Dietary Collagen Intake and Sources for Support of Dense Connective Tissues in Athletes

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Bone broth unlikely to provide reliable concentrations of collagen precursors compared with supplemental sources of collagen used in collagen research.	**Four**	Study design, sample preparation, statistical analysis, results interpretation, drafting, reviewing the initial manuscript, manuscript revision. 70%	1. Louise Burke: Study design, results interpretation, drafting, reviewing the initial manuscript, manuscript revision. 12.5% 2. Greg Shaw: Study design, results interpretation, drafting, reviewing the initial manuscript, manuscript revision. 12.5% 3. Marijke Welvaert: Statistical analysis 5%	1) Australian Catholic University 2) Australian sports commission 3) Swimming Australia 4) University of Canberra	Published *International journal of sports nutrition and exercise metabolism*
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No difference in material or mechanical properties of engineered ligaments, treated with serum obtained after the consumption of collagen and dairy proteins, and a media reflective of physiological levels.	**Seven**	Study design, sample preparation, statistical analysis, results interpretation, drafting, reviewing the initial manuscript, manuscript revision. 65%	1. Louise Burke: Study design, results interpretation, drafting, reviewing the initial manuscript, manuscript revision. 10% 2. Greg Shaw: Study design, results interpretation, drafting, reviewing the initial manuscript, manuscript revision. 10% 3. Nicolin Tee: Study design, data collection, drafting, results interpretation, reviewing the initial manuscript, manuscript revision. 15%	1) Australian Catholic University 2) Australian sports commission 3) Swimming Australia 4) University of California, Davis	In preparation for publication

Although minimal, there has been some restructuring of the final accepted versions of the published papers within thesis to ensure consistency in regards to formatting. Where available, the full published papers have been included as appendices.

I hereby certify that the above declaration is a correct declaration of the work contributions.

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Chapter Five: Alcock, RD., Shaw, GC., Tee, N., Welvaert, M., & Burke, LM. RAPID COMMUNICATION: Urinary Hydroxyproline Is Only Suitable As a Biomarker for Acute Intake, Up to 6 hr Post ingestion of Collagen Proteins in "Free-Living," Healthy, Active Males. *Int J Sport Nutr Exerc Metab.* 2019 29(5): 461-465. Doi:10.1123/ijsnem.2019-0024

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Manuscripts in Preparation for Publication

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Thesis Format

The General Introduction **(Chapter One)** provides the background and rationale for the work contained within the thesis. Within this Chapter, there is justification for the specific approach taken within the thesis, guided by an early review of the literature and discussions with experts in the field. Given the novelty of the research topic, a narrative review of the available literature is contained in **Chapter Two** which explores considerations relevant to the topic area and the available literature pertaining to the role of nutrition, specifically the intake of collagen-containing proteins, in the synthesis of collagen within ligaments and tendons. **Chapter Three** consists of the extended methods section and explores the data collection involved in all four experimental studies. **Chapter Four** contains the first experimental study which investigated the variability in the amino acid composition of different sources of bone broth, a food commonly promoted for the health of collagenous tissues, with special attention to those purported to promote collagen synthesis in ligaments and tendons. **Chapter Five** contains the second experimental study which aimed to assess whether a metabolite of proline (hydroxyproline) could be used as a biomarker of collagen intake. **Chapter Six** contains the third experimental study which investigated plasma kinetics of amino acids purported to have a role in the synthesis of collagen following the consumption of various protein sources. The final experimental study; **Chapter Seven** investigated the effects of consuming different protein sources (hydrolysed and non-hydrolysed collagen protein, and a hydrolysed leucine-rich dairy protein) on collagen synthesis using an *in vitro* engineered ligament model modified by the inclusion of a diluted media aimed to meet fasting physiological serum levels. These studies are followed by a general discussion chapter (**Chapter Eight)**, which provides a summary of the findings, the practical implications, limitations and future directions of the research contained within this thesis. **Chapter Nine** contains personal reflections of the

research process and PhD progression and challenges. **Chapter Ten** and **Chapter Eleven** contains references, the candidate's research portfolio and appendices.

Chapters Four, Five and **Six** are presented as they appear in published peer reviewed journals, with reformatting to maintain consistency throughout the thesis. **Chapter Seven** is presented within the manuscript as it has been submitted in the peer-reviewed journal. Due to journal requirements, there may be some language discrepancies (e,g. US vs AUS spelling conventions). A full list of abbreviations, figures and tables are provided prior to **Chapter One.**

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Abbreviations and Nomenclature

^{2}H	Hydrogen 2
^{13}C	Carbon 13
^{14}C	Carbon 14
^{15}N	Nitrogen 15
^{18}O	Oxygen 18
3D	Three-dimensional
AA	Amino acid
ACL	Anterior cruciate ligament
Ala	Alanine
ANOVA	Analysis of variance
Arg	Arginine
Asp	Aspartic acid
AUC	Area under the curve
BB	Bone broth
BL	Baseline
BM	Body mass
Cas	Caseinate
CEAA	Conditionally-essential amino acid
CFI	Collagen fibril index
C_{max} OBS	Observed maximum concentration

CPS	Collagen protein synthesis
Cre	Creatinine
CSA	Cross-sectional area
CTGF	Connective tissue growth factor
CTX	Cross-linked C-telopeptide of type I collagen
D_2O	Deuterium oxide
DDFT	Deep digital flexor tendon
DMEM	Dulbecco's modified eagle medium
EAA	Essential amino acids
ECM	Extra cellular matrix
FSR	Fractional synthetic rate
GAG	Glycosaminoglycan
Gel	Gelatin
GH	Growth hormone
Glu	Glutamic acid
Gly	Glycine
HCas	Hydrolysed caseinate
His	Histidine
Hyl	Hydroxylysine
Hyp	Hydroxyproline
ICTP	Carboxyterminal telopeptide of type I collagen
IGF-1	Insulin like growth factor

IL-6	Interleukin-6
LCL	Lateral collateral ligament
LCol	Liquid collagen
LDET	Long digital extensor tendon
Leu	Leucine
LOX	Lysly Oxidase
LYS	Lysine
MAD	Mass-average diameter
MAPK	Mitogen-activated protein kinase
MCL	Medial collateral ligament
Met	Methionine
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
MTL	Maximum tensile load
mTOR	Mammalian target of rapamycin
NEAA	Non-essential amino acids
NTX	N-terminal crosslinked telopeptide of type I collagen
Pep	Peptide
PCL	Posterior collateral ligament
Phe	Phenylalanine
PICP	Procollagen I Carboxyterminal propeptide
PINP	Procollagen I Amino-terminal propeptide

Pro	Proline
PT	Patella tendon
RM	Repetition maximum
SDFT	Superficial digital flexor tendon
Ser	Serine
TAA	Total amino acids
Tau	Taurine
TGF-β1	Transforming growth factor beta-1
Thr	Threonine
Tyr	Tyrosine
UTS	Ultimate tensile strength
Val	Valine

Abstract

Sporting success is determined by a multitude of factors and their interactions. Physical function is a major determinant of the athlete's ability to train optimally and with consistency, as well as to achieve the work requirements of their competitive event. Ligaments and tendons are types of dense connective tissue which play a major role in locomotion and stability. These tissues are at risk of being injured, interfering with the athlete's training and competition availability. Furthermore, due to their slow rate of turnover, the management of injuries to these tissues requires a significant rehabilitation period at the expense of both the athlete and/or sporting team. Practical strategies that reduce the injury risk by enhancing the strength of these tissues and/or lessen the rehabilitation period by enhancing the healing process are of understandable interest to the sporting community as well as other populations. However, determining the impact of nutritional interventions on whole body collagen synthesis provides considerable challenges, including the lengthy intervention period needed to observe meaningful changes in the structure and function of ligaments and tendons. Additionally, the many gaps within the current literature prevent researchers from identifying candidate treatments that would justify the necessary levels of investment, time and resources needed to undertake such a study. Consequentially, this thesis aimed to gather background data and address methodological considerations that would assist in the future implementation of a well-designed intervention study. Specific questions were identified:

- Which food sources and/or supplements are most likely to provide precursors that support the synthesis of new collagen?
- Can a purported biomarker of the intake of these precursors be validated as tool to assess recent or habitual intakes of collagen-promoting foods and assist epidemiological or intervention studies in this area?

- What are the key characteristics of the absorption and digestion after the consumption protein sources proposed to be implicated in the synthesis of collagen?

The first study in the series **(Study 1)** sought to determine whether bone broth, a presently "*in vogue*" source of collagen protein, is able to provide adequate, and consistent levels of the amino acids (AAs) purported to support the synthesis of new collagen. A variety of "standardised" and "non-standardised" preparations of bone broth was prepared and/or obtained. The main outcome of this study was that AA concentrations in bone broth were too inconsistent to provide a therapeutically beneficial amount of the key AAs of interest.

The second study **(Study 2)** aimed to determine whether urinary excretion of hydroxyproline, a component of collagenous tissue, is suitable as a biomarker of collagen intake in free-living individuals. However, this biomarker was only present within the urine for up to 6 h after the consumption of a 20 g serve of collagenous protein and was not substantially elevated through usual dietary intake. These findings, in conjunction with the large inter-individual variability in urinary hydroxyproline concentrations, underpin the conclusion that it is an unsuitable biomarker of habitual dietary intake of collagen.

Ligaments and tendons are poorly vascularised tissues, which may require exercise-induced stimulation of blood flow to transport nutrients such as AAs that may be implicated in collagen synthesis. Exercise may provide a further integral component of this process by upregulating the machinery for protein synthesis. Therefore, the intake of nutritional support for collagen synthesis must be coordinated so that peak blood concentrations of AA coincide with exercise-induced optimisation of tissue perfusion and activation of synthetic machinery. This

necessitates an understanding of the AA responses to intake of different collagen protein sources. **Study 3** involved obtaining serial plasma samples from individuals over the three hours following the consumption of a 20 g serve of various protein sources (including both collagen and dairy proteins), and a 300 mL serve of bone broth. The main finding was the variability in the timing and magnitude of peak concentrations of various AAs according to the type of protein that was consumed. Specially, collagen-containing proteins provided the highest source of glycine, whilst dairy proteins provided the highest source of leucine. Meanwhile, although hydrolysed forms of dairy proteins produced higher peak concentrations of AAs than their non-hydrolysed counterparts, this was not the case for the collagen proteins.

The final study within the series **(Study 4)** built upon previous *in vitro* work by our group (Shaw et al., 2017) in which a 15 g dose of gelatin was found to increase the content and mechanical properties of collagen in an engineered ligament model. Information on the timing of plasma AA peaks gained from prior studies in the thesis informed when the blood samples would be collected for treatment of engineered ligaments. A further addition to the methodology involved the manipulation of the media used to prepare the ligaments to more closely reflect physiological concentrations of AA in fasted individuals. While we failed to find an effect of the treatments, this study facilitates further questioning around key methodological considerations of this model, and highlights the challenges of undertaking research on ligaments and tendons.

In summary, this body of work identified supplementation protocols that merit investigation for the support of collagen synthesis and the health/functioning of dense connective tissues and established some key considerations for use in future research.

Chapter One: Introduction, Aims and Overview

Participation in physical activity and/or sport carries a number of benefits including lowering the risk of chronic disease, improved mental health, improved tissue strength and improved injury protection (Warburton et al., 2017). In children and/or adolescents, physical activity plays an integral role in coordination, balance, flexibility, tissue development and social wellbeing (Janssen et al., 2010). In later life, the maintenance of tissue strength is particularly important given the age related declines in muscle mass (i.e. sarcopenia), which can contribute to an increased risk of falls, resulting in a significant amount of morbidity and mortality (Siracuse et al., 2012). Physical activity is therefore important throughout all life-stages and is a necessary consideration for the maintenance of health and general wellbeing. For the select few (e.g. elite athletes), participation in sport is a profession and their predominant source of personal income, which further supports a much larger group of other people employed within the sports industry. Although there is an obvious benefit to participation in sports both for recreational and elite populations, participation in sport comes with risk. Within Australia, as at 2011-12, soft tissue (e.g. muscle, tendon and ligament) injuries were the second most common type of injury sustained (AIHW: Kreisfield et al., 2011-12). In regard to dense connective tissues specifically, a recent study over a 5 year period reported that Australia had one of the highest incidences of ACL reconstructions globally (Janssen et al., 2012). Meanwhile, the number of primary ACL reconstructions in young Australians increased by 43% from 2000-01 to 2014-15 (Zbrojkiewicz et al., 2018). The direct monetary cost of ACL reconstruction surgery as at 2014-15 was estimated to be $142 million, with further indirect costs such as income lost due to time off work, and the future development of chronic disease such as osteoporosis (Zbrojkiewicz et al., 2018). More widely, it is estimated that 5.2 million Australians sustain sport injuries each year, with an increasing monetary cost, last estimated $2 billion per year in 2005 (Kreisfeld et al., 2014).

Connective tissues are the most widely abundant and diverse set of tissues present within the human body and are involved in a vast array of functions ranging from organ support to the transportation of blood and nutrients (Ovalle et al., 2013). Ligaments and tendons are a particular type of dense, regular connective tissue, predominantly responsible for stability and locomotion, and when functioning optimally, also play an important role in injury prevention (Birch et al., 2013). Specifically, ligaments connect bone to bone and prevent abnormal movement occurring within the joints, while tendons connect muscle to bone, and are responsible for the transmission of force from the muscle to the bone, thereby facilitating movement (Birch et al., 2013). Although ligaments and tendons have distinct roles, they are structurally similar tissues with a composition dominated by collagen protein (principally Type I), followed by elastin, proteoglycans and binding proteins (Kjær, 2004). Collagen is the key tensile element that contributes to the strength and structure of these tissues, allowing for the resistance of tensile, compressive and shear forces (Muiznieks et al., 2013). Research suggests that tissues with larger and/or more densely packed collagen fibrils have a greater ability to withstand force, thereby providing better protection against injuries such as tendinopathies and ligament tears (Galloway et al., 2013; Magnusson et al., 2016). By contrast, conditions which cause a reduction in collagen synthesis and/or disruptions to collagen processing (e.g. heritable disorders of connective tissue or genetic polymorphisms), result in weak tissues prone to injuries such as fractures, tendon ruptures and ligament tears (Grahame, 2000; Vaughn et al., 2017). Although these disorders are uncommon, amongst healthy individuals, current literature suggests that smaller tendons, presumably with a lower collagen content and/or disruptions to mechanical properties are accompanied by a higher injury risk (Arya et al., 2010; Couppe et al., 2013; Kongsgaard et al., 2010), thus illustrating the importance of collagen for the optimal health and functioning of dense connective tissues.

Contrary to the historical belief that ligaments and tendons are relatively inert tissues with little capacity for hypertrophy post puberty, literature suggests that the collagen protein within ligaments and tendons share the characteristics of myofibrillar protein, in being responsive to exercise, which results in distinct changes to morphological, material and mechanical properties (Tardioli et al., 2012; West et al., 2015; Wren et al., 2000). Indeed, it has recently been shown that basal protein synthetic rates within tendons and ligaments, are within the same range as skeletal muscle protein synthetic rates at 0.06 ± 0.01%/h, 0.04 ± 0.01%/h and 0.04 ± 0.01%/h, respectively (Smeets et al., 2019). While researchers are endeavouring to establish the key mechanisms responsible for the biochemical response of mechanical load to ligaments and tendons, others have focused on the potential supportive role that nutrition may play in the synthesis of new collagen (Scott et al., 2016; Tack et al., 2018). Despite the lack of a clear evidence base, several recommendations have surfaced with the aim of minimising injury risk in athletes and/or assisting their "return to play". These include the pre-exercise consumption of protein derived from collagenous animal tissue to enable the provision of key AAs to the target tissues at a time when blood flow is optimised and synthetic machinery is upregulated (Baar, 2017). Even though such recommendations are appealing to both the athlete and their support team, a systematic process of developing such recommendations (such as that outlined in Figure 1.1) is needed. Thus, the initial aim of the thesis was to work through a progression of "steps" to bridge any identified gaps with the end goal of implementing a valid and reliable intervention study to consolidate current recommendations. In particular, we wanted to explore considerations, such as AA availability, and the impact of the ingestion of various proteins (including both whole foods and supplemental sources) on collagen synthesis *in vitro* prior to implementing an *in vivo* study. However, due to early investigations, supported by discussions with experts in the field, a number of challenges were identified in tackling the full scale of this model. For example, there is currently no consensus on a valid, reliable, economically

feasible and ethical approach to measure the synthesis and incorporation of collagen into ligaments and tendons and the functional outcomes of this increased collagen content (Phase 5), reducing our confidence in the likelihood of being able to identify whether any intervention would achieve real-world benefit. Because of the need for a lengthy intervention period for a final study (Phase 6), requiring significant resources and effort from a research team, and a high level of interest and/or compliance of the subject population, it was deemed unethical, or at least wasteful, to embark on a study with minimal chance of achieving the desired endpoint.

In addition to the current lack of consensus on the optimal dietary interventions that are important for collagen synthesis (Phase 1 and 2), we recognised that the poor vascularisation of ligaments and tendons created a need to interrogate whether key dietary factors are able to reach the target tissue before implementing a chronic intervention (Phase 4). Preliminary *in vitro* data, produced by members of our research group, suggested both a rationale for interest in the topic and a working model for Phase 3 of the systematic series of studies. However, the invasiveness and required expertise involved in conducting the micro-dialysis or biopsy techniques needed to confirm whether any favourable conditions *in vitro* can be achieved *in vivo* placed further emphasis on the need to quantify dietary sources of potentially important precursors to collagen synthesis. On this basis, the current research program undertook a deliberate focus on the first steps (Phases 1-3) in the development and validation of dietary strategies to improve the health and function of collagenous tissues. By providing a robust interrogation of the early phases of this pathway, this program aimed to create a foundation of information that others could leverage to continue further investigation into the role of nutrition support for collagen synthesis in ligaments and tendons. The research program underpinning this thesis was undertaken to address the following questions:

- Can a putative food source of collagen precursors, currently being used by athletes and/or their support teams for injury prevention/treatment, provide a proposed therapeutic dose of AAs?
- Is urinary hydroxyproline, a previously identified biomarker of collagen intake, a valid and reliable option to assess the dietary intake of collagen in free-living individuals, which can be used to enable greater rigor in implementing acute or chronic interventions utilising dietary and/or supplemental forms of collagen (or epidemiological approaches to the effect of dietary collagen sources on the health and function of collagenous tissues)?
- What are the key kinetics around the intake of protein sources proposed to be involved in collagen synthesis which will inform consumption protocols to coincide with exercise (e.g. when synthetic machinery is upregulated and tissue perfusion is supported)?
- Can an *in vitro* model, reflective of physiological conditions, provide evidence that the consumption of specific proteins is of benefit to the synthesis and function of a collagenous tissue?

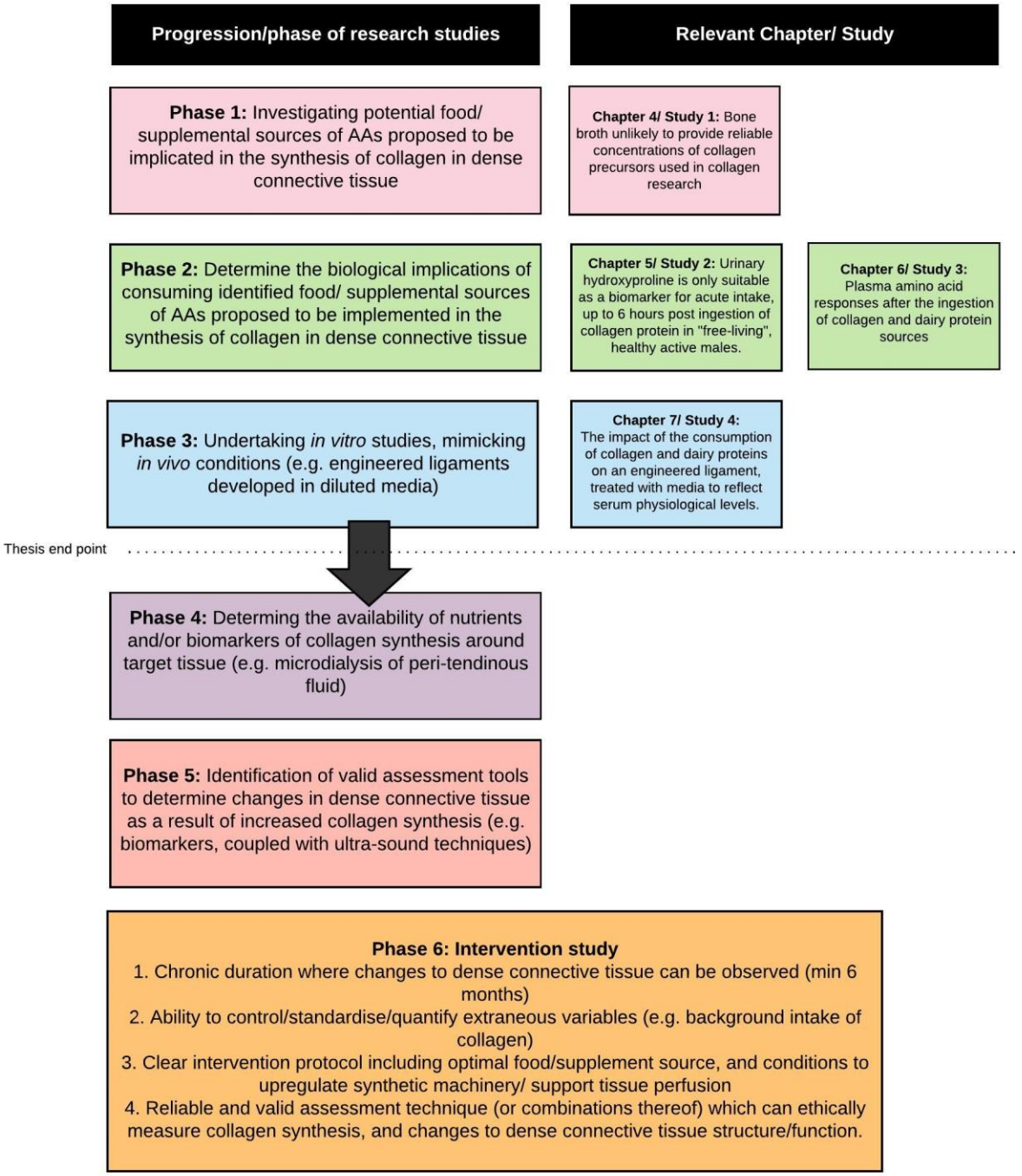

Figure 1.1: Theoretical model of studies required to optimise nutrition support for collagen synthesis in ligaments and tendons in athletes.

Chapter Two: Literature Review

2.1 Preface

The following literature review will summarise the function and structure of ligaments and tendons before moving to a discussion of the role of nutrition in their health and function. Themes that will be covered include: the importance of collagen protein; the biosynthesis of fibril-forming collagen; challenges in the measurement of collagen turnover, and the role of exercise in the synthesis of collagen. In addition, sex differences for connective health will be discussed, which ultimately led to the decision to exclude female participants from the ensuing data collection. Finally, the last section of the literature review will include the potential sources of collagen precursors in the Australian diet, and the background measurement of collagen intake. Whilst it is acknowledged that there are several nutrition strategies that may be associated with connective tissue repair (Curtis, 2016), including the provision of protein to prevent tissue atrophy (Berg et al., 1992; Farup et al., 2014) and the adequate intake of vitamins A and D (Berg et al., 1992), this literature review focused predominantly on nutritional strategies aimed to increase collagen synthesis within ligaments and tendons. Given the novel subject area and the availability of literature, the review will take on a narrative format, and include studies undertaken in animals and cell-culture models, where relevant. It is acknowledged that there are considerable differences in collagen turn-over between species (e.g. higher rates of growth in rodents), and challenges in the transferability of cell-culture models to *in vivo* outcomes (which will be further discussed in section 2.6.3). The literature included in this review was sourced from a number of databases including, but not limited to: PubMed, Medline via Ovid, and SPORTDiscus. Keywords used included: ligament, tendon, dense connective tissue, nutrition, collagen, amino acids, peptides, collagen synthesis.

2.2 Background

Although ligaments and tendons are predominantly composed of water, the majority of the dry weight of these tissues is made up of the structural protein, collagen (Magnusson et al., 2016). Collagen is the key tensile element in ligaments and tendons, and is responsible for the considerable strength that is necessary for their roles in locomotion and the stability of surrounding tissue (Gelse, 2003). The dominant type of collagen, known as fibril-forming collagen (principally type I), is composed of non-essential AAs (NEAA); chiefly proline and glycine (Kadler et al., 1996). Given its importance in the health and functioning of ligaments and tendons, there is an obvious interest in factors, including nutrition that may assist in collagen synthesis and integration within these tissue types. Skeletal muscle is often used as a reference point for the emerging interest in collagen health; indeed, there is considerable research on nutrition and myofibrillar protein synthesis (Stokes et al., 2018), and in particular, the pivotal role of essential AAs (EAA), particularly leucine (Dodd et al., 2012). However, similar research on dense connective tissue is made difficult by the challenges of measuring collagen turnover and incorporation into the insoluble pool of collagen in ligaments and tendons (Smith et al., 2007). Unlike other major collagen containing tissues in the body (e.g. skin, and bone), ligaments and tendons are notoriously slow to turnover, requiring a lengthy intervention period in order to detect any meaningful changes (Babraj et al., 2005a; Babraj et al., 2005b; Shoulders et al., 2009; Smith et al., 2007). Additionally, it remains difficult to determine actual changes to the turnover and integration of collagen in these tissues without causing long term damage, which may be considered both unethical and unfeasible in athletic populations (Movin, 2000). Finally, lengthy and difficult intervention studies may be considered superfluous to these population groups as some experts consider ligaments and tendons inert tissues with little capacity to turnover post puberty (Heinemeier et al., 2013).

Despite these challenges, a number of studies have provided insight into the role of exercise in the synthesis of collagen, and have established that ligaments and tendons are indeed dynamic tissues that respond to loading with a change in both material and mechanical properties (Kjaer et al., 2015; Kjaer et al., 2009). This process is driven by "mechanotransduction", a signalling cascade that occurs in response to a physical stimulus on the tissue, resulting in a biochemical milieu that enhances the synthesis of collagen (Chiquet et al., 2009; West et al., 2015). In addition to its role in the upregulation of collagen synthesis, exercise is also thought to enhance blood flow to these tissues which are otherwise poorly vascularised, thus assisting in the availability of nutrients when synthetic machinery is "turned on" (Bray et al., 2002). These issues may help to explain why previous studies have shown that nutrition alone appears unable to meaningfully increase collagen synthesis in tendons and ligaments (Babraj et al., 2005a).

While emerging research on the potentially supportive role of nutrition in the synthesis of collagen is still predominately limited to cell-culture work and/or animal models, it is speculated that, similar to other protein fractions, key AAs may have both a stimulatory function and provide the framework for collagen protein to be incorporated into ligaments and tendons (Baar, 2017). In response to the extensive study of skeletal muscle tissue growth, initial studies of nutrition interventions and changes to the material properties of ligaments and tendons have followed a similar interest in the potential role of leucine in the synthesis of collagen (Barbosa et al., 2012; Dideriksen et al., 2011; Kato et al., 2016). However, the available literature suggests that, in a fed state, leucine intake beyond that of dietary sufficiency does not appear to have any additional benefit (Barbosa et al., 2012; Kato et al., 2016). Furthermore, these findings may be confounded if an increase in muscle tissue in response to a leucine intervention increases the physical stimulus to neighbouring tendons and ligaments, creating an indirect increase in the synthesis of their collagen protein content (Miller et al.,

2005). Therefore, although leucine has strong physiological anabolic, and anti-catabolic effects on muscle tissue, which likely influences the synthesis of collagen in ligaments and tendons, its direct influence on collagen protein in ligaments and tendons remains inconclusive.

More recently, attention has been focussed on the role of non-essential amino acids (NEAA) in the synthesis of collagen, particularly when combined with an appropriate exercise stimulus (Baar, 2017). Although these AAs are considered, by definition, "non-essential", they are known to be the predominant AA within collagen protein. Furthermore, studies have suggested that NEAA may be required in higher amounts when endogenous production is inadequate to meet increased needs, such as during wound healing or where there are higher rates of turn-over (Meléndez-Hevia et al., 2009; Mussini et al., 2017; Stechmiller et al., 2005). It is possible that athletic populations have a higher turn-over rate of collagen protein within ligaments and tendons compared to their sedentary counterparts due to the effect of consistent high-level exercise. Furthermore, as injury to these tissues is common among sporting populations and is often accompanied by surgery, there may be an increased requirement for NEAAs during post-operative and/or rehabilitation periods. Whilst it is tempting to fortify individual AAs into the diet, the safety of this in humans is questionable, and may lead to AA imbalances, which has been shown in animal models to lead to adverse effects (Kurpad, 2018).

Despite the potential benefits of collagen intake on the health/function of connective tissues, a lack of information on the collagen content of foods within the Australian food supply prevents the ability to quantify the dietary intake of collagen-derived proteins within various populations. It is speculated that it is likely to have reduced in the modern era, given current consumer preferences for lean meats, void of collagenous tissues (e.g. cartilage and bone) (Williams et al., 2010; Wong et al., 2013). However, some subgroups within the community

potentially consume greater amounts of dietary collagen sources; this includes followers of the Palaeolithic diet for whom bone broth is a staple food choice because of its purported collagen content and alleged support for collagenous tissues in the body (MacSharry, 2012). In addition, collagen supplements are commercially available and are being marketed at athletes for the same purpose. These products include hydrolysed collagen formulas containing di- and tri-amino acid sequences (e.g. Pro-Hyp and Gly-Pro-Hyp). It has been suggested that these peptides have physiological functions beyond that of the intact or partially hydrolysed protein, and are able to reach the target tissue in their peptide form in a more rapid and pronounced manner (Aito-Inoue et al., 2007; Ichikawa et al., 2010; Iwai et al., 2005; Matsuda et al., 2006; Schunck et al., 2013). Such formulations have been shown to produce benefits to a range of collagen containing tissues with various outcomes including reduced inflammation, reduced tendon/joint pain and improved body composition within the elderly (Bello et al., 2006; Clark et al., 2008; Dressler et al., 2018; Oertzen-Hagemann et al., 2019; Oesser et al., 2016; Praet et al., 2019; Schunck et al., 2013; Zdzieblik et al., 2015). Although manufacturer claims promote these formulas as being more effective at increasing collagen synthesis than intact or partially-hydrolysed collagen proteins, these claims have not yet been substantiated within the literature.

2.3 The Function and Structure of Ligaments and Tendons, and the Importance of Collagen

Collagen is the most widely abundant protein present in the human body, accounting for ~ 1/3 of total body protein (Shoulders et al., 2009). Acting as the key tensile element of tissues, it contributes to strength and rigidity, and is a regulator of mechanical forces (Birch et al., 2013). Whilst 28 different types of collagen are currently known, type I, a fibril-forming collagen, is the main type present in the extra-cellular matrix (ECM) of ligaments and tendons (Shoulders et al., 2009). Furthermore, mechanical properties of ligaments and tendons, which are heavily

influenced by the degree off cross-linking within collagen fibrils (Magnusson et al., 2016), provide another important feature (Hansen et al., 2006; Muiznieks et al., 2013). Indeed, cross-linking of collagen molecules gives rise to tissues that are considerably strong and stable, as is necessary given their role in locomotion and stability of surrounding tissues (Rumian et al., 2007). Specifically, tendons are responsible for the transfer of force from the muscle to bone, enabling movement (Birch et al., 2013), while ligaments connect bone to bone and prevent excessive movement occurring within joints (Frank, 2004). The extracellular matrix (ECM) of dense connective tissues plays a pivotal role in their load bearing capacity (Chiquet et al., 2009). Within the ECM of ligaments and tendons there are different quantities of proteins, elastin, glycans and water, which is also distinctive to the tissue location (Table 2.1). Despite these differences, collagen protein predominates in all tissue types, accounting for ~55 – 80% of their dry weight (Rumian et al., 2007).

Table 2.1: Matrix composition differences within ligaments and tendons. Mean ± SD (n=3) Adapted from Rumian et al. (2007).

	CFI (%)	MAD (nm)	Water (% Total Weight)	GAG (µg/mg Dry Weight)	Collagen (% Dry Weight)
LCL	65.5 ± 3.9	126 ± 17	63.1 ± 1.0	5.1 ± 1.6	68.6 ± 5.3
MCL	67.8 ± 3.0	157 ± 22	59.9 ± 1.8	4.3 ± 0.6	71.4 ± 7.9
PCL	67.6 ± 2.9	197 ± 13	67.7 ± 0.7	34.3 ± 3.2	55.6 ± 4.3
ACL	67.1 ± 2.5	181 ± 14	64.6 ± 1.0	7.4 ± 1.3	58.9 ± 4.1
LDET	76.3 ± 2.6	187 ± 16	56.8 ± 1.0	6.2 ± 2.5	73.2 ± 3.6
SDFT	77.6 ± 2.7	240 ± 6	56.2 ± 1.0	1.7 ± 0.3	78.8 ± 13.4
PT	72.9 ± 3.6	202 ± 7	53.1 ± 0.8	2.3 ± 0.3	73.7 ± 1.4

Collagen Fibril Index (CFI); Mass-Average Diameter (MAD); Glycosaminoglycans (GAG); Lateral Collateral Ligament (LCL); Medical Collateral Ligament (MCL), Poster Cruciate Ligament (PCL), Anterior Cruciate Ligament (ACL), Long Digital Extensor Tendon (LDET), Superficial Digital Flexor Tendon (SDFT), Patellar Tendon (PT).

2.4 The Biosynthesis and Formation of Fibril-Forming Collagen

Fibroblasts are the major cells involved in the production of collagen within ligaments and tendons (Gelse, 2003). The biosynthesis of collagen is a complex, multi-step process (outlined in Figure 2.1), and commences with the typical process of gene transcription and translation within the cell nucleus of the fibroblast, before being transferred to the rough endoplasmic reticulum. This results in a pre-procollagen molecule with repeating units of Gly-X-Y; whereby Gly is occupied by glycine, while X and Y can be occupied by any other AA acid but generally consist of proline and hydroxyproline, respectively (Ramshaw et al., 1998). These pre-procollagen molecules then undergo hydroxylation of proline and lysine residues by the enzymes prolyl 3-hydroxylase, prolyl 4-hydroxylase and lysl hydroxylase. This is the first enzymatically-derived cross-linking process which utilises ascorbic acid (vitamin C) as a key co-factor (Gelse, 2003). The hydroxylated pre-procollagen molecules are then glycosylated with the addition of a sugar and disulphide bonds are formed. The pre-procollagen strands then wrap around each other to form a triple helical structure, through hydrogen bonding. The resulting molecule, known as procollagen, has the terminal ends: procollagen N terminal propeptide (PINP) and procollagen C terminal propeptide (PICP). The assembled procollagen chains are excreted into the ECM where they are cleaved at the terminal ends by N and C procollagen peptidases to form tropocollagen. Once this process is complete, collagen fibril assembly commences involving the maturation of the collagen molecule through covalent bonding, a copper-dependent hydroxylation of lysine residues utilising the key enzyme lyslyl oxidase (LOX) (Gelse, 2003). Finally, collagen molecules assemble in a hierarchical manner to form fibrils (~10-300 nm in diameter), then fibres (~1-20 μm), and may further associate into larger bundles (up to 500 μm) (Muiznieks et al., 2013).

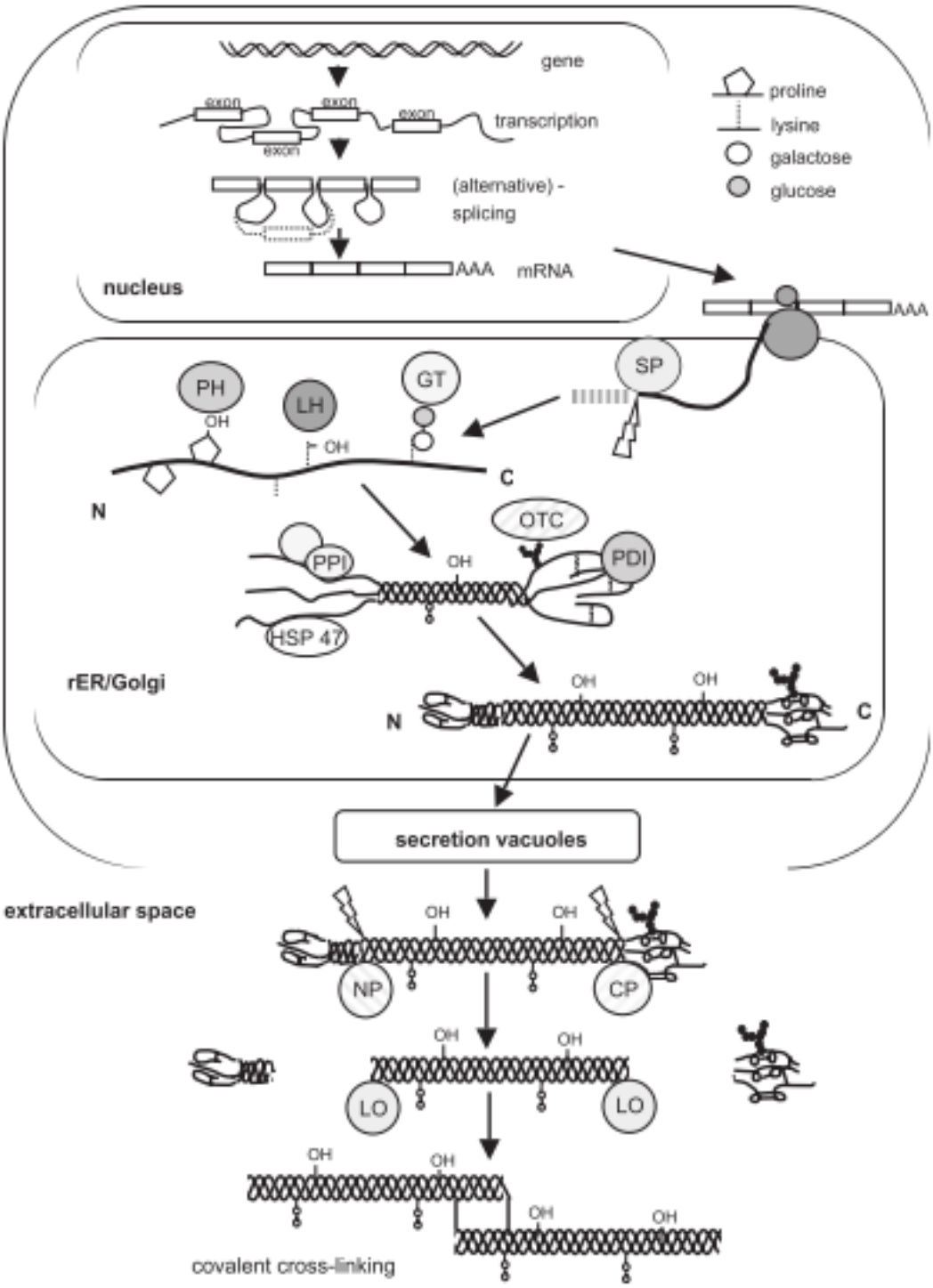

Figure 2.1: Schematic representation of collagen synthesis starting with transcription and translation within the fibroblast, followed by post-translational modifications, secretion, and finally extra cellular fibril formation. Source: Gelse, K., 2003.

2.5. Sex Differences for Connective Tissue Health

Women are said to be up to 10 times more likely to sustain an ACL rupture than men (Arendt et al., 1999; Gwinn et al., 2000). Although there remains no definitive consensus on factors that contribute to this increased risk, dynamic movement patterns appear to be the main contributing factor (Stijak et al., 2015). However, several studies have shown an association between the menstrual cycle phase and injury risk. It has been illustrated that the ACL is an androgen responsive tissue (Lovering et al., 2005), and although not yet fully understood, several studies have shown that fluctuations in circulating sex hormones such as progesterone, oestrogen and testosterone may play either a protective or non-protective role (Beynnon et al., 2005; Chidi-Ogbolu et al., 2018; Lee et al., 2015; Lovering et al., 2005; Stijak et al., 2015). It has been suggested that an increase in oestrogen results in an increased tissue laxity, which in turn increases knee displacement leading to a higher risk of injury (Beynnon et al., 2005; Chidi-Ogbolu et al., 2018). In a recent study by Lee et al. (2015), utilising a cell culture model, it was shown that oestrogen inhibited the activity of LOX, without changing LOX expression or the amount of collagen present within the graft (Lee et al., 2015). Given the role of LOX on the cross-linking of collagen molecules (section 2.4), this was found to reduce the mechanical function of the engineered ligaments (Lee et al., 2015). Although the increased risk of connective tissue injury in women and the influence of sex hormones on connective tissue health presents an important area for future investigations, there remains a lack of consensus within this area (Ireland, 2002; Stijak et al., 2015). Nonetheless, it can be said that differences in the hormonal milieu due to the menstrual cycle phase or contraceptive pill use, is a confounding factor in research exploring the impact of nutritional interventions on dense connective tissue health/performance, and as such it was decided to exclude females from the ensuing studies.

2.6 Challenges Associated with the Measurement of Collagen Turnover in Ligaments and Tendons

Presently, there is no "gold-standard", non-invasive technique to directly measure collagen metabolism within human dense connective tissue. Therefore, research within this particular field remains challenging. Furthermore, considerations such as the time period required for the turnover of ligaments and tendons in order to illustrate measurable change needs to be considered for reasons such as compliance, budget and participant/staff burden. As a result, researchers often use an assortment of techniques, including a number of direct and indirect approaches, dependant on the research question, the time and budget available, and the skill level of the research team (Smith et al., 2007). The following section will discuss a number of approaches currently utilised to measure collagen metabolism within human dense connective tissue. The challenges and/or considerations will be discussed, and are summarised in Table 2.2. Direct approaches will include those which determine the fractional synthetic rate (FSR) of collagen protein fractions within the target tissue utilising biopsy (Smith et al., 2007; Wilkinson et al., 2015; Wilkinson et al., 2014). Indirect approaches will include markers of collagen synthesis and breakdown within bodily fluids (e.g. procollagen monomers), and morphological changes measured through imaging techniques such as magnetic resonance imagining (MRI) and ultrasound (Hodgson et al., 2012). A more recent development, which utilises an *in-vitro* model to replicate human *in-vivo* conditions (3D cell culture) will also be discussed (Hagerty et al., 2012; Handsfield et al., 2017; Paxton et al., 2010; Paxton et al., 2012; Shaw et al., 2017).

2.6.1 Direct measures of collagen synthesis

Currently, tissue biopsy is the only direct way to measure collagen synthesis and incorporation within various collagen containing tissues in humans (Smith et al., 2007). The incorporation of isotopically labelled AAs (e.g. AAs containing ^{2}H, ^{18}O, ^{15}N or ^{13}C) into the tissue has traditionally been utilised to measure the FSR of various protein fractions in a range of musculoskeletal tissues (Babraj et al., 2005a; Babraj et al., 2005b; Dideriksen et al., 2013; Dideriksen et al., 2011). However, this technique has been criticised for its complex experimental procedure, required including bed rest, intravenous cannulation and the use of expensive tracers. Furthermore, the technique is limited in its ability to track changes in protein synthesis and AA incorporation over periods greater than 12 hrs. A time frame which may fail to truly capture the slow and transient nature of collagen synthesis in dense connective tissue (Movin, 2000; Wilkinson et al., 2015).

