) time to task failure (TTF) at 45% of MVIF force; ~78 s (Ahlborg *et al.*, 1972), and 2) the hold MVIF percentage based on the recorded TTF. The impulse (kN·s) of each IKET was calculated from the average force production, and the TTF of the fatigue hold contraction. For confirm the presence of muscle fatigue following the IKET, MVIF was recorded immediately following the IKET, declines in force generation indicated the presence of muscle fatigue (Hassanlouei *et al.*, 2012).

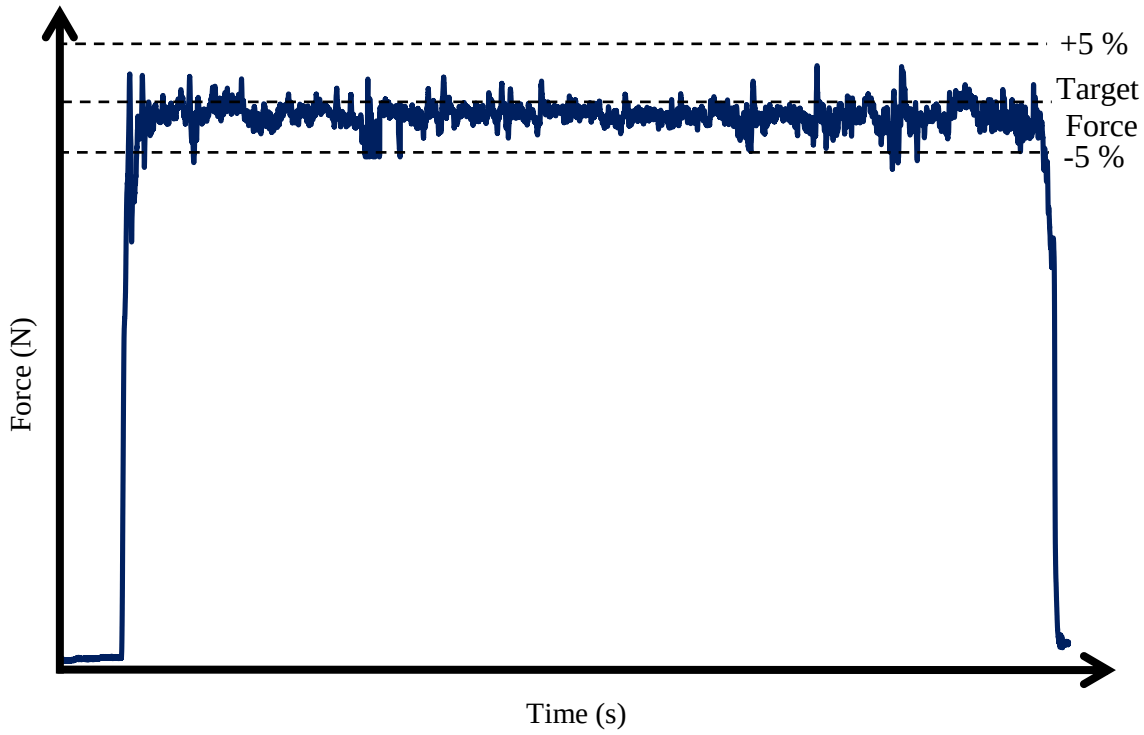

Fig. 3.6: Representative records from a single participant displaying the force responses of the knee extensors during an isometric knee extensor task, including three on-screen horizontal cursors depicting the target force (45% maximal isometric voluntary force) and 5% above and below the required target.

3.6.4 Twitches

Through the activation of a single action potential, via a 1 Hz (pulse per second) electrical impulse, the skeletal muscle contracts quickly, generating a small force response, referred to as a twitch contraction (Enoka, 2008; Fig. 3.7). Twitch contractions are the smallest contractile response in skeletal muscle and can last as little at 7.5 ms, yet can last ~100 ms (Martini, 1998). A twitch stimulus delivered in a voluntary passive state, generates a resting or un-potentiated twitch response, whilst stimulus delivered shortly following a voluntary muscle contraction, is referred to as a potentiated twitch (Rassier & MacIntosh, 2000; Hodgson *et al.,* 2005; Ebben, 2006; Fukutani *et al.*, 2013; Fig. 3.7). Potentiated twitches generally generate larger force responses compared to resting twitches, with increases up to 100% greater than those at rest (Kopman *et al.,* 2001). These increases are associated with the phosphorylation of the myosin regulatory light chain, leading to a larger number of attached cross brides at a given Ca^{2+} concentration (Sweeney *et al.,* 1993; Rassier & MacIntosh, 2000; Kamm & Stull, 2011). Potentiated twitches display increased peak force, twitch RFD and larger declines in TPT compared to resting twitches (Sweeney *et al.,* 1993; O'Leary *et al.,* 1997; Fig. 3.7). Potentiated twitches also demonstrate higher level of reproducibility compared to their resting twitch counterparts, with greater levels of sensitivity to fatigue (Kufel *et al.,* 2002).

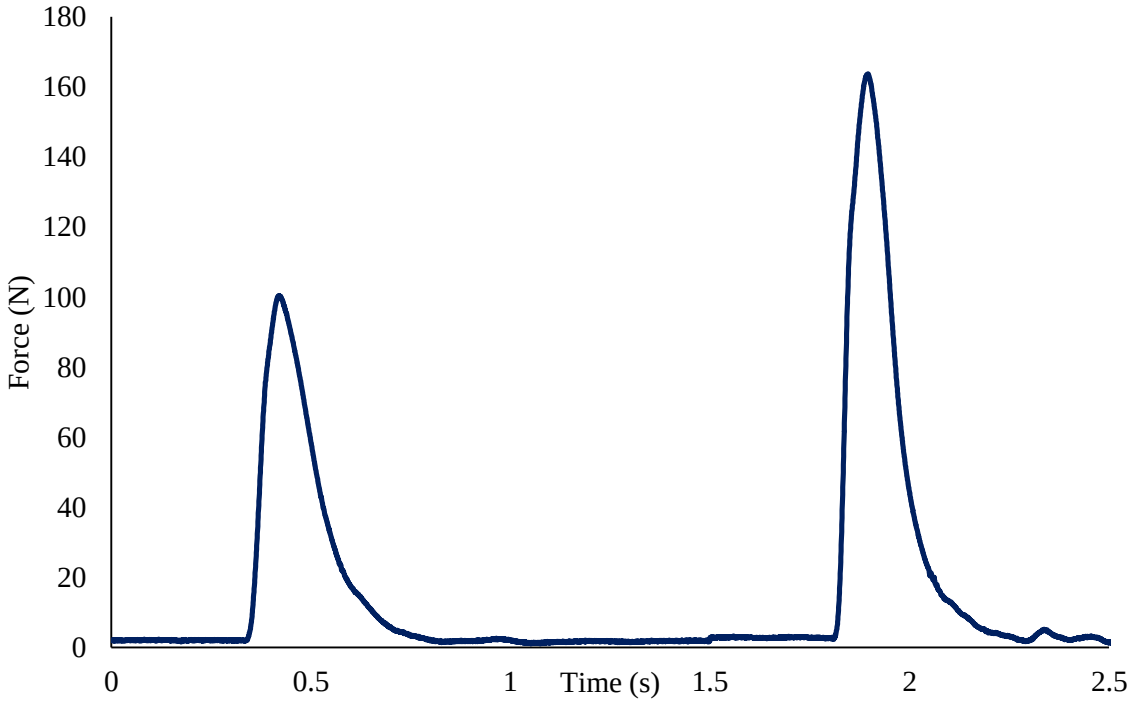

Fig. 3.7: Representative records from a single participant displaying force responses of the knee extensors following an evoked resting (before completion of a maximal voluntary isometric contraction) and a potentiated twitch (following a maximal voluntary isometric contraction).

3.6.4.1 Protocol

A twitch contraction as defined a single electrical impulse via femoral nerve stimulation, was delivered in stepwise increments, separated by greater than ten seconds to allow for neuromuscular recovery. Twitch contractions were elicited under rested conditions until a plateau in twitch force and M-wave amplitude were achieved. These plateaus indicated maximal depolarisation of the femoral nerve, to ensure supramaximal stimulation was achieved, the current was increased by 25%, to account for activity dependant changes in axonal excitability (Burke, 2001). Three discrete supramaximal stimuli were delivered to achieve a maximal resting twitch response. Resting twitches are defined as when the electrical stimuli are delivered under rested conditions. Following the completion of a MVIC, two femoral nerve supramaximal stimuli were delivered, the response to this stimulation resulted in a potentiated twitch response.

3.6.4.2 Assessed Variables

Resting and potentiated twitches were assessed for several force response variables including; peak twitch force, the time recorded between the activation of the first electrode (M-wave onset) and force onset (electromechanical delay; EMD; Cavanagh & Komi, 1979; Fig. 3.8), TPT (Fig. 3.9), RFD measured at 25 ms, 50ms and peak force, and HRT (Fig. 3.9). The M-wave area was recorded and averaged across all quadricep muscle sites (*m. rectus femoris*, *m. vastus medialis* and *m. vastus lateralis*), with the mean M-wave area of the three supramaximal stimuli defined as the M_{max} (Fig. 3.10). The M-wave area is a function of and incorporates both the amplitude and the duration of the assessed muscle. This representation of the M-wave has been suggested to be a more accurate estimate of the number of axons or muscle fibres firing, compared to assessing amplitude in isolation (Weiss *et al.*, 2015), thus providing a better overall representation.

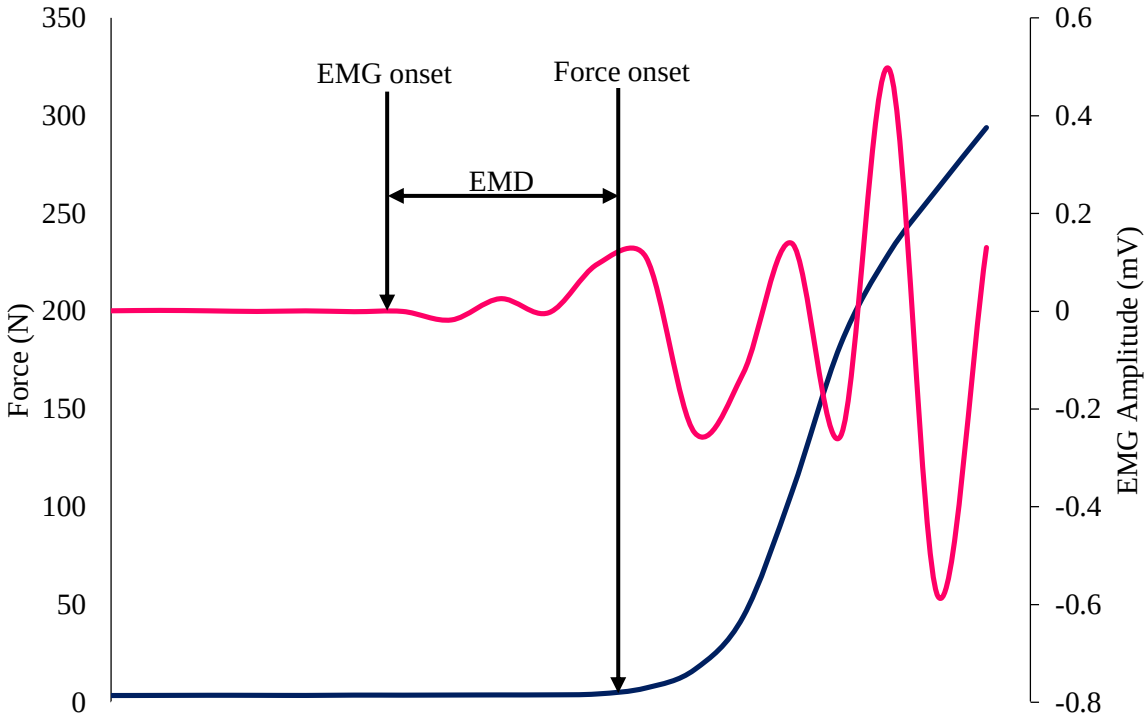

Fig. 3.8: Representative records from a single participant displaying knee extensor force (blue line) and agonist surface electromyography (EMG) (pink line) from the *m. rectus femoris* during the onset of an isometric explosive contraction. Electromechanical delay (EMD) is reported as the time delay between onset of EMG activity and force, identified manually.

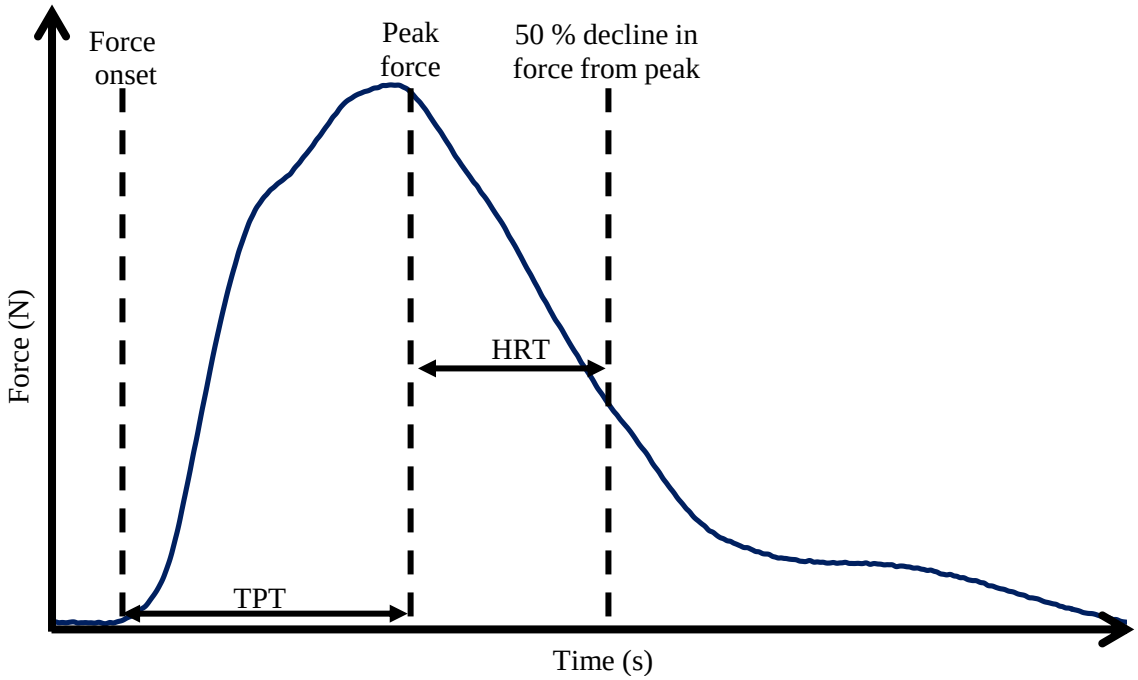

Fig. 3.9: Representative record from a single participant displaying force responses of the quadricep muscle following an electrically evoked resting twitch. Time-to-peak tension (TPT), peak force and half relaxation time (HRT) are reported.

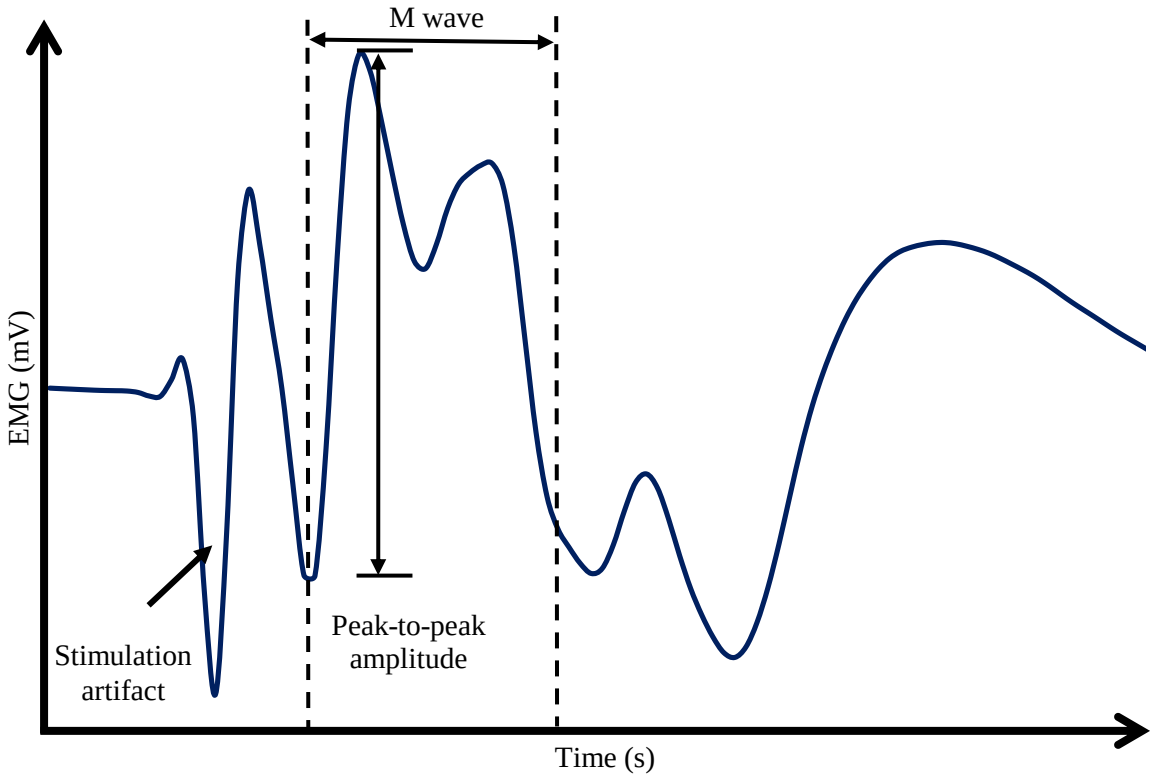

Fig. 3.10: Representative record from a single participant displaying surface electromyography (EMG) response from the *m. rectus femoris* following an evoked resting twitch. Muscle action potential (M-wave), stimulation artefact and peak-to-peak amplitude are reported.

3.6.5 Doublets

Doublet discharges are two closely spaced action potentials (<10 ms; Simpson, 1969; Christie & Kamen, 2006), which can be evoked by femoral nerve electrical stimulation (Gorassini *et al.,* 2000). In human skeletal muscle, doublets are shown to increase in frequency following exercise training, with a parallel increase in the RFD of the muscle (Van Cutsem *et al.*, 1998). Eliciting an electrical evoked doublet generates a nonlinear increase in force, larger than the sum of two twitches (Duchateau & Hainaut, 1986).

3.6.5.1 Protocol

Electrically evoked doublet contractions (two impulses at 100 Hz) were achieved through femoral nerve stimulation, initially delivered at a supramaximal current (+25%) with force and RFD recorded. Between subsequent stimuli, current intensity was increased (~5%), until no further increases were shown, confirming that maximal force and RFD had been obtained. If the first stimuli produced a submaximal response, a fourth stimuli were delivered to ensure three maximal responses. A maximum of four stimuli were elicited to prevent muscle damage.

3.6.5.2 Assessed Variables

Doublets contractions were assessed for peak force (Fig. 3.9), EMD (Fig. 3.8), TPT, and HRT (Fig. 3.9). All measurements were averaged across the three final contractions.

3.6.6 Octets

Electrically evoked supramaximal octet contractions (eight pulses at 300 Hz; Fig. 3.11) provide a reliable method of examining the maximal evoked explosive performance of the motor tendon unit and more reliable than the twitch for assessing peak RFD (de Ruiter, 2004). Octet RFD between 0 - 50 ms has been reported as the primary determinant of the voluntary RFD between 50 - 100 ms, accounting for 68% of the variance once agonist activation was greater than 50 ms (Folland, Buckthorpe & Hannah, 2013). Evoking octet contractions declines the inter-individual variability noted during voluntary contractions, with voluntary responses two to three times higher than those of evoked octet contractions (Folland, Buckthorpe & Hannah, 2013).

3.6.6.1 Protocol

Electrically evoked octet contractions (Fig. 3.11) were achieved through femoral nerve stimulation, initially delivered at a supramaximal current (+25%) with force and RFD recorded. Between subsequent stimuli current intensity was increased (~5%), until no further increases were seen, confirming that maximal force and RFD had been obtained. If the first stimuli produced a submaximal response, a fourth stimuli was delivered to ensure three maximal responses. A maximum of four stimuli were elicited to prevent muscle damage.

3.6.6.2 Assessed Variables

Octet contractions were assessed for peak force (Fig. 3.9), EMD (Fig. 3.8), TPT, and HRT (Fig. 3.9). All measurements were averaged across the three final contractions.

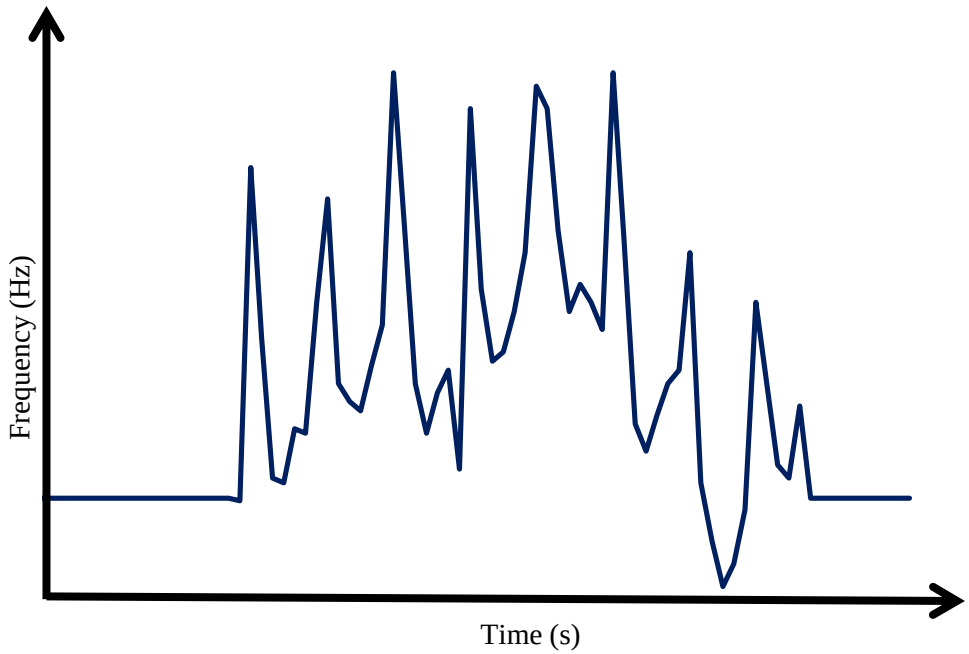

Fig. 3.9: Representative records from a single participant displaying force response of the quadricep muscles to an eight impulse 300 Hz octet contraction delivered via electrically evoked femoral nerve stimulation.

3.6.7 Submaximal contractions / Force-electromyography relationship

The relationship between force and EMG amplitude provides a greater understanding of the association between changes in activation and force (Tillin *et al.*, 2010; Saito & Akima 2013). The characteristics of the force-EMG relationship demonstrates the neuromuscular properties of the assessed contracting muscle (De Luca, 1997), with the movements in this relationship related to the recruitment and firing rate of the muscle motor units (Saito & Akima, 2013). The EMG-force relationship has been assessed in several muscles, including the knee extensors (Pincivero & Coelho, 2000; Pincivero *et al.*, 2003; Watanabe & Akima, 2009). The relationship between force and EMG amplitude is directly proportional with increased force, resulting in increased EMG signal, this has however, been widely disputed (Bilodeau *et al.*, 2003; Gerdle *et al.*, 1991; Herzog *et al.*, 1998; Karlsson & Gerdle, 2001; Moritani & Muro, 1987). The discrepancies in findings are likely due to the limitations of EMG assessments, including cross talk between signals, location of the electrode, and in most experiments the catchment area of the electrode does not extend sufficiently to detect the signal generated across the entire muscle volume (De Luca, 1997; Siegler *et al.*, 1985). There are also known differences between muscle recruitment during voluntary contractions, assessment of EMG during these contractions provides a way to verify differences in the activation behaviour of the different quadriceps muscles (Pincivero *et al.*, 2003). During voluntary isometric contractions, the *m. vastus lateralis* contributes the most to the muscle contraction compared to the *m. vastus medialis* or the *m. rectus femoris*, with the lowest contribution from the *m. vastus medialis* (Pincivero *et al.*, 2003).

3.6.7.1 Protocol

Participants were instructed to produce submaximal voluntary contractions to set target forces (15, 30, 45, 60, 75 and 90% MVIF) as identified using on-screen horizontal cursors. Individuals were asked to reach the target force as quickly as possible and to maintain the contraction for ~3 s in duration, with each voluntary contraction separated by greater than 20 s (Fig. 3.12).

3.6.7.2 Assessed Variables

The RMS was calculated during each voluntary contraction, with force and EMG measured over a 500 ms stable part of the voluntary contraction. The EMG RMS values were normalised to M_{max} and plotted against the respective force values. Linear regression was used to assess the slope and intercept of the force-EMG relationship incorporating all data between 15 - 90% MVIF.

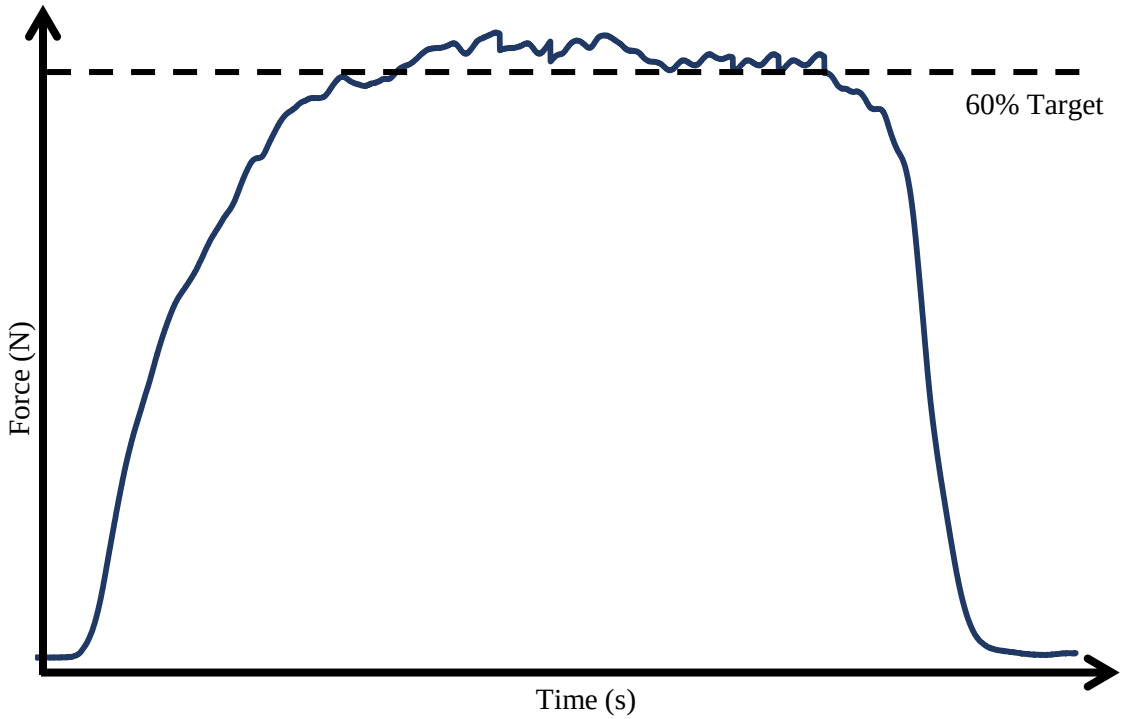

Fig. 3.11: Representative records from a single participant displaying force responses of the quadricep muscle during a submaximal contraction held at 60% maximal isometric voluntary force.

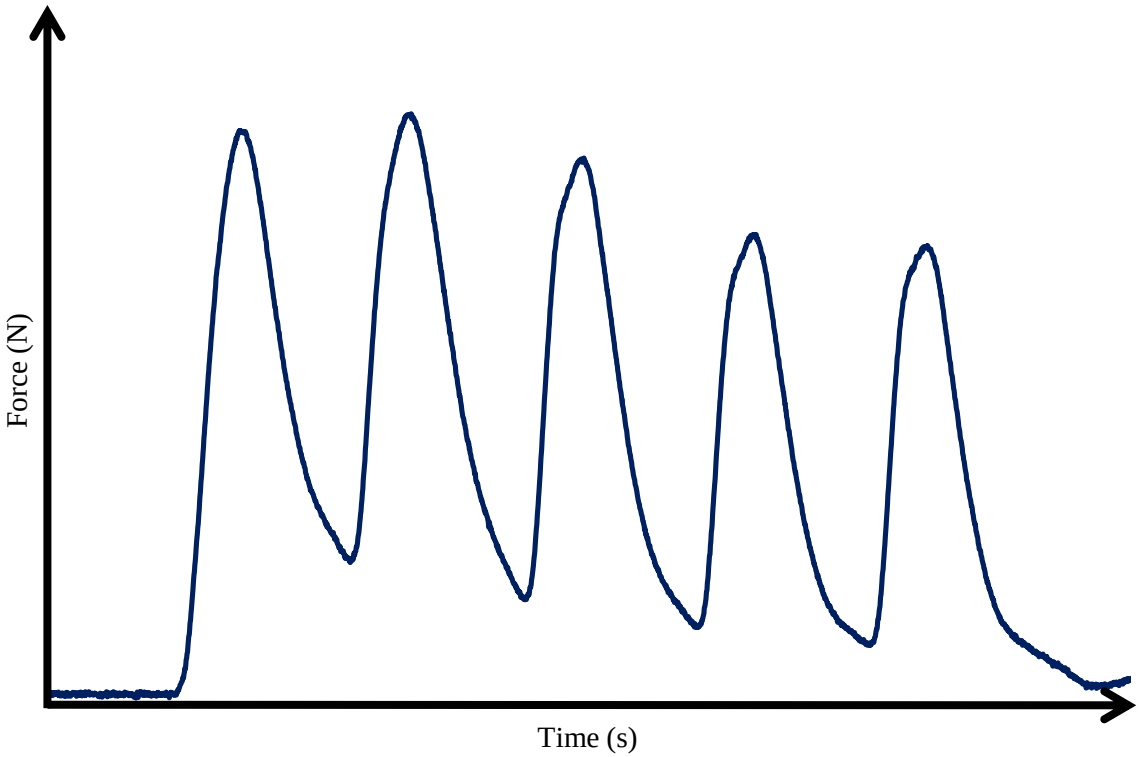

Fig. 3.11: Representative records from a single participant displaying force response of the quadricep muscle during a 5 Hz percutaneous stimuli, evoking tetanic contraction.

3.6.8 Force-frequency relationship

The force-frequency relationship is the association between skeletal muscle force output and electrically evoked stimulation frequency. Stimulation of the assessed skeletal muscle occurs at frequency from 1 Hz to 100 Hz. At low levels of stimulation frequencies (<5 Hz) the quadriceps muscles can contract and reach near full relaxation between pulses. As stimulation frequency is increased, complete relaxation of the skeletal muscle is unable to occur, the twitch contractions therefore begin to summate, producing a greater skeletal muscle force output. The contractile responses when the muscle is unable to completely relax prior to the following stimulus, is referred to as a tetanic contraction (MacIntosh *et al.*, 2006). Eventually maximal summation occurs, with the force output from the assessed muscle plateauing at maximal force (Binder-Macleod & McDermond, 1992). The slope and the half-maximal stimulation frequency become defining characteristics of the force-frequency relationship. To compare individual performances, force data is normalised by dividing the peak force produced at each frequency by the maximum peak force produced during the task, normally (but not always) produced during the 100 Hz contraction (Binder-Macleod & McDermond, 1992). The force-frequency relationship provides a mechanism to describe the contractile properties of the assessed muscle. Leftward shifts in the slope demonstrate the skeletal muscle force has become slower, whilst shifts in the force-frequency curve to the right denotes an increase skeletal muscle speed (Jones, 1996). One such example of the force-frequency curve shifting is during the ageing process, the relaxation properties of the triceps, quadriceps and adductor pollicis muscles slow, shifting the force-frequency curve to the left (Davies & White, 1983; Narici, Bordini & Cerretlli, 1991; Roos *et al.*, 1999; Stevens *et al.*, 2001). Alterations to the force-frequency relationship are suggested to be caused by Ca^{2+} kinetics, through adjustments to the sensitivity of the contractile properties to Ca^{2+} (Jones, 1981) or the amount of Ca^{2+} released from the SR (Westerblad *et al.*, 1993).

3.6.8.1 Protocol

Percutaneous electrical stimulation was used to evoke tetanic contractions of the quadriceps muscles (*m. rectus femoris*, *m. vastus medialis*, and *m. vastus lateralis*). Initially an evoked contraction at 100 Hz was delivered, with progressively increased intensity until the current elicited a skeletal muscle contraction at a force output of 50% MVIF (Fig. 3.13). The current was then maintained to evoke a 1 Hz contraction of the assessed skeletal muscle, followed by nine single contractions at increasing intensities (5, 10, 15, 20, 30, 40, 50, 80, 100 Hz) of one second duration, each contraction was separated by around 30 s.

3.6.8.2 Assessed variables

For each evoked stimulation (1 – 100 Hz), peak force was defined as the greatest instantaneous force during the whole tetanic contraction. Thereafter, the force values at each

stimulation frequency were normalized to the force produced during the 100 Hz contraction. The force-frequency relationship was fitted with a Hill curve and evaluated for frequency at 50% of the maximum force response (Dutka *et al.*, 2012).

Chapter 4: Effect of β-alanine supplementation on neuromuscular performance in fresh skeletal muscle

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Stannard, R.L., Hannah, R., Minshull, C., Artioli, G.G., Harris, R.C., Sale, C. (2015). β-alanine Supplementation Enhances Human Skeletal Muscle Relaxation Speed but Not Force Production Capacity. STAR conference, Nottingham Trent University.

Disclosure:

It should be noted that data collection was performed by Dr. Ricci Hannah (15 participants) and myself (8 participants), with 23 participants completing the whole protocol.

4.1 Introduction

Carnosine was first described as an intracellular pH buffer in 1938 (Bate-Smith, 1938; Deutsch & Eggleton, 1938), due to its molecular structure, the role of carnosine as an intracellular pH buffer is undisputable (Bate-Smith *et al.*, 1938; Sale *et al.*, 2010; 2013; Fig. 2.7), suitable over the whole exercise induced intramuscular pH transit-range (Bate-Smith *et al.*, 1938; Harris *et al.*, 2006). It is unsurprising that improvements in high-intensity exercise performance following increased skeletal muscle carnosine content, via β-alanine supplementation, have been associated with improved intracellular buffering capacity, resulting in improved exercise performance (see review; Sale *et al.*, 2013). Several other potential mechanisms for the enhancement in exercise performance following increased carnosine content via β-alanine supplementation have been proposed (see review; Begum *et al.*, 2005; Sale *et al.*, 2010). One such mechanism is the suggested influence of carnosine on Ca^{2+} kinetics within the skeletal muscle. Altering skeletal muscle carnosine content may influence the amount of Ca^{2+} released during skeletal muscle contraction, decreasing the inhibitory effect of low Mg^{+} concentrations (Batrukova & Rubtsov, 1997). Evidence in skinned animal muscle fibres suggested that carnosine influenced the sensitivity of the contractile apparatus to Ca^{2+} (Lamont & Miller, 1992; Dutka & Lamb, 2004), rather than increasing the amount of Ca^{2+} released. In-line with these data, skinned human *m. vastus lateralis* muscle fibre research has shown similar increases in contractile property Ca^{2+} sensitivity following increased carnosine concentrations (Dutka *et al.*, 2011). Raising skeletal muscle carnosine content, via β-alanine supplementation, could influence the amount of Ca^{2+} released during skeletal muscle contraction and/or the sensitivity of contractile properties to Ca^{2+}, thus alleviating the decline in *in-vivo* exercise performance (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.*, 2011; Everaert *et al.*, 2013). Research in nine male mice was the first to examine the influence of increased skeletal muscle carnosine content on *in-vivo* muscle contractile properties, through supplementation of drinking water with β-alanine (Everaert *et al.*, 2013). Supplementation with β-alanine for eight-weeks significantly increased skeletal muscle carnosine content and relative force of the *m. extensor digitorum longus* by 10 to 31%, inducing a leftward shift of the force–frequency relationship (Everaert *et al.*, 2013). These data highlight that increased skeletal muscle carnosine content, via β-alanine supplementation, may alter *in-vivo* human contractile properties and skeletal muscle performance via a Ca^{2+} kinetics mechanism. Nonetheless, it remains unclear if the relationship between skeletal muscle carnosine content and Ca^{2+} kinetics is apparent within a human *in-vivo* scenario.

As such, the current investigation examined the effect of β-alanine supplementation on intrinsic *in-vivo* knee extensor force production and skeletal muscle contractility in human skeletal muscle. It was hypothesised that β-alanine supplementation would improve intrinsic contractile properties, creating a leftward shift in the force-frequency relationship, enhancing peak and

explosive force responses, and thereby increasing explosive voluntary force production. Furthermore, it was hypothesised that the altered contractile properties would lead to a shift in the force-EMG relationship towards lower EMG levels for a given level of force.

4.2 Methodology

4.2.1 Participants

Twenty-six male participants provided written informed consent and were stratified and allocated into the two supplementation groups [placebo or β-alanine] based on MVIF values recorded during the familiarisation session. Three participants withdrew from the investigation (two from placebo and one from β-alanine); one due to a lack of tolerance of electrical stimulation and two provided no reason. As such, 23 participants completed all aspects of the investigation (Table 4.1). This research was approved by the Nottingham Trent University Human Ethical Review Committee (Application #265). All participants were 'moderately active' based on their short-form International Physical Activity Questionnaire (IPAQ) physical activity score with no significant difference between groups at baseline (β-alanine: 3368 ± 1600 Metabolic Equivalent of Task (METs); Placebo: 2429 ± 877; independent sample t-test, P = 0.13).

Table 4.1: Characteristics of participants (n = 23).

	Placebo (n = 11)	**β-alanine (n = 12)**
Age (years)	26 ± 6	26 ± 7
Body mass (kg)	79.1 ± 13.0	82.4 ± 15.2
Height (m)	1.80 ± 0.07	1.79 ± 0.06
MVIF (N)	602 ± 108	617 ± 111

No significant differences were reported. MVIF: Maximal voluntary isometric force. Data are mean ± 1SD.

4.2.2 Experimental design

Over a five-week period, three experimental sessions were undertaken by each participant; familiarisation sessions were held around seven days prior to the baseline session, with the final session conducted following 28-days of supplementation with either β-alanine or placebo. Participants were familiarised with the voluntary and electrical evoked knee extensor contractions, with all assessments undertaken on the dominant leg. Baseline and the follow-up session involved identical protocols, performed in accordance to a strict schedule, and undertaken at a consistent time of day. Participants were instructed to abstain from alcohol and strenuous/unaccustomed exercise for 36 h before each session, with caffeine prohibited on the day of the protocol. Compliance with these requests was confirmed verbally with the participant before commencement of the protocol. All raw data analyses, exclusions, and statistical analyses were undertaken by an experimenter blind to supplemental group, ensuring double-blind status.

4.2.3 Experimental protocol

Upon arrival to the familiarisation session participant's height and weight were recorded. Participants were seated in a rigid, custom-built isometric dynamometer (Fig. 3.5.3; page 36) with hip and knee joint angles of approximately 95° and 100° (180° = full extension), as previously described (Hannah *et al.*, 2012; 2013). Isometric knee extension force and EMG signals were recorded during all sessions, the details of which have been described in sections 3.5.3 and 3.5.4, page 38-39. Several isometric voluntary and electrically evoked contractions were undertaken during each session, including:

a) MVIC; as described in section 3.6.1, page 42.
b) Explosive voluntary contraction; as described in section 3.6.2, page 44.
c) Twitches (resting and potentiated); as described in section 3.6.4, page 49.
d) Octet contractions; as described in section 3.6.6, page 54.
e) Force-EMG relationship; as described in section 3.6.7, page 55.
f) Force-frequency relationship; as described in section 3.6.8, page 57.

4.2.4 Supplementation

Participants were supplemented with 6.4 $g \cdot day^{-1}$ of either β-alanine or a matched placebo in tablet form over 28-days. The supplementation protocol consisted of two 800 mg tablet ingested four times per day, at 2 - 4 h intervals. Compliance with the supplementation period was monitored with compliance logs, both groups reported a high degree of compliance, reported at 91 ± 7% in the β-alanine group (total dose of 163.1 ± 12.5 g) and 88 ± 10% in the placebo group (total dose of 157.7 ± 1.8 g; independent sample t-test, P = 0.60). Participants supplemented with β-alanine would expect an increase in skeletal muscle carnosine content of ~15 $mmol \cdot kg^{-1}$ dry muscle (~65%) from baseline based on comparable β-alanine supplementation protocols (Harris *et al.*, 2006). There were no reported symptoms of paraesthesia from any of the participants during the supplementation period.

4.2.5 Statistical analysis

The G* Power 3.1.6 software programme was used to calculate an *a priori* power calculation for sample size, having as parameters: statistical test (ANOVA), $\alpha = 0.05$, $\beta = 0.80$ (power of the sample), number of groups analysed (2 = β-alanine and placebo), and the effect of sample size based on previous findings (Hobson *et al.*, 2012). According to the calculations, a minimum of 22 participants would be required for the study, with 26 participants being recruited to allow for dropouts. A three-way analysis of variance (ANOVA) (group × session × time point) was used to assess dependent variables (force and EMG during explosive voluntary contractions, evoked twitch, and octet force) and the force-frequency relationship (group × session × frequency).

MVIF, HRT, TPT, the force-EMG relationship, and the force-frequency relationship (at 50%) were evaluated using a two-way ANOVA (group × session). Assessed variables were tested for normality using the Shapiro-Wilks test, and for homogeneity using the Fevene test. A Greenhouse-Geisser correction was applied when the ANOVA assumption of sphericity was violated, and significant interaction effects were followed-up by independent sample *t*-tests on the individual percentage change values for each condition. ES for multiple comparisons was calculated using partial (η_p^2) and generalised (η_g^2) eta squared (Lakens, 2013). Providing two ESs is suggested to yield a greater understanding of a specific effect (Preacher & Kelly, 2011). *Post hoc* comparisons to explain any significant interactions are reported with Cohen's *d* and Hedges *g* ES. An ES of 0.2 – 0.5 was defined as small, 0.5 – 0.8 as medium and ≥ 0.8 as a large effect (Schünemann *et al.*, 2008). Intra-individual variability was calculated using the mean intra-individual coefficient of variation (CV) between the pre- and post-supplementation sessions for the placebo group [(SD / mean) × 100]. Statistical analyses were completed using SPSS version 22 (SPSS Inc., Chicago, IF, USA) and Microsoft Excel (Microsoft Inc., USA). Statistical significance was accepted at $P \leq 0.05$, with data presented as mean ± 1 SD.

4.3 Results

4.3.1 Maximum and explosive voluntary contractions

MVIF was unaffected by 28-days of supplementation in fresh skeletal muscle (P = 0.17; f (1, 21) = 1.99, η_p^2 = 0.1, η_g^2 < 0.1; Fig. 4.1). The mean CV within the placebo group was 3%. Correspondingly, β-alanine supplementation showed no effect on explosive voluntary force measured at 25 ms intervals (P = 0.82; f (5, 105) = 0.44, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.56; f (1, 21) = 0.36, η_p^2 = 0.2, η_g^2 < 0. 1 (group × session); Fig. 4.1). The mean CV in the placebo group, across 25 - 50 ms window was 13 - 17%, decreasing to 4 - 7% in the 75 - 150 ms window.

Agonist EMG normalized to M_{max} during MVICs and explosive contractions were not influenced by supplementation (P = 0.69; f (3, 36) = 0.50, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.55; f (1, 21) = 0.36, η_p^2 < 0.1, η_g^2 < 0.1 (group × session); Fig. 4.1). The mean CV for the placebo group was 26% for the 0 - 50 ms window, 23% for the 50 - 100 ms window, 9% for the 100 - 150 ms window, and 13% for MVIF.

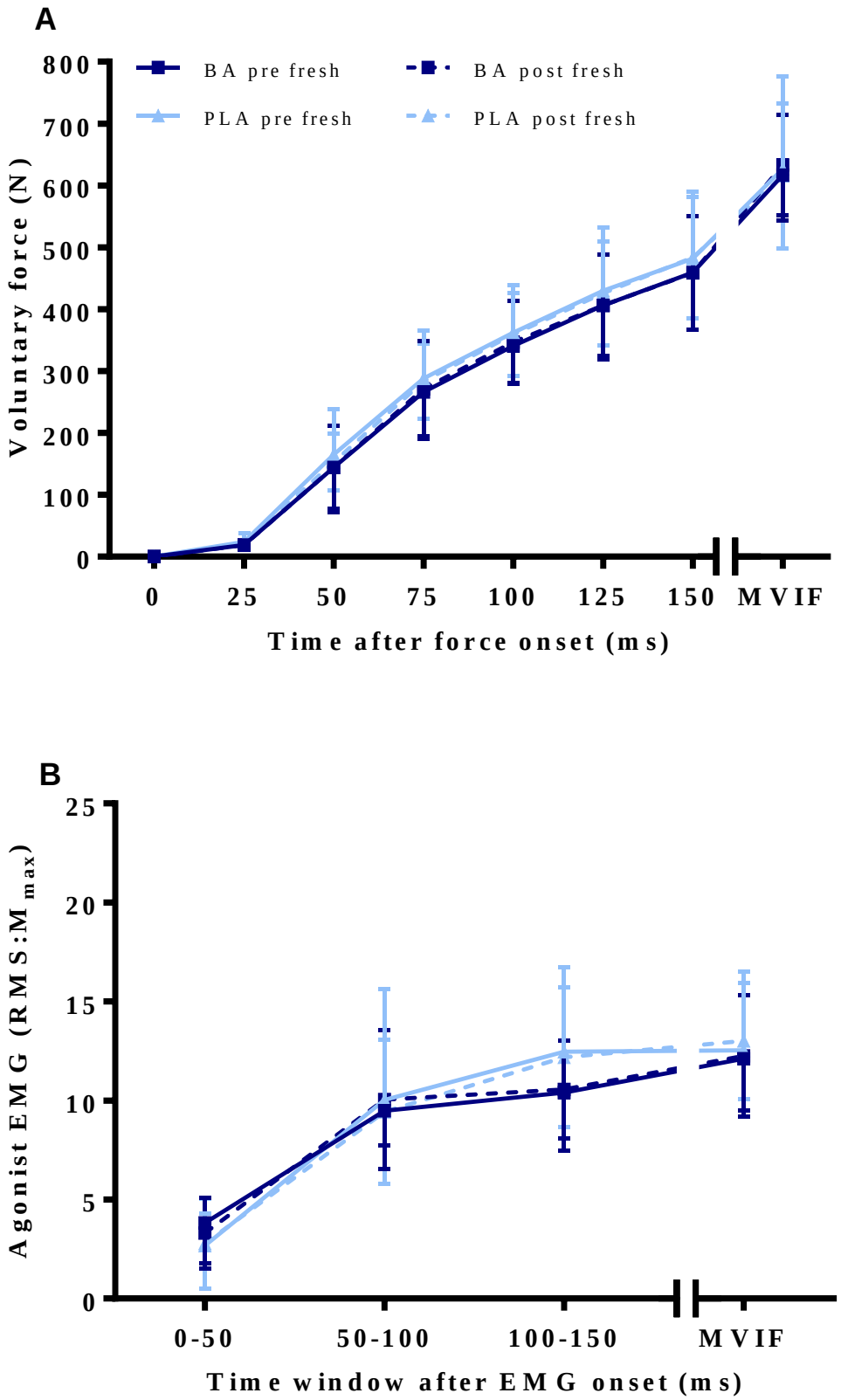

Fig. 4.1: Explosive and maximal isometric voluntary force (MVIF) (A), and agonist surface electromyography (EMG) normalised to maximum muscle action potential (M_{max}) during explosive contractions (0 - 50, 50 - 100 and 100 - 150 ms from onset) and at MVIF (B) for the β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation. RMS: Root mean square. Data are mean ± 1SD.

4.3.2 Twitches

Resting twitches: Supplementation did not significantly influence resting twitch force (P = 0.45; *f* (2, 40) = 0.82, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.70; *f* (1, 21) = 0.16, η_p^2 < 0.1, η_g^2 < 0.1 (group × session); Table 4.2), EMD (P = 0.55; *f* (1, 20) = 0.38, η_p^2 < 0.1, η_g^2 < 0.1; Table 4.2), or TPT (P = 0.79; *f* (1, 20) = 1.12, η_p^2 < 0.1, η_g^2 = 0.1 Table 4.2) in fresh skeletal muscle. There was, however, a significant group × session interaction for HRT (P = 0.02; *f* (1, 20) = 6.68, η_p^2 = 0.2, η_g^2 < 0.1; Fig. 4.2). *Post hoc* analysis showed that the percentage change in HRT was greater for the β-alanine group (-8 ± 10 ms; -10 ± 11%) compared with the increased responses in the placebo group (+3 ± 13 ms; 5 ± 12%; P = 0.006; *t* (21) = -3.0; 95% confidence interval [-24.3, -4.5], Cohen's *d* = 1.3; Hedge's *g* = 1.2). The confidence interval ES indicated that there was an 81% chance of a randomly selected pair of individuals would experience a decline in HRT following β-alanine supplementation. Mean CV values for fresh skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 14, 9 and 8%, EMD was 7%, TPT was 3% and HRT was 7%.

Potentiated twitches: Supplementation did not significantly influence resting twitch force (P = 0.43; *f* (2, 42) = 0.87, η_p^2 < 0.1, η_g^2 < 0.1; Table 4.2 (group × session × time point)), EMD (P = 0.47; *f* (1, 21) = 0.54, η_p^2 < 0.1, η_g^2 < 0.1; Table 4.2), or TPT (P = 0.29; *f* (1, 21) = 1.17, η_p^2 = 0.1, η_g^2 < 0.1 Table 4.2). There was, however, a significant group × session interaction for HRT (P = 0.04; *f* (1, 21) = 5.27, η_p^2 = 0.2, η_g^2 < 0.1; Fig. 4.2). *Post hoc* analysis showed that the change in HRT was greater for the β-alanine group (-12 ± 28 ms; -7 ± 11%) compared with the increased responses in the placebo group (+1 ± 5 ms; 1 ± 8%; P = 0.05 *t* (21) = -1.9; 95% confidence interval [-32.1, 0.9], Cohen's *d* = 0.8; Hedge's *g* = 0.8). The confidence interval ES indicated that there was a 71% chance of a randomly selected pair of individuals would experience a decline in HRT following β-alanine supplementation. Mean CV values for fresh skeletal muscle in the placebo group: force at 25 and 50ms and peak were 6, 3 and 3%, EMD was 6%, TPT was 3% and HRT was 4%.

4.3.3 Octet contractions

In fresh skeletal muscle, supplementation did not significantly alter octet EMD or TPT (Table 4.2). There was, however, a trend towards significance for HRT (P = 0.08; n_p^2=0.1; η_g^2=0.1). In fresh skeletal muscle, supplementation did not significantly alter octet force at 25 ms, 50 ms and peak (P = 0.62; f (2, 42) = 0.48, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × time point); P = 0.45; f (1, 21) = 0.60, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session); Table 4.2). Mean CV for the placebo group were 10, 3 and 4% for force at 25 ms, 50 ms and peak.

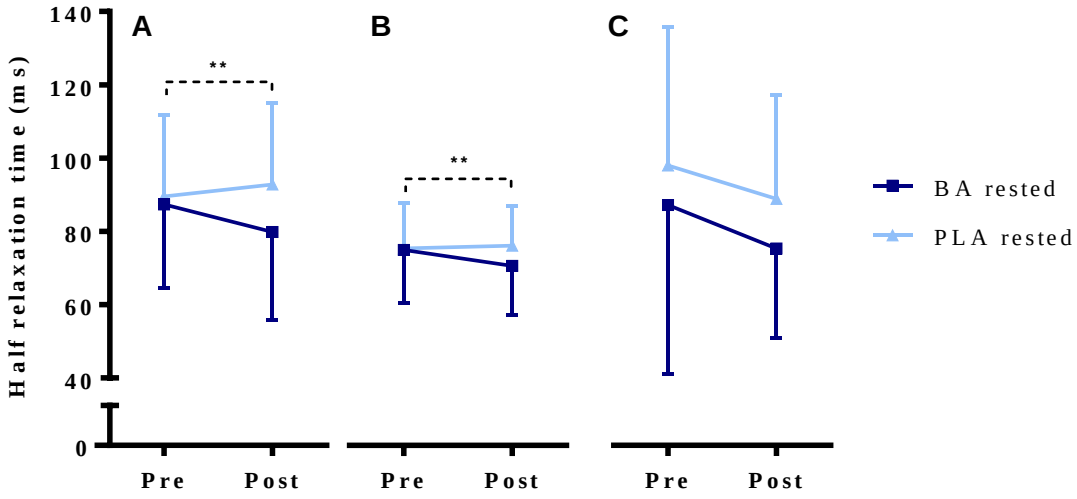

Fig. 4.2: Electrically evoked half relaxation time of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle during: resting twitch (a), potentiated twitch (b), and octets (c). Data are mean ± 1SD. **P = 0.006 and *P = 0.04 for *post hoc* independent t-test between β-alanine and placebo groups.

Table 4.2: Electrically evoked force responses, time-to-peak tension (TPT) and electromechanical delay (EMD) in β-alanine and placebo groups pre- and post-supplementation, in fresh skeletal muscle.

			Pre-supplementation					Post-supplementation				
			Force (N)			EMD (ms)	TPT (ms)	Force (N)			EMD (ms)	TPT (ms)
			25 ms	50 ms	Peak			25 ms	50 ms	Peak		
Resting Twitch	**Fresh**	**β-alanine**	31 ± 7	98 ± 21	127 ± 27	10 ± 1	86 ± 11	32 ± 7	100 ± 21	126 ± 25	10 ± 1	83 ± 7
	Fresh	**Placebo**	34 ± 12	107 ± 34	135 ± 43	10 ± 2	82 ± 5	36 ± 9	106 ± 24	130 ± 30	10 ± 1	80 ± 3
Potentiated Twitch	**Fresh**	**β-alanine**	69 ± 17	159 ± 29	193 ± 39	8 ± 1	82 ± 11	70 ± 18	164 ± 27	199 ± 34	9 ± 1	82 ± 11
	Fresh	**Placebo**	81 ± 23	171 ± 37	203 ± 45	9 ± 1	80 ± 9	79 ± 18	172 ± 33	204 ± 43	9 ± 1	83 ± 8
Octet	**Fresh**	**β-alanine**	86 ± 16	241 ± 29	384 ± 56	13 ± 1	134 ± 8	82 ± 16	244 ± 29	390 ± 57	12 ± 2	135 ± 9
	Fresh	**Placebo**	87 ± 18	238 ± 41	372 ± 89	14 ± 2	122 ± 18	79 ± 27	216 ± 76	344 ± 127	14 ± 3	118 ± 39

Data are mean ± 1SD.

4.3.4 Force-electromyography relationship

The slope and y-intercept of the force-EMG relationship were unaffected by supplementation in fresh skeletal muscle (*slope:* P = 0.13; f (1, 21) = 2.55, η_p^2 = 0.1, η_g^2 = 0.1; *y-intercept*: P = 0.39; f (1, 21) = 0.79, η_p^2 < 0.1, η_g^2 < 0.1 (group × session); Fig. 4.3). The mean CV in the placebo group for the slope of the force-EMG relationship was 15%.

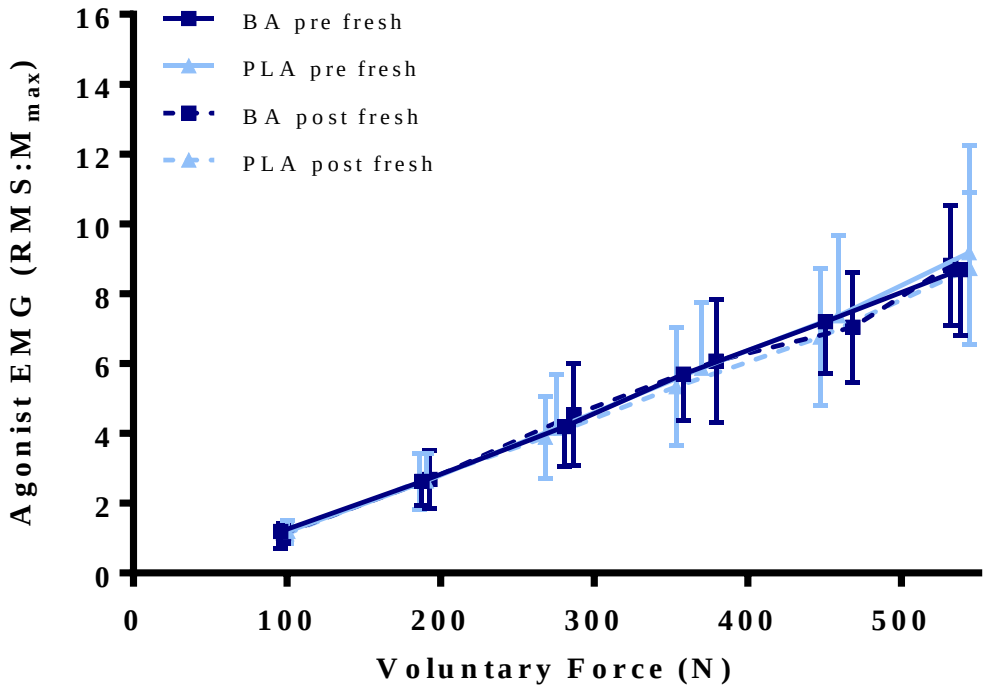

Fig. 4.3: Force-surface electromyography (EMG) relationship measured during submaximal voluntary contractions (15 – 90% maximal voluntary isometric force) for the β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation. M_{max}: Maximum muscle action potential; RMS: Root mean square. Data are mean ± 1SD.

4.3.5 Force-frequency relationship

Supplementation did not significantly influence peak force at each frequency of stimulation (P = 1.31; f (9, 189) = 6.24, η_p^2 = 0.1, η_g^2 < 0.1 (group × session × frequency); P = 0.50; f (1, 21) = 0.48, η_p^2 < 0.1, η_g^2 < 0.1 (group × session); Fig. 4.4). The frequency at 50% of the force response (Table 4.3) in fresh skeletal muscle was unaffected by supplementation. The mean CV for the placebo responses for relative force were 6 – 8% at 1 - 10 Hz, 1 – 3% at 15 – 80 Hz, and 6% at the maximum 100 Hz (50% of MVIF).

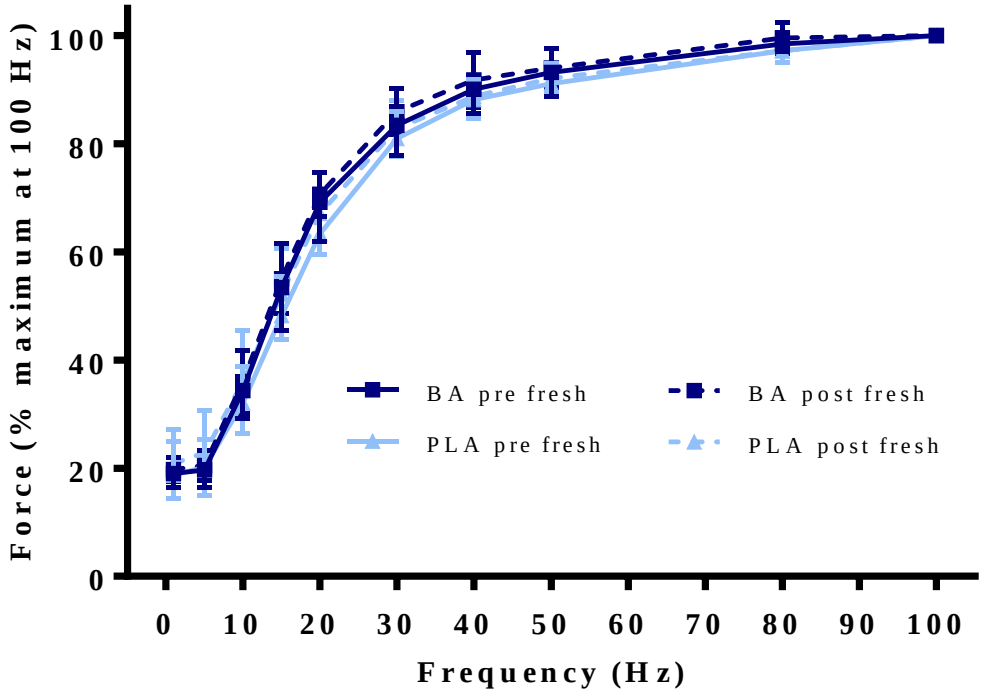

Fig. 4.4: Force-frequency relationship measured during percutaneous contractions pre- and post-supplementation with either β-alanine (BA) or placebo (PLA). Data are mean ± 1SD.

Table 4.3: Characteristics of the force-frequency and force-electromyography (EMG) relationships pre- and post-supplementation with either β-alanine or placebo.

	Pre-supplementation		Post-supplementation	
	β-alanine	**Placebo**	**β-alanine**	**Placebo**
Force-frequency relationship				
Frequency at 50% of response, Hz	17.3 ± 2.4	18.8 ± 1.5	16.8 ± 1.7	18.0 ± 1.9
Force-EMG relationship				
Intercept (RMS:M_{max})	-0.49 ± 0.74	-0.70 ± 0.47	-0.53 ± 0.91	-0.56 ± 0.43
Slope (RMS:M_{max}/N)	0.018 ± 0.005	0.018 ± 0.004	0.017 ± 0.004	0.017 ± 0.004

RMS: Root mean square. M_{max}: Maximum muscle action potential. Data are mean ± 1SD.

4.4 Discussion

There was no effect of 28-days β-alanine supplementation on the force-frequency relationship, the *in-vivo* analogue of the force-Ca^{2+} relationship (Balnave & Allen, 1996), at either relatively low (1 - 15 Hz) or high (20 - 80 Hz) frequencies. Supplementation with β-alanine resulted in no significant alterations to voluntary force responses during maximal and explosive contractions, or electrically evoked resting and potentiated twitches or octet contraction variables (EMD, peak force and TPT). These *in-vivo* data imply that β-alanine supplementation to increase skeletal muscle carnosine content, does not significantly influence Ca^{2+} kinetics or skeletal muscle performance. β-alanine supplementation did significantly decline skeletal muscle HRT, during electrically evoked resting and potentiated twitches (7 - 12%). The percentage change in skeletal muscle relaxation speed was greater for the β-alanine group than the placebo group, displaying a large effect in both resting and potentiated twitch responses (Cohen's d = 0.8 and 1.3).

The current findings are in contrast with previous research, where increased skeletal muscle carnosine content resulted in enhanced Ca^{2+} sensitivity in both human (Dutka *et al.,* 2012) and murine (Everaert *et al.,* 2013) skeletal muscle fibres. The discrepancies in outcomes may be associated with the nature of these investigations. The present research was conducted in *in-vivo* human skeletal muscle, whilst previous research reporting enhanced Ca^{2+} sensitivity with increased carnosine content were undertaken in *in-vitro* skinned muscle fibres (Dutka *et al.,* 2012; Everaert *et al.,* 2013). The manner in which *in-vitro* muscle fibres respond to increased carnosine content could have been directly influenced by the method and distribution of carnosine, influencing the contractile property responses. The proposed alterations to Ca^{2+} kinetics reported in *in-vitro* may have minor impact in an *in-vivo* scenario (Dutka *et al.,* 2012). The current investigation is limited by the lack of a direct skeletal muscle carnosine measurement following the β-alanine supplementation protocol. Nonetheless, it can be expected that participants would have experienced a 65% increase in skeletal muscle carnosine content from baseline, based on previous research (Harris *et al.,* 2006).

Voluntary contraction variables remained unaffected by β-alanine supplementation, which was unsurprising, due to the lack of effect that improved Ca^{2+} sensitivity of the contractile apparatus and/or the amount of Ca^{2+} released would potentially have on MVIF production (MacIntosh & Willis, 2000). There was also no significant influence of β-alanine supplementation on explosive force or the force-EMG relationship. Any potential changes in these measures could have been indicative of modifications to the neuromuscular activation required to produce a set amount of skeletal muscle force. Due to the lack of change in neural drive during both voluntary maximal and explosive contraction, it can also be suggested that β-alanine supplementation did not alter neuromuscular activation. The voluntary and electrically evoked data implied that increased

skeletal muscle carnosine content, achieved via β-alanine supplementation does not significant influence Ca^{2+} kinetics within a human *in-vivo* model.

There was, however, a significant decline in skeletal muscle relaxation speed, with a 7 – 12% decrease in HRT during both electrically evoked resting and potentiated twitch contractions. The interaction effect between groups over the supplementation period resulted in a medium effect during both resting and potentiated twitch contractions, with the percentage change in skeletal muscle relaxation speed greater for the β-alanine group than the placebo group. Alterations to the relaxation speed of the contracting skeletal muscle could prove beneficial, in regard to reducing the total energy expenditure and improving efficiency of joint movements experienced during high-intensity cyclic exercises. The speed at which skeletal muscle relaxes can be impacted by the rate of: (i) dissociation of Ca^{2+} from troponin (Little *et al.*, 2011); (ii) translocation of Ca^{2+} to near the site of entry into the SR (Muntener *et al.*, 1995); and (iii) re-uptake of Ca^{2+} into the SR by Ca^{2+} pumps (Nogueira *et al.*, 2013). No previous research has shown that carnosine influences these aspects of excitation-contraction coupling, although, Everaert *et al.*, (2013) did show decreased in fatigue related increases in skeletal muscle relaxation times following β-alanine supplementation in murine *m. soleus*. These findings in murine muscles, however, did not show any changes in resting rates of relaxation, and thus these findings could have been impacted by enhanced buffering capacity, rather than improved Ca^{2+} kinetics. Due to this unexpected finding, future research should examine the reproducibility of this proposed mechanism. There also needs to be greater examination of the impact of skeletal muscle fatigue on the contractile properties, providing improved understanding of the influence of β-alanine supplementation on skeletal muscle contractility, and the implications for metabolic and movement efficiency.

4.5 Conclusion

There was no effect of 28-days β-alanine supplementation, on the force-frequency relationship, suggesting a lack of influence of increased skeletal muscle carnosine concentration on the improved Ca^{2+} sensitivity of the contractile apparatus and/or the amount of Ca^{2+} released. There was no significant influence of β-alanine supplementation on voluntary and evoked force responses. These findings suggested that the improvements in exercise performance and capacity following β-alanine supplementation are not due to alterations to Ca^{2+} kinetics (sensitivity and/or release) in the skeletal muscle. There was, however, a significant decline in resting and potentiated twitch HRT following β-alanine supplementation. The reduction in skeletal muscle HRT could be explained by enhanced reuptake of Ca^{2+} into the SR, a factor known to alter muscle relaxation speed.

Chapter 5: Effect of β-alanine supplementation on neuromuscular performance in fresh and fatigued skeletal muscle

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5.1 Introduction

The data reported in study 1 (Chapter 4) was the first research to comprehensively examine the effect of 28-days of β-alanine supplementation on the electrically evoked contractile properties and voluntary force production of human skeletal muscle *in-vivo*. These data proposed that β-alanine supplementation did not alter the force-frequency relationship at either relatively low (1 - 15 Hz) or high (20 - 80 Hz) frequencies, with no significant alteration to voluntary force responses during maximal and explosive contractions, or electrically evoked resting or potentiated twitches and octets variables (EMD, peak force and TPT). These findings were in-line with the hypothesis arising from previous exercise performance studies (Sale *et al.,* 2010; 2013), that the main physiological role for carnosine in improving high-intensity exercise performance related to intracellular pH buffering, and not increased Ca^{2+} sensitivity of the *in-vivo* contractile apparatus. An unexpected finding was the significant reduction in skeletal muscle HRT during both resting and potentiated twitches following β-alanine supplementation. The finding was not an *a-priori* hypothesis, making it important to confirm these findings before exploring potential underlying mechanisms.

Swietach *et al.,* (2013) suggested that there may also be a mediating effect of pH on the interaction between L-histidine containing dipeptides (such as carnosine) and Ca^{2+}. High-intensity exercise leads to a more pronounced accumulation of H^+, a metabolic factor that might be involved in skeletal muscle fatigue, but in combination with other fatigue-induced changes or in an indirect manner (Westerblad, 2016). It should also be highlighted that skeletal muscle fatigue is multi-factorial phenomenon and can include alterations in neuromuscular transmission, muscle action potential propagation, excitation-contraction coupling and related contractile mechanisms (Boyas & Guevel, 2011). Skeletal muscle fatigue is generally accompanied by a marked slowing of relaxation (Allen, Lamb & Westerblad, 2008). H^+ are proposed to directly or indirectly inhibit sarcoplasmic Ca^{2+} release during skeletal muscle contraction (Laver, Eager, Taoube & Lamb, 2000; Laver, O'Neill & Lamb, 2004). Carnosine has the potential to serve as a cytoplasmic regulator of Ca^{2+} and H^+ coupling, since it binds to both ions (Baran, 2000). As such, it could be hypothesised that increasing skeletal muscle carnosine content, via β-alanine supplementation, would have a more pronounced beneficial effect on HRT when the skeletal muscle is fatigued.

Therefore, the present study aimed to examine the effect of β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and skeletal muscle contractility in both fresh and fatigued human skeletal muscle. It was hypothesised that 28-days of β-alanine supplementation would significantly decline in skeletal muscle HRT in fresh skeletal muscle, similar to findings in study 1 (Chapter 4). Additionally, due to the relationship between H^+ and

Ca^{2+} kinetics within the skeletal muscle it is hypothesised that there will be a decline in skeletal muscle HRT under fatigued conditions.

5.2 Methodology

5.2.1 Participants

Twenty-four male participants provided written informed consent and were then stratified and allocated into the two supplementation groups [placebo or β-alanine] based on MVIF values recorded during the familiarisation session. One participant withdrew from the investigation (placebo group) with no reason provided. As such, 23 participants completed all aspects of the investigation (Table 5.1). This research was approved by the Nottingham Trent University Human Ethical Review Committee (Application #369). Self-reported sleep quality categorised by the PSQI (Pittsburgh Sleep Quality Index), reported participants as good sleepers, with no differences between groups or following supplementation (Table 5.2). There was no significant difference between groups or pre- to post-supplementation regarding participant mood scores (Brunel University Mood Scale), or physical activity levels (International Physical Activity Questionnaires) with all participants categorised as 'moderately active' (Table 5.2). These additional questionnaires were included in-line with the development of the university sport science laboratory regulations, due to the potential impact of sleep, mood and physical activity of an individual's performance.

Table 5.1: Characteristics of participants (n = 23).

	Placebo (n = 11)	**β-alanine (n = 12)**
Age (years)	22 ± 1	22 ± 2
Body mass (kg)	81.4 ± 14.2	76.0 ± 7.3
Height (m)	1.83 ± 0.06	1.80 ± 0.05
MVIF (N)	600 ± 149	565 ± 86

No significant differences were reported. MVIF: Maximal voluntary isometric force. Data are mean ± 1SD.

5.2.2 Experimental design

Over a five-week period, three experimental sessions were undertaken by each participant; familiarisation sessions were held around seven days prior to the baseline session, with the final session conducted following 28-days of supplementation with either β-alanine or PLA. Participants were familiarised with the voluntary and electrical evoked knee extensor contractions, with all assessment undertaken on the dominant leg. Baseline and the follow-up session involved identical protocols, performed in accordance to a strict schedule, and undertaken at a consistent time of day. Participants were instructed to abstain from alcohol and strenuous/unaccustomed exercise for 36 h before each session, with caffeine prohibited on the day of the protocol. Compliance with these requests was confirmed verbally with the participant before commencement of the protocol. All

raw data analyses, exclusions, and statistical analyses was undertaken by an experimenter blind to supplemental group, ensuring double-blind status.

5.2.3 Experimental protocol

Upon arrival to the familiarisation session participant's height and weight were recorded. Participants were seated in a rigid, custom-built isometric dynamometer (Fig. 3.5.3; page 36) with hip and knee joint angles of approximately 95° and 100° (180° = full extension), as previously described (Hannah *et al.*, 2012; 2013). Isometric knee extension force and EMG signals were recorded during all sessions, the details of which have been described in sections 3.5.3 and 3.5.4, page 38-39. Several isometric voluntary and electrically evoked contractions were undertaken during each session, including:

a) MVIC; as described in section 3.6.1, page 42.
b) Explosive voluntary contraction; as described in section 3.6.2, page 44.
c) IKET; as described in section 3.6.7, page 47.
d) Twitches (resting and potentiated); as described in section 3.6.4, page 49.
e) Octet contractions; as described in section 3.6.6, page 54.
f) Force-EMG relationship; as described in section 3.6.7, page 55.
g) Force-frequency relationship; as described in section 3.6.8, page 57.

Fingertip blood lactate was recorded via fingertip capillary blood samples taken at rest, immediately prior to and 5 minutes following the IKET (as described in 3.5.2, page 37). The IKET was completed in accordance to the description within section 3.6.3, page 47.

5.2.4 Supplementation

Participants were supplemented with 6.4 $g \cdot day^{-1}$ of either β-alanine (sustained-release CarnoSyn™, NAI, USA) or a matched placebo (maltodextrin; NAI, USA) in tablet form over 28-days. All supplements were provided in identical white tubs by an individual not directly involved in testing or data analysis. The supplementation protocol consisted of two 800mg tablet ingested four times per day, at 2 - 4 h intervals. Compliance with the supplementation period was monitored with compliance logs, both groups reported a high degree of compliance, reported at 91 ± 7% in the β-alanine group (total dose of 163.1 ± 12.5 g) and 88 ± 10% in the placebo group (total dose of 157.7 ± 1.8 g; independent sample t-test, $P < 0.60$). Participants supplemented with β-alanine would expect an increase in skeletal muscle carnosine content of ~15 $mmol \cdot kg^{-1}$ DM (~65%) from baseline based on comparable β-alanine supplementation protocols (Harris *et al.*, 2006). There were no reported symptoms of paraesthesia from any of the participants during the supplementation period.

Table 5.2: Participant questionnaire scores.

	Placebo (n = 11)		**β-alanine (n = 12)**	
	Pre-supplementation	**Post-supplementation**	**Pre-supplementation**	**Post-supplementation**
PSQI	3 ± 1	3 ± 1	3 ± 1	3 ± 1
BRUMS				
Anger	6 ± 2	6 ± 2	5 ± 1	5 ± 1
Confusion	6 ± 2	6 ± 2	5 ± 1	4 ± 1
Depression	5 ± 1	5 ± 2	5 ± 2	5 ± 1
Fatigue	8 ± 1	7 ± 2	7 ± 2	7 ± 2
Tension	6 ± 2	6 ± 2	6 ± 3	6 ± 3
Vigour	12 ± 3	12 ± 4	12 ± 3	12 ± 3
IPAQ (METs)	2599 ± 1897	2770 ± 1979	3257 ± 1769	2876 ± 1760

No significant differences were reported. PSQI: Pittsburgh Sleep Quality Index. BRUMS: Brunel University Mood Scale. IPAQ: International Physical Activity Questionnaires. MET: Metabolic equivalent of task. Data are presented mean ± 1SD.

5.2.5 Statistical analysis

The G* Power 3.1.6 software programme was used to calculate an *a priori* power calculation for sample size, having as parameters: statistical test (ANOVA), $\alpha = 0.05$, $\beta = 0.80$ (power of the sample), number of groups analysed (2 = β-alanine and placebo), and the effect of sample size based on previous findings (Hobson *et al.*, 2012). According to the calculations, a minimum of 22 participants would be required for the study, with 24 participants being recruited to allow for dropouts. Dependent variables (MVIF, EMD, HRT, TPT, slope and intercept of force-EMG relationship, frequency at 50% of force response for the force-frequency relationship) were evaluated using a two-way mixed-model (group × session) ANOVA. Dependent variables measured over several time points (force and EMG during explosive voluntary contractions, evoked twitch, and octet force) were analysed using a three-way mixed-model (group × session × time) ANOVA. All variables were assessed during both fresh and fatigued conditions. IKET variables (TTF and impulse) were analysed using a two-way mixed-model ANOVA.

The impact of the IKET contraction on dependant variables (MVIF, EMD, HRT, TPT, slope and intercept of force-EMG relationship, frequency at 50% of force response for the force-frequency relationship) were analysed using a three-way mixed-model (group × session × fatigue) ANOVA. Dependent variables measured over several time points (force and EMG during explosive voluntary contractions, evoked twitch and octet force) were analysed using a three-way mixed-model (group × session × percentage change) ANOVA based on percentage change between fresh and fatigued values.

Assessed variables were tested for normality using the Shapiro-Wilks test, and for homogeneity using the Fevene test. A Greenhouse-Geisser correction was applied when the ANOVA assumption of sphericity was violated, and significant interaction effects were followed-up by independent sample *t*-tests on the individual percentage change values for each condition. ES for multiple comparisons was calculated using η_p^2 and η_g^2 eta squared (Lakens, 2013). Providing two ESs is suggested to yield a greater understanding of a specific effect (Preacher & Kelly, 2011). *Post hoc* comparisons to explain any significant interactions are reported with Cohen's *d* and Hedges *g* ESs. An ES of 0.2 – 0.5 was defined as small, 0.5 – 0.8 as medium and ≥ 0.8 as a large effect (Schünemann *et al.*, 2008). Intra-individual variability was assessed using the mean intra-individual CV between the pre- and post-supplementation sessions for the placebo group [(SD / mean) × 100]. Statistical analyses were completed using SPSS version 22 (SPSS Inc., Chicago, IF, USA) and Microsoft Excel (Microsoft Inc., USA). Statistical significance was accepted at $P \leq 0.05$, with data presented as mean ± 1 SD.

5.3 Results

5.3.1 Maximum and explosive voluntary contractions

Twenty-eight days of β-alanine supplementation had no effect on MVIF in fresh (P = 0.71; f (1, 21) = 0.29, η_p^2 < 0.1, η_g^2 < 0.1) or fatigued skeletal muscle (P = 1.0; f (1, 21) < 0.001, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 5.1A). The mean CV in the placebo group was 3% and 5% in fresh and fatigued skeletal muscle. Following the completion of the IKET, MVIF significantly declined (P < 0.001; f (1, 21) = 216.85, η_p^2 = 0.9, η_g^2 = 0.2), with no differences between sessions (P = 0.73; f (1, 21) = 0.12, η_p^2 < 0.1, η_g^2 < 0.1) or groups (P = 0.78; f (1, 21) = 0.08, η_p^2 < 0.1, η_g^2 < 0.1) (β-alanine: 17 – 18%; PLA: 21%; Fig. 5.1A). There was no effect of β-alanine supplementation on force measures at 25 ms intervals during explosive voluntary contractions in either fresh (P = 0.54; f (5, 105) = 0.82, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.91; f (1, 21) = 0.01, η_p^2 < 0.1, η_g^2 < 0.1 (group × session)) or fatigued skeletal muscle (P = 0.31; f (5, 105) = 1.20, η_p^2 = 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.67; f (1, 21) = 0.18, η_p^2 < 0.1, η_g^2 < 0.1 (group × session); Fig. 5.1A). The percentage change in explosive force between fresh and fatigued conditions was not altered between sessions (P = 0.75; f (1, 21) = 0.46, η_p^2 < 0.1, η_g^2 = 0.1) with no group × session interaction (P = 0.50; f (1, 21) = 0.46, η_p^2 < 0.1, η_g^2 < 0.1). The mean CV in the placebo group during explosive contractions was 15 - 20% at 25 - 50 ms and 4 - 8% from 75 - 150 ms during a fatigued condition and 18 - 22% at 25-50 ms and 6 - 12% from 75 - 150 ms during the fatigued condition.

Agonist EMG normalised to M_{max} during MVICs and explosive contraction was uninfluenced by β-alanine supplementation in fresh (P = 0.86; f (3, 63) = 1.39, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.90; f (1, 21) = 0.02, η_p^2 < 0.1, η_g^2 < 0.1 (group × session)) and fatigued skeletal muscle (P = 0.10; f (3, 51) = 6.95, η_p^2 < 0.1, η_g^2 = 0.1 (group × session × time point); P = 0.26; f (1, 17) = 1.36, η_p^2 = 0.1, η_g^2 < 0.1 (group × session); Fig. 5.1B). The mean CV values in the placebo group for agonist EMG in fatigued skeletal muscle were 26% at 0 - 50 ms, 21% at 50 - 100 ms and 20% at 100 - 150 ms time windows, and 12% at MVIF. The mean CV values in the placebo group for agonist EMG in fatigued skeletal muscle were 27% at 0 - 50 ms, 29% 50 - 100 ms and 21% at 100 - 150 ms time windows, and 19% at MVIF. The percentage change in agonist EMG normalised to M_{max} during MVICs and explosive contraction was not significantly altered between sessions (P = 0.29; f (1, 21) = 0.59, η_p^2 < 0.1, η_g^2 < 0.1), with no group × session interaction (P = 0.77; f (1, 17) = 0.09, η_p^2 < 0.1, η_g^2 < 0.1).

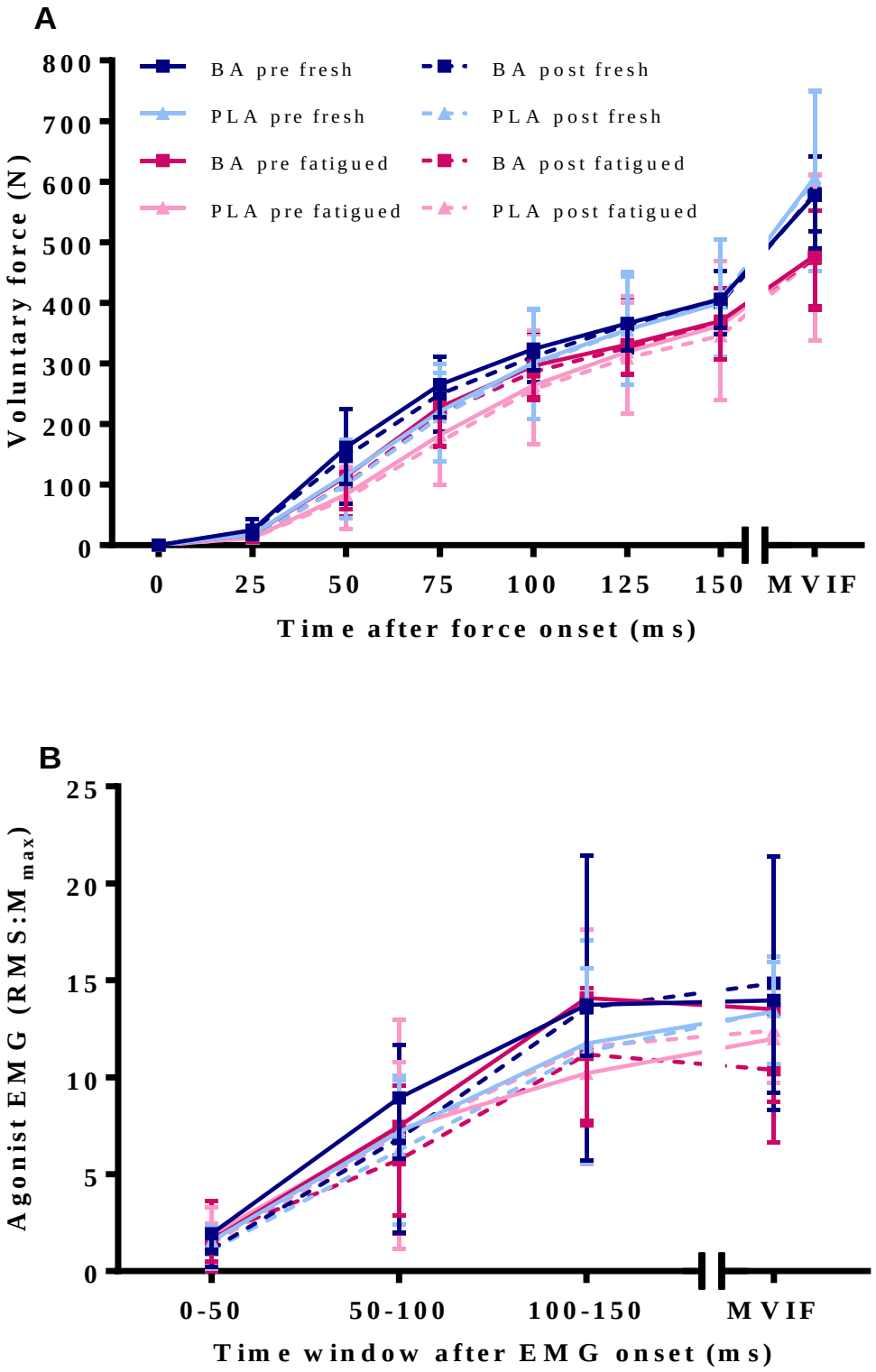

Fig. 5.1: Explosive and maximal voluntary isometric force (MVIF) voluntary force (A), and agonist Surface electromyography (EMG) normalised to maximum muscle action potential (M_{max}) during explosive contractions (0 - 50, 50 - 100 and 100 - 150 ms from onset) and at MVIF (B) for the β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle. RMS: Root mean square. Data are mean ± 1SD.

5.3.2 Twitches

Resting twitches: Supplementation did not significantly influence fresh or fatigued twitch force (*Fresh*: P = 0.88; *f* (2, 42) = 0.13, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.66; *f* (2, 42) = 0.42, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); *Fresh*: P = 0.66; *f* (2, 42) = 0.12, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.21; *f* (2, 42) = 1.67, η_p^2 = 0.1, η_g^2 < 0.1 (group × session); Table. 5.3), EMD (*Fresh*: P = 0.21; *f* (1, 21) = 1.71, η_p^2 = 0.1, η_g^2 = 0.2; *Fatigued*: P = 0.84; *f* (1, 21) = 0.04, η_p^2 < 0.1, η_g^2 = 0.4; Table. 5.3) or TPT (*Fresh*: P = 0.35; *f* (1, 21) = 0.91, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.95; *f* (1, 21) = 0.004, η_p^2 < 0.1, η_g^2 < 0.1; Table. 5.3). There was, however, a significant group × session interaction for HRT in both fresh (P = 0.04; *f* (1, 21) = 4.88, η_p^2 = 0.2, η_g^2 < 0.1) and fatigued skeletal muscle (P = 0.03; *f* (1, 21) = 5.57, η_p^2 = 0.2, η_g^2 = 0.1; Fig. 5.2). *Post hoc* analysis showed that the percentage change in fresh skeletal muscle HRT was not significantly different between the β-alanine (-2 ± 10 ms; -3 ± 13%) and placebo group (+8 ± 16 ms; 8 ± 16%) with a large effect reported (P = 0.06; *t* (21) = -2.0; 95% confidence interval [-32.8, 1.1], Cohen's *d* = 0.9; Hedge's *g* = 0.8). The confidence interval ES indicated that there was a 72% chance of a randomly selected pair of individuals would experience a decline in HRT following β-alanine supplementation. In fatigued skeletal muscle, *post hoc* analysis showed that the percentage change in fatigued skeletal muscle HRT was significantly different between the β-alanine (-25 ± 34 ms; -19 ± 26%) and placebo group (8 ± 16 ms; 0 ± 15%) with a large effect reported (P = 0.05; *t* (21) = -2.1; 95% confidence interval [-37.6, -0.9], Cohen's *d* = 0.9; Hedge's *g* = 0.9). The confidence interval ES indicated that there was a 74% chance of a randomly selected pair of individuals would experience a decline in HRT following β-alanine supplementation. Mean CV values for fresh skeletal muscle in the placebo group: force at 25 and 50ms and peak were 12, 11, and 12%, EMD was 5%, TPT was 4% and HRT was 13%. Mean CV values for fatigued skeletal muscle in the placebo group: force at 25 and 50ms and peak were 13, 16, and 15%, EMD was 13%, TPT was 7% and HRT was 10%.

The percentage difference between fresh and fatigued resting twitch force remained similar between sessions (P = 0.20; *f* (1, 21) = 1.74, η_p^2 = 0.1, η_g^2 < 0.1) with no group × session interaction (P = 0.69; *f* (1, 21) = 0.17, η_p^2 < 0.1, η_g^2 < 0.1). Resting twitch TPT declined following completion of the IKET (P = 0.001; *f* (1, 21) = 14.80, η_p^2 = 0.4, η_g^2 = 0.2; Table. 5.3), with no group × fatigue (P = 0.31; *f* (1, 21) = 1.09, η_p^2 = 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.38; *f* (1, 21) = 0.82, η_p^2 < 0.1, η_g^2 < 0.1). EMD remained similar following the IKET (P = 0.35; *f* (1, 21) = 0.91, η_p^2 < 0.1, η_g^2 < 0.1) with no group × fatigue (P = 0.43; *f* (1, 21) = 0.65, η_p^2 < 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.69; *f* (1, 21) = 0.16, η_p^2 < 0.1, η_g^2 < 0.1; Table. 5.3). Resting twitch HRT significantly increased following the completion of the IKET (P < 0.001; *f* (1,

21) = 41.36, η_p^2 = 0.7, η_g^2 = 0.3; Fig. 5.2). There was no group × fatigue (P = 0.11; *f* (1, 21) = 2.86, η_p^2 = 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.75; *f* (1, 21) = 0.10, η_p^2 < 0.1, η_g^2 < 0.1).

Potentiated twitches: Supplementation with β-alanine did not significantly influence fresh or fatigued skeletal muscle twitch force (*Fresh*: P = 0.31; *f* (2, 42) = 1.19, η_p^2 = 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.97; *f* (2, 42) = 0.03, η_p^2 < 0.1, η_g^2 < 0.1; (group × session × time point); *Fresh*: P = 0.32; *f* (1, 21) = 1.03, η_p^2 = 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.29; *f* (1, 21) = 1.18, η_p^2 = 0.1, η_g^2 < 0.1 (group × session); Table. 5.3), EMD (*Fresh*: P = 0.47; *f* (1, 21) = 0.53, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.56; *f* (1, 21) = 0.35, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 5.2D) or TPT (*Fresh*: P = 0.78; *f* (1, 21) = 0.08, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.68; *f* (1, 21) = 0.17, η_p^2 < 0.1, η_g^2 < 0.1; Table. 5.3). There was, however, a significant group × session interaction for HRT in both fresh (P = 0.03; *f* (1, 21) = 5.27, η_p^2 = 0.2, η_g^2 < 0.1) and fatigued skeletal muscle (P = 0.03; *f* (1, 21) = 5.20, η_p^2 = 0.2, η_g^2 < 0.1; Fig. 5.2). *Post hoc* analysis showed that the percentage change in fresh skeletal muscle HRT was not significantly different between the β-alanine (0 ± 9 ms; +1 ± 15%) and placebo group (+7 ± 10 ms; (+12 ± 15%) with a medium effect reported (P = 0.10; *t* (21) = -1.7; 95% confidence interval [-23.9, 2.5], Cohen's *d* = 0.7; Hedge's *g* = 0.7). The confidence interval ES indicated that there was a 69% chance of a randomly selected pair of individuals would experience a decline in HRT following β-alanine supplementation. In fatigued skeletal muscle, *post hoc* analysis showed that the percentage change in fatigued skeletal muscle HRT was significantly different between the β-alanine (-2.7 ± 16 ms; -2 ± 20%) and placebo group (12 ± 12 ms; 16 ± 17%) with a large effect reported (P = 0.03; *t* (21) = -2.2; 95% confidence interval [-33.8, -1.4], Cohen's *d* = 1.0; Hedge's *g* = 0.9). The confidence interval ES indicated that there was a 75% chance of a randomly selected pair of individuals would experience a decline in HRT following β-alanine supplementation. Mean CV values for fresh skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 10, 8, and 9%, EMD was 7%, TPT was 6% and HRT was 9%. Mean CV values for fatigued skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 17, 15, and 15%, EMD was 11%, TPT was 6% and HRT was 10%.

The percentage change in potentiated twitch force was unaffected by supplementation (P = 0.85; *f* (1, 21) = 0.03, η_p^2 < 0.1, η_g^2 < 0.1) with no group × session interaction (P = 0.08; *f* (1, 21) = 3.21, η_p^2 < 0.1, η_g^2 < 0.1). Potentiated twitch TPT declined following completion of the IKET (P < 0.001; *f* (1, 21) = 18.80, η_p^2 = 0.5, η_g^2 = 0.5; Table. 5.3), with no group × fatigue (P = 0.25; *f* (1, 21) = 1.38, η_p^2 = 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.88; *f* (1, 21) = 0.02, η_p^2 < 0.1, η_g^2 < 0.1). Potentiated EMD was significantly prolonged following the IKET (P = 0.001; *f* (1,

21) = 16.82, η_p^2 = 0.5, η_g^2 = 0.2) with no group × fatigue (P = 0.42; *f* (1, 21) = 0.67, η_p^2 < 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.99; *f* (1, 21) = 0.00, η_p^2 < 0.1, η_g^2 < 0.1). Potentiated HRT significantly increased following the completion of the IKET (P < 0.001; *f* (1, 21) = 42.35, η_p^2 = 0.7, η_g^2 = 0.4; Fig. 5.2). There was no group × fatigue (P = 0.72; *f* (1, 21) = 0.14, η_p^2 = 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.47; *f* (1, 21) = 0.54, η_p^2 < 0.1, η_g^2 < 0.1).

5.3.3 Octet contractions

In both fresh (P = 0.40; *f* (2, 42) = 0.95, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.16; *f* (1, 21) = 2.15, η_p^2 < 0.1, η_g^2 < 0.1 (group × session)) and fatigued (P = 0.84; *f* (2, 42) = 0.18, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); P = 0.83; *f* (1, 21) = 0.05, η_p^2 < 0.1, η_g^2 < 0.1 (group × session)) skeletal muscle, supplementation did not significantly alter octet force, EMD or TPT (Table. 5.3). There was, however, a significant group × session interaction for octet HRT in both fresh (P = 0.05; n_p^2 = 0.2; η_g^2 = 0.1) and fatigued (P = 0.01; n_p^2 = 0.3; η_g^2 = 0.2) skeletal muscle (Fig. 5.2). *Post hoc* analysis showed that the percentage change in fresh skeletal muscle was significantly different between the β-alanine (-26 ± 30 ms; -20 ± 22%) and placebo (0 ± 32 ms; 1 ± 34%) group with a large effect reported (P = 0.05, Cohen's *d* = 0.8). In fatigued skeletal muscle, *post hoc* analysis showed that HRT percentage change was significantly different between the β-alanine (-11 ± 20 ms; -11 ± 20%) and placebo (12 ± 19 ms; 7 ± 13%) groups with a large effect reported (P = 0.01, Cohen's *d* = 1.2; Fig. 5.2).

The percentage change in octet force between fresh and fatigued conditions was not significantly affected by supplementation (P = 0.20; *f* (1, 21) = 1.74, η_p^2 = 0.1, η_g^2 < 0.1), with no group × session interaction (P = 0.69; *f* (1, 21) = 0.17, η_p^2 < 0.1, η_g^2 < 0.1). Octet TPT (P = 0.05, η_p^2 = 0.2, η_g^2 = 0.1; Table. 5.3) and HRT (P = 0.008, η_p^2 = 0.3, η_g^2 = 0.2; Fig. 5.2), declined following completion of the IKET, with no group × fatigue or group × session × fatigue interactions. Octet EMD was not significantly influenced following the IKET, with no group × fatigue or group × session × fatigue interactions (Table. 5.3). Mean CV values for fresh skeletal muscle in the placebo group were 15, 12 and 12% for force at 25, 50 ms and peak. Mean CV values for fatigued skeletal muscle in the placebo group were 20, 13 and 17% for force at 25, 50 ms and peak.

Table 5.3: Electrically evoked force responses, time-to-peak tension (TPT) and electromechanical delay (EMD) of β-alanine and placebo groups pre- and post-supplementation, in fresh and fatigued skeletal muscle.

			Pre-supplementation					Post-supplementation				
			Force (N)			EMD	TPT	Force (N)			EMD	TPT
			25 ms	50 ms	Peak	(ms)	(ms)	25 ms	50 ms	Peak	(ms)	(ms)
Resting Twitch	**Fresh**	**β-alanine**	23 ± 5	73 ± 14	88 ± 17	10 ± 1	79 ± 11	24 ± 6	73 ± 17	90 ± 19	10 ± 1	81 ± 9
	Fresh	**Placebo**	28 ± 4	79 ± 19	99 ± 26	11 ± 1	82 ± 8	29 ± 5	82 ± 16	104 ± 24	10 ± 1	83 ± 9
	Fatigued	**β-alanine**	22 ± 7	61 ± 22	68 ± 25	11 ± 2	72 ± 16	26 ± 8	71 ± 22	79 ± 23	10 ± 1	74 ± 13
	Fatigued	**Placebo**	25 ± 6	58 ± 20	65 ± 24	11 ± 1	69 ± 8	24 ± 4	60 ± 15	67 ± 18	10 ± 2	71 ± 9
Potentiated Twitch	**Fresh**	**β-alanine**	55 ± 13	142 ± 24	159 ± 26	9 ± 1	79 ± 6	53 ± 8	136 ± 14	155 ± 9	9 ± 1	81 ± 6
	Fresh	**Placebo**	60 ± 16	138 ± 37	163 ± 43	9 ± 1	79 ± 9	59 ± 10	142 ± 34	167 ± 38	9 ± 1	82 ± 8
	Fatigued	**β-alanine**	37 ± 11	92 ± 27	101 ± 29	11 ± 1	74 ± 9	40 ± 9	101 ± 19	110 ± 18	10 ± 1	73 ± 10
	Fatigued	**Placebo**	42 ± 10	93 ± 25	105 ± 30	10 ± 1	74 ± 4	38 ± 9	93 ± 23	106 ± 28	10 ± 1	74 ± 8
Octet	**Fresh**	**β-alanine**	52 ± 15	165 ± 42	223 ± 47	7 ± 2	117 ± 25	64 ± 18	196 ± 37	274 ± 53	7 ± 2	120 ± 24
	Fresh	**Placebo**	66 ± 17	190 ± 62	285 ± 94	6 ± 1	127 ± 19	64 ± 17	199 ± 60	296 ± 94	7 ± 2	137 ± 7
	Fatigued	**β-alanine**	62 ± 23	191 ± 49	221 ± 72	7 ± 2	100 ± 23	62 ± 17	182 ± 47	244 ± 68	7 ± 2	114 ± 25
	Fatigued	**Placebo**	69 ± 20	191 ± 49	273 ± 72	7 ± 1	123 ± 14	64 ± 16	198 ± 49	283 ± 79	7 ± 2	125 ± 15

Data are mean ± 1SD.

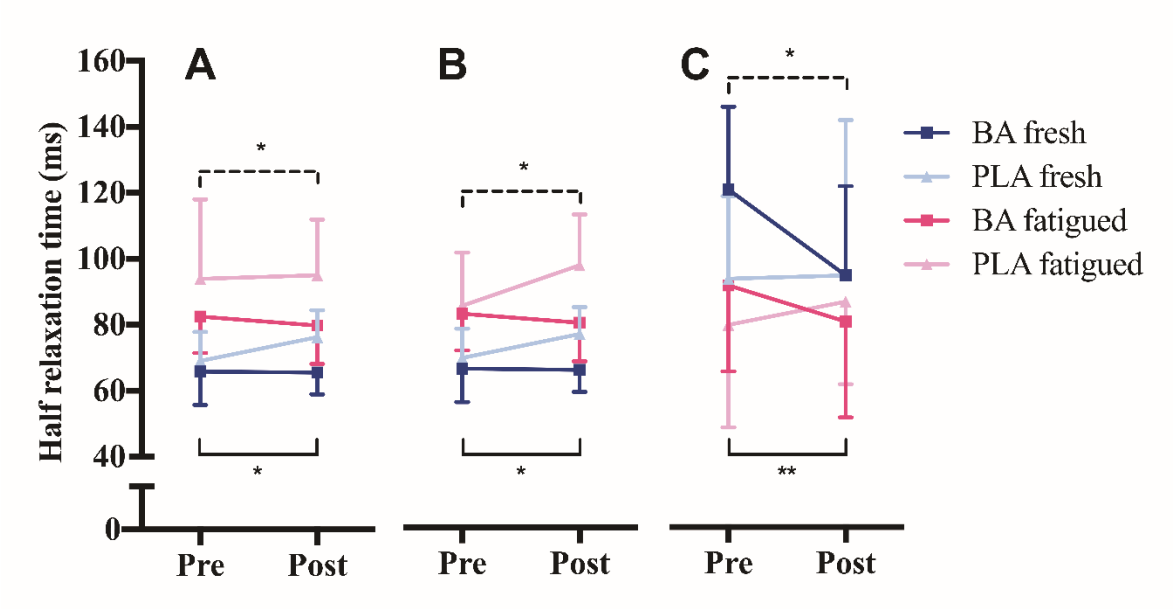

Fig. 5.2: Electrically evoked half relaxation time of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle during: resting twitch (a), potentiated twitch (b), and octets (c). Data are mean ± 1SD. **P≤0.01 and *P≤0.05 for *post hoc* independent t-test between β-alanine and placebo groups.

5.3.4 Force-electromyography relationship

The slope and y-intercept of the force-EMG relationship were unaffected by supplementation in both fresh (*slope:* P = 0.80; f (1, 21) = 0.07, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *y-intercept*: P = 0.52; f (1, 21) = 0.44, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session)) and fatigued skeletal muscle (*slope:* P = 0.74; f (1, 21) = 0.11, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *y-intercept*: P = 0.51; f (1, 21) = 0.46, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$) (Fig. 5.3 and Table 5.4). There was also no influence of the IKET on the slope and y-intercept of the force-EMG relationship (*slope:* P = 0.32; f (1, 21) = 1.03, $\eta_p^2 = 0.1$, $\eta_g^2 = 0.1$; *y-intercept*: P = 0.17; f (1, 21) = 2.05, $\eta_p^2 = 0.1$, $\eta_g^2 < 0.1$), with no group × fatigue interaction (*slope:* P = 0.32; f (1, 21) = 1.03, $\eta_p^2 = 0.1$, $\eta_g^2 = 0.1$; *y-intercept*: P = 0.63; f (1, 21) = 0.08, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). The mean CV in the placebo group for the slope of the force-EMG relationship was 17% (fresh skeletal muscle) and 15% (fatigued skeletal muscle).

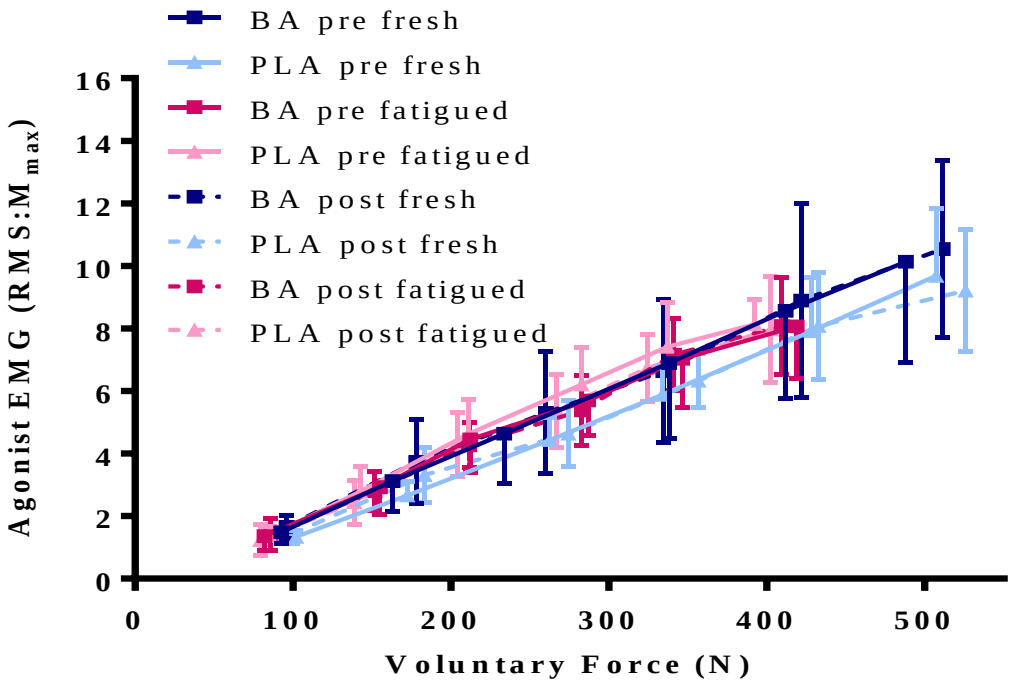

Fig. 5.3: Force-surface electromyography (EMG) relationship measured during submaximal voluntary contractions (15 - 90% maximal voluntary isometric force) for the β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle. M_{max}: Maximum muscle action potential; RMS: Root mean square. Data are mean ± 1SD.