More recently, orally administered deuterium oxide (D_2O), in combination with biopsy, has been utilised to quantify the day-to-day metabolism of multiple protein fractions (Wilkinson et al., 2014), and acute measures of MPS in as little as~3 h (Wilkinson et al., 2015). Consumed orally, D_2O equilibrates rapidly in body water (~1-2 h), which creates a homogenous labelled precursor pool with a slow-turnover rate (half-life of ~9-11 days). The deuterium is exchanged with hydrogen and can be incorporated into metabolic pools and tissues, and thus provides a way to measure the FSR of various protein fractions (Wilkinson et al., 2017). This technique is less invasive, more cost effective, can be utilised in “free-living” individuals and can assess changes in collagen synthesis over periods > 12 h, thus being able to determine more chronic outcomes of interventions than isotopically labelled AAs (Wilkinson et al., 2015; Wilkinson et al., 2014). Despite advancements to this technique, biopsy remains an invasive protocol and may not be appropriate in certain populations, such as athletes who cannot afford the down-

time associated with such a procedure. Finally, the site of measurement may impact on the findings. For example, a recent study investigating levels of the 14-carbon isotope (^{14}C) remaining in the core of the tendon in individuals born during periods of high atmospheric levels (~ 20 – 40 years ago, following the explosion of atomic bombs) led to the belief that such tissues do not turnover after pubertal development (Heinemeier et al., 2013). However, given that there is substantial evidence to suggest that ligaments and tendons are in fact dynamic and respond to multiple exercise modalities (see section 2.7.), others have suggested that ligaments and tendons grow at other regions of the tissue (e.g. periphery, proximal and/or distal ends) and therefore a biopsy of the tissue core may not appropriately reflect these changes (Kjaer et al., 2015; Mackey et al., 2008).

2.6.2 Indirect measures of collagen synthesis

There are several biomarkers that can be obtained from biological fluids to indicate collagen turnover. Collagen monomers (e.g. PINP and PICP), resulting from the formation of tropocollagen (see section 2.4), are commonly utilised to measure collagen synthesis (Koivula et al., 2012; Smith et al., 2007). Meanwhile, type I collagen fragments (e.g. Cross-linked C-telopeptide of type I collagen and Cross-linked C-telopeptide of type I collagen: ICTP and CTX), derived during the process of cross-linking to stabilise collagen fibrils, have been used to indicate collagen breakdown (Eyre et al., 2008; Smith et al., 2007). Finally, hydroxyproline, a metabolite of proline, unable to be re-incorporated into newly synthesised collagen, may also be utilised to measure collagen breakdown (Prockop et al., 1961). While these techniques offer useful information with regard to the turnover of collagen protein, there are several considerations in utilising these measures. First, it has been suggested that there are two "pools" of collagen including an immature, soluble, fast pool, and an insoluble, mature, slow-turnover pool. The immature pool may degrade prior to maturation and therefore never fully incorporate

into the tissue, thus overstating the impact of an increase in collagen synthesis (Smith et al., 2007). To overcome this issue, the measurement of markers of both collagen synthesis and breakdown may be warranted. Although this does not conclusively determine whether newly synthesised collagen is indeed incorporated into the target tissue, it allows for consideration of whether there is a net change in collagen metabolism within the body. However, it is also stated that serum measures are more likely indicative of bone collagen turnover given that bone has a higher rate of turnover (Eyre et al., 2008; Koivula et al., 2012). In order to overcome uncertainty around metabolic fates, obtaining dialysate from directly around the target tissue (e.g. peritendinous fluid) may be useful (Langberg et al., 2001; Langberg et al., 2000; Henning Langberg et al., 1999). However, this technique is also limited in its application due to greater invasiveness of procedure and the need for specialised equipment and/or personnel.

Other indirect measures of collagen turnover include the measurement of morphological changes to the tissue through magnetic resonance imaging (MRI) and/or ultrasound, and have been utilised by a number of researchers for longer term investigations into the synthesis and incorporation of collagen into dense connective tissues (Couppe et al., 2008; Couppe et al., 2013; Hodgson et al., 2012; Westh et al., 2008; Wiesinger et al., 2015; Zhang et al., 2015). Imaging techniques can illustrate tissue hypertrophy resulting from an increase in collagen content and are currently utilised to measure changes to collagen organisation or changes to tissue size which may imply an increase in collagen content or changes to fibril organisation associated with improved tissue health (Hodgson et al., 2012; van Schie et al., 2010). However, there are several limitations in the use of these techniques including high incidence of operator error and poor reproducibility (Hodgson et al., 2012). Moreover, morphological changes to tissue size necessitate a considerable time period to illustrate any measurable change given the slow turnover rate of such tissues. Additionally, changes to cross-sectional area (CSA) may not

equate to an increase in collagen integration. Indeed, an acute response of the tissues to injury (i.e. reactive tendinopathy) can result in a conditional hypertrophy due to an accumulation of water and ground substance (Cook et al., 2009). Thus imaging techniques should be used in conjunction with other measures of collagen turnover.

2.6.3 Cell-culture models

The use of 3D cell cultures, such as engineered ligaments and tendons have shown recent promise (Baar, 2017; Handsfield et al., 2017; Paxton et al., 2010; Paxton et al., 2012; West et al., 2015). Developed from primary fibroblast cells isolated from a human donor, or established cell lines, this model provides a way to determine the response of these tissues to various interventions, in a well-controlled environment, without the need for invasive techniques (Ravi et al., 2015). This model has recently been utilised to provide information about biochemical changes that may occur as a result of exercise and/or nutrition interventions (Handsfield et al., 2017; Paxton et al., 2010; Paxton et al., 2012; Shaw et al., 2017; West et al., 2015). After the 3D cell culture model is grown to a particular strength, it is then treated for a period of time with the specific intervention, which may include human serum and/or plasma obtained after an exercise or nutrition intervention. The treated engineered ligaments are then tested for material (e.g. collagen content) and mechanical properties (e.g. modulus, ultimate tensile strength, and maximal tensile load). Although this approach offers several benefits as outlined above, it is unlikely that this *in vitro* work truly reflects *in vivo* conditions. For example, cell culture requires above-optimal levels of various components (e.g. hormones and/or nutrients) in order to facilitate and support the growth of cells (Yao et al., 2017). In real-world conditions, tendons and ligaments are known to be poorly vascularised (Bray et al., 2002; Tempfer et al., 2015), and therefore nutrient delivery is likely sub-optimal in comparison to engineered tissues bathed in nutrients and hormones. In addition, the media composition used to develop and treat

the engineered tissues contains supra-physiological levels of AAs which means results obtained from this technique may not be directly translatable to *in vivo* conditions (Bray et al., 2002; Fenwick et al., 2002; Yao et al., 2017).

While each of these techniques offer their own distinct advantages and disadvantages, it could be argued that there is indeed no "gold standard" technique in which to monitor the whole tissue response both acutely and chronically to interventions aimed at increasing collagen metabolism within dense connective tissues in human subjects. Presently, tissue biopsy is the only direct way to measure collagen metabolism within dense connective tissues, however, even with advancements to this technique, it remains invasive and potentially unsuitable for use in athletic populations. Whilst a number of indirect markers are able to be utilised which are less invasive than tissue biopsy, there are several complications with the interpretation of these markers. This includes the consideration that biomarkers of collagen metabolism are only able to give a "snap-shot" of the acute whole body response. Whilst imaging techniques may be utilised as an adjunct to give an indication of changes to tissue size over the longer-term, an increase in collagen content of the tissue can only be inferred from such measures. Regardless of the intervention(s) chosen, the slow turnover rate of dense connective tissues must be taken into consideration as this will have implications in regards to cost, and participant compliance to nutritional interventions. Lastly, whilst cell culture models provide a way to measure the potential response of dense connective tissues to various interventions in a well-controlled laboratory environment, this model is not necessarily reflective of *in vivo* conditions, and therefore caution needs to be taken with its interpretation, and application to human subjects.

Table 2.2: Comparison of measurement techniques of collagen turnover in ligaments and tendons.

Measure	Rationale	Considerations
Direct		
FSR measured utilising biopsy	Direct measure of the synthesis and incorporation of collagen into the target tissue.	- Invasive. - Anecdotal reports of ongoing tissue pain, and the slow turnover rate of these tissues means its use needs to be considered in specific populations whom rely on the functioning of these tissues (e.g. athletes).
The incorporation of isotopically labelled AAs	Acute measure.	- Only able to be used acutely (up to 12 h) given the complex experimental procedure (i.e. bed rest, cannulations, laboratory control). - Multiple cannulations required for infusion of AAs. - Significant cost. - Cannot be utilised in "free-living" individuals.
Orally administered D_2O	Acute and chronic measure.	- Able to be utilised acutely (~ 3 h), and more chronically (up to 8 days), thus may illustrate changes which would not yet be picked up by imaging techniques. - More suitable for use in "free-living" populations. - Although less biopsies required compared to above, still invasive in nature.
Indirect		
Biomarkers of collagen synthesis in body fluids:		- Multiple measures may strengthen the validity of outcomes and give an indication of collagen synthesis and incorporation, rather than just synthesis.
Procollagen monomers e.g. PICP and PINP within the blood	Involved in the process of collagen synthesis (refer to section 2.4).	- Only semi-quantitative due doubts surrounding origin, site of processing and efficiency of the rates of transfer to sites of measurement i.e. tissue fluid, plasma, serum or urine. - PINP thought to be mainly reflective of bone turnover. - Reflects sum of whole body collagen turnover.
Serum or urine cross-linked telopeptides C or N terminal (CTX, NTX), pyridium crosslinks, Type I collagen fragments (ICTP) within the blood	Measurements of collagen breakdown or resorption.	- Little known about the metabolic fate or relationship between production and appearance. - More likely reflect acute changes in fast-turnover collagen pool.
Hydroxyproline excretion in urine	Hydroxyproline not re-utilized as there is no hydroxy-prolyl- tRNA.	- May be influenced by several other factors e.g. dietary intake, exercise, collagen related disease.
Micro dialysis of above	Measured closer to the site of the target tissue.	- Less likely to be contaminated with collagen synthesis of other tissues. - May still only be indicative of collagen synthesis and not necessarily integration into the tissue. - Not reflective of acute (within hours) synthesis.

Imaging techniques *Magnetic resonance imaging (MRI) and ultrasound*	Changes in size of tissue may be as a result of an increased collagen content.	- Only gives qualitative information. - May not give indication of collagen content, particularly at the injured tissue due to reactive tendinopathy: accumulation of ground substance. - Potentially expensive. - Can only infer changes to collagen content and/or fibril organisation; not a direct measure.
Cell culture		
Engineered ligaments/tendons	- Ability to measure outcome of interventions without the need for invasive techniques. - Collagen content of the tissue determined through measurement of hydroxyproline content as it accounts for ~ 13% of the collagen monomer. - Tissue mechanics (e.g. ultimate tensile strength, modulus etc.) measured by custom built force transducer.	- Cell donor may impact on tissue mechanics (e.g. post mortem cells may be weaker than those obtained during tissue reconstruction surgery). - For the cells to have the best chance of survival, conditions need to be "above optimal" e.g. media used to develop and treat ligaments contains up to four times fasting physiological levels of AAs. - Not likely to reflect *in vivo* conditions e.g. nutrient availability at the site of the tissue due to considerations such as blood flow.

2.7 The Impact of Exercise on the Synthesis of Collagen in Ligaments and Tendons

Whether there is capacity to influence the growth of ligaments and tendons beyond that of initial stages of development remains a topic of dispute. A study by Heinemeier and colleagues observed high levels of ^{14}C in the core of the tendon in individuals born at a time of high atmospheric levels but not within skeletal muscle tissue, suggesting substantially slower turnover of tendons (Heinemeier et al., 2013). However, within the same study, some individuals of a similar age were found to have low tendon ^{14}C levels, suggesting a variable capacity for tendon tissue turnover. In line with these findings, several others have found that tendons and ligaments respond both acutely and chronically to physical stimulus (Kjaer et al., 2015). Thus, it plausible that these responses may have been as a result of higher physical stimulus over the individuals' lifetime. By contrast, immobilisation has been shown to lead to atrophy of collagen containing tissues, via a marked reduction in circulating growth factors with a resultant reduction in collagen synthesis and further tissue mechanical properties (Boesen et al., 2013; de Boer et al., 2007; Kannus et al., 1997; Wren et al., 2000). Thus, it would appear that ligaments and tendons are in fact dynamic tissues that respond to loading.

2.7.1 Mechanotransduction and fibroblast response to exercise

It has been well documented that connective tissues respond to training through alterations to tissue mechanics (e.g. stiffness) and increased tissue size. Conversely, the absence of physical load results in tissue atrophy through a marked reduction of collagen synthesis (Magnusson et al., 2016). Mechanical stimulus (e.g. compression, tension and sheer) results in the activation of several intracellular signalling pathways and the expression of mechanosensitive genes leading to protein synthesis within the ECM of connective tissue (Figure 2.2) (Ackermann et al., 2016). Fibroblasts bind to the ECM via matrix adhesion contacts on the cell surface, while

integrin receptors act as the major transmembrane proteins. The matrix adhesions are responsible for the transmission of forces from the ECM to the intra-cellular environment, which results in a signalling cascade, known as “mechanotransduction” thought to drive collagen synthesis (Chiquet et al., 2009). Integrins, and integrin-associated proteins (e.g. collagen XII and tenascin-C), at the cell surface are believed to be key sensors of mechanical strain, and are important in linking the ECM to the cytoskeleton (Gauthier et al., 2018). Although several intracellular signalling pathways have been suggested to play a role in mechanotransduction, mitogen-activated protein kinase (MAPK) is said to be crucial in the conversion of mechanical stimulus to tissue adaptation (Kjær, 2004). Meanwhile, cell culture studies have illustrated that fibroblasts respond to mechanical stimulus by increasing their production and secretion of several growth factors and hormones, with a corresponding increase in mRNA expression of type I collagen (Kjaer et al., 2015). Growth factors and hormones believed to be involved in the process include transforming growth factor-1 (TGF-β1), connective tissue growth factor (CTGF), insulin-like growth factor (IGF-1) and growth hormone (GH) (Magnusson et al., 2016).

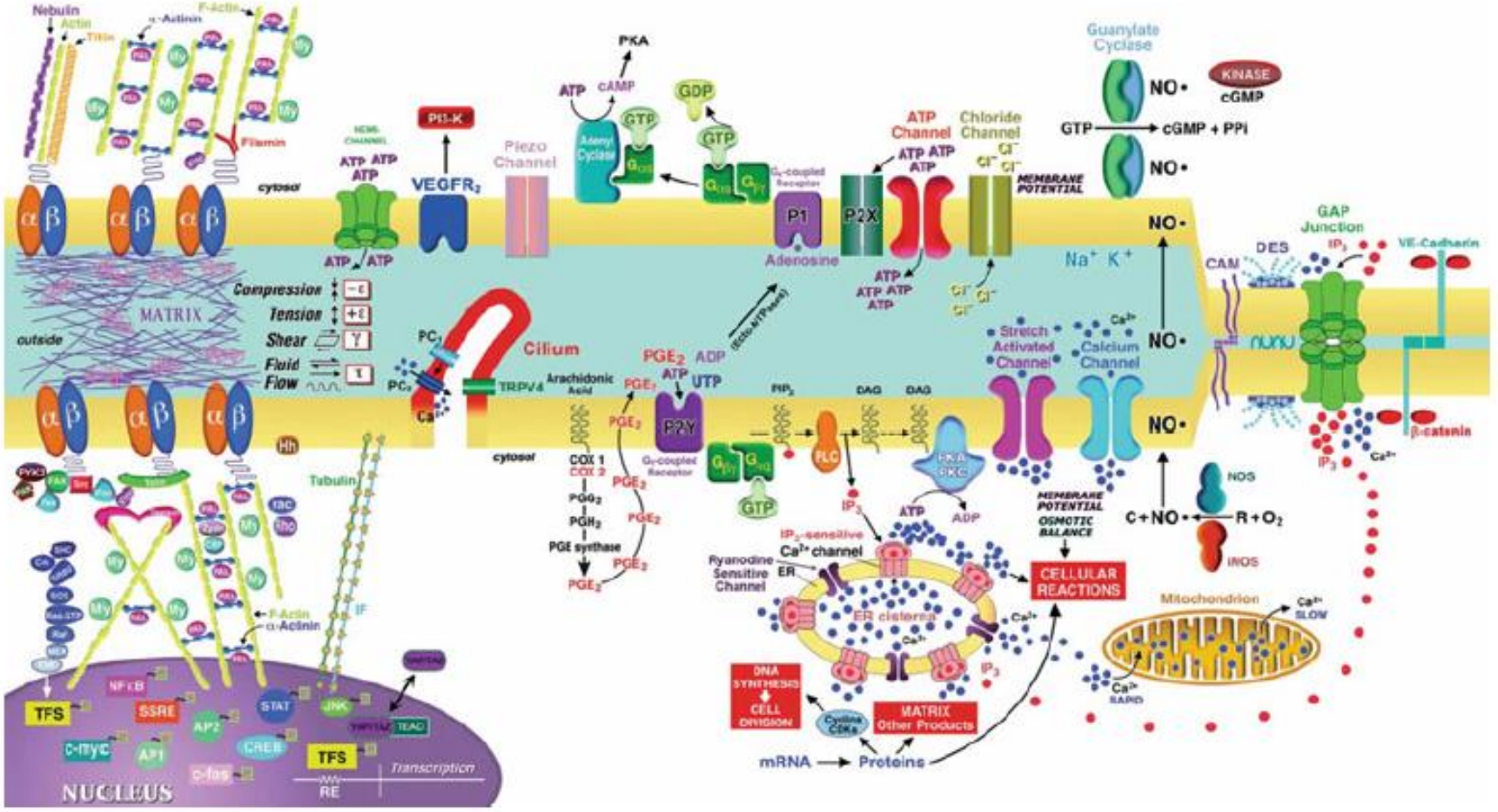

Figure 2.2: Cell signalling in tenocytes in response to mechanical load. Source: Ackermann et al., 2016.

2.7.2 The response of ligaments and tendons to exercise

While the exact mechanisms responsible for the adaptation of connective tissue in response to mechanical stimulus are complex and remain poorly understood, several studies have shown that dense connective tissue responds both acutely and chronically to exercise. Indeed, an increase in type I collagen biomarkers (e.g. ICTP, PICP) and/or growth factors (e.g. GH, TGF-β1) in response to acute exercise have been illustrated in several studies (Dideriksen et al., 2013; Heinemeier et al., 2003; Kjaer et al., 2014; Miller et al., 2005; Moerch et al., 2013). Meanwhile, these biomarkers have also been illustrated to be present in the peri-tendinous fluid surrounding the Achilles tendon in response to both acute (Langberg et al., 2001; Henning Langberg et al., 1999) and prolonged exercise (Langberg et al., 2000). Utilising a 3D cell culture model, Paxton and colleagues illustrated that mechanical stimulus resulted in an increased collagen content, interface strength and sinew modulus compared to control (Paxton et al., 2010). Furthermore, West and colleagues used a similar technique to show that engineered ligaments treated with serum obtained from young, healthy men after exercise resulted in more collagen and improved tensile strength compared to serum obtained from resting men (West et al., 2015). Although, as outlined in section 2.6.3, these models are not necessarily reflective of *in vivo* conditions. Moore and colleagues, utilising a tracer, biopsy method, were able to show an increase in muscle collagen synthesis after an acute bout of resistance exercise (Moore et al., 2005). Although the same outcome was not found in a similar study looking at patellar tendon collagen synthesis after an acute bout of resistance exercise (Dideriksen et al., 2013). As outlined in section 2.6.1, the component of connective tissue biopsied may have affected these findings. Finally, others have shown that habitual loading of tendons results in an increased CSA across varying sections of the tendon which is presumably as a result of an increased collagen content (Wiesinger et al., 2015; Zhang et al., 2015). Although there are several challenges with interpreting each of these data in isolation, taken

collectively, this evidence suggests that it is highly plausible that exercise plays a pivotal role in the synthesis, and incorporation of collagen protein within ligaments and tendons.

2.7.3 The role of exercise in nutrient delivery to ligaments and tendons

Adequate blood flow to tissues is necessary for the delivery of oxygen and nutrients (Pittman, 2011). Although variability occurs with the tissue location, ligaments and tendons are said to be poorly supplied by blood, and therefore the delivery of nutrients are suboptimal, particularly at rest (Fenwick et al., 2002; Petersen et al., 1999; Tempfer et al., 2015; Yang et al., 2013). Poor blood supply can lead to a reduced healing capacity, and in the case of ligaments and tendons results in an increased length of time to heal, in comparison to other tissue types (Tempfer et al., 2015). Post-surgery, blood flow to the tissue is essential, and an inadequate supply can lead to cell and tissue necrosis. It should be noted that, under some conditions (e.g. chronic tendinopathy and degeneration), there is an increased vascularity, the purpose of which is currently unknown but does not appear to be associated with tendon healing, and may in fact play a role in pain presentation, potentially as a result of tissue inflammation (Fenwick et al., 2002). In addition to blood supply, synovial fluid has also been proposed to play a major role in the delivery of nutrients to dense connective tissue (Fenwick et al., 2002). Therefore, increasing blood flow and synovial fluid through exercise, improves tissue perfusion and delivery of nutrients, maximising the conditions required for connective tissue growth..

2.8 The Role of Nutrition in the Synthesis of Collagen in Ligaments and Tendons

As outlined in section 2.4, there are several processes which require adequate nutrition for the formation of fibril-forming collagen within ligaments and tendons. First, as a substrate for the protein synthetic machinery that elongate and synthesise collagenous protein (i.e. AAs), second, as co-factors in key enzymatic processes necessary for the formation of collagen cross-links, resulting in a strong and rigid tissue (e.g. ascorbic acid and copper) (Scott et al., 2016). Adequate nutrition support is vital for the optimal functioning of all tissues in the human body, while malnutrition, such as inadequate dietary protein intake, can promote and/or accelerate losses to the integrity and structure of tissues (McCormick, 1989). Although recent research exploring the potential for nutrition to increase the synthesis and incorporation of collagen is scarce, it is well established that nutrition deficiencies can disrupt collagen synthesis and lead to poor wound healing and weak connective tissues (McCormick, 1989; Mussini et al., 2017). Indeed, given the role of ascorbic acid in the synthesis and stability of collagen molecules, its deficiency results in a loose ECM of collagen containing tissues, leading to the symptoms characteristic of scurvy such as bleeding gums, poor wound healing and retardations to normal tissue development (Boyera et al., 1998; Gelse, 2003). While it appears highly plausible that exercise plays a central role in the synthesis of collagen in ligaments and tendons (refer to section 2.7), the role of nutrition is still being determined beyond that of initial growth and development, and in states of malnutrition. The relevant studies are summarised in Tables 2.3-2.6.

Indeed, the effect of feeding on collagen synthesis remains inconclusive and appears to differ dependant on the tissue type (± the addition of exercise). In muscle, it has been illustrated that an AA infusion alone did not lead to an increased collagen synthesis (Mittendorfer et al., 2005).

Similarly, Babraj et al. illustrated that, unlike bone collagen, collagen synthesis within the ECM of connective tissues is not upregulated post-prandially by nutrition alone (Babraj et al., 2005a; Babraj et al., 2005b). However, Moore et al., 2005 illustrated that an increased EAA availability, through the consumption of a protein supplement drink, every 30 min (0.1g/kg/h of carbohydrate and protein) in addition to resistance exercise led to an increased collagen synthesis in muscle (Moore et al., 2005). Meanwhile, Miller and colleagues have illustrated a coordinated response of skeletal muscle and tendon collagen synthesis following a combination of exercise and nutrition support every 30 min over 4 h (Miller et al., 2005). By contrast, a more recent study by Holm et al., 2010 found that although there was a clear effect of exercise on skeletal muscle collagen synthesis, intermittent feeding had no additive effect (Holm et al., 2010). Thus, although not conclusive, it would appear that nutrition alone is unable to stimulate collagen synthesis within muscle and the ECM of dense connective tissue, and likely plays a supportive, rather than central, role.

2.8.1 Essential, non-essential and conditionally essential amino acids

Amino acids are necessary for the synthesis of all protein fractions within the human body, and have historically been defined as either EAAs or NEAAs, determined through nitrogen balance and growth (Rose, 1967). EAAs are those unable be endogenously synthesised and therefore exogenous sources are required to sustain tissue integrity via the consumption of suitable dietary protein sources. NEAAs, on the other hand, are synthesised endogenously, and therefore are not reliant on exogenous sources for adequate supply (Rose, 1967). It has been well documented that EAAs are necessary to support muscle protein synthesis (MPS) with inadequacies in the consumption of EAA resulting in atrophy of muscle tissue (Kumar et al., 2009). Meanwhile, the EEA, leucine, has received considerable attention for its ability to further stimulate MPS when combined with an appropriate exercise stimulus (Churchward-

Venne et al., 2012). The mechanism of action for leucine is thought to be the mammalian target of rapamycin (mTOR), which causes a signalling cascade resulting in MPS (Dodd et al., 2012).

Given the important role of leucine in the synthesis of myofibrillar protein, some research groups have explored whether leucine may also play a role in the synthesis of collagen. Barbosa et al. (2012) illustrated that, although malnourished rats fed a leucine rich diet had a higher collagen content and improved tissue mechanics of the digital flexor tendon, which was further amplified by exercise, the high leucine diet failed to have the same effect in rats that were not malnourished (Barbosa et al., 2012). Meanwhile, Kato and colleagues showed that the ingestion of leucine-enriched EAA after exercise did not result in increased muscle collagen protein synthesis in rats (Kato et al., 2016). This suggests that leucine may be beneficial to maintain protein synthesis and tissue integrity in a malnourished state, however under conditions of dietary adequacy, there may not be any added benefit to the intake of additional leucine. Although leucine has not yet been established as a key player in the synthesis of collagen, recent research has suggested that AAs, such as those that predominate in collagen (e.g. proline and glycine) (see Figure 2.3.), may play a more distinctive role in its synthesis (Baar, 2017).

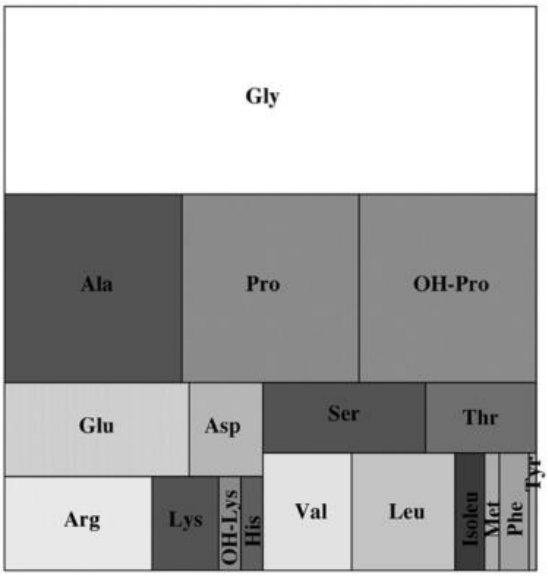

Figure 2.3: Schematic representation of the amino acid composition of collagen. Source: Barbul et al., 2008.

Although proline and glycine have previously been considered NEAAs, emerging evidence suggests that some NEAA may be conditionally essential (CEAA) and are required in higher amounts under certain conditions. An example of this is L-arginine, which has been shown to be required in higher amounts in situations where endogenous production does not meet the requirements for synthesis (e.g. during periods of metabolic stress and growth/development) (Demling, 2009; Mussini et al., 2017; Russell, 2001). L-arginine is utilised to synthesise nitric oxide, which, in dermal wound healing, has been shown to stimulate fibroblast proliferation and collagen synthesis, essential for scar formation (Fujiwara et al., 2014; Schaffer et al., 1996).

Glycine occupies one third of the AA residues in collagen, and has traditionally been thought of as a NEAA (Wang et al., 2013). However, increasing evidence suggests that glycine may, in fact, be a CEAA, and similar to L-arginine, its requirements may not be able to be met endogenously under certain conditions (de Paz-Lugo et al., 2018; Li et al., 2018; Meléndez-Hevia et al., 2009; Wang et al., 2013). Furthermore, glycine is thought to have a strong anti-inflammatory influence, which may also play a role in maintaining tissue integrity under certain conditions (e.g. tendinopathy) (Wang et al., 2013). Indeed, a study by Vieira et al. (2015) found the inflamed tendons in rats which were fed a diet containing glycine achieved a higher hydroxyproline content, and GAG concentrations than those of an inflamed group fed a glycine free diet, and values similar to that of an un-inflamed control group (Vieira et al., 2015). However, whether the administration of glycine under normal conditions results in an increased synthesis of collagen in ligaments and/or tendons is yet to be explored.

After glycine, proline (or its derivative, hydroxyproline) is the next most prevalent AA in collagen, comprising ~ 23% of AA content of collagen (Albaugh et al., 2017). Proline is a potent regulator of many biochemical and physiological processes in cells, while both proline

and hydroxyproline are essential for collagen structure and strength (Barbul, 2008). The hydroxylation of proline occurs post-translationally, utilising oxygen, iron and ascorbate as cofactors; once hydroxylated, it cannot be resynthesised into new collagen (Albaugh et al., 2017). For wound healing, there are several pathways in which proline can be metabolised, through the utilisation of other AAs, including arginine (Albaugh et al., 2017). However, it has been shown that supplementation with either proline or hydroxyproline does not result in increased wound breaking strength or increased collagen deposition, likely due to the tight regulatory control of proline availability and the inability of hydroxyproline to be incorporated into new collagen (Barbul, 2008). With regard to dense connective tissue, several researchers have shown the importance of proline in the synthesis of collagen. Indeed, Dideriksen et al. (2011) found a trend towards a higher fractional synthetic rate of muscle collagen in elderly individuals after the consumption of casein, compared to whey protein. It is possible that this is due to casein providing a higher source of proline at 10.0 grams compared to 4.6 grams per 100 grams for whey protein (Dideriksen et al., 2011). In support of these findings, recent *in vitro* work by Paxton and colleagues was able to illustrate that the treatment of engineered ligaments with proline (50 μM), in addition to ascorbic acid (50 μM) resulted in an increased collagen content, interface strength and sinew modulus compared to untreated ligaments (Paxton et al., 2010).

Utilising a combined *in vitro* and *in vivo* design Shaw et al. (2017) illustrated that the ingestion of 15 grams of gelatin (denatured collagen containing high levels of both glycine and proline), equating to a concentration of 40 – 45 μM of proline in growth media, in addition to 48 mg of vitamin C increased the collagen content and mechanical properties of an engineered ligament *in vitro*. Meanwhile, the *in vivo* arm of the study showed a doubling of blood concentrations of the collagen synthesis marker PINP following three days of a repeated (3 times/day) protocol

of gelatin ingestion, one hour prior to 6 minutes of skipping (Shaw et al., 2017).

Of course, as is previously outlined (section 2.6), there are several challenges with the interpretation of this data such as uncertainty around metabolic fates, and the challenges in transferring *in vitro* models, or animal studies, to human applications. Nonetheless, taken collectively, these studies illustrate that there is indeed potential for collagen precursors to support exercise in the synthesis of collagen in ligaments and tendons. Furthermore, it is also possible that during periods of increased metabolic demand and/or tissue turnover, the endogenous production of some AAs involved in the synthesis of collagen may be inadequate to meet requirements, and therefore may be required in the diet in higher amounts, however further investigations are required to confirm this hypothesis. Finally, more work needs to be done to determine the benefit of the intake of essential AAs (i.e. leucine) for ligaments and tendons, in a well fed state, beyond that of dietary sufficiency.

Table 2.3: The impact of dairy derived proteins on collagen synthesis and mechanical properties of dense connective tissues: Animal models.

Author/Year/Title	Subjects and design	Intervention		Measures	Outcomes	Commentary
		Nutrition	Exercise			
Barbosa et al., 2012. A Leucine-rich diet and exercise affect the biomechanical characteristics of the digital flexor tendon in rats after nutritional recovery	Male Wistar rats, 21 days old (n=56), maintained in collective cages at 22 ± 2 ℃, under 12 h light/dark cycles. Evenly separated into the following groups: • Control diet only: C • Control diet + 5 weeks swim exercise: CT • Malnourished only: M • 6 weeks malnourished + 5 weeks control diet: MRC • 6 weeks malnourished + 5 weeks control diet + swim exercise: MRT • 6 weeks malnourished + 5 weeks leucine rich diet: MRL • 6 weeks malnourished + 5 weeks leucine enriched diet + swim exercise: MRLT	Free access to semi purified diets + water • Control diet: 18% protein. • Malnourished diet: 6% protein. • Leucine rich diet: 15% protein - 3% L-Leucine.	Swimming after 6 weeks for 5 days starting with 15 min and progressively increasing 5 min per day until reaching 1 h of exercise per day.	• Mechanical tensile strain (maximal load, displacement, stress and strain). • Cross-sectional area (CSA). • Collagen content of the deep digital flexor tendon (DDFT) (determined by hydroxyproline content).	• Malnutrition produced a significant reduction in tendon CSA. • Exercise, especially when associated with nutritional recovery, increased the diameter of the tendon fibres as compared with the control group • Higher collagen content in leucine treated groups compared to sedentary and trained groups in tension region of the DDFT. • Higher collagen content in groups M and all nutritional recovery animals compared C in compression region of the DDFT. • In nutritional recovery groups, mechanical tensile strain were comparable to C. • Trained nutritionally recovered groups presented similar values to CT group.	• Malnutrition possible lead to reduced CPS, and in turn CSA. • Exercise increased CPS, leading to increased CSA. • Different regions of the DDFT appeared to be affected differently by nutrition states. • Nutritional recovery and/or exercise lead to improved tensile strain compared to control groups. • Appears that LEAA administration in the fed state does not lead to increased CPS.
Kato et al., 2016. Leucine-Enriched Essential Amino Acids Augment Mixed Protein Synthesis, But Not Collagen Protein Synthesis, in Rat Skeletal Muscle after Downhill Running	Female Wistar rats, 7 weeks old, housed in a temperature-controlled room on 12 h light/dark cycles. Groups: • Sedentary (n=30)	• Orally administered distilled water (Con), immediately, 3 h, 1 day, 2 days, 4 days, and 7 days after completion of the exercise (Con). • Leucine-enriched EAAs (LEAA)	• Intermittent running on a motor-driven treadmill, speed = 17 m/min, for a total of 130 min on downhill tracks. • During the exercise, 26 repetitions of five - min running bouts	30 min after oral administration, rats were injected with flooding dose of proline for 30 min at which time vastus lateralis muscle was removed and used for analysis.	• Collagen protein synthesis (CPS) in vastus lateralis muscle was elevated 1 and 2 days after the exercise, compared to the sedentary group. • LEAA administration did not further	Although CPS increased by exercise, increased EAA availability does not lead to further increases.

	• Active (n-64)	mixture (1 g/kg) immediately and 1 day after the exercise. • The LEAAs mixture consisted of: histidine, 2%; isoleucine, 11%; leucine, 40%; lysine, 17%; methionine, 3%; phenylalanine, 7%; threonine, 9%; tryptophan, 1% and valine, 11%. • Except for the elevated leucine, this mixture contains the ratio of EAAs found in whey Protein.	were separated by 2-min intervals.	increase CPS 1 h or 1 day after exercise.

Table 2.4: The impact of dairy derived proteins on collagen synthesis and mechanical properties of dense connective tissues: *In vivo* studies.

Author/Year/Title	Subjects and design	Intervention		Measures	Outcomes	Commentary
		Nutrition	Exercise			
Babraj et al., 2005. Collagen synthesis in human musculoskeletal tissues and skin	EAA group: • Healthy young men, 28 ± 6 y, BMI 24 ± 3 kg/m^2 (n=8). • Healthy elderly men, 70 ± 6 y, 26 ± 4 kg/m^2 (n=8) Mixed nutrient drink group: • Healthy young men 25 ± 1 y, 22 ± 2 kg/m^2	• 20 g of EAA • Mixed nutrient Drink: 15% Protein, 64% carbohydrate, and 21% fat over 280 min to provide the equivalent of 1.4 BMR	Nil	• Musculoskeletal collagen FSR measured by the incorporation of isotopically labelled proline or leucine. • Muscle biopsies at rest and 3 h post ingestion. • Patella tendon biopsies 2 h post flooding dose.	• 20 g of EAA ↑ young and elderly myofibrillar protein synthesis. • No difference on muscle collagen synthesis. • No difference on tendon collagen synthesis.	• Similar tendon and ligament collagen synthetic rates, which were higher than muscle collagen or myofibrillar protein. Suggesting higher tendon/ligament remodelling than previously thought. • Compared to similar study in bone tissue (Babraj et al., 2005) feeding independent of exercise had no effect.
Dideriksen at al., 2011. Stimulation of muscle protein synthesis by whey and caseinate ingestion after resistance exercise in elderly individuals	24 healthy elderly men (n = 15) and women (n = 9) (mean ± SEM; 68 ± 1 y) Groups: 1. Casein pre: 71 ± 3 y, 80.5 ± 6.5kg 31.1 ± 3.0 % body fat (BF) (n = 6). 2. Casein post: 70 ± 2 y, 75.8 ± 6.1 kg, 27.6 ± 4.0 % BF (n = 6). 3. Whey post 64 ±1 y, 76.1 ± 4.4 kg, 26.2 ± 0.6 % BF (n = 6). 4. Control: 68 ± 2 y, 71.9 ± 5.2 kg, 23.0 ± 3.2 % BF (n = 6).	Labelled protein dissolved in 300mL water was 0.45 g/kg lean body mass (LBM), resulting in individualized drinks with a total content of 15.6–30.4 g Protein, 7.8–14.6 g EAAs, and 1.5–3.4 g leucine 1. Calcium caseinate ingestion 30 min before exercise. 2. Calcium caseinate immediately after exercise. 3. Whey immediately after exercise 4. Non-caloric control drink ingestion immediately after exercise.	5 sets of 8 x 80% 1 RM unilateral knee-extension and bilateral leg-press with 3 min rest between sets	• Muscle myofibrillar and collagen fractional synthetic rates measured by isotopically labelled leucine. • Muscle biopsy 30 + 390 min after exercise • Venous blood samples to evaluate changes in insulin and AA concentrations.	• Myofibrillar protein ↑ after pre-casein ingestion. • Tendency for ↑ myofibrillar protein for post-casein and post-whey ingestion. • Insignificant tendency for ↑ muscle collagen synthesis for pre and post casein compared to post-whey ingestion and control.	• Trend toward a higher FSR with ingestion of casein compared to whey protein, possibly due to proline content of casein.

Farup et al., 2014. Whey protein hydrosylate augments tendon and muscle hypertrophy independent of resistance exercise contraction mode.	Healthy recreationally active males: 23.9 ± 0.8 y, 78.1 ± 1.8 kg, 181.5 ± 1.5 cm, 16.0 ± 0.9% BF (n = 22) Equally allocated to the following groups: 1. Placebo (PLA) (n = 11). 2. Whey protein hydrolysate + carbohydrate (WHD) (n=11).	Drinks (consumed ½ pre training and ½ post training): • Whey-carbohydrate (CHO): 663 KJ; 19.5 g whey protein. hydrolysate + 19.5 g CHO. • Placebo: 39 g CHO.	Knee extensions training program 3 x per week for 12 weeks (1 leg concentric + 1 leg eccentric): Exercise progression: • Week 1 6 x 10-15 repetition max (RM) • Week 2-3 8 x 10 – 15 RM • Week 6-8 10 x 10-15 RM • Week 8-10, 12 x 6–10 RM • Week 11-12, 8 x 6-10RM (2 sec tempo and 2 min rest between sets).	• Quadriceps muscle and patella tendon magnetic resonance imaging (MRI) CSA.	• WHD group ↑ CSA of quadriceps muscle independent of exercise mode. • PLA group ↑ CSA of quadriceps muscle independent of mode (but to a lesser extent than WHD). • WHD group ↑ CSA of patellar tendon. independent of mode • PLA group ↑ CSA of eccentric leg, but not concentric. • Significant correlation between quadriceps CSA and patella tendon CSA and quadriceps CSA and patella tendon CSA (when combining distal, mid and proximal levels).	Given the correlation between quadriceps muscle increase and patella tendon increase in CSA, the impact that whey protein has on MPS, and that load has on increased collagen synthesis and tendon hypertrophy, it is likely that the increased loading of the muscle on the tendon led to the increased CSA.
Miller et al., 2005. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise.	Healthy young men. Separated into two groups: Group 1. 25±1 y, 186±9 cm, 76±8 kg (n=8). Group 2 2. 29±1 y, 181±9 cm, 76±6 kg (n=8).	Consumed every 30 min for 3 hours: • Nutrient drink: 15 % protein, 64 % carbohydrate, 21 % fat. • Energy = 1.4 x Cunningham BMR • Feeding started with a double dose to bring subjects rapidly into a fed state.	Group 1: 1 of 1 legged kicking at 67% W_{max} vs Resting leg Group 2: 1 h of 1 legged kicking at 67% $W_{max.}$ R and L legs separated by 18 h. One leg: 6 x 10 max lengthening contractions w/ 2.5 min rest b/w sets. Other leg: 6 x 10 max shortening contractions + extra to match workload w/ 2.5 min rest b/w set.	• Incorporation of isotopically labelled AAs (proline and leucine) into patella tendon, and quadriceps muscle. • Muscle biopsy at 24, 48 and 72 h post exercise. • Muscle collagen at rest, 48 and 72 h after exercise • Tendon collagen synthesis at rest, fell thereafter but still significantly elevated 72 h after exercise.	• ↑ In muscle collagen synthesis at 6 and 24 h, return to BL by 72 h. • ~1.7 fold ↑ in tendon collagen synthesis at 6 and 24 hours, which continued at 72 h.	Increase in collagen synthesis in tendon potentially as a result of an increase in quadriceps size placing load upon the tendon.

Table 2.5: The impact of collagen derived proteins on collagen synthesis and mechanical properties of dense connective tissues: Cell culture models.