5.3.5 Force-frequency relationship

Supplementation did not significantly influence peak force at each frequency of stimulation (*fresh:* P = 0.26; f (9, 189) = 1.28, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *fatigued:* P = 0.94; f (9, 189) = 0.38; $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × frequency); *fresh:* P = 0.72; f (9, 189) = 1.81, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *fatigued:* P = 0.97; f (9, 189) = 1.91; $\eta_p^2 = 0.1$, $\eta_g^2 < 0.1$ (group × session); Fig. 5.4). The frequency at 50% of the force response (Table 5.4) in fresh (*peak force*: P = 0.93; f (1, 21) = 0.01, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *frequency at 50%:* P = 0.22; f (1, 21) = 1.58, $\eta_p^2 = 0.1$, $\eta_g^2 < 0.1$ (group × session)) and fatigued skeletal muscle were unaffected by supplementation (*peak force*: P = 0.76; f (1, 21) = 0.09, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *frequency at 50%:* P = 0.61; f (1, 21) = 0.27, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). Following the completion of the IKET peak force significantly declined (P = 0.001; f (1, 21) = 111.97, $\eta_p^2 = 0.8$, $\eta_g^2 = 0.2$), with no group × fatigue interaction (P = 0.52; f (1, 21) = 0.45, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). The frequency at 50% of the force response remained unaffected by the IKET (P = 0.60; f (1, 21) = 0.28, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$), with no group × fatigue interaction (P = 0.97; f (1, 21) = 0.00, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$).

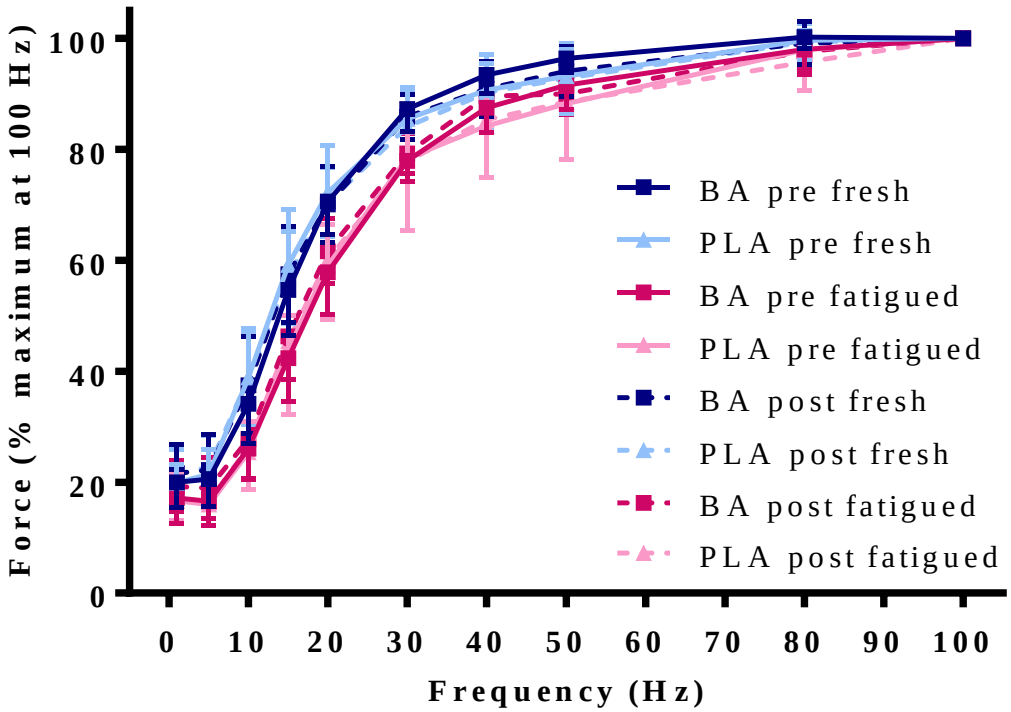

Fig. 5.4: Force-frequency relationship assessed during submaximal percutaneous stimulation for β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle. Data are mean ± 1SD.

Table 5.4: Characteristics of the force-frequency and force-electromyography (EMG) relationships in β-alanine and placebo groups in fresh and fatigued skeletal muscle, pre- and post-supplementation.

	Pre-supplementation				**Post-supplementation**			
	β-alanine		**Placebo**		**β-alanine**		**Placebo**	
	Fresh	**Fatigued**	**Fresh**	**Fatigued**	**Fresh**	**Fatigued**	**Fresh**	**Fatigued**
Force-frequency relationship								
Frequency at 50% of response, Hz	14.3 ± 2.5	14.2 ± 2.6	13.9 ± 2.2	13.6 ± 2.0	14.1 ± 2.4	14.0 ± 2.3	13.5 ± 2.5	13.6 ± 2.6
Force-EMG relationship								
Intercept (RMS:M_{max})	-0.52 ± 0.91	-0.19 ± 0.62	-0.88 ± 1.07	-0.35 ± 0.61	-0.25 ± 0.42	-0.36 ± 0.59	-0.34 ± 0.35	-0.33 ± 0.46
Slope (RMS:M_{max}/N)	0.022 ± 0.007	0.021 ± 0.007	0.022 ± 0.006	0.024 ± 0.007	0.021 ± 0.006	0.022 ± 0.006	0.020 ± 0.005	0.023 ± 0.007

Data are mean ± 1SD. M_{max}: Maximum muscle action potential; RMS: Root mean square.

5.3.6 Sustained isometric knee extensor endurance test

There was no group × session interaction for TTF (β-alanine: pre: 63 ± 13 s; post: 63 ± 15 s; Placebo: pre: 77 ± 25 s; Post: 75 ± 19 s; P = 0.85; f (1, 21) = 0.04, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 5.5). Based upon the recorded TTF, the Rohmert equation (Rohmert, 1960) was used to calculate estimated percentage of MVIF. Individuals within the β-alanine group were estimated to be holding the IKET at ~51% MVIF (~6% greater than asked), as denoted by slightly shorter hold times of~63 s. Individuals within the placebo group were estimated to be holding the IKET contraction at ~45% MVIF, with a hold time of ~77 s. There was no group × session interaction for impulse values (β-alanine: pre: 16 ± 4 $kN \cdot s^{-1}$; post 16 ± 5 $kN \cdot s^{-1}$; Placebo: pre: 20 ± 4 $kN \cdot s^{-1}$; post 19 ± 3 $kN \cdot s^{-1}$; P = 0.58; f (1, 21) = 0.30, η_p^2 < 0.1, η_g^2 < 0.1).

Blood lactate concentrations displayed a significant session × time × group interaction (P = 0.05; f (2, 20) = 3.41, η_p^2 = 0.3, η_g^2 = 0.1). Blood lactate concentrations significantly increased 5 minutes following the IKET compared to both rest and prior to values (P < 0.001; f (2, 20) = 38.17, η_p^2 = 0.8, η_g^2 = 0.6), with no influence of session (P = 0.20; f (2, 20) = 1.90, η_p^2 = 0.2, η_g^2 < 0.1) or group (P = 0.73; f (1, 10) = 0.13, η_p^2< 0.1, η_g^2 < 0.1; Table 5.5).

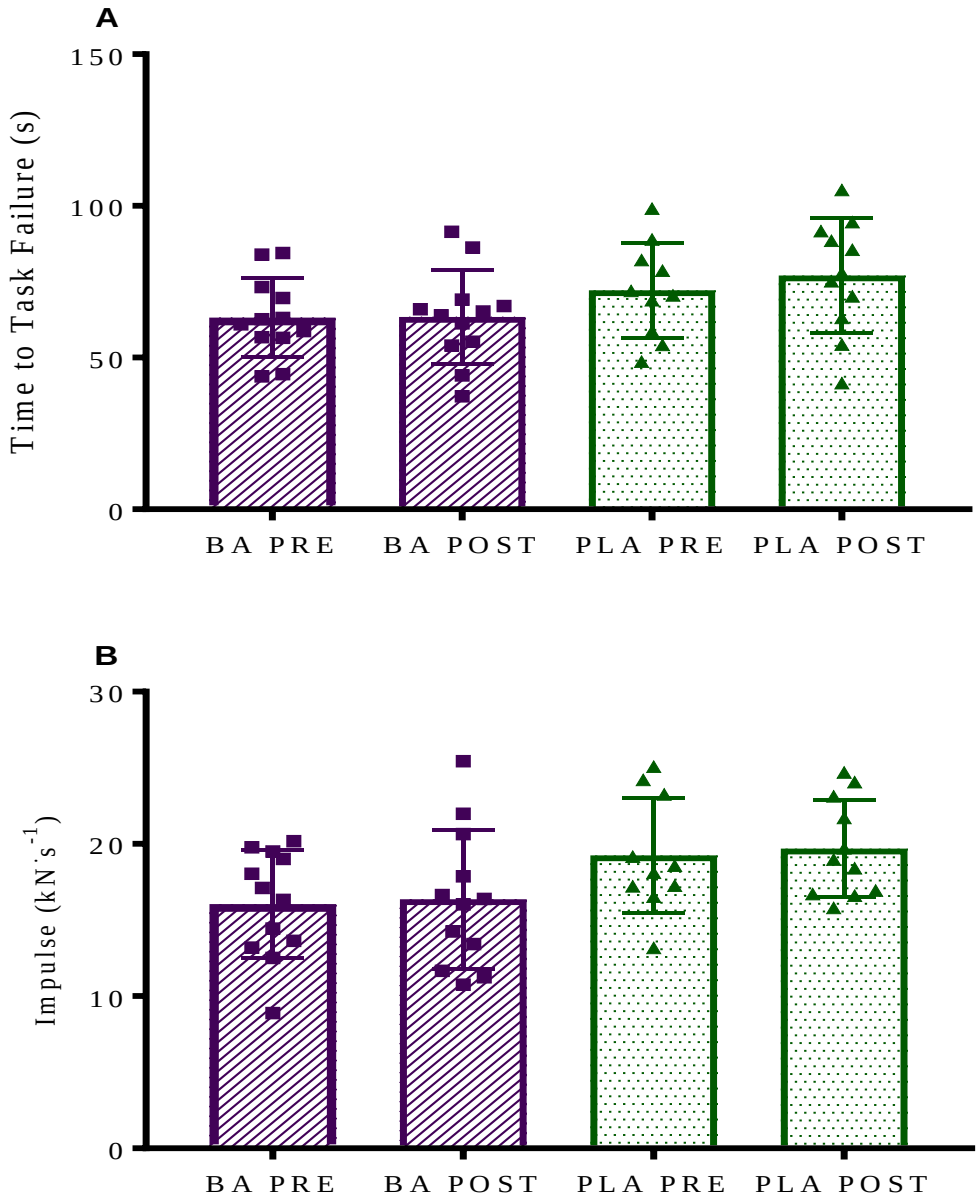

Fig. 5.5: Time to task failure (s; A) and impulse (kN·s; B) of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation. Data are mean ± 1SD. Individual data are presented as squares (BA), and triangles (PLA).

Table 5.5: Blood lactate concentrations ($mmol \cdot l^{-1}$) for the β-alanine and placebo groups pre- and post-supplementation, at rest, prior to and 5 mins following the completion of the isometric knee extensor task (+5 mins). Significant differences between concentrations are denoted by * (rest and +5 mins) and ^{X} (prior to and +5 mins).

	Pre-supplementation			**Post-supplementation**		
	Rest ($mmol \cdot l^{-1}$)	**Prior to ($mmol \cdot l^{-1}$)**	**+5 mins ($mmol \cdot l^{-1}$)**	**Rest ($mmol \cdot l^{-1}$)**	**Prior to ($mmol \cdot l^{-1}$)**	**+5 mins ($mmol \cdot l^{-1}$)**
β-alanine	1.0 ± 0.3	1.1 ± 0.2	3.8 ± 1.1^{*X}	1.2 ± 0.3	1.3 ± 0.4	3.7 ± 1.3^{*X}
Placebo	1.0 ± 0.3	1.1 ± 0.4	3.8 ± 1.3^{*X}	1.3 ± 0.3	1.2 ± 0.2	4.2 ± 1.0^{*X}

Data are mean ± 1SD.

5.4 Discussion

The main findings from the current investigation are a) β-alanine supplementation had no effect on isometric force production capacity in fresh or fatigued skeletal muscle, b) 28-days of β-alanine supplementation altered fresh skeletal muscle relaxation speed, confirming the data reported in Study 1 (Chapter 4), and c) reduced relaxation speed was also evident in fatigued skeletal muscle following β-alanine supplementation, with no change to peak force production or contraction time. The present research is the first to comprehensively examine the effect of 28-days β-alanine supplementation on voluntary and electrically evoked contractile properties of fatigued *in-vivo* human skeletal muscle.

β-alanine supplementation had no effect on voluntary isometric force production during fresh and fatigued conditions, including maximal and explosive force variables. Consistent with the lack of change in voluntary peak force, electrically evoked peak force responses, reported during twitch and octet contractions, were unaffected by 28-days of β-alanine supplementation during both fresh and fatigued conditions. Neural drive responses during both MVICs and explosive contractions in fresh and fatigued skeletal muscle pre- and post-supplementation remained similar. The present data in fresh skeletal muscle are consistent with the findings reported in Study 1 (Chapter 4 if β-alanine supplementation influenced Ca^{2+} related function, improvements in explosive voluntary force and/or alterations in the force-EMG relationship would be apparent.

There was no leftward shift of the force-frequency curve, the associated measure of intracellular Ca^{2+} levels (Batrukova & Rubstov, 1997), in either fresh or fatigued *in-vivo* human skeletal muscle, implying that elevated skeletal muscle carnosine content did not significantly alter Ca^{2+} related function. There may, however, have been a decrease in SR Ca^{2+} release which was not recognised in the force-frequency curve, due to an associated increase in Ca^{2+} sensitivity of the myofibrils, thereby equating to the same skeletal muscle force production. The data in fresh skeletal muscle is in-line with previous *in-vivo* research reported in this thesis (Study 1; Chapter 4), where increased skeletal muscle carnosine content following 28-day of β-alanine supplementation did not alter the force-frequency curve. It should be highlighted, that during both investigations there were no direct measures of skeletal muscle carnosine content. Numerous investigations have demonstrated increased skeletal muscle carnosine content following 28-days β-alanine supplementation in this adult population, almost without exception, on an individual-by-individual basis (Harris *et al.,* 2006; Hill *et al.,* 2007). It is therefore likely the current 28-day β-alanine supplementation protocol, significantly increased skeletal muscle carnosine concentration.

There was no significant influence of increased skeletal muscle carnosine content on TPT and force production following 28-days β-alanine supplementation. Based on previous *in-vitro*

research in chemically skinned muscle fibres from frogs (Lamont & Miller, 1992), mechanically skinned rat muscle fibres (Dutka & Lamb, 2004) and, Type I and Type II human skeletal muscle fibres (Dutka *et al.,* 2012), an alteration to submaximal action potential-mediated force responses via increased Ca^{2+} sensitivity, could have been expected. There was also no significant alteration to potentiated twitch contractions following β-alanine supplementation. Given that the phosphorylation of the myosin head during these twitch contractions, is due to the increased sensitivity of the contractile properties to Ca^{2+}, any alterations to Ca^{2+} sensitivity via increased carnosine content would have been apparent during these contractions. *In-vitro* data in mice showed that increased carnosine (+156%) and anserine content (+46%) in the *m. extensor digitorum longus*, alongside a marked leftward shift of the force-frequency relationship following β-alanine supplementation (Everaert *et al.,* 2013). These *in-vitro* data in skinned muscle fibre are interesting, yet due to the current *in-vivo* research (Studies 1 and 2; Chapter 4 and 5) it is suggested that these responses might not be significant enough to effect whole muscle contraction. It is also important to consider that the *in-vitro* research is performed outside the normal intracellular environment, with several *in-vitro* protocols performed with free magnesium, an inhibitor of skeletal muscle RyR (Laver, O'Neill & Lamb, 2004). During *in-vitro* investigations, solutions are added to regulate the pH level allowing analysis of the isolated effect of carnosine, whilst this is important, current research has yet to examine the impact of carnosine content and varying pH levels. The current *in-vivo* research should be considered separate from the *in-vitro* data, since it might be unrealistic to expect similar outcomes between research designs.

Twenty-eight days of β-alanine supplementation to increase skeletal muscle carnosine content, resulted in a shorter HRT (relative to equivalent placebo times) in fresh and fatigued skeletal muscle during both resting and potentiated twitch contractions. The beneficial effect of β-alanine supplementation on skeletal muscle HRT evident, are in-line with those reported in Study 1 (Chapter 4; 7 - 12%). Muscle relaxation speed has been associated with both Ca^{2+} removal from the myoplasm and Ca^{2+} dissociation from troponin followed by cross-bridge detachment (Westerblad *et al.,* 1997). Westerblad and Allen (1993) suggested that declines in skeletal muscle relaxation speed, apparent under fatigued conditions, reflected either reduced cross-bridge kinetics, or potentially a combination of impaired cross-bridge kinetics and Ca^{2+} kinetics. Muscle relaxation speed has been associated with Ca^{2+} re-uptake by the SERCA pump (Gafni & Yuh, 1989), the proposed rate-limiting step in skeletal muscle relaxation (Gillis, 1985; Dux, 1993). Acting on Ca^{2+} kinetics proteins directly or via other molecules provided a mechanism by which Ca^{2+} signalling can be inhibited or excited (Swietach *et al.,* 2013). Examination of isolated rat heart muscle, demonstrated improved contractile function and increased free intracellular Ca^{2+} concentrations in the presence of carnosine (Zaloga, Roberts & Nelson, 1996). Although speculative, these data propose that increased carnosine content might alter Ca^{2+}-channel activity by interacting with the

Ca^{2+}-channel itself (Batrukova & Rubstov, 1997), possibly via the existence of saturable binding site(s) for carnosine on the Ca^{2+}-channel. These *in-vitro* analysis are, however, limited by several methodological factors, including but not limited to, the lack of a Ca^{2+} buffer, the overloading of the SR with Ca^{2+}, concentrations ~10 times greater than normal, and the addition of un-physiological magnesium concentrations. Therefore, consideration needs to be taken regarding interpretations based on these data. Alternatively, the beneficial effect of increased carnosine content on skeletal muscle relaxation time, evident within the current investigation, may also be associated with an indirect mechanism. Carnosine may modify the protein interactions with the Ca^{2+}-channels and/or bind with the proteins themselves, given the quantity of additional proteins that bind to Ca^{2+}-channels (Berchtold, Brinkmeier & Muntener, 2000); both mechanisms could impact the activity of the Ca^{2+}-channel.

The mechanism for reduced skeletal muscle relaxation time following β-alanine supplementation remains uncertain, yet, could be beneficial for exercise performance, specifically those involving short, repeated contractions, where skeletal muscle relaxation comprises an important proportion of total energy consumption (Bergström & Hultman, 1988). The current findings are predominantly significant for activities where fast, repetitive contractions and relaxations occur, with no period of rest. It would be beneficial to replicate these data in an older adult population, where pre-existing declines in skeletal muscle function are evident, meaning that any improvement in skeletal muscle function would be proportionally more valuable. Due to the possible interaction between carnosine and SERCA activity, exploring the impact of increased skeletal muscle carnosine content on SERCA activity could provide a number of clinical avenues of research. It could be speculated that increased skeletal muscle carnosine content via β -alanine supplementation, may benefit individuals with Brody disease, where SERCA1 activity is significantly reduced (Guglielmi *et al.,* 2013) or Duchenne Muscular Dystrophy, where an excess of cytosolic Ca^{2+} occurs (Ohlendieck, 2000).

The IKET hold times within the present investigation were not significantly influenced by 28-days β-alanine supplementation, directly conflicting previous research, which demonstrated a 13% (10 ± 9 s) increase in 45% MVIF hold-times (Sale *et al.,* 2012). The reason for a lack of a significant effect is uncertain, given that IKET hold times reported by both investigations were similar and aligned to hold times predicted by the Rohmert equation (78 s; Ahlborg *et al.,* 1972). Blood lactate sampled from the finger 5 min post-exercise is indicative of the lower extremity lactate release (Comeau *et al.,* 2011), with both supplemental groups in the present research displaying similar levels of lactate accumulation. To further understand the relationship between skeletal muscle relaxation, skeletal muscle carnosine content and skeletal muscle fatigue, additional research examining dynamic fatiguing protocols are required, since contractile slowing (*i.e.*,

prolonged half-relaxation time) would affect shortening velocity and power output (Jones *et al.*, 2006).

5.5 Conclusion

The current research demonstrated that 28-days of β-alanine supplementation improved skeletal muscle relaxation, with declines in half relaxation time in both fresh and fatigued conditions. Reducing skeletal muscle relaxation rates is associated with improvements in skeletal muscle power output and exercise performance. It remains unclear however, whether the reported improvements in skeletal muscle relaxation time would be sufficient to result in improved exercise performance, particularly in the absence of any changes to the force-frequency relationship, peak force production, or contraction time. The mechanism for the ergogenic effect on skeletal muscle relaxation following increased carnosine content remains unclear. It could, however, be proposed that Ca^{2+} reuptake via direct or indirect mechanisms associated with SERCA pump activity is involved, as this is the rate-limiting step of skeletal muscle relaxation (see Study 4; Chapter 7).

Chapter 6: Effect of β-alanine supplementation on neuromuscular performance in fresh and fatigued skeletal muscle in older adults

The trial is registered with Clinicaltrials.gov, ID number NCT03111979

6.1 Introduction

The research reported within this thesis (Studies 1 and 2; Chapters 4 and 5) has examined the influence of 28-days of β-alanine supplementation on the electrically evoked contractile properties and voluntary force production of human skeletal muscle *in-vivo* in young healthy males. No alterations to maximal or explosive voluntary isometric force production, or the force-frequency relationship under both fresh and fatigued conditions following β-alanine supplementation was shown. Given that explosive voluntary force and the force-EMG relationship are influenced by Ca^{2+} related functions, if β-alanine supplementation did alter Ca^{2+} kinetics in the skeletal muscle, improvements in these measures would have been apparent. It can therefore be implied that increased carnosine content does not significantly influence Ca^{2+} related function. There was, however, a significant reduction in skeletal muscle resting and potentiated twitch HRT following 28-days of β-alanine supplementation during both fresh and fatigued conditions (Studies 1 and 2; Chapters 4 and 5). Additionally, β-alanine supplementation significantly reduced skeletal muscle octet HRT in both fresh and fatigued conditions (Study 2; Chapter 5). One explanation for an improvement in the skeletal muscle relaxation speed is the reuptake of Ca^{2+} in the skeletal muscle (Gafni & Yuh, 1989), with enhanced relaxation rates associated with improved skeletal muscle power output and exercise performance. Data reported in Studies 1 and 2 (Chapters 4 and 5) suggested that increased skeletal muscle carnosine content, via β-alanine supplementation, may be able to alter exercise performance through a Ca^{2+} kinetics mechanism, specifically during skeletal muscle relaxation phase.

Throughout the ageing process, there is a significant decline in skeletal muscle cross-sectional area, muscle fibre quantity, and carnosine concentrations (Lexell, 1995; Stuerenburg & Kunse, 1999; Doherty 2003; Tallon *et al.*, 2007; Verdijk *et al.*, 2010). The amalgamation of these factors increases the sense of frailty in older adults, impairing balance, gait speed, and increasing risk of falling (Madureira *et al.*, 2010), with one in three older individuals falling annually (Tinetti *et al.*, 1988). Declines in skeletal muscle power and strength are most notable in the lower-extremities, including the knee extensor muscles (Sieri & Beretta, 2004; Skelton *et al.*, 2002). Maintaining lower-extremity muscular strength is vital in preserving independence and quality of life in older adults. Although limited research exists, β-alanine supplementation has also been shown to increase skeletal muscle carnosine content in older adults, by 85% in twelve weeks (del Favero *et al.*, 2012). Ingestion of β-alanine in this older population has been associated with significant positive improvements in exercise performance (Stout *et al.*, 2008; del Favero *et al.*, 2012; McCormack *et al.*, 2013; Glenn *et al.*, 2015; 2016). Research by Stout *et al.*, (2008) reported improvements in the PWC_{FT} that were two-fold greater in older individuals than those reported in younger participants (younger; 12 – 15% vs. older; 29%; Stout *et al.*, 2006). Highlighting the possibility that older adults may experience a greater beneficial effect of β-alanine supplementation

compared to younger counterparts. Nonetheless, disparity remains, since β-alanine supplementation in older adults has not significantly improved measures of skeletal muscle function or quality of life (del Favero *et al.,* 2012). Due to the limited research in this population, it remains unclear how β-alanine supplementation alters skeletal muscle performance, and whether the mechanisms associated with increased skeletal muscle carnosine content in younger adults are similar to those in older individuals.

The current investigation investigated the effects of 28-days of β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and skeletal muscle contractility in both fresh and fatigued human skeletal muscle in healthy older adults (60 – 80 y). It was hypothesised that increased skeletal muscle carnosine content, via β-alanine supplementation, would subsequently improve the speed of skeletal muscle relaxation compared to the responses following placebo supplementation.

6.2 Methodology

6.2.1 Participants

Sixteen participants provided written informed consent and were then stratified and allocated into the two supplementation groups [placebo or β-alanine] based on MVIF values recorded during the familiarisation session. All participants completed all aspects of the study (Table 6.1), which was approved by the Nottingham Trent University Human Ethical Review Committee (Application #343). Self-reported sleep quality categorised by the PSQI, reported participants as good sleepers, with no differences between groups or following supplementation (Table 6.2). There were no significant differences between groups or pre- to post-supplementation regarding participant mood scores (BRUMS), physical activity levels (Modified Baecke Questionnaire for Older Adults), depression (Geriatric Depression Scale), falls efficacy (Falls Efficacy Scale) or pain scores (Global Pain Scale) (Table 6.2).

Table 6.1: Characteristics of participants (n = 16).

	Placebo (n = 8)	**β-alanine (n = 8)**
Age (years)	64 ± 3	65 ± 3
Body mass (kg)	83.4 ± 10.4	74.0 ± 11.2
Height (m)	169 ± 11	171 ± 12
MVIF (N)	334 ± 117	321 ± 132
Gender (M/F)	4/4	3/5

Data are mean ± 1SD. No significant differences were reported. MVIF: Maximal voluntary isometric force

6.2.2 Experimental design

Over a five-week period, three experimental sessions were undertaken by each participant; familiarisation sessions were held around seven days prior to the baseline session, with the final session conducted following 28-days of supplementation with either β-alanine or placebo. Participants were familiarised with the voluntary and electrical evoked knee extensor contractions, with all assessment undertaken on the dominant leg. Baseline and the follow-up session involved identical protocols, performed in accordance to a strict schedule, and undertaken at a consistent time of day. Participants were instructed to abstain from alcohol and strenuous/unaccustomed exercise for 36 hr before each session, with caffeine prohibited on the day of the protocol. Compliance with these requests was confirmed verbally with the all participants before commencement of the protocol. All raw data analyses, exclusions, and statistical analyses was undertaken by an experimenter blind to supplemental group, ensuring double-blind status.

6.2.3 Experimental protocol

Upon arrival to the familiarisation session participant's height and weight were recorded. Participants were seated in a rigid, custom-built isometric dynamometer (Fig. 3.5.3; page 36) with hip and knee joint angles of approximately 95° and 100° (180° = full extension), as previously described (Hannah *et al.,* 2012; 2013). Isometric knee extension force and EMG signals were recorded during all sessions, the details of which have been described in sections 3.5.3 and 3.5.4, page 38-39. Several isometric voluntary and electrically evoked contractions were undertaken during each session, including:

a) MVIC; as described in section 3.6.1, page 42.
b) Explosive voluntary contraction; as described in section 3.6.2, page 44.
c) IKET; as described in section 3.6.3, page 47.
d) Twitches (resting and potentiated); as described in section 3.6.4, page 49.
e) Doublet contractions; as described in section 3.6.5, page 53.
f) Force-EMG relationship; as described in section 3.6.7, page 55.
g) Force-frequency relationship; as described in section 3.6.8, page 57.

Table 6.2: Participant questionnaire scores.

	Placebo (n = 8)		β-alanine (n = 8)	
	Pre-supplementation	**Post-supplementation**	**Pre-supplementation**	**Post-supplementation**
PSQI	4 ± 3	5 ± 3	5 ± 3	5 ± 3
BRUMS				
Anger	5 ± 1	5 ± 1	5 ± 1	5 ± 1
Confusion	4 ± 2	4 ± 1	5 ± 2	4 ± 2
Depression	4 ± 2	4 ± 2	4 ± 1	4 ± 1
Fatigue	5 ± 2	4 ± 1	7 ± 3	6 ± 2
Tension	5 ± 2	5 ± 1	5 ± 2	4 ± 1
Vigour	14 ± 2	13 ± 4	13 ± 3	13 ± 3
MBQOA Physical activity				
Household	22 ± 5	21 ± 5	23 ± 6	23 ± 4
Sport	4 ± 2	4 ± 2	2 ± 2	2 ± 2
Leisure	3 ± 4	3 ± 3	2 ± 3	3 ± 3
Overall	29 ± 8	28 ± 7	27 ± 7	27 ± 5
Generic depression score	0 ± 0	0 ± 1	0 ± 1	1 ± 1
Falls efficacy scale score	10 ± 0	10 ± 0	10 ± 1	10 ± 1
Global Pain Score	3 ± 2	3 ± 6	6 ± 6	6 ± 4

No significant differences were reported. PSQI: Pittsburgh Sleep Quality Index. BRUMS: Brunel University Mood Scale. MBQOA: Modified Baecke questionnaire for older adults. Data are mean ± 1SD.

6.2.4 Supplementation

Participants were supplemented with 4.8 $g \cdot day^{-1}$ of either β-alanine or a matched placebo in tablet form over 28-days. All supplements were provided in identical white tubs by an individual not directly involved in testing or data analysis. The supplementation protocol consisted of two 800mg tablet ingested three times per day, at 2 - 4 h intervals. Compliance with the supplementation period was monitored with compliance logs, both groups reported a high degree of compliance, reported at 95 ± 4% in the β-alanine group (total dose of 128 ± 6 g) and 93 ± 4% in the placebo group (total dose of 125 ± 5 g; independent sample t-test, P = 0.36). Participants consumed a total dose of ~130 g β-alanine, increasing skeletal muscle carnosine content by an expected ~40% from baseline (del Favero *et al.*, 2012). There were no reported symptoms of paraesthesia from any of the participants.

6.2.5 Statistical analysis

The G* Power 3.1.6 software programme was used to calculate an *a-priori* power calculation for sample size, having as parameters: statistical test (ANOVA), α = 0.001, β = 0.50 (power of the sample), number of groups analysed (2 = β-alanine and placebo), and the effect of sample size based on previous findings two investigations (Stout *et al.*, 2008; Hobson *et al.*, 2012). Based on the effect size reported in Stout *et al.*, (2008) a minimum of 8 participants would be required. Dependent variables (MVIF, EMD, HRT, TPT, slope and intercept of force-EMG relationship, frequency at 50% of force response for the force-frequency relationship) were evaluated using a two-way mixed-model (group × session) ANOVA. Dependent variables measured over several time points (force and EMG during explosive voluntary contractions, evoked twitch, and doublet force) were analysed using a three-way mixed-model (group × session × time) ANOVA. All variables were assessed during both fresh and fatigued conditions. IKET variables (TTF and impulse) were analysed using a two-way mixed-model ANOVA.

The impact of the fatigue hold contraction on dependant variables (MVIF, EMD, HRT, TPT, slope and intercept of force-EMG relationship, frequency at 50% of force response for the force-frequency relationship) was analysed using a three-way mixed-model (group × session × fatigue) ANOVA. Dependent variables measured over several time points (force and EMG during explosive voluntary contractions, evoked twitch and doublet force) were analysed using a three-way mixed-model (group × session × percentage change) ANOVA based on percentage change between fresh and fatigued values.

Assessed variables were tested for normality using the Shapiro-Wilks test, and for homogeneity using the Fevene test. A Greenhouse-Geisser correction was applied when the ANOVA assumption of sphericity was violated, and significant interaction effects were followed-

up by independent sample *t*-tests on the individual percentage change values for each condition. Effect size for multiple comparisons was calculated using η_p^2 and η_g^2 eta squared (Lakens, 2013). Providing two effect sizes is suggested to yield a greater understanding of a specific effect (Preacher & Kelly, 2011). *Post hoc* comparisons to explain any significant interactions are reported with Cohen's *d* and Hedges *g* effect sizes. An effect size of 0.2 – 0.5 was defined as small, 0.5 – 0.8 as medium and ≥ 0.8 as a large effect (Schünemann *et al.,* 2008). Intra-individual variability was assessed using the mean intra-individual CV between the pre- and post-supplementation sessions for the placebo group [(SD / mean) × 100]. Statistical analyses were completed using SPSS version 22 (SPSS Inc., Chicago, IF, USA) and Microsoft Excel (Microsoft Inc., USA). Statistical significance was accepted at $P \leq 0.05$, with data presented as mean ± 1 SD.

6.3 Results

6.3.1 Maximum and explosive voluntary contractions

Twenty-eight days of supplementation had no effect on MVIF in fresh (P = 0.85; f (1, 14) = 1.23, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$) or fatigued skeletal muscle (P = 0.15; f (1, 14) = 2.30, $\eta_p^2 = 0.1$, $\eta_g^2 = 0.1$; Fig. 6.1A). The mean CV in the placebo group was 5 and 6% in fresh and fatigued skeletal muscle. MVIF was significantly lower following the completion of the IKET (P < 0.001; f (1, 14) = 30.54, $\eta_p^2 = 0.7$, $\eta_g^2 < 0.1$), with no differences between sessions (P = 0.51; f (1, 14) = 0.45, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$) or groups (P = 0.90; f (1, 14) = 0.02, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$) (β-alanine: 14 – 18%; placebo : 11 - 12%; Fig. 6.1A). There was no effect of supplementation on force measures at 25 ms intervals during explosive voluntary contractions in fresh (P = 0.71; f (5, 70) = 0.59, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × time point); P = 0.45; f (1, 14) = 0.60, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session)) or fatigued skeletal muscle (P = 0.90; f (5, 70) = 0.32, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × time point); P = 0.37; f (1, 14) = 0.87, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session); Fig. 6.1A). The percentage change in explosive force between fresh and fatigued conditions was not altered between sessions (P = 0.27; f (1, 14) = 1.34, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$) with no group × session interaction (P = 0.80 f (1, 14) = 0.07, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). The mean CV in the placebo group during explosive contractions was 12 - 18% at 25 - 50 ms and 5 - 10% from 75 - 150 ms during a fatigued condition and 17 - 19% at 25-50 ms and 6 - 17% from 75 - 150 ms during the fatigued condition.

Agonist EMG normalised to M_{max} during MVICs and explosive contraction remained uninfluenced by supplementation in fresh (P = 0.30; f (3, 42) = 1.26, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × time point); P = 0.85; f (1, 14) = 0.04, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session)) and fatigued skeletal muscle (P = 0.98; f (3, 30) = 0.05, $\eta_p^2 < 0.1$, $\eta_g^2 = 0.1$ (group × session × time point); P = 0.68; f (1, 10) = 0.18, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session); Fig. 6.1B). The mean CV values in the placebo group for agonist EMG in fatigued skeletal muscle were 44% at 0 - 50 ms, 33% at 50 - 100 ms and 41% at 100 - 150 ms time windows, and 39% at MVIF. The mean CV values in the placebo group for agonist EMG in fatigued skeletal muscle were 20% at 0 - 50 ms, 30% 50 - 100 ms and 21% at 100 - 150 ms time windows, and 25% at MVIF. The percentage change in agonist EMG normalised to M_{max} during MVICs and explosive contraction was not significantly altered between sessions (P = 0.24; f (1, 14) = 1.53, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$), with no group × session interaction (P = 0.73; f (1, 14) = 0.12, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$).

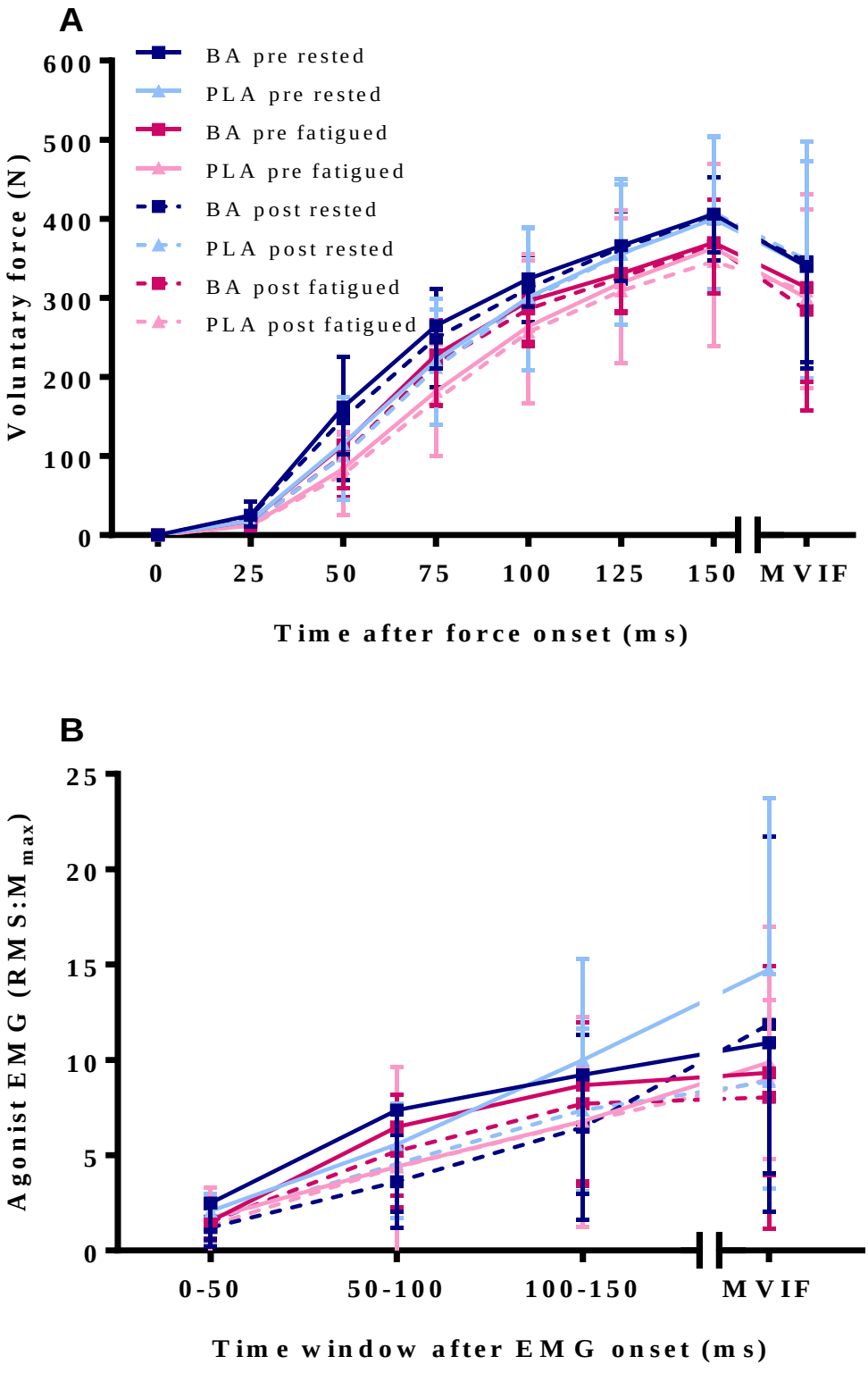

Fig. 6.1: Explosive and maximal isometric voluntary force (MVIF) (A), and agonist surface electromyography (EMG) normalised to maximum muscle action potential (M_{max}) during explosive contractions (0 - 50, 50 - 100 and 100 - 150 ms from onset) and at MVIF (B) for the β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle. RMS: Root mean square. Data are mean ± 1SD.

6.3.2 Twitches

Resting twitches: Supplementation did not significantly influence fresh or fatigued skeletal muscle twitch force (*Fresh*: P = 0.07; *f* (2, 28) = 2.89, η_p^2 < 0.1, η_g^2 = 0.1 *Fatigued*: P = 0.94; *f* (2, 28) = 0.06, η_p^2 < 0.1, η_g^2 < 0.1 (group × session × time point); *Fresh*: P = 0.25; *f* (1, 14) = 1.45, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.39; *f* (1, 14) = 0.80, η_p^2 < 0.1, η_g^2 < 0.1 (group × session); Table 6.3). There was no significant group × session interaction for EMD (*Fresh*: P = 0.67; *f* (1, 14) = 0.16, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.62; *f* (1, 14) = 0.26, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3), TPT (*Fresh*: P = 0.60; *f* (1, 14) = 0.28, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.54; *f* (1, 14) = 0.39, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3) or HRT (*Fresh*: P = 0.27; *f* (1, 14) = 1.32, η_p^2 = 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.43; *f* (1, 14) = 0.09, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 6.2). Under fresh conditions, HRT within the β-alanine group declined by 6 ± 14% (6 ± 14 ms) pre- to post-supplementation, whilst the placebo group increased by 4 ± 16% (3 ± 19 ms). Under fatigued conditions, HRT within the β-alanine group declined by 5 ± 18% (10 ± 31 ms) pre- to post-supplementation, whilst the placebo group increased by 15 ± 58% (5 ± 37 ms). Mean CV values for fresh skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 12, 15 and 12%, EMD was 15%, TPT was 5% and HRT was 9%. Mean CV values for fatigued skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 15, 17, and 15%, EMD was 16%, TPT was 16% and HRT was 7%.

The percentage difference in resting twitch force pre- to post-supplementation was not affected (P = 0.75; *f* (1, 14) = 0.13, η_p^2 < 0.1, η_g^2 < 0.1) with no group × session interaction (P = 0.80; *f* (1, 14) = 0.07, η_p^2 < 0.1, η_g^2 < 0.1). Resting twitch TPT was unaffected by the completion of the IKET (P = 0.19; *f* (1, 14) = 1.94, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3) with no group × fatigue (P = 0.81; *f* (1, 14) = 0.06, η_p^2 < 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.82; *f* (1, 14) = 0.05, η_p^2 < 0.1, η_g^2 < 0.1). EMD remained similar following the IKET (P = 0.72; *f* (1, 14) = 0.13, η_p^2 < 0.1, η_g^2 < 0.1) with no group × fatigue (P = 0.94; *f* (1, 14) = 0.06, η_p^2 < 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.49; *f* (1, 14) = 0.51, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3). Resting twitch HRT significantly increased following the completion of the IKET (P = 0.04; *f* (1, 14) = 5.42, η_p^2 = 0.3, η_g^2 = 0.1; Fig. 6.2). There was no group × fatigue (P = 0.09; *f* (1, 14) = 3.42, η_p^2 = 0.2, η_g^2 = 0.1) or group × session × fatigue interactions (P = 0.71; *f* (1, 14) = 0.14, η_p^2 < 0.1, η_g^2 < 0.1).

Potentiated twitches: Supplementation did not significantly influence fresh or fatigued skeletal muscle twitch force (*Fresh*: P = 0.46; *f* (2, 28) = 0.81, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.48; *f* (2, 28) = 0.76, η_p^2 < 0.1, η_g^2 < 0.1; (group × session × time point); *Fresh*: P = 0.59; *f* (1, 14) = 0.30, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.80; *f* (1, 14) = 0.07, η_p^2 = 0.1, η_g^2 < 0.1 (group × session); Table 6.3). There was no significant group × session interaction for EMD (*Fresh*: P = 0.66; *f* (1, 14) = 0.20, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.95; *f* (1, 14) = 0.005, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3), TPT (*Fresh*: P = 0.94; *f* (1, 14) = 0.007, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.95; *f* (1, 14) = 0.005, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3) or HRT (*Fresh*: P = 0.59; *f* (1, 14) = 0.32, η_p^2 < 0.1, η_g^2 < 0.1; *Fatigued*: P = 0.38; *f* (1, 14) = 0.84, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 6.2). In fresh skeletal muscle, there was a significant decline in EMD pre- to post-supplementation (P = 0.03; *f* (1, 14) = 6.13, η_p^2 = 0.1, η_g^2 = 0.1), this was evident in both conditions with no group × session effect. Mean CV values for fresh skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 14, 16, and 12%, EMD was 11%, TPT was 3% and HRT was 6%. Mean CV values for fatigued skeletal muscle in the placebo group: force at 25 and 50 ms and peak were 12, 20, and 14%, EMD was 13%, TPT was 4% and HRT was 5%.

The percentage change in potentiated twitch force was unaffected by supplementation (P = 0.38; *f* (1, 14) = 0.81, η_p^2 < 0.1, η_g^2 < 0.1) with no group × session interaction (P = 0.66; *f* (1, 14) = 0.20, η_p^2 < 0.1, η_g^2 < 0.1). Potentiated twitch TPT declined following completion of the IKET (P = 0.07; *f* (1, 14) = 3.85, η_p^2 = 0.2, η_g^2 < 0.1; Table 6.3), with no group × fatigue (P = 0.50; *f* (1, 14) = 0.49, η_p^2 < 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.90; *f* (1, 14) = 0.02, η_p^2 < 0.1, η_g^2 < 0.1). Potentiated EMD significantly prolonged following the IKET (P = 0.03; *f* (1, 14) = 6.38, η_p^2 = 0.3, η_g^2 < 0.01) with no group × fatigue (P = 0.17; *f* (1, 14) = 2.15, η_p^2 = 0.1, η_g^2 = 0.1) or group × session × fatigue interactions (P = 0.82; *f* (1, 14) = 0.05, η_p^2 < 0.1, η_g^2 < 0.1; Table 6.3). Potentiated HRT remained similar following the IKET (P = 0.87; *f* (1, 14) = 0.03, η_p^2 < 0.1, η_g^2 < 0.1). There was no group × fatigue (P = 0.61; *f* (1, 14) = 0.27, η_p^2 < 0.1, η_g^2 < 0.1) or group × session × fatigue interactions (P = 0.94; *f* (1, 14) = 0.007, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 6.2).

6.3.3 Doublet force

In both fresh (P = 0.82; f (2, 28) = 0.20, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × time point); P = 0.93; f (1, 14) = 0.009, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session)) and fatigued (P = 0.83; f (2, 28) = 0.19, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × time point); P = 0.81; f (1, 14) = 0.06, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session)) skeletal muscle, supplementation did not significantly alter doublet force at 25, 50ms and peak (Table 6.3). The percentage change in doublet force between fresh and fatigued conditions was not significantly affected by supplementation (P = 0.44; f (2, 28) = 0.85, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$), with no group × session interaction (P = 0.32; f (1, 14) = 1.07, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). Mean CV values for fresh skeletal muscle in the placebo group were 11, 14 and 13% for force at 25, 50 ms and peak. Mean CV values for fatigued skeletal muscle in the placebo group were 11, 5 and 5% for force at 25, 50 ms and peak.

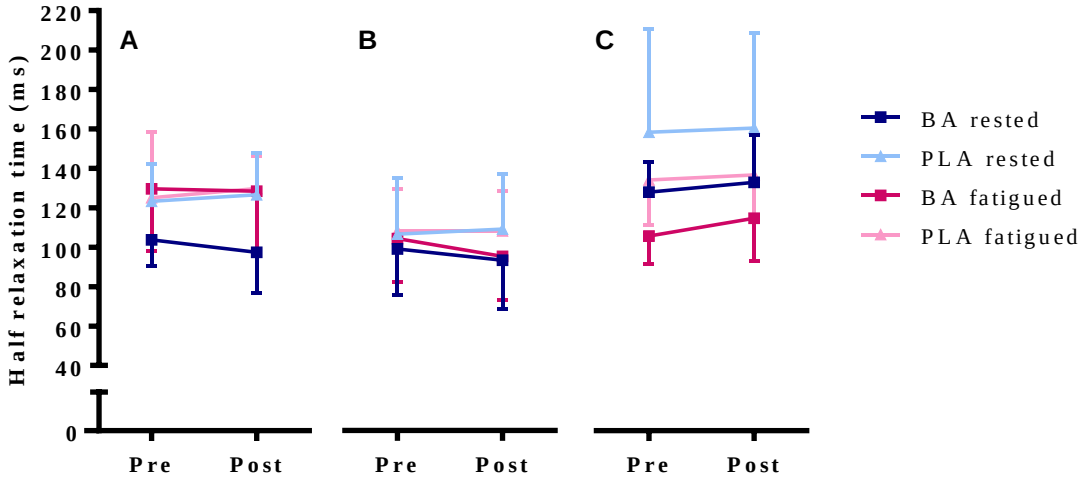

Fig. 6.2: Electrically evoked half relaxation time of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle during: resting twitch (a), potentiated twitch (b), and doublets (c). Data are mean ± 1SD.

Table 6.3: Electrically evoked force responses, time-to-peak tension (TPT) and electromechanical delay (EMD) of β-alanine and placebo groups pre- and post-supplementation, in fresh and fatigued skeletal muscle.

			Pre-supplementation					Post-supplementation				
			Force (N)					Force (N)				
			25	50	Peak	EMD (ms)	TPT (ms)	25	50	Peak	EMD (ms)	TPT (ms)
Resting Twitch	**Fresh**	**β-alanine**	14 ± 5	42 ± 16	60 ± 12	11 ± 1	89 ± 17	16 ± 6	46 ± 17	68 ± 15	10 ± 2	86 ± 19
	Fresh	**Placebo**	13 ± 4	41 ± 13	57 ± 16	13 ± 3	87 ± 12	14 ± 4	43 ± 12	57 ± 11	13 ± 2	88 ± 13
	Fatigued	**β-alanine**	16 ± 7	49 ± 20	64 ± 24	11 ± 2	84 ± 21	19 ± 6	55 ± 16	66 ± 22	11 ± 3	82 ± 21
	Fatigued	**Placebo**	16 ± 7	52 ± 19	65 ± 23	13 ± 4	82 ± 16	15 ± 6	52 ± 18	63 ± 19	13 ± 2	86 ± 25
Potentiated Twitch	**Fresh**	**β-alanine**	26 ± 13	71 ± 38	97 ± 35	12 ± 3	84 ± 16	29 ± 14	79 ± 33	96 ± 39	10 ± 2	81 ± 23
	Fresh	**Placebo**	26 ± 16	75 ± 27	97 ± 30	12 ± 3	79 ± 11	27 ± 11	88 ± 35	110 ±42	11 ± 3	77 ± 18
	Fatigued	**β-alanine**	23 ± 10	58 ± 31	77 ± 28	12 ± 2	76 ± 9	24 ± 12	63 ± 25	73 ± 28	11 ± 3	76 ± 20
	Fatigued	**Placebo**	22 ± 11	60 ± 27	79 ± 27	14 ± 4	75 ± 12	21 ± 10	65 ± 16	84 ± 28	12 ± 3	75 ± 16
Doublet	**Fresh**	**β-alanine**	28 ± 10	92 ± 31	126 ± 33	14 ± 3	100 ± 31	34 ± 9	108 ± 28	142 ± 32	14 ± 2	96 ± 19
	Fresh	**Placebo**	30 ± 7	100 ± 24	135 ± 29	14 ± 3	105 ± 37	31 ± 6	116 ± 26	152 ± 36	14 ± 1	108 ± 35
	Fatigued	**β-alanine**	30 ± 12	95 ± 33	122 ± 36	15 ± 2	93 ± 23	33 ± 11	101 ± 29	128 ± 35	15 ± 1	92 ± 22
	Fatigued	**Placebo**	30 ± 11	99 ± 32	130 ± 34	15 ± 4	98 ± 42	33 ± 10	104 ± 32	140 ± 27	15 ± 3	97 ± 25

Data are mean ± 1SD.

6.3.4 Force-electromyography relationship

The slope and y-intercept of the force-EMG relationship were unaffected by supplementation in both fresh (*slope:* P = 0.86; f (1, 14) = 0.03, η_p^2 < 0.1, η_g^2 < 0.1; *y-intercept*: P = 0.20; f (1, 14) = 1.77, η_p^2 = 0.1, η_g^2 < 0.1 (group × session)) and fatigued skeletal muscle (*slope:* P = 0.43; f (1, 14) = 0.67, η_p^2 < 0.1, η_g^2 < 0.1; *y-intercept*: P = 0.31; f (1, 14) = 1.10, η_p^2 < 0.1, η_g^2 < 0.1) (Fig. 6.3 and Table 6.4). There was also no influence of the IKET on the slope and y-intercept of the force-EMG relationship (*slope:* P = 0.62; f (1, 14) = 0.26, η_p^2 < 0.1, η_g^2 < 0.1; *y-intercept*: P = 0.94; f (1, 14) = 0.006, η_p^2 < 0.1, η_g^2 < 0.1), with no group × fatigue interaction (*slope:* P = 0.41; f (1, 14) = 71, η_p^2 < 0.1, η_g^2 < 0.1; *y-intercept*: P = 0.70; f (1, 14) = 0.16, η_p^2 < 0.1, η_g^2 < 0.1). The mean CV in the placebo group for the slope of the force-EMG relationship was 31% (fresh skeletal muscle) and 1% (fatigued skeletal muscle).

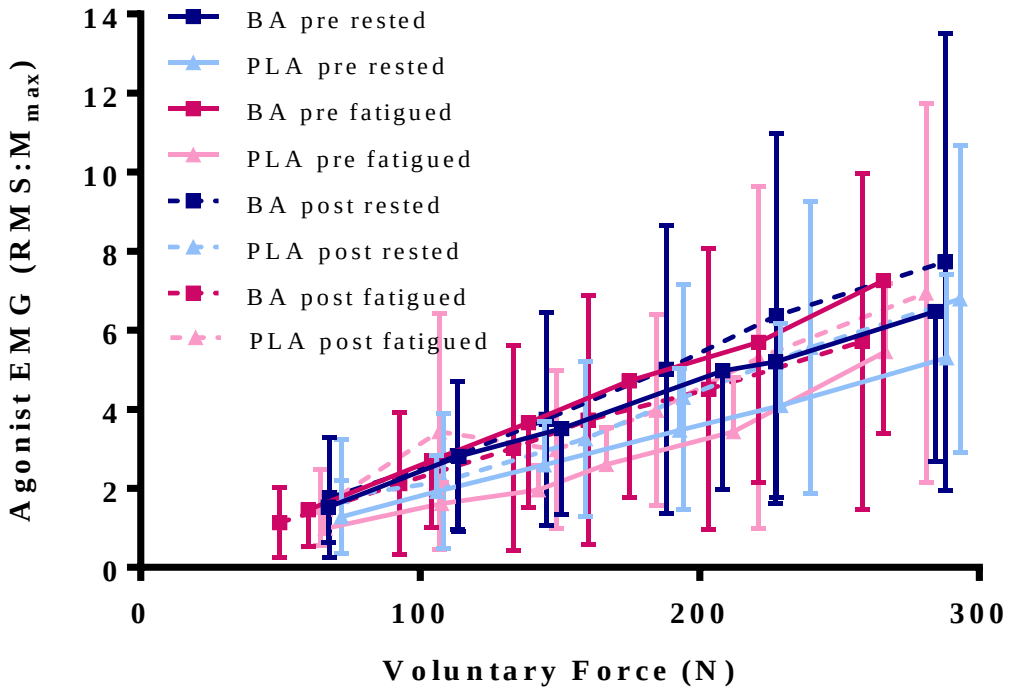

Fig. 6.3: Force-surface electromyography (EMG) relationship measured during submaximal voluntary contractions (15 - 90% MVIF) for the β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle. M_{max}: Maximum muscle action potential; RMS: Root mean square. Data are mean ± 1SD.

6.3.5 Force-frequency relationship

Supplementation did not significantly influence peak force at each frequency of stimulation (*fresh:* P = 0.99; f (9, 108) = 0.2, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *fatigued:* P = 0.52; f (9, 108) = 0.91; $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session × frequency); *fresh:* P = 0.61; f (9, 108) = 0.28, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *fatigued:* P = 0.64; f (9, 108) = 0.23; $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session); Fig. 6.4). The frequency at 50% of the force response (Table 6.4) in fresh (*peak force*: P = 0.98; f (1, 14) < 0.01, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *frequency at 50%:* P = 0.31; f (1, 14) = 1.12, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$ (group × session)) and fatigued skeletal muscle was unaffected by supplementation (*peak force*: P = 0.81; f (1, 14) = 0.06, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$; *frequency at 50%:* P = 0.30; f (1, 14) = 1.15, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). Peak force significantly declined following the completion of the IKET (P = 0.03; f (1, 14) = 6.39, η_p^2 = 0.3, η_g^2 = 0.3), with no group × fatigue interaction (P = 0.48; f (1, 14) = 0.52, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$). The frequency at 50% of the force response remained unaffected by the IKET (P = 0.46; f (1, 14) = 0.57, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$), with no group × fatigue interaction (P = 0.68; f (1, 14) = 0.18, $\eta_p^2 < 0.1$, $\eta_g^2 < 0.1$).

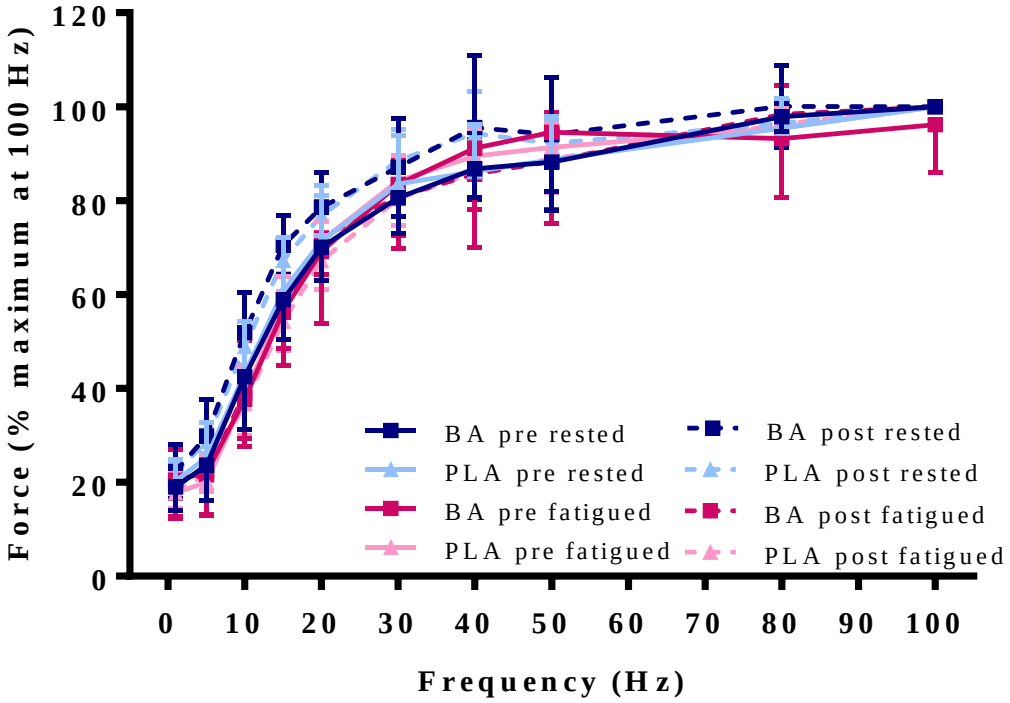

Fig. 6.4: Force-frequency relationship assessed during submaximal percutaneous stimulation for β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued skeletal muscle. Data are mean ± 1SD.

Table 6.4: Characteristics of the force-frequency and force-electromyography (EMG) relationships of β-alanine and placebo groups in fresh and fatigued skeletal muscle, pre- and post-supplementation.

	Pre-supplementation				**Post-supplementation**			
	β-alanine		**Placebo**		**β-alanine**		**Placebo**	
	Fresh	**Fatigued**	**Fresh**	**Fatigued**	**Fresh**	**Fatigued**	**Fresh**	**Fatigued**
Force-frequency relationship								
Frequency at 50% of response, Hz	13.6 ± 2.8	14.0 ± 2.4	12.9 ± 2.4	13.8 ± 3.3	14.3 ± 3.6	14.1 ± 3.4	15.0 ± 4.2	14.7 ± 3.0
Force-EMG relationship								
Intercept (RMS:M_{max})	-0.16 ± 0.62	-0.33 ± 0.56	-0.13 ± 0.52	-0.67 ± 0.69	-0.44 ± 0.87	-0.02 ± 0.56	-0.10 ± 0.55	0.29 ± 1.79
Slope (RMS:M_{max}/N)	0.023 ± 0.012	0.027 ± 0.014	0.019 ± 0.006	0.024 ± 0.012	0.027 ± 0.020	0.020 ± 0.013	0.025 ± 0.014	0.021 ± 0.015

Data are mean ± 1SD. M_{max}: Maximum muscle action potential; RMS: Root mean square.

6.3.6 Sustained isometric knee extensor endurance test

There was no group × session interaction for TTF (β-alanine: pre: 131 ± 65 s; post: 148 ± 63 s; Placebo: pre: 133 ± 46 s; Post: 154 ± 79 s; P = 0.82; f (1, 14) = 0.05, η_p^2 < 0.1, η_g^2 < 0.1; Fig. 6.5). Based upon the recorded TTF, the Rohmert equation (Rohmert, 1960) was used to calculate an estimated percentage of MVIF. Individuals within both the β-alanine and placebo group were holding the IKET at ~31% MVIF (~14% lower than asked), as denoted by longer hold times of ~65 s. There was no group × session interaction for impulse values (β-alanine: pre: 19 ± 8 $kN \cdot s^{-1}$; post 21 ± 7 $kN \cdot s^{-1}$; Placebo: pre: 19 ± 6 $kN \cdot s^{-1}$; post 21 ± 7 $kN \cdot s^{-1}$; P = 0.68; f (1, 14) = 0.18, η_p^2 < 0.1, η_g^2 < 0.1).

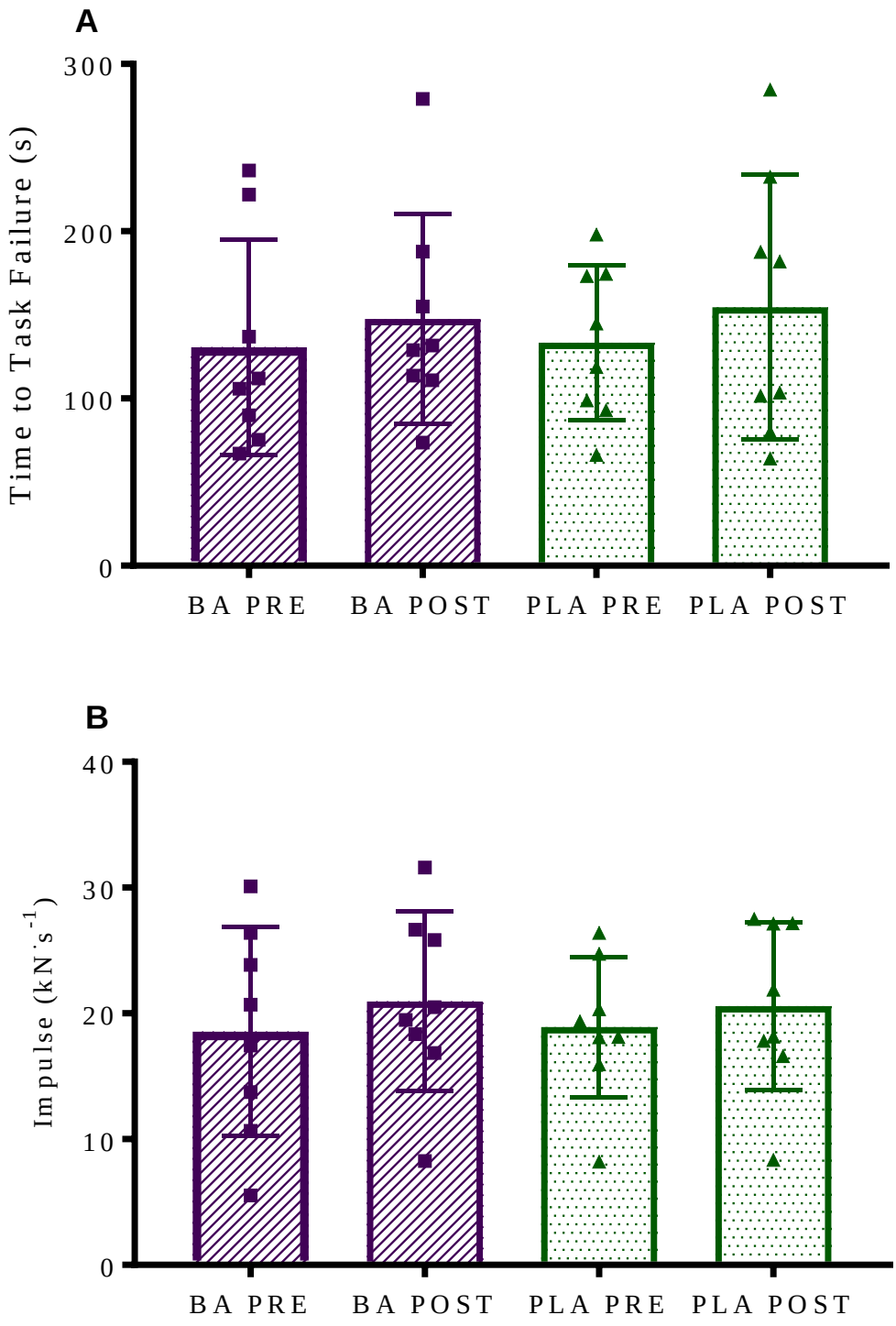

Fig. 6.5: Time to task failure (s; A) and impulse (kN·s; B) of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation. Data are mean ± 1SD. Individual data are presented as squares (BA), and triangles (PLA).

6.4 Discussion

The current investigation demonstrated that increased skeletal muscle carnosine content via β-alanine supplementation in healthy older adults (60 - 80 y) does not significantly influence force production capacity in either fresh or fatigued skeletal muscle conditions. In contrast to previous findings in young adults (Studies 1 and 2; Chapters 4 and 5), there was no decline in skeletal muscle HRT following 28-days of β-alanine supplementation in either fresh or fatigued skeletal muscle conditions. The current research indicated that increased skeletal muscle carnosine content in older healthy adults, does not alter Ca^{2+} kinetics, a proposed mechanism associated with improved exercise performance (Dutka & Lamb, 2004; Dutka *et al.*, 2012; Everaert *et al.*, 2013). These data extend the limited literature focused on increasing skeletal muscle carnosine content via β-alanine supplementation in older individuals, and is the first to comprehensively examine the effect of β-alanine supplementation on voluntary and electrically evoked contractile properties of *in-vivo* human skeletal muscle in older adults.

In-line with the findings in young healthy adults reported in Studies 1 and 2 (Chapters 4 and 5), β-alanine supplementation did not significantly influence voluntary force production, including maximal and explosive force variables, with similar neural drive responses during contractions, pre- and post-supplementation. Based on the current data it is proposed that 28-days of β-alanine supplementation (4.8 $g \cdot d^{-1}$) does not significantly influence skeletal muscle Ca^{2+} related function in older healthy adults. There was no leftward shift of the force-frequency curve, the associated measure of intracellular Ca^{2+} levels (Balnave & Allen, 1996) in either fresh or fatigued conditions following β-alanine supplementation. These *in-vivo* data contrast previously reported *in-vitro* research, where increased carnosine content improved the sensitivity of the muscle contractile apparatus to Ca^{2+} conducted in animal and human skeletal muscle fibres (Lamont & Miller, 1992; Dutka *et al.*, 2012). The current investigation, along with the research in young healthy adults (Studies 1 and 2; Chapter 4 and 5) suggests that the reported alterations to skinned muscle fibre and associated Ca^{2+} kinetics evident with increased carnosine content (Lamont & Miller, 1992; Dutka *et al.*, 2012), are not substantial enough to alter whole skeletal muscle contraction in either young or older healthy adults.

Surprisingly, increased skeletal muscle carnosine content via β-alanine supplementation in older adults did not significantly alter twitch concentration HRT, in either fresh or fatigued skeletal muscle conditions. These data contrast previous *in-vivo* research in younger adults (Studies 1 and 2; Chapters 4 and 5), where 28-days of β-alanine supplementation (6.4 $g \cdot d^{-1}$) resulted in significant reductions in skeletal muscle HRT in both fresh and fatigued muscle conditions (Studies 1 and 2; Chapters 4 and 5). It was hypothesised that the older adults would experience a similar, if not greater, reduction in skeletal muscle HRT following 28-days of β-alanine supplementation

compared to younger counterparts, due to the already apparent detrimental influence of the ageing process. The ageing process is associated with a negative impact on twitch contraction time and HRT in skeletal muscle (Allman, Cheng & Rice, 2004; Vandervoort & McComas, 1986; Narici, Bordini & Cerretelli, 1991; Hunter *et al.,* 1999), with HRT increased by 20 – 43% in older adults (Vandervoort & McComas, 1986; Cupido *et al.,* 1992; Doherty & Brown, 1997; Hunter *et al.,* 1999). Alterations to these phases of skeletal muscle concentration can influence the efficiency of the older adult to perform muscular activities (Kirkendall & Garrett, 1998), are functional parameters directly attributed to SR function (Coyle *et al.,* 1979; Larsson & Salviati, 1989) and indicate a reduction of SR efficiency to re-uptake Ca^{2+} (Klitgaard *et al.,* 1989; Petrella *et al.,* 1989). In-line with the declined skeletal muscle relaxation speed, there is also a reduction in the maximal rate of SERCA uptake and activity in older individuals (Hunter *et al.,* 1999). The apparent lack of alteration to skeletal muscle HRT following increased skeletal muscle carnosine content, via β-alanine supplementation, may be attributed to the supplementation protocol. The current investigation supplemented older individuals over 28-days with a daily dose of 4.8 $g \cdot day^{-1}$ β-alanine, currently the largest daily dose of β-alanine in this older population (del Favero *et al.,* 2012; Glenn *et al.,* 2015; 2016). No feelings of paraesthesia were reported following supplementation, supporting the ingestion of this larger 4.8$g \cdot d^{-1}$ dose of β-alanine in older adults (60 y). Yet, it is important to note that there are no direct measurements of skeletal muscle carnosine content. Although participants were supplemented with β-alanine at a daily dose of 4.8 $g \cdot day^{-1}$, the total β-alanine dose over the 28-days was ~134 g, around half of that reported previously (del Favero *et al.,* 2012). Due to the dose-dependent effect of β-alanine supplementation on carnosine content, and the reported 85% increase in skeletal muscle carnosine content following ingestion of ~280 g of β-alanine (del Favero *et al.,* 2012), the expected increase in skeletal muscle carnosine concentration following 134 g β-alanine would be approximately 40%. Older adults ingesting 28-days β-alanine supplementation, albeit at a lower daily and total doses (3.2 $g \cdot d^{-1}$; total dose; 89.6 g), have shown improvements in muscular performance, as indicated by peak torque and work completed during an isokinetic performance task (Glenn *et al.,* 2016). There were no direct measurements of skeletal muscle carnosine content, however, the authors proposed that increased skeletal muscle carnosine content from exogenous β-alanine consumption were responsible for the beneficial effects on performance (Glenn *et al.,* 2016). Based on the existing, although limited research, in older individuals it can be hypothesised that β-alanine supplementation resulted in increased skeletal muscle carnosine content, yet the exact extent of this increase warrants further investigation.

There was not effect of supplementation on IKET performance, with similar hold times reported pre- and post-supplementation for both the placebo and β-alanine groups. The lack of change in IKET hold times in older healthy individuals following β-alanine supplementation is consistent with the data previously reported in young healthy adults (Study 2; Chapter 5). The

IKET hold times in the older participants were ~70 s (~53%) greater than those reported in young healthy adults (Study 2; Chapter 5), implying that the older individuals were not completing the IKET at 45% MVIF. At 45% MVIF occlusion of the contracting skeletal muscle occurs, resulting in maximal accumulation of lactate, pyruvate, and H^+ (Ahlborg *et al.,* 1972). Due to the longer hold times, it can be estimated with the Rohmert equation (Rohmert, 1960), that the older participants were completing the IKET at around 30% MVIF, much lower than that instructed (45% MVIF). Completion of the IKET at 30% MVIF would not result in the same physiological responses as those evident during a contraction at 45% MVIF, indicating that the current IKET was not generating the same levels of muscle fatigue in these older adults, as evident in the younger adults. As such, it remains unlikely that the IKET protocol in older adults allowed examination of the effect of β-alanine supplementation on electrically evoked contractile properties and voluntary force production of fatigued *in-vivo* skeletal muscle in older adults. The IKET hold times in healthy older adults (Study 3; Chapter 6) and those in younger individuals (Study 2; Chapter 5) are in-line with IKET performance times reported by McPhee *et al.,* (2014). Older individuals completing an IKET at 50% MVIF, demonstrated hold times ~20 s longer in duration than those of younger adult (Young: 71.2 ± 5 s; Older: 91.5 ± 5 s; P = 0.001; McPhee *et al.,* 2014). The authors proposed that the differences in IKET hold times were associated with dissimilar contractile properties of the two populations (McPhee *et al.,* 2014), with older individuals having a greater proportion of slow contracting Type I muscle fibres compared to their younger counterparts (Lexell *et al.,* 1988). During the current investigation, the Rohmert equation (Rohmert, 1960) was used to estimate IKET hold times, in-line with previous research within this thesis (Study 2; Chapter 5). It should be highlighted, however, that the Rohmert equation (Rohmert, 1960) was designed to evaluate skeletal muscle contraction performance in young healthy adults. Therefore, the Rohmert equation (Rohmert, 1960) may not be applicable to accurately predict IKET performance times in older individuals, especially due to the potential impact of the ageing process, and declines in both the size and number of Type II muscle fibres (Lexell *et al.,* 1995), key contributors to skeletal muscle performance.

6.5 Conclusion

The present investigation was the first to comprehensively examine the effect of 28-days of β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and muscle contractility in fresh and fatigued human skeletal muscle in an older population (60 – 80 y). In-line with previous research in young adults (Study 2; Chapter 5), 28-days of β-alanine supplementation in older individuals reported no significant alterations to voluntary force responses or electrically evoked resting or potentiated twitches and octets variables (EMD, peak force and TPT). Surprisingly, there was no significant beneficial effect of β-alanine supplementation on skeletal muscle HRT, unlike that reported in young adults (Study 2; Chapter 5). These data imply that β-

alanine supplementation did not alter skeletal muscle the amount of Ca^{2+} released and/or sensitivity of the contractile properties to Ca^{2+} in older adults. These data extended the limited research examining the impact of β-alanine supplementation on skeletal muscle performance in older adults. Based on the present data, it can be proposed that older adults (60 – 80 y) can undertake β-alanine supplementation protocols, with a daily dose of 4.8 $g \cdot d^{-1}$, without experiencing paraesthesia, maintaining the double-blind nature of the investigation.

Chapter 7: The examination and manipulation of skeletal muscle carnosine content in rat tissue, and the effect on ATPase activity

The research reported within the current chapter are supported by the development of several methodological processes, as reported in appendix 1

7.1 Introduction

The research previously reported within the current thesis was the first to examine the effect of increased skeletal muscle carnosine content via 28-days of β-alanine supplementation, on the electrically evoked contractile properties and voluntary force production of human skeletal muscle *in-vivo*. This research proposed that in young healthy adult males (18 – 30 y) 28-days of β-alanine supplementation reduced skeletal muscle HRT (relative to equivalent placebo times) in fresh (Studies 1 and 2; Chapters 4 and 5) and fatigued (Study 2; Chapter 5) resting and potentiated twitch and octet contractions. These data were extended, examining the influence of 28-days β-alanine supplementation in older individuals (60 - 80 y), surprisingly there were no significant changes in skeletal muscle HRT under fresh or fatigued conditions. As discussed in Study 3 (Chapter 6), there were several potential reasons for the disparately in these findings. The declines in skeletal muscle HRT in young healthy adults (Studies 1 and 2; Chapters 4 and 5) are in-line with the hypothesis that increased carnosine content alters Ca^{2+} kinetics within the skeletal muscle, a proposed mechanism associated with the improvement in exercise performance (Dutka and Lamb, 2004; Everaert *et al.,* 2013; Guglielmi *et al.,* 2013; Sale *et al.,* 2013). Skeletal muscle relaxation speed can be impacted by several factors associated with Ca^{2+} kinetics (Muntener *et al.,* 1995; Allen, Lamb & Westerblad, 2008; Little *et al.,* 2011; Nogueira *et al.,* 2013), whether that be adaptations to the sensitivity of the contractile properties to Ca^{2+} and/or the amount of Ca^{2+} released from the SR lumen to the myofibrils. Working together, Ca^{2+} pumps and Ca^{2+} release channels form ubiquitous Ca^{2+} regulatory systems in muscle and non-muscle cells (McMullan *et al.,* 1997). The Ca^{2+}-dependent ATPase, SERCA, maintains the Ca^{2+} gradient across the membrane of intracellular vesicles of all cells (MacLennan & Kranias, 2003; Rossi & Dirksen, 2006). SERCA pump activity is not only associated with the speed at which the skeletal muscle relaxes (Gafni & Yuh, 1989), the rate-limiting step of skeletal muscle relaxation (Dux, 1993; Gillis *et al.,* 1985).

Carnosine is historically referred to as a cytosolic dipeptide, due to its role in neutralising the extensive formation of lactic acid during high-intensity anaerobic exercise within the cytosolic of the cell (Abe, 2000), and the prominent level (98%) of carnosine synthetase activity undertaken in the cytosolic (Harding & Fallon, 1979). Nonetheless, for carnosine to alter Ca^{2+} kinetics of the skeletal muscle, there needs to be a direct or indirect connection to the Ca^{2+}-dependent ATPases. Ca^{2+} pumps, such as the SERCA, are located on the longitudinal SR of the cardiac and skeletal muscle cells (MacLennan & Kranias, 2003), meaning that, to be most effective in this role carnosine needs to be apparent within the microsomal fraction of the skeletal muscle. Research examining skeletal muscle carnosine content in both humans and rats, is generally examined by muscle biopsy, or ^{1}H-MRS techniques (Steurenburg & Kunze, 1999; Harris *et al.,* 2006; Hill *et al.,* 2007; Derave *et al.,* 2007; Kendrick *et al.,*2008; 2009; Baguet *et al.,* 2010; Stellingwerff *et*

al.,2011; del Favero *et al.,* 2012; Bex *et al.,* 2014; Chung *et al.,* 2014; Gross *et al.,* 2014; Stegen *et al.,* 2014; Danaher *et al.,* 2014). These carnosine content are of the whole skeletal muscle fibre, there remains no research examining the distribution of the carnosine molecule across subcellular compartments. It can be hypothesised that if the carnosine molecule is reported within other subcellular compartments, in addition to the cytosolic as historically proposed (Harding & Fallon, 1979; Abe, 2000), the potential roles of carnosine extend above that of purely an intracellular buffer (see review; Sale *et al.,* 2010; 2013).

Upon initiation of skeletal muscle relaxation, Ca^{2+} ions are pumped out of the cytosolic fraction by PMCAs and pumped into intracellular compartments by SERCAs, where Ca^{2+} is stored and released upon necessity (Kochegarov, 2003). The SERCA pump serves as a dual function, (a) lowering the cytosolic Ca^{2+} to induce muscle relaxation, and (b) concurrently restoring the SR Ca^{2+} store required for following muscle contraction (MacLennan, 1970; Gommans *et al.,* 2002; Rossi & Dirksen, 2006; Periasamy & Kalyanasundraam, 2007; Bers, 2008). Through conformational changes, SERCA pumps transfer two Ca^{2+} ions from the cytoplasm into the endoplasmic lumen per molecule of ATP hydrolysed (Baba-Aissa *et al.,* 1998; Moller *et al.,* 2010). Following the transport of Ca^{2+} ions into the lumen, phosphate is released as Pi, quantification of the Pi during ATP-γ-P hydrolysis can be evaluated as a measure of SERCA activity (McMullen *et al.,* 2012). By acting on Ca^{2+} kinetics proteins directly or via other molecules, Ca^{2+} signalling can be inhibited or excited (Swietach *et al.,* 2013). This physiological process can, therefore, provide an understanding of the functionality of the Ca^{2+}-dependent ATPase activity. Carnosine has already been shown to maintain Ca^{2+} release pump activity at a pH of 6.0, where a complete decline in activity is evident (Zaloga, Roberts & Nelson, 1996). Increasing the availability of carnosine around the Ca^{2+}-dependent ATPase pumps, therefore, may impact Ca^{2+}-channel activity by interacting with the Ca^{2+}-channel itself (Batrukova & Rubstov, 1997), possibly via the existence of saturable binding site(s) for carnosine on the Ca^{2+}-channel.

The present investigation aimed to examine the distribution of the carnosine molecule across subcellular fractions (nucleic, mitochondrial, cytosolic and microsomal) within rat skeletal muscle tissue. It was hypothesised that carnosine, or its constituents, β-alanine and L-histidine, would need to be evident in both the cytosolic and microsomal (containing the SR) fractions of the skeletal muscle to support the mechanisms of carnosine as an intracellular buffer, and as a manipulator of skeletal muscle Ca^{2+} kinetics. Furthermore, these data investigated the impact of increased carnosine availability on ATPase activity, a measure associated with skeletal muscle relaxation, estimated by Pi generation. It was hypothesised based on the improvements in skeletal

muscle HRT *in-vivo* (Studies 1 and 2; Chapter 4 and 5), that increased carnosine availability would improve SERCA activity, as estimated by Pi generation.

7.2 Methodology

7.2.1 Materials and chemicals

The current investigation complied with the operations of the Animals (Scientific Procedures) Act 1986 (Amendment Regulations 2012, SI 2012/3039) and did not require institutional ethical approval (see Section 3.2; Page 35). Skeletal muscle tissue was obtained from the hind limbs of nine young (10 weeks, ~300 g in weight) International Genetic Standard Wistar rats (Table 7.1). Rats were kept in a light-controlled (12 h light/dark cycle) temperature-controlled (22ºC) environment and fed a pellet rodent diet, *ad libitum*, and had unrestricted access to water. Before experiments, the animals were killed by cervical dislocation followed by decapitation, the skeletal muscles from the hind limbs were then dissected out and placed in phosphate buffered saline (PBS). The Bicinchoninic acid (BCA) assay protein assay kit was purchased from Cheshire Sciences Limited (Chester, UK). Amino acid lithium citrate buffers were purchased from Biochrom (Cambourne, UK). BIOMOL Green phosphate assay reagent was purchased from Enzo Life Sciences (UK) Ltd. All other chemicals were purchased from Sigma Aldrich (St. Louis, MA, USA).

Table 7.1: Characteristics of the rat skeletal muscle tissue samples, weight of skeletal muscle is reported as wet muscle tissue in phosphate buffered saline.

Rat	Skeletal muscle weight (g)	Gender	Age (weeks)	Date collected
1	21.1	Male	10	8.11.16
2	23.4	Male	10	8.11.16
3	25.4	Male	10	8.11.16
4	22.3	Male	10	8.11.16
5	17.8	Male	10	8.11.16
6	17.6	Male	10	21.11.16
7	20.6	Male	10	21.11.16
8	22.1	Male	10	21.11.16
9	24.3	Male	10	21.11.16

7.2.2 *In-vitro* skeletal muscle tissue preparation

Fresh rat skeletal muscle tissue was rinsed in a homogenisation medium (PBS), trimmed of fat, connective tissue and minced. The muscle tissue was homogenised in an ice-cold homogenisation buffer (25 mM Potassium chloride, 20 mM HEPES pH 7.4, 1 mM Ethylenediaminetetraacetic acid, 50 mM Sucrose, 200 mM Mannitol, 1:300 dilution of protease inhibitor) using an electronic homogeniser (Ultra-turrax, IKA laboratory technology England Ltd, Oxford, UK). Homogenisation was undertaken at maximum speed for 1.5 min (Wrzosek, 2014), bringing the pestle to the bottom at least eight times (Wilkie & Schirmer, 2008). The homogenate was filtered through layers of cheesecloth (pore size = 75 μM) to remove cellular debris and connective tissue (Fig. 7.1). The filtrate was centrifuged at 1000 g for 10 min at 4^{o}C (Harrier 18/80 Refrigerated benchtop centrifuge, MSE Ltd, London, UK; Fig. 7.2; Fig. 7.3). The resulting pellet containing the nucleic fraction, was resuspended in a storage medium (25 mM potassium chloride, 20 mM Hepes pH 7.4, 250 mM sucrose, 5 mM magnesium chloride) and frozen at -80^{o}C. Prior to additional analysis, the nucleic pellet was defrosted and resuspended in 1 mL of storage buffer, the samples were vortexed for 15 s and spun at 500 g for 15 min (Dimauro *et al.,* 2012). The resulting pellet containing the nucleic fraction was re-suspended in 2 mL storage buffer, vortexed for 15 s and spun at 1,000 g for 15 min, the final pellet was removed and stored in 2 mL storage buffer (Dimauro *et al.,* 2012).

The separated supernatant was ultra-centrifuged at 20,000 g for 20 min at 4^{o}C in a Fixed angle (Type 70 Ti) Beckman rotor (Beckman Coulter, Fullerton, CA, USA; Fig. 7.2; Fig. 7.3). The resulting pellet containing the mitochondrial fraction, was resuspended in a storage medium and frozen at -80^{o}C. Prior to additional analysis, the mitochondrial pellet was defrosted and resuspended in 1 mL of storage buffer, the samples were vortexed for 15 s and spun at 11,000 g for 10 min. The resulting pellet was resuspended in 2 mL of storage buffer (Dimauro *et al.,* 2012).

The remaining supernatant was ultra-centrifuged at 100,000 g for 40 min at 4 ^{o}C in a Fixed angle (Type 70 Ti) Beckman rotor (Beckman Coulter, Fullerton, CA, USA; Fig. 7.2; Fig. 7.3). This step pellets the microsomal fraction derived from SR membranes, reducing soluble protein content and lowering the volume required for subsequent fractionation (Wilkie & Schirmer, 2008). The supernatant containing the cytosolic fraction was carefully decanted from the pellet and frozen at -80^{o}C. The resulting pellet containing the microsomal fraction, was resuspended in a storage medium and frozen at -80^{o}C.

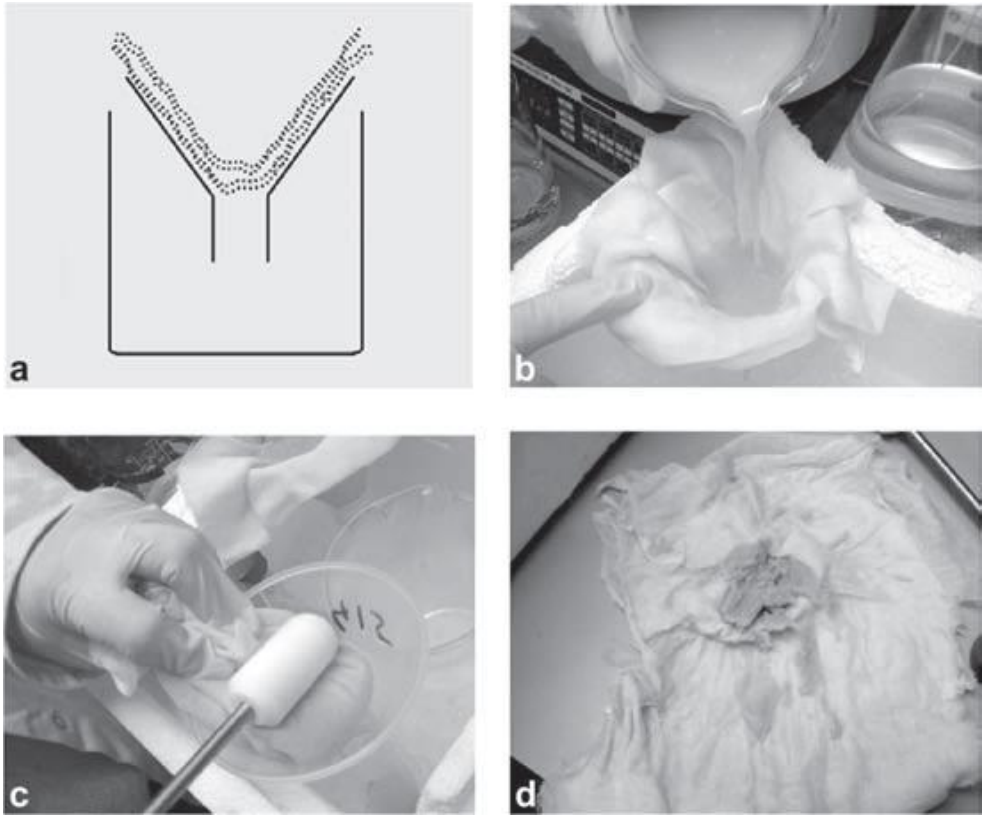

Fig. 7.2: Filtration of muscle homogenate. (a) Cheesecloth in a funnel suspended in a beaker. (b) Homogenate poured into the cheesecloth, drained into a beaker. (c) The cheesecloth is squeezed (d) A large amount of fibrous material should remain in the cheesecloth after all liquid has been recovered. From Wilkie & Schirmer (2008).

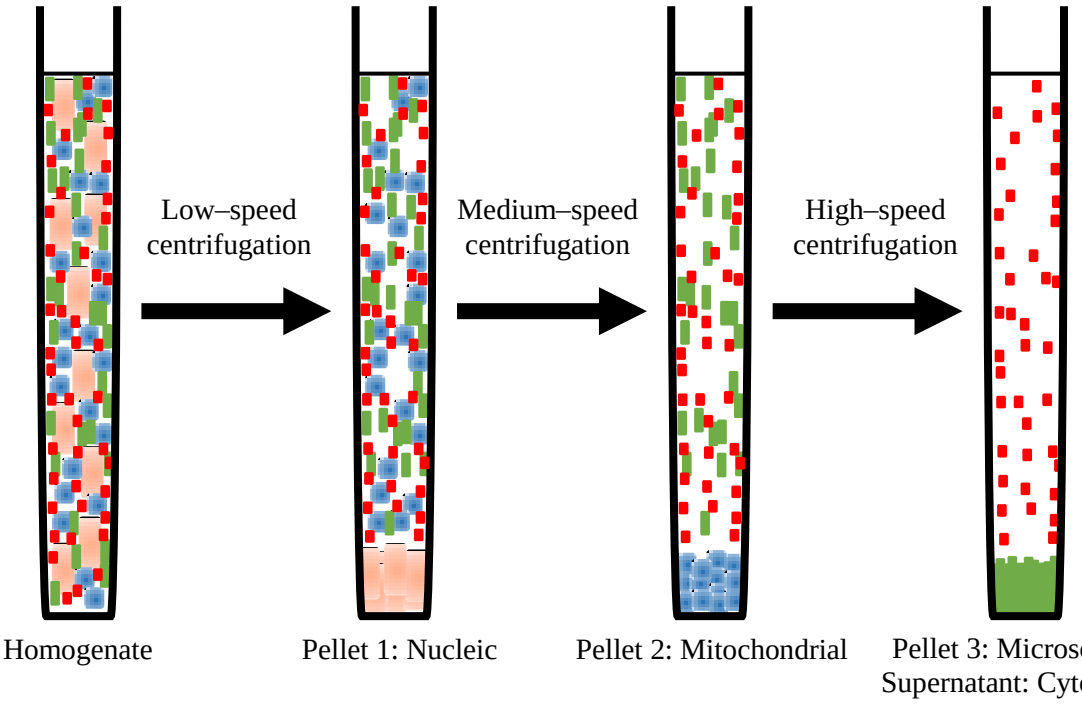

Fig. 7.1: Pictorial representation of the centrifugation process produces large centrifugal forces, causing the particles in the sample to sediment, the progressively higher the speeds and longer duration will pellet distinct subcellular fractions.

7.2.3 Protein assay

A fresh set of protein standards from 20 $\mu g \cdot mL^{-1}$ to 2 $mg \cdot mL^{-1}$ were diluted from the stock solution in the storage medium (25 mM potassium chloride, 20 mM Hepes pH 7.4, 250 mM sucrose, 5 mM magnesium chloride). The BCA working reagent was prepared for the standard protocol through the addition of 1 part of reagent B to 50 parts of reagent A, the volume of working reagent was calculated based on the number of standards and samples required. The working reagent was then allowed to reach room temperature. Once prepared, 25 µL of each standard, control and sample (10% dilution) were pipetted into microplate wells in triplicate where 200 µL of BCA assay working reagent was added. The microplate was then incubated for 30 min at 37^{o}C. Following incubation, the microplate was cooled to room temperature and optical density was read at 562 nM.

The values for each BCA standard were plotted against the protein concentration ($\mu g \cdot mL^{-1}$) of the sample. A curvilinear regression analysis was undertaken to produce the standard curve, in this case a 2nd order polynomial equation. The protein concentration of the assessed sample was then plotted as the dependant variable (y-axis) and the absorbance as the independent variable (X-axis), values were then placed into the equation $y = ax^2 + bx + c$, where solving y determines the protein concentration of the sample. The protein concentrations were then adjusted based on dilution value and converted to $mg \cdot mL^{-1}$.

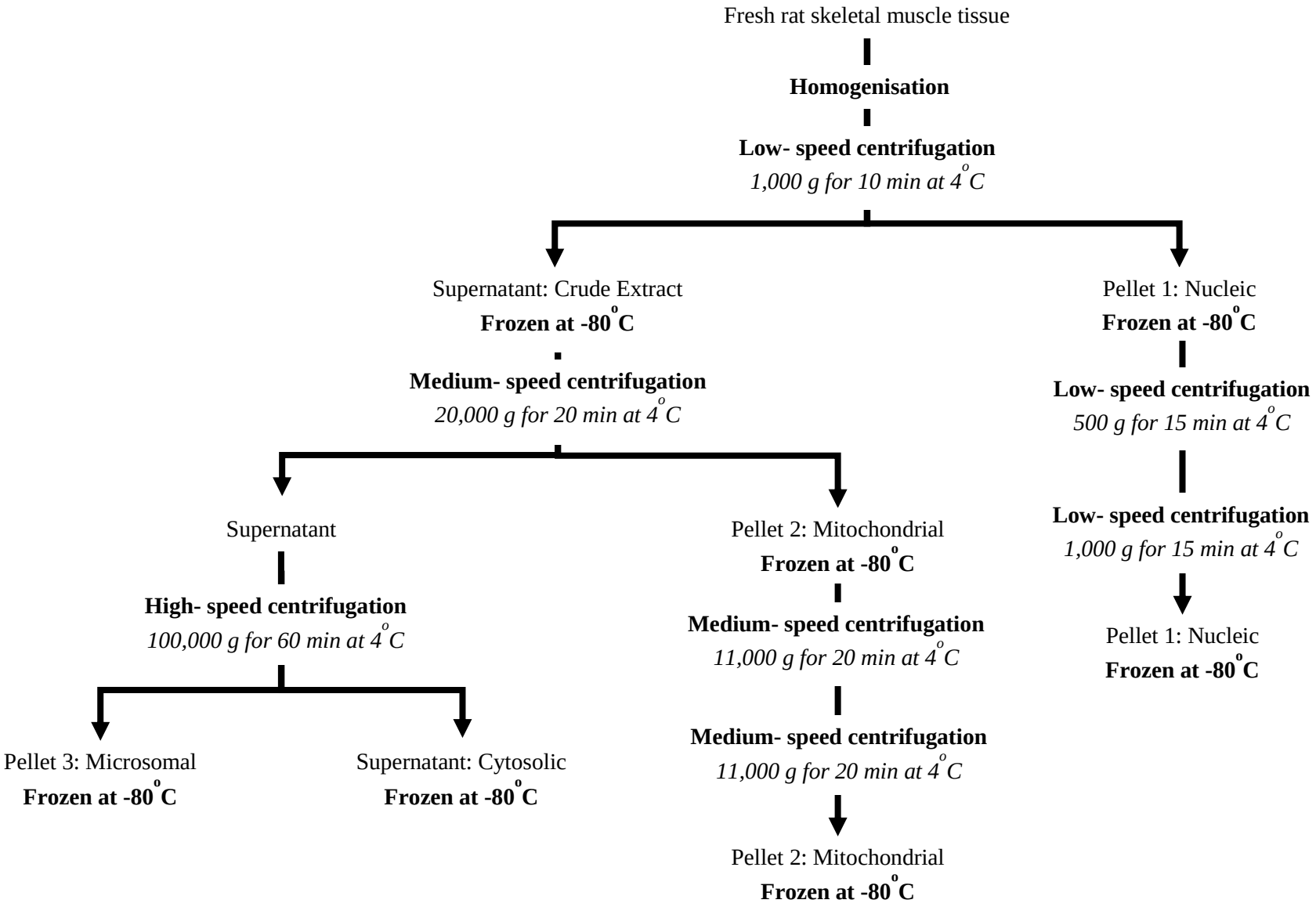

Fig. 7.3: The centrifugation protocol implemented within the current thesis, increasing in centrifugal forces to separate the sample into distinct subcellular fractionations.

7.2.4 Carnosine, β-alanine and L-histidine content

The physiological samples were thawed prior to deproteinisation achieved through the addition of a 5% 5-Sulphosalicylic acid solution (10 nmol 5-Sulphosalicylic acid; 20 μL L-Norleucine) to the samples. The contents were mixed and allowed to stand for 30 min at 4^{o}C. The precipitate was centrifuged at 10,000g for 5 min at 4^{o}C until the supernatant was clear, and finally filtered through a 0.2 μM membrane filter to remove any remaining particulate material. Solutions were analysed in an Amino Acid Analyser (Biochrom 30, Pharmacia Biochrom Ltd) by using ion-exchange chromatography. The amino acids were separated by using standard lithium citrate buffers of pH 2.80, 3.00, 3.15, 3.50, and 3.55. The post column derivatisation was performed with ninhydrin. Quantitation was performed by calibrating the peak heights to the internal standard, L-Norleucine. The programme EZ Chrom Elite (Biochrom, Cambourne, UK) was used to examine peak height, with ratio to internal standard calculated on Microsoft Excel 2013 (Microsoft Inc., Redmond, WA, USA).

7.2.5 BIOMOL green phosphate assay

Microsomal subcellular fractions were taken from nine skeletal rat muscle samples, and mixed with a buffer solution (100 mM Potassium Chloride, 20 mM HEPES pH 7.4 and 2 mM Magnesium Chloride) and varying levels of ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) and Ca^{2+}. The addition of EGTA and Ca^{2+} allowed the manipulation of Ca^{2+} (μM) and calculated free Ca^{2+} (μM) values within the samples. The standards of the BIOMOL Green phosphate assay ranged from 0 to 2 nmol, with incubation times of 15 min, the samples were diluted to 25% with storage buffer (see Appendix 1). Samples were incubated for 30 min at 25 ^{o}C, following which 100 μL of sample was mixed with 100 μL of BIOMOL Green, stopping the reaction. The samples were incubated at room temperature for 15 min to allow development of the green colour. The amount of Pi released during the Ca^{2+} uptake assay was determined by reading sample absorbance at 640 nM. The values for each BIOMOL Green phosphate assay standard were plotted against the Pi concentration (nmol) of the sample. A linear regression analysis was undertaken to produce the standard curve. The Pi concentration of the assessed sample was then plotted and then placed into the equation $y = ax^2 + b$, where solving y determines the Pi concentration of the sample.

Ca^{2+}-dependent ATPase activity: To calculate the amount of Ca^{2+}-dependent ATPase activity, the amount of Pi released during the 0 μM Ca^{2+} condition was subtracted from the 1 μM Ca^{2+} condition.

SERCA activity: Thapsigargin is a non-competitive inhibitor of SERCA activity, preventing uptake of Ca^{2+}, thereby emptying the intracellular Ca^{2+} stores (Thastrup, 1990). As such, thapsigargin has found popularity as an agent both to define and to manipulate intracellular Ca^{2+} pools (Bian *et al.,* 1991; Foskett *et al.,* 1991; Verma *et al.,* 1990; Kwan *et al.,* 1990). Inhibiting SERCA pump activity with thapsigargin can allow the isolation of other ATPases which generate Pi within the microsomal fraction, including the PMCA and the Golgi apparatus secretory protein ATPases (McMullen *et al.,* 2012). Thapsigargin can inhibit the PMCA, the Golgi apparatus secretory protein ATPases, although the concentration required to do so is 10 to 300 times greater than that needed to inhibit SERCA activity (Treiman *et al.,* 1998; Harper *et al.,* 2005). Pi generated from SERCA pump activity is defined as the difference between Pi generated the condition without thapsigargin, minus the Pi generated in the same condition in the presence of thapsigargin.

7.2.6 Statistical analysis

Dependent variable protein content was evaluated using a one-way mixed-model ANOVA. Dependent variables measured over several subcellular fractions (amino acid/dipeptide content relative to protein concentration) were analysed using a two-way mixed-model (amino acid/dipeptide × Subcellular fraction) ANOVA. Carnosine, β-alanine and L-histidine concentration relative to protein concentratin in each subcellular compartment was analysed using a one-way mixed-model ANOVA. Dependent variable (ATPase activity) was analysed using a three-way mixed-model (thapsigargin × Ca^{2+} × carnosine) ANOVA. Dependent variables (Ca^{2+}-dependent ATPase and SERCA activity) were analysed using a two-way mixed-model (thapsigargin × carnosine; Ca^{2+} × carnosine) ANOVA. Effect size for multiple comparisons was calculated using η_p^2 and η_g^2 eta squared (Lakens, 2013). Providing two effect sizes is suggested to yield a greater understanding of a specific effect (Preacher & Kelly, 2011). An effect size of 0.2 – 0.5 was defined as small, 0.5 – 0.8 as medium and ≥ 0.8 as a large effect (Schünemann *et al.,* 2008). Statistical analyses were completed using SPSS version 23 (SPSS Inc., Chicago, IF, USA) and Microsoft Excel (Microsoft Inc., USA). Statistical significance was accepted at $P < 0.05$, with data presented as mean ± 1 SD.

7.3 Results

7.3.1 Protein assay

The protein concentration for each subcellular fraction was assessed from nine skeletal muscle rat samples (Fig. 7.4). All samples were recorded in triplicate with the average reported. There was a significant main effect of subcellular fraction on protein concentration (P = 0.004; f (3, 24) = 40.6, η_p^2 = 0.9, η_g^2 = 0.9). The nucleic fraction had significantly greater protein content than all other subcellular fractions; mitochondrial (P = 0.008), cytosolic (P = 0.001) and microsomal (P = 0.002). The microsomal fraction had a similar protein concentration to both the mitochondrial and cytosolic fractions (P > 0.05). There were, however, significantly greater protein concentrations in the mitochondrial fraction compared to the cytosolic fraction (P = 0.003).

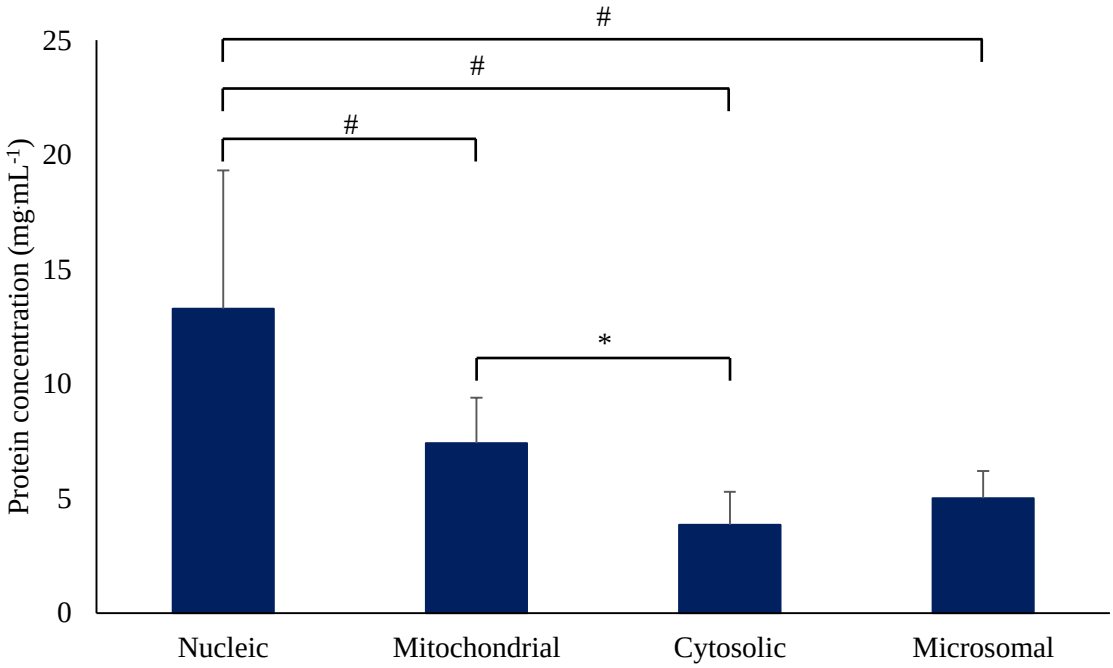

Fig. 7.4: Protein concentration ($mg \cdot mL^{-1}$) for the assessed subcellular fractions from nine rat skeletal muscle samples; nucleic, mitochondrial, cytosolic and microsomal. Data are mean ± 1SD. Post hoc analysis; # significantly different to nucleic subcellular fraction (P < 0.008). * Significantly different to mitochondrial fraction (P = 0.003).