Author/Year/Title	Subjects and design	Intervention		Measures	Outcomes	Commentary
		Nutrition	Exercise			
Paxton et al., 2010. Engineering an In Vitro Model of a Functional Ligament from Bone to Bone	Engineered ligament developed utilising embryonic chick fibroblast cells.	Constructs fed every 2-3 days with DMEM media supplemented with 10% fetal bovine serum, and then supplemented with ascorbic acid (50 µM) , proline (50 µM), and/or TGF-β1 (2.5 ng/mL) on day 7 after plating for 2 and 4 weeks. Groups included: • Constructs untreated at 2 and 4 weeks. • Constructs treated with ascorbic acid + proline at 2 weeks. • Constructs treated with ascorbic acid + proline + TGF-β1 at 2 weeks.	TGF-β1 acts as an exercise stimulus as it is upregulated after resistance training.	• Tissue mechanics (Young's modulus, ultimate tensile strength). • Collagen content (%) (measured through hydroxyproline assay).	Untreated: • Collagen content: 1.45 ± 0.32 Ascorbic acid + proline: • Collagen content: 8.34 ± 0.90, • 22 fold increase in young's modulus compared to untreated controls. Ascorbic acid + proline + TGF-β1: • Collagen content: 11.25 ±0.82 • 34 fold increase in young's modulus.	• DMEM media utilised to develop and treat ligaments are at supra-physiological levels for some AAs particularly proline and leucine, whilst tendons/ligaments are poorly supplied by blood so unlikely that these levels world be reflective of *in vivo* conditions. • The vitamin C added to constructs was considerably high, and therefore led to high tissue mechanics.
Shaw et al., 2017. Vitamin C–enriched gelatin supplementation before intermittent activity augments collagen synthesis.	Eight healthy, recreationally active males: 27 ± 6 y, 79.6 ± 12 kg (mean ± SEM). Engineered ligaments developed utilising human ACL cells.	0, 5 or 15 grams of gelatin. Consumed 1 h before each rope skipping session.	6 min of skipping rope and then rest for 4 h. After leaving lab instructed to complete 3 sessions of 6 min skipping, with at least 6 h between each bout of exercise.	• Blood draws at -1h and 0 h time points (before exercise) were used for engineered ligament treatment. • Bloods draws at 24, 48 and 72 h after the initial bout of exercise were used to assess PINP. • Blood draws at -1, -0.5 h, 0, 0.5, 1, 2 and 4 h for AA analysis • Mechanical. properties: ultimate tensile stress (UTS), maximal tensile load (MTL).	• Proline and glycine peaked at 1 h within the blood. • There was an increased collagen content corresponding to dose of gelatin. • There was an increased collagen % of dry mass. corresponding to dose without a change in CSA suggesting increased density of collagen fibrils. • MTL of ligaments increased for all treatment groups.	• As a collagenous food source was utilised within the study, unable to ascertain which AA exactly had an impact. • PINP is more reflective of bone collagen synthesis. • As above re DMEM media composition.

			• Increased UTS and tensile strength. • Increase in PINP for treatment groups, doubling in 15 g gelatin group.

Table 2.6: The impact of collagen derived proteins on collagen synthesis and mechanical properties of dense connective tissues: Animal models.

Author/Year/Title	Subjects and design	Intervention		Measures	Outcomes	Commentary
		Nutrition	Exercise			
Vieira et al., 2015. Glycine Improves Biochemical and Biomechanical Properties Following Inflammation of the Achilles Tendon.	Young adult male (60 days old) Wistar rats, housed under 12 h light/dark cycle with free access to standard chow and water prior to treatment with glycine, and control diet.	The animals were divided into five groups: • C: Control group • G1 and G3: Inflamed and control diet, and; • G2 and G4: Inflamed and supplied diet containing 5% glycine.	Tendinitis induced via injection into Achilles tendon	• Collagen content (assessed via determination of hydroxyproline) • CSA • Biomechanics (MTL, MPa)	• Greater concentration of hydroxyproline in G4 compared to other inflamed and treated groups: similar to control group. The effect of glycine on collagen content occurred within the first 7-21 days as there was no differences in the first 7 days of treatment • G4 withstood a larger load than other groups: similar to control • No differences in CSA between groups in 21 days. • Epitenon of G2 was thicker than other groups. • No difference in MPa	These results indicate that the glycine rich diet may rectify the degradation and disorganisation associated with inflammation results in biomechanics and collagen content similar to non-inflamed tendons. However, there was no non-inflamed glycine diet group, thus it is not possible to determine the impact of glycine in a healthy tendon within this study.

2.8.2 Potential sources of collagen precursors in the Australian diet

It has been estimated that American populations consume between 5.3 - 22.6 and 3.3 - 12.7 grams of collagen protein per day for males and females respectively, which is predominantly provided through the intake of highly processed meats (Paul et al., 2019). Meanwhile, there is no reliable information available within Australia in regard to the collagen content of naturally-occurring or processed foods, and as such, the average consumption cannot be estimated. As collagen contributes to a large proportion of protein found in animal tissues, dietary collagen is be obtained through the consumption of meat, particularly those with the skin, bone or connective tissue still attached (Eastoe, 1955) (Table 2.7). However, recent trends in Australia in regards to meat and poultry consumption has prompted a move towards isolated, lean cuts at the expense of the purchase and intake of whole carcasses or "lower quality" cuts that are often a valuable source of collagen (i.e. bone and cartilage) (Williams et al., 2010; Wong et al., 2013).

Table 2.7: Amino acid composition of the 5 major mammalian collagens and gelatin in comparison to human collagen.

	Animal Collagen				Human Collagen	
Amino Acid	Ox-hide	Gelatin	Pig skin	Ox-bone	Bone	Tendon
Alanine	11.0	11.3	10.7	10.5	10.9	10.3
Glycine	27.5	27.2	26.4	25.3	25.8	25.4
Proline	16.4	15.5	16.2	14.7	15.3	15.2
Glutamic acid	11.4	11.6	11.3	11.9	11.4	11.1
Hydroxyproline	14.1	13.3	13.5	14.1	14.1	12.6

Amounts given in grams per 100 gram of dry ash-free Protein (adapted from Eastoe, 1955).

Although, there is no information regarding the collagen content of processed meats in Australia, it seems reasonable that a higher processed meat consumption would also result in a higher dietary collagen intake. However, processed meats are associated with an increased risk of several forms of cancer, and therefore, excess consumption is not recommended (Australian Government, 2015). Data from 2011-12 from the Australian Government indicated a typical average daily intake of 14.5 and 9.7 grams of processed meats for males and females respectively (Australian Government, 2017). This is in comparison to an average daily intake of 31.2 and 17.0 grams for males and females respectively, in America in 2001-04 (Paul et al., 2019). Thus, within Australia there is a lower consumption of processed meat, potentially leading to a lower consumption of collagen compared to American populations. Another dietary source of collagen is gelatin which is commonly utilised in processed foods as a thickening or gelling agent; this is manufactured from ground animal collagen, most commonly porcine (Gomez-Guillen et al., 2011). Unfortunately, the gelatin content of foods such as ice-cream, yoghurt and other desserts, or jelly confectionary is generally not specified since Australian labelling laws do not require amounts to be stated (Australian Government, 2016). Thus, the typical dietary intake of collagen in the contemporary Australian diet is thought to be low, or mostly unknown. This contrasts with increasing interest within some lifestyle and diet philosophies (e.g. the Paleo diet) which promote the intake of collagen-containing food sources, but supports the potential need for specialised food products, such as hydrolysed collagen powders (refer to section 2.8.3). Although there may be increasing interest surrounding supplemental sources of collagen, a food source may be preferential provided it supplies the potential collagen precursors without exposing the individual to known harmful dietary constituents often found within highly processes meats. The use of a food source of dietary collagen has the potential to tackle a number of sports nutrition and general health goals simultaneously, whilst having a lower risk of banned substance contamination (Geyer et al.,

2008).

One particular food source of collagen to receive considerable attention in lay literature and alternative health circles is bone broth. Bone broth differs from meat stock and broth in that it is made by simmering bones, and other collagen-containing tissues, such as cartilage for lengthy periods (usually in excess of 24 hours) to enable the collagen protein that predominates in these tissue types to be leeched into the liquid (MacSharry, 2012). Bone broth is claimed to have several health benefits including boosting the immune system, treating disease including cancer, arthritis and inflammatory bowel disease, and addressing malnutrition (MacSharry, 2012). It is also said to be beneficial for ailments affecting connective tissues (MacSharry, 2012). However, there is a lack of evidence to substantiate these claims. Although bone broth is suggested to be a constant source of dietary collagen (and therefore the AAs that predominate), it may in fact describe a range of products including those that are home-made or commercially produced, and in varying forms including liquid and dehydrated powder. In addition, recipes may differ according to the source of the collagenous tissues (e.g. beef or chicken), the type of bone (e.g. standard soup/butcher bones, or marrow bones), the duration of cooking and the addition of acidic components such as vinegar which anecdotally, improves the solubility of bone and other collagenous tissue. As a result, it is likely that there is considerable variation in the amount and type of AAs present in bone broth (Murphy et al., 2000). Therefore, while there has been increasing interest, and potentially increased intake of collagenous food sources within some lifestyle groups, it remains difficult to quantify intake due to a lack of information within food composition databases, a lack of labelling requirements, and differences in the preparation methods of collagenous food sources.

2.8.3 Hydrolysed proteins and bioactive peptides

Supplemental forms of dietary proteins provide convenient, specific and timely sources of key nutrients, and may be of benefit when the consumption of whole foods cannot meet therapeutic requirements. Hydrolysed collagen and/or "bioactive peptide formulas" are steadily increasing on the market and are accompanied by claims that they are superior to other forms of dietary and supplemental collagen. Furthermore, due to the hydrolysis of cross-linking bonds, hydrolysed collagen powders are highly water soluble, which may result in increased palatability compared to partially hydrolysed formulations (i.e. gelatin). Additionally, recent research by Paul et al., (2019) suggested that it was possible to supplement the diet with a level as high as 36% of collagen peptides as a protein substitution without impacting on the overall protein quality of the diet (Paul et al., 2019). Some studies have shown that the ingestion of peptide formulas may be beneficial in a range of situations including a reduction of joint and tendon pain (Clark et al., 2008; Oesser et al., 2016; Praet et al., 2019) and improved body composition (Zdzieblik et al., 2015). The superiority of hydrolysed collagen and/or "bioactive peptide formulas" is proposed to be due to the high percentage of two and three hydroxyproline containing AA peptide sequences (e.g. Pro-Hyp and Pro-Hyp-Gly) which have the ability to rapidly pass the mucosal barrier, and are in turn suggested to have a higher bioavailability at the tissue matrix after their ingestion (Aito-Inoue et al., 2007; Ichikawa et al., 2010; Iwai et al., 2005; Wang et al., 2016). However, these peptides have also been shown to appear in the blood after the ingestion of gelatin (Wang et al., 2015). In addition, it has also been suggested that peptide formulas have bioactive properties which influence physiological functions, including the upregulation of collagen synthesis, beyond that of the partially hydrolysed or intact proteins (Edgar et al., 2018; Paul et al., 2019; Rutherfurd-Markwick, 2012). Indeed, a number of studies have shown that the ingestion of hydrolysed collagen leads to an increased collagen synthesis in dermal, ligament, tendon and bone tissue (Elango et al., 2019; Matsuda et al., 2006; Oesser

et al., 2003; Schunck et al., 2013). In support of this, a recent study by Oertzen-Hagemann et al., (2019) identified that resistance exercise, in combination with the consumption of hydrolysed collagen, resulted in a higher upregulation of collagen related proteins and pathways, compared to a placebo control (Oertzen-Hagemann et al., 2019). Thus, the consumption of hydrolysed and/or bioactive peptide formulas may have several beneficial influences on collagen containing tissues compared to non-hydrolysed and/or intact proteins, although more work is necessary to confirm this.

2.8.4 Quantifying habitual intake of collagen

Given the lack of information on the collagen content of various food stuffs in Australia, it is difficult to account for background dietary intake of collagen in both epidemiological and intervention studies (Weaver et al., 2017). As outlined in section 2.6.2, hydroxyproline is a metabolite of proline, and cannot be incorporated into the synthesis of new collagen (Albaugh et al., 2017). Hydroxyproline constitutes ~14% $^{w}/_{w}$ of collagenous protein, and its measurement in bodily fluids (e.g. blood and urine) can be used to assess the breakdown of collagen protein as a result of collagen related disease and/or disorders (e.g. Marfan's syndrome) (Eastoe, 1955; Sjoerdsma, 1965). In such protocols, the intake of dietary collagen is restricted prior to testing, to prevent this source of hydroxyproline in bodily fluids from influencing the interpretation of data (Prockop et al., 1961; Sjoerdsma, 1965). Indeed, early studies by Prockop et al., (1961) found a four- to five-fold increase in excretion of urinary hydroxyproline when 28 grams of gelatin (containing 4 grams of Hyp) was consumed (Prockop et al., 1961). More recent research has shown that the consumption of 30 grams of gelatin led to the urinary excretion of 14 mg of hydroxyproline, while a parallel study by the same author showed an increase in hydroxyproline excretion corresponding to an increased load of gelatin consumed (up to ~60

μM after the consumption of 10 grams of gelatin) (Knight et al., 2006). Given that the intake of collagenous proteins lead to an increased excretion of hydroxyproline in the urine, it may be possible to use urinary hydroxyproline as a biomarker for collagenous intake in healthy individuals, although this has not yet been explored.

2.9 Summary and Conclusion

The capacity for nutrition to influence collagen synthesis in ligaments and tendons is of obvious interest as it may play a role in the health/functioning, injury prevention and supporting the rehabilitation of these tissues. The limited, but accumulating, data suggest that nutrition interventions may support exercise in increasing the synthesis and integration of collagen into ligaments and tendons. However, there are challenges and/or gaps within the current literature that need to be considered prior to embarking on a lengthy, costly, and challenging nutrition intervention study. These include determining the optimal food source and/or supplement containing AAs that may support collagen synthesis, and the bioavailability of various collagen food sources and/or supplements, including hydrolysed vs. non-hydrolysed formulations. Finally, determining a suitable biomarker to assess collagen intake may provide rigidity to nutrition intervention studies by accounting for background intake of collagen proteins. The studies contained within this thesis will attempt to address some of the gaps unearthed within this literature review.

Chapter Three: Extended Method

3.1 Overview

As is outlined in Chapter One, this body of work involved a series of studies aimed at progressing the knowledge gained in each, with the goal of "filling in" literary gaps prior to the future implementation of a well-designed *in vivo* intervention study (see Figure 1.1). Detailed methods of each study are available in the respective chapters. Study 1 was completed independently of Studies 2-4, with a comprehensive outline of the methods available in Chapter Four. The data collection process for Studies 2-4 was undertaken over two phases; this section will give an overview of the rationale, process and timeline of this data collection.

3.2 Rationale for Data Collection Process

Given that there are several benefits of utilising food over supplemental sources of nutrients, Study 1 "Bone broth unlikely to provide reliable concentrations of collagen precursors used in collagen research" investigated whether bone broth is a reliable source of AAs that may be involved in the synthesis of collagen in amounts that are equivalent to supplemental sources. These data informed the decision not to utilise bone broth in the final study, as it was found to be an unreliable source of AAs. Study 2 "Urinary hydroxyproline is only suitable as a biomarker for acute intake, up to 6 hours post ingestion of collagen proteins in free living, healthy active males" was developed to determine whether urinary hydroxyproline could be used as a reliable biomarker for background dietary collagen intake, to ultimately enhance the robustness of nutritional intervention studies. Study 3 "Plasma amino acid responses after the ingestion of dairy and collagen protein, in healthy active males" was developed to characterise the plasma AA response to the intake of collagen and dairy protein sources that might be used to support the synthesis of collagen in ligaments and tendons. Study 4 "The consumption of collagen and dairy proteins does not affect material and mechanical properties of engineered

ligaments treated with a media reflective of fasting physiological amino acids" represented the culmination of information derived from previous studies, utilising serum obtained after the consumption of dairy and collagen proteins to treat engineered human ligaments *in vitro*.

Data collection for Study 3 was undertaken in two phases (see Figure 3.1). Part A, was undertaken during September/October 2017 and also included the data collection for Study 2. Part B data collection occurred during February/March 2018 and also gathered samples for Study 4, with the information from Part A used to inform the optimal timing (i.e. time of peak concentrations of AAs of interest) for the blood draw used in the treatment of engineered ligaments. This sequencing of data collection allowed sufficient time to analyse the relevant samples to inform the methodology of the subsequent study, while also providing an efficient way to reduce the total number of participants in all three studies.

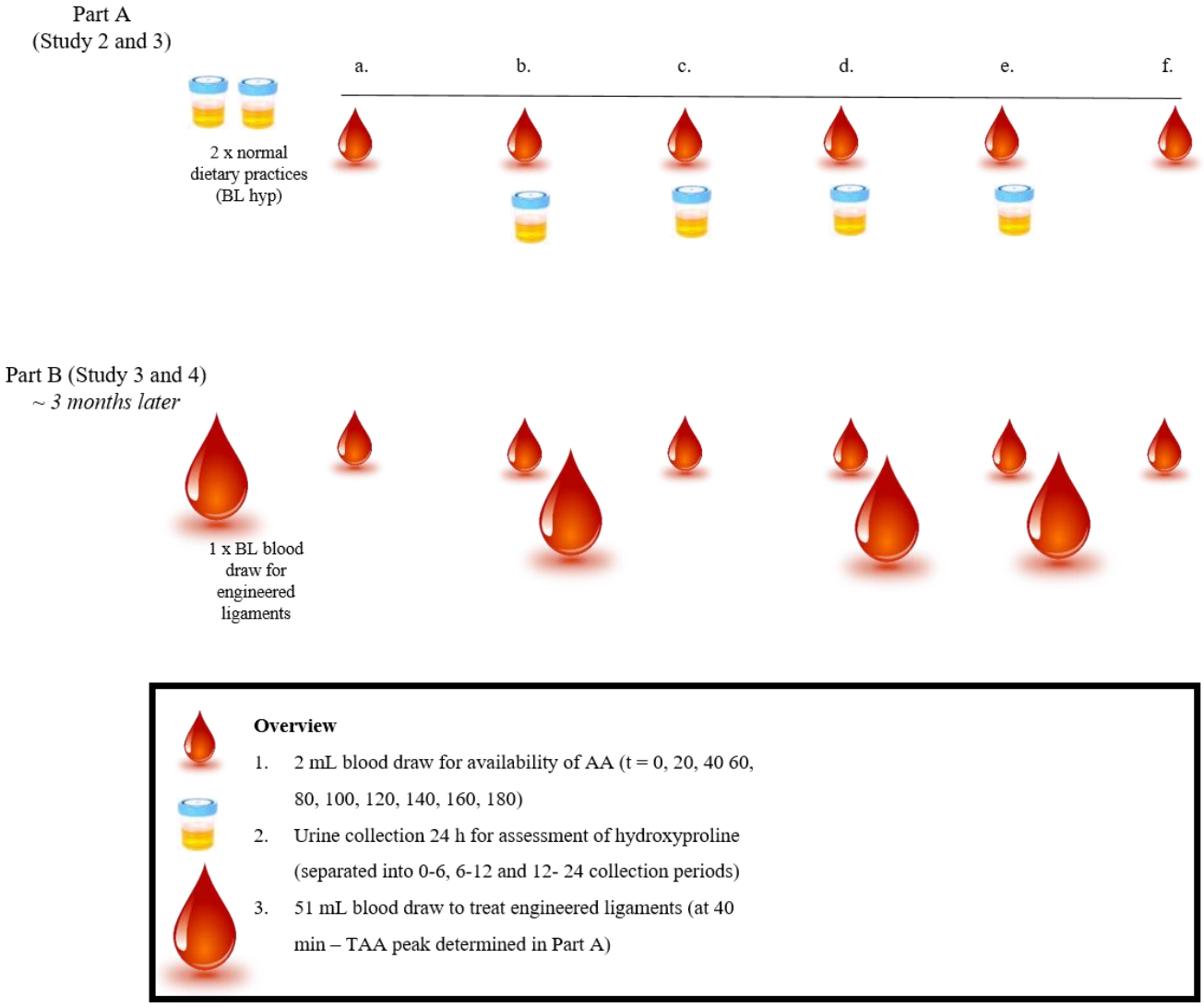

Figure 3.1: Overview of data collection protocol.

3.3 Participants

A total of 15 healthy, active males (30 ± 5 years; 80 ± 8 kg BM) were recruited for Studies 2, 3 and 4, with data collection occurring over two phases (as outlined above). The following exclusion criteria was specified:

- Females: As is outlined in section 2.5, variations in serum oestrogen concentrations associated with female reproductive function have been suggested to influence the mechanical properties of engineered ligaments via its action on LOX (Lee et al., 2015). This would add a confounding variable to the study and therefore we decided to control for this by excluding female subjects.
- Individuals with allergy/intolerance to porcine and/or bovine gelatin, milk and/or milk products and/or chicken/beef/seafood.
- Individuals with diabetes: Increased blood glucose has been shown to influence non-enzymatic cross-linking in collagen (Couppe et al., 2016).
- Individuals with a history of recent (< 1 month) or current tendinopathies: Inflammatory cytokines have been shown to influence fibroblast activity and collagen synthesis and subsequent cross-linking (Kjaer et al., 2013).

The sample size (of n = 15) for Study 3 was chosen using power estimation determined in previous, similar studies of the AA response to dietary protein intake (Burke et al., 2012; van Loon et al., 2000). Sample size estimates for Study 4 were informed by previous work by our group utilising a similar protocol. With the goal of being able to detect a 2.4 µg change in engineered ligament collagen content, and assuming a SD of 1 µg with 80% power at the 0.05 level, a sample size of eight subjects was deemed appropriate (Shaw et al., 2017). The first data collection (Part A) included eight participants for Study 2 and formed the first cohort of Study

3. The second period of data collection (Part B) involved recruiting the final seven participants for Study 3 (to reach a total of n = 15 as determined by power calculations) and eight participants for Study 4 (with one participant re-recruited from Part A). The data collection protocol was submitted to the Australian New Zealand Clinical Trials Registry (ANZCTR12617000923369), and approval was granted by the Australian Institute of Sport Ethics Committee (20170607).

3.4 Protein Food Source or Supplement

Four collagen and two dairy protein sources were selected for Study 3 and administered in a counter-balanced order. These included a hydrolysed collagen peptide powder (Tendoforte, Gelita, Eberbach, Germany); a non-hydrolysed collagen powder (Gelatin, McKenzies, VIC, Australia); a hydrolysed dairy protein powder (Hydrolysed casein) and a non-hydrolysed dairy protein powder (Calcium Caseinate) (both from Professional whey, NSW, Australia); a liquid collagen supplement (GBR nutrition, Lancashire, UK), and a bone broth (Elemental Café, Braddon, ACT, Australia) sourced for Study 1, and frozen until needed for use in the relevant studies (refer to Table 4.1, for further details). We chose a standard dose of 20 g protein for all powdered supplements and 60 mL of liquid collagen (equivalent to 20 g of collagen protein as per manufacturer information). This dose was chosen to build on previous work (Shaw et al., 2017), with a slightly higher dose to increase AA availability (Imaoka et al., 1992; Iwai et al., 2005). As we found substantial variability in the AA content of bone broth (Alcock et al., 2018), we decided on a 300 mL serve, as we deemed this to be an acceptable amount to consume with the prescribed 5 minutes. To maintain consistency all other protein sources were made up to 300 mL and served lukewarm.

3.5 Data Collection Protocol

3.5.1 General protocol

For each data collection (Part A and B); a total of six trials were undertaken with the ingestion of a different test protein food source/supplement, in a counter-balanced order, with a minimum of 48 h between trials. Participants arrived to the laboratory (The Australian Institute of Sport) after an overnight fast (> 10 h) and in a rested state, to ensure that no significant elevations to heart rate would increase blood flow. On arrival to the lab, a 22G indwelling cannula was inserted into the antecubital vein for blood collection by a trained phlebotomist, and a baseline (BL) blood sample (2 mL) was collected into lithium heparin Vacuette tubes (Greiner Bio-One, Kremsmünster, Austria). Immediately after cannulation, the protein source was prepared and given to participants who were instructed to consume it within 5 min, sipping slowly throughout. Completion of the 5 min period was considered as t = 0. To standardise gastric emptying, no other fluid was available for 60 min following consumption; thereafter *ad libitum* water consumption was permitted. Participants then remained resting while 2 mL blood samples were collected every 20 minutes over the succeeding three hours. Immediately after each blood collection, samples were centrifuged at 1500 xg for 10 min at 4°C. The resulting plasma was separated into 500 µL Eppendorf cryotubes and stored at -80°C until required for analysis. Whilst at the lab, participants were given a feedback form to complete. The form provided four questions related to acceptability and palatability of the protein source in the format of a Likert scale (Likert, 1932), and one question with a yes or no response (further details can be seen in Chapter Six). Once the three hour period was concluded, the cannula was removed and participants were allowed to leave the laboratory.

3.5.2 Part A specific protocol

After the consumption of the hydrolysed and non-hydrolysed dairy, and collagen proteins (outlined above), participants were required to collect their urine into clean plastic bottles (2 L capacity) over a 24 h period, divided into three collection periods (t = 0-6, 6-12 and 12-24 h). The subjects also completed 24 h urine collections on 2 baseline (BL) days when no supplement was consumed, and normal eating patterns were maintained using breakfast as t = 0 and continuing the same collection periods. Participants were instructed to keep urine in a cool, dark place and return samples to the laboratory as soon as possible after collection. Urine volumes were measured, and aliquots of each sample were stored at -80°C until analysis. Since hydroxyproline: creatinine ratio is a commonly accepted way to standardise the reporting of hydroxyproline values, hydroxyproline and creatinine were analysed, and the hydroxyproline mmol: creatinine mol ratio was calculated.

At the commencement of the study, subjects were instructed by a Sports Dietitian to maintain their habitual dietary practices while recording their meal on the night prior to each trial and their food and fluid intake during the period of the 24 h urine collection. Food diaries were analysed for total protein intake (g/meal and g/day) using dietary analysis software (Foodworks v9, Xyris Software, Australia). As the excretion of hydroxyproline has been shown to be upregulated for >24 h following exercise (Brown et al., 1997), exercise in the 24 h period prior to collection, was recorded by participants in terms of the duration of the bout and a sessional rating of perceived exertion (RPE). A standardised metric to describe/compare exercise sessions was then calculated by multiplying these factors (Foster et al., 2017).

3.5.3 Part B specific protocol

On this occasion, an additional 55mL of blood was collected on three occasions, 40 min after the ingestion of three different supplements; a non-hydrolysed collagen (gelatin), a hydrolysed collagen (gelita) and a hydrolysed dairy protein (casein). In addition, a BL blood draw was also taken on one occasion before any supplements were consumed. After collection, the blood was allowed to clot for 30 min at room temperature then centrifuged at 1500 xg for 10 min with the resultant serum separated into 2 x ~12 mL, aliquots, under sterile conditions. The samples were then stored at -80°C until further analysis. Once all samples were collected, they were then sent to the University of California, Davis Proteomics Core facility to develop and treat the engineered ligaments.

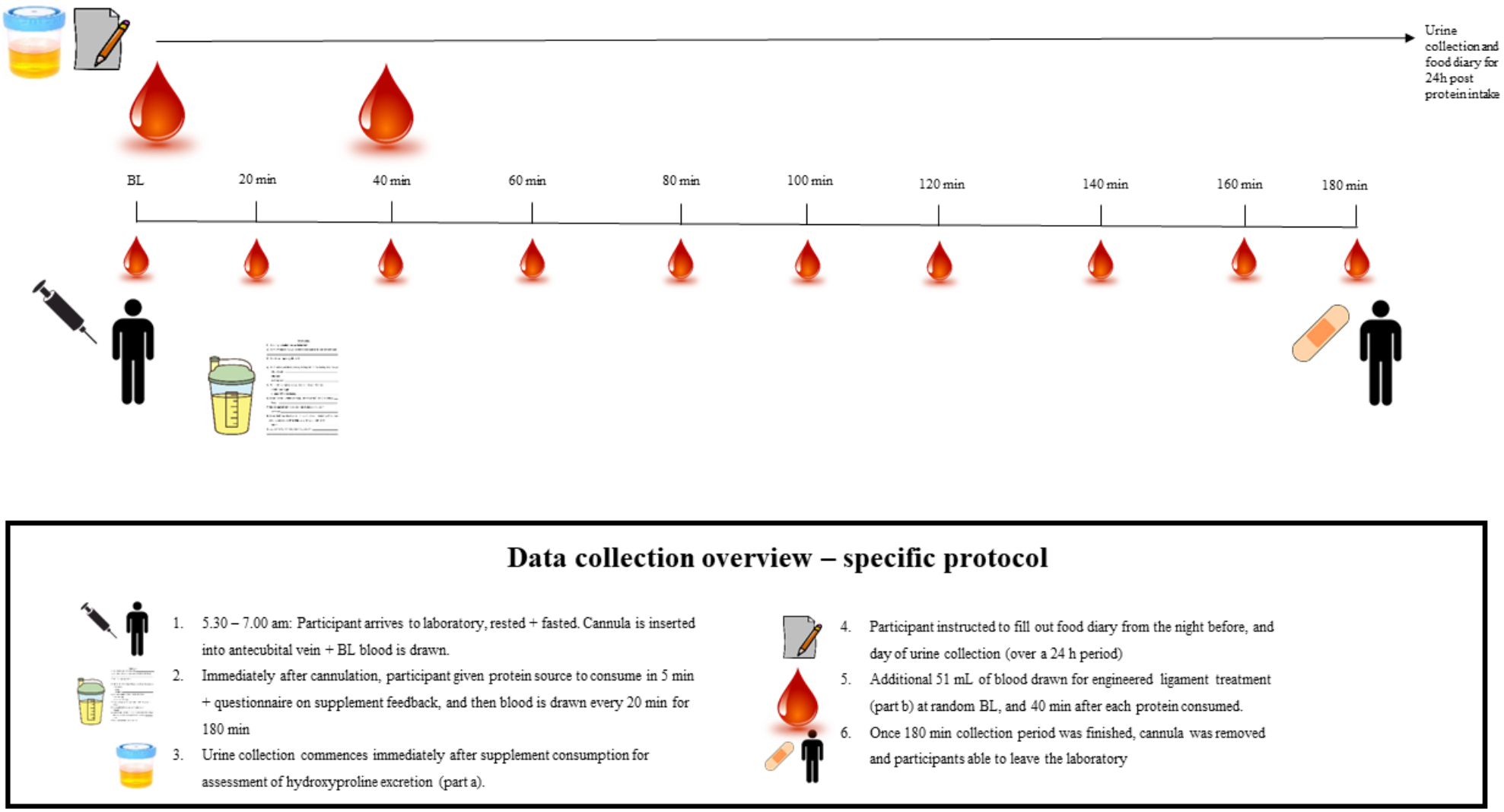

Figure 3.2: Specific data collection protocol.

3.6 Specimen Processing and Analysis

This section will provide extended information in regard to processing and analysis that is not available within some, or all, of the Chapters. Where there is extensive information provided within the respective Chapters, this information has not been reproduced in this section.

3.6.1 AA analysis of protein sources

Protein sources were analysed for full AA profiles at an independent facility (Australian Proteome Analysis Factory, Macquarie University, NSW) using Ultra Performance Liquid Chromatography (Waters AccQTag Ultra, Waters Corporation, MA, USA). Where necessary (such as for bone broth), prior to analysis, samples were briefly warmed to 37°C to liquefy any fatty material and then mixed to provide a homogenous sample. Samples underwent a 24 h liquid hydrolysis in 6 M HCl at 100°C and were analysed in duplicate with results provided as a mean value (acceptance of variation of 10%).

Hydroxyproline analysis

Urinary hydroxyproline was measured using a colorimetric hydroxyproline assay kit according to manufacturer's instructions (abcam, Melbourne, Australia). Briefly, in a counter-balanced order, 100 µL urine was mixed with 100 µL of 10 N NaOH in a pressure-tight vial and hydrolysed at 120°C for 1 hour. Vials were then cooled on ice and neutralised by adding 100 µL of 10 N concentrated HCl. Samples were then decolourised by adding 4 mg of activated charcoal to the neutralised hydrolysate. Vials were then centrifuged at 10,000 xg for 5 min. Collected, clarified supernatant was then evaporated in a 96 well plate (Corning, NY, USA) at 65°C. 100 µL of an oxidation reagent mix was added to each well, and incubated at room temperature for 20 min. 50 µL of development solution was then added to each reaction well

and incubated at 37°C for an additional 5 min. 50 µL DMAB concentrate solution was then added to each reaction well, and the plate was sealed, and incubated at 65°C for 45 min. Absorbance was then measured at 560 nm on a microplate reader (FLOUstar omega, BMG LABTECH Pty. Ltd., Mornington, Vic, Australia). The hydroxyproline concentration was then calculated from a curve of known standards.

3.6.2 Creatinine analysis

Urine was diluted with deionised water 1:20 and then analysed for creatinine by an enzymatic colorimetric assay on an automated clinical chemistry analyser (COBAS Integra 400, Roche Diagnostics, Risch-Rotkreuz, Switzerland) and the Hyp: Cre mmol: mol ratio was calculated.

3.6.3 Plasma AA

For analysis of AA concentrations in food and plasma, samples were sent to M3 Research Unit (Muscle Metabolism Maastricht, Human Movement Sciences, Maastricht University, Netherlands) and analysed using liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). Briefly, samples were prepared according to manufacturer's instructions, with an internal standard added. 50 µL of plasma was deproteinised using 100 µL of 10% sulfosalicyclic acid with 50 µM of metabolomics AA mix standard (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralised water was added and samples were centrifuged. After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 µL of AccQ-Tag derivatising reagent solution (Waters, Saint-Quentin, France) was added, and the solution was heated to 55°C for 10 min. Of this 100 µL derivative 1 µL was injected and measured using UPLC-MS.

Preface to Chapter Four

Chapter Two identified a range of potential sources of collagen precursors within the Australian diet. Bone broth is an "*in vogue"* source of dietary collagen which is touted for its benefits in a range of tissues, including ligaments and tendons. The use of food sources to provide a therapeutic benefit is of obvious interest for sports nutrition professionals given the potential to provide other beneficial nutrients, and to avoid the risk of inadvertent doping. However, there is a lack of research substantiating many of the claims surrounding bone broth. In particular the AA content of this putative collagenous protein source, and how it compares to reference supplements which have preliminary evidence suggesting the capacity to support exercise in the upregulation of collagen synthesis in ligaments and tendons. The following Chapter (Study 1) sought to determine whether bone broth is comparable to reference supplements in its AA content, and whether differences in preparation methods would increase the availability of AA within bone broth.

Chapter Four: Bone Broth is Unlikely to Provide Reliable Concentrations of Collagen Precursors Compared with Supplemental Sources of Collagen Used in Collagen Research

Statement of publication

The chapter is comprised of a currently published manuscript, with slight adjustments made to formatting for the purpose of this thesis. A published version is available in the *International Journal of Sports Nutrition and Exercise Metabolism.*

Alcock RD, Shaw, GC, Burke, LM. Bone Broth Unlikely to Provide Reliable Concentrations of Collagen Precursors Compared With Supplemental Sources of Collagen Used in Collagen Research. *Int J Sport Nutr Exerc Metab*. 2018 Sep 26:1-8. Doi: 10.1123/ijsnem.2018-0139.

4.1 Abstract

Intake of dietary sources of collagen may support the synthesis of collagen in varying tissues, with the availability of key amino acids being a likely contributor to its effectiveness. This study analyzed commonly consumed preparations of bone broth (BB) to assess the amount and consistency of its amino acid content. Commercial and laboratory prepared samples, made with standardized and variable (non-standardized) protocols were analyzed for key amino acids (glycine, lysine, proline, leucine, hydroxyproline and hydroxylysine). The main finding of the study was that amino acid concentrations in BB made to a standardized recipe were significantly lower for hydroxyproline, glycine, proline; $P = 0.003$ and hydroxylysine, leucine and lysine; $P = 0.004$ than those provided by a potentially therapeutic dose (20 g) of reference collagen supplements ($P > 0.05$). There was large variability in the amino acid content of BB made to non-standardized recipes, with the highest levels of all amino acids found within the café prepared varieties. For standardized preparations, commercial BB were lower in all amino acids than the self-prepared varieties. There were no differences ($P > 0.05$) in the amino acid content of different batches of BB when prepared according to a standardized recipe. If the intake of collagen precursors is proven to support the synthesis of new collagen in vivo, it's unlikely that bone broth can provide a consistently reliable source of key amino acids. Focus on the provision of key amino acids from dietary sources should continue to focus on the standard sources currently being researched.

Keywords: proline; glycine; gelatin; protein; tendon; ligament

4.2 Introduction

Collagen is the most abundant body protein, constituting around one third of total protein stores. Within the extra cellular matrix of musculoskeletal and connective tissues, collagen contributes to force transmission and joint stability, providing resistance to forces and sudden directional changes that might otherwise contribute to injury (Baar, 2015). Both the overall collagen content of the tissue and the cross-linking of collagen molecules contribute to a greater level of tissue stiffness and stability (Kadler et al., 2007; Kjaer et al., 2009). Connective tissues with a higher cross-sectional area, and presumably higher collagen content and/or density, may be more resistant to strain and more capable of withstanding higher loads (Couppe et al., 2008). Consistent evidence of a beneficial interaction between exercise and nutrition on skeletal muscle protein synthesis has led to explicit recommendations to optimize this process; these involve the timing of intake and dose of specific amino acids in combination with exercise, with leucine providing a separate stimulus for protein synthesis and other amino acids servicing as precursors for the building of the new muscle proteins (Atherton et al., 2012; Phillips et al., 2011). Whether nutritional strategies can enhance the growth and/or assist in the repair of collagenous tissues is currently under investigation and may have implications both within ageing populations for the treatment of age related declines in muscle tissue (i.e. sarcopenia) (Zdzieblik et al., 2015), and athletic populations for the prevention and treatment of connective tissue injury (Baar, 2015, 2017). Although whey protein has been shown to enhance patella tendon hypertrophy in young males when combined with a 12 week resistance exercise training program (Farup et al., 2014), it is currently unclear whether this resulted from direct upregulation of collagen synthesis or in response to hypertrophy of the quadriceps, due to the provision of key amounts of the amino acid, leucine. Indeed, according to the ‘leucine’ trigger Hypothesis, the provision of targeted amounts (~3 g) of leucine, as found in whey protein, in conjunction with other amino acid building blocks may maximize the synthesis of skeletal

muscle protein in response to exercise. (Atherton et al., 2017; Phillips, 2014). Alternatively, amino acids abundant in collagen protein, such as proline, and glycine have been shown to be essential for the synthesis of new collagen (Li et al., 2018) with in vitro and in vivo work demonstrating the potential benefit of glycine and proline availability at times around exercise when synthetic machinery is up- regulated. Recently, we reported increases in procollagen I Intact N terminal (PINP), following a 3-d protocol involving consumption of 15 $g.d^{-1}$ of gelatin (a dietary form of collagen) and rope-skipping exercise compared to a placebo control. While further in vitro work supported these findings, with higher serum concentrations of glycine and proline increasing the collagen content and mechanical properties of an engineered ligament exposed to a stretch stimulus (Shaw et al., 2017). Meanwhile, other investigations have demonstrated the benefit of hydrolyzed collagen supplementation in a range of collagen containing tissues (Daneault et al., 2017; Oesser et al., 2016; Oesser et al., 2003; Schunck et al., 2013). Although research to date has focused on supplemental and refined sources of collagenous proteins, some cultures and exercise groups (e.g. traditional Chinese medicine and Cross-fit athletes) are known to consume food forms of collagen for therapeutic purposes (Nelson, 2015; Wu et al., 2013). While, it is currently unclear as to which nutrients are specifically involved in promoting the health of collagenous tissues, there is also evidence that sports nutrition professionals are recommending the intake of collagenous protein sources (such as gelatin) to athletes with tendon and ligament injuries to aid in repair and accelerate return to play after injury. The proposed mechanism underpinning such a strategy is that the increase in blood concentrations of the amino acids and related compounds found in collagen will support an increase collagen synthesis (Baar, 2015).

Bone broth (BB) is a dietary collagen source which has received anecdotal support for a variety of health claims, including enhancement of bone, connective tissues, skin and nails. It is made

by simmering bone and cartilaginous meat sources for lengthy periods (> 24 h), purportedly to enable collagen protein and bone-related minerals to be released into the liquid (McGruther, 2014). However, BB composition may vary substantially due to differences in preparation protocols, such as the animal source and type of the bones/meat, the method and duration of cooking, addition of ingredients that may aid the release of key nutrients from animal tissue (e.g. vinegar), and any processing associated with the end product (e.g. dehydration to powder form). We hypothesized that even though BB is considered a single foodstuff that can be used to provide collagen precursors, variability in its production techniques would lead to significant variation in key amino acids.

Accordingly, the aim of the current study was to assess the amino acid composition between and within examples of commonly consumed BB preparations. Although, BB is a source of nutrients suggested to influence connective tissue health, such as saturated fat and copper (Scott et al., 2016), we focused on the provision of amino acids which are putative precursors for collagen synthesis (glycine, lysine, proline, and leucine) or indicators of collagen content; hydroxyproline and hydroxylysine. We compared both the absolute amount and variability of these constituents in a standard serve of bone broth (set at 250 ml) with several supplements that could provide a potentially therapeutic dose of collagen precursors. We chose a therapeutic dose of 20 g for these reference supplements, noting it to be slightly larger than the 15 g dose involved in our earlier studies (Shaw et al., 2017), since amino acid availability has been shown to improve with increased intake (Imaoka et al., 1992; Iwai et al., 2005).

4.3 Methods

We identified a range of preparations that represent typical ways in which BB might be consumed within the general population, including homemade and commercially prepared broths that were made to either standardized or non-standardized protocols. Standardized broths were made to the same recipe (i.e. ingredients and cooking techniques) each time they were prepared, while non-standardized broths varied by one or more of the following: the source of the bone (i.e. chicken vs beef), the type of bone (i.e. standard soup bone vs marrow bones), cooking methods (duration of cooking, stove top vs slow cooker, addition of vinegar). Table 4.1 summarizes the final range of bone broth samples included in the study, along with gelatin and hydrolyzed collagen products which acted as reference supplements.

Samples were prepared or purchased from commercial sources according to the summary (Table 4.1.) Each bone broth was prepared or collected on at least two separate occasions to produce duplicate or triplicate samples of the standardized recipe. The non-standardized bone broths included one which was prepared by a café chef on one occasion and on another by the authors according to the "chef provided generic" recipe, and then deliberately manipulated to change the type of bones or to remove the fat after cooking. In the case of self-prepared broths, the recipe yield was also documented to allow determination of mg of amino acids extracted per kg of bone. Two separate samples of gelatin (varying by brand) and collagen peptide powder (two different batches) were purchased, as well as one sample of liquid collagen and a hydrolyzed collagen powder (different batches were unavailable). In total, 28 samples were analyzed.

Table 4.1: Bone broth sample details and sources.