7.3.2 Carnosine, β-alanine and L-histidine content

There was a significant subcellular fraction × dipeptide/amino acid interaction for muscle content ($P < 0.001$; f (6, 48) = 171.85, $\eta_p^2 = 1.0$, $\eta_g^2 = 0.9$; Fig. 7.5). There was a significant main effect of both dipeptide/amino acid ($P < 0.001$; f (2, 16) = 291.11, $\eta_p^2 = 0.8$, $\eta_g^2 = 0.5$) and subcellular fraction ($P < 0.001$; f (3, 24) = 184.99, $\eta_p^2 = 1.0$, $\eta_g^2 = 0.9$). Pairwise comparison analysis showed that there was significantly more carnosine across the subcellular fractions than both of its components, β-alanine ($P < 0.001$) and L-histidine ($P < 0.001$), although there was no difference between β-alanine and L-histidine contents. Dipeptide/amino acid contents in the cytosolic fraction were significantly greater than the microsomal fraction ($P < 0.001$), with both the cytosolic and microsomal amino acid contents significantly greater than those in the nucleic and mitochondrial fractions (all $P < 0.001$). There was no significant difference between the nucleic and mitochondrial fractions for dipeptide/amino acid contents ($P < 0.05$).

Carnosine contents were significantly different across subcellular fractions ($P = 0.001$; f (3, 24) = 180.51, $\eta_p^2 = 1.0$, $\eta_g^2 = 0.9$; Fig. 7.5). The largest contents were shown in the cytosolic fraction (192 ± 109 µM.g protein), with 69% of the total carnosine evident in this compartment, significantly greater than all other fractions (all $P < 0.001$). Carnosine contents in the microsomal fraction (64 ± 15 µM.g protein; 27% of total carnosine) were significantly larger than the nucleic and mitochondrial compartments ($P < 0.001$). There was no significant difference in carnosine contents between the nucleic and mitochondrial fractions (4 ± 3 µM.g protein; 5 ± 2 µM.g protein), each fraction containing around 2% of total carnosine contents.

There were significantly different β-alanine contents across subcellular fractions ($P = 0.001$; f (3, 24) = 44.18, $\eta_p^2 = 1.0$, $\eta_g^2 = 0.9$; Fig. 7.5). The largest contents evident in the cytosolic fraction (11 ± 5 µM.g protein), with 66% of the total β-alanine evident in this compartment,, significantly greater than the contents in the nucleic (0.5 ± 0.2 µM.g protein; $P < 0.001$), mitochondrial (1 ± 1 µM.g protein; $P < 0.001$) and microsomal (4 ± 2 µM.g protein; $P < 0.02$) compartments. The microsomal fraction β-alanine contents were 25% of the total β-alanine contents, significantly greater than the nucleic and mitochondrial fractions ($P = 0.007$; $P = 0.02$). No significant difference between nucleic and mitochondrial contents, each fraction containing only small percentages of total β-alanine contents (3 and 5%).

L-histidine contents were significantly different across subcellular fractions ($P < 0.001$; f (3, 24) = 49.91, $\eta_p^2 = 0.9$, $\eta_g^2 = 0.9$; Fig. 7.5). Significantly greater than the nucleic and mitochondrial fractions (both 1 ± 1 µM.g protein; $P < 0.001$), the contents of L-histidine in the cytosolic fraction (12 ± 5 µM.g protein) contained 59% of total L-histidine contents. These were not, however,

significantly greater than those in the microsomal fraction (7 ± 3 µM.g protein; P = 0.21). Microsomal L-histidine contents were significantly greater than the nucleic and mitochondrial fractions (P = 0.002; P = 0.001), containing 32% of the total L-histidine contents. There was no significant difference between L-histidine contents in the nucleic and mitochondrial fractions, each compartment containing over a small percentage of total L-histidine content (3 and 6%).

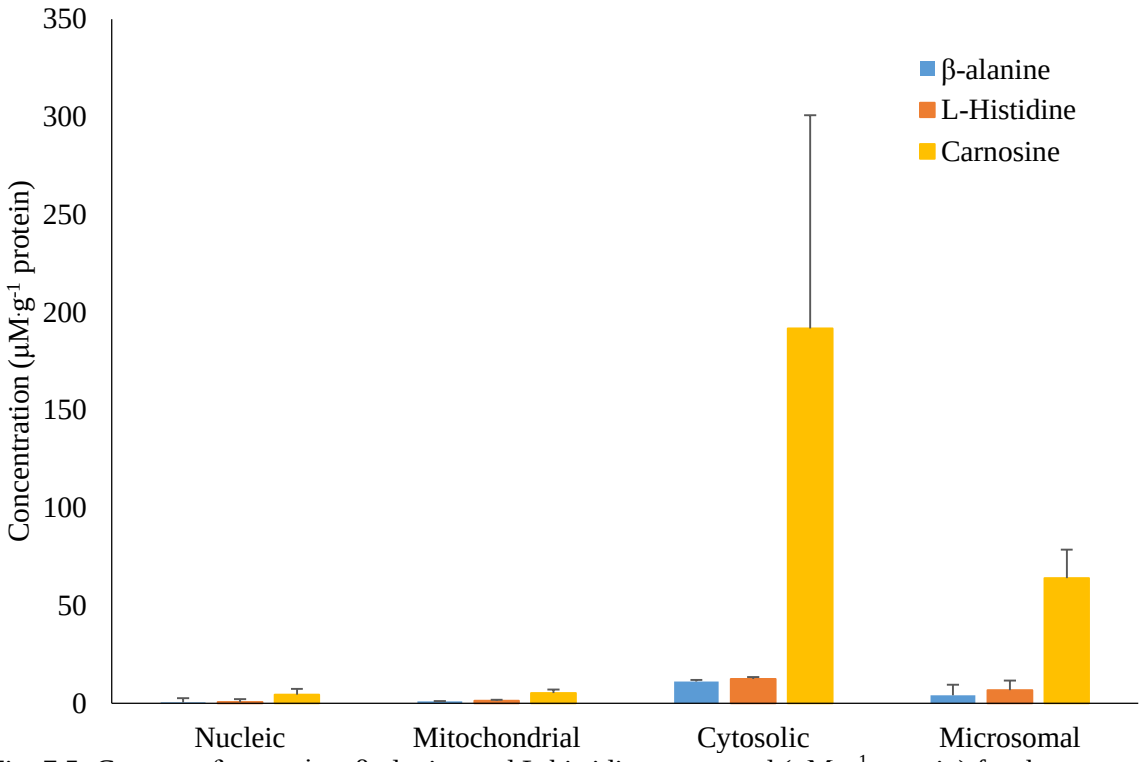

Fig. 7.5: Content of carnosine, β-alanine and L-histidine expressed (µM·g^{-1} protein) for the assessed subcellular fractions from nine rat skeletal muscle samples; nucleic, mitochondrial, cytosolic and microsomal fractions. Data are mean ± 1SD.

7.3.3 BIOMOL green phosphate assay

ATPase activity: There was no significant thapsigargin × Ca^{2+} × carnosine interaction, or carnosine × thapsigargin main effect of Pi generation via ATPase activity, all data are represented relative to subcellular protein content (Fig. 7.6). There was a significant main interaction between thapsigargin and Ca^{2+} (P = 0.002; f (1, 8) = 18.87, η_p^2 = 0.7, η_g^2 = 0.4), Ca^{2+} increased Pi generation with and without the addition of thapsigargin. Thapsigargin significantly decreased ATPase activity, with lower Pi generation via ATPase activity (P = 0.002; f (1, 8) = 19.93, η_p^2 = 0.7, η_g^2 = 0.4). Both Ca^{2+} (P = 0.004; f (1, 8) = 15.64, η_p^2 = 0.7, η_g^2 = 0.4) and carnosine (P = 0.004; f (1, 8) = 15.39, η_p^2 = 0.7, η_g^2 = 0.4) significantly increased ATPase activity, as highlighted by increased Pi generation (Fig. 7.6).

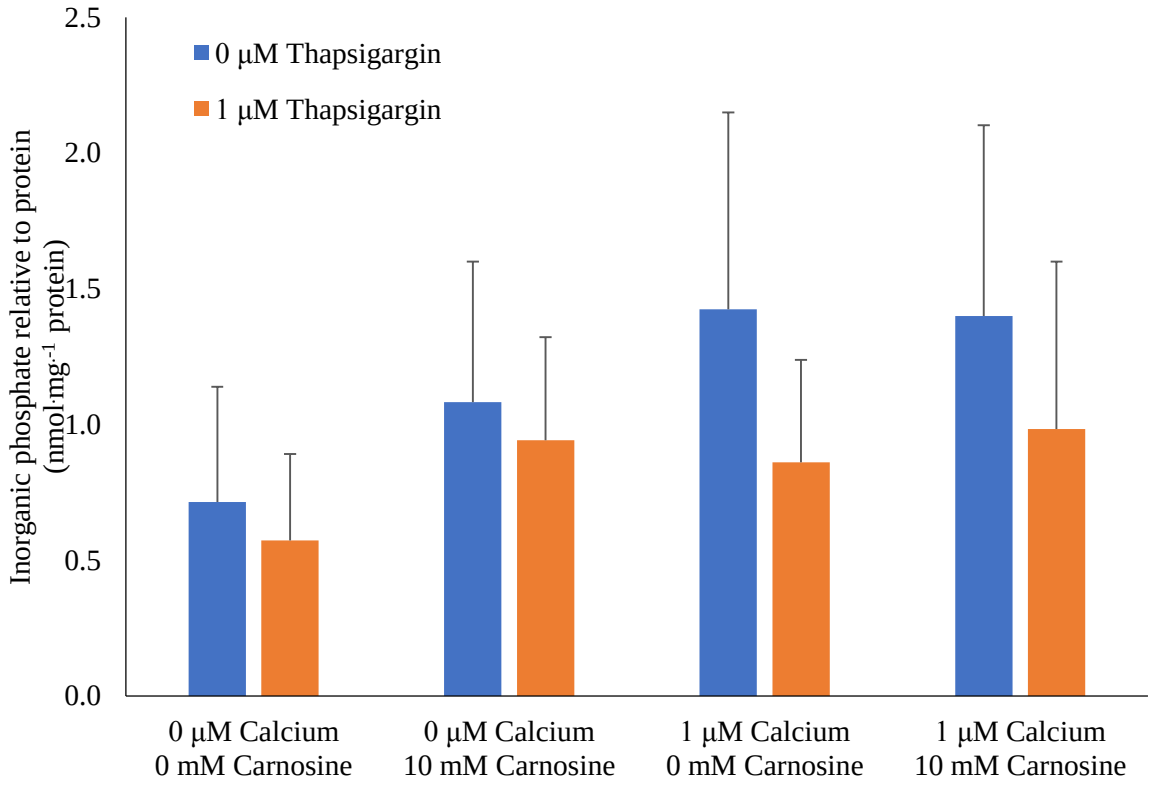

Fig. 7.6: Inorganic phosphate (nmol) generation under 0 µM or 1 µM calcium conditions, with and without the addition of 10 mM carnosine, and 1 µM thapsigargin.

Ca^{2+}-dependent activity: There was no significant thapsigargin × carnosine interaction on Ca^{2+}-dependent ATPase activity (P = 0.14; f (1, 8) = 1.29, η_p^2 = 0.1, η_g^2 < 0.1). All data was represented relative to subcellular protein content (Fig 7.7). The addition of both 1 μM thapsigargin (P = 0.005; f (1, 8) = 14.26, η_p^2 = 0.6, η_g^2 = 0.2) and 10 mM carnosine (P = 0.006; f (1, 8) = 14.01, η_p^2 = 0.6, η_g^2 = 0.2) significantly reduced Ca^{2+}-dependent ATPase activity, as noted by significantly less Pi generation during these conditions.

SERCA activity: There was no significant Ca^{2+} × carnosine interaction on SERCA activity (P = 0.30; f (1, 8) = 1.24, η_p^2 = 0.1, η_g^2 < 0.1). All data was represented relative to subcellular protein content (Fig 7.8). The addition of 1 μM Ca^{2+} significantly increased SERCA activity (P = 0.006; f (1, 8) = 14.11, η_p^2 = 0.6, η_g^2 = 0.3) as expected, however, there was no influence of increasing carnosine concentrations by 10 mM on SERCA activity (P = 0.60; f (1, 8) = 0;.30, η_p^2 < 0.1, η_g^2 < 0.1).

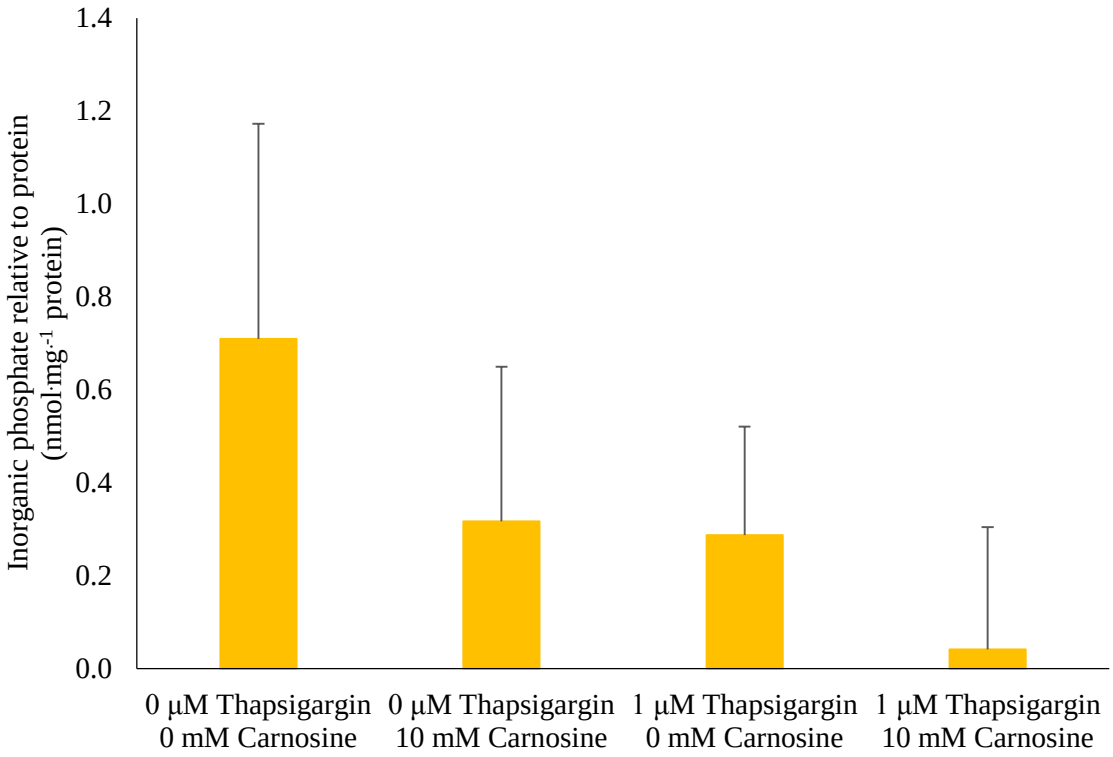

Fig. 7.7: Inorganic phosphate generation relative to protein content from calcium ATPase dependent activity, with and without the addition of 10 mM carnosine, and 1 µM thapsigargin.

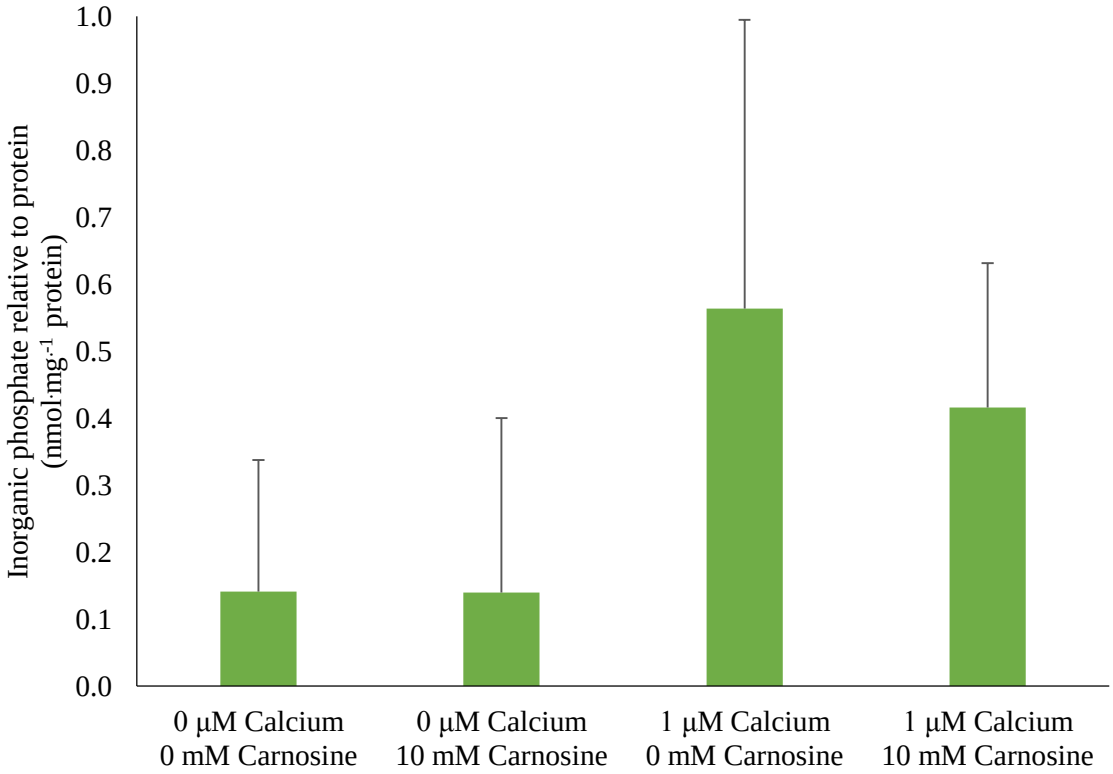

Fig. 7.8: Inorganic phosphate generation relative to protein content from sarcoplasmic/endoplasmic reticulum calcium ATPase activity, with and without the addition of 10 mM carnosine, and 1 µM calcium.

7.4 Discussion

The main findings from the present investigation are: a) carnosine was evident in all assessed subcellular fractions, predominantly located in the cytosolic compartment; b) β-alanine and L-histidine were reported in all assessed subcellular fractions, yet at lower content than carnosine (both <50 μM); c) the addition of carnosine does not affect *in-vitro* SERCA pump activity, estimated from Pi generation; and d) in the presence of carnosine, overall ATPase activity increased, whilst Ca^{2+}-dependent ATPase activity declined. The lack of change to *in-vitro* Ca^{2+}-dependent ATPase and SERCA activity is unexpected, due to the previously reported declines in *in-vivo* skeletal muscle relaxation time following β-alanine supplementation (Studies 1 and 2; Chapters 4 and 5). The current investigation is the first to examine the distribution of carnosine, β-alanine and L-histidine across separate subcellular fractions of the skeletal muscle. Furthermore, the present *in-vitro* analysis is the first to examine the impact of increased carnosine content on ATPase activity, specifically SERCA and Ca^{2+}-dependent ATPases.

Due to the role of carnosine as an intracellular pH buffer (see review; Sale *et al.*, 2010; 2013), it had been proposed that carnosine was predominantly evident within the cytosolic compartment of the skeletal muscle (Harding & Fallon, 1979; Abe, 2000). These data demonstrated that carnosine, and it constituents β-alanine and L-histidine, are indeed predominantly evident within the cytosolic fraction of the skeletal muscle. That said, carnosine, β-alanine and L-histidine were also shown across all assessed subcellular fractions. For carnosine to be apparent within these compartments, the molecules needs to be transported between subcellular compartments, and/or the carnosine synthase enzyme is present within these subcellular fractions, although this is yet to be determined. The distribution of carnosine across several cellular compartments, does not allow for any of the proposed mechanisms, associated with increased skeletal muscle carnosine concentration on improved exercise performance (see section 2.3; Page 21), to be contested. It does, however, allow for speculation about the potential roles of carnosine within the skeletal muscle. The presence of carnosine and its constituents within the microsomal fraction, indicates the possibility of these molecules interacting with the SR of the skeletal muscle, and potentially ATPase activity. The existence of carnosine within the mitochondrial compartment of the skeletal muscle, implies that carnosine may be able to alter the antioxidant abilities of the tissue, as previously proposed (Neifakh, 1966). Since carnosine was apparent within both the cytosolic and microsomal fractions of the skeletal muscle, there is the potential of carnosine to manipulate Ca^{2+} kinetics, by increasing the sensitivity of the contractile properties to Ca^{2+} and/or the amount of Ca^{2+} released from the SR lumen to the myofibrils (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.*, 2011; Everaert *et al.*, 2013). The current data is the first to examine the distribution of carnosine and its components, across several subcellular fractions of the skeletal

muscle, indicating potential direct and indirect interactions with skeletal muscle functions. That said, the current data does not mean that the content of carnosine, β-alanine and L-histidine are physiologically relevant, and thus important to skeletal muscle function.

Overall ATPase activity, estimated from Pi generation, significantly increased in the presence of 10 mM carnosine. Surprisingly, the alterations to overall ATP activity were not associated with improved Ca^{2+}-dependent ATPases or SERCA activity. It could be hypothesised that the improvement in overall ATPase activity in the presence of carnosine, are associated with greater levels of Pi production by PMCA and the Golgi apparatus secretory protein ATPases. The current *in-vitro* data are unexpected, based on the reported improvements in skeletal muscle relaxation, following β-alanine supplementation (Studies 1 and 2; Chapters 4 and 5). The *in-vitro* data implies that the improvements to *in-vivo* skeletal muscle HRT (Studies 1 and 2; Chapters 4 and 5), and might be due to improved PMCA and/or the Golgi apparatus secretory protein ATPases activity, rather than improved Ca^{2+} -dependent ATPase activity. As hypothesised, increasing the availability of Ca^{2+} significantly improved overall ATPase, Ca^{2+}-dependent ATPase and SERCA activity. Additionally, thapsigargin, a non-competitive inhibitor of SERCA activity (Thastrup, 1990) significantly reduced overall ATPase and Ca^{2+}-dependant ATPase activity. It is important to consider that the current *in-vitro* research is performance in rat skeletal muscle tissue, and may not be representative of the findings in human skeletal muscle. Potential interactions with other molecules, such as anserine (in rodent tissue) and taurine may be apparent. Taurine is found in high concentrations in the skeletal muscle (Huxtable, 1992) and has been shown to increase skeletal muscle twitch force in rat fast-twitch skeletal muscle fibres (Goodman *et al.*, 2000), and influence intracellular Ca^{2+} regulation, via increased SR Ca^{2+} uptake and total storage capacity of the SR vesicles (Huxtable & Bressler, 1973).

The isolation of the skeletal muscle subcellular fractions was achieved using centrifugation, a commonly used preparative method that enables maintenance of the protein intracellular context (de Araujo & Huber, 2007). The current centrifugation protocol was based on previously published methodologies (Wilkie & Schirmer, 2008; Dimauro *et al.*, 2012; Wilson & Walker, 2015), that said, no direct assessment of compartment purity was undertaken. A certain amount of contamination of one fraction to other may occur, in which small amounts of proteins from neighbouring compartments contaminate the fraction of interest. Cross-contamination of subcellular compartments would result in false positives being reported, (*i.e.*, contamination of microsomal fraction could potentially affect ATPase activity, and impact interaction of increased carnosine on ATPase activity). To provide support for these *in-vitro* analysis purity conformational analysis is required to establish the degree of cross-contamination of the subcellular fractions.

7.5 Conclusion

The current investigation was the first to report carnosine, β-alanine and L-histidine across the nucleic, mitochondrial, cytosolic and microsomal subcellular compartments of rat skeletal muscle tissue. Although *in-vitro* SERCA pump activity was unaffected, the presence of 10 mM carnosine significantly increased overall ATPase activity. Supplementary examination of PMCA and the Golgi apparatus secretory protein ATPases in the presence of increased carnosine content, provide greater understanding of the impact of carnosine on skeletal muscle ATPase activity. These *in-vitro* data does not does not allow for any of the proposed mechanisms, associated with increased skeletal muscle carnosine concentration on improved exercise performance to be contested. The current data does not mean that the content of carnosine, β-alanine and L-histidine are physiologically relevant, and thus important to skeletal muscle function.

Chapter 8: General discussion

The improvements in exercise performance following β-alanine supplementation, specifically those at high-intensities have been associated with the role of skeletal muscle carnosine as an intracellular pH buffer (Bate-Smith, 1938; Deutsch & Eggleton, 1938). This does not, however, exclude the possibility that carnosine may have other physiological roles. *In-vitro* research indicated that increasing the carnosine content in the skeletal muscle, via β-alanine supplementation, could alter the Ca^{2+} properties of the muscle (Lamont & Miller, 1992; Batrukova & Rubtsov, 1997; Dutka & Lamb, 2004; Dutka *et al.*, 2011; Everaert *et al.*, 2013). The aim of this thesis was to investigate the effect of β-alanine supplementation on voluntary and electrically evoked contractile properties of *in-vivo* human skeletal muscle.

To examine this aim, Study 1 (Chapter 4) investigated the effect of 28-days β-alanine supplementation on the intrinsic contractile properties of human skeletal muscle *in-vivo*, as well as on voluntary muscle function in young (18 - 30 y) healthy males. It was hypothesised that 28-days of β-alanine supplementation would significantly alter the force-frequency relationship, the *in-vivo* analogue of the force-Ca^{2+} relationship (Balnave & Allen, 1996). These data, however, showed no significant influence of β-alanine supplementation on voluntary and electrically evoked force responses, or the force-frequency relationship in fresh skeletal muscle. It was suggested that 28-days of β-alanine supplementation, to increase muscle carnosine content, does not significantly influence the Ca^{2+} kinetics in the skeletal muscle, and thus overall muscle performance. There was, however, a significant decline in skeletal muscle HRT during electrically evoked octet contractions, and resting and potentiated twitches. The improvements in skeletal muscle relaxation speed indicated that raising carnosine content may potentially alter Ca^{2+}-channel activity by interacting with the Ca^{2+}-channel itself (Batrukova & Rubstov, 1997), possibly via the existence of saturable binding site(s) for carnosine on the Ca^{2+}-channel.

Study 1 (Chapter 4) was conducted in fresh skeletal muscle, where there were no perturbations of pH, implying that the positive effect on HRT following increased carnosine content was due to altered Ca^{2+} kinetics, rather than a pH mediated effect. Swietach *et al.*, (2013) suggested that there may also be a mediating effect of pH on the interaction between L-histidine containing dipeptides (such as carnosine) and Ca^{2+}. H^+ are also proposed to directly or indirectly inhibit sarcoplasmic Ca^{2+} release during skeletal muscle contraction (Laver, Eager, Taoube & Lamb, 2000; Laver, O'Neill & Lamb, 2004). Furthermore, carnosine has the potential to serve as a cytoplasmic regulator of Ca^{2+} and H^+ coupling, since it binds to both ions (Baran, 2000). As such, it was hypothesised that increased muscle carnosine content, via β-alanine supplementation, would have a more pronounced beneficial effect on HRT when the muscle was fatigued. To investigate this hypothesis, Study 2 examined the effect of β-alanine supplementation on intrinsic *in-vivo*

isometric knee extensor force production and muscle contractility in both fresh and fatigued human skeletal muscle. An IKET at 45% MVIC was implemented to occlude blood flow to the active muscle, (de Ruiter *et al.,* 2007), obstructing circulation to the contracting muscle with minimal efflux on lactate and H^+ (Ahlborg *et al.,* 1972). In-line with the findings from Study 1 (Chapter 4), there was no significant influence of 28-days of β-alanine supplementation on voluntary and electrically evoked force responses, or the force-frequency relationship under fresh conditions. There was also no influence of β-alanine supplementation on voluntary and electrically evoked force responses, or the force-frequency relationship under fatigued conditions, following completion of the IKET at 45% MVIC. β-alanine supplementation, however, significantly altered both fresh and fatigued HRT during electrically evoked octet contractions, and resting and potentiated twitches. In the current investigation, there was a significant benefit of β-alanine supplementation on skeletal muscle HRT relative to placebo, although the declines in fresh muscle HRT in the current investigation in resting and potentiated twitches were slightly less than those reported previously (Study 1; Chapter 4). These data, however, demonstrate a significantly valuable effect of β-alanine supplementation on skeletal muscle during both fresh and fatigued conditions, suggesting that raising carnosine content might alter Ca^{2+}-channel activity, by interacting with the Ca^{2+}-channel itself (Batrukova & Rubstov, 1997). The reduction in skeletal muscle relaxation time could be beneficial for exercise performance specifically those involving short, repeated muscle contractions, where muscle relaxation comprises an important proportion of total energy consumption (Bergström & Hultman, 1988). The improvement in skeletal muscle function, may also prove beneficial to older adults, were pre-existing declines in muscle function are evident due to the ageing process (Lexell, 1995; Stuerenburg & Kunse, 1999; Doherty 2003; Tallon *et al.,* 2007; Verdijk *et al.,* 2010). Due to the importance of maintaining lower-extremity muscular strength in older adults, thus preserving independence and quality of life, the effect of 28-days of β-alanine supplementation on neuromuscular performance was investigated in older adults (60 – 80 y).

Increased skeletal muscle carnosine content, via 28-days of β-alanine supplementation, in older individuals, did not benefit voluntary or electrically evoked skeletal muscle contractions, including HRT, in either fresh or fatigued muscle conditions (Study 3; Chapter 7). These data directly contrast the declines in skeletal muscle HRT evident in young adults (Studies 1 and 2; Chapter 4 and 5). The lack of alteration to skeletal muscle HRT may be attributed to several methodological factors, including the supplementation protocol with the older adults ingesting 4.8 $g \cdot d^{-1}$ of β-alanine for 28-days, as opposed to the 6.4 $g \cdot d^{-1}$ ingested by younger adults. Based on previous research (del Favero *et al.,* 2012), it was hypothesised that there was a 40% increase in skeletal muscle carnosine content from baseline following the current supplementation protocol.

That said, the expected relative 40% increase in carnosine content, may not have been adequate in increasing absolute carnosine levels to a content that would have resulted in improved skeletal muscle function, specifically HRT. It could also be suggested that older individuals may not be able to achieve the same carnosine content as that of younger adults, following the decline in Type II muscle fibres during ageing (Lexell *et al.,* 1995). Since these Type II are the predominant store of skeletal muscle carnosine content (Dutka *et al.,* 2012; Stellingwerff *et al.,* 2011; Harris *et al.,* 2012), it could be proposed that that declines in absolute carnosine content in the skeletal muscle is irreversible. Futuremore, as part of the current investigation, all older adults completed a medical screening questionnaire to ensure they were 'medically stable' to participate (Greig *et al.,* 1994). This process may have indirectly led to a bias in participant selection, with individuals whom are more likely to be physically active accepted, therefore the declines in skeletal muscle function in these individuals may not be as extensive as that of the general population, and almost certainly not to the declines evident in the frail elderly. It could be hypothesised that supplementing frail individuals with a similar amount of β-alanine would potentially improve skeletal muscle function, even when not apparent in healthy older adults.

Previous research examining the effect of β-alanine supplementation on skeletal muscle carnosine content and physical exercise capacity in older adults (Stout *et al.,* 2008; del Favero *et al.,* 2012; McCormack *et al.,*2013; Glenn *et al.,* 2015; 2016), has been conducted with daily doses of β-alanine between 1.6 and 3.2 $g \cdot d^{-1}$. The current investigation supplemented older individuals with a daily dose of β-alanine (4.8 $g \cdot day^{-1}$), currently the largest reported daily dose of β-alanine in this older population. Importantly, there were no symptoms of paraesthesia reported by any participant, a key consideration for any future research undertaken with this daily dose of β-alanine in this population. These data indicated that older adults (60 – 80 y) can complete β-alanine supplementation protocols, with a daily dose of 4.8 $g \cdot d^{-1}$, without experiencing paraesthesia, maintaining the double-blind nature of the investigation. It is important to note that the current investigation is limited by the lack of direct measurements of skeletal muscle carnosine content.

Presently there is a limited number of investigations undertaking assessment of voluntary and electrically evoked contractions in an older population (60 – 80 y), the current investigation found all participants to be very compliant with the study protocol, and completed all required experimental tasks, with no participants withdrawing from the investigation. On reflection of the research process it should be noted, that the recruitment process for older adults, specifically those defined as medically stable (Greig *et al.,* 1994), took a considerable amount of time, this should be factored into future research projects. Additionally, recruitment should be directed to more face-to-face contact, and word of mouth, with less recruitment being successfully achieved via internet

resources. That said, the participants within the current investigation were all keen to be involved and understood the importance of completing research within this population, and all were willing to complete additional research investigations, albeit not specifically those involving electrical stimulation to the current intensity and duration.

Both younger and older individuals during studies 2 and 3 (Chapters 5 and 6) completed an IKET fatigue protocol to examine the effect of 28-days β-alanine supplementation on intrinsic *in-vivo* isometric knee extensor force production and muscle contractility in fatigued skeletal muscle. Participants were instructed to complete the IKET at 45% MVIC, an intensity anticipated to occlude blood flow to the active muscle becomes through increased intramuscular pressure (de Ruitter *et al.*, 2007). That said, the IKET hold times reported in older adults were ~70 s (~53%) greater than both those reported in younger adults (Study 2; Chapter 5) and those predicted by the Rohmert equation (Rohmert, 1960). Using the Rohmert equation, it was estimated that the older adults completed the IKET at a much lower intensity of 30% MVIF, rather than 45% MVIF, as instructed. Completion of the IKET at 30% MVIF would not result in the same physiological responses as those evident during a 45% MVIF hold. It can therefore, be hypothesised that the current IKET was not generating the same levels of muscle fatigue in these older adults, as evident in the younger adults. Due to the lack of change in skeletal muscle HRT in this older population, it was important to fully examine the declines in skeletal muscle HRT in young adults following 28-days β-alanine supplementation.

Based on the current data in young adults, reporting a reduction in skeletal muscle HRT, following 28-days of β-alanine supplementation (Studies 1 and 2), it was proposed that there was a direct or indirect mechanism associated with activity of the SERCA pump (Gafni & Yuh, 1989), the proposed rate-limiting step of muscle relaxation (Gillis, 1985; Dux, 1993). Acting on Ca^{2+} kinetic proteins directly or via other molecules provides a mechanism by which Ca^{2+} signalling can be inhibited or excited (Swietach *et al.*, 2013). Study 4 (Chapter 7) investigated the impact of increased carnosine availability on *in-vitro* skeletal muscle ATPase activity, through quantification of Pi generation. *In-vitro* analysis of ATPase activity revealed that SERCA activity, the pump associated with skeletal muscle relaxation speed, was unaffected by increased carnosine content, although there was a significant increase in overall ATPase activity (Study 4; Chapter 7). The current *in-vitro* data are unexpected, based on the previously reported improvements in skeletal muscle relaxation (Studies 1 and 2; Chapters 4 and 5). The enhanced ATPase activity evident following the addition of carnosine could be due to improved PMCA and/or the Golgi apparatus secretory protein ATPases activity, rather than altered Ca^{2+}-dependent ATPases. Due to the novelty of these data, it is also important to consider other mechanisms that may explain the beneficial

effect of β-alanine supplementation on skeletal muscle HRT in young adults (Studies 1 and 2; Chapter 4 and 5), including the potential of indirect mechanism of carnosine, such as modification of the protein interactions with the Ca^{2+}-channels and/or binding with the proteins themselves (Berchtold, Brinkmeier & Muntener, 2000). It is important to also consider that these *in-vitro* data was performed in rat skeletal muscle tissue, and may not be representative of the findings in human skeletal muscle.

There is a large body of evidence documenting skeletal muscle carnosine content in both humans and rats (Steurenburg & Kunze, 1999; Harris *et al.,* 2006; Hill *et al.,* 2007; Derave *et al.,* 2007; Kendrick *et al.,*2008; 2009; Baguet *et al.,* 2010; Stellingwerff *et al.,*2011; del Favero *et al.,* 2012; Bex *et al.,* 2014; Chung *et al.,* 2014; Gross *et al.,* 2014; Stegen *et al.,* 2014; Danaher *et al.,* 2014). That said, these data were conducted on whole muscle fibres, using the muscle biopsy, or via ^{1}H-MRS techniques. Examination of specific subcellular fractions was required to provide support for the roles associated with increased carnosine content, on skeletal muscle performance. It was hypothesised that if carnosine and/or its constituents were not present in the subcellular fraction, it would be unlikely that these molecules would exhibit alterations to cellular functions of the compartment. To investigate if carnosine, β-alanine and L-histidine, had the ability to directly influence the SR of the skeletal muscle, as proposed in Study 2 (Chapter 5), the distribution of carnosine and its constituents were examined in several subcellular fractions (Study 4; Chapter 7). Carnosine, β-alanine and L-histidine were ubiquitously distributed across the nucleic, mitochondrial, cytosolic and microsomal fractions of rat skeletal muscle, albeit in varying contents. β-alanine and L-histidine contents were significantly lower than those of carnosine, across all assessed subcellular fractions. The distribution of carnosine across several cellular compartments, does not allow for any of the proposed mechanisms, associated with increased skeletal muscle carnosine content on improved exercise performance, to be contested. It does, however, allow for speculation about the potential roles of carnosine within the skeletal muscle. Since carnosine was apparent within both the cytosolic and microsomal subcellular fractions, the current data implies that there is the potential of carnosine to manipulate the Ca^{2+} properties of the skeletal muscle, although the exact mechanism remains unclear. These data are the first to report the distribution of carnosine, β-alanine and L-histidine across different subcellular fractions of the skeletal muscle. The current data does not however, mean that the content of carnosine, β-alanine and L-histidine are physiological relevant, and therefore important to skeletal muscle function.

The overall aim of this thesis was to investigate the effect of β-alanine supplementation on voluntary and electrically evoked contractile properties of *in-vivo* human skeletal muscle. In both young and older adults, 28-days of β-alanine supplementation did not significantly influence

voluntary and evoked force responses, or the force-frequency relationship, in either fresh or fatigued skeletal muscle (Studies 1-3, Chapter 4-7). In young adults, there were significant improvements in skeletal muscle HRT following β-alanine supplementation, during electrically evoked octet contractions, resting and potentiated twitches (Studies 1 and 2; Chapters 4 and 5). These alterations to skeletal muscle HRT following β-alanine supplementation were not evident in healthy older adults. Carnosine, β-alanine and L-histidine are ubiquitously distributed across the nucleic, mitochondrial, cytosolic and microsomal fractions of rat skeletal muscle. Nonetheless, carnosine was the most prominent molecule, with the largest content shown in the cytosolic compartment of the skeletal muscle. These data allow for speculation about the potential roles of carnosine within the skeletal muscle. The exact mechanism associated with the *in-vivo* decline in skeletal muscle HRT remains unclear, yet raising the availability of carnosine *in-vitro*, does improve overall ATPase activity, although not Ca^{2+}-dependent or SERCA activity.

8.1 Overview of the key findings

1. In young adults, 28-days of β-alanine supplementation did not significantly influence voluntary and evoked force responses, or the force-frequency relationship, the *in-vivo* analogue of the force-Ca^{2+} relationship, in either fresh (Studies 1 and 2; Chapters 4 and 5) or fatigued (Study 2; Chapter 5) skeletal muscle.
2. There was, a significant decline in skeletal muscle HRT in young adults, during electrically evoked octet contractions, and resting and potentiated twitches (Studies 1 and 2; Chapters 4 and 5).
3. Older adults demonstrated no beneficial effect of 28-days β-alanine supplementation on voluntary or electrically evoked muscle contractions (Study 3; Chapter 6).
4. Based on the *in-vivo* research, it was proposed that there was a direct or indirect mechanism associated with SERCA activity, the proposed rate-limiting step of skeletal muscle relaxation.
5. *In-vitro* analysis of ATPase activity demonstrated no significant alteration in SERCA activity following increased carnosine content, although there was a significant increase in overall ATPase activity (Study 4; Chapter 7).
6. Carnosine, β-alanine and L-histidine are ubiquitously spread across the nucleic, mitochondrial, cytosolic and microsomal subcellular fractions of rat skeletal muscle, albeit in varying contents. *In-vitro* findings allowed for speculation about the potential roles of carnosine within the skeletal muscle.

8.2 Future research

Due to the highlighted limitations of the present IKET in older adults (Study 3; Chapter 6), future investigation should focus on the development of an isometric fatigue protocol where occlusion of blood flow to the active muscle is evident. It should be considered that the Rohmert equation (Rohmert, 1960) may not be applicable to predict IKET performance times in older individuals, due to the significant decline in skeletal muscle cross-sectional area, muscle fibre quantity and Type II fibres, evident during the ageing process.

Older individuals within the current thesis (Study 3; Chapter 6), ingested β-alanine supplementation at a daily dose of 4.8 $g \cdot d^{-1}$, without experiencing paraesthesia. This is the first investigation to supplement older individuals with this dose of β-alanine. That said, it should be noted that there was no direct assessment of skeletal muscle carnosine content. To determine if older individuals experience the same dose-response relationship of β-alanine supplementation on skeletal muscle carnosine content, further research is required. This additional research may allow for the ingestion of larger daily doses of β-alanine in this population, thereby reducing the duration of supplementation protocols to significantly increase skeletal muscle carnosine content.

The isolation of the skeletal muscle subcellular fractions in Study 4 (Chapter 7) was achieved using centrifugation; during this process tiny amounts of proteins from neighbouring compartments may contaminate the fraction of interest. To provide support for these *in-vitro* data, examination of the cross-contamination between subcellular compartments is required. Western blot analysis is a widely implemented and important technique used in cell and molecular biology, allowing the identification of specific proteins from a complex mixture of proteins extracted from cells (Mahmood & Yang, 2012).

The ubiquitous distribution of carnosine, β-alanine and L-histidine across the nucleic, mitochondrial, cytosolic, and microsomal fractions of rat skeletal muscle, albeit in different content, provides essential information regarding the potential roles of carnosine within the skeletal muscle. That said, to understand the impact of increased carnosine content on human muscle performance, these *in-vitro* analysis should be continued in human skeletal muscle fibres. Additionally, due to the potential influencing factors of other amino acids, such as anserine (in rodents) and taurine, examination of these molecule contents within the subcellular fractions of the skeletal muscle is required

In-vitro analysis demonstrated that carnosine, β-alanine and L-histidine are evident in all assessed subcellular fractions of rat skeletal muscle cells. It does, however, remain uncertain how/if

these molecules are transported throughout each subcellular fraction, and whether carnosine is transported across the subcellular fractions as a whole molecule, or synthesised within the fraction from β-alanine and L-histidine. Analysis of the molecular transporters, Tau-t and PEPT2, and the enzyme that catalyses the formation of carnosine, carnosine synthase, within each subcellular fraction would enable the examination of these mechanisms. These data would provide greater understanding of the how the carnosine molecule is transported and synthesised within the skeletal muscle. Additionally, these analyses would provide knowledge on the mechanisms associated with β-alanine and L-histidine, examining the potentially differing functions of these constituents in separate compartments.

Ca^{2+} pumps together with Ca^{2+} release channels are present within the skeletal muscle and non-muscle cells, forming a ubiquitous Ca^{2+} regulatory system (McMullen *et al.,* 1997). The addition of carnosine has been shown to interact with ATPase activity *in-vitro* (Study 4; Chapter 7). Carnosine is reported in excitable tissues, including the cardiac muscle and neuronal tissue (Everaert *et al.,* 2013), it remains unclear if the current alterations to *in-vitro* ATPase activity evident with increased carnosine concentration would be apparent in other non-muscle cells. The ATPase, cardiac SERCA, regulates intracellular Ca^{2+} movement, fulfilling a crucial role in initiating cardiac contraction and relaxation, as well as facilitating the storage and distribution of Ca^{2+} in the SR (Frank *et al.,* 2003). The current *in-vitro* data indicated the possibility of altered skeletal muscle ATPase activity with the addition of carnosine (Study 4; Chapter 7), extension of these analyses is required to examine if similar effects are shown in cardiac muscle. These data would be of great clinical significance, since abnormalities of the cardiac muscle contraction and relaxation coupling, specifically Ca^{2+} cycling, has been reported in the failing heart (Hasenfuss, 1998; Bers, 2001; 2011). That said, carnosine may not have the same functions in different tissues (Boldyrev *et al.*, 2013).

Brody's disease is a very rare inherited human muscle disorder, impacting the SR Ca^{2+} uptake (MacLennan, 2000). The main clinical feature of Brody's disease experienced by individuals is impairment of muscle relaxation and muscle stiffness (Brody, 1969; Karpati *et al.,* 1986; Danon *et al.,* 1988). Individuals experiencing Brody's disease are reported to have Ca^{2+}-ATPase activity of around 50% compared to control individuals, examined in both whole muscle homogenates prepared from biopsies and muscle cell cultures (Benders *et al.,* 1994). These individuals are also showed to have extremely low Ca^{2+} transport activity at around five percent when compared to controls (Menke & Jockusch, 1995). The *in-vivo* data reported within the current thesis indicated that increased carnosine availability, via β-alanine supplementation, improved young adults skeletal muscle HRT, which could be vital for individuals suffering with Brody's

disease. Examining the impact of increased skeletal muscle carnosine content, via β-alanine supplementation, in individuals suffering from Brody's disease, where impairment to skeletal muscle relaxation is evident, may provide a number of clinically relevant discoveries.

It was hypothesised following *in-vivo* analysis, that increased carnosine content would improve skeletal muscle SERCA activity, the pump associated with skeletal muscle relaxation speed (Study 2; Chapter 5). Study 4 (Chapter 7) showed no improvement in SERCA activity following increase carnosine content, yet there was improved overall ATPase activity, as demonstrated with increase Pi generation. It was suggested that these improvements to overall ATPase activity were associated with increased Pi generation by PMCA and golgi apparatus secretory protein ATPase. Inhibition of these ATPases can been achieved using thapsigargin, although the content required to do so is 10 to 300 times greater than those needed to inhibit SERCA activity (Treiman *et al.*, 1998; Harper *et al.*, 2005). Isolation of SERCA, PMCA and the golgi apparatus secretory protein ATPases is required to fully examine the influence of carnosine on the activity of each ATPase. This research would provide a clearer understanding of the direct impact on ATPase activity *in-vitro* that said, any alterations of *in-vitro* ATPase activity may not result in alterations to *in-vivo* muscle function.

Chapter 9: References

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Appendix 1: Methodological development

The research reported within the current appendix provides supporting experimental background and development for study 4 (Chapter 7).

A1.1 Introduction

There are several protocols documented within study 4 (Chapter 7) of the current thesis, that needed additional support and methodological development above that reported within the specific chapter. The current appendix will document the development of these methodologies.

A1.2 *In-vitro* skeletal muscle tissue preparation

A1.2.1 Background

The cell lysate was disrupted to disaggregate the cells and breaks them open, whilst applying minimal damage to the internal components of the cell (Clayton & Shadel, 2015; Pryor, 2015). The method of cell disruption (homogenisation), blending, is ideal for the disruption in mammalian and plant tissue (Wilson & Walker, 2015), undertaken with the samples placed in an ice-cold isotonic buffer (Wilkie & Schirmer, 2008). Blending protocols are conducted based on duration of blending, or the number of times the blender is placed in the samples (Wilkie & Schirmer, 2008; Wrzosek, 2014). Following homogenisation of mammalian tissue excess material, such as, incompletely homogenised connective and/or vascular tissue and small fragments of non-homogenised tissue (Wilson & Walker, 2015) need to be filtered. The filtering of the sample can be achieved via cheesecloth, muslin, surgical gauze or a single layer of nylon mesh (75 μM pore size) (Graham & Rickwood, 2002), although the use of cheesecloth is the easiest way to complete this process (Wilson & Walker, 2015; Fig. 7.1). The homogenisation protocol is completed with all samples on ice, whilst the experimenters wear protective gloves to reduce heat transfer to the samples (Graham & Rickwood, 2002). The remaining solution is the lysate or homogenate.

The process of subcellular fractionation is flexible, adjustable (both in cell and tissues), and allows the analysis of proteins in their physiologic/intracellular context, therefore it is the most commonly used preparative/enrichment method (de Araujo & Huber, 2007). Cellular fractionation involves the development of pure fractions of cell components based on the density and size differences (Michelsen & Hagen, 2009). Fractionation of a sample can be achieved via biological centrifugation, a method which uses centrifugal forces to separate and purify mixtures of biological particles in a liquid medium (Wilson & Walker, 2015). Centrifugation occurs by the rapid rotation of a rotor which produces large centrifugal forces, causing the particles in the sample to sediment. In general, the largest units experience the largest centrifugal force and move the most rapidly in the sample. Repeated centrifugation at progressively higher speeds and longer centrifugation periods divide the muscle homogenate into distinct fractions (Wilson & Walker, 2015; Fig. 7.2). At relatively low speeds, large components such as the nucleic sediment to form a pellet at the bottom of the centrifuge tube. At slightly higher speed, a mitochondrial pellet is deposited, with even higher speeds and longer periods of centrifugation, isolating the small closed vesicles and then the

ribosomes. Fixed-angle rotors are an ideal tool for pelleting during the differential separation of biological particles where sedimentation rates differ significantly (Wilson & Walker, 2015). Protocols vary with typical centrifugation steps of 10 min for 1000 g to pellet the nucleic and cellular debris, 10 min for 10,000g to pellet the contractile apparatus, 20 min at 20,000 g to pellet a fraction enriched in mitochondria, and 1 h at 100000 g to separate the microsomal and cytosolic fractions (Wilson & Walker, 2015; Fig. 7.2).

A1.2.2 Methodological development

The rat skeletal muscle tissue was initially separated into nucleic, mitochondrial, cytosolic and microsomal fractions, using the following centrifugation protocol;

1) The homogenised filtrate was centrifuged at 1000 g for 10 min at 4^{o}C (Harrier 18/80 Refrigerated benchtop centrifuge, MSE Ltd, London, UK). The resulting pellet containing the nucleic fraction was resuspended in 2 mL storage medium (25 mM potassium chloride, 20 mM Hepes pH 7.4, 250 mM sucrose, 5 mM magnesium chloride) and frozen at -80^{o}C.
2) The supernatant was ultra-centrifuged at 20,000 g for 20 min at 4^{o}C in a fixed angle (Type 70 Ti) Beckman rotor (Beckman Coulter, Fullerton, CA, USA). The resulting pellet containing the mitochondrial fraction was suspended in 2 mL storage medium and frozen at -80^{o}C.
3) The remaining supernatant was ultra-centrifuged at 100,000 g for 40 min at 4 ^{o}C in a fixed angle (Type 70 Ti) Beckman rotor (Beckman Coulter, Fullerton, CA, USA). The supernatant containing the cytosolic fraction was carefully decanted from the pellet and frozen at -80^{o}C. The resulting pellet containing the microsomal fraction was suspended in 2 mL storage medium and frozen at -80^{o}C.

Based on further examination of the current literature (Dimauro *et al.*, 2012), following thawing of the nucleic and mitochondrial samples, several additional centrifugation protocols were undertaken (Fig. 7.3). These additional protocols were undertaken to increase the purity of these subcellular fractions. The nucleic pellet was defrosted and resuspended in 1 mL of storage medium, the samples were vortexed for 15 s and spun at 500 g for 15 min (Dimauro *et al.*, 2012). The resulting pellet containing the nucleic fraction was re-suspended in 2 mL of storage medium, vortexed for 15 s and spun at 1,000 g for 15 min. The final pellet was removed and stored in 2 mL of storage medium.

The mitochondrial pellet was defrosted and resuspended in 1 mL of storage medium, vortexed for 15 s and spun at 11,000 g for 10 min (Dimauro *et al.*, 2012). The resulting pellet was resuspended in 2 mL of storage medium. The protein concentrations of these samples following the additional centrifugation processes were then determined (see section A1.3.2.2).

A1.3 Protein assay

A1.3.1 Background

The protein content within each subcellular fraction was quantified by the BCA colorimetric assay, involving the reduction of copper oxide to copper by proteins in an alkaline medium. Using defined time and temperature, examination of the intense purple colour produced through this process can allow examination of protein concentrations. Absorbance of the sample is directly proportional to the protein concentration, with the protein concentration calculated based on a reference curve obtained from a standard protein solution.

A1.3.2 Methodological development

A1.3.2.1 Standard vs. enhanced assay protocols

The current analysis was undertaken to determine the standard protein concentration protocol required for the rat skeletal muscle subcellular fractions. The BCA has two sets of protocols; standard, with concentrations from 20 $\mu g \cdot mL^{-}1$ to 2 $mg \cdot mL^{-1}$, or enhanced, with concentrations from 500 $\mu g \cdot mL^{-}1$ to 2 $mg \cdot mL^{-1}$. A fresh set of protein concentrations for both the standard and enhanced protocols were formed, and diluted in the stock solution in the storage medium (25 mM potassium chloride, 20 mM Hepes pH 7.4, 250 mM sucrose, 5 mM magnesium chloride). The BCA working reagent was prepared for both protocols (standard and enhanced) through the addition of 1 part of reagent B to 50 parts of reagent A, the volume of working reagent was calculated based on the number of standards (8 standard points essential on a microplate assay, including triplicates) and samples required. The working reagent was then allowed to reach room temperature. Once prepared, 25 µL of each standard, control and sample were pipetted into microplate wells in triplicate where 200 µL of BCA assay working reagent was added. The microplate was then placed incubator for 30 min at either 37^{o}C (standard protocol) or 60^{o}C (enhanced protocol). Following incubation, the microplate was cooled to room temperature and optical density was read at 562 nM, against the average blank standard sample (water + BCA assay reagent).