Source	Type	Sample	Details
Standardized bone broth			
Laboratory prepared	Chicken bone broth	CB	Prepared by author (RDA) as per standardized recipe below, chicken bones
		CBv	As above + addition of vinegar (2 tablespoons)
	Beef bone broth	BB	Prepared by author (RDA) as per standardized recipe below, beef bones
		BBv	As above + addition of vinegar (2 tablespoons)
Broth bliss, South Yarra, VIC, AUS	Long life liquid chicken broth	BBC	Commercial, packaged liquid broth made from whole chicken frames and wings
	Long life liquid beef broth	BBB	Commercial, packaged liquid broth made from beef marrow bones and joints
Broth of life, Cromer, NSW, AUS	Dehydrated chicken broth powder,	BBC	Commercial, powdered broth, made from whole chicken frames and reconstituted according to instructions (3 g/100 ml)
	Dehydrated beef broth powder	BBB	Commercial, powdered broth made from beef marrow bones – neck and leg and reconstituted according to instructions (3 g/100 ml)
Non-standardized bone broth			
Elemental Cafe, Braddon, ACT, AUS	Café prepared beef broth	Ca	Commercial broth, prepared by Café chef each day "to taste" from a general recipe (see below)
Laboratory replication	Café prepared beef broth	Ca(2)	Prepared by author (RDA) from recipe supplied by Café
	Standardised beef broth using different ingredients	BBm(1)	Prepared by author (RDA) from standardized recipe (below), but using beef marrow bones
	Beef broth using different preparation method	BBm(2)	As above, allowed to cool, and fat scraped from the top (to remove fat from the solution).
Reference supplements			
McKenzies, Altona, VIC, AUS	Powdered gelatin	GEL(1)	Commercial gelatin powder used in household cooking/food preparation

Dr Oetker, Mentone, VIC, AUS	Powdered gelatin	GEL(2)	Commercial gelatin powder used in household cooking/food preparation
Tendeforte, Gelita, Eberbach, Germany	Hydrolyzed collagen peptide powder	PEP	Commercial supplement promoted for support of connective tissue
GBR Nutrition, Lancashire, UK	Liquid collagen supplement	LCol	Commercial supplement promoted for anti-ageing and joint protection benefit. 60 ml liquid used to provide 20 g collagen as per manufacturer's instructions
Professional whey, NSW, Australia	Hydrolyzed collagen powder	HYD	Commercial supplement

Standardized bone broth recipe (adapted from wellnessmama.com)

Ingredients	*Method*
2 carrots, roughly chopped 2 celery stalks , roughly chopped 2 medium onion , roughly chopped 7 garlic cloves , crushed 1 kg chicken frames + 500 g wing tips (for chicken broth) or 1.5 kg beef bones (for beef broth) 2 tablespoons apple cider vinegar 1 teaspoon salt	1. Turn on the slow cooker to low 2. Place vegetables on the bottom of the slow cooker 3. Lay bones over the top 4. Top with 2.5 L water, apple cider vinegar and salt 5. Close the lid of the slow cooker, and cook for 24 h 6. After 24 h strain the broth through a sieve to remove any solids

Café bone broth recipe (supplied by chef)

Ingredients	*Method*
~5.5 kg beef marrow bones – neck and leg (5.4 kg used in self-prepared recipe) Cherry tomato* Carrot * Celery * Onion * Garlic* 3-6 tablespoons. apple cider vinegar* 4.8-5.5 L water (enough to cover bones), top up as fluid reduces for final yield of 7.5-8 L *NB. Amounts not specified, used 1 x Punnett cherry tomatoes, and other vegetables as above.	1. Place bones in roasting trays between 190-210 degrees for 20-35 mins, until bones are roasted but not burnt 2. Remove bones from oven, add to pot with vegetables and water 3. Place pot on the stove and bring to a simmer 4. Remove from the stove and place into electric oven at ~95 degrees 5. Cook for 72 h, checking occasionally to determine if fluid requires topping up. 6. After 72 h, strain liquid, and set in fridge 7. Place bones back into pot, and cover with water, cook for an extra 24 h 8. Mix with liquid from first batch, place liquid back on the stove with extra carrot, onion, celery, bay leaves, thyme and peppercorns (to taste). 9. Cook until liquid is reduced back down to 7.5-8 L. 10. Strain broth through a sieve to remove any solids

Once prepared/obtained (see Table 4.1 for further details), BB were mixed to ensure a homogenous sample, before transferring 50 mL of each liquid to a collection pot for freezing at -20°C. With the exception of the dehydrated bone broth powder, which was prepared according to the manufacturer's instructions and analyzed as a liquid, powdered products were measured into 10 g samples. Twelve hours prior to transportation, samples were transferred to a -80°C freezer before being couriered on dry ice to an analytical laboratory (Australian Proteome Analysis Factory, Macquarie University, Sydney, NSW) where they were stored at -20°C freezer until analysis.

Full amino acid profiles were analyzed using Ultra Performance Liquid Chromatography (Waters AccQTag Ultra). Where necessary, prior to analysis, BB samples were briefly warmed to 37°C to liquefy any fatty material and then mixed to provide a homogenous sample. Samples underwent a 24 h liquid hydrolysis in 6 M HCl at 100°C and were analyzed in duplicate with results provided as a mean value (acceptance of variation of 10%). For the purposes of this study only selected amino acids (glycine, lysine, proline, leucine, hydroxyproline and hydroxylysine) are reported. Full amino acid profiles can be found in supplementary Table 11.2.1.

All data are reported as a range, except where only one sample was analyzed. Data were analyzed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA). After visually assessing for normality using a Q-Q plot, a Kruskal-Wallis test followed by Dunnett's multiple comparisons test was utilized to compare the means values from standardized and non-standardized bone broths against the reference supplements. Unpaired t-tests were used to compare batches of the standardized broths. Statistical significance was set at $P< 0.05$.

4.4 Results

Table 4.2 summarises the amino acid content per typical serve of product, and where applicable, the amount (mg/kg) of amino acid extracted from the bone. Comparisons between standardized and non-standardized BB and the reference supplements (Figure 4.1.) revealed that standardized preparations were significantly lower in amino acid content than the reference supplements (hydroxyproline, glycine, proline; $P = 0.003$ and hydroxylysine, leucine and lysine; $P = 0.004$), while the non-standardized preparations were similar to the reference supplements ($P > 0.05$). Non-standardized BB had greater variability in amino acid content than standardized broths or reference supplements and the highest levels of amino acids across all samples were present in the café prepared bone broths, although the exact factors contributing to this outcome are unknown (Table 4.2.). For the standardized BB: commercial varieties were lower in all amino acids than the self-prepared varieties. There were no differences ($P > 0.05$) in the amino acid content of different batches of the same bone broth when prepared according to a standardized recipe. While none of the BB or collagen supplements were able to provide a therapeutic dose of leucine; indeed, most were below 500 mg, and all were below 2 g (Figure 4.1.).

The addition of vinegar did not influence the amino acid content of the broths. Meanwhile the source of the bones was associated with differences a higher amino acid content for all amino acids for the laboratory-prepared broths (beef > chicken), but not for the commercially sourced broths. Differences in the type of bones (long bones containing marrow vs smaller bones) altered the amino acid content (“marrow bones” > normal bones), while the removal of the fat from bone broth reduced the amino acid content (Table 4.2.).

Table 4.2: Range of amino acid (mg) provided per serve of laboratory prepared and commercial varieties of bone broth and reference supplements and per kg of animal tissue for laboratory prepared varieties

mg of Amino acid provided per serve								
Bone broths-standardized recipe								
	Self-prepared				**Commercial**			
	CB	**BB**	**CBv**	**BBv**	**BBC**	**BBB**	**BOLC**	**BOLB**
No of samples	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
HYP	573-635	918-975	443-610	790-908	385-445	470-500	220-246	230-279
GLY	1018-1108	1633-1713	780-1063	1405-1598	685-783	830-878	432-477	470-532
PRO	628-680	1000-1048	480-650	848-968	428-480	503-538	288-316	314-342
HYL	78-83	125-135	60-80	113-130	55-65	55-58	27-29	29-31
LYS	288-300	343-368	218-280	293-328	195-203	180-188	186-211	170-195
LEU	265-268	370-398	190-250	318-353	170-188	178-185	201-218	197-232
Bone broths – non-standardized recipe					***Reference supplements***			
	Ca	**Ca(2)**	**BBm(1)**	**BBm(2)**	**GEL**	**PEP**	**Lcol**	**HYD**
No of samples	**3**	**1**	**1**	**1**	**2**	**2**	**1**	**1**
HYP	2825-5450	2250	850	625	2010-2030	2026-2100	1770	2260
GLY	4900-9450	3925	1500	1100	3610-3642	3726-3694	3204	3830
PRO	2975-5850	2400	1048	675	2246-2282	2296-2332	2028	2322
HYL	275-475	250	125	100	186-190	182-186	162	168
LYS	850-1475	650	275	200	680-610	652 3	552	678
LEU	850-1650	675	300	225	498-508	504-504	444	532

mg of Amino acid extracted per kg of animal tissue

	CB	BB	CBv	BBv	Ca(2)	BBm(1)	BBm(2)
HYP	3817-4233	6117-6500	2950-4967	5267-6050	10000	5667	4167
GLY	6783-7383	10883-11417	5200-7083	9367-10650	17444	10000	7333
PRO	4183-4533	6667-6983	3200-4333	5650-6450	10667	6167	4500
HYL	517-550	833-900	400-533	750-867	1667	833	667
LYS	1917-2000	2283-2450	1450-1867	1950-2183	2889	1833	1333
LEU	1767-1783	2467-2650	1267-1667	2117-2350	3000	2000	1500

HYP: Hydroxyproline; GLY: Glycine; Pro: Proline; HYL: Hydroxylysine; LYS: Lysine; LEU: Leucine.

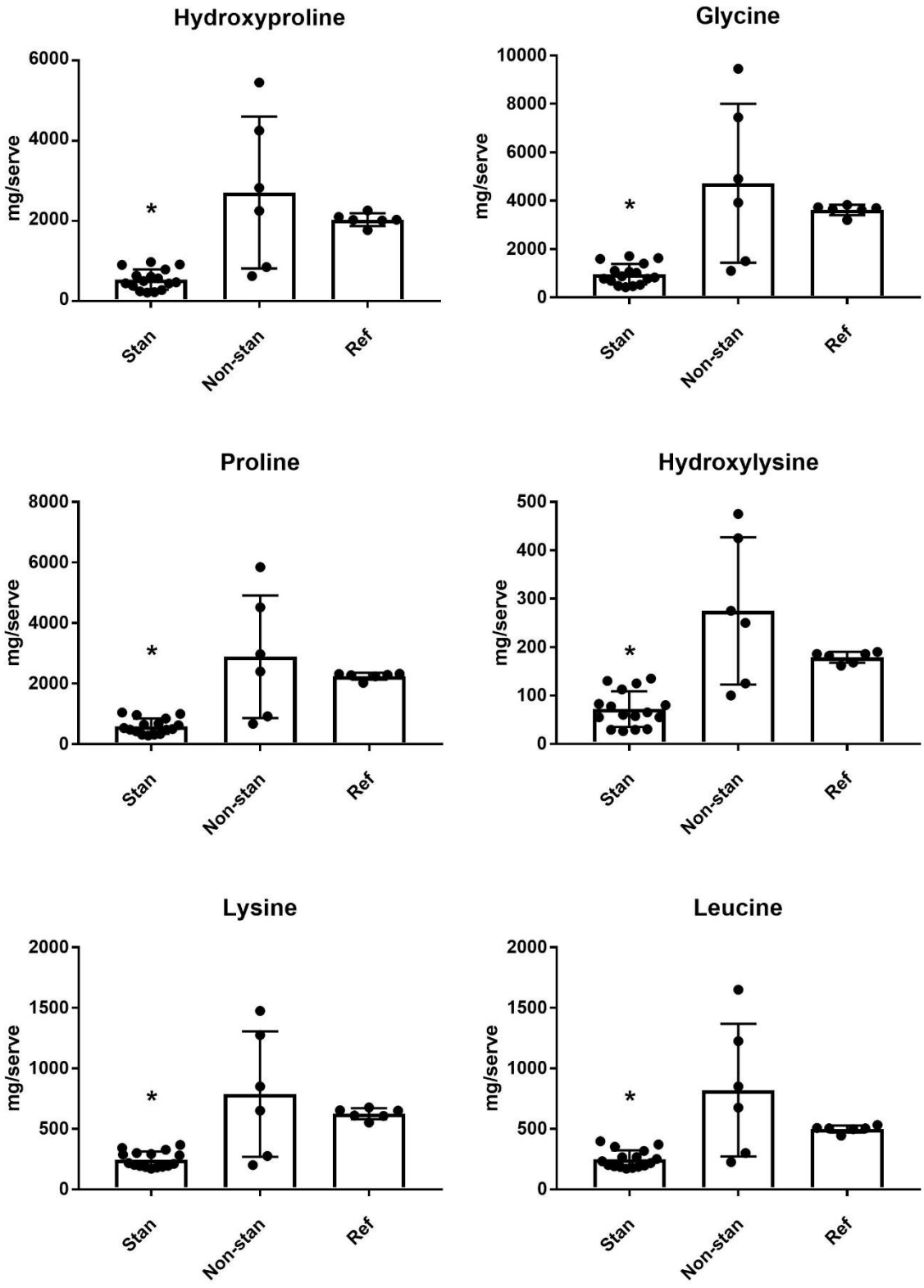

Figure 4.1: Kruskal-Wallis test comparing standardized (Stan) and non-standardized (Non-Stan) broths to reference standards (Ref). *P <0.05 vs Ref

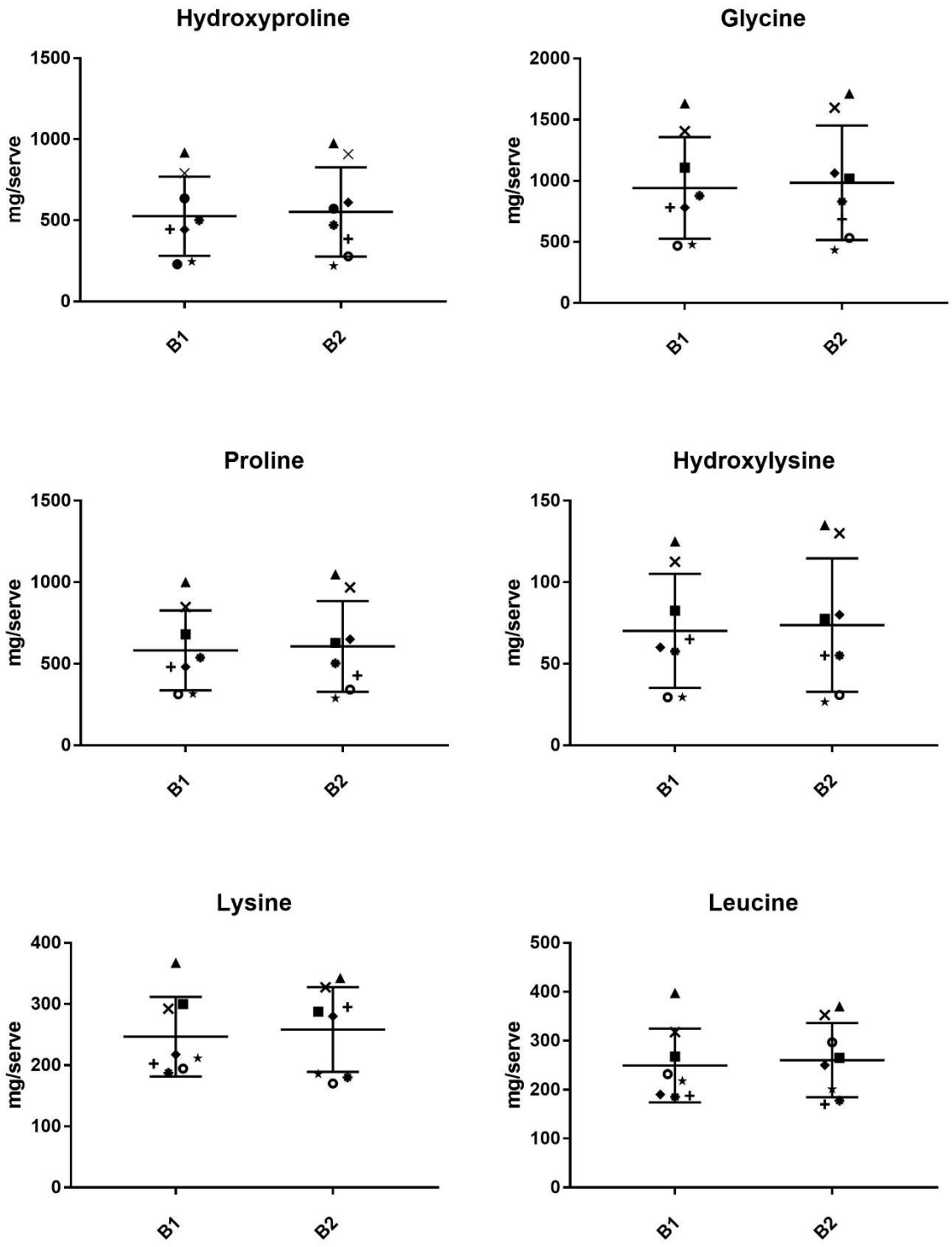

Figure 4.2: Unpaired t-test between batch 1 (B1) and batch 2 (B2) of laboratory prepared bone broth

4.5 Discussion

This study addressed the potential role of BB as a dietary source of amino acids necessary for collagen synthesis. We compared the content of specific amino acids (glycine, lysine, leucine and proline) and collagen components (hydroxyproline and hydroxyproline) within a typical serve of homemade and commercial BB preparations in comparison to reference supplements which have been used in existing collagen synthesis research. Our chief findings were 1) that a typical serve of BB, made according to standardized protocols, provided these constituents in amounts that were consistent but below those found in supplemental products supplying a 20 g therapeutic dose. 2) While some batches of the non-standardized BB had comparable amino acid profiles to supplemental products, their composition was varied (Figure 4.1.). Indeed, the variability of the amino acid content of the non-standard broths was greater than that of the standardized preparations (Figure 4.2.). 3) BB and supplements targeting collagen synthesis are low in leucine, the amino acid that has been proven to promote muscle protein synthesis. Therefore, if a therapeutic dose of 20 g of collagenous protein is proven to support the health of collagenous body tissues, it is unlikely that BB could provide a reliable source for such treatment due to generally low levels of key amino acids in a standard serve and the inconsistency of its ability to provide a therapeutic dose when some, as yet unidentifiable, factors are involved in its preparation. Furthermore, all dietary and/or supplemental sources of collagenous tissue are inadequate in providing the amino acid known to promote the activation of protein synthetic machinery and enhance actual protein synthesis within skeletal muscle.

While definitive evidence for the effectiveness of specific amino acids on connective tissue health and performance is still emerging, we were interested to explore a parallel to the story of skeletal muscle, exercise and protein intake. Evidence to support the consumption of protein for skeletal muscle growth has evolved through a hierarchy of sources: from purified amino acids (i.e. leucine) to intact protein (i.e. whey) to everyday food sources (i.e. chocolate milk)

(Lunn et al., 2012). Specifically, we were interested to see if a culturally valued food source (BB) which has anecdotal support for a variety of health claims (McGruther, 2014) could provide a practical form of the nutrient intake recommendations that may emerge from collagen research. We recognize that there are other scenarios in which food sources of nutrients are unable to support optimal protocols of use. These include creatine supplementation in which the typical provision from meat is too low to meet targets for rapid loading (Harris et al., 1992) and caffeine, whose concentration in coffee is too variable and unpredictable to make it an optimal choice for targeted use as an aid for sports performance (Desbrow et al., 2012) . Although findings within the current study indicate that it is possible to obtain leucine, collagenous amino acids (glycine, proline and lysine) and other factors (hydroxylated proline and lysine), similar to that provided by a 20 g dose of reference supplements promoted for collagen therapy, it is challenging to determine which characteristics of ingredient or preparation lead to the greater amino acid content.

Our results demonstrate that the source of animal tissue utilized influenced the amino acid concentration of the end product, with beef providing higher amino acid concentrations for all amino acids than chicken for the standardized laboratory prepared varieties of BB (Table 4.2.) irrespective of the volume of animal tissue used. Different characteristics of the bones such as amount of meat, fat or marrow remaining on the bone after butchering may have accounted for this outcome. For example, marrow is proposed to be a rich source of collagen and therefore utilizing bone with more marrow would theoretically result in a higher amino acid concentration (McGruther, 2014). Meanwhile, the presence of fat (of which animal skin is a rich source) may alter the concentration of amino acids. In the current study, when a BB was prepared to a standardized recipe, changing only the type of bone (i.e. marrow vs. standard butcher bone with minimal marrow) we found similar, if not lower values of amino acids (Table

4.2). However, leaving a BB preparation “as is” (with the fat still included) resulted in a high concentration of amino acids than when the fat was removed (Table 4.2.).

Recent literature suggests that adding ingredients (i.e. vinegar) suggested to provide a more acidic environment to BB, or prolonging the cooking duration, may assist with the extraction of nutrients into BB (Hsu et al., 2017). In this instance, the addition of vinegar did not appear to have any effect on amino acid content of the broth. However, the BB associated with the greatest content of amino acids (Café prepared, non-standardized) was reportedly cooked for 72 h, in comparison to standardized BB which were cooked for 24 h. This suggests longer cooking times may increase amino acid extraction; indeed, when the café prepared broth recipe was replicated in the lab under standardized conditions, the amino acid content was lower than the purchased form, but higher than that of 24 h-cooked preparations (Table 4.2). This finding is supported by the comparison of amino acid content of broths obtained per kg of animal tissue used in the recipe (Table 4.2.) and indicates that some unidentified factor may have been involved in the preparation of the Café product.

We acknowledge that we have compared the broth contents against a 20 g serve of supplemental collagen, a dose currently unproven in the literature as an optimal dose. Indeed, the manufacturer’s instructions related to these reference products recommended intakes of 15-30 g (hydrolyzed collagen) and 10 g (liquid collagen) as a single dose, while our previous study which demonstrated benefits of gelatin intake, provided a 15 g dose (Shaw et al., 2017). It should be noted, however, that our previous work focused on providing a dose of gelatin that could be blinded in comparison to a placebo, rather than the dose providing an optimal amount of key amino acids. Nevertheless, previous evidence supports an increasing amino acid availability with the ingestion of greater amounts of gelatin (Iwai et al., 2005) and we chose

20 g of our reference collagen supplements as a balance between optimal availability, practicality and palatability. Although it could be possible to achieve similar amino acid intake by consuming larger volumes of BB, practical considerations may be self-limiting. For example, it is unlikely that an athlete would regularly consume 500 ml of beef broth in the 30 min period prior to exercise should future work show that the combination of increased blood content of collagen containing amino acids (through dietary intake) and increased blood flow to ligament/ tendon (via exercise) enhance the synthesis of collagen in these tissues.

In conclusion, this study demonstrates that it is possible to consume similar amounts of key amino acids found in collagen protein from certain BB preparations in similar amounts as those provided in a therapeutic dose of collagen supplements, however it is an unlikely and potentially unrepeatable outcome. There is significant variability in the amino acid content of various forms of BB, changing according to differences in ingredients and preparation techniques. Indeed, the amino acid content of a range of home-made and commercial forms of BB were consistently lower than our reference supplements. Whilst a BB prepared in a local café was found to provide concentrations of amino acids equal to, or higher than those found in the selected reference supplements, we were unable to match these levels when prepared to a recipe provided by the café in our laboratory. This suggests that compared to supplemental sources of collagen protein it is unlikely that BB will be able to provide a consistently reliable and optimal source of collagen-derived amino acids. Furthermore collagenous foods and supplements are a poor source of leucine.

Novelty statement

This is the first study to consider the ability of bone broth to provide a potentially therapeutic dose of key amino acids to support collagen synthesis, as per the popular claims for this food source.

Practical application statement

Currently available commercial and self-prepared forms of beef broth are unlikely to provide a reliable source of key amino acids in doses simulating those supplements specifically targeting the support of collagen synthesis. If a therapeutic dose of these amino acids can be defined, further work will be needed to find standardized sources capable of replicating these amino acid contents. Presently, despite its promoted benefits, bone both does not appear to provide a consistent or optimal source of such nutrient support.

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Author Contributions

R.D.A, L.M.B and G.C.S were involved in study design, results interpretation, drafting, reviewing and revising the initial manuscript. R.D.A. was involved in sample preparation and

statistical analysis.

Conflicts of Interest

The authors declare no conflict of interest.

Preface to Chapter Five

Hydroxyproline is a metabolite of proline and is a significant component of collagen protein, but cannot be incorporated into the synthesis of new collagen. Previously, hydroxyproline has been utilised to assess collagen metabolism disease, with the restriction of the intake of dietary collagen to prevent an interference with the interpretation of data. For Study 2 (Chapter Five), we were interested to determine if hydroxyproline could be utilised as a biomarker of collagen intake within “free-living”, healthy, active males. If found to be a sensitive biomarker, urinary hydroxyproline could be assessed as a proxy for background intakes of collagen, which are otherwise unable to be quantified due to the lack of food composition data on dietary collagen sources. This would in-turn allow for more accurate interpretation of dietary intervention studies looking to assess the impact of dietary collagen intake on the health and functioning of dense connective tissue.

Chapter Five: Urinary Hydroxyproline is Only Suitable as a Biomarker for Acute Intake, Up to 6 hr Post ingestion of Collagen Proteins in "Free-Living", Healthy, Active Males

Statement of publication

The chapter is comprised of a currently published manuscript, with slight adjustments made to formatting for the purpose of this thesis. A published version is available in the *International Journal of Sports Nutrition and Exercise Metabolism.*

Alcock, R. D., Shaw, G. C., Tee, N., Welvaert, M., & Burke, L. M. Urinary Hydroxyproline Is Only Suitable As a Biomarker for Acute Intake, Up to 6 hr Post ingestion of Collagen Proteins in "Free-Living", Healthy, Active Males. *Int J Sport Nutr Exerc Metab.* 2019 29(5), 461-465. doi:10.1123/ijsnem.2019-0024

5.1 Abstract

Urinary excretion of hydroxyproline (Hyp), abundant in collagen protein, may serve as a biomarker of habitual collagen intake, assisting with investigations of the current interest in the role of dietary collagen intake in supporting the synthesis of collagenous body tissues. This study investigated the time course of urinary Hyp excretion in "free-living", healthy active males following the ingestion of a standardized bolus (20 g) of collagenous (gelatin and gelita) and dairy (calcium caseinate and hydrolyzed casein) proteins. Excretion of Hyp was assessed over a 24 h period, separated into 0-6, 6-12 and 12-24 h collection periods. Hyp was elevated for 0-6 h after consumption of collagen-containing supplements (gelatin, 31.3 ± 8.8 mmol: mol and hydrolyzed collagen, 33.7 ± 22.0 mmol: mol vs baseline (BL); 2.4 ± 1.7 mmol: mol and 2.8 ± 1.5 mmol: mol, $p < 0.05$) but not for dairy protein supplements (calcium caseinate, 3.4 ± 1.7 mmol: mol and hydrolyzed casein, 4.0 ± 3.7 mmol: mol, $p > 0.05$). Therefore, urinary Hyp reflects acute intake of collagenous protein but is not suitable as a biomarker for quantifying habitual collagen intake, provided through regular dietary practices in "free-living", healthy, active males.

Keywords: Gelatin, nutrition, connective tissue, ligament, tendon

5.2 Introduction

Musculoskeletal injuries are a common occurrence within athletic populations and may place a significant burden on the professional athlete and/or their respective team (Kreisfeld et al., 2014). Collagen, which makes up approximately one third of total body protein (Shoulders et al., 2009), serves as a key tensile element within connective and structural tissues such as muscles, ligaments, tendons and bone, due to cross-linking within its triple helical structure (Kadler et al., 2007). Although in its infancy, developing research suggests that it may be possible to nutritionally support collagen synthesis, potentially enhancing the growth and/or repair of connective tissues (Curtis, 2016). Among the nutrients known to be involved in collagen synthesis, there is emerging evidence for the key roles played by amino acids abundantly present in collagen protein (such as proline and glycine) as well as unique di- and tri-peptides (Baar, 2017; Curtis, 2016; Schunck et al., 2013; Shaw et al., 2017; Smith et al., 2007). This has contributed to the marketing of "collagen support" products for tendon/ligament health or repair, with guidance that athletes should consume supplemental or dietary sources of collagen, peptides and specific amino acids in concert with exercise that stimulates blood flow to the specific tissues (Baar, 2017). Furthermore, it has been suggested that hydrolyzed collagen may be superior to un-hydrolyzed forms in its role in collagen metabolism and provide a more timely dose of amino acids and/or peptide (Schunck et al., 2013). Although further evidence is needed to support the benefits of these strategies, adjunct research, and research tools, are needed to understand background dietary intakes of collagenous proteins. A sensitive and reliable biomarker of habitual collagen intake would allow for improved epidemiological research investigating correlations between connective tissue health and optimal intakes in various populations. Current consumer preferences within Western societies (such as Australia) for leaner, filleted cuts suggest that the intake of collagen from traditional dietary sources is low (Williams et al., 2013). However, the use of collagen-

derived gelatin in food technology as a texture modifier is widespread but unquantified. Indeed, even the most comprehensive food composition databases do not provide complete information of collagen, gelatin, or hydroxyproline (Hyp) content in commonly consumed foods (Gomez-Guillen et al., 2011; Khong et al., 2016; USDA ARS, 2018). This renders the use of dietary analysis for identifying habitual or period-specific intakes of collagenous proteins to, at best, a qualitative tool.

Hyp, a metabolite of proline which constitutes ~ 14% of the total amino acid content of collagen protein, has historically been measured in urine to measure collagen turnover and/or perturbations to collagen metabolism (Prockop et al., 1961). In such cases, collagen intake prior to testing is restricted to prevent its contribution to Hyp excretion from interfering with the validity and interpretation of data (Sjoerdsma, 1965). More recently, exercise has been suggested to lead to acute collagen protein turn-over which may lead to a transient increase in the excretion of Hyp (Tardioli et al., 2012). However, to date, no studies have determined if urinary Hyp is a reliable and valid biomarker of habitual or an acute intake of collagen in "free-living" individuals, engaging in normal dietary and exercise practices. Accordingly, the aims of the current study were to determine the time course of urinary Hyp excretion over a 24 h period following the intake of 20 grams of either collagen or dairy proteins, and a habitual diet to determine whether urinary Hyp is a suitable marker of habitual or acute intake of dietary collagen protein in "free-living" healthy, active males.

5.3 Methods

Subjects and study overview

Eight healthy, male subjects (31 ± 5 years; 79 ± 10 kg body mass) with no current collagen-related disease or known collagen or milk protein allergies were recruited for the study. Approval was granted by the Australian Institute of Sport Ethics Committee (20170607) and written consent was received from participants prior to commencing the study. A randomized, cross-over design was utilized, with at least 48 h between trials.

Supplement selection, preparation and consumption

The current study was carried out during part of a larger research project investigating plasma amino acid availability after the consumption of various protein sources (Alcock et al., 2019). For the current study data were collected after the consumption of four different protein sources, and two occasions where a normal breakfast was consumed (BL) in a counterbalanced, randomized order, with a minimum of 48 h washout period between trials. The protein sources included two collagen supplements: gelatin (McKenzie's gelatin, Altona, VIC, Australia), and a hydrolyzed collagen powder (Tendeforte, Gelita, Eberbach, Germany), and two dairy protein supplements that did not contain any collagen: calcium caseinate and hydrolyzed casein (both from Professional whey, NSW, Australia). Participants reported to the laboratory between 5-7 am after a > 10 h overnight fast and were given five minutes to consume 20 g of supplement dissolved in 300 mL of warm water. To standardize gastric emptying, no further fluid was allowed for 60 minutes after the supplement ingestion and no food was consumed, or exercise performed for 3 h, after which participants returned to their normal dietary and exercise habits. The dose of 20 g was chosen to build on our previous work (Shaw et al., 2017) and to increase the availability of key amino acids as per other dosing studies (Ichikawa et al., 2010; Imaoka et al., 1992). Supplements were analyzed at an independent facility (Australian Proteome

Analysis Factory, Macquarie University, Sydney, NSW) for levels of Hyp via Ultra Performance Liquid Chromatography (Waters AccQTag Ultra, Waters Corporation, MA, USA).

Urine collection and analysis

Following the ingestion of the supplement, urine was collected into clean plastic bottles (2 L capacity) for a period of 24 h, divided into three collection periods (t = 0-6, 6-12 and 12-24 h). The subjects also completed 24 h urine collections on 2 baseline (BL) days when no supplement was consumed, and normal eating patterns were maintained using breakfast as t = 0 and continuing the same collection periods. Subjects were instructed to keep urine in a cool, dark place and return samples to the laboratory as soon as possible after collection. Urine volumes were measured, and aliquots of each sample were stored at -80°C until analysis. Urinary Hyp was measured using a colorimetric hydroxyproline assay kit, according to manufacturer's instructions (abcam, Melbourne, Australia). Since the Hyp: creatinine ratio is a commonly accepted way to standardize the reporting of Hyp values Hyp: Cre, creatinine (Cre) was analyzed on a COBAS Integra 400 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and the Hyp: Cre mmol: mol ratio was calculated.

Food, fluid and exercise diaries

At the commencement of the study, subjects were instructed by a Sports Dietitian to maintain and record habitual dietary and exercise practices for specific periods. Participants were instructed to record their meal on the night prior to each trial, and to record their food and fluid intake during the period of the 24 h urine collection. Food diaries were analyzed for total protein intake (g/ meal and g/day respectively) using dietary analysis software (Foodworks v9, Xyris Software, Australia). As the excretion of Hyp has been shown to be upregulated (>24 h)

following exercise (Brown et al., 1997), exercise in the 24 h period prior to collection, was recorded by participants in terms of the duration of the bout and a sessional rating of perceived exertion (RPE). A standardized metric to describe/compare exercise sessions was then calculated multiplying these factors (Foster et al., 2017).

Statistics

We assumed that a 5 mg change in urinary Hyp excretion represented a minimal worthwhile change based on previous variability estimates of Hyp excretion (Prockop et al., 1961). The simulated power to detect a significant effect while allowing for a 5% Type I error rate based on 1000 replications of our study design with 8 participants was calculated to be 94. The data were analyzed with a General Linear Mixed Model using the R package lme4 (Bates et al., 2015; R Core Team, 2017). A random intercept for subjects was included to account for intra-individual dependencies and inter-individual heterogeneity. This also allowed for individual baseline adjustment. All models were estimated using Restricted Maximum Likelihood. Assumptions of normality and homoscedasticity were assessed via visual inspection of residual QQ-plots. The plots showed signs of heavy tails in the distribution, but this was deemed to be within the margin of deviation to which the model is known to be robust (Jacqmin-Gadda et al., 2007). To account for a potential distributional impact, confidence intervals were generated using a bootstrapping procedure (B. et al., 1994). P-values were obtained using Type II Wald F tests with Kenward-Roger degrees of freedom as implemented in the R package car (Fox et al., 2011). Results are reported as mean estimates and 95% confidence intervals.

A Pearson's correlation was performed to determine the association between exercise in the 24 h prior to urine collection and the 0-6 h excretion of urinary Hyp. Differences in protein intake the night prior and the day of urine collection were analyzed using a One way Anova. Both

analyzes were made using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California, USA).

5.4 Results

Collagen containing supplements provided 2010 and 2026 mg of Hyp for gelatin and hydrolyzed collagen, respectively. Full amino acid profiles are available in Supplementary table 11.2.1. Hyp was not detected in the dairy protein supplements. The excretion of Hyp normalized to creatinine is displayed in Figure 5.1. Urinary Hyp remained stable for both BL measures throughout all of the collection periods (3.0 ± 0.9 mmol: mol). Hyp was elevated for 0-6 h following the consumption of collagen proteins (gelatin: 31 ± 8.8 and hydrolyzed collagen 33.7 ± 22.0 mmol: mol) compared to BL values of the same time period (2.6 ± 0.2 mmol: mol) ($p < 0.05$). Furthermore, there was considerable variability in the excretion of Hyp between individuals ranging from 22.9 to 47.5 mmol: mol and 2.0 to 50.5 mmol: mol, 0-6 h after the consumption of gelatin and hydrolyzed collagen, respectively These levels returned to BL values by the 6-12 h collection period ($p > 0.05$). Dairy protein supplements (calcium caseinate and hydrolyzed casein) did not elevate Hyp excretion at any time point compared to BL values ($p > 0.05$). There was no difference in urinary Hyp excretion between participants for any of the BL measures ($p > 0.05$).

Dietary protein intake (night before and day of urine collection) and sRPE are displayed in Table 5.1. There was no difference in protein intake the night before, or the day of urine collections for any conditions ($p > 0.05$). Although there was large variation in sRPE across different conditions exercise in the 24 h prior to urine collection was not correlated with the 0-6 h excretion of Hyp for either dairy supplements, collagen containing supplements, or BL measures ($p >0. 05$).

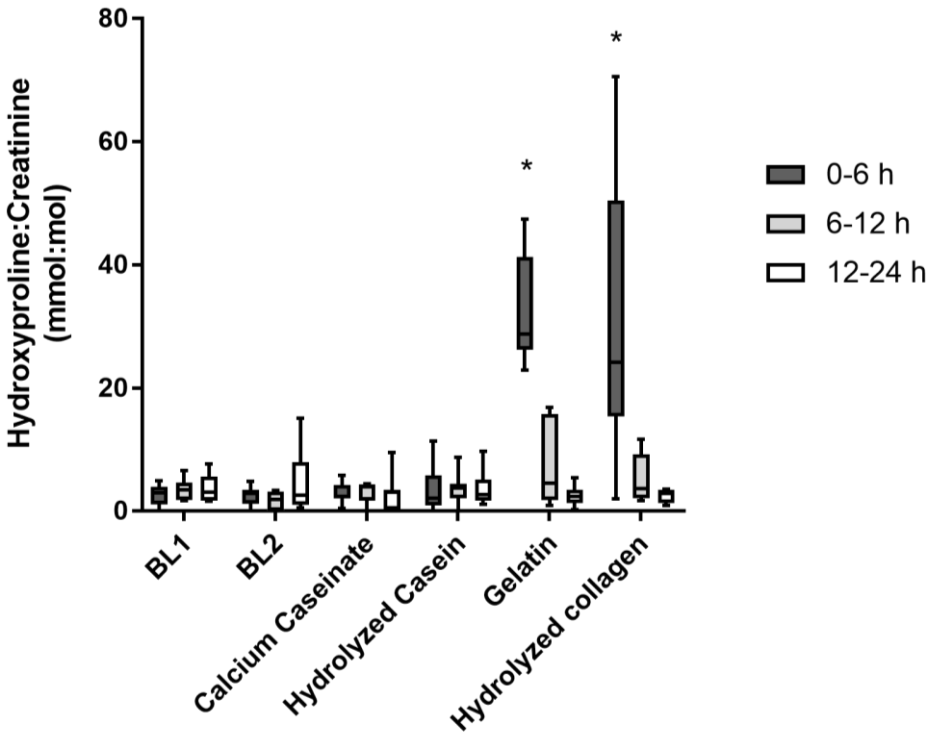

Figure 5.1: Urinary excretion of hydroxyproline (normalized to creatinine) over 0-6, 6-12 and 12-24 hours after the consumption of a habitual breakfast (BL1 and BL2) or 20 grams of either collagen containing (gelatin & hydrolyzed collagen) or, dairy (calcium caseinate & hydrolysed casein) supplements. Line shows median, boxes represent 25th-75th percentiles and whiskers show maximum and minimum values. * P< 0.05 compared to 0-6 h BL1 and BL2.

Table 5.1: Dietary protein intake and exercise

	Protein intake			**Exercise (n = 8)**
	Day of urine collection		**Night prior to urine collection**	**In the 24 h prior to urine collection**
Mean ± SD	**Grams/kg BM/day**	**Grams/day**	**Grams/meal**	**sRPE x duration**
BL1	1.8 ± 0.7 (n=7)	146 ± 62 (n=7)	60 ± 27 (n=7)	175 ± 300
BL2	1.6 ± 0.4 (n = 7)	127 ± 36 (n=7)	51 ± 19 (n=7)	707 ± 616
Calcium caseinate	1.7 ± 0.5 (n=7)	138 ± 49 (n=7)	62 ±16 (n=7)	405 ± 562
Hydrolysed casein	2.0 ±0.7 (n =6)	159 ± 37 (n=6)	68 ±17 (n=7)	444 ± 689
Gelatin	1.6 ±0.5 (n=6)	121 ± 35 (n=6)	62 ± 38 (n=6)	647 ± 1167
Hydrolysed collagen	2.0 ±0.6 (n=7)	158 ± 47 (n=7)	68 ± 22 (n=5)	84 ± 143

5.5 Discussion

Our findings demonstrate that urinary Hyp is elevated for the period of 0-6 h after ingesting a 20 g dose of collagen in supplemental form (~ 2000 mg of Hyp), with levels returning to BL within 12 h. There was no difference in the excretion of Hyp when dairy protein supplements or when a "normal breakfast" was consumed. Additionally, there was no difference in protein intake for either the night prior to or the day of urine collections for any of the collection periods, which would indicate normal dietary intake of protein was not responsible for the differences observed in urinary excretion of Hyp. Our results showed no association between exercise in the previous 24 h period and the 0-6 h Hyp excretion for either BL measures or when a dairy or collagen protein was consumed. Therefore, in practical terms, while urinary Hyp may serve as a suitable biomarker for the ingestion of a single large serving (>20 g) of collagenous protein for 0-6 h post ingestion in free-living individuals, on waking, urinary Hyp is unlikely to be a suitable measure of habitual intake or collagen consumed > 6 h prior. Additionally, a 24 h urine collection is only likely to document the intake of a substantial dose of collagen consumed within a relatively short period before collection, which is unlikely to be achievable through normal dietary sources (e.g. meat) (USDA ARS., 2018).

Of interest, we found considerable inter-individual variability in the excretion of Hyp 0-6 h after the consumption of collagen proteins. While it has been suggested that exercise may increase collagen turnover, and thereby the excretion of Hyp (Brown et al., 1997), despite the large variability in the exercise patterns reported by individuals during the collection periods, we did not find any association between exercise over the 24 h prior to urine collection and the excretion of Hyp in the following 0-6 h. Similarly, although previous data suggests that smaller, regular intake of collagenous proteins may lead to a cumulative rise of plasma Hyp (Knight et al., 2006), the normal dietary practices of our participants (BL) failed to show any changes in

urinary Hyp. This suggests habitual intake of collagenous protein sources in our population were not substantial enough to influence Hyp excretion. Indeed, current consumer trends indicate a preference for meats void of large amounts of collagenous tissue, containing ~ 0.1 mg/ 100 g of Hyp (USDA ARS, 2018; Wong et al., 2013). It is possible that individual differences in capacity to metabolize collagenous protein exists and may account for the differences of excretion found within individuals (Gardner, 1988). This may be an important area for further study, as difficulty in metabolizing collagen proteins consumed specifically for health and rehabilitation purposes may affect the availability of amino acids proposed to play a role in the synthesis of new collagen.

In summary, while urinary Hyp is a suitable biomarker of bolus collagen intake for up to 6 h after its consumption, Hyp is not suitable as a sensitive biomarker for habitual intake of dietary collagen in "free-living" individuals.

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Author Contributions

R.D.A, N.T, L.M.B and G.C.S were involved in study design, results interpretation, drafting, reviewing and revising the manuscript. R.D.A and N.T were involved in data collection and

data analysis, and R.D.A and M.W were involved in statistical analysis.