The values for each BCA standard were plotted against the protein concentration ($\mu g \cdot mL^{-1}$) of the sample. A curvilinear regression analysis was undertaken to produce the standard curve, in this case a 2nd order polynomial equation. The protein concentration of the assessed sample was then plotted as the dependant variable (y-axis) and the absorbance as the independent variable (X-axis), values were then placed into the equation $y = ax^2 + bx + c$, where solving y determines the protein concentration of the sample. The protein concentrations were then adjusted based on dilution value and converted to $mg \cdot mL^{-1}$.

The current experiment compared the standard and enhanced BCA protein protocols. Based on the current observations, the standard protocol is sufficient in examining the protein contents in the subcellular fractions produced in the current thesis. All additional optimisation will be conducted with a standard protein curve.

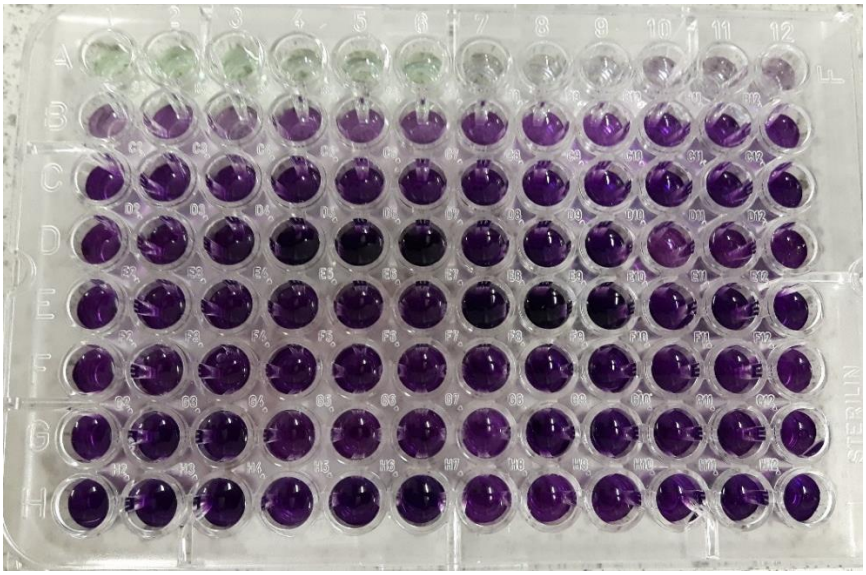

Fig. A1.1: Image of the BCA assay in a microplate, with no dilution of the sample.

A1.3.2.2 Sample dilution

No dilution: A total of four rat skeletal muscle samples were analysed, with the protein concentrations of the nucleic, mitochondrial, cytosolic and microsomal compartments examined, for each skeletal muscle sample. Each sample was recorded in triplicate, with the mean reported. During the current experiment, the protein concentrations for the samples were much greater than the protein standards, above the maximum standard (2 $mg \cdot mL^{-1}$). These high protein concentrations were apparent visually by the very dark purple colour in the microplate wells (Fig. A1.1). Based on the current analysis, dilution of the subcellular fractions is required to examine protein concentrations within the standard range of the BCA assay.

25% dilution: Subcellular compartments were diluted to a 25% dilution, one part sample, three parts storage media. A total of four rat skeletal muscle samples were analysed, with the protein concentrations of the nucleic, mitochondrial, cytosolic and microsomal compartments examined, for each skeletal muscle sample. Each sample was recorded in triplicate, with the mean reported. The protein concentration within the assessed samples remained above that of the highest standard (2 $mg \cdot mL^{-1}$), when examining several samples, specifically the nucleic and mitochondrial fractions. Several samples were within the standards range, that said, the protein concentrations

remained nearer the upper end of the standard range, which was not ideal. Based on the current data, a greater dilution of the samples is required to examine the protein concentration within the standard range of the BCA assay.

10% dilution: Subcellular compartments were diluted to a 10% dilution, one part sample, and nine parts storage media. A total of four rat skeletal muscle samples were analysed, with the protein concentrations of the nucleic, mitochondrial, cytosolic and microsomal compartments examined, for each skeletal muscle sample. Each sample was recorded in triplicate, with the mean reported. The protein concentrations of the samples at a 10% dilution remained within the range of the BCA assay standards (< 2 $mg \cdot mL^{-1}$; Fig. A1.2). Based on the current analysis, all samples will be diluted to a 10% dilution prior to the completion of the BCA assay, to examine protein concentrations.

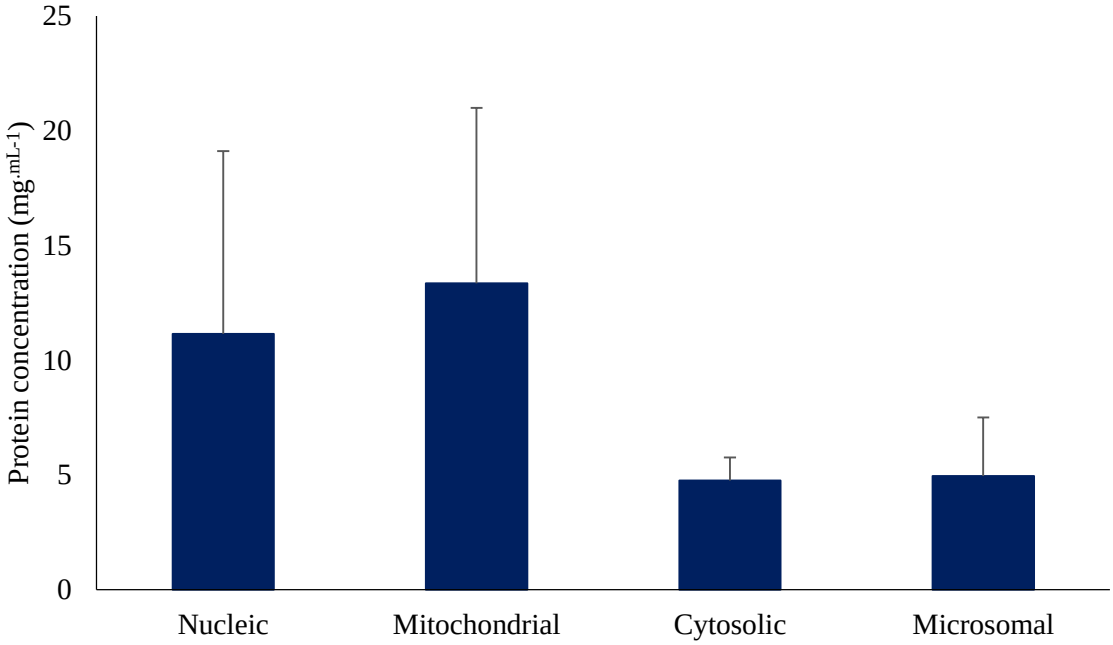

Fig. A1.2: Protein concentration ($mg \cdot mL^{-1}$) for each assessed subcellular fraction of four skeletal muscle rat tissue samples. Data are mean ± 1SD.

10% dilution following additional centrifugation: Following the completion of additional centrifugation processes, as reported in Section A1.2, the protein concentrations of the subcellular fractions was re-examined. Samples were assessed at a 10% dilution; one part sample, nine parts storage media. A total of four rat skeletal muscle samples were analysed, with the protein concentrations of the nucleic, mitochondrial, cytosolic and microsomal compartments examined, for each skeletal muscle sample. Each sample was recorded in triplicate, with the mean reported. The protein concentrations of the samples at a 10% dilution remained within the range of the BCA assay standards (Fig. A1.3)

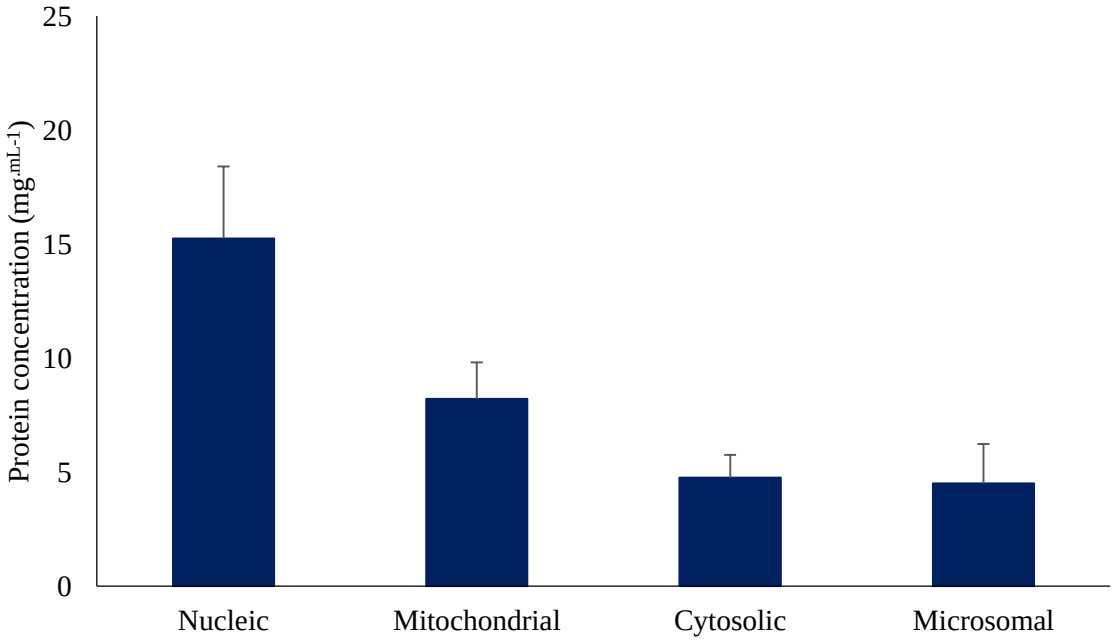

Fig. A1.3: Protein concentration ($mg \cdot mL^{-1}$) for each assessed subcellular fraction of four skeletal muscle rat tissue samples following additional centrifugation of the nucleic and mitochondrial fractions. Data are mean ± 1SD.

A1.4 Fluorescence spectroscopy

A1.4.1 Background

Fluorescence spectroscopy is the analysis of samples via the excitation of the electrons in the molecules via a beam of light (photon). At room temperature, most molecules occupy the lowest vibrational level of the ground electronic state, on absorption of light they are elevated to produce excited states. When the module returns to its ground state energy is released, thus emission of a photon occurs carrying energy, this emission is detectable. The optical density of a sample can be determined using the Lambert-Beer law, in which the absorption of a material is defined as the logarithmic relative decrease if intensity before and after the sample. If a solution in a cuvette has OD = 1, this states that only 10% of the light pass (*i.e.* 90% are absorbed). If the concentration of a solution is unknown, it can be obtained by plotting the absorption against a series of known concentrations or a reference sample. Fluorescence spectroscopy is undertaken by using either cuvettes or microplates, although the optics used between each technique are different (Lakowicz, 2006). Additionally, although microplates are typically used to perform fluorescence spectroscopy, cuvettes are advantageous as they allow for right-angle observations (Lakowicz, 2006). The microplate technique involves a Xenon flash lamp as a light source for the samples, with the excitation wavelength selected using an excitation monochromator. The fluorescence from the sample is then directed towards the detector optics. Normally, the microplate is moved to position each well in the observation path.

A1.4.2 Methodological development

A1.4.2.1 Carnosine absorbance spectrum

To characterise the absorbance properties of the carnosine molecule in different pH levels (6.1 and 7.0), two 50 mM carnosine solutions were prepared in a phosphate buffer. Full-spectrum absorbance measurements were performed with filter settings of emission between 220 – 1000 nM at a resolution of 1 nM on a FLUOstar® Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). All measurements were recorded at room temperature. The scanning of the samples gave absorption spectrum values of 3.20 nM (pH 6.1) and 3.00 nM (pH 7.0). The current analysis suggested that there was no clear maximum absorption for carnosine at a 50 mM concentration (Fig. A1.4).

A1.4.2.2 Carnosine fluorescence emission

The fluorescence emission spectra were recorded with a DeltaRAM X High Speed Random Access Monochromator (Photon Technology International, New Jersey, USA) equipped with a 75 W xenon arc lamp and a photomultiplier tube R955 detection system (Hamamatsu, Japan) using a motorised slit band-passes for excitation and the emission monochromators, increment of 1 nM for

the emission monochromator, integration times of 0.1 s. The emission spectra were corrected for the lamp, the monochromators and the detector response. Fluorescence emission spectra at excitation wavelengths of 300, 350, 400, 450, 500, 550 and 600 nM were measures in a 1 cm quartz cuvette. The emission wavelengths ranged from 340 to 700 nM, with a total of seven trials (340 – 700, 390 – 700, 440 – 700, 490 – 700, 540 – 700, 590 – 700, 640 – 700 nM) for each 50 mM carnosine solution. The fluorescence emission spectra were recorded for the phosphate buffer solution without any carnosine content at excitation wavelength of 600 nM and excitation wavelengths of 640 – 700 nM.

Florescence emission spectra was produced for the phosphate buffer solution with no difference in emission between the 50 mM carnosine solutions and the phosphate buffer solution at excitation wavelength of 600 nM and excitation wavelengths of 640 – 700 nM (Fig. A1.5). Fluorescence emission spectra of 50 mM carnosine at pH 6.1 and pH 7.0 are shown in Fig. A1.6A and A1.6B. There was no spike in fluorescence emission indicating that a 50 mM carnosine concentration at either a pH of 6.1 or 7.0 did not emit light. The current experimental data suggested that the carnosine molecule on its own displays no fluorescence properties.

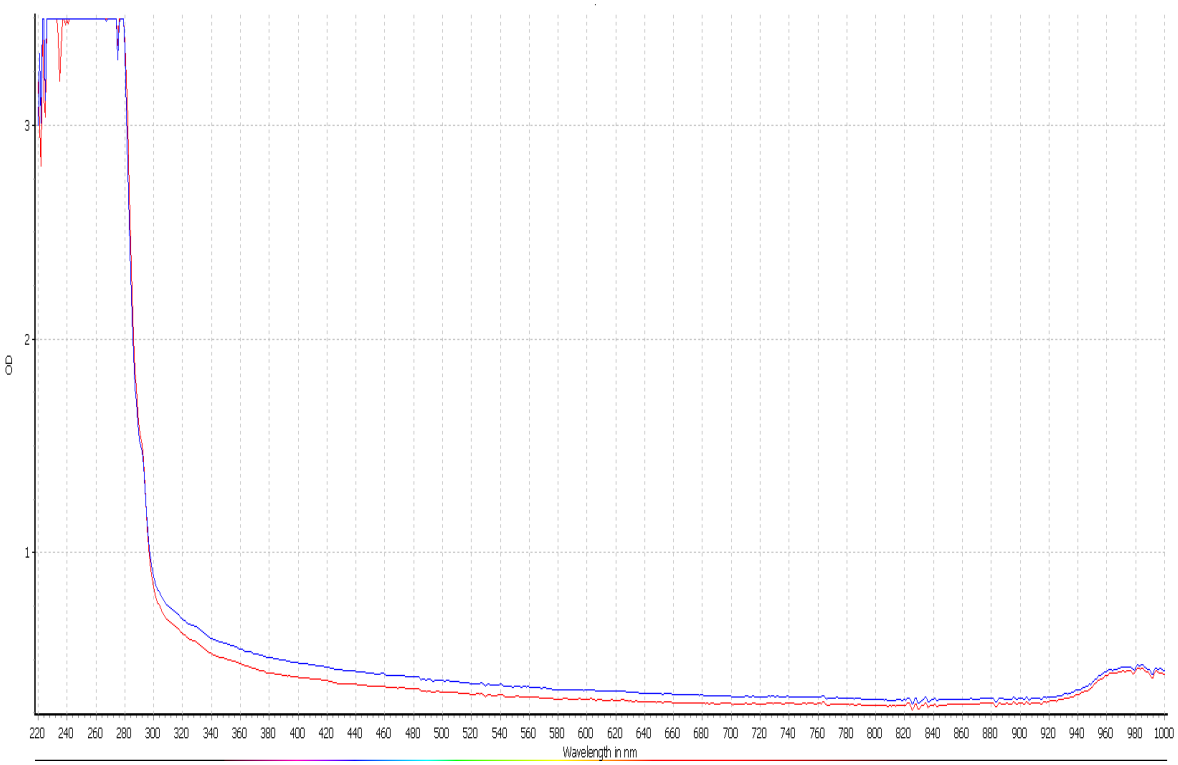

Fig. A1.4: Full-spectrum absorbance measurements performed on a 50 mM carnosine solution at a pH of 6.1 (red line) and 7.0 (blue line) in a phosphate buffer. OD: Optical density

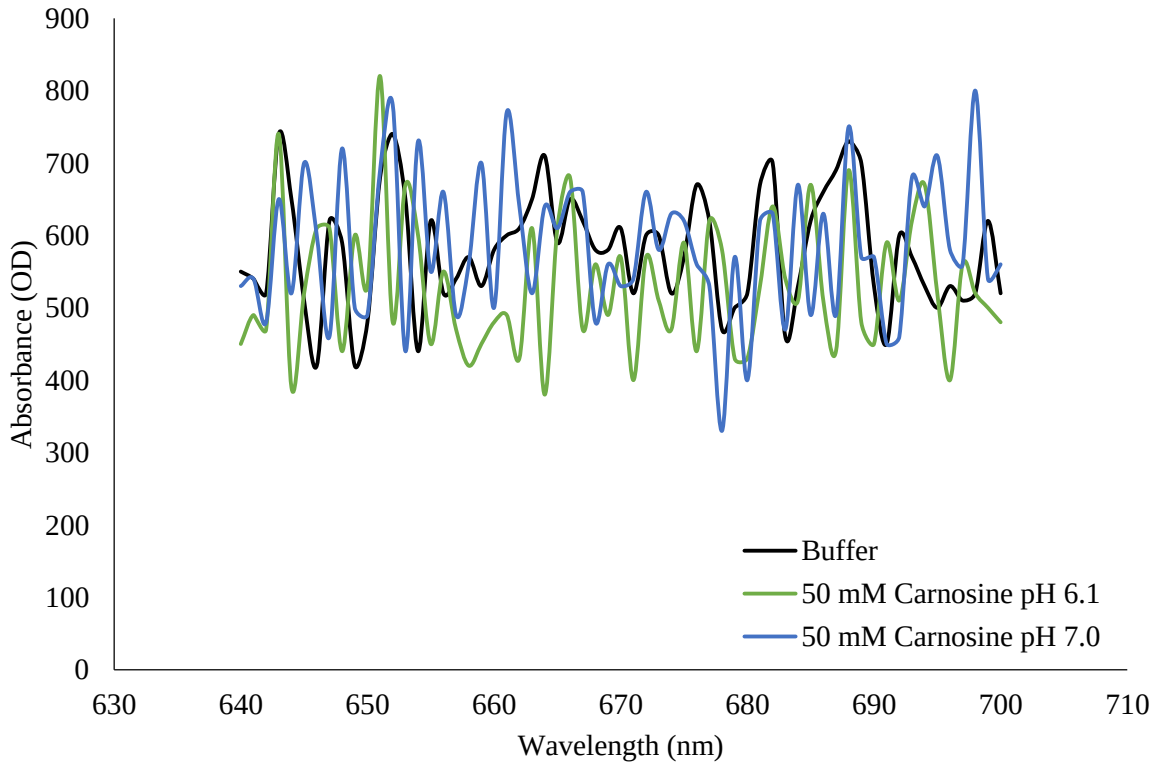

Fig. A1.4: Fluorescence emission spectra of phosphate buffer solution and 50 mM carnosine solutions at pH 6.1 and pH 7.0. Excitation wavelength of 600 nM and excitation wavelengths of 640 – 700 nM. OD: Optical density

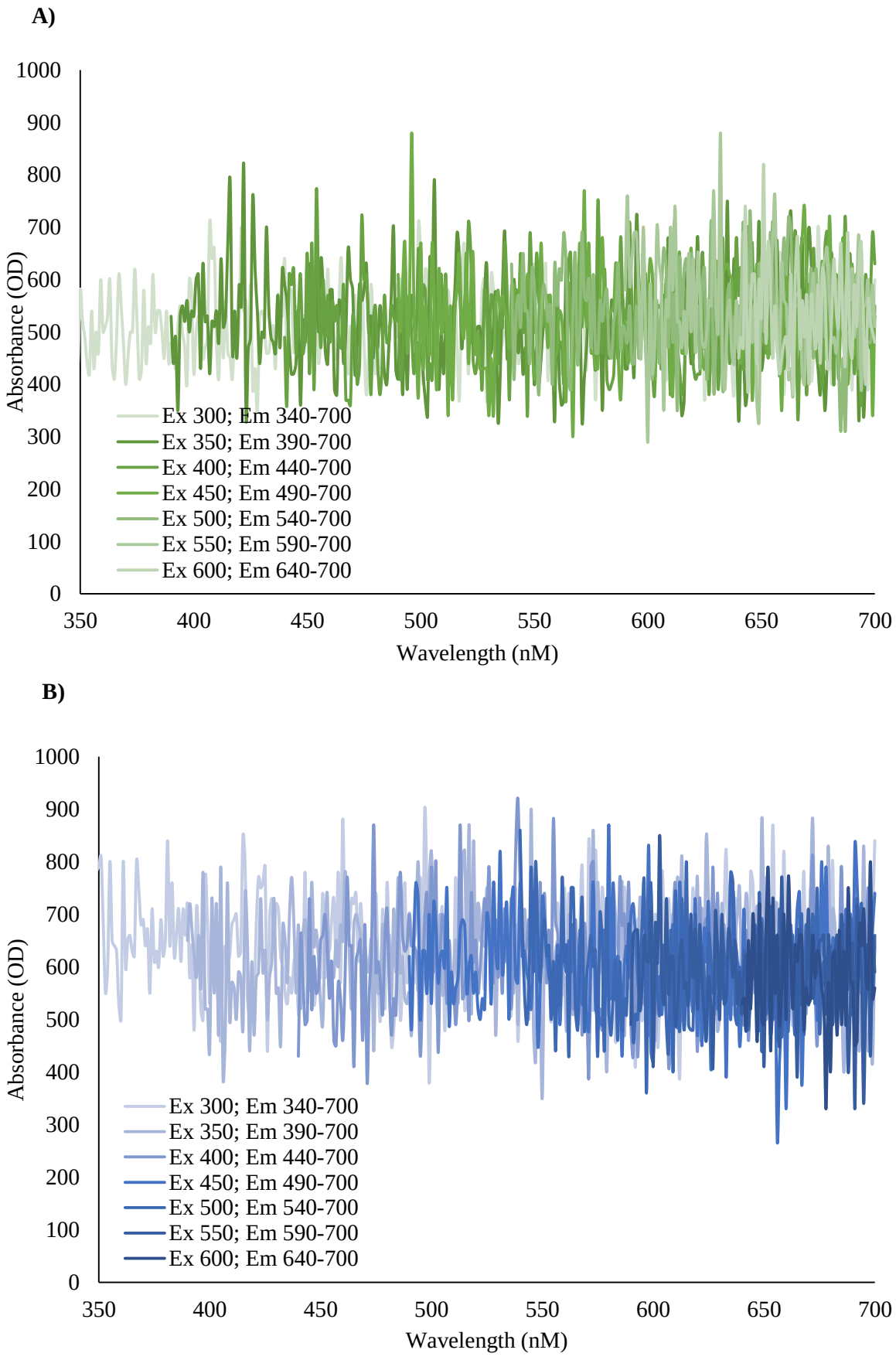

Fig. A1.5: Fluorescence emission spectra of 50 mM carnosine solutions at pH 6.1 (A) and pH 7.0 (B). OD: Optical density

A1.4.2.3 Carnosine and calcium binding

The fluorescence emission spectra were recorded with a FLUOstar® Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). Fluorescence emission wavelength was 485 nM and excitation was 520 nM. The gain or implication of the FLUO Omega microplate reader was adjusted to obtain observable signals, it was found that 1318 gain was too high, whilst 400 gain was too low to detect a clear emission. All samples were sampled at a value of 700 gain. To all samples 2.5µL of a Ca^{2+} sensitive probe (FLUO 4 penta-potassium salt; Thermofisher, UK) was added.

Low calcium buffer: A 2 mM carnosine concentration was added to a low Ca^{2+} cytosolic buffer (140 mM Potassium chloride, 10 mM Sodium chloride, 1 mM Magnesium chloride, 20 mM Hepes, with an overall pH of 7.2), samples containing carnosine had a decline in fluorescence emission properties, indicating a potential binding to Ca^{2+}. An additional test was run with 0.5 mM and 10 mM carnosine concentrations included. Fluorescence emission was the greater in the 0.5 mM carnosine concentration sample compared to the 2 mM and 10 mM samples. Yet, emission in the 2 mM carnosine sample displayed the lowest emission properties, representing the greatest binding of Ca^{2+} to carnosine at this concentration, greater than that reported in the 10 mM concentration. It should be noted that the current samples were conducted in a cytosolic like buffer, although Ca^{2+} was low within this buffer, there was Ca^{2+} still present. The experiment was halted here.

Low calcium Ca^{2+} buffer with no sodium chloride or magnesium chloride: To further explore the relationship between carnosine and Ca^{2+}, 100 mM Ca^{2+} was added to samples containing an intracellular-like buffer (140 mM Potassium chloride, 20 mM Hepes, with an overall pH of 7.2) with varying concentrations of EGTA and carnosine. EGTA is a chelating agent which has a lower affinity for magnesium, making it more selective for Ca^{2+}, also known as a Ca^{2+} collator, and thus it was predicted that fluorescence would decline with increasing EGTA levels.

Pilot of calcium chelation: Data reported in Table A1.1 displays the fluorescence of assessed samples containing 200, 140 or 100 mM EGTA with the addition of 0, 2 or 10 mM carnosine. All samples were assessed in triplicate, with mean and SD reported. Predicted free Ca^{2+} concentrations are reported based on the concentration of EGTA within the sample. The current data displayed a decline in fluorescence with increasing EGTA concentration from 100 mM to 200 mM (Table A1.1), supporting the role of EGTA as a Ca^{2+} chelator. Based on the current data, there is the potential influencing factor of carnosine on Ca^{2+} chelation, as highlighted by reduction in fluorescence when carnosine was added to the 200 mM EGTA condition. This reduction in

fluorescence implies that alongside the chelation of Ca^{2+} by EGTA, the carnosine molecule is also chelating any free Ca^{2+} within the solution. That said, both the 100 and 140 mM EGTA conditions displayed no decline in fluorescence with the addition of the carnosine solution. It is likely that during these conditions there was too much Ca^{2+} within the system; therefore all the Ca^{2+} binding sites were saturated.

Table A1.1: The fluorescence ($OD_{em\ 385,\ ex\ 520}$) of samples containing 200, 140 or 100 mM EGTA with 0, 2 or 10 mM carnosine solution.

	Predicted Free Ca^{2+} (μM)	0 mM Carnosine	2 mM Carnosine	10 mM Carnosine
200 mM EGTA	0.38	6616 ± 117	6424 ± 119	3852 ± 1086
140 mM EGTA	0.93	10702 ± 1254	11239 ± 633	11522 ± 455
100 mM EGTA	6.03	12378 ± 297	13419 ± 255	13769 ± 863

Data are mean ± 1SD. OD: Optical density

Ca^{2+} chelation: Based on the pilot data we could examine the effect of carnosine on Ca^{2+} chelation at a predicted free Ca^{2+} level of 0.38 μM, where the addition of carnosine indicated a decline in fluorescence, without saturating the Ca^{2+} binding sites. To further examine these data, an additional 20 samples were analysed at an EGTA concentration of 200 mM, with the addition of either 0 mM (n=10) or 10 mM (n=10) carnosine. The addition of carnosine reduced the fluorescence of the assessed samples from 2714 ± 442 to 1607 ± 321 $OD_{em\ 385,\ ex\ 520}$($P < 0.001$). These data support the previous notion that the addition of carnosine to the sample, provides a greater collation of the available Ca^{2+}, reducing the fluorescence, and highlighting the potential of carnosine to act as a Ca^{2+} buffer. It should be noted that the current experiment was conducted at a pH of ~7. The pH of the solutions was then adjusted to 7.4, where the analysis was repeated with a further 22 samples assessed at an EGTA concentration of 200 mM with the addition of either 0 mM (n=12) or 10 mM (n=12) carnosine. In-line with previous experimental data, carnosine significantly reduced the fluorescence of the samples, from 2181 ± 150 to 1639 ± 231 $OD_{em\ 385,\ ex\ 520}$ ($P < 0.001$).

The current analysis supports the role of EGTA as a Ca^{2+} collator, reducing the fluorescence of samples with increasing levels of EGTA. Interestingly, the current research proposes that the carnosine molecule is also able to act as a Ca^{2+} collator, decreasing fluorescence to a greater degree than with only EGTA. These analyses were conducted in the presence of both EGTA and carnosine; it could be proposed that the carnosine molecule may have additional Ca^{2+} chelation properties, above those reported in the current analysis, since it has to compete against the EGTA molecule, a known Ca^{2+} chelator.

A1.5 Carnosine, β-alanine and L-histidine content

A1.5.1 Background

Amino acid analysis is a method undertaken to determine the amino acid content of amino acid–, peptide- and protein-containing samples (Rutherfurd & Gilani, 2009) providing an important quantitative parameter in the characterisation of isolated protein or peptide samples (Ozols, 1990). An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The process by which the amino acid content is determined involved the samples being loaded a column of cation-exchange resin, where buffers of varying pH and ionic strength are pumped through the column to separate the various amino acids. The integrity of these samples is key to the accuracy of amino acid analysis (Tyler, 2000). The retention time of each peak on the output from the amino acid analyser identifies the specific amino acid, the area under the peak indicating the quantity of amino acid present.

A1.5.2 Methodological development

Amino acid analysis is a common technique to assess amino acid concentrations of blood plasma and urine samples (Decombaz *et al.*, 2012; Wilkinson *et al.*, 2013; Mitchell *et al.*, 2015). There are limited examinations of amino acid concentrations in skeletal muscle samples using an ion-exchange chromatography (Stuerenburg & Kunze, 1999; Hammarqvist *et al.*, 2005; Hammarqvist *et al.*, 2010; Teloh *et al.*, 2016). There is currently no research examining the amino acid concentrations in rat skeletal muscle subcellular fractions, specifically the amino acids, carnosine, β-alanine and L-histidine. Due to the novelty of this process, several repeatability experiments were completed using an Amino Acid Analyser (Biochrom 30, Pharmacia Biochrom Ltd). Standard area and ratios, and sample concentration (uM) measurements were analysed using paired t-tests, intra-class correlations (ICC, 2-way fixed, repeated measures, absolute model), systematic bias, limits of agreement (LoA; Bland & Altman, 1986) and CV (SPSS v23, IBM, USA).

Inter-reliability: Eight standards and 15 samples were assessed for inter-reliability (Table A1.2; Table A1.3), with measures assessed on two separate occasions. There was no significant difference standard ratios or samples amino acid concentrations between assessments from the same sample on two separate occasions, with systemic bias of between -1.5 – 16.6% for standards and -4.5 - 14.0% for sample concentrations. The ICC for standards ranged from 0.99 to 1.0 with all significance values $P < 0.001$, the ICC for the sample amino acid concentrations range from 0.98 to 0.99, with all significance values below $P < 0.001$.

Intra-reliability: A total of eight standards and 15 samples were assessed for intra-reliability (Table A1.2; Table A1.3). There was no significant difference standard ratios or samples amino acid concentrations between repeated assessments from the same sample, with systemic bias of between -2 - 4.2% for standards and 2.1 - 2.5% for sample concentrations. The ICC for standards ranged from 0.99 to 1.0 with all significance values $P < 0.001$, the ICC for the sample amino acid concentrations range from 0.95 to 1.0, with all significance values below $P < 0.001$.

The aim of these data was to determine the inter- and intra-reliability of a selection of carnosine, β-alanine and L-histidine concentrations within rat skeletal muscle subcellular fractions. These data indicate that this technique is appropriate to provide detailed and accurate data on molecular concentrations in rat skeletal muscle samples. It should be highlighted that other amino acids examined using the amino acid analyser have not been examined, thus, additional analysis is required for further analysis. Based on the current information it will also be critical to conduct future reliability research using human skeletal muscle tissue.

Table A1.2: Intra- and inter- reliability measures for the carnosine, β-alanine and L-histidine variables from the amino acid standard solution.

	Standards (n=8)					
	Carnosine Area	**Carnosine ratio to Norleucine**	**β-alanine Area**	**β-alanine ratio to Norleucine**	**Histidine Area**	**Histidine ratio to Norleucine**
Measurements						
Measure 1	5094908 ± 5328802	1.2 ± 1.2	4842791 ± 4982160	1.1 ± 1.1	4664229 ± 4815631	1.1 ± 1.1
Measure 2	4896268 ± 4948687	1.2 ± 1.3	4634278 ± 4713535	1.1 ± 1.2	4517061 ± 4647409	1.1 ± 1.2
Measure 2 repeat	4929848 ± 5088289	1.2 ± 1.2	4897279 ± 4946592	1.1 ± 1.1	4562317 ± 4887141	1.1 ± 1.1
Intra-reliability						
Systemic Bias	33580 (-4.2%)	-0.02 (3.6%)	263001 (-2%)	0.01 (3.2%)	45256 (-7.1%)	0.03 (3.6%)
CV (%)	8.74	7.64	9.48	6.31	6.17	7.79
ICC (P value)	0.99 (P < 0.001)	1.0 (P < 0.001)	0.99 (P < 0.001)	1.0 (P < 0.001)	1.0 (P < 0.001)	1.0 (P < 0.001)
t-Test	0.83	0.46	0.14	0.61	0.66	0.42
Cohen's D	0.01	0.02	-0.04	-0.01	-0.01	-0.02
LoA Random Error	841423	0.16	828437	0.13	513404	0.16
LoA Lower Limit	-8075003	-0.19	-565436	-0.12	-468	-0.13
LoA Upper Limit	875002	0.14	1091438	0.15	558659	0.18
Inter-reliability						
Systemic Bias	-198640 (16.6%)	-0.01 (-1.5%)	-2085137 (2.6%)	-0.01 (4.6%)	-147167 (4.2%)	-0.04 (-9.2%)
CV (%)	10.52	8.4	7.14	4.95	4.73	7.19
ICC (P value)	0.99 (P < 0.001)	0.99 (P < 0.001)	1.0 (P < 0.001)	1.0 (P < 0.001)	1.0 (P < 0.001)	1.0 (P < 0.001)
t-Test	0.32	0.88	0.12	0.8	0.1	0.21
Cohen's D	0.03	0.004	0.03	-0.004	0.03	0.03
LoA Random Error	1030354	0.18	620128	0.1	398079	0.15
LoA Lower Limit	-1228996	-0.19	-828641	-0.1	-545247	-0.18
LoA Upper Limit	831714	0.18	411616	0.11	250911	0.11

.Measurement data are mean ± 1SD.

Table A1.3: Intra- and inter-reliability measures for carnosine, β-alanine and L-histidine concentrations from rat skeletal muscle subcellular fractions.

	Samples (n=15)		
	Carnosine (uM)	**Histidine (uM)**	**β-alanine (uM)**
Measurements			
Measure 1	338.3 ± 299.3	22.8 ± 15.3	18.2 ± 15.8
Measure 2	329.2 ± 297.6	22.2 ± 16.4	18.8 ± 16.3
Measure 2 repeat	331.4 ± 299.4	24.5 ± 19.3	19.0 ± 16.2
Intra-reliability			
Systemic Bias	2.14 (2.3%)	2.38 (2.5%)	0.21 (2.1%)
CV (%)	6.25	24.37	26.89
ICC (P value)	1.0 (P < 0.001)	0.96 (P < 0.001)	0.95 (P < 0.001)
t-Test	0.69	0.13	0.87
Cohen's D	-0.01	-0.11	-0.01
LoA Random Error	39.12	10.77	9.63
LoA Lower Limit	-36.98	-8.39	-9.41
LoA Upper Limit	41.26	13.15	9.84
Inter-reliability			
Systemic Bias	9.09 (7.3%)	0.63 (14.0%)	-0.62 (-4.5%)
CV (%)	11.59	15.96	13.78
ICC (P value)	0.99 (P < 0.001)	0.98 (P < 0.001)	0.99 (P < 0.001)
t-Test	0.38	0.51	0.36
Cohen's D	-0.02	-0.03	0.03
LoA Random Error	73.25	6.79	4.82
LoA Lower Limit	-64.16	-6.16	-5.44
LoA Upper Limit	82.34	7.43	4.21

Measurement data are mean ± 1SD.

A1.6 BIOMOL Green phosphate assay

A1.6.1 Background

The BIOMOL Green phosphate assay reagent (Enzo Life Sciences, Loerrach, Germany) is a non-radioactive method for assessing Pi released during enzymatic phosphatase. Highly sensitive methods for phosphate measurement are based on the change in the absorption spectra of basic dyes upon complex formation with phosphomolybdic heteropolyacid (Baykov *et al.,* 1987). Phosphate is released as Pi, quantification of the Pi generated during ATP-y-P hydrolysis there can be evaluated as a measure of SERCA activity (McMullen *et al.,* 2012).

A1.6.2 Methodological development

A1.6.2.1 Absorbance spectrum

An absorbance spectrum was run between 400 and 800 nM, and although the BIOMOL Green phosphate assay reagent was normally undertaken at 620 nM, based on the absorbance spectrum of our equipment, the most appropriate absorbance was 640 nM (Fig. A1.7).

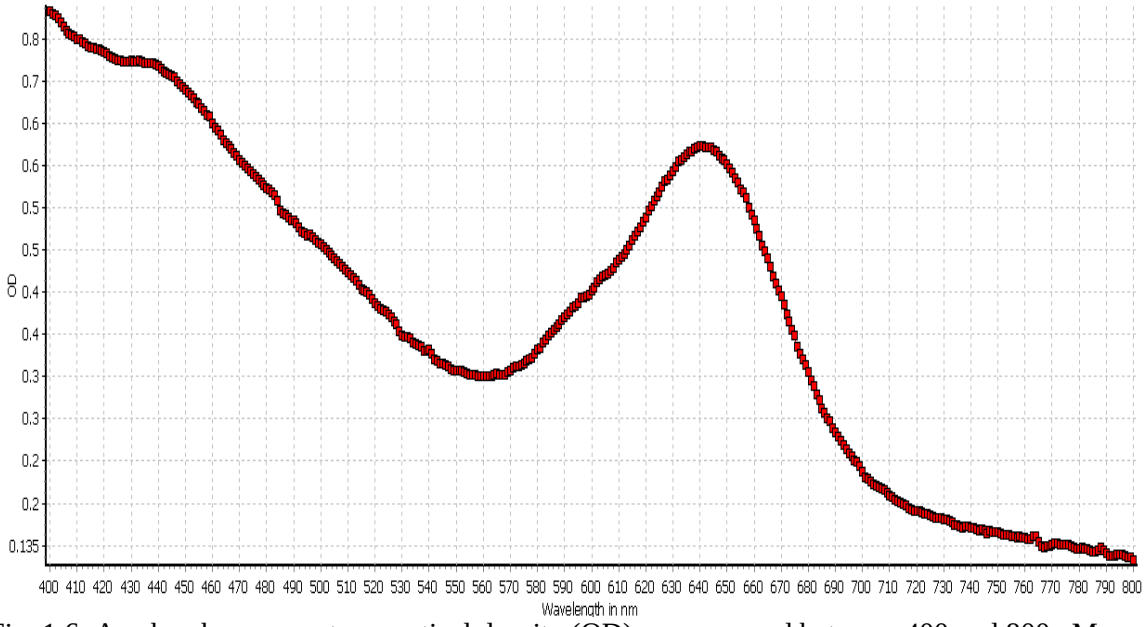

Fig. 1.6: An absorbance spectrum optical density (OD) was assessed between 400 and 800 nM.

A1.6.2.2 Optimisation of the assay

Test one: The samples were incubated for 30 min at 25 ^{o}C, following which 100 µL of sample was mixed with 100 µL of BIOMOL Green phosphate assay reagent, thus stopping the reaction, and allowing colour development to initiate. The samples were incubated at room temperature for 30 min to allow development of the green colour. The amount of Pi released during the Ca^{2+} uptake assay was determined by reading sample absorbance at 640 nM. The standards of the BIOMOL Green phosphate assay ranged from 0 to 2 nmol, with a linear curve fitted, reporting a R^2 value of 0.99 (Fig. A1.8). The absorbance values within the current experiment were largely above the protein concentration standard values, therefore this experiment was halted.

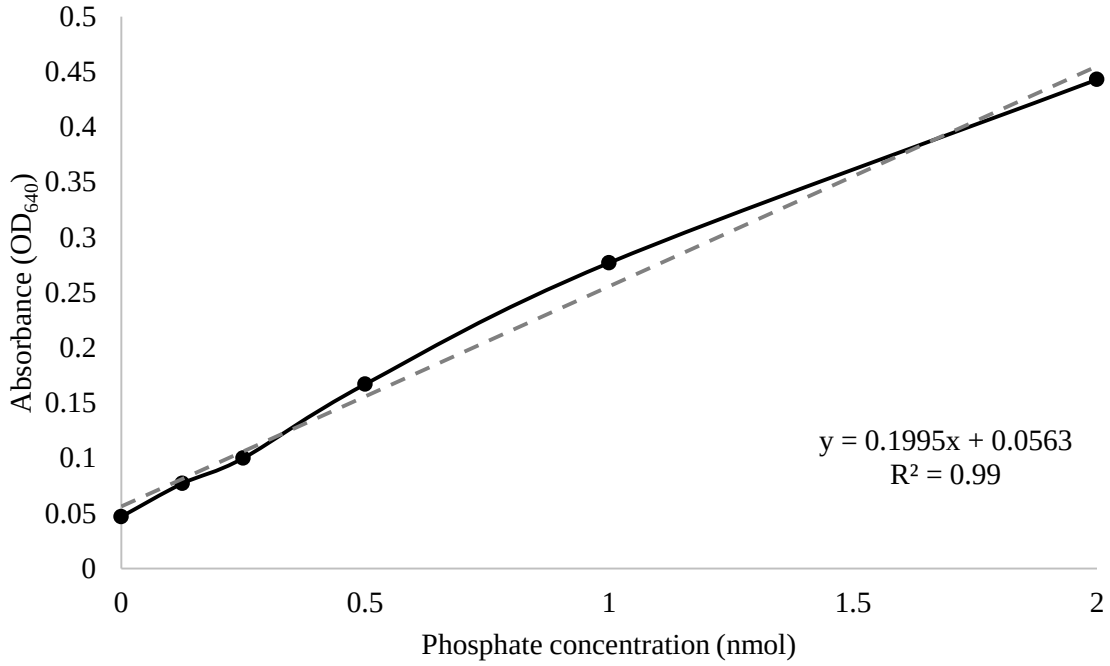

Fig. A1.7: The phosphate concentration (nmol) standard curve from test one, fitted within a linear curve against absorbance optical density (OD) of 640 nM.

Test two: The standards of the BIOMOL Green phosphate assay were increased to include 4 and 6 nmol, thereby ranging from 0 to 6 nmol. Additionally, the room temperature incubation time was reduced to 20 min. Samples were incubated for 30 min at 25 ^{o}C, following which 100 µL of sample was mixed with 100 µL of BIOMOL Green, thus stopping the reaction and allowing colour development to initiate. The samples were incubated at room temperature for 20 min to allow development of the green colour. The amount of Pi released during the Ca^{2+} uptake assay was determined by reading sample absorbance at 640 nM. The standards of the BIOMOL Green phosphate assay ranged from 0 to 6 nmol, with a linear curve fitted, reporting a R^2 value of 0.98. The absorbance values within the current experiment were still above the protein concentration standard values, even with the addition of the 6 nmol standard.

Test three: The samples were diluted to 50% with storage buffer, the standards of the BIOMOL Green phosphate assay ranged from 0 to 4 nmol, with incubation time decreased, and recorded at both 15 and 20 min. Samples were incubated for 30 min at 25 ^{o}C, following which 100 µL of sample was mixed with 100 µL of BIOMOL Green, thus stopping the reaction and allowing colour development to initiate. The samples were incubated at room temperature for 15 and 20 min to allow development of the green colour. The amount of Pi released during the Ca^{2+} uptake assay was determined by reading sample absorbance at 640 nM. The standards of the BIOMOL Green phosphate assay ranged from 0 to 4 nmol, with a linear curve fitted, reporting a R^2 value of 0.87 for both the 15 min and 20 min readings (Fig. A1.9). From examination of the standard curve is an apparent plateau in the standard concentrations between 2 and 4 nmol of phosphate. Furthermore, the absorbance values during analysis of the current subcellular fractions, remained at the top end of the phosphate standard curve. The current data supports the use of a shorter (15 min) sample incubation period, with no difference in standard curves or calculated phosphate concentrations compared to those of longer incubation periods.

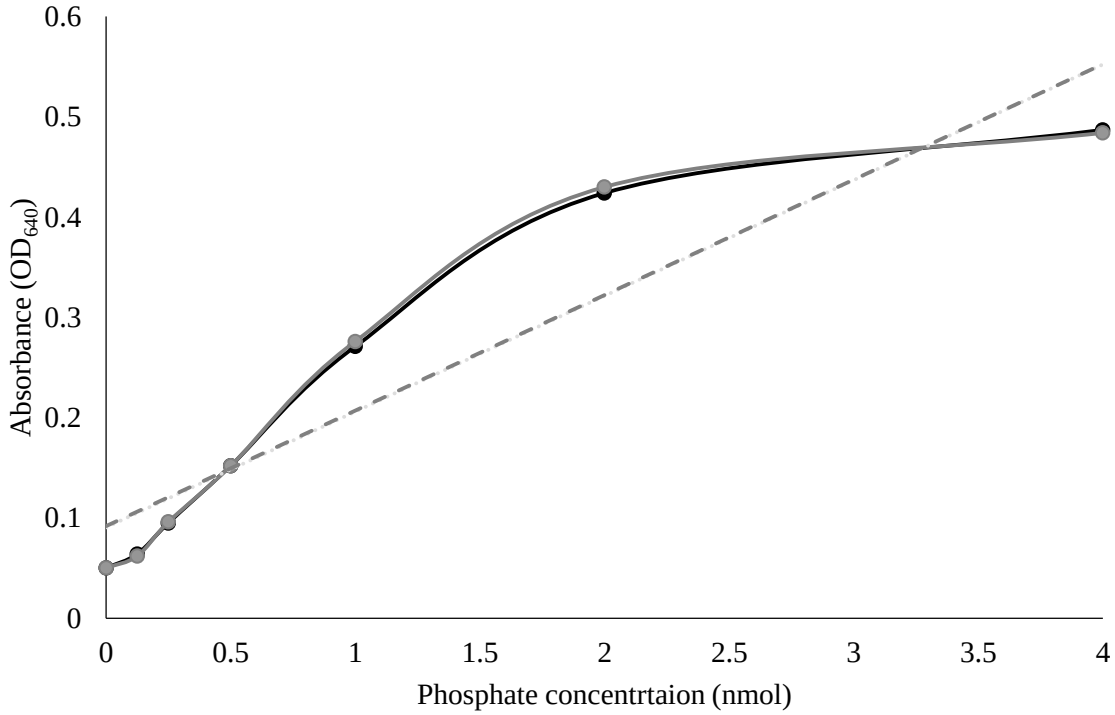

Fig. A1.8: The phosphate concentration (nmol) standard curve from test three at 15 min and 20 min of incubation at room temperature, fitted within a linear curve against absorbance optical density (OD) at 640 nM.

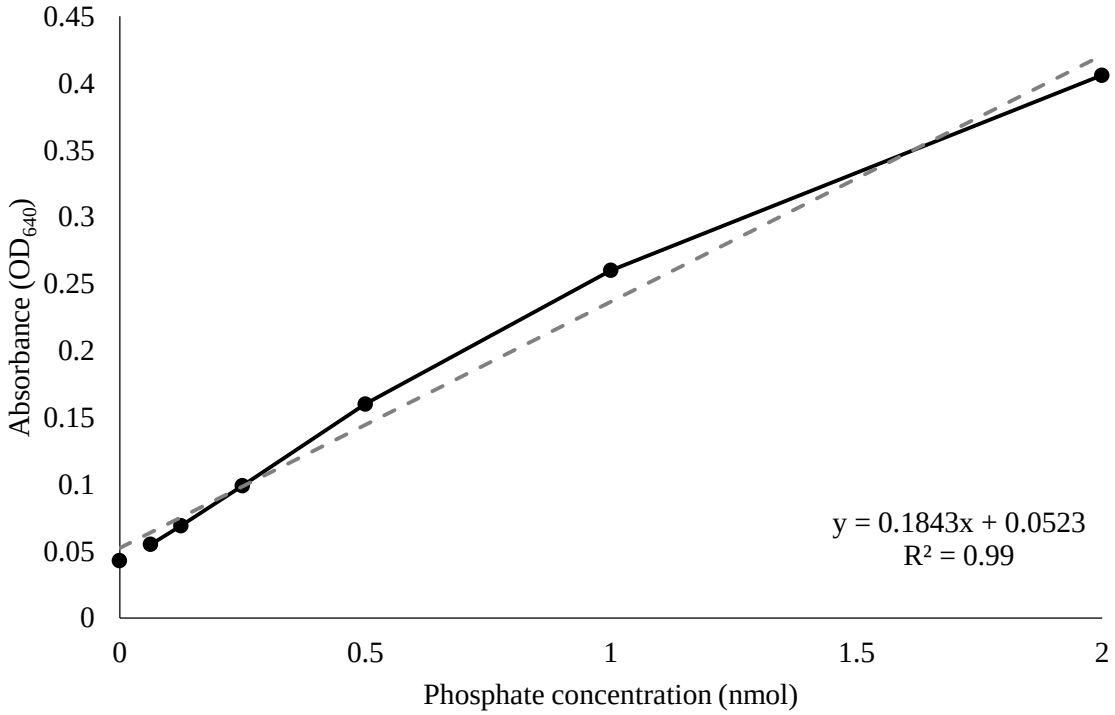

Fig. A1.9: The phosphate concentration (nmol) standard curve from test four at 15 min of incubation at room temperature, fitted within a linear curve against absorbance optical density (OD) at 640 nM.

Test four: The standards of the BIOMOL Green phosphate assay ranged from 0 to 2 nmol, with incubation times of 15 min, the samples were diluted to 25% with storage buffer (Fig. A1.10). The standard range was optimised for the current samples with two of the smaller concentrations (0.031 and 0.063 nmol Phosphate) removed, and three additional standards included (1.6, 1.2 and 0.8 nmol Phosphate). Samples were incubated for 30 min at 25 ^{o}C, following which 100 µL of sample was mixed with 100 µL of BIOMOL Green, thus stopping the reaction and allowing colour development to initiate. The samples were incubated at room temperature for 15 min to allow development of the green colour. The amount of Pi released during the Ca^{2+} uptake assay was determined by reading sample absorbance at 640 nM. The standards of the BIOMOL Green phosphate assay ranged from 0 to 2 nmol, with a linear curve fitted, reporting a R^2 value of 0.99 (Fig. A1.10). The absorbance values of the assessed skeletal muscle sample remained within the standards, demonstrating a clear measure of ATP activity within the microsomal samples collected from the rat skeletal muscle tissue.

Fig. A1.10: Image of the BIOMOL Green phosphate assay in a microplate, with a 25% dilution of the sample.

A1.6.2.3 Calcium dependent activity

The protocol undertaken in test four, with a 25% dilution of samples, and 15 min incubation period, was repeated on all remaining rat skeletal muscle microsomal fractions (n=9), under varying concentrations of free Ca^{2+}, as predicted by the volume of EGTA within the solution. The absorbance OD of each samples were used to calculate phosphate concentration. All samples were undertaken in triplicate, with the mean reported.

These data displayed a significant main effect of predicted free Ca^{2+} ($P < 0.001$; Fig. A1.12A). The solution containing 0 μM of predicted free Ca^{2+} released significantly less phosphate in comparison to all other conditions (0.097 μM, $P = 0.007$; 1 μM, $P = 0.002$, 10 μM, $P = 0.003$ and 100 μM, $P = 0.02$). Additionally, the solution containing 0.097 μM of predicted free Ca^{2+} released significantly less phosphate in comparison to 1 μM condition ($P = 0.004$), yet there was no significant difference between the other conditions. The 1 μM predicted free Ca^{2+} solution was the only concentration to be significantly higher than the 0 and 0.097 μM solutions.