Conflicts of Interest

The authors declare no conflict of interest

Preface to Chapter Six

As outlined in Chapter Two, there is evidence that nutrition plays a supportive, rather than central role in the synthesis of collagen protein within ligaments and tendons. However, it has been suggested that hydrolysed forms of proteins are superior to their non-hydrolysed counterparts because they deliver potentially beneficial peptides to the target tissue in a more rapid and pronounced manner. Thus, the premise of the following Chapter (Study 3) was to provide information on the plasma AA responses to the consumption of a selection of dairy and collagen protein sources. A direct comparison between hydrolysed and non-hydrolysed forms of some proteins was planned to determine whether the form influenced the apparent bioavailability of the source.

Chapter Six: Plasma Amino Acids after the Ingestion of Dairy and Collagen Supplements, in Healthy Active Males

Statement of publication

The chapter is comprised of a currently published manuscript in *Frontiers in Nutrition*, with slight adjustments made to formatting for the purpose of this thesis. A published version of the paper is available in the appendix section.

Alcock RD, Shaw, GC, Tee, N, Burke, LM. Plasma Amino Acid Concentrations after the Ingestion of Dairy and Collagen Proteins, in Healthy Active Males. *Front Nutr.* 2019 6(163). Doi: 10.3389/fnut.2019.00163

6.1 Abstract

Introduction: Recent evidence suggests that the consumption of essential amino acids (AA) and/or those abundantly present in collagen may have the capacity to influence the synthesis of new collagen in ligaments and tendons, when tissue perfusion is optimised (e.g. during exercise). However, little is currently known about the bioavailability of these AAs in blood after the consumption of various collagen and diary protein sources: such information is needed to develop potentially useful dietary and supplement intake strategies.

Objectives: The aim of the current study was to characterise blood AA concentrations in response to consumption of collagen and dairy protein sources; specifically, maximum concentrations, the timing of maximum concentration, and total (area under the curve) exposure above baseline.

Methods: A 20 g serve of various dairy and collagen proteins, and a 300 mL serve of bone broth were consumed by healthy, recreationally active males after an overnight fast. Blood samples were drawn every 20 min for a total of 180 min, for analysis of plasma AA concentrations. Total AA, essential AA and collagen specific AAs were analysed for maximum concentration, timing of peak, and area under the curve.

Results: In general, protein intake was associated with a similar increase in total and collagen specific AAs, except for collagen proteins being a superior source of glycine (683 ± 166 µmol/L) compared to 260 ± 65 µmol/L for dairy proteins ($P < 0.0001$), whilst dairy proteins were a superior source of leucine (267 ± 77 µmol/L) compared to 189 ± µmol/L for collagen proteins ($P < 0.04$). Although there were several differences in the bioavailability of hydrolysed compared to non-hydrolysed proteins, this only reached statistical significance within the dairy proteins, but not for collagen proteins.

Conclusions: The intake of collagen proteins result in higher plasma peaks of glycine, whilst the intake of dairy proteins result in higher plasma peaks of leucine. This information may

support further investigations, and identification of key AAs that may support exercise in the synthesis of collagen.

Keywords: Leucine, proline, glycine, hydroxyproline, tendon, ligament, connective tissue, athlete.

6.2 Introduction

Literature suggests that ligaments and tendons are similar to muscle in being dynamic structures that respond to mechanical loading with tissue hypertrophy (Couppe et al., 2013; Kongsgaard et al., 2007; Zhang et al., 2015). Larger tissues with more densely packed collagen fibrils have a greater capacity to withstand force, and thereby exert greater injury protection (Galloway et al., 2013). On the other hand, smaller tissues with disorganised collagen fibrils have been associated with higher injury risk, such as the development of tendinopathies (Couppe et al., 2013). Although the amino acids (AAs) required for myofibrillar tissue formation have been well documented (Morton et al., 2015; Morton et al., 2018; Phillips, 2006; Phillips, 2014; Phillips et al., 2011), evidence regarding the potential role of collagen precursors (e.g. proline, glycine and lysine) and/or stimulatory AAs (e.g. leucine) in the synthesis of new collagen in ligaments and tendons is still emerging. Furthermore, the requirement for an appropriate exercise stimulus to support tissue perfusion appears essential (Baar, 2019; Curtis, 2016; Kjaer et al., 2015; Langberg et al., 2001).

Leucine, an essential amino acid (EAA), has been the subject of extensive research for its role in stimulating myofibrillar protein synthesis (Morton et al., 2018). Leucine exerts its effect on the mammalian target of rapamycin (mTOR), which results in a signalling cascade leading to the synthesis of myofibrillar protein in muscle tissue (Dodd et al., 2012). In the context of connective tissue, work undertaken in an animal model has suggested an increase in collagen synthesis in the deep digital flexor tendon of malnourished rats in association with a leucine-rich diet, and further enhanced when combined with physical stimulation (Barbosa et al., 2012). Similarly, in humans, the ingestion of leucine-rich whey protein, coupled with resistance training, has been shown to lead to patellar tendon hypertrophy, with the proximal cross-sectional area increasing by 14.9 ± 3.1%, compared to 8.1 ± 3.2% for the placebo group (Farup

et al., 2014), but no change in distal or mid tendon CSA. However, it is highly plausible that this increase occurred secondary to a distinct increase in the size of the quadriceps muscle, acting as a stimulatory load on the tendon (Kjaer et al., 2009).

In addition to the stimulatory role of leucine on the protein synthetic machinery, it is likely that other AAs, such as those found in large quantities in collagen protein (e.g. glycine, proline and lysine) play a role in the synthesis of new collagen. Proline is a conditionally essential amino acid (CEAA), which plays a role in the formation and structural integrity of collagen fibrils (Li et al., 2018). Indeed, in older individuals, ingestion of leucine and proline in the form of casein protein, in combination with resistance training, resulted in a trend towards a higher fractional synthetic rate of collagen compared to whey protein (Dideriksen et al., 2011). In an engineered ligament model, it has been shown that the addition of 50 μM proline with 50 μM ascorbic acid, to a media rich in AAs such as leucine and glycine, increased ligament collagen content from 1.34% ± 0.2% to 8.34% ± 0.37% (Paxton et al., 2010). More recently, work from our group has shown that the consumption of 15 g of dietary collagen; one hour prior to intermittent exercise led to an increase in procollagen I N-terminal propeptide (P1NP), compared to a placebo control (Shaw et al., 2017). Additionally, an *in vitro* arm of the same study, displayed an increase in the collagen content and mechanical properties of an engineered ligament treated with serum obtained post ingestion of dietary collagen (Shaw et al., 2017). Thus, it is plausible that a combination of EAA, to upregulate synthetic machinery, and CEAA to supply AA building blocks is required during the synthesis of collagen protein. Although these findings are promising, it remains unclear as to the value of hyper-aminoacidemia in the synthesis of new collagen, and whether these amino acids work in isolation or synergistically.

In line with this, there is emerging evidence of benefits associated with the ingestion of collagen

peptides in a range of collagen containing tissues, including increased collagen synthesis (Daneault et al., 2017; Schunck et al., 2013), improved body composition (Oertzen-Hagemann et al., 2019; Zdzieblik et al., 2015), reduced pain (Clark et al., 2008; Oesser et al., 2016; Praet et al., 2019) and the slowing of degenerative diseases such as osteoarthritis (Bello et al., 2006; Van Vijven et al., 2012). It has been suggested that hydrolysis of collagen protein prior to ingestion allows two and three amino acid peptides to pass across the mucosal barrier equating to a higher expression and therefore biosynthesis within the tissue matrix (Aito-Inoue et al., 2007). This was illustrated in a recent study whereby the consumption of hydrolysed collagen proteins resulted in a higher bioavailability of AAs compared to non-hydrolysed collagen protein and a placebo control (Skov et al., 2019). Furthermore, it has recently been shown that the consumption of collagen peptides resulted in a higher expression of collagen signalling proteins, compared to a placebo control (Oertzen-Hagemann et al., 2019).

Accordingly, we aimed to determine the bioavailability [i.e. timing, maximum concentration, and area under the curve (AUC)] of TAA, EAA and key AAs proposed to support the synthesis of new collagen, after the consumption of a selection of hydrolysed and non-hydrolysed collagenous, and dairy proteins, and a collagenous food source. This data would be of benefit to allow further investigations into a range of practical questions:

1. The optimal food source/supplement, AA and/or combinations of AAs that may support exercise in increasing collagen synthesis.

2. The optimal timing to consume this food source/ supplement and/or AA(s) prior to an exercise bout.

3. Whether there is increased bioavailability from the consumption of hydrolysed vs non-hydrolysed forms of collagenous and/or high leucine proteins.

6.3 Materials and methods

Subjects and ethics

Fifteen healthy, recreationally active, male subjects (30 ± 5 years; 80 ± 8 kg BM) with no current collagen-related disease or known protein allergies were recruited for the study. The current study formed part of a larger study, which necessitated a male subject population given the influence of female sex hormones of ligament health (Stijak et al., 2015). Sample size was chosen using power estimation determined in previous, similar studies (Burke et al., 2012; van Loon et al., 2000). The Human Ethics Committee of the Australian Institute of Sport granted approval for this study (20170607) and written informed consent was received from participants prior to its commencement. The protocol was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR12617000923369).

Protein source and preparation

Four collagen and two high leucine dairy protein sources were selected for the study. These included a hydrolysed and non-hydrolysed collagen powder: Gelita (Pep) and gelatin (Gel) respectively; a hydrolysed and non-hydrolysed dairy protein supplement: calcium caseinate (Cas) and hydrolysed casein (HCas) respectively; a liquid collagen supplement (LCol) and one collagenous food source: bone broth (BBr). Further details of these protein sources can be seen in Table 6.1. We chose a standard dose of 20 g for all powdered supplements and 60 mL of liquid collagen (equivalent to 20g of collagen protein as per manufacturer information). This dose was slightly higher than used in previous work (Shaw et al., 2017), due to the benefits associated with to increased amino acid availability (Imaoka et al., 1992; Iwai et al., 2005). The BBr used in this study was chosen on the basis of the results of our previous work (Alcock et al., 2018) in which this preparation was found to have a higher protein content than other broths assessed, and within the range provided by the reference supplements in a standard serve

(further details of the broth used in this study; “chef prepared bone broth” are available elsewhere; Alcock et al., 2019). Furthermore, we decided on a 300 mL serve as a representative portion size that would be practical to consume within the prescribed time-period, as we would not ascertain total protein content of this broth prior to commencing the study. Analysis of the batch actually consumed in this study, subsequent to the trials, found that it was considerably higher than expected. Nevertheless, by purchasing a bulk volume of the broth (5 L) and mixing it well before dividing it into 300 mL serves, we achieved consistency between the serves consumed by subjects.

To maintain consistency across all experimental protocols, all protein sources were served warm, and in/with the same amount of fluid (300 mL) achieved via the addition of water. Supplements were provided in a counter-balanced, randomised fashion, with at least 48 h between trials. All protein sources were assessed for full amino acid profiles analysed using Ultra Performance Liquid Chromatography (Waters AccQTag Ultra) (Australian Proteome Analysis Factory, Macquarie University, Sydney, NSW).

Experimental protocol and analysis

Participants fasted overnight (> 10 h) prior to attending the laboratory at the Australian Institute of Sport between 5:30 and 7 am. On waking, participants were allowed to consume 250 mL of water to ensure adequate hydration for the blood collections but instructed to arrive to the lab in a rested state to avoid significant elevations to heart rate that would increase blood flow. On arrival, a 22G indwelling cannula was inserted into the antecubital vein for blood collection by a trained phlebotomist, and a baseline (BL) blood sample was collected. Immediately after, the protein source was prepared and given to participants who were instructed to consume it within 5 min, sipping slowly throughout. Completion of the 5 min period was considered as t = 0. To

standardise gastric emptying, no other fluid was available for 60 min following consumption, and then ad libitum water consumption was permitted. Immediately after the consumption of the protein source, participants were asked to fill out a feedback form (as outlined below). Blood samples were then collected every 20 min for 180 min into 2 mL lithium heparin Vacuette tubes (Greiner Bio-One, Kremsmünster, Austria). Immediately after each blood collection, samples were centrifuged at 1500 xg for 10 min at 4°C. The resulting plasma was separated and stored at -80°C until further analysis. Once the 180 min period was concluded the cannula was removed and participants were allowed to leave the laboratory.

Once all plasma samples were collected, they were transported to Maastricht University for analysis of full amino acid profiles using liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). Briefly, samples were prepared according to manufacturer's instructions, with an internal standard added. 50 µL of blood plasma was deproteinised using 100 µL of 10% sulfosalicyclic acid (SSA) with 50 µM of metabolomics amino acid mix standard (MSK A2) (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged. After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). Twenty µL of AccQ-Tag derivatising reagent solution (Waters, Saint-Quentin, France) was then added, and the solution was heated to 55°C for 10 min. Of this 100 µL derivative, 1 µL was injected and measured using UPLC-MS.

Due to the interests of this study, we have reported concentrations of TAA, EAA and a selection of AAs which have been suggested to be involved in the synthesis of collagen (including

proline, glycine, lysine and leucine) (Baar, 2017; Dideriksen et al., 2011; Farup et al., 2014; Shaw et al., 2017).

Protein source acceptability

The feedback form completed by subjects immediately after consuming a test product provided four questions in the format of a Likert scale (Likert, 1932), and one question with a yes or no response. The questions are outlined in Table 6.3 and included questions related to acceptability and palatability. While we acknowledge that this is not a validated questionnaire, it was intended that this feedback would provide practical insight into the use of this product for connective tissue health purposes.

Statistics

Trapezoidal rule (Yeh, 2002) adjusted to baseline concentration was applied to calculate the area under curve (AUC) of the amino acid concentration. Statistical analysis were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California, USA). Data were checked for normality using a D'Agostino & Pearsons normality test. A repeated-measures One-way ANOVA by General Linear Models (GLM) was used to compare the effects of different supplements on outcome variables (plasma amino acid content for: baseline; BL, observed maximum concentration; C_{max} Obs, timing of peak; T_{max} and AUC). Tukey's method was used to adjust for multiple comparisons between different groups.

6.4 Results

Total protein and amino acid content of selected collagen and dairy proteins

The composition per serve of the protein sources (total protein, TAA, EAA and selected AAs) is displayed in Table 6.1. Full amino acid profiles are available as supplementary material (see supplementary table 11.2.2). The supplements (Cas, HCas, Gel, Pep and LCol) provided 19.3 ± 0.8 grams protein per 20 gram serve, whereas BBr provided 52.2 grams of protein per 300 mL serve. Per serve, BBr contained the highest levels of all selected AAs, with the exception of leucine, which was provided in the highest amount in the dairy protein supplements, HCas and Cas at ~1540 mg per serve. AA profiles of proline, glycine, lysine and leucine of protein sources of similar derivatives (e.g. dairy and collagen proteins), were comparable in their amino acid content. Liquid collagen provided the lowest levels of all AA among the collagen protein sources in this study.

Observed maximum concentration (C_{max} Obs)

Figure 6.1. displays the time course of the appearance of TAA, EAA, and the selected AAs in the plasma over 180 min. Kinetic parameters of TAA, EAA and selected AAs after the consumption of collagen and dairy protein sources are displayed in Table 6.2., as means and SD. There were no differences in BL levels for TAA, EAA or any of the AAs between protein sources ($P > 0.2$).

In terms of TAA, C_{max} Obs was similar for all protein sources, except for Cas which was significantly lower ($P < 0.04$) than all protein sources and LCol which was significantly lower than Pep ($P = 0.03$). HCas was significantly higher for C_{max} Obs for EAA than all other protein sources ($P < 0.0001$) while Cas was only higher than BBr and LCol ($P < 0.04$). There was no difference in C_{max} Obs of EAA for any of the collagen protein sources ($P > 0.1$). The C_{max} Obs

of proline was similar (P > 0.1) for all protein sources other than Cas, which was significantly lower than the others (P < 0.001). HCas provided the highest C_{max} Obs for lysine compared to all other protein sources (P < 0.002). Collagen proteins provided a higher C_{max} Obs for glycine compared to Cas and HCas (P <0.0001) while there was no difference between the dairy protein sources (P = 0.8). Meanwhile, HCas provided the highest C_{max} Obs of leucine compared to all collagen proteins (P = 0.0001) and Cas also had a higher C_{max} Obs for leucine than these products (P < 0.04). However, differences in C_{max} Obs for leucine between any of the collagen proteins failed to reach significance (P > 0.06).

Timing of maximum concentration (T_{max})

Results of T_{max} in Table 6.2., illustrate a similar time course for TAA for all products except an earlier peak with HCas than BBr and Gel (P < 0.02). There was no difference in T_{max} of EAA with all protein sources peaking between ~ 30 and 50 min (P > 0.1). T_{max} of proline for BBr was slower than all other protein sources (P < 0.002). Although the time to peak for proline was slower for the non-hydrolysed proteins compared to the hydrolysed proteins, this did not reach statistical significance (P > 0.05). BBr was the slowest to peak for glycine, and was significantly slower than Pep and LCol (p <0.001). The T_{max} of glycine for Gel was also found to be slower than for Pep (P = 0.04). There was no difference in T_{max} for leucine between any of the protein sources, with all peaking between ~ 30 to 50 min (P > 0.04). The only difference for lysine T_{max} was a quicker peak with BBr than HCas (P = 0.001).

Area under the curve (AUC)

Figure 6.2 displays the AUC of amino acid concentrations in plasma after consumption of the protein sources. BBr provided the highest AUC for TAA at 91029 ± 29630 µmol/L/180 min, which was significantly higher than both dairy proteins [Cas and HCas at 27265 ± 14130

μmol/L/180 min and 36042 ± 12833 μmol/L/180 min, respectively (P < 0.0001)] but not significantly different to any of the collagen protein supplements [77052 ± 24304, 84664 ± 20582 and 71882 ± 19085 μmol/L/180 min for Gel, Pep and LCol, respectively (P > 0.2)]. However, as seen in Figure 6.1., plasma AAs after consumption of BBr remained elevated at the completion of 180 min, particularly for proline and glycine. Cas provided the lowest AUC for TAA, and was significantly lower than all collagen supplements (P <0.0002), but not HCas (P = 0.6). Although hydrolysed supplements appeared to have a higher AUC for TAA than their non-hydrolysed counterparts, this was not statistically different for either collagen or dairy proteins (P > 0.6). Collagen proteins had a higher AUC than dairy proteins for TAA (81157 ± 8419 and 31654 ± 6206 μmol/L/180 min, respectively, P <0.0001).

HCas provided the highest AUC for EAA at 55992 ± 15675 μmol/L/180 min, which was significantly greater than all other protein sources (P<0.003), except Cas which provided an AUC for EAA of 39587 ± 18573 μmol/L (P = 0.2). The AUC for EAA of Cas, was only significantly higher than LCol and Pep which provided 8806 ± 16410 and 12238 ± 13682 μmol/L/180 min, respectively (P < 0.006). The lowest AUC of EAA was provided by LCol at 8806 ± 16410 μmol/L/180 min, but this was not different to the other collagen protein sources (P > 0.8).

In terms of individual AA, the AUC of lysine was greater for HCas than Gel, Pep and LCol [AUC of 11187 ± 2764 μmol/L/180 min compared to 7003 ± 4340, 6310 ± 2714 and 5729 ± 3290 μmol/L/180 min, respectively (P <0.008)]. For leucine, dairy proteins had a higher AUC than all collagen proteins at 10187 ± 3023 μmol/L/180 min and 746 ±1043 μmol/L/ 180 min respectively (P < 0.001). Meanwhile, HCas provided a higher AUC for leucine than Cas (P = 0.003). Collagen proteins has a higher AUC than dairy proteins for proline (25102 ± 2345 and

13547 ± 3450 µmol/L/180 min, for Gel, Pep and LCol respectively $P < 0.01$), and glycine [(48563 ± 4429 and -1863 ± 2255 µmol/L/ 180 min ($P < 0.0001$).]

Collagen and dairy protein acceptability and palatability

Responses to the questions around acceptability and palatability for each protein source are displayed in Table 6.3. LCol had the highest scores individually, and on average, while HCas was rated the lowest. Despite these differences between products, only 53% of participants reported being willing to pay the specified price for either product. Meanwhile, the non-hydrolysed supplements (which were also the lowest in cost; Table 6.1.), scored 93% for willingness to pay, if it was found to be beneficial to connective tissues.

Table 6.1: Details of collagen and non-collagen (dairy) protein sources, and collagen specific amino acid profiles per serve of select food/supplement.

Type	**Dairy protein supplement**		**Collagen protein supplement**			**Collagen protein food source**
Brand	Professional whey, NSW, Australia	Professional whey, NSW, Australia	McKenzies, Altona, VIC, Australia	Tendeforte, Gelita, Eberbach, Germany	GBR Nutrition, Lancashire, UK	Elemental Cafe, Braddon, ACT, Australia
Details	Calcium caseinate	Hydrolysed casein powder	Powdered gelatin	Hydrolysed collagen peptide powder	Liquid collagen supplement	Chef made bone broth
Abbreviation	**Cas**	**HCas**	**Gel**	**Pep**	**LCol**	**BBr**
Serve	20 grams	20 grams	20 grams	20 grams	60 mL (equivalent to 20 grams)	300 mL
Cost per serve	$ 0.70	$ 1.24	$ 0.74	$ 3.00	$ 5.37	$ 4.50
Manufacturer stated total protein per serve (g)	20	20	20	20	20	N/A
Actual measured total protein per serve (g)*	19.2	19.2	20.1	20.6	17.9	52.2
TAA per serve (mg)*	19172	19378	20108	20594	178928	52260
EAA per serve (mg)*	8306	8368	4944	5858	4392	13320
Proline per serve (mg)*	2038	2060	2664	2722	2406	6420
Glycine per serve (mg)*	360	354	4750	4904	4212	11760
Lysine per serve (mg)*	1422	1468	696	746	630	1740
Leucine per serve (mg)*	1782	1786	588	584	516	1710

EAA: Essential Amino Acids; TAA: Total Amino Acids
* Calculation based on free amino acid molecular weight
Note: Cysteine and tryptophan have not been included in analysis, which account for < 5% in total.

Table 6.2: Kinetic parameters of total, essential, and collagen specific, amino acid concentration in plasma after the consumption of collagen and dairy protein sources. Data presented as mean ± SD.

		Cas	HCas	Gel	Pep	LCol	BBr	Significance
TAA	Baseline (µmol/L)	2156 ± 234	2233 ± 403	2155 ± 308	2178 ± 331	2151 ± 325	2254 ± 392	P = 0.6430
	C_{max} Obs (µmol/L)	3012 ± 407	3725 ± 641	3525 ± 631	3710 ± 670	3445 ± 641	3552 ± 561	P = 0.0004
	T_{max} (min) (min/max)	45 ± 35	35 ± 9	59 ± 28	48 ± 27	47 ± 39	64 ± 24	P = 0.0877
EAA	Baseline (µmol/L)	998 ± 116	1044 ± 170	1030 ± 135	1017 ± 148	1000 ± 147	1074 ± 142	P = 0.4621
	C_{max} Obs (µmol/L)	1457 ± 137	1946 ± 292	1311 ± 211	1313 ± 190	1270 ± 198	1347 ± 138	P <0.0001
	T_{max} min (min/max)	47 ± 36	33 ± 10	47 ± 24	35 ± 31	36 ± 42	51 ± 24	P = 0.3310
Proline	Baseline (µmol/L)	168 ± 47	177 ± 49	163 ± 55	173 ± 62	166 ± 49	170 ± 72	P = 0.7901
	C_{max} Obs (µmol/L)	282 ± 68	392 ± 71	381 ± 76	431 ± 113	388 ± 89	386 ± 85	P <0.0001
	T_{max} min (min/max)	56 ± 34	41 ± 9	61 ± 22	55 ± 27	52 ± 38	103 ± 29	P < 0.0001
Glycine	Baseline (µmol/L)	229 ± 67	235 ± 64	226 ± 172	215 ± 176	222 ± 143	229 ± 170	P = 0.5773
	C_{max} Obs (µmol/L)	267 ± 67	253 ± 64	674 ± 172	747 ± 176	646 ± 143	666 ± 170	P <0.0001
	T_{max} min (min/max)	67 ± 49	39 ± 49	71 ± 25	48 ± 20	53 ± 26	88 ± 24	P = 0.0103
Lysine	Baseline (µmol/L)	174 ± 33	178 ± 31	179 ± 28	178 ± 58	176 ± 33	183 ± 30	P = 0.8818
	C_{max} Obs (µmol/L)	273 ± 31	369 ± 65	269 ± 52	271 ± 50	262 ± 48	272 ± 41	P <0.0001
	T_{max} min	51 ± 36	29 ± 10	45 ± 19	41 ± 18	44 ± 33	51 ± 17	P = 0.1629

	(min/max)							
Leucine	Baseline (µmol/L)	136 ± 17	144 ± 27	145 ± 26	144 ± 28	140 ± 29	158 ± 29	P = 0.1640
	C_{max} Obs (µmol/L)	230 ± 31	351 ± 59	184 ± 29	190 ± 34	181 ± 30	203 ± 28	P <0.0001
	T_{max} min (min/max)	47 ± 36	29 ± 10	36 ± 23	29 ± 31	33 ± 41	33 ± 12	P = 0.3768

Table 6.3: Likert scale of protein source feedback and palatability. Data presented as median (range).

Question (1-7)	**Cas**	**HCas**	**Gel**	**Pep**	**LCol**	**BBr**	**Significance**
Serve size	20 g	20 g	20 g	20 g	60 mL	300 mL	P < 0.001
1	4 (1-7)	3 (1-5)	4 (1-7)	5 (2-7)	6 (307)	5 (2-7)	P < 0.001
2	6 (3-7)	5 (2-6)	6 (2-7)	6 (2-7)	7 (4-7)	6 (2-7)	P = 0.032
3	6 (2-7)	5 (1-7)	6 (2-7)	6 (2-7)	7 (5-7)	6 (2-7)	P = 0.044
4	6 (3-7)	4 (1-7)	6 (1-7)	6 (2-7)	7 (4-7)	6 (2-7)	P = 0.006
5 (% Yes)	93	53	93	60	53	73	-

*Question 1: The palatability (taste, texture) of the product was acceptable
*Question 2: The volume of the product (300mL) was acceptable to consume in the time specified (5 mins)
*Question 3: The volume of the product would be acceptable to consume within 20-40 minutes prior to exercise
*Question 4: I would be willing to consume this product at least twice a day as part of a rehabilitation program
**Question 5: Would you be willing to pay the specified amount for one serve of the product if it was beneficial to connective tissue health?

1 = strongly agree, 7 = strongly disagree

** Out of 1 (strongly agree) to 7 (strongly disagree)*

*** % out of 100*

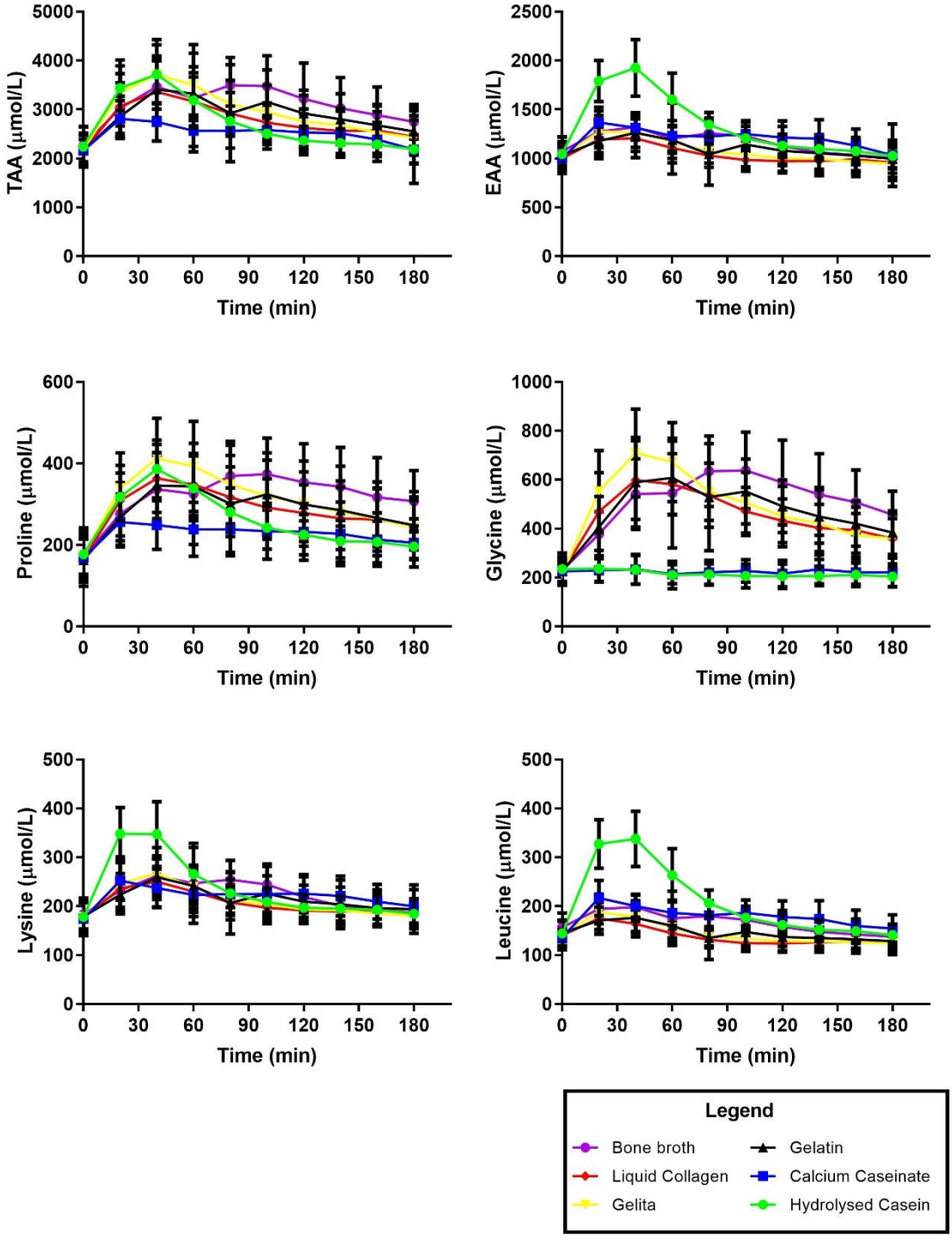

Figure 6.1: Time course of plasma amino acid concentrations over 180 min after the consumption of collagen or dairy protein sources. Data presented as mean ± SD.

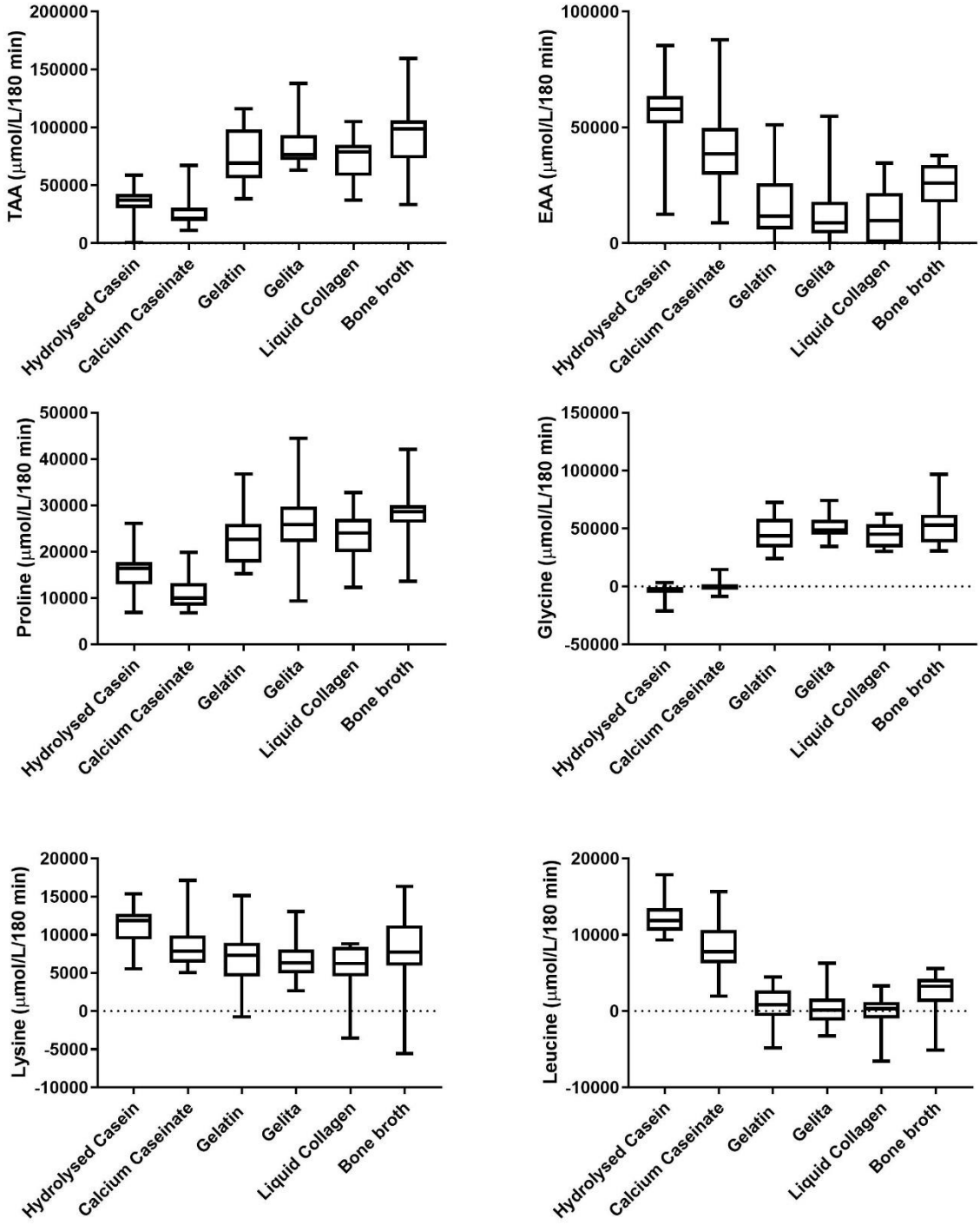

Figure 6.2: Area under the concentration – time curve (AUC) of plasma amino acids over 180 minutes after the consumption of dairy and collagen protein sources. Middle line shows mean, boxes represent 25th – 75th percentile and whiskers represent min and max values.

6.5 Discussion

A number of AAs have been suggested to play a complementary role in the synthesis of collagen in ligaments and tendons, when combined with an appropriate mechanical stimulus (Baar, 2017). There is emerging evidence that the ingestion of key AAs (including proline, glycine, lysine and leucine), or combinations thereof, provide enhanced availability to support the synthesis of collagen when combined with an appropriate exercise protocol (Scott et al., 2016). Accordingly, the aim of the present study was to characterise the bioavailability (e.g. timing of appearance, maximum concentration, and AUC) of TAA, EAA and key AAs that may be involved in the synthesis of collagen, after the ingestion of a selection of collagen and dairy protein sources.

In general, plasma AA responses reflected the AA profiles of the consumed supplement, except in the case of the casein, and bone broth. These both resulted in a lower and more prolonged appearance of AAs in the blood over the 180 min following consumption (See Table 6.1. and 6.2. and Figure 6.2.). This is likely due to various components known to slow gastric emptying such as the fat content of the BBr (Lin et al., 1996), and the clotting of casein in the stomach after ingestion (Boirie et al., 1997). However, it is also plausible that the delayed gastric emptying of BBr may be related to the volume was consumed. As can be seen in Figure 6.1., the plasma concentration of AAs after consumption of BBr remained elevated at the 180 min mark, and most likely would have continued to be available at a higher amounts in the blood beyond the 3 hours that were monitored for this study.

It has been proposed that AAs play a supportive, rather than stimulatory role in the synthesis of collagen within ligaments and tendons (Baar, 2017; Babraj et al., 2005a; Shaw et al., 2017). Meanwhile, exercise has been shown to be a potent regulator of collagen turnover resulting in

an upregulation of collagen synthesis for a period of up to 72 h (Langberg et al., 2000). Exercise results in an increase in hormones that have been shown to stimulate the synthesis of collagen in connective tissue (e.g. growth hormone, and insulin-like growth factor 1) (West et al., 2015). As ligaments and tendons are poorly vascularised tissues, it may be sensible to isolate the provision of AAs to scenarios involving the exercise-induced enhancement of blood flow (Henning Langberg et al., 1999). Therefore protein sources that achieve higher AA peaks over a shorter period of time (e.g. HCas) may be considered optimal, whereas a slower release of key AAs over an extended period of time (e.g. with BBr) may not be as easily matched to enhanced tissue blood flow. Literature to date suggests that there is dose related response to the key AA involved in the synthesis of collagen. Indeed, Shaw et al. (2017) illustrated that a 15 g dose of gelatin resulted in an increased availability of AAs and collagen synthesis than a 5 g dose, resulting in improvements to tissue mechanics in engineered ligaments *in vitro*. Peak blood concentrations of glycine and proline in the current study (~650-750 and 350-450 µmol/L, respectively) were slightly higher than those reported in previous work (i.e. 448 ± 165 and 238 ± 77 µmol/L in (Shaw et al., 2017). This is to be expected given the slightly higher dose within our current protocol. However, others have shown similar plasma values after the ingestion of 35 grams of collagenous protein (Skov et al., 2019) which they suggest shows an upper threshold to the AA availability of collagen proteins. In general since all AAs appeared to peak between 30 and 60 mins (Table 6.2.), the consumption of ~ 20 grams of protein within the 30 – 60 mins prior to exercise would ensure the optimal availability of AAs at a time when synthetic machinery is upregulated, and tissue perfusion supported (H. Langberg et al., 1999; Langberg et al., 2001; Langberg et al., 2000). It should be noted, however, that whether the increased availability of AAs within the plasma results in an increased availability of AAs around the target tissue (e.g. the peritendinous fluid), where it as able to be utilised and integrated into the tissue is yet to be determined.

While the maximum concentrations of total AA present within the plasma after the ingestion of collagen and high leucine dairy proteins were comparable, dairy proteins provided a larger C_{max} Obs and AUC of leucine, while hydrolysed casein provided a higher C_{max} Obs of EAA than both dairy and collagen proteins, and collagen proteins were superior in terms of glycine. Such differentiation mean that no protein source was superior in terms of all AAs that are potentially implicated in collagen synthesis. If subsequent research identifies the benefits of EAA as well as collagen precursors (e.g. proline and glycine), a case could be made for the consistent intake of EAA (i.e. the current guidelines for regular intake of high-quality protein sources over the day), with a supplemental and/or food source of collagenous protein consumed at key periods i.e. prior to exercise. The lack of such a pattern may also detract from the health of connective tissues.

It has been proposed that hydrolysed proteins are superior to non-hydrolysed forms in providing a higher AA bioavailability; more specifically, being able to reach a target tissue more quickly and with a higher peak concentration (Aito-Inoue et al., 2007; Koopman et al., 2009; Skov et al., 2019). Within the present study, the plasma characteristics of hydrolysed and non-hydrolysed dairy proteins showed some differences, but these were not seen with the collagen proteins. Our findings are similar to the study of Koopman and co-workers where ingestion of 35 grams of hydrolysed casein protein resulted in an increased bioavailability and incorporation rate of AAs into skeletal muscle protein than intact casein (Koopman et al., 2009). However, while another more recent study showed that the ingestion of 35 grams of enzymatically hydrolysed collagen resulted in a higher bioavailability of several AAs compared to non-enzymatically hydrolysed collagen (Skov et al., 2019), we did not find any difference in the bioavailability of gelatin compared to a hydrolysed peptide powder. It is

possible that differences in processing methods accounted for this finding as it has been suggested to influence digestibility and therefore bioavailability of AAs (Dallas et al., 2017).

In terms of the limitations of this study, we acknowledge that the questionnaire relating to acceptance and palatability of the protein sources used in this study was not a validated tool. Nevertheless, it provided insight into factors that are likely to affect the compliance of use of protein sources of potential benefit for collagenous tissues. Indeed, it indicated that despite the superiority of HCas over Cas in terms of AA availability, its poor scores for taste/texture are likely to affect consumer uptake and compliance with regular use. On the other hand, non-hydrolysed supplements scored well in terms of acceptability in taste and cost; this makes gelatin a potentially useful product since it provided similar peak and total AA exposure than other collagen products. Furthermore, our inability to measure the composition of the protein sources actually used in this study until after its conduct meant that we relied on previously collected data, or that stated by the manufacturer which differed to the final measured content (Table 6.1.). The subsequent discovery of the higher protein of this broth makes it difficult to compare the bioavailability based on their actual protein intake. However, since the serves used in this study are the commonly consumed or recommended amounts of these protein sources, it did allow real-life insight into the AA profiles.

In summary, our study characterised the blood AA profiles following the intake of a range of dairy and collagen supplements providing 20 g of protein, and a common serve of beef broth of higher total protein. While protein sources of similar origin contained a comparable total AA profile, consumption of dairy proteins provided a more pronounced amount of EAA and leucine than collagen sources, with hydrolysed casein showing increased bioavailability of AAs than an intact form. Intake of collagen proteins achieved a greater peak concentration of

glycine, with small differences between hydrolysed and non-hydrolysed forms. Although the total AA content of the beef broth was greater, a lower and more sustained blood AA profile seems likely. This information may help to inform protocols for achieving ideal blood AA responses once the optimal support for the synthesis of collagenous tissues is identified. However, the cost and palatability of these dietary and supplement sources should be considered.

Contribution to the field statement

The current study profiled plasma AA responses to the intake of a number of potentially valuable protein sources, including high leucine dairy-proteins and dietary and supplemental forms of collagenous proteins in intact and hydrolysed forms. Information on the bioavailability and consumer acceptance of these sources will be of value to ongoing research around the synthesis of collagenous proteins. In addition, it will also help to inform practical diet and exercise strategies that emanate from future *in vitro* and *in vivo* work.

Author contribution statement

R.D.A, L.M.B and G.C.S were involved in study design. R.D.A and NT was involved in data collection, data analysis and statistical analysis R.D.A, L.M.B, G.C.S and NT were involved in results interpretation, drafting, reviewing and revising the initial manuscript.

Conflict of interest statement

This study, funded by a grant administered by the Centre for Exercise and Nutrition, Mary Mackillop Institute for Health Research, Australian Catholic University, and provided by the ACT Brumbies Super Rugby Team, was conducted at the Australian Institute of Sport. Whilst

the ACT Brumbies provided the funding for the study; they had no direct role in any part of the study. All authors declare no conflict of interest.

Acknowledgements

Amino acid analysis was facilitated using infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS).

Preface to Chapter Seven

As outlined in Chapter Two, previous studies have demonstrated that the consumption of collagenous protein (in concert with an exercise stimulus) can result in an increased collagen content and improved tissue mechanics in an *in vitro* engineered ligament model. The final Chapter (Study 4) aimed to build on this work using information obtained within the initial studies of the thesis including the timing and appearance of AAs after the consumption of a selection of collagen and dairy proteins. A previously established engineered ligament model was updated with the consideration that the usual media used in this protocol is four times more concentrated than physiological serum levels and may, therefore, not represent the *in vivo* environment experienced by tendons and ligaments. Thus, an additional aim of the study was to determine the true effect of the differing AA responses to the intake of collagen and dairy proteins against a background that would more closely reflect physiological levels.