These phosphate concentration data were presented relative to the protein concentration of the subcellular fraction (Fig. A1.12B). In-line with the above data, there was a significant main effect of predicted free Ca^{2+} ($P < 0.001$) on phosphate relative to protein concentrations. The solution containing 0 μM of predicted free Ca^{2+} released significantly less phosphate relative to protein concentrations in comparison to all other conditions (0.097 μM, $P = 0.02$; 1 μM, $P = 0.008$, 10 μM, $P = 0.007$ and 100 μM, $P = 0.03$). Additionally, the solution containing 0.097 μM of predicted free Ca^{2+} released significantly less phosphate in comparison to 1 μM condition ($P = 0.007$), yet there was no significant difference between the other conditions. The 1 μM predicted free Ca^{2+} solution was the only concentration to be significantly higher than the 0 and 0.097 μM solutions.

Due to the significant increase in the phosphate released during the 1 μM condition, above the phosphate concentrations in the 0 and 0.097 μM conditions, the 1 μM solution was used for further analysis, allowing for comparison to the 0 μM condition. To calculate the amount of Ca^{2+}-dependent ATPase activity, the amount of phosphate released during the 0 μM condition was subtracted from the 1 μM condition. These data are displayed in both absolute phosphate concentrations (Fig. A1.13A) and phosphate concentrations relative to protein concentration (Fig. A1.13B).

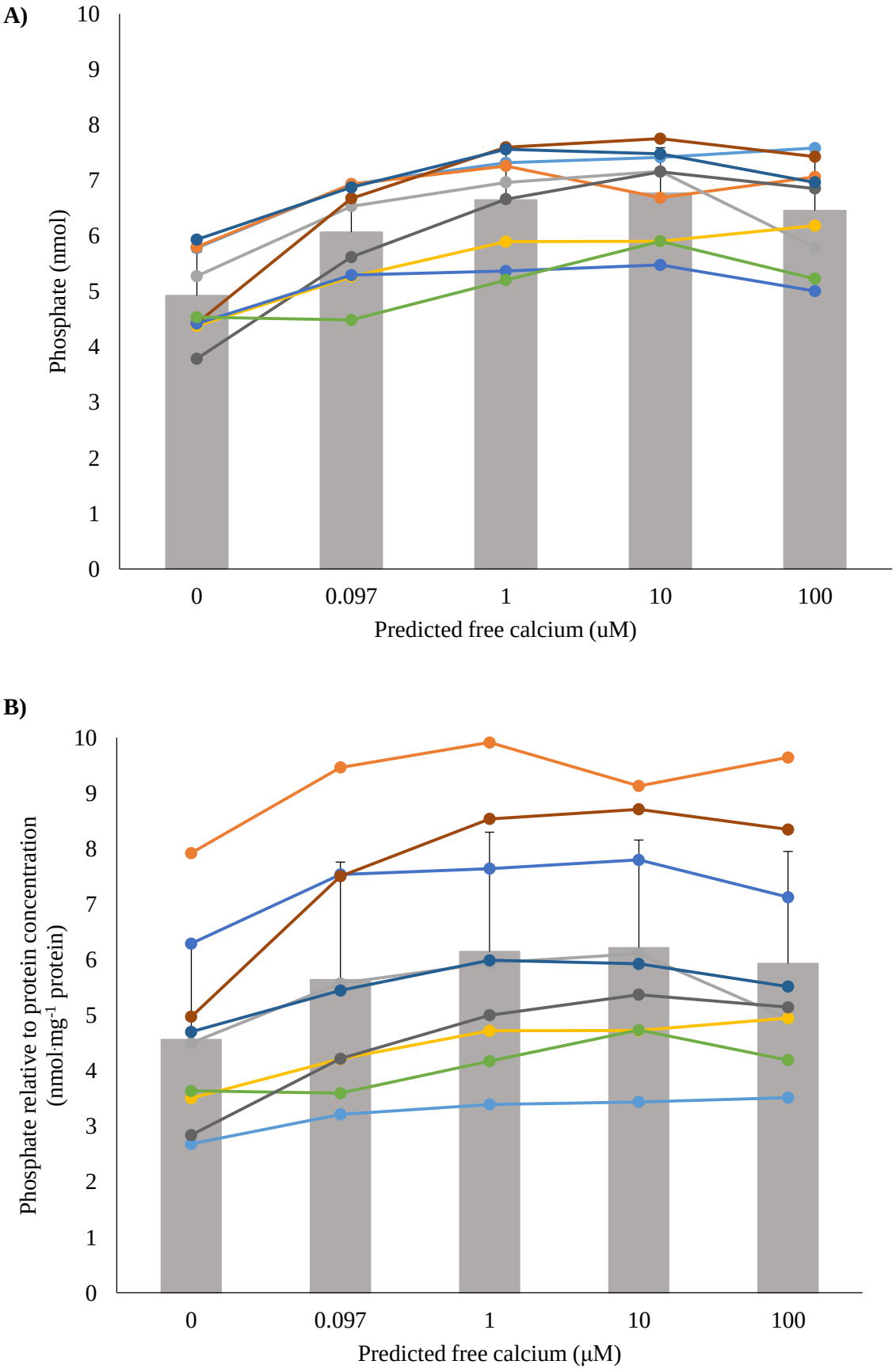

Fig. A1.11: The absolute phosphate concentration (nmol; A) and phosphate concentrations relative to protein concentration (nmol·mg^{-1} protein; B) of microsomal fraction from nine rats at predicted free calcium concentrations from 0 to 1 µM. Bar chart data are mean ± 1SD. Line graph data are individual skeletal muscle samples.

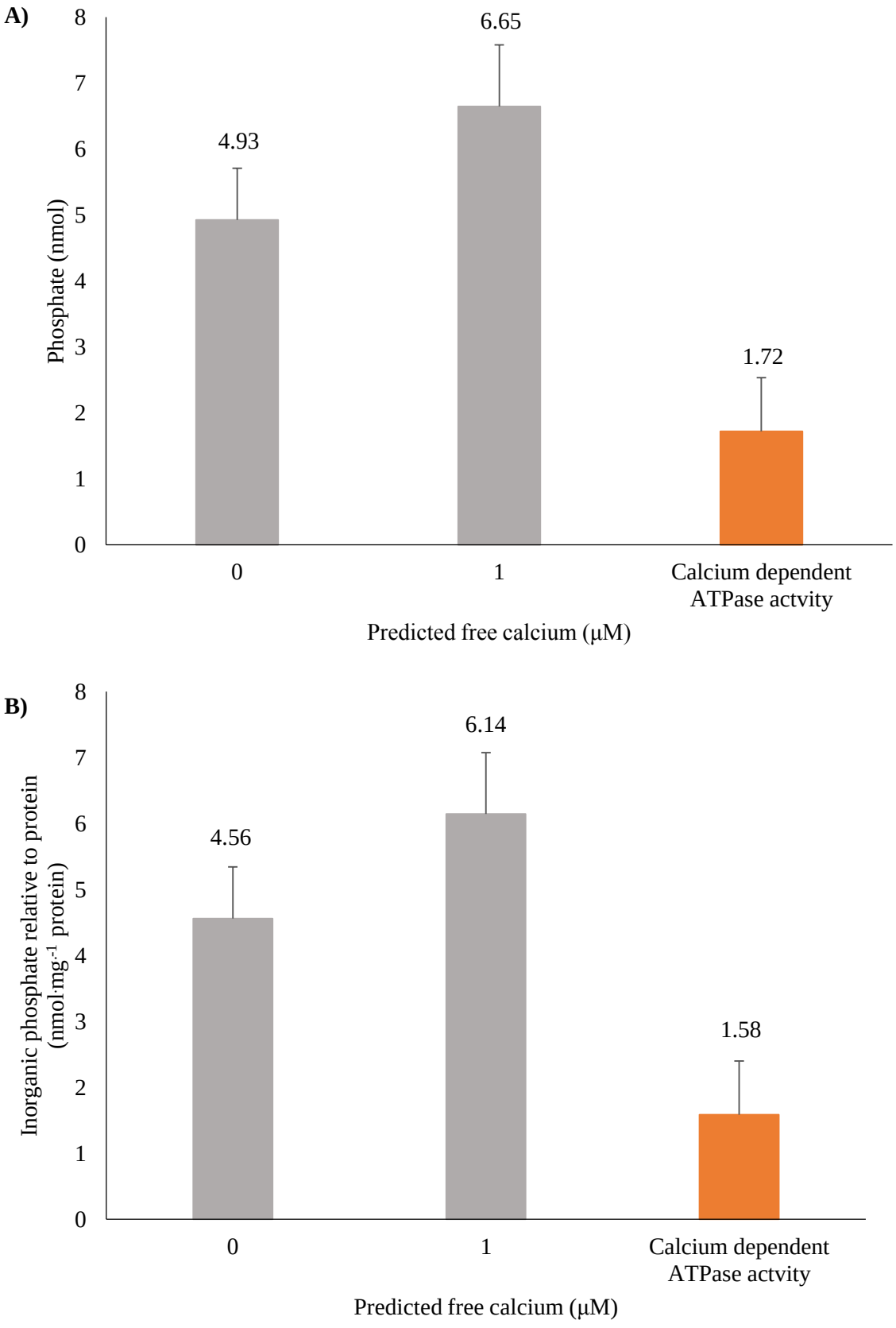

Fig. A1.12: The absolute phosphate concentration (nmol; A) and phosphate concentrations relative to protein concentration (nmol·mg^{-1} protein; B) of the microsomal fraction from nine rats at 0 to 1 µM predicted free calcium concentrations. The calcium dependent ATPase activity is the phosphate concentrations at 1 µM predicted free calcium concentrations minus those generated at 0 µM predicted free calcium concentrations. Representative data from one microsomal sample.

A1.6.2.4 Controls

Storage medium: To control for the potential effects of the storage medium on phosphate generation during each condition, protein concentrations were recorded in solutions where the sample volume was replaced with additional storage medium. ATP remained within the given solutions, maintaining the same solution volume. These analyses demonstrated very low levels of phosphate were generated during the conditions with additional storage medium (1.38 ± 0.92 nmol). The current experiments indicate that the storage medium was not generating phosphate, and therefore not influencing the samples.

ATP: To control for the potential effect of the ATP on phosphate generation during each condition, protein concentrations were recorded in solutions where the some of the storage medium was replaced with additional ATP. The sample remained within the given solutions, maintaining the same solution volume. These analyses demonstrated slightly greater levels of phosphate generations during the conditions with additional ATP (2.55 ± 0.39 nmol), with a trend be significantly greater than those in the storage medium ($P = 0.08$). Therefore, based on the current analysis, ATP concentrations need to be maintained across all samples, since it increased the generation of phosphate within the samples.

EGTA: To control for the potential effect of the high EGTA on phosphate generation during each condition, protein concentrations were recorded in solutions where some of the storage medium was replaced with EGTA. The sample remained within the given solutions, maintaining the same solution volume. In the presence of additional EGTA, additional phosphate was generated (4.31 ± 1.31 nmol), significantly above phosphate concentrations in the two previous conditions (Storage medium: $P = 0.002$; ATP: $P = 0.003$). This data indicated the EGTA need to be maintained across samples, since it increased the generation of phosphate within the samples.

Appendix 2: Journal articles

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β-Alanine supplementation enhances human skeletal muscle relaxation speed but not force production capacity

Ricci Hannah,[1] Rebecca Louise Stannard,[1] Claire Minshull,[1] Guilherme Giannini Artioli,[2] Roger Charles Harris,[3] and Craig Sale[1]

[1]*Sport, Health and Performance Enhancement (SHAPE) Research Group, School of Science and Technology, Nottingham Trent University, United Kingdom;* [2]*Laboratory of Applied Nutrition and Metabolism, School of Physical Education, University of São Paulo, São Paulo, Brazil; and* [3]*Junipa, Limited, Newmarket, Suffolk, United Kingdom*

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Hannah R, Stannard R, Minshull C, Artioli GG, Harris RC, Sale C. β-Alanine supplementation enhances human skeletal muscle relaxation speed but not force production capacity. *J Appl Physiol* 118: 604–612, 2015. First published December 24, 2014; doi:10.1152/japplphysiol.00991.2014.—β-Alanine (BA) supplementation improves human exercise performance. One possible explanation for this is an enhancement of muscle contractile properties, occurring via elevated intramuscular carnosine resulting in improved calcium sensitivity and handling. This study investigated the effect of BA supplementation on in vivo contractile properties and voluntary neuromuscular performance. Twenty-three men completed two experimental sessions, pre- and post-28 days supplementation with 6.4 g/day of BA ($n = 12$) or placebo (PLA; $n = 11$). During each session, force was recorded during a series of knee extensor contractions: resting and potentiated twitches and octet (8 pulses, 300 Hz) contractions elicited via femoral nerve stimulation; tetanic contractions (1 s, 1–100 Hz) via superficial muscle stimulation; and maximum and explosive voluntary contractions. BA supplementation had no effect on the force-frequency relationship, or the force responses (force at 25 and 50 ms from onset, peak force) of resting or potentiated twitches, and octet contractions ($P > 0.05$). Resting and potentiated twitch electromechanical delay and time-to-peak tension were unaffected by BA supplementation ($P > 0.05$), although half-relaxation time declined by 7–12% ($P < 0.05$). Maximum and explosive voluntary forces were unchanged after BA supplementation. BA supplementation had no effect on evoked force responses, implying that altered calcium sensitivity and/or release are not the mechanisms by which BA supplementation influences exercise performance. The reduced half-relaxation time with BA supplementation might, however, be explained by enhanced reuptake of calcium, which has implications for the efficiency of muscle contraction following BA supplementation.

β-alanine; muscle contractile properties; electrical stimulation; force-frequency relationship

CARNOSINE (β-ALANYL-L-HISTIDINE) is a cytoplasmic dipeptide synthesized from β-alanine (BA) and histidine and is found in high concentrations within mammalian skeletal muscle. Carnosine is formed, primarily in skeletal and brain tissue, by bonding histidine and BA in a reaction catalyzed by carnosine synthase (23, 40). The availability of BA in the human diet is the rate-limiting factor for carnosine synthesis in human skeletal muscle (for a brief review see Ref. 20). Long-term (4–10 wk) dietary supplementation with BA significantly increases human skeletal muscle carnosine content (19, 21, 24). Interest in elevating carnosine levels through BA supplementation has dramatically increased since it was first shown that doing so increased high-intensity cycling capacity (21). Since then, it has been well established that BA supplementation can improve high-intensity exercise performance (e.g., 2,000-m rowing performance and 100- to 200-m swimming performance) and capacity during exercise of ~1–6 min (see reviews in Refs. 22, 34). However, the physiological mechanisms for these ergogenic effects remain poorly understood.

Carnosine is suggested to have several physiological roles in muscle, which are pertinent to muscle function and performance. For example, its molecular structure makes it well suited to act as a pH buffer (36). The pKa of its imidazole ring is 6.83, placing it right in the middle of the pH transit range of exercising muscle. This means that an increase in carnosine content within the skeletal muscles also results in an expansion of the imidazole ring content, concomitantly increasing the muscle buffering capacity. As a result, performance improvements in high-intensity exercise (particularly when hydrogen cation accumulation is likely to limit performance) have largely been ascribed to increases in intracellular buffering (see review in Refs. 34, 35).

Alternative mechanisms for the enhancement of exercise performance following BA supplementation have been proposed. For example, previous work in rat skeletal muscle suggested a role for carnosine in increasing the sensitivity of the contractile apparatus to calcium ions (Ca^{2+}) (10). More recent work in skinned human medialis (m.) vastus lateralis fiber preparations showed a similar increase in Ca^{2+} sensitivity (11). Although only slight changes were shown in the maximum Ca^{2+}-activated force (≤3%), a significant leftward shift in the force-calcium concentration relationship was shown, indicating that force for a given submaximal Ca^{2+} concentration was increased in the presence of higher carnosine levels in type I and II fibers. Elevated carnosine levels also increased Ca^{2+} release from the sarcoplasmic reticulum of type I fibers, whereby carnosine appeared to enhance the Ca^{2+} sensitivity of ryanodine receptors and potentiated Ca^{2+}-induced Ca^{2+} release (11). Thus it was suggested that elevated carnosine after BA supplementation could alleviate the decline in contractile performance during fatiguing contractions by countering factors that might cause reduced calcium sensitivity and release (11).

A recent study provided the first evidence that dietary BA supplementation may influence the muscle contractile properties of mice (13), potentially via elevated intramuscular carnosine and its effect on calcium sensitivity and handling (11). BA supplementation was associated with a leftward shift in the electrically evoked force-frequency relationship of excised muscle, which is analogous to the force-calcium concentration

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relationship (5, 27), eliciting a 10–31% increase in the force produced at low stimulation frequencies (13). However, the possibility that dietary BA supplementation might change in vivo human muscle contractile properties, and thus voluntary muscle performance, has not been investigated. There is a need to examine this possibility given that we would expect a wider range of performance effects of carnosine than has currently been shown if improved calcium handling were the major physiological role of carnosine in human skeletal muscle (34).

As such, we examined the effects of 28-day BA supplementation on the intrinsic contractile properties of human skeletal muscle in vivo, as well as on voluntary muscle function. Intrinsic contractile properties were assessed via the force-frequency relationship in response to muscle stimulation and the evoked twitch and octet (8 pulses at 300 Hz, which drives the muscle at its maximum capacity for rapid or "explosive" force production; Ref. 8) responses to supramaximal nerve stimulation. We hypothesized that BA supplementation would enhance intrinsic contractile properties, producing a leftward shift in the force-frequency relationship, increasing the peak and explosive force responses to twitch and octet stimulation, and thus enhance explosive voluntary force production. In addition, we hypothesized that the altered contractile properties would lead to changes in motor control, reflected as a shift in the force-electromyography (EMG) relationship towards lower EMG levels for a given level of force.

METHODS

Participants

Twenty-six participants were recruited to the study and were stratified and allocated to the two supplement groups [placebo (PLA) or BA] on the basis of maximum knee extensor strength [maximum voluntary force (MVF); see below] values recorded during the familiarization session, such that the two groups were matched for knee extensor strength. However, three participants withdrew from the study (2 from PLA and 1 from BA), one during familiarization due to a lack of tolerance of electrical stimulation and two following baseline testing with no reason provided. As such, 23 participants completed all aspects of the study (PLA group: $n = 11$; age, 25.6 ± 5.6 yr; body mass, 79.1 ± 13.0 kg; height, 1.80 ± 0.07 m; BA group: $n = 12$; age, 26.1 ± 7.4 yr; body mass, 82.4 ± 15.2 kg; height, 1.79 ± 0.06 m). All participants provided written informed consent and completed this study, which was approved by the Institutional Human Ethical Review Committee. None of the participants had taken any nutritional supplements in the previous 6 mo. Participants had no injuries to the lower limbs, were not involved in any systematic physical training, and were categorized as having moderate habitual levels of physical activity using the International Physical Activity Questionnaire Short Format (http://www.ipaq.ki.se/ipaq.htm; Ref. 7). Throughout the study participants were requested to maintain similar levels of physical activity and dietary intake; this was verbally confirmed at the start of each session. None of the subjects were vegetarian or vegan, and therefore, they would likely have encountered small amounts of BA in their diet (1).

Study Design

This was a double-blind placebo-controlled experiment. Participants completed three experimental sessions over a 5-wk period: a familiarization session, which preceded a baseline session by 7 days, and a follow-up session after 28 days of supplementation with either BA or PLA. Participants were instructed to abstain from alcohol and strenuous/unaccustomed exercise for 36 h before measurement sessions, with caffeine prohibited on the day of measurement sessions. Compliance with these requests was confirmed verbally with each participant before commencing each session. Measurement sessions were completed at a consistent time of day, with recordings of force and surface EMG during a series of voluntary and involuntary (electrically evoked) isometric contractions of the knee extensors of the dominant leg. The familiarization session involved all the voluntary and evoked contractions, except the evoked octet contractions. The baseline and follow-up sessions involved an identical protocol performed according to a strict schedule. All raw data analyses, exclusions, and statistical analyses were completed blind to supplement group.

Supplementation

Participants received 6.4 g/day of either BA (sustained-release Carnosyn) or a matched PLA (maltodextrin) for 28 days (2 × 800 mg tablets, ingested 4 times per day). The sustained-release formulation used in this study has been shown to reduce or remove the paraesthesia often experienced by participants following doses of free BA powder (9). We would expect the increase in muscle carnosine content to be close to 15 mmol/kg dry muscle (an increase of ~65% in a participant eating a mixed diet), given that Harris et al. (19) reported this level of increase following a similar but slightly lower total dose of BA. None of the participants reported any feelings of paraesthesia during the study. Throughout supplementation participants completed a log to verify supplement compliance, with similar compliance reported at 91 ± 7% in the BA group and 88 ± 10% in the PLA group (independent sample t-test, $P = 0.60$).

Supplements were provided to each participant in identical white tubs by an individual not directly involved in testing or data analysis to maintain the double-blind. BA tablets were tested by the manufacturer before release for the study and conformed to the label claim for BA content. In addition, BA and PLA supplements were independently tested by HFL Sports Science, UK, before use to ensure no contamination with steroids or stimulants according to International Organization for Standardization (ISO) 17025 accredited tests.

Experimental Setup

Knee extension force. Participants were seated in a rigid, custom-built dynamometer, as adapted from previous studies (17, 18), with knee and hip joint angles of ~95 and 100° (180° = full extension). Adjustable strapping across the pelvis and shoulders prevented extraneous movement during muscle activation. An ankle cuff was attached to the dominant leg of the participant ~2 cm proximal to the medial malleolus and was in series with a linear strain gauge (615; Tedea-Huntleigh, Herzliya, Israel) oriented perpendicular to the tibia. Dynamometer configuration was established during the familiarization session and replicated thereafter. The force signal was amplified (×1,000) in the frequency range of 0–500 Hz and sampled at 2,000 Hz using an external A/D converter (1401; CED, Cambridge, UK), interfaced with a personal computer (PC) using Spike 2 software (CED). Force data were low-pass filtered in both directions at 450 Hz using a fourth-order zero-lag Butterworth filter before analysis. Baseline resting force was subtracted from all force recordings to correct for the effects of gravity.

Electromyography. EMG signals were recorded from the superficial quadriceps: m. rectus femoris (RF), m. vastus medialis (VM), and m. vastus lateralis (VL). After preparation of the skin by shaving, light abrasion, and cleaning with alcohol, bipolar surface electrodes (2.5-cm interelectrode distance; silver/silver chloride, 95-mm^2 area; Ambu Blue Sensor; Ambu, Ballerup, Denmark) were attached over each muscle at standardized percentages of thigh length, as measured from the knee joint space to the greater trochanter: RF, 55%; VM, 25%; and VL, 45%. These sites were selected to avoid the innervation zones of each of the assessed muscles (32). A reference electrode was placed on the patella of the same limb. EMG signals were preamplified by active EMG leads (input impedance: 100 MΩ; common mode

rejection ratio: >100 dB; base gain: 500; 1st order high-pass filter set to 10 Hz; Noraxon, Scottsdale, AR) connected in series to a custom-built junction box and subsequently to the same A/D converter and PC software that enabled synchronization with the force data. The signals were sampled at 2,000 Hz. EMG data were band-pass filtered in both directions between 20 and 450 Hz using a fourth-order zero-lag Butterworth filter before analysis.

Electrical stimulation. A constant current variable voltage stimulator (DS7AH; Digitimer, Welwyn Garden City, UK) was used to assess knee extensor contractile properties while the participant was voluntarily passive. Square-wave pulses (0.2-ms duration) were delivered via: *1*) supramaximal femoral nerve stimulation to evoke maximal resting twitch, potentiated twitch, and octet contractions; and *2*) percutaneous submaximal muscle stimulation to evoke contractions at a range of frequencies (1 to 100 Hz) to assess the force-frequency relationship. Femoral nerve stimulation involved a cathode stimulation probe (1-cm diameter; Electro-Medical Supplies, Wantage, UK) firmly pressed into the skin over the femoral nerve in the femoral triangle and an anode (7 × 10 cm carbon rubber electrode; Electro-Medical Supplies) coated with electrode gel and taped to the skin over the greater trochanter. The precise location of the cathode was determined as the position that evoked the greatest twitch response for a particular submaximal electrical current (typically 30–50 mA). For percutaneous stimulation, the surfaces of two carbon rubber electrodes (14 × 10 cm; Electro-Medical Supplies) were coated with electrode gel and secured over the proximal and distal surface of quadriceps at standardized percentages of thigh length, as measured from the patella to the anterior superior iliac spine (ASIS): proximal electrode placed 20% distal to the ASIS; distal electrode placed 10% proximal to the patella.

Protocol and Measurements

Measurements were completed in the following order, according to a consistent time schedule including ≥3 min rest between successive measurements.

Force and EMG onsets for all evoked and voluntary contractions were identified manually using visual identification by the same investigator, in accordance with a previously published method (16, 37). This approach is considered more valid than the use of automated methods of identification (38).

Resting twitches. Resting twitches were evoked following ≥15 min passive sitting to remove any lingering potentiation, which incorporated the time for securing the participant in the dynamometer and preparing them for EMG and electrical stimulation. Single electrical impulses were delivered with stepwise increments in the current, separated by 10 s to allow for neuromuscular recovery, until a plateau in the amplitude of twitch force and compound muscle action potentials (M-waves) were reached. The stimulus intensity was then increased by 25% above the value required to elicit a plateau to ensure supramaximal stimulation, and three discrete supramaximal stimuli separated by 10 s were then delivered to elicit maximal twitch responses and M-waves.

The time difference between M-wave onset (1st electrode site to be activated) and twitch force onset was defined as the electromechanical delay (EMD). Twitch force was measured at 25 and 50 ms from onset, as markers of the explosive force production during the rising slope, and at the peak of the force response. The time-to-peak tension (TPT) and half-relaxation time (HRT) were also recorded. All measurements were averaged across the three maximal twitch contractions. The M-wave response for the three quadriceps electrodes was measured for M-wave area, from EMG onset to the point where the signal returned to baseline, and averaged across the three sites. The mean M-wave area of the three supramaximal stimuli was defined as the maximal M-wave area (M_{max}) and was used for normalization of voluntary quadriceps EMG (6).

Maximum voluntary contractions and potentiated twitches. A brief warm-up of three submaximal knee extension contractions at 50, 75, and 90% of the participants' perceived maximal force were performed; contractions lasted ~3 s each and were separated by ~20 s. Participants then completed four maximum voluntary contractions (MVCs) of the knee extensors ≥60 s apart, during which they were instructed to contract "as hard as possible" for 3–4 s. During and after each contraction they received strong verbal encouragement reiterating the instructions, together with online feedback of the force signal and a marker of their maximum force during that session displayed onscreen. Supramaximal stimulation of the femoral nerve, using the same configuration and stimulus intensity as for resting twitches, was used to elicit a maximal potentiated twitch ~1 s after each of the MVCs. The greatest instantaneous force during either the knee extensor MVCs or explosive voluntary contractions (see below) of that trial was defined as MVF. The root mean square (RMS) of the EMG signal for each muscle (RF, VM, and VL) was calculated over a 500-ms epoch surrounding MVF (250 ms either side) and normalized to the corresponding M_{max} (6), before averaging across all three sites to calculate a mean quadriceps value. The EMD, force at 25 and 50 ms from onset, peak twitch force, TPT, and HRT were averaged across the four maximal potentiated twitch contractions.

Explosive voluntary contractions. The protocol followed previously published procedures (6, 16). Participants completed ≥10 isometric explosive voluntary knee extensions, each separated by ~20 s. Starting from a completely relaxed state, they were instructed to respond to an auditory signal by extending their knee "as fast and hard as possible" for ~1 s, with an emphasis on "fast." An on-screen cursor was used to provide online feedback on their explosive performance, displaying the maximum rate of force development (2-ms time constant) of their best attempt. Strong verbal encouragement was provided to participants to exceed this target during each subsequent contraction. A second visual marker on the screen depicted 80% of the peak force recorded during MVCs, which participants were expected to achieve or exceed during each explosive contraction. Resting force was also displayed on a sensitive scale during all explosive contractions to aid the detection of pretension or countermovement. The explosive contractions were performed until 10 contractions, with no prior countermovement or pretension, had been recorded.

The three contractions with the greatest maximum rate of force development, meeting the following criteria, were used for analysis: *1*) no prior countermovement or pretension, and *2*) peak force ≥80% MVF. Analyses involved measurement of the force-time and EMG-time traces in short periods after their onsets. Explosive force was measured at 25-ms intervals up to 150 ms after force onset. The RMS of the EMG signal from each muscle was measured over three consecutive 50-ms time periods from EMG onset of the first agonist muscle to be activated (i.e., 0–50, 50–100, and 100–150 ms). Thereafter, RMS EMG at each EMG site was normalized to M_{max} and averaged to provide a mean quadriceps value. All measurements were averaged across the three selected contractions.

Force-EMG relationship (via voluntary incremental knee extension contractions). A series of submaximal knee extension contractions were performed at 15% increments of MVF, in ascending order, up to 90%. Horizontal cursors on the screen in front of participants depicted the target levels of force. Participants were instructed to reach the target quickly and maintain the level of force as accurately as possible for ~3 s. Contractions were separated by ~20 s. The RMS of the EMG and average force over a stable 500-ms part of the force trace (minimal standard deviation of the force trace for that contraction) were analyzed at each of the contraction intensities. The EMG RMS values were normalized to M_{max} and plotted against the respective force values. Linear regression was used to evaluate the slope and intercept of the force-EMG relationship incorporating all data between 15 and 90% MVF.

Octet contractions. Octet contractions (8 impulses at 300 Hz; Ref. 8) were evoked via supramaximal stimulation of the femoral nerve.

First, a brief series of single stimuli were administered, and twitch force and M-wave amplitudes were monitored to confirm that the stimuli were supramaximal. The current was increased if necessary to ensure supramaximal stimulation. Then three discrete pulse trains (≥15 s apart) were delivered with a supramaximal current (+25%) to evoke maximal octet contractions. The current increased by ~5% after each pulse train to confirm a plateau in both the peak force and maximum rate of force development. On some occasions, where the first pulse train elicited a submaximal response, a fourth pulse train was delivered to ensure three maximal responses. The octet force response was measured at 25 and 50 ms from force onset, as well as at the peak. All measurements were averaged across the three analyzed contractions.

Force-frequency relationship. Surface EMG electrodes were removed and carbon rubber electrodes were attached over the quadriceps, taking ~5 min. The force-frequency relationship was then evaluated during tetanic contractions elicited via submaximal percutaneous electrical stimulation (3, 15).

Initially, 100-Hz contractions were evoked at increasing current intensities, ≥30 s apart, to determine the current that elicited 50% of MVF. This current (typically 110–200 mA) was then used for the following force-frequency measurements. The final calibration contraction at 100 Hz and the subsequent measured contractions were separated by ≥60 s. The force-frequency relationship contractions consisted of two twitch contractions (1 Hz), followed by single contractions of 1-s duration at each of nine different frequencies (5, 10, 15, 20, 30, 40, 50, 80, and 100 Hz) performed in ascending order with ~30 s between contractions. Peak force was defined as the greatest instantaneous force. Thereafter, the force values at each stimulation frequency were normalized to the force obtained at 100 Hz. The force-frequency relationship was fitted with a Hill curve and evaluated for frequency at 50% of the maximum force response (11).

Statistical Analysis

Dependent variables measured over several time points/periods (force and EMG during explosive voluntary contractions, evoked twitch, and octet force) were analyzed using a three-way (group × session × time point) ANOVA. Similarly, the force-frequency relationship was assessed by a three-way (group × session × frequency) ANOVA. Other dependent variables (MVF, HRT, TPT, slope and intercept of force-EMG relationship, frequency at 50% of force response for the force-frequency relationship) were evaluated using two-way ANOVA (group × session). A Greenhouse-Geisser correction was applied when the ANOVA assumption of sphericity was violated, and significant interaction effects were followed-up by independent sample *t*-tests on the individual percentage change values for each condition. The change in group mean values was used to calculate the percentage change values presented. Intraindividual variability was assessed using the mean intraindividual coefficient of variation (CV) across the two measurement sessions for the PLA group [(means ÷ SD) × 100]. Statistical analyses were completed using SPSS version 21 (SPSS, Chicago, IL), and statistical significance was accepted at $P \leq 0.05$. Data are presented as means ± 1SD.

RESULTS

Electrically Evoked Contractile Properties

Resting twitches. There was no influence of supplementation on resting twitch force ($P = 0.46$ and 0.70 for group × session and group × session × time point interactions; Fig. 1*A*), EMD ($P = 0.63$; Fig. 2*A*), or TPT ($P = 0.29$; Fig. 2*B*), although there was a group × session interaction for HRT ($P = 0.018$; Fig. 2*C*). Post hoc analysis showed that the change in HRT was greater for the BA group (−12 ± 10%) compared with the PLA group (+2 ± 11%; $P < 0.01$). Mean CV values for the PLA group: force at 25 and 50 ms and peak were 14, 9, and 8%; EMD was 7%; TPT was 3%; and HRT was 7%.

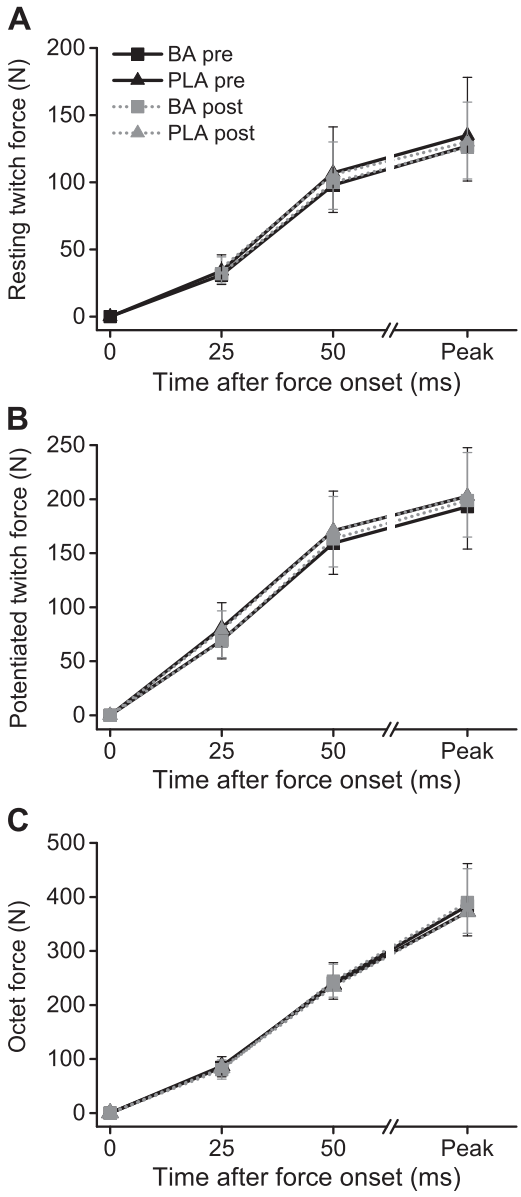

Fig. 1. Electrically evoked force of β-alanine (BA) and placebo (PLA) groups pre- and postsupplementation: resting twitch force (*A*), potentiated twitch force (*B*), and octet force (*C*). Data are means ± 1SD.

Potentiated twitches. There was no influence of supplementation on potentiated twitch force ($P = 0.44$ and 0.52; Fig. 1*B*), EMD ($P = 0.48$ Fig. 2*D*), or TPT ($P = 0.32$; Fig. 2*E*). However, there was a group × session interaction for HRT ($P = 0.041$; Fig. 2*F*) and post hoc analysis showed that the change in HRT was greater for the BA group (−7 ± 11%) compared with the PLA group (+1 ± 8%; $P = 0.050$). Mean CV values for the PLA group: force at 25 and 50 ms and peak were 6, 3, and 3%; EMD was 6%; TPT was 3%; HRT was 4%.

Octet contractions. Supplementation did not influence resting octet force at any time point (Fig. 1*C*). Mean CV values for the PLA group: force at 25 and 50 ms and peak were 10, 3, and 4%.

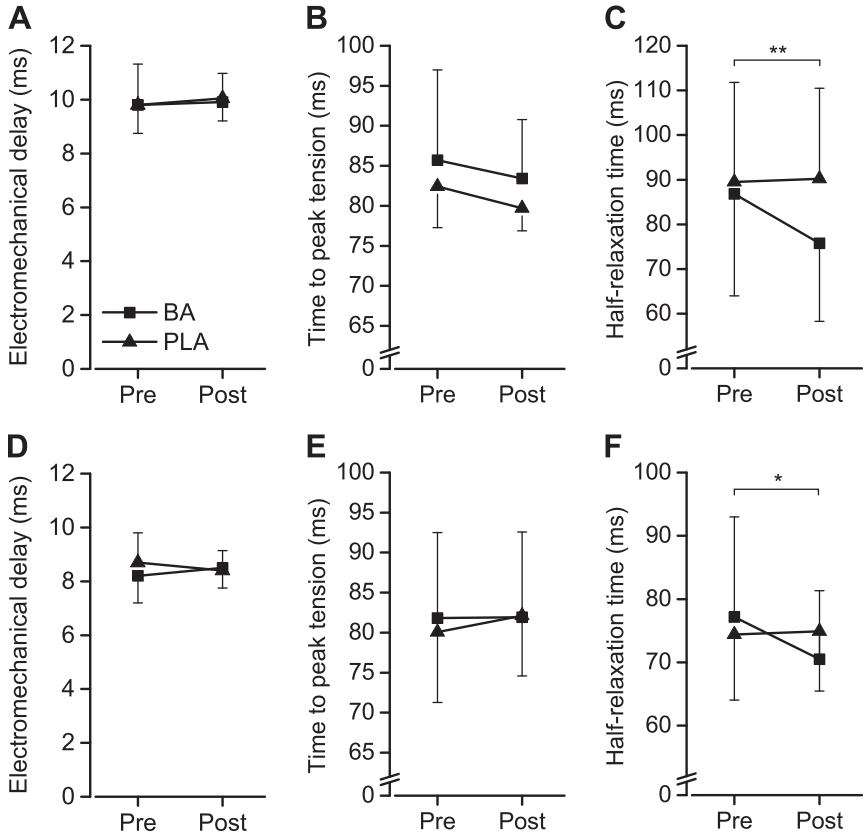

Fig. 2. Contraction times during twitch contractions evoked via femoral nerve stimulation for BA and PLA groups pre- and post-supplementation. Resting twitch: electromechanical delay (EMD; *A*), time-to-peak tension (TPT; *B*), and half-relaxation time (HRT; *C*). Potentiated twitch: EMD (*D*), TPT (*E*), and HRT (*F*). Data are means ± 1SD. $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ for post hoc independent *t*-test on %change values in BA and PLA groups.

Force-frequency relationship. The peak force at each frequency of stimulation (Fig. 3) and the frequency at 50% of the force response (Table 1) were both unaffected by supplementation. Mean CV values for relative force (%maximum at 100 Hz) in PLA group were 6–8% at 1–10 Hz, 1–3% at 15–80 Hz, and 6% for the frequency at 50% of force response.

Maximum and Explosive Voluntary Force Production

There was no affect of supplementation on MVF (Fig. 4*A*). The mean CV for MVF in the PLA group was 3%. Similarly, there was no influence of supplementation on force measured at 25-ms intervals during explosive voluntary contractions (Fig. 4*A*). The mean CV values for voluntary force production in the PLA group were 13–17% at 25–50 ms and 4–7% from 75–150 ms.

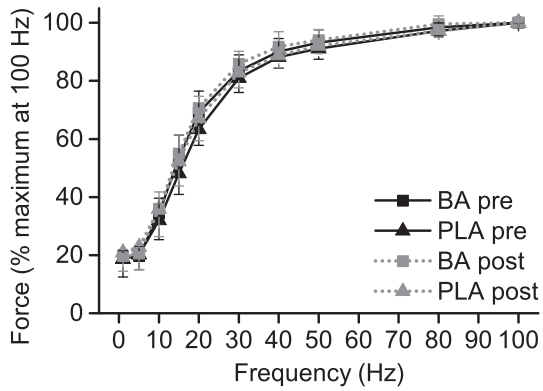

Fig. 3. Force-frequency relationship assessed during submaximal percutaneous stimulation for BA and PLA groups pre- and post-supplementation. Data are means ± 1SD.

Neuromuscular Activation

Agonist neuromuscular activation during maximum voluntary and explosive voluntary contractions. Agonist EMG normalized to M_{max} during MVCs and explosive contractions was not affected by supplementation (Fig. 4*B*), indicating that neuromuscular activation was consistent across measurement sessions. The mean CV values for agonist EMG in the PLA group were 26, 23, and 9% in the 0- to 50-ms, 50-, to 100-ms, and 100- to 150-ms time windows, and 13% at MVF.

Force-EMG relationship. The slope and *y*-intercept of the force-EMG relationship were unaffected by supplementation (Fig. 5 and Table 1). The mean CV value for slope of the force-EMG relationship in the PLA group was 15%. Although the CV was very high for the intercept of the relationship (80%) as a consequence of intercept values being close to zero, the mean difference between sessions was actually very low when expressed as a percentage of maximal EMG at MVF (4%).

DISCUSSION

The present study is the first to comprehensively examine the influence of BA supplementation on the electrically evoked contractile properties of human skeletal muscle in vivo. BA supplementation had no effect on the force-frequency relationship, evaluated during submaximal muscle stimulation. Similarly, BA did not influence the EMD, explosive force (at 25 and 50 ms), peak force or TPT of resting twitch, or potentiated

Table 1. *Characteristics of the force-frequency and force-EMG relationships of BA and PLA groups pre- and postsupplementation*

	Pre		Post	
	BA	PLA	BA	PLA
Force-frequency relationship				
Frequency at 50% of force response, Hz	17.3 ± 2.4	18.8 ± 1.5	16.8 ± 1.7	18.0 ± 1.9
Force-EMG relationship				
Intercept (RMS:M_{max})	−0.49 ± 0.74	−0.70 ± 0.47	−0.53 ± 0.91	−0.56 ± 0.43
Slope (RMS:M_{max}/N)	0.0175 ± 0.0054	0.0180 ± 0.0035	0.0174 ± 0.0037	0.0170 ± 0.0037

Data are means ±1SD. BA, β-alanine; PLA, placebo; RMS, root mean square; M_{max}, M-wave area.

twitch or octet contractions elicited by supramaximal stimulation of the femoral nerve. In line with these findings, there were no changes in maximum or explosive voluntary force production following BA supplementation. The only significant effect of BA was a 12 and 7% reduction in HRT during resting and potentiated twitch contractions.

Knee Extensor Intrinsic Contractile Properties

The force-frequency relationship of the knee extensors was evaluated during submaximal muscle stimulation at a range of frequencies (1–100 Hz) to evaluate potential effects of BA supplementation on calcium handling and sensitivity, since an association between intracellular calcium levels and force production in response to different stimulation frequencies has previously been shown (5). BA supplementation, however, had no effect on knee extensor force production at relatively low (1–15 Hz) or high (20–80 Hz) frequencies of muscle stimulation, corresponding to relatively low (19–53% force at 100 Hz) and high (63–95% force at 100 Hz) levels of force. Previous in vitro research showed that increasing cytoplasmic carnosine levels from those normally present to levels approaching those attained after supplementation produced a marked enhancement in Ca^{2+} sensitivity (i.e., an increased force response to submaximal Ca^{2+} levels) of fibers from human VL, as well as enhanced Ca^{2+} release in type I fibers (11). Thus the present data showing no effect of BA supplementation on the force-frequency relationship, the in vivo analog of the force-calcium concentration relationship (5, 27), responses might therefore be taken to imply that supplementation did not grossly influence Ca^{2+} sensitivity (10, 11) or Ca^{2+} release (11, 33).

The present force-frequency data are supported by the findings that force and contraction time responses to supramaximal nerve stimulation at low (resting and potentiated twitch) and high frequencies (300 Hz, octet) were not affected by BA supplementation. Improved Ca^{2+} sensitivity or release would be expected to be particularly beneficial in situations where calcium saturation is submaximal; during resting twitch contractions evoked by a single nerve impulse, for example. Combined evaluation of resting and potentiated twitch responses might have been expected to reveal any influence of BA supplementation on these processes, since the mechanisms for potentiation include the phosphorylation of myosin, which increases the sensitivity of the contractile elements to Ca^{2+}, as well as altered Ca^{2+} handling (39). However, neither peak force, TPT, nor explosive force (force at 25

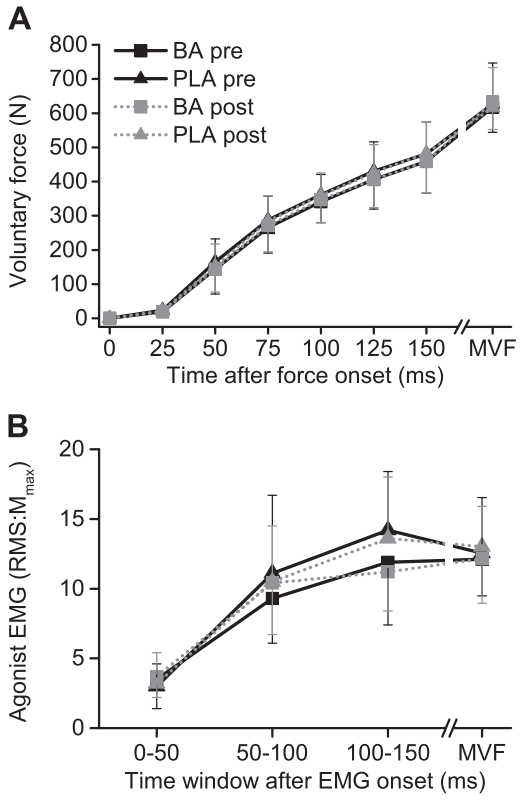

Fig. 4. Explosive and maximum voluntary force (MVF) of BA and PLA groups pre- and post-supplementation (*A*). Agonist EMG normalized to M-wave area (M_{max}) during explosive contractions (0–50, 50–100, and 100–150 ms from onset) and at MVF for the BA and PLA groups pre- and postsupplementation (*B*). RMS, root mean square. Data are means ± 1SD.

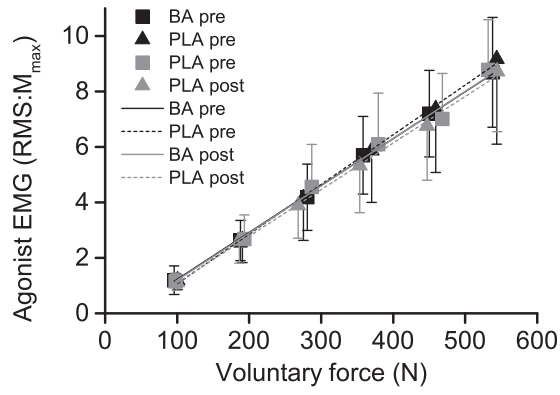

Fig. 5. Force-EMG relationship measured during submaximal voluntary contractions (15–90% MVF) for BA and PLA groups pre- and postsupplementation. Data are means ± 1SD.

and 50 ms) of resting and potentiated twitches was affected by BA supplementation. Similarly, the resting and potentiated twitch EMD, which reflects the time for excitation-contraction coupling processes and for muscle shortening to remove slack from the muscle tendon unit (29, 31), was unaltered following BA supplementation. These data further imply that BA supplementation had little influence on Ca^{2+} sensitivity or release.

The current data appear at odds with the human single fiber data mentioned above (11) and recent findings in mouse muscle where 10–31% increases in force were shown at frequencies between 25 and 125 Hz, but not at 1 Hz, following BA supplementation (13). Several factors could explain the present results and apparent contrast with the previous data. Firstly, there are obvious differences with the study of Dutka et al. (11), including the manner by which they increased carnosine levels (acute exposure to a carnosine containing solution), the conditions of the muscle (skinned fibers devoid of connective tissue and not attached to bone), and the manner in which it was activated (exposure to Ca^{2+}-buffered solutions), all of which bear little resemblance to the present in vivo study. Secondly, although enhanced Ca^{2+}-induced Ca^{2+} release was observed following exposure to carnosine in single fiber preparations (11), the authors concede that this may not occur in vivo, since this mechanism might have limited relevance to the control of Ca^{2+} release through ryanodine receptors by the dihydropyridine receptors (12, 25). Thirdly, species differences in carnosine metabolism and histidine-containing dipeptide content (4, 13) could explain the discrepancy between the data of Everaert et al. (13) in mice and the data from the present study. The potential for interspecies differences is suggested by the fact that previous human data showed no fiber-type differences in the carnosine-related changes in Ca^{2+} sensitivity (11), while there was some suggestion of fiber type differences in mice (i.e., differences in the response of "slow" soleus vs. "fast" extensor digitorum longus muscles to BA supplementation) (13). It should be noted that we did not measure muscle carnosine content in the present study and so we cannot confirm the actual change due to BA supplementation or whether this directly relates to the individual responses in muscle contractile properties. It is likely, given the previous data on the topic (e.g., Ref. 19), that the increase in muscle carnosine would be ~15 mmol/kg dry muscle or +65% in these participants, with this supplementation regimen.

While the majority of the evoked contractile properties showed no change in response to BA supplementation, HRT decreased by 7–12% during resting and potentiated twitch contractions. Muscle relaxation is initiated by a reduction in sarcoplasmic reticulum Ca^{2+} concentration. The rate of relaxation may be influenced by *1*) the rate of dissociation of Ca^{2+} from troponin (26); *2*) the rate of translocation of Ca^{2+} to a site close to the sarcoplasmic reticulum (28); and *3*) the rate of reuptake of Ca^{2+} into the sarcoplasmic reticulum by ATPase driven Ca^{2+} pumps (30). At present, there do not appear to be any reports of carnosine influencing these aspects of excitation-contraction coupling. Interestingly, however, Everaert et al. (13) reported an attenuation of the fatigue-related increases in relaxation times after BA supplementation in murine soleus muscle. While their finding in this case could be a consequence of enhanced buffering capacity, during the repeated contractions, since BA supplementation had no influence on resting rate of relaxation, their report further highlights the functional implications of the present data. During fatigue, the rate of muscle relaxation slows as a consequence of a reduced rate of cross-bridge dissociation or impaired Ca^{2+} pumping into the sarcoplasmic reticulum (2). The latter is energetically costly (30), and, as such, any improvements in Ca^{2+} handling with BA supplementation could reduce the total energy expenditure during high-intensity cyclic joint movements by reducing that energy cost and also by improving the efficiency of joint movements by reducing co-contraction. Future research should attempt to confirm the present findings and extend them by investigating the changes in evoked contractile properties during fatigue to better understand the influence of BA supplementation on muscle contractility and implications for metabolic and movement efficiency during exercise.

Voluntary Force Production and Motor Control

BA supplementation had no effect on MVF, a finding consistent with the lack of changes in electrically evoked twitch or tetanic (octet) peak force in the present study. Maximum isometric force is not affected by either increased Ca^{2+} sensitivity or increased myoplasmic Ca^{2+} concentration (27), and previous studies reported minimal effects of carnosine on maximum calcium-activated force (0–3% increase) (11) and of BA supplementation on maximal twitch and tetanic force (13). Improved Ca^{2+} sensitivity or release would be expected to be beneficial for force production in situations where calcium saturation is submaximal (e.g., during the rising phase of voluntary force production where neuromuscular activation is submaximal; Ref. 14) and during sustained submaximal contractions. Thus one might have expected improvements in explosive voluntary force and/or alterations in the force-EMG relationship, indicative of the change in neuromuscular activation required to produce a given change in force, if BA supplementation had influenced these Ca^{2+}-related functions. However, in accordance with the lack of changes in the force-frequency relationship, as well as the force responses during twitch and octet contractions, BA supplementation did not influence voluntary explosive force or the force-EMG relationship. The similar neural drive during both the MVCs and explosive voluntary contractions confirm that the voluntary force measurements were not confounded by changes in neuromuscular activation over time.

Conclusions

The results of the present study showed that BA supplementation had no effect on the force-frequency relationship, implying a lack of any effect on muscle Ca^{2+} sensitivity or release. In support of these data, there was no effect of BA supplementation on force responses to resting and potentiated twitches and octet contractions. As such, the study findings do not support the idea that exercise performance and capacity improvements after BA supplementation are due to enhanced Ca^{2+} sensitivity or release. We do, however, show a reduction in HRT with BA supplementation, which might possibly be explained by enhanced reuptake of Ca^{2+} into the sarcoplasmic reticulum. This has potentially important implications for the efficiency of muscle contraction following BA that should be

explored in future studies, since this could conceivably contribute to the ergogenic potential of BA supplementation during high-intensity exercise involving rapid muscle contractions.

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GRANTS

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DISCLOSURES

R. Harris is an independent paid consultant of Natural Alternatives International (San Marcos, CA) and is named as an inventor on patents held by Natural Alternatives International.