Chapter Seven: The Consumption of Collagen and Dairy Proteins Does Not Affect Material and Mechanical Properties of Engineered Ligaments Treated With a Media Reflective of Fasting Physiological Amino Acids.

The chapter is comprised of a paper in preparation for publication in *The American Journal of Clinical Nutrition*.

7.1 Abstract

Background: Three-dimensional (3D) cell culture models present an opportunity to measure changes to dense connective tissues as a result of nutritional interventions, without the need for invasive techniques such as biopsy. However, this methodology may not be representative of *in vivo* synthesis due to supra-physiological levels of several amino acids (AA) proposed to be implicated in the synthesis of collagen.

Objectives: The aim of this study was to measure changes in material and mechanical properties of 3D engineered ligaments within a physiologically relevant media, combined with serum collected after the consumption of collagen and dairy proteins.

Design: Serum obtained from individuals after the consumption of 20 grams of collagen and dairy proteins, was added to a media developed to reflect physiological levels of AAs and utilized to treat 3D engineered ligaments. Once fully formed (8 - 10 days after plating), ligaments were treated for an additional 6 days in the serum media, after which time ligaments were tested for material and mechanical properties.

Results: Despite differences in the AA profiles of the plasma after the consumption of collagen and dairy proteins ($P < 0.007$), there were no significant differences in collagen content ($P = 0.09$), collagen % dry mass ($P = 0.3$), CSA ($P = 0.2$), MTL ($P = 0.2$), UTS ($P = 0.3$), or modulus ($P = 0.5$) for any of the treatment groups.

Conclusions: Enhanced AA availability did not improve mechanical or material properties of 3D engineered ligaments treated with a media reflective of physiological AA availability.

Keywords: Leucine, proline, glycine, hydroxyproline, tendon, ligament, connective tissue, athlete

7.2 Introduction

A wide range of factors have been shown to influence the health and function of dense connective tissues including mechanical stimulus (Bohm et al., 2015), menstrual cycle phase (Morrison et al., 2015), inflammation (Tang et al., 2018) and metabolic disease (Abate et al., 2013). Collagen, the main protein present within these tissues, is integral to their health and functioning. Conditions which lead to perturbations in the production of collagen protein (e.g. Ehlers-Danlos syndrome) result in tissues with poor integrity that are prone to injury (Myllyharju et al., 2009). Furthermore, it has been suggested that tendinopathies are accompanied with a lower collagen content and/or disruptions to mechanical properties, compared to healthy tendons (Arya et al., 2010; Couppe et al., 2013).

While the impact of mechanical stimulus on collagen production and/or alterations to mechanical properties of collagen containing tissues has been thoroughly explored, there is only a small amount of research available seeking to determine whether nutrition may also impact material and/or mechanical properties of collagen containing tissues. Promising candidates include essential amino acids (EAAs) (e.g. leucine) (Barbosa et al., 2012; Dideriksen et al., 2011; Farup et al., 2014; Kato et al., 2016), those enriched in collagen protein (e.g. proline and glycine) (Baar, 2019; Barbul, 2008; Li et al., 2018; Paxton et al., 2012; Shaw et al., 2017; Vieira et al., 2015), or a combination of both (Dideriksen et al., 2011). Meanwhile, hydrolysed collagen and/or peptide formulations have shown promise in a range of collagen containing tissues including the reduction of joint and tendon pain (Oesser et al., 2016; Praet et al., 2019). Hydrolysed collagen formulations are suggested to have a higher bioavailability of bi- and tri- hydroxyproline containing peptides (e.g. gly-pro-hyp), resulting in an increased expression of collagen synthetic genes, which may in turn increase the expression and synthesis

of collagen (Aito-Inoue et al., 2007; Koopman et al., 2009; Oertzen-Hagemann et al., 2019; Schunck et al., 2013; Skov et al., 2019). While there is some support for these claims, more work is necessary before they merit implementation in resource-intensive longitudinal studies of ligament and tendon health.

The slow regenerative capacity of dense connective tissues (Heinemeier et al., 2013) renders research on connective tissue health/function as lengthy and challenging. Indeed, the only direct measure of both the synthesis and incorporation of collagen into dense connective tissue requires a biopsy of the tendon with the incorporation of isotopically labelled AAs (Smith et al., 2007). However, invasive measures to collect data on dietary interventions within athletic populations whom are heavily reliant on the day to day functioning of such tissues are not appropriate, as they can take a significant period to heal. In the absence of a non-invasive and precise measure of collagen synthesis and its incorporation into connective tissues, an *in vitro* three dimensional (3D) engineered ligament model has recently been developed (Paxton et al., 2010). Through the use of this model, *in vivo* conditions as a result of exercise and/or nutrition interventions can tested in *in vitro* environments. This allows interventions to be undertaken in a well-controlled environment and reduces the premature need for invasive measures such as biopsies. Utilising this model, it has been shown that ligaments treated with serum obtained from exercising individuals resulted in an enhanced collagen content and mechanical properties, compared to ligaments treated with serum obtained from sedentary individuals (West et al., 2015). More recently engineered ligaments exposed to serum obtained after the ingestion of 15 g of supplemental gelatin, resulted in an increased collagen content and improved tissue mechanics compared to 5 g of an isocaloric placebo (Shaw et al., 2017). Although these findings are indeed promising, the application of such findings to “real world” conditions is questionnable.

Indeed, the media [Dulbecco's Modified Eagle Medium (DMEM)], that is traditionally utilised in the development and treatment of 3D cell cultures contains up to four times the fasting physiological levels of specific AAs, such as leucine and glycine (Alcock et al., 2019; Sarwar et al., 1991; Yao et al., 2017). Although necessary for the success of *in vitro* models, such high levels of nutrients may provide an artefact compared to real-life *in vivo* models where these tissue types are known to be poorly vascularised and may not receive such high levels of nutrient availability (Boushel et al., 2000a; Bray et al., 2002). Accordingly, the aim of the current study was to develop engineered ligaments according to established methods (Paxton et al., 2010), and once fully formed, treat them with a media reflective of physiological levels, with the addition of serum obtained from human participants after the interventions of interest. The specific targets of this work were the consumption of a standardised amount of hydrolysed and non-hydrolysed collagen proteins, and a high leucine hydrolysed dairy protein. It was hypothesised that this would allow for a more physiologically relevant interpretation of the influence of various protein sources on the mechanical and material properties of engineered ligaments.

7.3 Subjects and Methods

Subjects and blood collection

Eight healthy, recreationally active males (29 ± 6 years; 81 ± 4 kg BM) with no known protein allergies or collagen related disease were recruited for the study. The sample size was chosen using similar power size estimations as outlined in (Shaw et al., 2017). The Australian Institute of Sport (AIS) human ethics committee granted ethics approval (#20170607) and written consent was received from participants prior to commencement of the study. This study was conducted as part of a larger investigation, with the entire study protocol submitted to the Australian New Zealand Clinical Trials Registry (ANZCTR12617000923369).

Subjects arrived at the Australian Institute of Sport physiology laboratory after an overnight fast (> 10 h) on 4 separate occasions, with at least 48 h separating trials. On arrival, a cannula was placed into the antecubital vein of the arm, and an initial 2 mL blood sample was collected into lithium heparin collection tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) for baseline (BL) plasma amino acid analysis. On one occasion only for each participant in random order an additional 51 mL blood sample was collected into 6 x 8.5 mL serum-separator tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) for the treatment of BL ligaments. Immediately after the BL blood collections, subjects were given the treatment to consume and then instructed to rest for 40 minutes, at which time a 2 mL blood sample was collected into lithium heparin collection tubes for plasma amino acid analysis and a 51 mL blood sample was taken for the treatment of the engineered ligaments. The time of blood collection was chosen in light of the results of an earlier study which identified the plasma concentrations of TAA peak at this time (Alcock et al., 2019). Blood was immediately centrifuged (1500 x g; 4°C; 10 min) and the resulting plasma was separated and stored at -80°C for later plasma amino acid analysis. Meanwhile, the blood for the engineered ligament testing was allowed to clot, before centrifuging, processing and storage using aseptic technique. The resulting serum was frozen at -80°C until all samples were collected. The samples were then transported to the University of California, Davis for use in engineered ligament treatment.

Supplement selection and preparation.

Three supplements were chosen for the study; two collagen based supplements including a readily available commercial variety of ground porcine collagen (partially hydrolysed) (Gelatin; McKenzies, Altona, VIC, Australia), and a specific collagen peptide powder (hydrolysed) (Gelita; Tendoforte, Eberbach, Germany). A hydrolysed form of dairy protein

(Hydrolysed Casein; Professional Whey, NSW, Australia) was also chosen. In a recent study by our group we determined that whilst the three supplements chosen in the current study had a similar bioavailability of TAAs and proline, the collagen supplements provided a significantly higher amount of glycine, whilst hydrolysed casein provided a significantly higher amount of leucine at the timing of the peak (Alcock et al., 2019). Thus by choosing these supplements we were able to further explore the impact of specific AAs proposed to play a role in collagen synthesis. Although we did not find any difference in the availability of the AAs between hydrolysed and partially hydrolysed collagen supplements, within the current we were interested to determine if other factors that we did not capture such as peptide bioavailability may result in improved ligament outcomes. A 20 gram dose for each supplement powder was chosen to optimise AA availability (Alcock et al., 2019; Imaoka et al., 1992; Iwai et al., 2005). The supplements were mixed with 300 mL lukewarm water, and subjects were instructed to consume the liquid within 5 minutes.

Amino acid composition of protein sources

All protein sources were assessed for full amino acid profiles analysed using Ultra Performance Liquid Chromatography (Waters AccQTag Ultra) (Australian Proteome Analysis Factory, Macquarie University, Sydney, NSW).

Plasma amino acid content

Plasma samples were transported to Maastricht University for analysis of full amino acid profiles using liquid chromatograph mass spectrometry as reported previously (Alcock et al., 2019).

Engineered ligament formation

Primary human anterior cruciate ligament (ACL) cells were isolated from a young, male donor (18 y old) post-mortem, and used to construct engineered ligaments using a protocol approved by the Institutional Review Board at University of California, Davis. Cells were collected as described previously (Paxton et al., 2010). The ACL cells were expanded in DMEM growth media containing 10% foetal bovine serum and 1% penicillin and frozen at -200°C. When required, frozen cells were thawed, expanded in growth media, and passaged at least five times prior to the formation of the engineered ligaments. Engineered ligaments were formed as described previously (Paxton et al., 2010). Constructs were grown for 8-10 days in feed media containing 2 mL DMEM, 200 µM ascorbic acid, 50 µM proline and 3 ng/mL TGF- β per construct.

Engineered ligament treatment and analysis

Once constructs were formed fully, they were treated for 6 days with the media that was developed by diluting the DMEM media, and adding back in the relevant AAs to mimic physiological levels (Sarwar et al., 1991). The composition of the developed media can be found in Supplementary Table 11.2.3, and included 100 mL DMEM, 400 mL PBS, 1800 mg glucose, 9 mg glycine, 1% penicillin, 80 mg calcium chloride, 39 mg magnesium sulphate, 200 µM ascorbic acid, 50 µM proline, 3 ng/mL TGF- β and 50 mL treatment serum collected from participants. A minimum of 3 constructs per condition were treated, with the mean being used for analysis. The tissue engineer treating the ligaments was blinded to the serum treatments.

After treatment, constructs were tested as described previously (Paxton et al., 2010). Ligaments' width and length were measured using digital callipers and cross-sectional area (CSA) was calculated. The anchors of the engineered constructs were then placed into a stepper

motor and force transducer and stretched using a custom LabView program (National Instrument) at a rate of 0.4 mm/s until failure. Maximal tensile load (MTL), ultimate tensile stress (UTS) and modulus (stiffness) were calculated. Once tensile testing was completed, ligaments were analysed for hyp content, from which collagen content was determined (Paxton et al., 2010). Briefly, constructs were hydrolysed in 6 N HCl for 3 hours and dried at 120°C. The resulting sample was resuspended in hyp buffer consisting of citric acid, acetic acid, sodium acetate and sodium hydroxide solution (approximate pH 6 - 6.5). An aliquot was diluted 1:20, combined with 150 μL of 14.1 mg/mL chloramine-T solution and incubated for 20 minutes at room temperature. Subsequently, aldehyde-perchloric acid was added and incubated for 15 minutes at 60°C and cooled for 10 minutes at room temperate. 200 μL of each sample was added to a 96 well plate and absorbance was read at 550 nm.

Statistics

Although a sample size of 8 subjects was intended, a vial of serum for one participant was contaminated during transportation. Therefore, this subject was excluded from the study. Statistical analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California, USA). Prior to analysis, data was assessed for normality using a D'Agostino & Pearson's normality test. Engineered ligament data were normalised to baseline values and then analysed using a linear mixed model with Tukey's post hoc analysis. Differences in amino acid content of supplements was compared using a one-way ANOVA (if a full data set was available) or mixed-effect analysis with Tukey's post hoc analysis. Correlations between plasma amino acid concentrations and ligament outcomes were assessed using a Pearson's correlation coefficient. The significance level was set at $P < 0.05$ for all comparisons.

7.4 Results

Amino acid content of protein sources

The AA content of the various protein sources have been reported elsewhere (Alcock et al., 2019). Although there were similarities in TAA concentrations, the dairy protein contained higher levels of EAA, leucine and lysine, whilst the collagen proteins were superior sources of glycine. Hydroxyproline and hydroxylysine were not detected in the dairy supplement. There was no significant difference in proline content between the protein sources.

Amino acid availability in the plasma

Plasma AA data are displayed in Table 7.1. Plasma AA concentrations were significantly higher for TAA, EAA, lysine and proline after the consumption of all supplements at 40 min compared to BL ($P < 0.003$). Compared to BL measures, the ingestion of the collagen supplements led to significantly higher plasma concentration of glycine ($P < 0.007$), after 40 min, while the ingestion of hydrolysed casein led to a significantly higher plasma concentration of leucine and lysine at 40 min compared to BL ($P < 0.0001$). Across plasma concentrations at 40 min; the ingestion of collagen supplements led to significantly higher plasma concentrations of glycine, hydroxylysine and hydroxyproline compared to hydrolysed casein ($P < 0.002$). Meanwhile, the ingestion of hydrolysed casein led to substantially higher plasma concentrations of EAA and leucine compared to collagen supplements ($P < 0.0001$), and higher plasma lysine ($P = 0.02$). There were no difference between collagen supplements in terms of AA availability at 40 min after their ingestion ($P > 0.1$).

Collagen content and mechanics of engineered ligaments

There was no difference in collagen content ($P = 0.09$), collagen % dry mass ($P = 0.3$), CSA ($P = 0.2$), MTL ($P = 0.2$), UTS ($P = 0.3$), or modulus ($P = 0.5$) for any of the treatment groups

(Figure 7.1). However, there was considerable inter-individual variability within these measures which is illustrated by individual data points in Figure 7.1.

Correlation of amino acid availability and material and mechanical properties

There was a weak inverse correlation between collagen % dry mass and plasma EAA ($r = -0.46$, $P = 0.05$), and proline ($r = -0.47$, $P = 0.001$). There were no other correlations found between plasma AA concentrations and collagen content ($P > 0.2$), collagen % dry mass ($P > 0.05$), CSA ($P > 0.4$), MTL ($P > 0.3$), UTS ($P > 0.3$) or modulus ($P > 0.05$).

Table 7.1: Amino acid content (mg) per 20 gram serving of dairy and collagen proteins, and plasma amino acid concentrations 40 min after their consumption, presented as mean ± SD.

Supplement	Hydrolysed casein powder			Powdered gelatin			Hydrolysed collagen peptide powder		
Amino acid content	Per serve (mg)	Plasma concentration (µmol/L)		Per serve (mg)	Plasma concentration (µmol/L)		Per serve (mg)	Plasma concentration (µmol/L)	
	-	BL	40 min	-	BL	40 min	-	BL	40 min
TAA	19378	2193 ± 244	3629 ± 455	20108	2111 ± 324	3399 ± 615	20594	2143 ±489	3695 ± 919
EAA	8368	998 ± 112	1849 ± 159×^	4944	972 ± 140	1281 ± 241	5858	1008 ±206	1281 ± 241
Pro	2060	178 ± 33	386 ± 64	2664	160 ± 49	336 ± 95	2722	163 ± 68	425 ± 144
Gly	354	234 ± 38	225 ± 45	4750	232 ± 38	635 ± 173*	4904	222 ± 51	744 ± 248*
Lys	1468	172 ± 28	331 ± 52 ×^	696	174 ± 35	263 ± 46	746	178 ± 42	279 ± 56
Leu	1786	139 ± 21	329 ± 30 ×^	588	172 ± 29	172 ± 29	584	147 ± 35	182 ± 38
Hyl	N.D.	N.D.	N.D.	210	N.D.	17 ± 3*	208	N.D.	22 ±10*
Hyp	N.D.	18 ± 9	19 ± 8	2330	20 ± 11	139 ± 42*	2434	17 ± 6	197 ± 105*

TAA: Total amino acids, EAA: Essential amino acids, Pro: Proline, Gly: Glycine, Lys: Lysine, Leu: Leucine, Hyl: Hydroxylysine, Hyp: Hydroxyproline, N.D.: Not detected.

Note: Cysteine and tryptophan have not been included in analysis, which account for < 5% in total. mg per serve calculated based on free amino acid molecular weight.

Plasma concentrations presented as mean ± SD. Significantly higher than:* hydrolysed casein, × Gelatin, ^ hydrolysed collagen ($P < 0.05$)

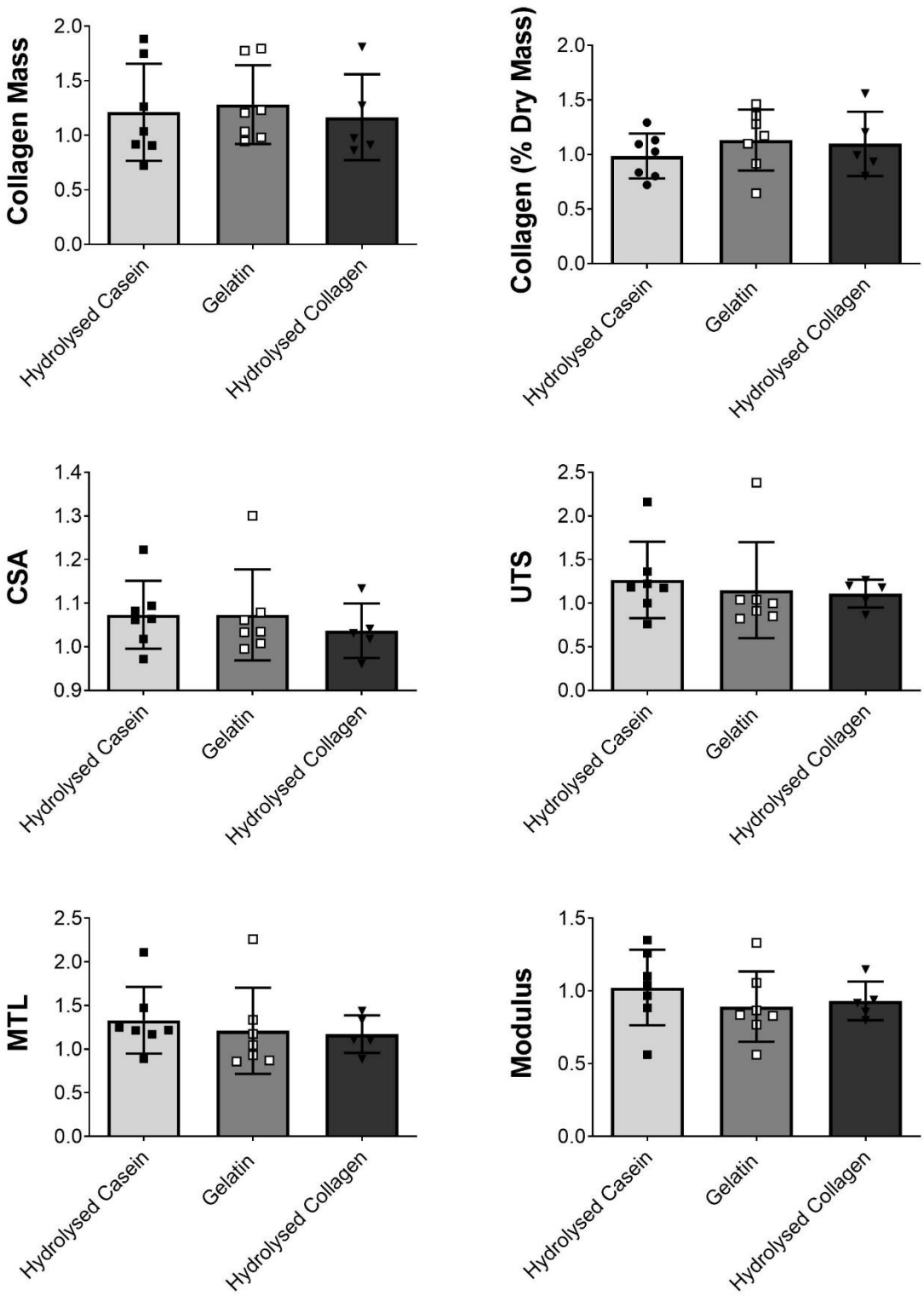

Figure 7.1: Material and mechanical properties of engineered ligaments treated with serum obtained after the consumption of collagen and dairy proteins, and a physiologically relevant media. Column shows mean, lines represent SD and shapes represent individual data points. All data are normalised to BL and are presented unit-less.

7.5 Discussion

The main finding of the current study was that, despite differences in blood AA availability following the ingestion of dairy and collagen proteins, we failed to find significant differences in the material or mechanical properties of the engineered ligaments when they were exposed to this nutrient support. Furthermore, there was only a weak inverse correlation between plasma EAA and proline and collagen % dry mass, while there were no other correlations between plasma AA concentrations and ligament properties. These findings are in contrast to previous work by our group in which we found enhanced collagen content in engineered ligaments treated with normal DMEM and serum collected after the ingestion of increasing amounts of gelatin (Shaw et al., 2017).

Although the recent development of 3D models have allowed for insight into the impact of various nutrition and exercise interventions without the need for invasive techniques, there is criticism as to their "real-world" applicability, particularly in terms of the composition of media utilised to grow and treat the constructs (Ackermann et al., 2019; McKee et al., 2017; Ravi et al., 2015; Yao et al., 2017). Indeed, DMEM was initially developed to supply cancer cells with nutrients essential for their continuous proliferation (Ackermann et al., 2019). As such, DMEM contains nutrients in supra-physiological levels including glucose, minerals such as calcium and phosphorus, and AAs such as leucine and lysine (Ackermann et al., 2019; Yao et al., 2017). Our study followed the concept implemented by several other researchers of manipulating constituents of the media to be more reflective of physiological levels (Baar, 2015; Cantor et al., 2017; Paxton et al., 2010; Vande Voorde et al., 2019), with the specific focus on AAs that differ within dairy, and collagen proteins, specifically EAAs, glycine, hydroxyproline and hydroxylysine.

Proline and ascorbic acid

Although DMEM contains supra-physiological levels of some nutrients, others, such as proline, and ascorbic acid are not present in the media. Proline constitutes a large component of collagenous protein and has been shown to be essential for collagen production (Wu et al., 2011). Meanwhile, ascorbic acid is an essential cofactor for prolyl-4-hydroxylysase, which catalyses the formation of hydroxyproline. Therefore, deficiencies in the intake of ascorbic acid interferes with the development of collagen protein and leads to poor connective tissue strength (Boyera et al., 1998; Peterkofsky, 1991). In optimising the engineered ligament model, Paxton et al. (2010), determined that the addition of proline (50 µM), and ascorbic acid (50 µM) led to an increased collagen content and strength in their constructs (Paxton et al., 2010). Later, Hagerty et al. (2012), determined that the addition of 200 µM ascorbic acid and other growth factors led to further increases in the collagen content of engineered ligaments. Furthermore, recent work by our group determined that the intake of increasing amounts of gelatin led to an increased proline availability, potentially leading to collagen content of the engineered ligaments (Shaw et al., 2017). Although in the current study we allowed our base media to contain optimal amounts of ascorbic acid and proline levels associated with the viability of engineered ligaments (West et al., 2015), we still expected to see differences in blood proline concentrations following the intake of our experimental supplements. In fact, there was no difference in availability of proline between protein sources after consumption. Thus, it is possible that the lack of additional availability differences in proline and/or ascorbic acid from the intake of collagen and dairy proteins led to the lack of differences seen in material and mechanical properties of the engineered ligaments. It is also possible that the addition of these components to the media had such a large impact on the outcome measures, that any subtle differences due to other AA concentrations in the subjects' plasma were unable to be detected. Further work is therefore required to determine whether proline and/or ascorbic acid were the

key determining factors that accounted for the lack of differences seen within the ligament properties despite receiving different levels of other AAs within the treatment media.

Glycine

Glycine is an important AA in collagen synthesis protein, providing ~ 1/3 of the protein content of a collagen molecule (Li et al., 2018). Although glycine has been defined as a non-essential AA (NEAA), it has been suggested that its biosynthesis within the body does not meet the demands for collagen synthesis requirements in all scenarios (Meléndez-Hevia et al., 2009; Wang et al., 2013). In addition, glycine has been shown to exert a range of anti-inflammatory effects in a range of tissues including the liver, kidney, heart, intestine and skeletal muscle in varying states of disease and malnutrition (Zhong et al., 2003). In an animal model, a high glycine diet has been shown to increase collagen synthesis in an inflamed deep digital flexor tendon (DDFT), to a similar degree to that of a non-inflamed DDFT (Vieira et al., 2015). Within the current study, plasma glycine concentrations at the time point of sampling for the ligament treatment (40 min post-ingestion) were 744 ± 248 and 635 ± 173 µmol/L for hydrolysed collagen, and gelatin, respectively; this is similar to the concentrations reported in our previous study for the high (15 g; 687 µmol/L) gelatin dose (Shaw et al., 2017). We note, therefore, that our higher doses of collagen proteins (20 g) did not appear to result in increased availability of glycine, suggesting a threshold in regard to the amount of gelatin that is able to be absorbed or possibly utilised. However, we acknowledge that blood concentrations provide a blunt snapshot of the dynamic kinetics between appearance and tissue uptake of nutrients. Furthermore, we note that the serum used to treat the engineered ligaments was obtained at a time point informed by an earlier part of data collection of the larger study as the mean time to reach peak plasma AA concentrations and further data collection/analysis showed variability in time to peak between individuals (Alcock et al., 2019). Thus, it is possible that the peak

concentration of glycine was not captured for all individuals within the current study. This also provides a case for further work to determine differences in BL levels and/or absorption of key AAs that may be associated with collagen synthesis in dense connective tissues.

Hydroxyproline and hydroxylysine

Other AAs of quantitative importance in collagen proteins, but absent within the dairy proteins include glycine, hydroxyproline and hydroxylysine. Hydroxyproline and hydroxylysine are metabolites unique to collagen protein and are produced during the process of collagen synthesis (Shoulders et al., 2009). Within the current study, both were found to be present in plasma following the consumption of collagen-derived protein supplements (Table 7.1.). Although they contribute to the strength and rigidity of formed collagen molecules, our current understanding is that plasma hydroxyproline and hydroxylysine are incapable of being incorporated into new collagen (Shoulders et al., 2009; Sjoerdsma, 1965; Yamauchi et al., 2012). Therefore, differences in their bioavailability should have little influence on the collagen content or mechanical properties of engineered ligaments, unless they have a unique bioactive role that may upregulate the synthesis of collagen in certain tissues as has been previously suggested (Edgar et al., 2018; Matsuda et al., 2006; Rutherfurd-Markwick, 2012; Schunck et al., 2013).

Essential AAs and leucine

EAAs are necessary for the maintenance and integrity of muscle tissue and inadequate intakes lead to perturbations of growth and development and lean mass atrophy (Hou et al., 2015). Considerable attention has focused on the role of one particular EAA, leucine for its role in stimulating myofibrillar protein synthesis via the mammalian target of rapamycin (Dodd et al., 2012). Indeed, leucine has been shown to amplify the growth of muscle tissue when combined

with an appropriate exercise stimulus (Phillips, 2014; Phillips et al., 2011). In extension of their importance in myofibrillar protein synthesis, researchers have sought to determine whether EAA may also play a role in the synthesis of collagen with equivocal results. Barbosa et al., (2012) reported increased collagen content and tissue mechanics of the digital flexor tendon, amplified by exercise, when malnourished rats consumed a high leucine diet. However, these results were not seen in a nutrient replete cohort (Barbosa et al., 2012), and a separate study involving rodents failed to see an increase in skeletal muscle collagen when leucine enriched EAA was consumed immediately, and 1 day, after exercise (Kato et al., 2016). In the current investigation, although the ingestion of hydrolysed casein resulted in a higher plasma availability of leucine compared to the intake of collagen derived proteins, this increased availability did not result in any differences in material or mechanical properties of the engineered ligaments. This suggests that leucine may not play a critical role in collagenous protein synthesis within the engineered ligament model compared to that which is well-established in myofibrillar proteins (Churchward-Venne et al., 2012; Dodd et al., 2012).

Hydrolysed and non-hydrolysed collagens

Suggestions that hydrolysed protein sources are superior to whole protein sources due to higher bioavailability and bioactive functions (Aito-Inoue et al., 2007; Iwai et al., 2005) have not been well documented within the literature. In the present study, we did not see any differences in apparent availability (i.e. higher or more rapidly achieved increased in blood concentrations) of any AAs between the hydrolysed, and partially hydrolysed collagen protein sources at the chosen timepoint of 40 min post-ingestion (Table 7.1). Although we did not measure peptide availability, if there was a significant biological effect of hydrolysed collagen on collagen synthesis compared to the partially hydrolysed collagen, we would then expect to see significant differences in material or mechanical properties of the engineered ligaments after

their consumption, however this was not the case (Figure 7.1). This suggests that within the current study hydrolysed collagen was not superior to partially hydrolysed collagen in terms of general bioavailability or impact on collagen synthesis.

Individual variability

As was evidenced in Table 7.1, there was between-subject variability in plasma AA response to the consumption of dairy and collagen protein sources, suggesting differences in individual capacity to digest, absorb and/or the transportation of varying protein sources. Furthermore, although the loss of one subject's data weakened the study power and our ability to probe individual "responsiveness", there was a visual difference in the inter-individual results around the material and mechanical properties of the engineered ligaments (Figure 7.1). However, there were only weak inverse correlations found between plasma EAAs and proline and collagen % dry mass. Thus, differences in AA availability between individuals were potentially too small to equate to significant differences in material or mechanical properties, or the particular AAs which presented differences in availability were not implicated in collagen synthesis.

Limitations and future directions

There were several limitations to the current study. The desired sample size was not achieved due to an accident in the sample handling; this reduced the statistical power of the study and decreased the likelihood of detecting real-world significance. Further the inter-individual variability suggested that other factors such as exercise habits, hormonal milieu and/or regular dietary intake may influence an individual's AA concentrations in response to an ingested protein dose. In addition, although all plasma/serum was obtained after an over-night fast, with a minimum of 48 h between trials, we did not capture the exercise and/or regular dietary intake

of subjects during this preparation phase. Differences in background diet and exercise, as well as other unidentified factors may have contributed to the considerable inter-individual variability in the changes in the properties of the engineered ligaments following our interventions. Although it is difficult to ascertain what led to the lack of differences in the material and mechanical properties of engineered ligaments, it is possible that low levels of AAs present in the treatment media did not support the optimal growth of the engineered ligaments as is necessitated in cell-culture work (Yao et al., 2017). As such, it is possible that other components of the development media i.e. the fibrinogen gel may have been broken down and utilised as an additional source of AAs to support ligament growth. Another limitation of the current study was that although proline was reduced when initially diluting the media, we decided to follow the standard media enrichment protocol whereby 50 µM of proline was added to the growth and treatment media (Hagerty et al., 2012). Had we not added extra proline to the treatment media, we may have seen slight differences in the material and/or mechanical properties of the engineered ligaments as a result of differences in proline availability after the consumption of the protein sources. Although we adapted our treatment media to simulate physiological values for serum AA concentrations, it may be more appropriate to match the composition of the media to that of peritendinous fluids after the consumption of specific protein sources and/or AAs. However, to our knowledge this information is not yet available, thus would be of benefit for future research. Further research on manipulations of treatment media is needed to find a balance between mimicking *in vivo* conditions and supporting the viability of *in vitro* work. It is possible that if there we had more participants, and thereby power to the current study, this may have overcome differences in inter-individual variability, and led to significant outcomes. A final limitation to the current study, is that no comparison between characteristics of the engineered tissues from pre- to post-treatment could made due to the destructive nature of the testing process.

Conclusion

Although several AAs have been proposed to influence collagen synthesis, the differences in concentrations of AAs in the plasma at 40 min post consumption of a hydrolysed diary and collagen protein, and a non-hydrolysed collagen protein, in addition to a media to reflect fasted, rested physiological levels did not influence the material or mechanical properties of engineered ligaments. Despite these findings, this study highlights several challenges in utilising *in vitro* cell culture models to investigate conditions found *in vivo* models. Thus, care should be taken when translating findings obtained from cell culture work to whole body outcomes. Furthermore, extraneous variables such as habitual protein intake, exercise, and potentially the ability to digest/absorb proteins should be considered where possible.

Author contributions statement

R.D.A, L.M.B, G.C.S and K.B. were involved in study design. R.D.A and NT were involved in data collection. R.D.A, N.T, K.B and D.S were involved in data analysis and statistical analysis. R.D.A, L.M.B, N.T, G.C.S, K.B and D.S were involved in results interpretation, drafting, reviewing and revising the initial manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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Chapter Eight: Discussion

8.1 General Discussion and Summary

Maintaining the health and function of dense connective tissues is of obvious interest to all populations. As discussed in Chapter One, the incidence and cost of connective tissue injury is of significant concern to all exercising individuals from recreational, to elite athletes. Furthermore, given the potentially slow rate of turnover of these tissue types, injuries can require lengthy rehabilitation periods to ensure full recovery. Therefore, strategies that can enhance the strength and/or accelerate healing of dense connective tissues would be of significant value (Baar, 2015).

Early research on the synthesis of collagen in ligaments and tendons investigated whether EAAs, such as leucine, would extend their role in the synthesis of myofibrillar protein to the dense connective tissues (Scott et al., 2016). However, it remains unclear whether the intake of leucine beyond that of dietary sufficiency for normal growth and development is of benefit to ligaments and tendons. Separate lines of investigation of the strategic intake of the AAs that make up the bulk of collagen protein (e.g. glycine and proline) have shown promise in introductory models involving cell cultures (Paxton et al., 2010; Shaw et al., 2017). However, confirmation of the transferability of these findings to "real-world" conditions requires further investigation involving *in vivo* protocols.

As discussed in Chapter Two, longitudinal intervention studies pose significant challenges, including the lengthy intervention period required to observe meaningful changes in ligaments and tendons, and the difficulty of measuring the synthesis and incorporation of collagen into these tissues. Furthermore, the initial scoping activities associated with this body of work identified uncertainty around the optimal protein source and/or AAs, the bioavailability of AAs

after the consumption of various protein sources and the lack of ability to control or measure background intake of collagenous protein. The combination of this uncertainty with the level of resourcing required for a lengthy intervention study led to the decision that the priority needs in this area were to systematically determine and confirm the best candidates for a chronic supplementation study, and to identify any factors that might confound the outcomes or the interpretation of the data. Thus, the intention of this thesis was to create a foundation of information that could assist in the future implementation of more invasive quantitative intervention study. The specific areas investigated within this thesis and the outcomes of these investigations are outlined in Figure 8.1. The main findings of these studies are:

1) Bone broth, a source of collagen-derived protein, commonly used in various sporting and/or interest groups (e.g. Cross-fit, and traditional Chinese medicine) has a widely variable AA composition, which is likely to be associated with differences in preparation methods (e.g. animal and/or bone type, cooking time etc.). The principle cause of this variability was not obvious and therefore, could provide confounding factors for future studies. On this basis, bone broth should not be considered a consistent and therapeutically beneficial source of collagen-specific AAs.
2) Urinary hydroxyproline is not suitable as a sensitive biomarker of habitual collagen intake in "free-living", healthy, active males, as it is rapidly excreted from the body (within ~ 6 h), and there is large inter-individual variation potentially due to differences in collagen digestion and/or collagen turnover.
3) In contrast to the claims of superior bioavailability of commercial hydrolysed collagen protein formulas (e.g. Gelita Tendoforte) compared to partially-hydrolysed collagen sources (e.g. gelatin), plasma AA responses to the consumption of several collagen-derived proteins was similar. The consumption of dairy-based and collagenous proteins produced a similar bioavailability of proline. Meanwhile, there was a higher blood

bioavailability of EAAs and leucine after the consumption of dairy proteins, and higher concentrations of glycine associated with collagen-derived protein.

4) The treatment of engineered ligaments with serum collected in response to the consumption of collagen and dairy proteins, and a media to reflect physiological nutrient levels did not result in any significant differences in collagen content or mechanical properties. The reason for this outcome is largely unknown but may be due to insufficient AA availability in the treatment media, or due to the lack of differences in the proline content between the dietary treatments. This study illustrates the limitations and care that needs to be taken when designing and utilising an *in vitro* model to infer *in vivo* outcomes.

Overall, these findings highlight the complexity of research within this area and that careful consideration is necessary prior to engaging in costly, complex and lengthy intervention studies aiming to increase our knowledge of nutrition support for dense connective tissues.

8.1.1 Thesis outcomes

Chapter Four (Study 1) summarises an investigation to determine whether a food source of collagen, bone broth, would provide specific AAs in amounts that are similar to reference supplements currently involved in collagen research. This aligns with the "food first" approach of sports nutrition professionals and recognises that the provision of nutrient needs through food sources has the capacity to meet several sports specific and health goals simultaneously while avoiding the risk of inadvertent doping associated with supplement use (Geyer et al., 2008). A variety of bone broth sources were assessed, including both commercial and home-made varieties, with both standardised and non-standardised recipes. The home-made varieties of bone broth included a number of preparation methods claimed to extract higher levels of key AAs (e.g. the addition of vinegar) and the use of various different animal sources and/or bone

types. Analysis of the broths showed consistency in the AA composition of batches prepared to the same recipe (made by the author) but inconsistencies between batches prepared according to different recipes. However, although a bone broth prepared by an external source (i.e. the "chef prepared bone broth") contained comparable (although highly variable) amounts of key AAs to the reference supplements, a further batch prepared by the author to the same recipe was unable to match the level of AAs. There was no consistency across factors involved in the different preparation methods of home-made bone broths to explain the differences in the AA content. This led to the conclusion that bone broth is too variable in composition to be utilised therapeutically for the purpose of supporting collagen synthesis in ligaments and tendons. We recognise that there was a practical limit to the number of variations in both broth recipes that could be included in the study. Therefore, it is plausible that different types of bones and/or cooking methods may achieve increased levels of AAs in some bone broths, and therefore increase the suitability of this food source if prepared to a known and standardised recipe. Nevertheless, the variability in AA content and the general findings of insubstantial amounts of potentially important AAs reduces the widespread viability of bone broth as a vehicle for supplying doses associated with therapeutic uses.

Chapter Five (Study 2) was undertaken in recognition of the lack of data on the background intake of collagen-derived protein in the Australian diet, due to the lack of information on the collagen content of various food sources. The inability to account for background dietary intake of nutrients may confound the validity of research on dietary support for the health/performance of dense connective tissues, both in epidemiological settings and in the standardisation of intervention studies (Weaver et al., 2017). Hydroxyproline is a metabolite of proline, which is released during collagen breakdown but not incorporated into new collagen (Prockop et al., 1965). Historically, urinary hydroxyproline concentrations have been measured

as a marker of hydroxyproline excretion, secondary to the breakdown of collagen proteins in the body (Prockop et al., 1961). As the urinary excretion of hydroxyproline is increased after the consumption of dietary collagen, when this method is used to screen for the presence of conditions with atypical collagen breakdown, the intake of collagenous food sources is usually restricted to prevent interference with the validity and interpretation of data (Prockop et al., 1961). However, no studies have sought to determine whether urinary hydroxyproline could be indicative of dietary collagen intake in "free-living" individuals, and the time course of its appearance within the urine after its intake. The results of this study showed that urinary hydroxyproline (standardised to creatinine excretion) was increased for up to six hours following the consumption of a large bolus of collagen proteins (31 ± 8.8 mmol: mol after the consumption of gelatin and 33.7 ± 22.0 mmol: mol after the consumption of hydrolysed collagen). However, usual dietary intake (i.e. a normal breakfast) failed to create a detectable change in urinary concentrations. Furthermore, there was also considerable variability between individuals, ranging from 22.9 to 47.5 mmol: mol and 2.0 to 50.5 mmol: mol, 0-6 h after the consumption of gelatin and hydrolysed collagen, respectively. This variability could not be accounted for with the measured variables. Indeed, there was no association found between general protein intake or exercise performed and the excretion of hydroxyproline. Therefore, other factors must influence urinary hydroxyproline concentrations between, and within, individuals. This might include individual capacity to digest collagen protein (Gardner, 1988), which has implications in terms of how these individuals may respond to dietary interventions utilising collagen protein.

Previous literature suggests that, unlike other tissues with high rates of turn over (e.g. bone, and muscle), nutrition is likely to play a supportive, rather than stimulatory role, in collagen synthesis within ligaments and tendons (Babraj et al., 2005a; Babraj et al., 2005b). This is

likely due to dense connective tissue being reliant on mechanical stimulus in order to facilitate growth, and the need to encourage blood flow (through exercise) through tissues which are otherwise poorly vascularised, in order to improve AA availability at the site of the tissue. Therefore, Chapter Six (Study 3) aimed to answer several questions with regard to the bioavailability of AAs after the consumption of various collagen and dairy protein sources. These included the timing and magnitude of peak blood concentrations of the AAs putatively involved in the synthesis of collagen; such information would help to inform prescriptions for the optimal timing and amount of the protein source(s) prior to exercise. Furthermore, given the lack of clarity around which AAs are involved in enhancing the synthesis of collagen (see section 2.8, we endeavoured wished to determine blood AA profiles following the intake of a protein dose considered to be of therapeutic value for collagen synthesis as well as a commonly consumed serve of bone broth (although there were limitations using this approach; see section 8.3).

It has been suggested that hydrolysed collagen proteins are superior to intact, partially or non-hydrolysed collagen proteins. Such formulations are proposed to have a more pronounced and rapid ability to appear in the blood and reach the target tissue, as well as providing physiological functions beyond that of the whole, intact proteins (Aito-Inoue et al., 2007; Edgar et al., 2018; Matsuda et al., 2006; Oesser et al., 2003; Rutherfurd-Markwick, 2012). The use of these hydrolysed collagen formulations that contain bioactive peptides have been reported to enhance the health/function of collagen-containing tissues within the body, with outcomes ranging from reduced joint pain, to improved body composition (Edgar et al., 2018; Oertzen-Hagemann et al., 2019; Oesser et al., 2016; Oesser et al., 2003; Praet et al., 2019; Schunck et al., 2013; Zdzieblik et al., 2015). However, these products have not been compared to other dietary and/or supplemental forms of collagen. This study determined that dairy proteins

provided higher plasma EAA, and leucine concentrations, while collagen proteins provided higher plasma glycine concentrations. Meanwhile, although increased bioavailability (i.e. greater AA appearance in blood following intake of the protein) was observed with the hydrolysed dairy proteins compared to non-hydrolysed dairy proteins this difference was not seen between the hydrolysed and non-hydrolysed collagen formulas. This finding contrasted with the results of other similar studies, and may have resulted from differences in processing methods to hydrolyse protein sources (Dallas et al., 2017).

Chapter Seven (Study 4) was informed by the first part of the data collection for study 3 (part A), in which 8 of the 15 subjects' plasma was analysed for AA bioavailability after the consumption of collagen and dairy protein sources (refer to Chapter Three). This allowed an indication of when AAs were at their peak in the blood, which would inform when to obtain the blood used to treat the engineered ligaments. Three supplements were utilised for this study including a hydrolysed and partially hydrolysed form of collagen protein, and a hydrolysed dairy protein. Engineered ligaments were grown from human fibroblasts in a full strength media for 8-10 days, and treated with serum collected from the participants after the consumption of dairy and collagen proteins and mixed into a more physiologically reflective media (Sarwar et al., 1991) for 6 days. This media was manipulated as the full strength media utilised to develop and treat engineered ligaments contains concentrations of AAs four times that which is found in the blood (Yao et al., 2017). Therefore, it is not reflective of "real-world" AA availability within the body, and in particular within these tissue types which are known to be poorly vascularised (Boushel et al., 2000a; Boushel et al., 2000b; Bray et al., 2002; Petersen et al., 1999). After treatment, the ligaments were tested for material and mechanical properties using a variety of methods. However, there were no significant differences in collagen content and/or mechanical properties found between the protein sources. Unfortunately, we were

unable to ascertain what led to the lack of significant findings, however it could have been attributable to a lack of AA availability within the treatment media that was purposefully diluted, or that there was equivalent proline availability across all treatments. It was also possible that the addition of ascorbic acid may have reduced the likelihood of detecting small differences in ligament properties. Finally, there was also considerable inter-individual variability, which may have been confounded by other variables not captured within this study. Whilst we were unable to ascertain what led to either the lack of significant findings or individual variability, this study does illuminate potential challenges of this model in its application to *in vivo* conditions.

Therefore, collectively, this body of work was able to fill in several literary gaps and provide further insight into methodological considerations that will assist in the development of further research and ultimately a well-designed intervention study. However, several other gaps were highlighted, and further highlights the difficulty undertaking research within this area.

8.2 Limitations and Considerations

It is important to recognise the global limitations of this body of work, by summarising and integrating the limitations of the separate studies. Study 1 aimed to investigate the diversity in the amino acid content of different types and batches of bone broth. Although we tried to include a large range in the types of broth, there are a multitude of different combinations of individual meat types and/or meat products, amounts of ingredients, the inclusion or exclusion of varying ingredients, such as vinegar, and treatment of the end product (e.g. with, or without fat, strained, or unstrained and so forth). Had we included a greater range of collagenous-rich protein sources (for example chickens feet, tendon, tripe etc.), it may have been possible to

reach higher amounts of collagen precursors, comparable to the doses present in the reference supplements. Indeed, we note that the café-provided bone broth was able to match, and even exceed, the level of AAs in the reference supplements; presumably, this was attributable to some unidentified component of preparation. Furthermore, the dose of reference supplements (20 grams), may be higher than the optimal dosage, meaning that we may have overlooked the benefits of some broth sources.

In Study 2, we used 20 g doses of collagen-rich protein supplements to assess urinary hydroxyproline excretion as a potential biomarker of its intake. Although we concluded that spot collections of urine provide only a short-term observation of a relatively substantial dietary intake (e.g. 20 grams of collagen powder), further interrogation could include analysis of the response to different dietary protein sources and its interaction with a range of different factors such as individual body composition/lean mass or volume, intensity and mode of exercise that may contribute to the individual variability of urinary hydroxyproline excretion. It should be noted that due to challenges with data collection for dietary intake records, the sample size on some occasions was reduced to 5. This reduced our ability to determine if any of these factors had a significant impact on hydroxyproline excretion. However, given that hydroxyproline appeared to be excreted within a 6-hour period, it is unlikely that dietary intake the night prior (> 6 hours) would have had an impact. Furthermore, given that hydroxyproline excretion was not significantly increased on days where the collagen supplement was not consumed, it is unlikely that protein intake during the day would have had any correlation with hydroxyproline excretion, unless of course a substantial source of dietary protein intake came from collagenous sources, which (as mentioned in section 2.8.2) is unable to be quantified.

As outlined in Chapter Three, the data collection for Study 3 was split into two phases and participant cohorts. This was necessary to enable the proposed studies to be completed in a timely manner. More specifically, in utilising this systematic approach, it enabled information from the first eight participants to inform data collection for the final study: to determine the time point of peak AA concentrations at which the large blood sample, needed for the engineered ligament protocol, would be drawn. This occurred before receiving the complete data set from the chemical analysis of the AA and protein content of the study food sources, bone broths and reference supplements was received. We were reliant on using the initial analyses, and manufacturer-derived information on the protein content, of the doses of the liquid collagen and bone broth used in Study 3. In some cases, the actual AA content deviated from our expectations/initial data. This prevented us from being able to truly compare the bioavailability of true 20-gram protein serves of these products. Due to financial constraints and limits to the volume of blood that could be collected from subjects over multiple trials in a short time frame (particularly the 51 ml sample), we were restricted to collecting blood at 20 min intervals for a total of three hours only. It is possible that more regular sample collection and/or for a longer period of time, may have given more accurate outcome measures, such as time to peak, and AUC. Furthermore, it is possible that had we engaged in more than one data collection for each protein source per participant, we may have seen differences in intra-individual variability on different days. This may have had implications for the ensuing study.

Finally, with regard to the cell culture work undertaken in Study 4, there is a considerable time required from passaging cells to growing the optimal set of at least 4 engineered ligaments per individual per treatment (approximately 4 – 6 months). This meant that there was insufficient time to develop another set of ligaments to be treated with full-strength media, against which the ligaments treated with the media manipulated to reflect physiological levels could have

been compared. Perhaps the most significant limitation was the decision to adhere to previous protocols including standardised addition of proline and ascorbic acid to treatment media. This may have reduced the likelihood to observe any differences resulting from the treatments per se, given that both proline and ascorbic acid are heavily involved in collagen production (Barbul, 2008; Boyera et al., 1998).

8.3 Future Research Directions

This body of work was able to address several gaps in the current literature including: a) whether a common food source of collagen, currently used by several cultural populations is able to provide a reliable and therapeutically beneficial amount of key AAs proposed to be involved in the synthesis of collagen; b) whether it is possible to use urinary hydroxyproline as a suitable biomarker of collagen intake in "free-living", healthy, active males and; c) the bioavailability of AAs after the consumption of various collagen and dairy protein sources, including intact, hydrolysed and partially hydrolysed forms. Although the final study did not have the intended outcome and allow for the interpretation of the various treatments, it did highlight the challenges in the transferability of these models to "real-world" conditions. As a result of these findings, several future research directions require further investigation (Figure 8.1.). These include:

1) The analysis of the collagen content of various food sources, including varying cuts of meat and offal determined through the analysis of their hydroxyproline content, to allow for the development of more detailed nutrient composition tables for the quantification of collagen intake in dietary intake studies. This could provide added rigidity to studies within this area and/or allow for epidemiological research.

2) Further investigations into individuals' ability to digest collagen proteins, including those consuming a vegetarian diet, and the impact of these differences on various outcomes such as collagen synthesis and incorporation, and/or dense connective tissue health.
3) Investigations into whether athletes have a higher rates of collagen turnover compared to sedentary individuals and whether different tissues within the body have higher rates of collagen turnover.
4) Investigations as to the optimal dosage of collagen, whether there is a threshold in regards to bioavailability after the consumption of collagen proteins, and whether this is dependent on body mass.
5) Investigations into whether regular, consistent intake of collagen results in increased availability of AAs in the blood, and the impact that this might have on dense connective tissue material and its' mechanical properties.
6) Microdialysis study investigating the availability of key nutrients and presence of biomarkers of collagen turnover in peri-tendinous fluid after the consumption of collagen.
7) Engineered ligament/cell culture studies mimicking *in vivo* conditions, including proline and ascorbic acid consumed, rather than added to the treatment media.
8) Engineered ligament/cell culture studies mimicking *in vivo* conditions, compared to traditional conditions (e.g. physiologically relevant compared to full strength media), and whether increased EAA content with media matched to peritendinous fluid (as above) would have an impact.
9) Engineered ligament/cell culture studies treated with individual AAs, and/or combinations thereof in order to determine which AA/combination of AAs are involved in collagen turnover, in dense connective tissues.

10) Validation of assessment techniques for collagen synthesis and integration in dense connective tissues *in vivo*.
11) Further investigations into gender differences for connective tissue health/function and injury risk as a result of fluctuations in sex hormones, and the nutritional management of these potential differences.
12) A well-designed intervention study (as outlined in Table 8.1, below).

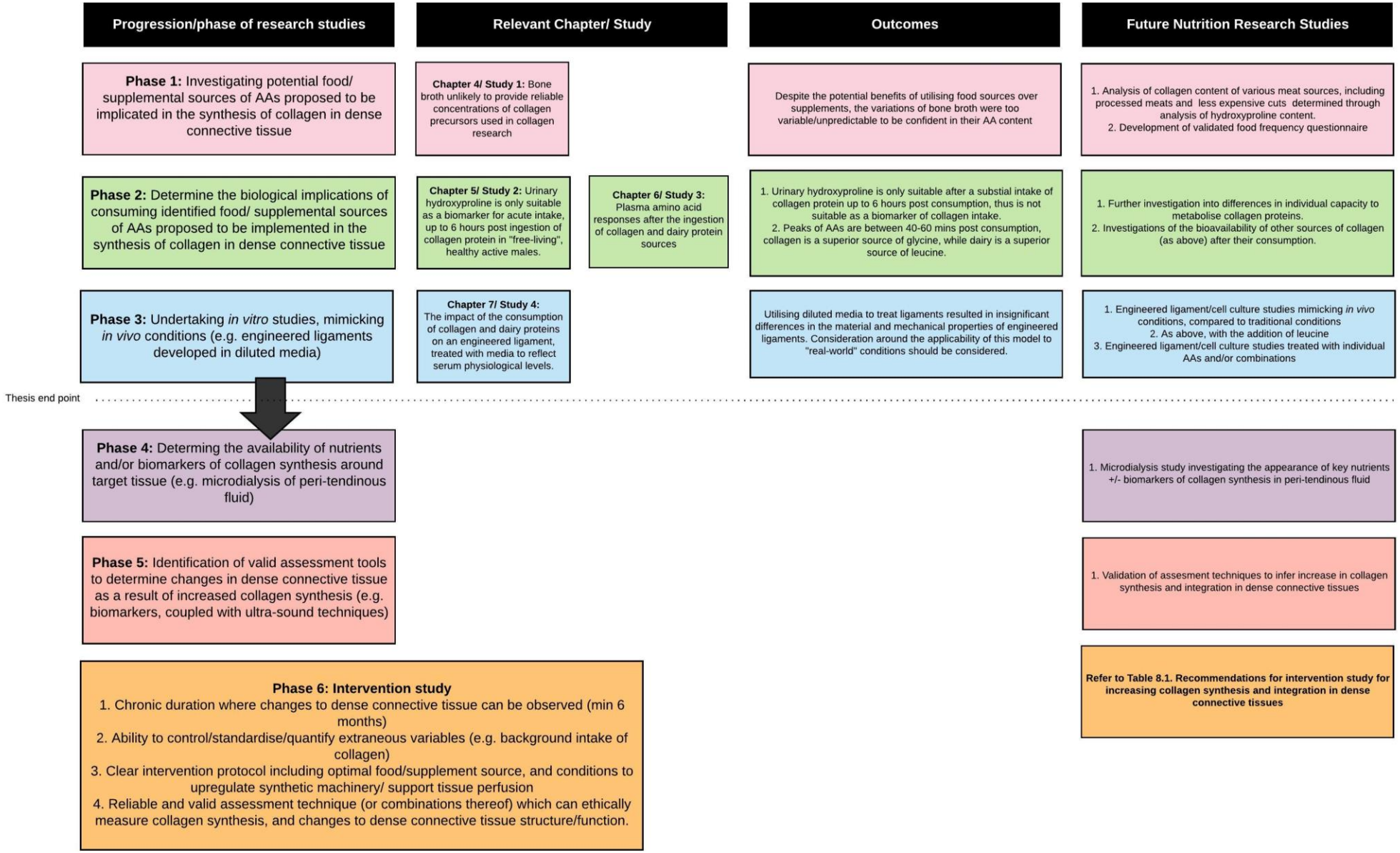

Figure 8.1: Research phases, summary of outcomes from studies within this thesis, and recommendation for further research studies.

Table 8.1: Recommendations for intervention study for increasing collagen synthesis and integration in dense connective tissues.

Component of study design	Methodological considerations	Other considerations
Participant selection and recruitment	• Should initially consist of male participants given the effect of female sex hormones on dense connective tissue health and injury risk. • Should be athletes who utilise explosive movements or are prone to connective tissue injury given nature of sport and/or separate athletes in different positions. • Responders vs. non-responders should be assessed through the excretion of hydroxyproline or blood availability of AAs after the consumption of protein sources in order to assist the interpretation of research outcomes. • Exclusion criteria includes low or non-meat eaters; not recommended as this may impact on collagen digestion. • Need to quantify or standardise exercise habits. • Use power calculation as minimum number of participants required with an additional 10-20% in case of any data loss. • Should utilise trained population as it is likely that athletes have higher rate of collagen turnover than sedentary individuals (need further studies to confirm).	Likely need to determine other factors that may increase or decrease hydroxyproline excretion between individuals and/or differences in ability to metabolise collagenous proteins
Length of intervention	• Minimum of 6 months. • This may depend on the dense connective tissue being targeted e.g. those with higher turnover compared to those with lower turnover.	Cost will likely affect how long the study can be undertaken.
Dietary intervention and adjunct measures	• Bioactive peptide formula vs hydrolysed collagen vs gelatin (need further studies to confirm). • Compare against placebo (e.g. vegetarian collagen as this is void of collagen specific AAs), also compare against high leucine protein sources. • Document vitamin C intake and assess within the blood. Recommend dietary adequacy rather than supplemental forms. • May be worth utilising single AAs and/or combinations thereof (need further studies to confirm) • Consume within 40-60 mins prior to exercise as per AA paper dependant on AA targeting. • Standardised diet likely not possible given the length of the study, therefore, dietary intake should be monitored, suggested, and/or repeated where possible. • Protein intake in particular should be consistent between individuals to ensure adequate availability of EAAs. • Minimum EI should also be recommended to ensure prevention of LEA which may impact on connective tissue health.	• Challenges in measuring dietary intake, need to utilise several adjunct measures, and ensure participants comply and do not lose food diaries. • Athlete compliance also needs to be taken into consideration and should also form part of data collection.
Exercise Protocol	• 6 minutes skipping protocol added to athlete's current training? • Imbed connective tissue specific programming for participants to encourage blood flow to the target tissue, and/or increase or decrease tissue mechanics.	Must control/document exercise undertaken outside of study, or give strict protocols.
Assessment of collagen turnover (synthesis and breakdown), incorporation into target tissue and functional outcomes	In lieu of direct measures (i.e. biopsy) several measures are necessary, including biomarkers (preferentially at the site of the tissue of interest), and changes to tissue size.	Need to complete micro dialysis study to confirm availability of AAs and biomarkers at the target tissue site.

8.4 Practical Implications

The potential to have a positive impact on the health/function of ligaments and tendons is of obvious interest, and therefore, perhaps prematurely, several guidelines have surfaced around the intake of collagen, with the intention to prevent, or accelerate healing from, soft tissue injuries. Presently, as a result of *in vitro* and *in vivo* work, it is recommended that 15 grams of gelatin, with a source of vitamin C be consumed 1 hour prior to exercise to assist in the upregulation of collagen synthesis and to improve tissue mechanics. Furthermore, several groups have recommended that hydrolysed/bioactive peptide formulations be consumed, as they have better bioavailability and bioactivity than non-, or partially hydrolysed forms. Meanwhile, others have recommended the consumption of bone broths for connective tissue support. The studies contained within this thesis have found that 20 grams of collagenous proteins achieved higher plasma AAs than those found in previous work utilising 15 grams, thus it is possible that consumption at this amount is of further benefit in regards to increasing the synthesis of collagen in dense connective tissue. However, we were unfortunately unable to confirm this with the *in vitro* work performed as part of this thesis, thus it has been recommended as an area for further research (see section 8.3). It was also discovered that, in the case of a hydrolysed peptide formula, and a non-hydrolysed collagen, there was no difference in amino acid bioavailability. Although there was a higher bioavailability of AAs in hydrolysed, compared to non-hydrolysed, dairy proteins, the low scores in regard to acceptability/palatability meant that it is unlikely to be consumed, even for the benefit of connective tissue health.

In regard to the non-hydrolysed collagen (gelatin), as it is considered a food product, there is also low risk of inadvertent doping with the consumption of this product. Furthermore, it is also at a substantially lower cost than hydrolysed collagen, thus compliance may be improved.

This was reflected in palatability/acceptability scores in study 2. Meanwhile, bone broth was found to be too variable in its AA content, and therefore, would not likely be able to consistently provide adequate levels of AA to support collagen synthesis/incorporation. Although it is not yet established whether outcomes determined *in vitro* will be reflected *in vivo*, given the potential of consumed collagen to increase the synthesis of collagen in dense connective tissue, the recommendations that have eventuated from this thesis for dense connective tissue health/injury prevention and/or to assist in tissue rehabilitation are as follows:

- During periods of connective tissue repair, consume 15-20 grams of collagen protein (either gelatin or hydrolysed collagen), 40 – 60 minutes prior to any exercise that will encourage/support blood flow to the target tissue, and "switch-on" synthetic machinery.
    - As collagen is metabolised and excreted within 6 hours; intake at least two times per day is potentially of further benefit than intake once per day.
- Given the anti-inflammatory properties of glycine, that is highly available within collagen protein sources, collagen intake may also assist with pain management in chronically inflamed dense connective tissues and/or joint pain.
- In times of increased metabolism (e.g. post-surgery), dietary adequacy of vitamin C and protein should be encouraged, with collagen precursors consumed as soon as possible after surgery.
- Within athletes, inadequate protein intake may put them at risk of connective tissue injury due to the poor availability of AAs necessary for the synthesis of collagen (particularly if they are found to have higher turnover rates of collagenous tissues). Thus, regular and adequate intake of protein should be encouraged.
- Whether collagen supplementation is useful as a preventative measure is currently unknown, however in athletes who have higher rates of exercise, and potentially a higher rate of collagen tissue turnover, collagen supplementation and/or inclusion of

collagen rich food sources within the diet may be of benefit, and are likely of low risk, particularly when consumed as a food source.

- Further, in athletes who have had previous connective tissue injury, utilising collagen daily may be of benefit, given dietary intake may be insufficient to support collagen containing tissues, and there may be a higher requirement within these athletes. It is possible that regular intake may also increase individual's capacity to digest these protein sources, thus regular intake may also assist with increased bioavailability.
- Gelatin may be a preferential option in some groups given its equivalent bioavailability to hydrolysed-collagen, reduced risk of inadvertent doping, and increased acceptability and palatability.

8.5 Conclusion

Although further work is necessary to confirm the outcome of dietary collagen and/or high leucine dairy proteins on dense connective tissue health/performance *in vivo*, this body of work provides a significant contribution to the literature. Furthermore, this work also highlights limitations and/or considerations in regard to the applicability of *in vitro* work to *in vivo* conditions when utilising a cell culture model. Ultimately, this thesis provides relevant information that will assist in the development of a well-designed intervention studies and inform professional practice recommendations.

Chapter Nine: Personal Reflection

When you commence a PhD, you are told that you will complete it and become an expert in your field of research. You start with this false confidence that even though it is incredibly difficult when you start, in a mere three years you will emerge as an expert researcher (a.k.a., a butterfly), complete with a super hero ability to foresee any difficulties or limitations that may arise in future research. Three and a half years on, I feel as though I have more questions than when I started, and I have realised that the beauty in research is not when everything runs smoothly and you get the outcome that you are looking for, it is when things don't go to plan that you experience the most learning and growth. In essence, it made me realise that I am still a caterpillar, waiting to get my wings. It has also made me realise that no matter how much control you think you have, there are some aspects of research that you just can't control. Instead, you are forced to go with the flow, and be comfortable in the uncontrollable mess that is sometimes characteristic of both life and research.

Perhaps naively, upon commencing this thesis, I had thought that everything would run smoothly, with a clear progression of questions that would be answered, which would ultimately lead to the implementation of a well-designed and well-controlled intervention study. However, upon thoroughly immersing myself in the literature, in addition to discussions with several experts within the field, it soon became clear that this would not be the case. For example, within dense connective tissue research, the only direct way to measure not only the synthesis, but also incorporation of collagen into these tissues is through biopsy. However, although not documented within the literature, anecdotally, there are reports of ongoing tendon pain/ damage in utilising the biopsy technique. So, the next step was to find a non-invasive way to measure changes to collagenous tissues. After leveraging some of the expertise at the now disbanded AIS, and playing around with ultra-sound machines. I was on my way to discuss the use of the UTC at a lab in Melbourne thoroughly involved in its research. However, when

asking the question “so what do these outcome measures actually mean”, and being met with a “well we don’t actually know that yet”, I realised, it was probably best to look for a plan C. Enter the “micro dialysis study” idea which involved an initial meeting with several experts at the AIS. However, this was another technique which required specific expertise, along with purpose-built tubes to enable the correct particles to be able to be obtained from the dialysate (i.e. collagen synthesis biomarkers; PINP and PICP). After a few weeks of researching this potential option I/we were met with another dead end. Although this was frustrating and felt like a purposeless exercise, I now appreciate that value of these so called “dead-ends”, and the role that they play in your future research planning.

In regard to the studies that were eventually decided on, and undertaken, there were also several difficulties/considerations which emerged. During the entirety of the thesis, two days per week were involved in servicing a professional rugby union team. It was intended that this would serve two purposes; to assist in the funding of the research studies contained within this thesis, and, to have a captive audience of which to implement phase 5; the intervention study. However, this did create some difficulty in regard to time constraints, and therefore, as outlined in Chapter Three, several data collections were completed in an over-lapping manner (specifically studies 2-4). Whilst this did reduce the total time commitment, cost, staff and participant burden, and avoided the need to recruit 3 different participant groups, it did restrict how data was able to be obtained, in the fact that we decided on utilising the one methodological protocol for several studies. Further, on some occasions, due to time constraints it meant that some trials had to be commenced prior to getting “the full picture” (e.g. total information on the peak of AAs, and total protein content for some of the samples).

Finally, the last study was commenced without a full understanding of cell-culture work which resulted in a considerable amount of limitations, and potentially a "non-significant finding". However, it wasn't until I physically went to the laboratory at University of California, Davis, and developed and treated the engineered ligaments myself, and completed the entire study, that I would have a complete understanding of the complexity of this work.

To conclude, whilst this PhD has taught me that although you can be incredibly thorough in your planning, there are some lessons in research that you won't be apparent until you go through those perceived failures. However, as aforementioned, it is during those times where you experience the most growth. To quote the great Thomas Edison: *"I have not failed, I have just found 10,000 ways that won't* work".

Chapter Ten: References

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Chapter Eleven: Research Portfolio and Appendices

11.1 Published Papers

Due to copyright restrictions, the following papers are not available within this thesis, please use the links below to find the final published versions.

Alcock RD., Shaw, GC., Burke, LM. Bone Broth Unlikely to Provide Reliable Concentrations of Collagen Precursors Compared With Supplemental Sources of Collagen Used in Collagen Research. *Int J Sport Nutr Exerc Metab*. 2018 (26):1-8. Doi: 10.1123/ijsnem.2018-0139.

Available at: https://www.ncbi.nlm.nih.gov/pubmed/29893587

Alcock, RD., Shaw, GC., Tee, N., Welvaert, M., & Burke, LM. RAPID COMMUNICATION: Urinary Hydroxyproline Is Only Suitable As a Biomarker for Acute Intake, Up to 6 hr Post ingestion of Collagen Proteins in "Free-Living," Healthy, Active Males. *Int J Sport Nutr Exerc Metab*. 2019 29(5): 461-465. Doi:10.1123/ijsnem.2019-0024

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Plasma Amino Acid Concentrations After the Ingestion of Dairy and Collagen Proteins, in Healthy Active Males

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Introduction: Recent evidence suggests that the consumption of essential amino acids (AA) and/or those abundantly present in collagen may have the capacity to influence the synthesis of new collagen in ligaments and tendons, when tissue perfusion is optimized (e.g., during exercise). However, little is currently known about the bioavailability of these AAs in blood after the consumption of various collagen and diary protein sources: such information is needed to develop potentially useful dietary and supplement intake strategies.

Objectives: The aim of the current study was to characterize blood AA concentrations in response to consumption of collagen and dairy protein sources; specifically, maximum concentrations, the timing of maximum concentration, and total (area under the curve) exposure above baseline.

Methods: A 20 g serve of various dairy and collagen proteins, and a 300 mL serve of bone broth were consumed by healthy, recreationally active males after an overnight fast. Blood samples were drawn every 20 min for a total of 180 min, for analysis of plasma AA concentrations. Total AA, essential AA and collagen specific AAs were analyzed for maximum concentration, timing of peak, and area under the curve.

Results: In general, protein intake was associated with a similar increase in total and collagen specific AAs, except for collagen proteins being a superior source of glycine (683 ± 166 μmol/L) compared to 260 ± 65 μmol/L for dairy proteins ($P < 0.0001$), whilst dairy proteins were a superior source of leucine (267 ± 77 μmol/L) compared to 189 ± μmol/L for collagen proteins ($P < 0.04$). Although there were several differences in the bioavailability of hydrolysed compared to non-hydrolysed proteins, this only reached statistical significance within the dairy proteins, but not for collagen proteins.

Conclusions: The intake of collagen proteins result in higher plasma peaks of glycine, whilst the intake of dairy proteins result in higher plasma peaks of leucine. This information may support further investigations, and identification of key AAs that may support exercise in the synthesis of collagen.

Keywords: leucine, proline, glycine, hydroxyproline, tendon, ligament, connective tissue, athlete

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INTRODUCTION

Literature suggests that ligaments and tendons are similar to muscle in being dynamic structures that respond to mechanical loading with tissue hypertrophy (1–3). Larger tissues with more densely packed collagen fibrils have a greater capacity to withstand force, and thereby exert greater injury protection (4). On the other hand, smaller tissues with disorganized collagen fibrils have been associated with higher injury risk, such as the development of tendinopathies (1). Although the amino acids (AAs) required for myofibrillar tissue formation have been well-documented (5–9), evidence regarding the potential role of collagen precursors (e.g., proline, glycine and lysine) and/or stimulatory AAs (e.g., leucine) in the synthesis of new collagen in ligaments and tendons is still emerging. Furthermore, the requirement for an appropriate exercise stimulus to support tissue perfusion appears essential (10–13).

Leucine, an essential amino acid (EAA), has been the subject of extensive research for its role in stimulating myofibrillar protein synthesis (6). Leucine exerts its effect on the mammalian target of rapamycin (mTOR), which results in a signaling cascade leading to the synthesis of myofibrillar protein in muscle tissue (14). In the context of connective tissue, work undertaken in an animal model has suggested an increase in collagen synthesis in the deep digital flexor tendon of malnourished rats in association with a leucine-rich diet, and further enhanced when combined with physical stimulation (15). Similarly, in humans, the ingestion of leucine-rich whey protein, coupled with resistance training, has been shown to lead to patellar tendon hypertrophy, with the proximal cross-sectional area (CSA) increasing by 14.9 ± 3.1%, compared to 8.1 ± 3.2% for the placebo group (16), but no change in distal or mid tendon CSA. However, it is highly plausible that this increase occurred secondary to a distinct increase in the size of the quadriceps muscle, acting as a stimulatory load on the tendon (17).

In addition to the stimulatory role of leucine on the protein synthetic machinery, it is likely that other AAs, such as those found in large quantities in collagen protein (e.g., glycine, proline, and lysine) play a role in the synthesis of new collagen. Proline is a conditionally essential amino acid (CEAA), which plays a role in the formation and structural integrity of collagen fibrils (18). Indeed, in older individuals, ingestion of leucine and proline in the form of casein protein, in combination with resistance training, resulted in a trend toward a higher fractional synthetic rate of collagen compared to whey protein (19). In an engineered ligament model, it has been shown that the addition of 50 μM proline with 50 μM ascorbic acid, to a media rich in AAs such as leucine and glycine, increased ligament collagen content from 1.34 ± 0.2% to 8.34 ± 0.37% (20). More recently, work from our group has shown that the consumption of 15 g of dietary collagen; 1 h prior to intermittent exercise led to an increase in procollagen I N-terminal propeptide (P1NP), compared to a placebo control (21). Additionally, an *in vitro* arm of the same study, displayed an increase in the collagen content and mechanical properties of an engineered ligament treated with serum obtained post ingestion of dietary collagen (21). Thus, it is plausible that a combination of EAA, to upregulate synthetic machinery, and CEAA to supply AA building blocks is required during the synthesis of collagen protein. Although these findings are promising, it remains unclear as to the value of hyper-aminoacidemia in the synthesis of new collagen, and whether these amino acids work in isolation or synergistically.

In line with this, there is emerging evidence of benefits associated with the ingestion of collagen peptides in a range of collagen containing tissues, including increased collagen synthesis (22, 23), improved body composition (24, 25), reduced pain (26–28) and the slowing of degenerative diseases such as osteoarthritis (29, 30). It has been suggested that hydrolysis of collagen protein prior to ingestion allows two and three amino acid peptides to pass across the mucosal barrier equating to a higher expression and therefore biosynthesis within the tissue matrix (31). This was illustrated in a recent study whereby the consumption of hydrolysed collagen proteins resulted in a higher bioavailability of AAs compared to non-hydrolysed collagen protein and a placebo control (32). Furthermore, it has recently been shown that the consumption of collagen peptides resulted in a higher expression of collagen signaling proteins, compared to a placebo control (24).

Accordingly, we aimed to determine the bioavailability [i.e., timing, maximum concentration, and area under the curve (AUC)] of TAA, EAA, and key AAs proposed to support the synthesis of new collagen, after the consumption of a selection of hydrolysed and non-hydrolysed collagenous, and dairy proteins, and a collagenous food source. This data would be of benefit to allow further investigations into a range of practical questions:

- The optimal food source/supplement, AA and/or combinations of AAs that may support exercise in increasing collagen synthesis.
- The optimal timing to consume this food source/ supplement and/or AA(s) prior to an exercise bout.
- Whether there is increased bioavailability from the consumption of hydrolysed vs. non-hydrolysed forms of collagenous and/or dairy proteins.

MATERIALS AND METHODS

Subjects and Ethics

Fifteen healthy, recreationally active, male subjects (30 ± 5 years; 80 ± 8 kg BM) with no current collagen-related disease or known protein allergies were recruited for the study. The current study formed part of a larger study, which necessitated a male subject population given the influence of female sex hormones of ligament health (33). Sample size was chosen using power estimation determined in previous, similar studies (34, 35). The Human Ethics Committee of the Australian Institute of Sport granted approval for this study (20170607) and written informed consent was received from participants prior to its commencement. The protocol was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR12617000923369).

Protein Source and Preparation

Four collagen and two high leucine dairy protein sources were selected for the study. These included a hydrolysed and non-hydrolysed collagen powder: Gelita (Pep) and gelatin (Gel), respectively; a hydrolysed and non-hydrolysed dairy protein supplement: calcium caseinate (Cas) and hydrolysed casein (HCas), respectively; a liquid collagen supplement (LCol) and one collagenous food source: bone broth (BBr). Further details of these protein sources can be seen in **Table 1**. We chose a standard dose of 20 g for all powdered supplements and 60 mL of liquid collagen (equivalent to 20 g of collagen protein as per manufacturer information). This dose was slightly higher than used in previous work (21), due to the benefits associated with increased amino acid availability (36, 37). The bone broth used in this study was chosen on the basis of the results of our previous work (38) in which this preparation was found to have a higher protein content than other broths assessed, and within the range provided by the reference supplements in a standard serve [further details of the broth used in this study; "chef prepared bone broth" are available elsewhere; (38)]. Furthermore, we decided on a 300 mL serve as a representative portion size that would be practical to consume within the prescribed time-period, as we would not ascertain total protein content of this broth prior to commencing the study. Analysis of the batch actually consumed in this study, subsequent to the trials, found that it was considerably higher than expected. Nevertheless, by purchasing a bulk volume of the broth (5 L), and mixing it well before dividing it into 300 mL serves, we achieved consistency between the serves consumed by subjects.

To maintain consistency across all experimental protocols, all protein sources were served warm, and in/with the same amount of fluid (300 mL) achieved via the addition of water. Supplements were provided in a counter-balanced, randomized fashion, with at least 48 h between trials. All protein sources were assessed for full amino acid profiles analyzed using Ultra Performance Liquid Chromatography (Waters AccQTag Ultra) (Australian Proteome Analysis Factory, Macquarie University, Sydney, NSW).

Experimental Protocol and Analysis

Participants fasted overnight (>10 h) prior to attending the laboratory at the Australian Institute of Sport between 5:30 and 7 a.m. On waking, participants were allowed to consume 250 mL of water to ensure adequate hydration for the blood collections but instructed to arrive to the lab in a rested state to avoid significant elevations to heart rate that would increase blood flow. On arrival, a 22 G indwelling cannula was inserted into the antecubital vein for blood collection by a trained phlebotomist, and a baseline (BL) blood sample was collected. Immediately after, the protein source was prepared and given to participants who were instructed to consume it within 5 min, sipping slowly throughout. Completion of the 5 min period was considered as $t = 0$. To standardize gastric emptying, no other fluid was available for 60 min following consumption, and then *ad libitum* water consumption was permitted. Immediately after the consumption of the protein source, participants were asked to fill out a feedback form (as outlined below). Blood samples were then collected every 20 min for 180 min into 2 mL lithium heparin Vacuette tubes (Greiner Bio-One, Kremsmünster, Austria). Immediately after each blood collection, samples were centrifuged at 1,500 ×g for 10 min at 4°C. The resulting plasma was separated and stored at −80°C until further analysis. Once the 180 min period was concluded the cannula was removed and participants were allowed to leave the laboratory.

Once all plasma samples were collected, they were transported to Maastricht University for analysis of full amino acid profiles using liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). Briefly, samples were prepared according to manufacturer's instructions, with an internal standard added. Fifty microliters of blood plasma was deproteinised using 100 µL of 10% sulfosalicyclic acid (SSA) with 50 µM of metabolomics amino acid mix standard (MSK A2) (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged. After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). Twenty µL of AccQ-Tag derivatising reagent solution (Waters, Saint-Quentin, France) was then added, and the solution was heated to 55°C for 10 min. Of this 100 µL derivative, 1 µL was injected and measured using UPLC-MS.

Due to the interests of this study, we have reported concentrations of TAA, EAA, and a selection of AAs which have been suggested to be involved in the synthesis of collagen (including proline, glycine, lysine and leucine) (16, 19, 21, 39).

Protein Source Acceptability

The feedback form completed by subjects immediately after consuming a test product provided four questions in the format of a Likert scale (40), and one question with a yes or no response. The questions are outlined in **Table 3** and included questions related to acceptability and palatability. While we acknowledge that this is not a validated questionnaire, it was intended that this feedback would provide practical insight into the use of this product for connective tissue health purposes.

Statistics

Trapezoidal rule (41) adjusted to baseline concentration was applied to calculate the area under curve (AUC) of the amino acid concentration. Statistical analysis were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California, USA). Data were checked for normality using a D'Agostino & Pearsons normality test. A repeated-measures One-way ANOVA by General Linear Models (GLM) was used to compare the effects of different supplements on outcome variables (plasma amino acid content for: baseline; BL, observed maximum concentration; C_{max} Obs, timing of peak; T_{max} and AUC). Tukey's method was used to adjust for multiple comparisons between different groups.

TABLE 1 | Details of collagen and non-collagen (dairy) protein sources, and collagen specific amino acid profiles per serve of select food/supplement.

Type	Dairy protein supplement		Collagen protein supplement			Collagen protein food source
Brand	Professional whey, NSW, Australia	Professional whey, NSW, Australia	McKenzies, Altona, VIC, Australia	Tendeforte, Gelita, Eberbach, Germany	GBR Nutrition, Lancashire, UK	Elemental Cafe, Braddon, ACT, Australia
Details	Calcium caseinate	Hydrolysed casein powder	Powdered gelatin	Hydrolysed collagen peptide powder	Liquid collagen supplement	Chef made bone broth
Abbreviation	Cas	HCas	Gel	Pep	LCol	BBr
Serve	20 g	20 g	20 g	20 g	60 mL	300 mL
Cost per serve	$ 0.70	$ 1.24	$ 0.74	$ 3.00	$ 5.37	$ 4.50
Manufacturer stated total protein per serve (g)	20	20	20	20	20	N/A
TAA per serve (mg)*	19,172	19,378	20,108	20,594	17,892	52,260
EAA per serve (mg)*	8,306	8,368	4,944	5,858	4,392	13,320
Proline per serve (mg)*	2,038	2,060	2664	2,722	2,406	6,420
Glycine per serve (mg)*	360	354	4,750	4,904	4,212	11,760
Lysine per serve (mg)*	1,422	1,468	696	746	630	1,740
Leucine per serve (mg)*	1,782	1,786	588	584	516	1,710

EAA, Essential Amino Acids; TAA, Total Amino Acids.
**Calculation based on free amino acid molecular weight.*
Cysteine and typtophan have not been included in analysis, which account for < 5% in total.

RESULTS

Total Protein and Amino Acid Content of Selected Collagen and Dairy Proteins

The composition per serve of the protein sources (total protein, TAA, EAA and selected AAs) is displayed in **Table 1**. Full amino acid profiles are available as **Supplementary Table 1**. The supplements (Cas, HCas, Gel, Pep, and LCol) provided 19.3 ± 0.8 g protein per 20 gram serve, whereas BBr provided 52.2 g of protein per 300 mL serve. Per serve, BBr contained the highest levels of all selected AAs, with the exception of leucine, which was provided in the highest amount in the dairy protein supplements, HCas and Cas at ∼1,540 mg per serve. AA profiles of proline, glycine, lysine and leucine of protein sources of similar derivatives (e.g., dairy and collagen proteins), were comparable in their amino acid content. Liquid collagen provided the lowest levels of all AA among the collagen protein sources in this study.

Observed Maximum Concentration (C_{max} Obs)

Figure 1 displays the time course of the appearance of TAA, EAA, and the selected AAs in the plasma over 180 min. Kinetic parameters of TAA, EAA, and selected AAs after the consumption of collagen and dairy protein sources are displayed in **Table 2**, as means and SD. There were no differences in BL levels for TAA, EAA or any of the AAs between protein sources ($P > 0.2$).

In terms of TAA, C_{max} Obs was similar for all protein sources, except for Cas which was significantly lower ($P < 0.04$) than all protein sources and LCol which was significantly lower than Pep ($P = 0.03$). HCas was significantly higher for C_{max} Obs for EAA than all other protein sources ($P < 0.0001$) while Cas was only higher than BBr and LCol ($P < 0.04$). There was no difference in C_{max} Obs of EAA for any of the collagen protein sources ($P > 0.1$). The C_{max} Obs of proline was similar ($P > 0.1$) for all protein sources other than Cas, which was significantly lower than the others ($P < 0.001$). HCas provided the highest C_{max} Obs for lysine compared to all other protein sources ($P < 0.002$). Collagen proteins provided a higher C_{max} Obs for glycine compared to Cas and HCas ($P < 0.0001$) while there was no difference between the dairy protein sources ($P = 0.8$). Meanwhile, HCas provided the highest C_{max} Obs of leucine compared to all collagen proteins ($P = 0.0001$) and Cas also had a higher C_{max} Obs for leucine than these products ($P < 0.04$). However, differences in C_{max} Obs for leucine between any of the collagen proteins failed to reach significance ($P > 0.06$).

Timing of Maximum Concentration (T_{max})

Results of T_{max} in **Table 2** illustrate a similar time course for TAA for all products except an earlier peak with HCas than BBr and Gel ($P < 0.02$). There was no difference in T_{max} of EAA with all protein sources peaking between ∼ 30 and 50 min ($P > 0.1$). T_{max} of proline for BBr was slower than all other protein sources ($P < 0.002$). Although the time to peak for proline was slower for the non-hydrolysed proteins compared to the hydrolysed proteins, this did not reach statistical significance ($P > 0.05$). BBr was the slowest to peak for glycine, and was significantly slower than Pep and LCol ($P < 0.001$). The T_{max} of glycine for Gel was also found to be slower than for Pep ($P = 0.04$). There was no difference in T_{max} for leucine between any of the protein sources, with all peaking between ∼ 30 to 50 min ($P > 0.04$). The only difference for lysine T_{max} was a quicker peak with BBr than HCas ($P = 0.001$).