AUTHOR CONTRIBUTIONS

Author contributions: R.H., C.M., G.G.A., R.C.H., and C.S. conception and design of research; R.H. and R.L.S. performed experiments; R.H. and R.L.S. analyzed data; R.H., R.L.S., and C.S. interpreted results of experiments; R.H. prepared figures; R.H. and C.S. drafted manuscript; R.H., R.L.S., C.M., G.G.A., R.C.H., and C.S. edited and revised manuscript; R.H., R.L.S., C.M., G.G.A., R.C.H., and C.S. approved final version of manuscript.

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ORIGINAL ARTICLE

β-alanine supplementation improves in-vivo fresh and fatigued skeletal muscle relaxation speed

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Abstract
Purpose In fresh muscle, supplementation with the rate-limiting precursor of carnosine, β-alanine (BA), results in a decline in muscle half-relaxation time (HRT) potentially via alterations to calcium (Ca^{2+}) handling. Accumulation of hydrogen cation (H^+) has been shown to impact Ca^{2+} signalling during muscular contraction, carnosine has the potential to serve as a cytoplasmic regulator of Ca^{2+} and H^+ coupling, since it binds to both ions. The present study examined the effect of BA supplementation on intrinsic in-vivo isometric knee extensor force production and muscle contractility in both fresh and fatigued human skeletal muscle assessed during voluntary and electrically evoked (nerve and superficial muscle stimulation) contractions.
Methods Twenty-three males completed two experimental sessions, pre- and post- 28 day supplementation with 6.4 g.day^{-1} of BA ($n=12$) or placebo (PLA; $n=11$). Isometric force was recorded during a series of voluntary and electrically evoked knee extensor contractions.
Results BA supplementation had no effect on voluntary or electrically evoked isometric force production, or twitch electromechanical delay and time-to-peak tension. There was a significant decline in muscle HRT in fresh and fatigued muscle conditions during both resting ($3\pm13\%$; $19\pm26\%$) and potentiated ($1\pm15\%$; $2\pm20\%$) twitch contractions.
Conclusions The mechanism for reduced HRT in fresh and fatigued skeletal muscle following BA supplementation is unclear. Due to the importance of muscle relaxation on total energy consumption, especially during short, repeated contractions, BA supplementation may prove to be beneficial in minimising contractile slowing induced by fatigue.
Trial registration The trial is registered with Clinicaltrials.gov, ID number NCT02819505.

Keywords Contractile properties · Electrical stimulation · Muscle fatigue · Carnosine

Abbreviations

η_g^2	Generalised eta squared
η_p^2	Partial eta squared
ANOVA	Analysis of variance
BA	β-alanine
Ca^{2+}	Calcium
CV	Coefficient of variation
EMD	Electromechanical delay
EMG	Surface electromyography
H^+	Hydrogen cation
HRT	Half-relaxation time
M_{max}	The mean M-wave area of the three supramaximal stimuli
M-wave	Muscle action potential
MVIC	Maximal voluntary isometric contraction
MVIF	Maximal voluntary isometric force

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PLA	Placebo
RF	Rectus femoris
RMS	Root mean squared
TPT	Time-to-peak tension
TTF	Time-to-task failure
VL	Vastus lateralis
VM	Vastus medialis

Introduction

Carnosine (β-alanyl-$_L$-histidine) is a cytoplasmic dipeptide, synthesised from β-alanine (BA) and histidine and is found in high concentrations in skeletal muscle. The synthesis of carnosine is limited by the availability of BA from the diet, while supplementation with BA over a number of weeks results in significant increases in the skeletal muscle carnosine content (Harris et al. 2006; Hill et al. 2007). BA supplementation has been shown to consistently increase human skeletal muscle carnosine concentrations, and by an equal amount in both type I and II muscle fibres (Hill et al. 2007), with increases of 40–80% evident depending upon dose (3.2–6.4 g day^{-1}) and duration of administration (4–10 weeks) (Harris et al. 2006; Hill et al. 2007). Increasing skeletal muscle carnosine concentrations via BA supplementation in both upper and lower limbs has consistently been shown to benefit high-intensity exercise capacity and performance, as highlighted by several reviews (Sale et al. 2010, 2013) and a recent meta-analysis (Hobson et al. 2012).

With a pKa of 6.83 for the histidine imidazole ring when combined with BA and the abundance of carnosine within skeletal muscle, it has been proposed that the improvements in high-intensity exercise outcomes following BA supplementation are the result of increased muscle buffering capacity over the exercise pH transit range (Hill et al. 2007). Whilst the role of carnosine as an intracellular pH buffer is undisputable, other physiological roles for carnosine underlying high-intensity exercise improvements following BA supplementation have been proposed (Sale et al. 2010). Carnosine can increase the sensitivity of the calcium (Ca^{2+}) release channels in the sarcoplasmic reticulum and/or the sensitivity of the contractile apparatus in chemically skinned muscle fibres from frogs (Lamont and Miller 1992), mechanically skinned rat muscle fibres (Dutka and Lamb 2004) and type I and type II human skeletal muscle fibres (Dutka et al. 2012). Muscle Ca^{2+} release channels contain saturable binding sites for carnosine, indicating that carnosine has the potential to alter the Ca^{2+} channel itself (Batrukova and Rubstov 1997). Until recently, however, research has been limited to rodent and in-vitro models.

Recent work (Hannah et al. 2015) examined the effect of BA supplementation on human skeletal muscle contractile properties and force production capabilities in-vivo. BA supplementation did not alter maximal or explosive voluntary isometric force production, or the force–frequency relationship (Hannah et al. 2015), this relationship is the in-vivo analogue of the force–calcium concentration relationship (Batrukova and Rubstov 1997). These findings were in-line with the hypothesis arising from our previous exercise performance studies (Sale et al. 2010, 2013) proposing that the main physiological role for carnosine in improving high-intensity exercise performance related to intracellular pH buffering and not to increased Ca^{2+} sensitivity of the in-vivo contractile apparatus. An unexpected result was a significant decline in half-relaxation time (HRT) during both resting and potentiated twitches following BA supplementation, making it important to confirm these findings before exploring potential underlying mechanisms. Muscle relaxation speed can be impacted by the rate of: (1) dissociation of Ca^{2+} from troponin (Little et al. 2011); (2) the rate of translocation of Ca^{2+} to near the site of entry into the sarcoplasmic reticulum (Muntener et al. 1995); (3) reuptake of Ca^{2+} into the sarcoplasmic reticulum by Ca^{2+} pumps (Nogueira et al. 2013) and cross-bridge detachment (Allen et al. 2008). Slowing of skeletal muscle relaxation decreases power output and shortening velocity (Allen et al. 2008), thus limiting performance during dynamic exercise where rapidly alternating movements are performed (Allen et al. 1995). Improving the relaxation of skeletal muscle can be energetically beneficial by increasing the efficiency of joint movements by reduced co-contraction (Nogueira et al. 2013). This would be expected to contribute to enhanced dynamic exercise performance, especially where rapidly alternating movements are performed.

High-intensity exercise leads to a more pronounced accumulation of hydrogen cation (H^+), a metabolic factor which might be involved in skeletal muscle fatigue, but in combination with other fatigue-induced changes or in an indirect manner (Westerblad 2016). It should also be highlighted that muscle fatigue is multi-factorial phenomenon. Skeletal muscle fatigue is generally accompanied by a marked slowing of relaxation (Allen et al. 2008). H^+ are proposed to directly or indirectly inhibit sarcoplasmic Ca^{2+} release during skeletal muscle contraction (Laver et al. 2000, 2004). Carnosine has the potential to serve as a cytoplasmic regulator of Ca^{2+} and H^+ coupling, since it binds to both ions (Baran 2000). As such, it could be hypothesised that increasing muscle carnosine content, via BA supplementation, would have a more pronounced beneficial effect on HRT when the muscle is fatigued (Bergstrom and Hultman 1988).

The present study aimed to examine the effects of 28 days of BA supplementation on intrinsic in-vivo isometric

knee extensor force production and muscle contractility in both fresh (rested conditions) and fatigued human skeletal muscle.

Methods

Ethical approval

All participants were fully informed of any risks and discomforts associated with the study. Participants provided written informed consent and completed a health screen questionnaire prior to taking part in the study, which was first approved by the Nottingham Trent University Ethical Advisory Committee.

Participants

Twenty-four male participants were allocated to the two supplement groups [placebo (PLA) or BA] on the basis of maximal voluntary isometric force (MVIF) values recorded during familiarisation. One participant withdrew from the study (PLA group; $n=11$) with no reason provided, and consequently, 23 participants completed all aspects of the study (PLA; age, 22 ± 1 years, height, 1.83 ± 0.06 m, body mass, 81.4 ± 14.2 kg, MVIF, 600 ± 149 N; and BA; age, 22 ± 2 years, height, 1.80 ± 0.05 m, body mass, 76.0 ± 7.3 kg, MVIF, 565 ± 86 N). Participants had not ingested any nutritional supplements, had no injuries of the lower limb, and were not involved in any systematic physical training in the 6 months prior to the study. Participants were requested to maintain similar levels of physical activity and dietary intake, which was verbally confirmed at the start of each session. None of the participants were vegetarian or vegan, and therefore, they would likely have encountered small amounts of BA in their diet.

Study design

This was a double-blind, placebo-controlled study with all raw data analyses, exclusions, and statistical analyses undertaken blind to the supplement group. Participants undertook three experimental sessions; a familiarisation session, which preceded a baseline session by ~7 days, and a follow-up session after 28 days of supplementation. Participants were instructed to abstain from alcohol and strenuous/unaccustomed exercise for 36 h before measurement sessions, with caffeine prohibited on the day of testing. Compliance with these requests was confirmed verbally with participants before commencing each session. Measurement sessions recorded force and surface electromyography (EMG) during a series of voluntary and involuntary (electrically evoked) isometric contractions of the knee extensors of the dominant leg. All participants were first familiarised with the protocol measures, both baseline, and follow-up sessions involved an identical protocol performed according to a strict schedule.

Supplementation

Participants were provided with 6.4 g day^{-1} of either BA (sustained-release CarnoSyn™; NAI, Inc. San Marcos, USA) or a matched PLA (maltodextrin; NAI, Inc. San Marcos, USA) for 28 days (2×800 mg tablets, ingested 4 times per day). Based on similar BA supplementation protocols muscle carnosine content was expected to increase to ~38 mmol·kg^{-1} dry muscle (based upon a 65% increase from a baseline concentration of 23 mmol·kg^{-1} dry muscle) or 65% above the typical carnosine content of individual eating a mixed diet (Harris et al. 2006; Sale et al. 2013). The sustained-release formulation used in this study has been shown to reduce or remove the paraesthesia often experienced by participants following doses of free BA powder (Decombaz et al. 2012). Supplement compliance was verified with participant logs. Compliance was similar in both groups and was reported as 91 ± 6% (BA) and 92 ± 9% (PLA; independent sample t test, $P=0.68$); no feelings of paraesthesia were reported. Supplements were provided in identical white tubs by an individual blind to the supplement groups. BA tablets were tested by the manufacturer before release for the study and conformed to the label claim for BA content. To ensure no contamination with steroids or stimulants according to the International Organization for Standardization (IOS) 17,025 accredited tests, the BA and PLA supplements were independently tested by HFL Sports Science.

Experimental setup

The experimental setup for the determination of isometric knee extension force, EMG, and electrical stimulation in our laboratory has been described in detail previously (Hannah et al. 2015).

Isometric knee extension force

The participant was strapped into a custom-built dynamometer with knee and hip joint angles of ~95 and 100° (180° = full extension), and with an ankle cuff attached ~2 cm proximal to the medial malleolus secured around the participant's dominant leg, and which was in series with a linear strain gauge (Model 615; Tedea-Huntleigh, Herzliya, Israel). The chair position, strain gauge position, and strapping setup were recorded during the familiarisation session and replicated identically during subsequent testing sessions. Force signals were amplified (×1,000) in the

frequency range of 0–500 Hz, sampled at 2000 Hz using an external A/D converter (Model 1401; CED, Cambridge, UK), interfaced with a personal computer (PC) using the Spike 2 software (CED). Force data were low-pass filtered in both directions at 450 Hz using a fourth-order zero-lag Butterworth filter before analysis. Baseline resting force was subtracted from all force recordings to correct for the effects of gravity.

Electromyography

EMG signals were recorded from the superficial quadriceps: *m*. rectus femoris (RF), *m*. vastus medialis (VM), and *m*. vastus lateralis (VL). EMG signals were pre-amplified by active EMG leads (input impedance: 100 MΩ; common mode rejection ratio: >100 dB; base gain: 500; first order high-pass filter set to 10 Hz; Noraxon, Scottsdale, AR) connected in series to a custom-built junction box and subsequently to the same analogue–digital converter and PC software that enabled synchronisation with the force data. The signals were sampled at 2000 Hz. EMG data were band-pass filtered in both directions between 20 and 450 Hz using a fourth-order zero-lag Butterworth filter before analysis.

Electrical stimulation

Knee extensor contractile properties were assessed using a constant current variable voltage stimulator (DS7AH; Digitimer, Welwyn Garden City, UK). Square-wave pulses (0.2 ms duration) were delivered via: (1) supramaximal femoral nerve stimulation to evoke maximal resting twitch, potentiated twitch, and octet contractions; and (2) percutaneous submaximal muscle stimulation to evoke contractions at a range of frequencies (1 to 100 Hz) to assess the force–frequency relationship. A cathode stimulation probe (1 cm diameter; Electro-Medical Supplies, Wantage, UK) and an anode (7 × 10 cm carbon rubber electrode; Electro- Medical Supplies) were used to elicit femoral nerve stimulation. Two carbon rubber electrodes (14 × 10 cm; Electro-Medical Supplies) were used to elicit percutaneous stimulation.

Protocol and measurements

Identification of force and EMG onset for all evoked and voluntary contractions was conducted manually using visual identification (Hannah et al. 2012, 2015), which is considered more valid than the use of automated identification methods (Tillin et al. 2013). Voluntary and evoked contractions were elicited in accordance with a previously published method (Hannah et al. 2012, 2015; Tillin et al. 2013) and are described below in brief.

Resting twitches

A single electrical impulse was delivered with stepwise increments in the current to evoke a twitch response, until a plateau in the amplitude of twitch force and compound muscle action potentials (M-waves) was reached. To ensure supramaximal stimulation, stimulus intensity was increased by 25% above the value required to evoke a plateau. Three discrete supramaximal stimuli were then evoked to elicit maximal twitch responses and M-waves. Electromechanical delay (EMD) was defined as the time difference between M-wave onset (1st electrode site to be activated) and force onset. Twitch force at 25 and 50 ms from force onset, was measured as markers of the explosive force production, peak force, time-to-peak tension (TPT), and half-relaxation time (HRT) were also reported. All measurements were averaged across the three maximal twitch contractions. The M-wave response for the three quadriceps electrodes was measured for M-wave area, from EMG onset to the point where the signal returned to baseline, and averaged across the three sites. The mean M-wave area of the three supramaximal stimuli was defined as the maximal M-wave area (M_{max}) and was used for normalisation of voluntary quadriceps EMG.

Maximum voluntary contractions and potentiated twitches

Participants were instructed to produce four maximal voluntary isometric contractions (MVIC), "as hard as possible" for 3–4 s. Strong verbal encouragement reiterating the instructions was provided during and after each contraction, together with visual onscreen feedback. Following each MVIC, supramaximal stimulation of the femoral nerve at the same configuration and stimulus intensity as the resting twitches was elicited to evoke maximal potentiated twitch. MVIF was defined as the greatest instantaneous force during either the knee extensor MVICs or explosive voluntary contractions (see below). The root mean square (RMS) of the EMG signal for each muscle (RF, VM, and VL) was calculated over a 500 ms epoch surrounding MVIF (250 ms either side) and normalized to the corresponding M_{max}. All sites were then averaged to calculate a mean quadriceps value. EMD, force at 25 and 50 ms from onset, peak twitch force, TPT, and HRT were averaged across the four maximal potentiated twitch contractions.

Explosive voluntary contractions

Participants completed isometric explosive voluntary contractions, starting each contraction completely relaxed, contracting their knee "as fast and hard as possible" for ~1 s, with an emphasis on "fast". The three contractions with the greatest maximum rate of force development,

achieving the following criteria, were used for analysis: (1) no prior countermovement or pretension, and (2) peak force >80% MVIF. Explosive force was measured at 25 ms intervals up to 150 ms after force onset. The RMS of the EMG signal from each muscle was measured over three consecutive 50 ms time periods from EMG onset of the first agonist muscle to be activated (i.e., 0–50, 50–100, and 100–150 ms). Thereafter, RMS at each EMG site was normalized to M_{max} and averaged to provide a mean quadriceps value. All measurements were averaged across the three selected contractions.

Force–EMG relationship (via voluntary incremental knee extension contractions)

Submaximal knee extensor contractions were completed at 15% increments of MVIF, in ascending order, separated by ≥20 s. Force target levels were displayed on screen by horizontal cursors, with participants instructed to reach the target as quickly as possible, and then maintain this target force level as accurately as possible for ~3 s. During each contraction intensity, the RMS of the EMG and average force over a stable 500 ms part of the force trace (minimal standard deviation of the force trace for that contraction). The EMG RMS values were normalized to M_{max} and plotted against the respective force values. Linear regression was used to evaluate the slope and intercept of the force–EMG relationship incorporating all data between 15 and 90% MVIF.

Octet contractions

Octet contractions (8 impulses at 300 Hz) were evoked via supramaximal stimulation of the femoral nerve. In summary, three discrete pulses (≥15 s apart) were delivered with a supramaximal current (+25%) to evoke maximal octet contractions. The octet force response was measured at 25 and 50 ms from force onset, as well as at the peak. All measurements were averaged across the three analysed contractions.

Force–frequency relationship

Tetanic contractions were elicited via submaximal percutaneous electrical stimulation of the quadricep to examine the force–frequency relationship (Lamont and Miller 1992). 100 Hz contractions were evoked at increasing current intensities, ≥30 s apart, to determine the current that elicited 50% of MVIF. This current was then used for the following force–frequency measurements. The final calibration contraction at 100 Hz and the subsequent measured contractions were separated by ≥60 s. The force–frequency relationship contractions consisted of two twitch contractions (1 Hz), followed by single contractions of 1 s duration at each of nine different frequencies (5, 10, 15, 20, 30, 40, 50, 80, and 100 Hz) performed in ascending order with ≥30 s between contractions. Peak force was defined as the greatest instantaneous force. Thereafter, the force values at each stimulation frequency were normalized to the force obtained at 100 Hz. The force–frequency relationship was fitted with a Hill curve and evaluated for frequency at 50% of the maximum force response (Dutka and Lamb 2004).

Sustained isometric knee extensor hold

To induce H^+ accumulation within the quadricep muscles, participants were instructed to perform a voluntary isometric contraction at 45% of MVIF for "as long as possible". The start of the sustained fatigue hold was defined as the time when force was greater than 40% of MVIF, and terminated when force fell below 5% of the target force for more than 3 s, despite strong encouragement. Strong verbal encouragement was provided alongside visual feedback displayed onscreen. The time between start and end of the sustained fatigue was defined as the time-to-task failure (TTF), with average force recorded across this time. Impulse (kN.s) was calculated as the product of the average force and TTF. It has been estimated that TTF would be ~78 s for a contraction held at 45% of MVIC force (Ahlborg et al. 1972). Immediately upon completion of the sustained fatigue hold, participants repeated all voluntary and evoked contractions.

Blood samples

Fingertip capillary blood samples were taken at rest, immediately prior to and 5 min following the sustained fatigue hold. Fingertip capillary blood lactate measured 5 min post-exercise provides an estimate of lower limb blood lactate contractions (Comeau et al. 2011). Sampling involved the collection of 80 µL of whole blood into a heparin-coated clinitube; all samples were analysed immediately post-sampling (Radiometer Ltd, UK).

Statistical analysis

Based on an *a priori* power calculation, a minimum of 22 participants were required to achieve 92% power at $P < 0.05$. Calculations were based on the previous findings (Hobson et al. 2012), with 24 participants being recruited to allow for dropouts. Statistical analyses were completed using SPSS version 22 (SPSS Inc., Chicago, IL, USA), with statistical significance accepted at $P \leq 0.05$. Data are presented as means ± 1 standard deviation (SD). Dependent variables (MVIF, EMD, HRT, TPT, slope, and intercept

of force–EMG relationship, frequency at 50% of force response for the force–frequency relationship) were evaluated using a two-way mixed-model (group × session) analysis of variance (ANOVA). Dependent variables measured over several time points (force and EMG during explosive voluntary contractions, evoked twitch, and octet force) were analysed using a three-way mixed-model (group × session × time) ANOVA. All variables were assessed during both fresh and fatigued conditions. The sustained fatigue time-to-task failure (TTF) and impulse were analysed using a two-way mixed-model ANOVA.

The impact of the fatigue hold contraction on dependant variables (MVIF, EMD, HRT, TPT, slope, and intercept of force–EMG relationship, frequency at 50% of force response for the force–frequency relationship) was analysed using a three-way mixed-model (fatigue × group × session) ANOVA. Percentage change between fresh and fatigued values for the dependent variables measured over several time points (force and EMG during explosive voluntary contractions, evoked twitch, and octet force) were analysed using a three-way mixed-model (percentage change × group × session) ANOVA. A Greenhouse–Geisser correction was applied when the ANOVA assumption of sphericity was violated. Effect size for multiple comparisons was calculated using partial (η_p^2) and generalised (η_g^2) eta squared (Lakens 2013). Providing two effect sizes is suggested to yield a greater understanding of a specific effect (Preacher and Kelly 2011). *Post hoc* comparisons to explain any significant interactions are reported with Cohen's *d* effect size. An effect size of 0.2–0.5 was defined as small, 0.5–0.8 as medium, and ≥0.8 as large (Schünemann et al. 2008). Intra-individual variability was assessed using the mean intra-individual coefficient of variation (CV) across the two measurement sessions for the PLA group [(SD/mean) × 100], the current research CVs are in line with those reported previously using the same equipment (Hannah et al. 2015).

Results

Electrically evoked contractile properties

Resting twitches

Supplementation did not significantly influence twitch force, EMD, or TPT (Table 1). There was, however, a significant group × session interaction for HRT in both fresh ($P = 0.04$, $n_p^2 = 0.2$, $n_g^2 < 0.001$) and fatigued ($P = 0.03$, $\eta_p^2 = 0.2$, $\eta_g^2 = 0.1$; Figs. 1, 2) muscle. *Post hoc* analysis showed that the percentage change in fresh muscle HRT was not significantly different between the BA (−2 ± 10 ms; −3 ± 13%) and PLA group (+8 ± 16 ms; 8 ± 16%) with a large effect reported (P = 0.06; Cohen's d = 0.9). In fatigued muscle, *post hoc* analysis showed that HRT percentage change was significantly different between the BA (−25 ± 34 ms; −19 ± 26%) and PLA (8 ± 16 ms; 0 ± 15%) group with a large effect reported ($P = 0.05$, Cohen's $d = 0.9$).

Table 1 Electrically femoral nerve evoked force responses, time-to-peak tension (TPT), and electromechanical delay (EMD) of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued muscle. Data are means ± 1SD

		Pre-supplementation					Post-supplementation				
		Force (N)			EMD (ms)	TPT (ms)	Force (N)			EMD (ms)	TPT (ms)
		25	50	Peak			25	50	Peak		
Resting twitch											
Fresh	BA	23 ± 5	73 ± 14	88 ± 17	10 ± 1	79 ± 11	24 ± 6	73 ± 17	90 ± 19	10 ± 1	81 ± 9
Fresh	PLA	28 ± 4	79 ± 19	99 ± 26	11 ± 1	82 ± 8	29 ± 5	82 ± 16	104 ± 24	10 ± 1	83 ± 9
Fatigued	BA	22 ± 7	61 ± 22	68 ± 25	11 ± 2	72 ± 16	26 ± 8	71 ± 22	79 ± 23	10 ± 1	74 ± 13
Fatigued	PLA	25 ± 6	58 ± 20	65 ± 24	11 ± 1	69 ± 8	24 ± 4	60 ± 15	67 ± 18	10 ± 2	71 ± 9
Potentiated twitch											
Fresh	BA	55 ± 13	142 ± 24	159 ± 26	9 ± 1	79 ± 6	53 ± 8	136 ± 14	155 ± 9	9 ± 1	81 ± 6
Fresh	PLA	60 ± 16	138 ± 37	163 ± 43	9 ± 1	79 ± 9	59 ± 10	142 ± 34	167 ± 38	9 ± 1	82 ± 8
Fatigued	BA	37 ± 11	92 ± 27	101 ± 29	11 ± 1	74 ± 9	40 ± 9	101 ± 19	110 ± 18	10 ± 1	73 ± 10
Fatigued	PLA	42 ± 10	93 ± 25	105 ± 30	10 ± 1	74 ± 4	38 ± 9	93 ± 23	106 ± 28	10 ± 1	74 ± 8
Octet											
Fresh	BA	52 ± 15	165 ± 42	223 ± 47	7 ± 2	117 ± 25	64 ± 18	196 ± 37	274 ± 53	7 ± 2	120 ± 24
Fresh	PLA	66 ± 17	190 ± 62	285 ± 94	6 ± 1	127 ± 19	64 ± 17	199 ± 60	296 ± 94	7 ± 2	137 ± 7
Fatigued	BA	62 ± 23	191 ± 49	221 ± 72	7 ± 2	100 ± 23	62 ± 17	182 ± 47	244 ± 68	7 ± 2	114 ± 25
Fatigued	PLA	69 ± 20	191 ± 49	273 ± 72	7 ± 1	123 ± 14	64 ± 16	198 ± 49	283 ± 79	7 ± 2	125 ± 15

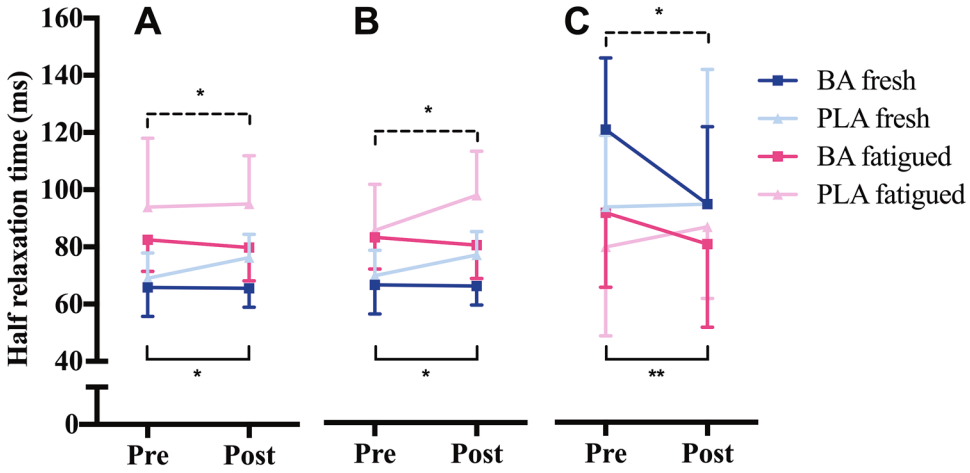

Fig. 1 Electrically evoked half-relaxation time of β-alanine (BA) and placebo (PLA) groups pre- and post-supplementation, in fresh and fatigued muscle during: resting twitch (**a**), potentiated twitch (**b**), and octets (**c**). Data are means ± 1SD. $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ for *post hoc* independent *t*-test between BA and PLA groups

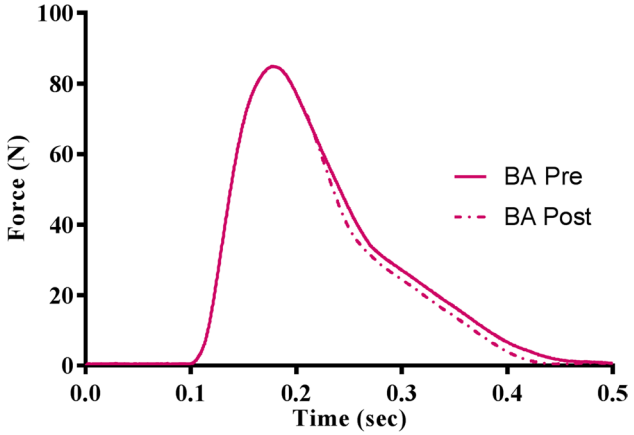

Fig. 2 Representative records of the force response during an electrically evoked resting twitch contraction pre- and post-supplementation with β-alanine under fresh conditions. These records are averaged records from three participants to provide an illustration of the decline in twitch half-relaxation time

The percentage difference between fresh and fatigued resting twitch force remained similar between sessions for both supplementation groups. Resting twitch TPT declined following completion of the sustained fatigue hold ($P = 0.001$, $\eta_p^2\eta_p^2 = 0.4$, $\eta_g^2 = 0.2$), although EMD remained similar with no difference between sessions or groups (Table 1). Resting twitch HRT significantly increased following the completion of the sustained fatigue hold ($P < 0.001$, $n_p^2 = 0.7$, $n_g^2 = 0.3$; Fig. 1) with no group × fatigue or group × session × fatigue interactions.

Potentiated twitches

Supplementation did not significantly influence twitch force, EMD or TPT (Table 1). There was, however, a significant group × session interaction for HRT in both fresh ($P = 0.03$, $n_p^2 = 0.2$, $n_g^2 < 0.001$) and fatigued muscle ($P = 0.03$, $\eta_p^2 = 0.2$, $\eta_g^2 < 0.001$; Figs. 1, 2). *Post hoc* analysis showed that the percentage change in fresh muscle HRT was not significantly different between the BA (0 ± 9 ms; +1 ± 15%) and PLA group (+7 ± 10 ms; +12±15%) with a medium effect reported ($P = 0.10$, Cohen's $d = 0.7$). In fatigued muscle, *post hoc* analysis showed that the percentage change in fatigued muscle HRT was significantly different between the BA (−2.7 ± 16 ms; −2 ± 20%) and PLA group (12 ± 12 ms; 16 ± 17%) with a large effect reported ($P = 0.03$, Cohen's $d = 1.0$).

The percentage difference between fresh and fatigued potentiated twitch force remained similar between sessions for both supplementation groups, with only a significant effect of time ($P = 0.001$, $\eta_p^2 = 0.5$, $\eta_g^2 = 0.5$). Potentiated twitch TPT declined following completion of the sustained fatigue hold ($P < 0.001$, $\eta_p^2 = 0.5$, $\eta_g^2 = 0.5$; Table 1). Potentiated EMD significantly prolonged following the fatigue hold ($P = 0.001$, $\eta_p^2 = 0.45$, $\eta_g^2 = 0.2$) with no group × fatigue or group × session × fatigue interactions. Potentiated HRT significantly increased following the completion of the sustained fatigue hold ($P < 0.001$, $\eta_p^2 = 0.7$, $\eta_g^2 = 0.4$; Fig. 1), with no group × fatigue or group × session × fatigue interactions.

Octet contractions

In both fresh and fatigued muscle, supplementation did not significantly alter octet peak force, EMD or TPT (Table 1). There was, however, a significant group × session interaction for octet HRT in both fresh ($P = 0.05$; $n_p^2 = 0.2$; $\eta_g^2 = 0.1$) and fatigued ($P = 0.01$; $n_p^2 = 0.3$; $\eta_g^2 = 0.2$; Figs. 1, 2) skeletal muscle. *Post hoc* analysis showed that the percentage change in fresh muscle was significantly different between the BA (−26±30 ms; −20 ± 22%) and PLA (0 ± 32 ms; 1 ± 34%) group with a large effect reported

($P = 0.05$, Cohen's $d = 0.8$). In fatigued muscle, *post hoc* analysis showed that HRT percentage change was significantly different between the BA (−11 ± 20 ms; −11 ± 20%) and PLA (12 ± 19 ms; 7 ± 13%) groups with a large effect reported ($P = 0.01$, Cohen's $d = 1.2$).

Octet force percentage change between fresh and fatigue was not significantly affected by supplementation. Octet TPT ($P = 0.05$, $\eta_p^2 = 0.2$, $\eta_g^2 = 0.1$; Table 1) and HRT ($P = 0.008$, $\eta_p^2 = 0.3$, $\eta_g^2 = 0.2$; Fig. 1), declined following completion of the sustained fatigue hold with no group × fatigue or group × session × fatigue interactions. Octet EMD was not significantly influenced following the fatigue hold, with no group × fatigue or group × session × fatigue interactions (Table 1).

Force–frequency relationship

Supplementation did not significantly influence peak force at each frequency of stimulation, and the frequency at 50% of the force response (Table 2) in either fresh or fatigued muscle. Following the fatigue hold, peak force significantly declined ($P = 0.001$, $\eta_p^2 = 0.8$, $\eta_g^2 = 0.2$), although the frequency at 50% of the force response remained unaffected.

Maximum and explosive voluntary force production

Supplementation had no effect on MVIF in fresh or fatigued muscle (Fig. 3a). Following the fatigue hold, MVIC significantly declined ($P < 0.001$, $\eta_p^2 = 0.9$, $\eta_g^2 = 0.2$), with no differences between session and groups (BA: 17–18%; PLA: 21%; Fig. 3a). There was no effect of supplementation on force measures at 25 ms intervals during explosive voluntary contractions in fresh and fatigued states (Fig. 3a). Explosive force percentage change between fresh and fatigue of force remained unaffected by supplementation.

Neuromuscular activation

Agonist neuromuscular activation during maximal and explosive voluntary contractions

Agonist EMG normalized to M_{max} during MVICs and explosive contraction remained uninfluenced by supplementation in fresh and fatigued muscles (Fig. 3b).

Force–EMG relationship

The slope and y-intercept of the force–EMG relationship were unaffected by supplementation in both fresh and fatigued muscles (Fig. 4; Table 2). The percentage change in agonist EMG normalized to M_{max} during MVICs and explosive contraction was not significantly altered between sessions.

Table 2 Force–frequency relationship assessed during submaximal percutaneous stimulation, and characteristics of the force–frequency and force–EMG relationships of BA and PLA groups in fresh and fatigued muscle, pre- and post- supplementation

			Force–frequency								Frequency at 50% of response, Hz	Force–EMG relationship	
			1	5	10	15	20	30	40	50	80	Intercept (RMS:Mmax)	Slope (RMS:Mmax/N)
Pre-	Fresh	BA	19.99 ± 4.52	20.55 ± 5.01	34.15 ± 7.21	54.67 ± 8.16	70.56 ± 5.98	87.29 ± 4.09	93.41 ± 3.41	96.40 ± 2.52	100.21 ± 2.04	14.3 ± 2.5	−0.52 ± 0.91	0.022 ± 0.007
	Fresh	PLA	19.87 ± 3.29	21.45 ± 4.39	38.88 ± 8.87	59.14 ± 10.04	72.24 ± 8.51	85.51 ± 5.68	90.69 ± 4.80	93.18 ± 4.79	99.60 ± 1.75	13.9 ± 2.2	−0.19 ± 0.62	0.021 ± 0.007
	Fatigued	BA	17.15 ± 4.60	16.53 ± 4.33	26.01 ± 5.59	43.35 ± 7.68	57.87 ± 7.68	77.93 ± 3.78	87.50 ± 4.46	91.57 ± 4.33	97.97 ± 4.60	14.2 ± 2.6	−0.88 ± 1.07	0.022 ± 0.006
	Fatigued	PLA	16.63 ± 4.72	15.98 ± 3.98	25.38 ± 5.52	43.80 ± 6.26	59.09 ± 7.32	78.33 ± 8.26	84.19 ± 8.51	88.25 ± 8.22	97.94 ± 3.54	13.6 ± 2.0	−0.35 ± 0.61	0.024 ± 0.007
Post-	Fresh	BA	21.57 ± 5.22	22.16 ± 6.37	37.45 ± 8.78	57.45 ± 8.64	70.06 ± 6.87	85.82 ± 4.07	90.91 ± 4.95	94.08 ± 4.51	99.12 ± 3.83	14.1 ± 2.4	−0.25 ± 0.42	0.021 ± 0.006
	Fresh	PLA	21.41 ± 4.63	22.70 ± 5.79	38.65 ± 8.23	56.85 ± 8.27	70.40 ± 6.28	84.10 ± 6.78	90.25 ± 6.82	92.82 ± 6.33	99.36 ± 3.37	14.0 ± 2.3	−0.36 ± 0.59	0.022 ± 0.006
	Fatigued	BA	19.25 ± 4.65	18.90 ± 5.60	27.72 ± 7.17	46.31 ± 7.76	61.60 ± 5.84	79.22 ± 3.61	89.52 ± 3.72	90.01 ± 3.69	97.63 ± 3.22	13.5 ± 2.5	−0.34 ± 0.35	0.020 ± 0.005
	Fatigued	PLA	16.58 ± 3.52	16.43 ± 4.18	26.39 ± 7.76	45.40 ± 13.12	60.03 ± 10.72	77.87 ± 12.44	85.31 ± 10.38	88.53 ± 10.30	95.73 ± 5.11	13.6 ± 2.6	−0.33 ± 0.46	0.023 ± 0.007

Data are means ± 1SD

BA β-alanine, *PLA* placebo, *RMS* root mean square, M_{max} M-wave area

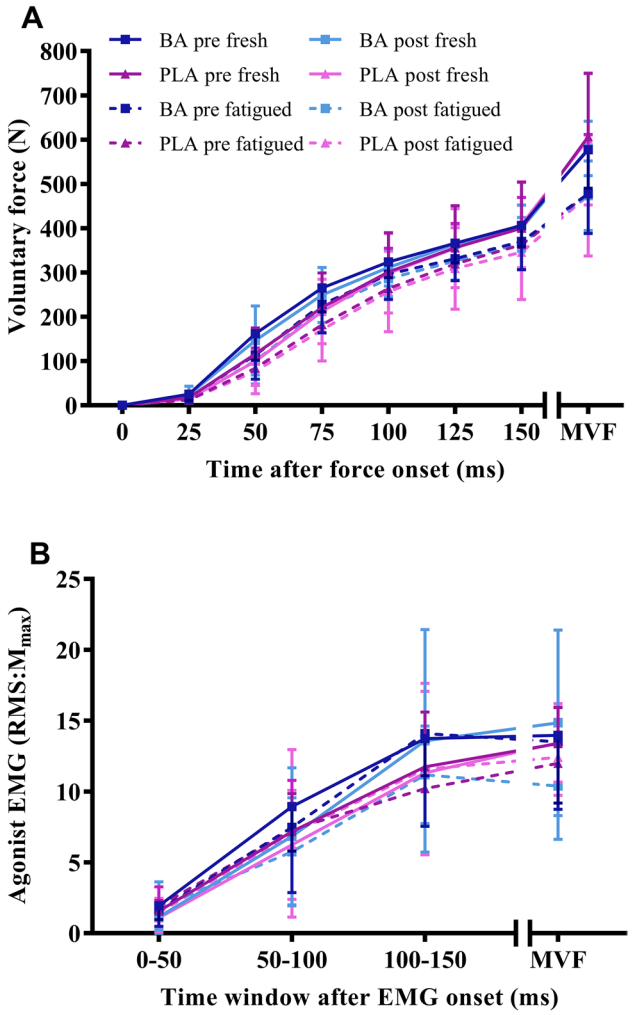

Fig. 3 Explosive and maximal voluntary isometric force (MVIF) responses (**a**), and agonist EMG normalized to M-wave area (M_{max}) during explosive contractions (0–50, 50–100, and 100–150 ms from onset) and at MVIF (**b**) for the BA and PLA groups pre- and post-supplementation, in fresh and fatigued muscles. Data are means ± 1SD

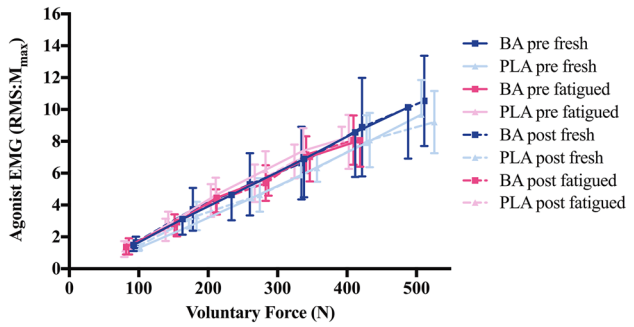

Fig. 4 Force–EMG relationship measured during submaximal voluntary contractions (15–90% MVIF) for the BA and PLA groups pre- and post-supplementation, in fresh and fatigued muscles. Data are means ± 1SD

Sustained isometric knee extensor hold

TTF was unaffected by BA (pre: 63.2 ± 13.0 s; post: 63.4 ± 15.3 s) or PLA supplementation (pre: 77.3 ± 24.8 s; Post: 75.3 ± 18.9 s). Impulse was also not significantly influenced by BA (pre: 16.1 ± 3.5 kN s^{-1}; post 16.4 ± 4.6 kN s^{-1}) or PLA supplementation (pre: 19.6 ± 4.4 kN s^{-1}; post 19.4 ± 3.2 kN s^{-1}). Blood lactate concentrations at rest and prior to the sustained fatigued hold were not significantly different (Table 3). Blood lactate concentrations significantly increased 5 min following the sustained isometric knee extensor hold compared to both rest and prior to values ($P < 0.001$, $\eta_p^2 = 0.8$, $\eta_g^2 = 0.6$), with no difference between sessions or group (Table 3).

Discussion

The key findings from the present study are: (a) no effects of BA supplementation on isometric force production capacity in either fresh or fatigued skeletal muscle, (b) the confirmation of our previous findings (Hannah et al. 2015) showing altered fresh muscle relaxation speed following 28 days of BA supplementation, and (c) that the skeletal muscle relaxation speed is also reduced by BA supplementation following muscle fatigue in the absence of any change to peak force production or contraction time compared to the PLA group. The current investigation examined the influence of BA supplementation on neuromuscular performance measures, associated with Ca^{2+} handling within the skeletal muscle, a proposed mechanism associated with the improvements in exercise performance (Dutka and Lamb 2004; Everaert et al. 2013; Guglielmi et al. 2013; Sale et al. 2013) in fresh and fatigued muscle conditions. These data are the first to comprehensively examine the effect of BA supplementation on voluntary and electrically evoked contractile properties of in-vivo fatigued human skeletal muscle.

During both fresh and fatigued conditions, BA supplementation has no effect on voluntary isometric force production including maximal and explosive force variables. Voluntary force peak data are consistent with the lack of change in electrically evoked peak force responses noted during twitch and octet contractions under both fresh and fatigued conditions. There were similar neural drive responses during both MVICs and explosive contractions pre- and post-supplementation in fresh and fatigued muscle. The current findings in fresh muscle are in-line with the previous findings (Hannah et al. 2015), it was proposed that if BA supplementation had influenced Ca^{2+} related function, improved explosive voluntary force and/or alterations in the force–EMG relationship would have been evident.

Table 3 Blood lactate concentrations (mmol·l^{-1}) for the BA and PLA groups pre- and post-supplementation, at rest, prior to, and 5 min following the completion of the sustained fatigue hold (+5 min)

	Pre-supplementation			Post-supplementation		
	Rest (mmol·l^{-1})	Prior to (mmol·l^{-1})	+5 min (mmol·l^{-1})	Rest (mmol·l^{-1})	Prior to (mmol·l^{-1})	+5 min (mmol·l^{-1})
BA	1.0 ± 0.3	1.1 ± 0.2	3.8 ± 1.1* ^{X}	1.2 ± 0.3	1.3 ± 0.4	3.7 ± 1.3* ^{X}
PLA	1.0 ± 0.3	1.1 ± 0.4	3.8 ± 1.3* ^{X}	1.3 ± 0.3	1.2 ± 0.2	4.2 ± 1.0* ^{X}

Significant differences between concentrations are denoted by * (rest and +5 min) and ^{X} (prior to and +5 min). Data are means ± 1SD

In both fresh and fatigued in-vivo human skeletal muscles, there was no leftward shift of the force–frequency curve, the associated measure of intracellular Ca^{2+} levels (Batrukova and Rubstov 1997). Thus suggesting that elevation of carnosine concentrations did not significantly alter Ca^{2+}-related function. That said, there may have been a decline in sarcoplasmic reticulum Ca^{2+} release which was not documented in the force–frequency curve, due to an associated increase in Ca^{2+} sensitivity of the myofibrils, resulting in the same skeletal muscle force. The current research in fresh muscle is in line with the previous in-vivo research (Hannah et al. 2015), where increased muscle carnosine concentration following a similar 28-day BA supplementation protocol did not alter the force–frequency curve. One potential limitation of both studies is that neither measured intracellular carnosine concentrations directly. That said there are many studies displaying increased muscle carnosine following BA supplementation, almost without exception, on an individual-by-individual basis (Harris et al. 2006; Hill et al. 2007). Thus, we are confident in assuming that a significant increase in muscle carnosine content would have occurred with the BA supplementation protocol implemented in the present study.

The lack of an effect of BA on TPT and force production following increased carnosine concentrations is interesting. Based on the previous in-vitro studies in chemically skinned muscle fibres from frogs (Lamont and Miller 1992), mechanically skinned rat muscle fibres (Dutka and Lamb 2004) and type I and type II human skeletal muscle fibres (Dutka et al. 2012), an alteration to submaximal action potential-mediated force responses via increased Ca^{2+} sensitivity could have been expected. Furthermore, the current investigation reported no alteration to potentiated twitch contractions. Given that the phosphorylation of the myosin head during these twitches is impacted by Ca^{2+} sensitivity, any impact of increased carnosine concentrations would have been displayed as a resultant effect on these measures. In-vitro data following BA supplementation in mice reported increased carnosine (+156%) and anserine content (+46%) in the extensor digitorum longus muscle and a marked leftward shift of the force–frequency relationship (Everaert et al. 2013). These alterations to skinned muscle fibre Ca^{2+} handling when exposed to increased carnosine concentrations are interesting, although both the previous (Hannah et al. 2015) and current in-vivo studies suggest that these responses might not be significant enough to be evident during whole muscle contraction. It might be that the differences between these in-vitro data (where carnosine can be indirectly elevated to a consistent level) and the data from the current study reflect differences in the magnitude of intramuscular carnosine elevation, which we, unfortunately, cannot confirm in the current study. It is also important to note that in-vitro research is conducted outside the normal intracellular environment, and importantly, a number of protocols include the use of free magnesium, an inhibitor of skeletal muscle ryanodine receptors (Laver et al. 2004). Furthermore, during in-vitro studies solutions are added to control pH levels allowing examination of the direct effect of carnosine, although this is important, these investigations have yet to examine the influence of carnosine concentration and varying pH levels. The current body of in-vivo research is completely separate from the in-vitro data, which might make it unrealistic to expect similar findings between research designs.

Increasing muscle carnosine concentrations with 28 days of BA supplementation resulted in a shorter HRT (relative to equivalent PLA times) in fresh and fatigued skeletal muscle during both resting and potentiated twitch contractions. These are in contrast with HRT in fresh and fatigued resting and potentiated twitch contractions following PLA supplementation. The altered muscle HRT values in the current investigation are in-line with those previously reported following the same BA supplementation protocol (7–12%; Hannah et al. 2015). Muscle relaxation speed has been associated with both Ca^{2+} removal from the myoplasm (Ca^{2+} component) and Ca^{2+} dissociation from troponin followed by cross-bridge detachment (cross-bridge component) (Westerblad et al. 1997). Research conducted by Westerblad and Allen (1993), in fatigued mouse muscle fibres, suggested that the slowing of muscle relaxation, apparent under fatigued conditions, was a reflection of slowed cross-bridge kinetics, rather than a reduction in the rate of Ca^{2+} decline at the end of the stimulation train. Yet, when these data were repeated in Xenopus muscle fibres, the slowing of muscle relaxation was associated with a combination of altered cross-bridge kinetics and impaired

Ca^{2+} handling, rather than just slowed cross-bridge kinetics alone. As such, the decline in skeletal muscle HRT following 28 days of BA supplementation shown in the current investigation may be associated with alterations to skeletal muscle cross-bridge kinetics. Alternatively, the decrease in HRT may be associated with the Ca^{2+} component of skeletal muscle relaxation speed, given that it has been proposed that Ca^{2+} re-uptake by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) is the rate-limiting step in muscle relaxation (Gillis 1985; Dux 1993). The transfer of Ca^{2+} into the SR lumen by the SERCA pump is accompanied by a counter-transport of H^+ out into the cytosol (Tran et al. 2009), by acting on Ca^{2+}-handling proteins directly or via other molecules, Ca^{2+} signalling can be inhibited or excited (Swietach et al. 2013). The presence of carnosine has already been shown to improve isolated rat heart muscle contraction and increases free intracellular Ca^{2+} concentrations (Zaloga et al. 1996). At a pH of 6.0, where a complete decline in Ca^{2+} release pump activity was evident, the presence of carnosine maintained ~30% of pump activity at the same pH. Although speculative, these data suggest that increasing carnosine concentrations might alter Ca^{2+}-channel activity by interacting with the Ca^{2+}-channel itself (Batrukova and Rubstov 1997), possibly via the existence of saturable binding site(s) for carnosine on the Ca^{2+}-channel. These data are, however, limited by a number of methodological factors, including but not limited to, the lack of a Ca^{2+} buffer, the overloading of the sarcoplasmic reticulum with Ca^{2+} concentrations approximately ten times greater than normal, and the addition of un-physiological magnesium concentrations. As such, care needs to be taken over the interpretation of these findings. Alternatively, the decrease in HRT may be mediated through improved pH control in the microenvironment of the Ca^{2+} release pump where rapid ATP hydrolysis will result in increased release of protons. There could also be an indirect mechanism to explain the beneficial effects displayed within the current investigation in regards to muscle relaxation. Given the number of other proteins that bind to Ca^{2+}-channels (Berchtold et al. 2000), carnosine may alter the protein interactions with the Ca^{2+}-channels and/or bind with the proteins themselves; both of these mechanisms could influence the activity of the Ca^{2+}-channel via increased carnosine concentrations.

Although the mechanism for reducing skeletal muscle relaxation time following BA supplementation remains unclear, such an outcome might be beneficial to exercise performance, especially during short, repeated muscle contractions where muscle relaxation comprises an important proportion of total energy consumption (Bergstrom and Hultman 1988). During concentric contractions, improvement of muscle recovery time has been shown to be critical to the amount of post-shortening force decrease (Edman 1975). Reducing relaxation rates may improve muscle power output and exercise performance. These findings are particularly important for activities where fast, repetitive contractions, and relaxations occur with no period of rest. Future research is essential to confirm an effect of BA supplementation and/or muscle carnosine accumulation on SERCA activity and to better understand how these isolated muscle effects might relate to repetitive sporting movements and overall performance. It would be of benefit to repeat these data in elite athletes where small changes to HRT might be advantageous. Equally we might speculate that benefits may occur in clinical populations such as Brody disease (where SERCA1 activity is significantly reduced; Guglielmi et al. 2013) or Duchenne Muscular Dystrophy (where an excess of cytosol Ca^{2+}occurs; Ohlendieck 2000).

Within the current investigation, isometric knee extensor fatigue hold times were not significantly influenced by BA or PLA supplementation, in direct contrast to our previous findings that showed a 13.2% (9.7 ± 9.4 s) increase in 45% hold times following BA supplementation (Sale et al. 2012). The reason for a lack of a significant effect in the current study is unclear, given that isometric knee extensor hold times reported by both investigations were similar and aligned to times predicted by the Rohmert equation at a 45% MVIC (78 s; Ahlborg et al. 1972). At 45% MVIC, blood flow is occluded and thus the active muscle fibres are largely dependent upon anaerobic energy provision (Ahlborg et al. 1972). Blood lactate sampled from the finger 5 min post-exercise is indicative of the lower extremity lactate release (Comeau et al. 2011), with groups in the current study displaying similar levels of lactate accumulation in the lower limb. To greater understand the relationship between skeletal muscle HRT, increased carnosine concentrations and muscle fatigue, further investigations implementing a dynamic fatiguing protocol are required, since contractile slowing (i.e., prolonged half-relaxation time) would affect shortening velocity and power output (Jones et al. 2006).

Conclusion

The current investigation showed that 28 days of BA supplementation enhanced muscle relaxation time in both fresh and fatigued skeletal muscle. Whilst this finding is of interest, it remains unclear as to whether it would be sufficient to result in improved exercise performance, particularly in the absence of any changes to the force–frequency relationship, peak force production, or contraction time. The mechanism for the ergogenic effect on muscle relaxation following increased carnosine content remains unclear. It could however, be proposed that Ca^{2+} re-uptake

via direct or indirect mechanisms associated with SERCA pump activity is involved, as this is the rate-limiting step of muscle relaxation.

Author contributions All experiments were performed at Nottingham Trent University (Department of Sport Science). The study was designed as part of a wider research project by RLJ, CTB, RH, WF and CS. Acquisition and analysis of data for the work by RLJ, JD, and BM; data interpretation and manuscript preparation were undertaken by RLJ, CTB, JD, BM, WF, RH, and CS. All authors approved the final version of the paper. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Compliance with ethical standards

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