Area Under the Curve (AUC)

Figure 2 displays the AUC of amino acid concentrations in plasma after consumption of the protein sources. BBr provided

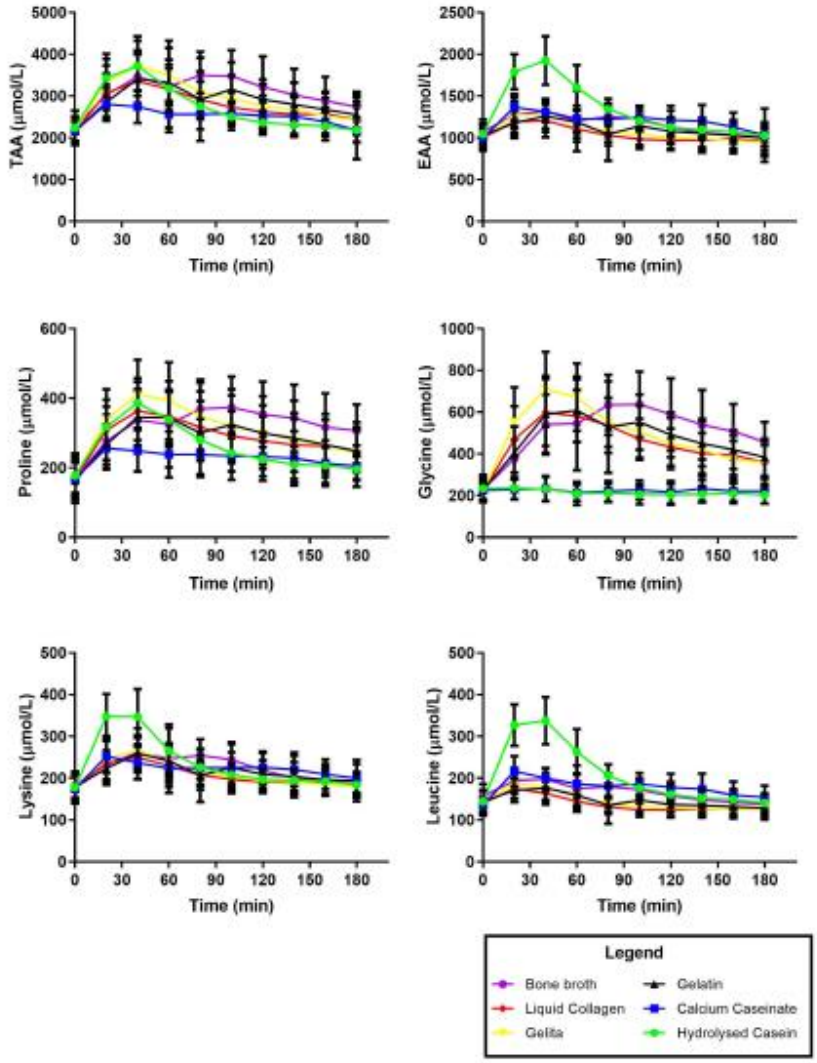

FIGURE 1 | Time course of plasma amino acid concentrations over 180 min after the consumption of collagen or dairy protein sources. Data presented as mean ± SD.

the highest AUC for TAA at 91,029 ± 29,630 μmol/L/180 min, which was significantly higher than both dairy proteins [Cas and HCas at 27,265 ± 14,130 μmol/L/180 min and 36,042 ± 12,833 μmol/L/180 min, respectively ($P < 0.0001$)] but not significantly different to any of the collagen protein supplements [7,7052 ± 2,4304, 84,664 ± 20,582 and 71,882 ± 19,085 μmol/L/180 min for Gel, Pep and LCol, respectively ($P > 0.2$)]. However, as seen in **Figure 1**, plasma AAs after consumption of BBr remained elevated at the completion of 180 min, particularly for proline and glycine. Cas provided the lowest AUC for TAA, and was significantly lower than all collagen supplements ($P < 0.0002$), but not HCas ($P = 0.6$). Although hydrolysed supplements appeared to have a higher AUC for TAA than their non-hydrolysed counterparts, this was not statistically different for either collagen or dairy proteins ($P > 0.6$). Collagen proteins had a higher AUC than dairy proteins for TAA (81,157 ± 8,419 and 31,654 ± 6,206 μmol/L/180 min, respectively, $P < 0.0001$).

HCas provided the highest AUC for EAA at 55,992 ± 15,675 μmol/L/180 min, which was significantly greater than all other protein sources ($P < 0.003$), except Cas which provided an AUC for EAA of 39,587 ± 18,573 μmol/L ($P = 0.2$). The AUC for EAA of Cas, was only significantly higher than LCol and Pep which provided 8,806 ± 16,410 and 12,238 ± 13,682

TABLE 2 | Kinetic parameters of total, essential, and collagen specific, amino acid concentration in plasma after the consumption of collagen and dairy protein sources.

		Cas	HCas	Gel	Pep	LCol	BBr	Significance
TAA	Baseline (μmol/L)	2 ± 156 , 234	2 ± 233 , 403	2 ± 155 , 308	2 ± 178 , 331	2,151 ± 325	2,254 ± 392	$P = 0.6430$
	C_{max} Obs (μmol/L)	3,012 ± 407	3,725 ± 641	3,525 ± 631	3,710 ± 670	3,445 ± 641	3,552 ± 561	$P = 0.0004$
	T_{max} (min) (min/max)	45 ± 35	35 ± 9	59 ± 28	48 ± 27	47 ± 39	64 ± 24	$P = 0.0877$
EAA	Baseline (μmol/L)	998 ± 116	1,044 ± 170	1,030 ± 135	1,017 ± 148	1,000 ± 147	1,074 ± 142	$P = 0.4621$
	C_{max} Obs (μmol/L)	1,457 ± 137	1,946 ± 292	1,311 ± 211	1,313 ± 190	1,270 ± 198	1,347 ± 138	$P < 0.0001$
	T_{max} min (min/max)	47 ± 36	33 ± 10	47 ± 24	35 ± 31	36 ± 42	51 ± 24	$P = 0.3310$
Proline	Baseline (μmol/L)	168 ± 47	177 ± 49	163 ± 55	173 ± 62	166 ± 49	170 ± 72	$P = 0.7901$
	C_{max} Obs (μmol/L)	282 ± 68	392 ± 71	381 ± 76	431 ± 113	388 ± 89	386 ± 85	$P < 0.0001$
	T_{max} min (min/max)	56 ± 34	41 ± 9	61 ± 22	55 ± 27	52 ± 38	103 ± 29	$P < 0.0001$
Glycine	Baseline (μmol/L)	229 ± 67	235 ± 64	226 ± 172	215 ± 176	222 ± 143	229 ± 170	$P = 0.5773$
	C_{max} Obs (μmol/L)	267 ± 67	253 ± 64	674 ± 172	747 ± 176	646 ± 143	666 ± 170	$P < 0.0001$
	T_{max} min (min/max)	67 ± 49	39 ± 49	71 ± 25	48 ± 20	53 ± 26	88 ± 24	$P = 0.0103$
Lysine	Baseline (μmol/L)	174 ± 33	178 ± 31	179 ± 28	178 ± 58	176 ± 33	183 ± 30	$P = 0.8818$
	C_{max} Obs (μmol/L)	273 ± 31	369 ± 65	269 ± 52	271 ± 50	262 ± 48	272 ± 41	$P < 0.0001$
	T_{max} min (min/max)	51 ± 36	29 ± 10	45 ± 19	41 ± 18	44 ± 33	51 ± 17	$P = 0.1629$
Leucine	Baseline (μmol/L)	136 ± 17	144 ± 27	145 ± 26	144 ± 28	140 ± 29	158 ± 29	$P = 0.1640$
	C_{max} Obs (μmol/L)	230 ± 31	351 ± 59	184 ± 29	190 ± 34	181 ± 30	203 ± 28	$P < 0.0001$
	T_{max} min (min/max)	47 ± 36	29 ± 10	36 ± 23	29 ± 31	33 ± 41	33 ± 12	$P = 0.3768$

Data presented as mean ± SD.

μmol/L/180 min, respectively ($P < 0.006$). The lowest AUC of EAA was provided by LCol at 8,806 ± 16,410 μmol/L/180 min, but this was not different to the other collagen protein sources ($P > 0.8$).

In terms of individual AA, the AUC of lysine was greater for HCas than Gel, Pep and LCol [AUC of 11,187 ± 2,764 μmol/L/180 min compared to 7,003 ± 4,340, 6,310 ± 2,714, and 5,729 ± 3,290 μmol/L/180 min, respectively ($P < 0.008$)]. For leucine, dairy proteins had a higher AUC than all collagen proteins at 10,187 ± 3,023 μmol/L/180 min and 746 ±1,043 μmol/L/ 180 min, respectively ($P < 0.001$). Meanwhile, HCas provided a higher AUC for leucine than Cas ($P = 0.003$). Collagen proteins has a higher AUC than dairy proteins for proline (25,102 ± 2,345 and 13,547 ± 3,450 μmol/L/180 min, for Gel, Pep, and LCol, respectively $P < 0.01$), and glycine [(48,563 ± 4,429 and −1,863 ± 2,255 μmol/L/ 180 min ($P < 0.0001$)].

Collagen and Dairy Protein Acceptability and Palatability

Responses to the questions around acceptability and palatability for each protein source are displayed in **Table 3**. LCol had the highest scores individually, and on average, while HCas was rated the lowest. Despite these differences between products, only 53% of participants reported being willing to pay the specified price for either product. Meanwhile, the non-hydrolysed supplements (which were also the lowest in cost; **Table 1**), scored 93% for willingness to pay, if it was found to be beneficial to connective tissues.

DISCUSSION

A number of AAs have been suggested to play a complementary role in the synthesis of collagen in ligaments and tendons, when combined with an appropriate mechanical stimulus (39).

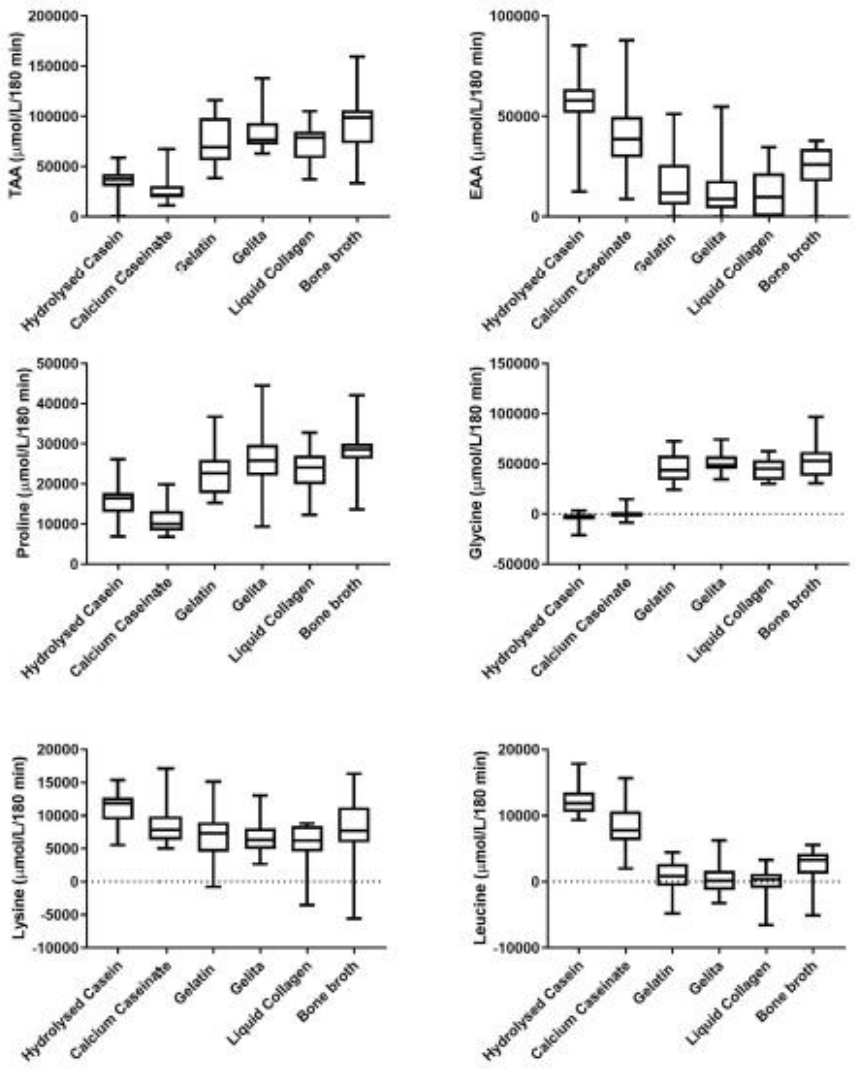

FIGURE 2 | Area under the concentration—time curve (AUC) of plasma amino acids over 180 min after the consumption of dairy and collagen protein sources. Middle line shows mean, boxes represent 25–75th percentile and whiskers represent min and max values.

There is emerging evidence that the ingestion of key AAs (including proline, glycine, lysine, and leucine), or combinations thereof, provide enhanced availability to support the synthesis of collagen when combined with an appropriate exercise protocol (42). Accordingly, the aim of the present study was to characterize the bioavailability (e.g., timing of appearance, maximum concentration, and AUC) of TAA, EAA, and key AAs that may be involved in the synthesis of collagen, after the ingestion of a selection of collagen and dairy protein sources.

In general, plasma AA responses reflected the AA profiles of the consumed supplement, except in the case of the casein, and bone broth. These both resulted in a lower and more prolonged appearance of AAs in the blood over the 180 min following consumption (See **Tables 1**, **2** and **Figure 2**). This is likely due to various components known to slow gastric emptying such as the fat content of the BBr (43), and the clotting of casein in the stomach after ingestion (44). However, it is also plausible that the delayed gastric emptying of BBr may be related to the volume was consumed. As can be seen in **Figure 1**, the plasma concentration of AAs after consumption of BBr remained elevated at the 180 min mark, and most likely would have continued to be available at a higher amounts in the blood beyond the 3 h that were monitored for this study.

TABLE 3 | Likert scale of protein source feedback and palatability.

Question (1–7)	Cas	HCas	Gel	Pep	LCol	BBr	Significance
Serve size	20 g	20 g	20 g	20 g	60 mL	300 mL	$P < 0.001$
1	4 (1–7)	3 (1–5)	4 (1–7)	5 (2–7)	6 (307)	5 (2–7)	$P < 0.001$
2	6 (3–7)	5 (2–6)	6 (2–7)	6 (2–7)	7 (4–7)	6 (2–7)	$P = 0.032$
3	6 (2–7)	5 (1–7)	6 (2–7)	6 (2–7)	7 (5–7)	6 (2–7)	$P = 0.044$
4	6 (3–7)	4 (1–7)	6 (1–7)	6 (2–70)	7 (4–7)	6 (2–7)	$P = 0.006$
5 (% Yes)	93	53	93	60	53	73	-

*Question 1: The palatability (taste, texture) of the product was acceptable.

*Question 2: The volume of the product (300 mL) was acceptable to consume in the time specified (5 min).

*Question 3: The volume of the product would be acceptable to consume within 20–40 min prior to exercise.

*Question 4: I would be willing to consume this product at least twice a day as part of a rehabilitation program.

**Question 5: Would you be willing to pay the specified amount for one serve of the product if it was beneficial to connective tissue health?

Data presented as median (range).
1, strongly agree; 7, strongly disagree.
**Out of 1 (strongly agree) to 7 (strongly disagree).*
*** % out of 100.*

It has been proposed that AAs play a supportive, rather than stimulatory role in the synthesis of collagen within ligaments and tendons (21, 39, 45). Meanwhile, exercise has been shown to be a potent regulator of collagen turnover resulting in an upregulation of collagen synthesis for a period of up to 72 h (46). Exercise results in an increase in hormones that have been shown to stimulate the synthesis of collagen in connective tissue (e.g., growth hormone, and insulin-like growth factor 1) (47). As ligaments and tendons are poorly vascularised tissues, it may be sensible to isolate the provision of AAs to scenarios involving the exercise-induced enhancement of blood flow (48). Therefore, protein sources that achieve higher AA peaks over a shorter period of time (e.g., HCas) may be considered optimal, whereas a slower release of key AAs over an extended period of time (e.g., with BBr) may not be as easily matched to enhanced tissue blood flow. Literature to date suggests that there is dose related response to the key AA involved in the synthesis of collagen. Indeed, Shaw et al. (21) illustrated that a 15 g dose of gelatin resulted in an increased availability of AAs and collagen synthesis than a 5 g dose, resulting in improvements to tissue mechanics in engineered ligaments *in vitro*. Peak blood concentrations of glycine and proline in the current study (~650–750 and 350–450 μmol/L, respectively) were slightly higher than those reported in previous work [i.e., 448 ± 165 and 238 ± 77 μmol/L in Shaw et al. (21)]. This is to be expected given the slightly higher dose within our current protocol. However, others have shown similar plasma values after the ingestion of 35 g of collagenous protein (32) which they suggest shows an upper threshold to the AA availability of collagen proteins. In general since all AAs appeared to peak between 30 and 60 min (**Table 2**), the consumption of ~ 20 g of protein within the 30–60 min prior to exercise would ensure the optimal availability of AAs at a time when synthetic machinery is upregulated, and tissue perfusion supported (13, 46, 49). It should be noted, however, that whether the increased availability of AAs within the plasma results in an increased availability of AAs around the target tissue (e.g., the peritendinous fluid), where it is able to be utilized and integrated into the tissue is yet to be determined.

While the maximum concentrations of total AA present within the plasma after the ingestion of collagen and high leucine dairy proteins were comparable, dairy proteins provided a larger C_{max} Obs and AUC of leucine, while hydrolysed casein provided a higher C_{max} Obs of EAA than both dairy and collagen proteins, and collagen proteins were superior in terms of glycine. Such differentiation mean that no protein source was superior in terms of all AAs that are potentially implicated in collagen synthesis. If subsequent research identifies the benefits of EAA as well as collagen precursors (e.g., proline and glycine), a case could be made for the consistent intake of EAA (i.e., the current guidelines for regular intake of high-quality protein sources over the day), with a supplemental and/or food source of collagenous protein consumed at key periods i.e., prior to exercise. The lack of such a pattern may also detract from the health of connective tissues.

It has been proposed that hydrolysed proteins are superior to non-hydrolysed forms in providing a higher AA bioavailability; more specifically, being able to reach a target tissue more quickly and with a higher peak concentration (31, 32, 50). Within the present study, the plasma characteristics of hydrolysed and non-hydrolysed dairy proteins showed some differences, but these were not seen with the collagen proteins. Our findings are similar to the study of Koopman and co-workers where ingestion of 35 g of hydrolysed casein protein resulted in an increased bioavailability and incorporation rate of AAs into skeletal muscle protein than intact casein (50). However, while another more recent study showed that the ingestion of 35 g of enzymatically hydrolysed collagen resulted in a higher bioavailability of several

AAs compared to non-enzymatically hydrolysed collagen (32), we did not find any difference in the bioavailability of gelatin compared to a hydrolysed peptide powder. It is possible that differences in processing methods accounted for this finding as it has been suggested to influence digestibility and therefore bioavailability of AAs (51).

In terms of the limitations of this study, we acknowledge that the questionnaire relating to acceptance and palatability of the protein sources used in this study was not a validated tool. Nevertheless, it provided insight into factors that are likely to affect the compliance of the use of protein sources that have potential benefit for collagenous tissues. Indeed, it indicated that despite the superiority of HCas over Cas in terms of AA availability, its poor scores for taste/texture are likely to affect consumer uptake and compliance with regular use. On the other hand, non-hydrolysed supplements scored well in terms of acceptability in taste and cost; this makes gelatin a potentially useful product since it provided similar peak and total AA exposure than other collagen products. Furthermore, our inability to measure the composition of the protein sources actually used in this study until after its conduct meant that we relied on previously collected data, or that stated by the manufacturer which differed to the final measured content (**Table 1**). The subsequent discovery of the higher protein of this broth makes it difficult to compare the bioavailability based on their actual protein intake. However, since the serves used in this study are the commonly consumed or recommended amounts of these protein sources, it did allow real-life insight into the AA profiles.

In summary, our study characterized the blood AA profiles following the intake of a range of dairy and collagen supplements providing 20 g of protein, and a common serve of beef broth of higher total protein. While protein sources of similar origin contained a comparable total AA profile, consumption of dairy proteins provided a more pronounced amount of EAA and leucine than collagen sources, with hydrolysed casein showing increased bioavailability of AAs than an intact form. Intake of collagen proteins achieved a greater peak concentration of glycine, with small differences between hydrolysed and non-hydrolysed forms. Although the total AA content of the bone broth was greater, a lower and more sustained blood AA profile seems likely. This information may help to inform protocols for achieving ideal blood AA responses once the optimal support for the synthesis of collagenous tissues is identified. However, the cost and palatability of these dietary and supplement sources should be considered.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Human Ethics Committee of the Australian Institute of Sport. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RA, LB, and GS were involved in study design. RA and NT was involved in data collection, data analysis, and statistical analysis RA, LB, GS, and NT were involved in results interpretation, drafting, reviewing, and revising the initial manuscript.

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This study, funded by a grant administered by the Center for Exercise and Nutrition, Mary Mackillop Institute for Health Research, Australian Catholic University, and provided by the ACT Brumbies Super Rugby Team, was conducted at the Australian Institute of Sport. Whilst the ACT Brumbies provided the funding for the study; they had no direct role in any part of the study.

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Amino acid analysis was facilitated using infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2019.00163/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

11.2 Supplementary Tables

11.2.1: Supplementary for study 1. Range of Amino acid (mg) provided per serve of laboratory prepared and commercial varieties of bone broth and reference supplements and per kg of animal tissue for laboratory prepared varieties

mg of Amino acid provided per serve								
Bone broths-standardized recipe								
	Self-prepared				Commercial			
	CB	**BB**	**CBv**	**BBv**	**BBC**	**BBB**	**BOLC**	**BOLB**
No of samples	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
HYP	573-635	918-975	443-610	790-908	385-445	470-500	220-246	230-279
GLY	1018-1108	1633-1713	780-1063	1405-1598	685-783	830-878	432-477	470-532
PRO	628-680	1000-1048	480-650	848-968	428-480	503-538	288-316	314-342
HYL	78-83	125-135	60-80	113-130	55-65	55-58	27-29	29-31
LYS	288-300	343-368	218-280	293-328	195-203	180-188	186-211	170-195
LEU	265-268	370-398	190-250	318-353	170-188	178-185	201-218	197-232
HIS	110-180	150-163	100-115	133-145	88-93	95-98	71-78	71-84
TAU	0-43	N.D.	35-38	N.D.	35-40	N.D.	15-17	3-3
SER	210-230	363-368	170-218	308-345	170-185	183-190	139-151	153-162
ARG	525-550	790-813	400-540	675-770	345-385	383-410	281-312	281-288
ASP	483-533	720-740	385-500	600-670	365-380	365-378	299-319	288-321
GLU	950-1025	1330-1360	745-960	1108-1278	678-740	738-740	507-557	521-564
THR	188-195	243-253	143-185	205-233	135-140	118-123	135-147	125-146
ALA	593-640	900-915	453-603	755-858	403-448	453-478	291-318	319-325
TYR	70-75	100-113	55-73	85-95	53-58	48-50	72-80	64-82
MET	98-103	115-123	78-103	95-115	60-80	53-63	71-81	61-76

VAL	188-193	290-308	255-280	130-138	38-148	138-148	154-165	153-178
ISO	143-145	188-193	105-135	150-170	95-100	90-95	126-134	112-134
PHE	173-180	258-273	130-173	223-248	120-128	125-133	133-144	130-147

Total protein (g)

	Bone broths – non-standardized recipe					***Reference supplements***		
	Ca	**Ca(2)**	**BBm(1)**	**BBm(2)**	**GEL**	**PEP**	**Lcol**	**HYD**
No of samples	**3**	**1**	**1**	**1**	**2**	**2**	**1**	**1**
HYP	2825-5450	2250	850	625	2010-2030	2026-2100	1770	2260
GLY	4900-9450	3925	1500	1100	3610-3642	3726-3694	3204	3830
PRO	2975-5850	2400	1048	675	2246-2282	2296-2332	2028	2322
HYL	275-475	250	125	100	186-190	182-186	162	168
LYS	850-1475	650	275	200	680-610	652 3	552	678
LEU	850-1650	675	300	225	498-508	504-504	444	532
HIS	275-525	175	75	75	134-140	152- 158	132	98
TAU	N.D.	N.D.	N.D.	N.D.	0-80	N.D.	N.D.	N.D.
SER	950-1700	750	325	225	668-672	692-702	582	656
ARG	2025-3550	1525	675	500	1584-1590	1638-1652	1380	1570
ASP	1775-3200	1525	675	500	1584	1638-1652	1380	1570
GLU	3325-6200	2450	1075	775	1930-1956	1970-1978	1788	2122
THR	550-1000	450	200	150	356-358	352-354	306	382
ALA	2525-3800	2000	775	575	1694-1702	1678-1682	1530	772
TYR	225-300	175	100	50	88-102	112-114	42	32
MET	250-500	200	75	50	160-170	150-154	138	112

VAL	725-1400	575	250	175	444-450	474	396	506
ISO	450-875	350	150	125	292- 300	250-252	264	292
PHE	650-1250	500	225	150	384-388	474-476	336	396

11.2.2: Supplementary for Study 3. Amino acid profiles of select food/supplements (mg/serve)

	Cas	HCas	Gel	Pep	LCol	BBr
Hyp	N.D.	N.D.	2330	2434	2052	5910
Gly	360	354	4750	4904	4212	11760
Pro	2038	2060	2664	2722	2406	6420
Hyl	N.D.	N.D.	210	208	186	570
Lys	1422	1468	696	746	630	1740
Leu	1782	1786	588	584	516	1710
His	546	480	140	158	132	450
Tau	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Ser	1086	1088	668	702	582	1680
Arg	706	700	1584	1652	1380	3840
Asp	1212	1238	1072	1088	1996	2970
Glu	3930	4040	1956	1970	1788	5670
Thr	810	812	356	352	306	990
Ala	540	540	1694	1678	1530	4560
Tyr	994	990	102	114	42	360
Met	550	554	160	154	138	420
Val	1234	1242	450	474	396	1260
Iso	986	986	300	252	264	810
Phe	976	978	388	402	336	1140
Total protein (g)	19.2	19.4	20	20	17.9	52.2

HYP: Hydroxyproline; GLY: Glycine; Pro: Proline; HYL: Hydroxylysine; Lys: Lysine; Leu: Leucine; HIS: Histidine; TAU: Taurine; SER: Serine; ARG: Arginine; GLU: Glutamic acid; THR: Threonine; ALA: Alanine; TYR: Tyrosine; MET: Methionine; VAL: Valine; ISO: Isoleucine; PHE: Phenylalanine; N.D.: Not detected.

mg of Amino acid extracted per kg of animal tissue							
	CB	**BB**	**CBv**	**BBv**	**Ca(2)**	**BBm(1)**	**BBm(2)**
HYP	3817-4233	6117-6500	2950-4967	5267-6050	10000	5667	4167
GLY	6783-7383	10883-11417	5200-7083	9367-10650	17444	10000	7333
PRO	4183-4533	6667-6983	3200-4333	5650-6450	10667	6167	4500
HYL	517-550	833-900	400-533	750-867	1667	833	667
LYS	1917-2000	2283-2450	1450-1867	1950-2183	2889	1833	1333
LEU	1767-1783	2467-2650	1267-1667	2117-2350	3000	2000	1500
HIS	733-783	1000-1083	667-767	883-967	778	500	500
TAU	0-283	N.D.	233-250	N.D.	N.D.	N.D.	N.D.
SER	1400-1533	2417-2450	1133-1450	2050-2300	3333	2167	1500
ARG	3500-3667	5267-5417	2667-3600	4500-5133	6778	4500	3333
ASP	3217-3550	4800-4933	2567-3333	40000-4467	5889	3833	2833
GLU	6333-6833	8867-9067	4967-6400	7383-8517	10889	7167	5167
THR	1250-1300	1617-1683	950-1233	1367-1550	2000	1333	1000
ALA	4267-3950	6000-6100	3017-4017	5033-5717	8889	5167	3833
TYR	467-500	667-750	367-483	567-633	778	667	333
MET	650-683	767-817	517-683	633-767	889	500	333
VAL	1250-1283	1933-2050	950-1250	1700-1867	2556	1667	1167

ISO	950-967	1250-1283	700-900	1000-1133	1556	1000	833
PHE	1150-1200	1717-1817	876-1150	1483-1650	2222	1500	1000

Abbreviations

HYP: Hydroxyproline; GLY: Glycine; Pro: Proline; HYL: Hydroxylysine; Lys: Lysine; Leu: Leucine; HIS: Histidine; TAU: Taurine; SER: Serine; ARG: Arginine; GLU: Glutamic acid; THR: Threonine; ALA: Alanine; TYR: Tyrosine; MET: Methionine; VAL: Valine; ISO: Isoleucine; PHE: Phenylalanine; N.D.: Not detected.

11.2.3: Supplementary Table for Study 4. mg per litre of amino acids in diluted DMEM media.

Amino acids	DMEM media	Population means	Final concentration of diluted media
Leucine	105	19	21
Glycine*	30	24	24
Proline*	0	30.2	30.2
Lysine*	146	38.5	38.6
Arginine	84	20.3	20.4
Cysteine	63	6	12.6
Glutamine	584	90	116.8
Histidine	42	18.8	18.8
Isoleucine	105	9	21
Methionine	30	5	6
Phenylalanine	66	14	14
Serine	42	4	8.4
Threonine	95	18	19
Tryptophan	16	17	3.2
Tyrosine	104	15	20.8
Valine	94	32	32

Other Components added to diluted media			
Glucose	-	-	3,600
Magnesium sulphate	-	-	78
Calcium chloride	-	-	160

* Indicates amino acids added to make final diluted media equivalent to population means.

11.3 Ethics Approval, Participant Information Forms and Informed Consent

The following ethics approval relates to all studies contained within this thesis, and was submitted as a complete project

11.3.1 Evidence of AIS ethics committee approval

Australian Government
Australian Sports Commission

TO:	Rebekah Alcock	CC:	Louise Burke
FROM:	Ms Kyira Cox		
SUBJECT:	Approval from AIS Ethics Committee	DATE:	7th July 2017

On the 6th July 2017, the AIS Ethics Committee gave consideration to your submission titled ***"The impact of collagen and non-collagen containing food sources and supplements on plasma amino acid response, in-vitro collagen synthesis and mechanical properties in an engineered ligament"***. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project is: 20170607

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30th April 2018 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577.

Sincerely

[redacted]

Kyira Cox
Secretary
AIS Ethics Committee

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/theais
/ausport

AIS

11.3.2 Evidence of minor variation to initial project

AIS
Australian Institute of Sport

MINUTE

TO:	Mr Nick Brown	**CC:**	
FROM:	Rebekah Alcock		
SUBJECT:	Amendment to AIS Ethics Approval		
DATE:	06/07/17		

Minor Variation to project # 20170607

"Insert Original Project Title Here" - Authors
The impact of collagen and non-collagen containing food sources and supplements on plasma amino acid response, in-vitro collagen synthesis and mechanical properties in an engineered ligament. *(Alcock, Burke, Shaw, Praet, Oesser, van Loon)*

What section of the approved Ethics submission does the amendment fall under?

1. Methodology/Specimen collection: Urinary hydroxypoline (HYP)
2. Information to participants

What is the justification behind the amendment? (Give reasons for the amendment and a little more detail for what is involved)

1. Due to the participant burden of collecting of urine for the assessment of HYP excretion at several specific time points (previously t = BL, 180, 420, 660, 900, 24H) for 7 test foods and 1 BL, and after further consultation/ consideration of budgeting, the time points for urine collection have been amended to 3 specific time points (t = AM, PM and 24h) for 4 BL and 4 test foods only.

2. On addressing considerations as per the ethics meeting in May, it was determined that there was not enough information included for participants around the data collection and methodology. There was an absence of information around the collection of urine for assessment of urinary HYP excretion. There was also not enough detail provided around the consumption of test foods, thus more detail was added to this section.

What is the proposed amendment? (In one or two paragraphs, state the amendment and how it differs from the original proposal. Eg The researchers wish to include a test to measure……)

1. Reducing the collection of urine for assessment of HYP excretion from 5 time points for 7 foods + 1 BL (total = 40) to 3 time points for 4 foods and 4 baseline (total = 24).

11.3.3 Information to participants

Research Title:

The impact of different Proline and Glycine containing test foods and supplements on serum amino acid and peptide availability in healthy active males

Principal Researcher:

Rebekah Alcock, Australian Institute of Sport

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Co-researchers:

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We would like to invite you to participate in this postgraduate research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand, why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Background:

Specific amino acids (i.e. Proline and Glycine) and/or sequences of these amino acids (i.e. peptides) play a supportive role in the synthesis of collagen, the predominant protein found within connective tissues, such as ligaments and tendons. Recent work by our team has shown a dose response relationship between amino acid availability, collagen content and tissue mechanics within engineered ligaments following the ingestion of Gelatin (a food form of collagen). However, it is not currently known whether other foods/supplements containing relevant amino acids/ peptides have the potential to support collagen synthesis. Furthermore, the bioavailability (i.e. timing and peaks of appearance within the blood) of varying collagen and non-collagen containing food sources is currently unknown.

Aim:

The aim(s) of this research project is to assess;

1. The bioavailability (appearance within the blood) of amino acids after consumption of collagen and non-collagen containing food sources and supplements.
2. Whether urinary hydroxyproline (HYP) may be used as an indicator of dietary collagen intake
3. The effect of three selected test supplements on collagen content and mechanics in engineered ligaments.

Benefits:

This study will help to provide information around the optimal food source/supplement and its' bioavailability (timing and peaks of appearance within the blood), to support the health/ function of connective tissues. Additionally, it may also assist in the development of a validated food frequency questionnaire to assess intake of dietary sources of collagen.

What is involved?

Note: There are three "parts" to this study (as per the aims outlined above), which will completed in 2 separate study groups. The total time commitment will be identical in each group, however in group 1 aims 1 and 2 will be explored and in group 2, aims 1 and 3 will be explored.

Part 1: A total of 7 trials will be undertaken in fasted, rested conditions with a 48 hour wash-out period between trials. Participants will arrive on testing days following an overnight fast (10 h). Participants will be cannulated and a baseline (BL) blood sample collected (2 ml). The participants will then consume the test food (as described below) and blood samples (2ml) will be obtained every 20 minutes over the Proceeding 3 hours for plasma amino acid assessment.

Participants will ingest 7 different Proline, Glycine and/or Leucine containing test foods (specifically whey protein powder, skim milk powder, casein protein powder, hydrolysed collagen powder, liquid gelatin, solid gelatin powder and a bone broth).

Participants will ingest an amount of product equivalent to 20 g protein. Where food protein concentrations are small and doses required to provide 20 g are impractical to consume within 5 minutes, an amount to an acceptable/ practical volume (i.e. 300ml) will be prescribed. After cannulation, and an initial blood draw is taken for baseline measures, participants will commence consumption of the specified food/supplement and encouraged to finish within 5 min. Fluid volume associated with product intake will be standardised to 300ml. No further fluid intake will be allowed for 60 min after ingestion of test food to standardise gastric emptying rates. While food will be withheld until completion of the trial.

Once the final blood sample is collected, the cannula will then be removed and pressure applied to the insertion site, after which participants will then be provided with a meal. The participant will be free to go and will return to the lab for subsequent testing after 48 h washout.

It is anticipated there will be time commitment at the lab of approximately 3.5 hours. However, participants are free to bring outside work and have access to wireless internet.

Part 2: Participants will be required to collect urine samples for 24 hours on 6 different occasions (2 baselines in the week prior to commencing the study on any day they wish, and for 4 of the test foods – these will be randomised in order). All of the urine MUST be collected at the following time points 0-6h after the test food (or breakfast on BL days), 6-12h after the test food (or breakfast on BL days), and 12-24h after the test food (or breakfast on BL days). A 24hr food and exercise diary, and information on food and fluid intake the night prior to urine collection will be required, which will then be verified by a trained Dietitian on the subsequent testing day. It is imperative that all urine is stored in a cool/dry place throughout collection.

Part 3: Participants will follow the same protocol as per part 1, with an additional 55ml of blood collected at BL and at the time point associated with the Proline and Glycine plasma peak after ingesting the supplement. This blood collection will occur for 3 of the 7 foods described above (no more than 75mls of blood will be taken in 1 day).

Supplements/Medication use

The supplements to be utilised within this research project are currently classified under the AIS Sports Supplement Framework as group C supplements. Further information can be found at: https://www.ausport.gov.au/ais/nutrition/supplements/groupc

The following supplements will be used:

1. Gelita Tendoforte: www.gelita.com
2. Professional Whey Hydrolysed casein: www.Professionalwhey.com.au
3. Professional Whey Calcium caseinate: www.Professionalwhey.com.au
4. GBR Nutrition Collagen Liquid: www.gbrnutrition.com

Who we are recruiting?

Healthy, active males, aged 18-40 years old, without a known food allergy or intolerance to pig and/or beef gelatin, milk and/or milk products and/or chicken/beef/seafood, non-diabetics and individuals without current tendinopathies.

Adverse Effects and Withdrawal:

Although it is expected that this study be low risk if you do happen to suffer any adverse reactions to cannulation, or the supplement/food sources provided the project doctor, will assess your symptoms and provide appropriate support. You will have the right to withdraw from the study at any stage.

Confidentiality:

Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by password. In addition, the participants will be identified by number only. All address lists will be kept securely in the primary investigator's care and only be available to the primary investigator and appropriate co-researchers. The presentation of results will not make reference to individual participants when the data are published. The raw data will be retained by the principal investigator for five years in a secure cabinet at the Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed.

Ethics Approval:

The project set out in the attached application, including the adequacy of its research design and compliance with recognised ethical standards, has the approval of the Australian Institute of Sport (AIS). If you have any concerns, please contact the secretary of the AIS Ethics Committee on 02 6214 1577.

Further information:

Please contact Rebekah Alcock at Rebekah.Alcock@ausport.gov.au or 0476291511 if you have any questions, concerns, or require further information in regards to any aspect of participating in this study.

11.3.4 Informed consent form

Project Title: The impact of collagen and non-collagen containing food sources and supplements on plasma amino acid response, in-vitro collagen synthesis and mechanical properties in an engineered ligament.

Principal Researchers: Rebekah Alcock, AIS

Nicolin Tee, AIS

Greg Shaw, AIS

Louise Burke, AIS

This is to certify that I, hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of .

The investigation and my part in the investigation have been defined and fully explained to me by and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.
- I have read and understand the product and policy information provided to me on surrounding the use of supplements/medications within the study (where applicable)

***Privacy Statement**: The information submitted will be managed in accordance with the ASC Privacy Policy.*

□ *I consent to the ASC keeping my personal information.*

Signature of Subject: _____ Date: ___/___/___

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: _____ Date: ___/___/___

11.4 Likert Scale for Supplement Palatability and Acceptability

Please fill out the following questionnaire in relation to the supplement/food product that you just consumed:

		Strongly disagree			Neutral			Strongly agree
	Question	1	2	3	4	5	6	7
1	The palatability (taste, texture) of the product was acceptable							
2	The volume of the product (300ml) was acceptable to consume in the time specified							
3	The volume of the product would be acceptable to consume within 40 – 20 minutes prior to exercise							
4	I would be wiling to consume this product at least twice per day as part of a rehabilitation program							
5	Would you be willing to pay the following amount for one serve of this product? $0.17			Yes		No		

11.5 Urine Collection Instructions Sheet

In order to assess the excretion of the urinary marker hydroxyproline (an amino acid which may be able to be used as a marker for intake of collagen containing food sources), we are collecting urine on 6 occasions – 2 baseline (on any day in the week before commencing the study), and with the intake of 4 foods (2 that contain collagen and 2 that don't – these will be randomised in order).

Please ensure you have a 10 hour fast (no food and keep fluid intake the night prior consistent - document on the next page), empty bladder on waking, then start collection after you have consumed breakfast or the test supplement. You will receive 3 bottles on each occasion labelled as followed:

- Time of commencement: Please document the time you finish your breakfast/ test supplement – this is when your 0-6h period (as described below commences).
- 0-6h: This is the 6 hour period after consumption of the food on the allocated collection day, and may be after breakfast on BL day.
- 6 – 12h: This is from 6 to 12 hours (inclusive) after consumption of the food on the allocated collection day, and from 6-12h (inclusive) after breakfast consumption.
- 12 – 24h: This is from 12 hours up until the equivalent time once your collection time commenced the first day i.e. if you finished the food/breakfast on the day prior at 7am, you should collect until 7am the following day.

Baseline day 1 (your choice)		**Date**	
Time of commencement	6-12 hours post BF/sup time	12-24 hours post BF/sup time	Time of completion
Baseline day 2 (your choice)		**Date**	
Time of commencement	6-12 hours post BF/sup time	12-24 hours post BF/sup time	Time of completion
Collection 1		**Date**	
Time of commencement	6-12 hours post BF/sup time	12-24 hours post BF/sup time	Time of completion
Collection 2		**Date**	

Time of commencement	6-12 hours post BF/sup time	12-24 hours post BF/sup time	Time of completion
Collection 3		**Date**	
Time of commencement	6-12 hours post BF/sup time	12-24 hours post BF/sup time	Time of completion
Collection 4		**Date**	
Time of commencement	6-12 hours post BF/sup time	12-24 hours post BF/sup time	Time of completion

Please collect all urine excreted into the bottles provided – the easiest way may be to keep the bottles next to the toilet.

11.6 24 Hour Food, Fluid and Exercise diaries

The purpose of collecting data around your intake and exercise will enable a better understanding of the factors that may have an impact of the excretion of hydroxyproline. It is imperative that these diaries are as comprehensive as possible, if you have any questions please contact Rebekah Alcock at 0476 291 511 or Rebekah.Alcock@ausport.gov.au.

Please read the instructions thoroughly before commencing:

Instructions

1. Please document every food and liquid that you consume, and any exercise that you do within the 24h period that coincides with your urine collection
2. Ensure that you document as much information as possible, including any toppings/ additions. For example;

   - Burgen bread, soy and linseed, 2 slices with 2 tsp meadowlea canola spread
   - Yoghurt – Yoplait vanilla 175 grams
   - 250 gram rump steak, fat removed, cooked in 1tblsp olive oil
3. Utilise measuring cups and/or spoons where possible to weigh out amounts.
4. Note the weight or volume of items that come in packages, and how much you consumed.
5. If you are consuming mixed dishes, please include the recipe and the amount that you consumed of each component i.e. spaghetti bolognaise (500 grams lean mince, 140 grams tinned tomatoes, 2 beef stock cubes, 2 tblsp tomato paste, 500 grams penne pasta) – consumed 1 cup sauce, 2 cups pasta (cooked), 1 tbs parmesan cheese.
6. Please document the amount of exercise, including any warm-ups and cool downs – including HR if possible.
7. Please document total fluid intake as accurately as possible – including all water consumed.

The below may assist you in documenting amounts where scales/measuring-ups are not available:

1 closed fist = 1 cup i.e. cereal, fruit, salad, pasta
1 palm = 100 grams meat
1 cupped hand = ½ cup i.e. pasta, rice, beans
2 cupped hands = 85 – 100 grams (i.e. popcorn, chips, pretzels)
1 thumb = 1 tsp i.e. butter, salad dressing

Example food diary

Meal/Time	Food/ fluid	Quantity	Comments
Breakfast 08:30am	Toast (multigrain) – Burgen brand Meadowlea canola spread Vegemite Water	2 pieces 2 tbslp canola spread 2 tblsp vegemite 1 x 250ml glass	
Morning tea 10:30am	Sunbright Corn thins Ricotta Honey	2 whole 1 tblsp 4 tsp	
Lunch 1:00pm	Bread (Burgen soy and linseed) Mixed lettuce leaves Cheese (koon light and tasty pre-packaged) slices Ham	2 slices ½ cup 3 slices 30 grams (3 thin slices)	
Afternoon tea 3:00pm	Uncle Toby's muesli bar forest fruit flavour	1 bar	
Dinner 6:00pm	Rump steak Mashed potato (3 whole potatoes, ½ cup milk, 1 tblsp butter) Peas	300 grams ½ cup ½ cup	Lean steak, cut off the fat

Dinner + fluid intake the night prior to urine collection day – please aim to consume at least 2.5L of water

Please allow at least 10 hours with no food intake (prior to the test food/breakfast) and document all fluid intake

Meal/Time	Food/ fluid	Quantity	Comments
Dinner			
Dessert			
Supper			
Fluid intake			

Food/fluid intake on urine collection day – please aim to consume at least 2.5L of water

Meal/Time	Food/ fluid	Quantity	Comments

Exercise the day prior to urine collection		**Date**
Exercise	**Time**	**Type/ intensity/ duration (include reps/ sets where possible)**
Exercise the day of urine collection		**Date**
Exercise	**Time**	**Type/ intensity/ duration (include reps/ sets where possible)**

End of Document