Benchmarking Native Collagen: Evaluation of Structural Differences between Tendon Types and across Animal Models

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A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Master of Science in Applied Science.

December 2023, Halifax, Nova Scotia

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Date: December 7th, 2023

Table of Contents

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by Amanda Lee

Abstract

Tendons perform two very distinct functions that require different mechanical properties and are categorized into two types based on these functions: positional (PTs) and energy storing (ESTs). Structural differences between PTs and ESTs enable these distinct functions. Within bovine and equine animal models, both structural and mechanical differences between PTs and ESTs have been noted. However, the extent of these differences is unclear, as is how well conserved any such differences are across species. The present research aimed to understand the structural differences and similarities between the most commonly used PTs and ESTs in three animal models: bovine, ovine, and rat. Tendons were structurally evaluated by four methods: Hydrothermal Isometric Tension (HIT), $NaBH_4$ reduction with HIT, Transmission Electron Microscopy, and Scanning Electron Microscopy. Overall, results from all four methods of analysis show that there are distinct differences between tendon types and across models: particularly between large and small animal models.

December 7th, 2023

Acknowledgments

I'd like to thank my supervisor, Dr. Samuel Veres, for all of his help over the past almost two and a half years. The support and patience that you have shown has been unparalleled. I am grateful that you gave me the opportunity to explore my interest in biomedical research and allowed me to delve into the world of biomedical engineering. I would also like to thank my supervisory committee, Dr. Danielle Tokarz and Dr. Tamara Franz-Odendaal, thank you for all of your questions and comments that helped to guide the final outcome of this project. I am thankful for your support and contributions.

Thank you to my labmates, Austin Yazbeck-Macdonad, Ted Lownie, and Kelsey Gsell. I am grateful for your assistance with various steps throughout this project as well as for your company in the lab, every day would have been a lot more boring without your presence. As well, thank you to Meghan Martin for your assistance in many of the stages of this project; everything would not have gone as smoothly without your help. I am also very grateful for your support and friendship. To Darren Cole and Mary Ann Trevors, thank you for all of your assistance in the completion of the experiments within this project. You were both incredibly helpful, and I am thankful for your support.

Thank you to Reid's Meats, Northumberlambs, and Dalhousie University for supplying tissue throughout this project. Thank you to Saint Mary's University and to Research Nova Scotia for providing the funding that allowed me to be able to complete this project. Additionally, to Saint Mary's University Faculty of Science for all of their support and to the graduate students and professors alike who work towards fostering a fantastic science community at Saint Mary's.

Lastly, thank you to my family. To my parents and sister, thank you for providing me with valuable advice and life lessons that led me to where I am today; I couldn't have done this without your support and encouragement.

List of Figures

List of Tables

List of Abbreviations

AFM	Atomic Force Microscopy
AGE	Advanced Glycation End-product
CDET	Common Digital Extensor Tendon
EMC	Electron Microscopy Core
EtOH	Ethanol
FWHM	Full Width at Half the Maximum
GAG	Glycosaminoglycan
HIT	Hydrothermal Isometric Tension
k	Slope of load decay during isothermal segment of a HIT experiment
$NaBH_4$	Sodium borohydride
PBS	Phosphate Buffered Saline
SEM	Scanning Electron Microscopy
SDFT	Superficial Digital Flexor Tendon
SLRP	Small leucine-rich proteoglycans
$t_{1/2}$	Half-time of load decay
T_d	Denaturation Temperature
TEM	Transmission Electron Microscopy
T_{Fmax}	Temperature at Maximum Force

Chapter 1 - Introduction

1.1 Tendon Anatomy

1.1.1 Structural Hierarchy

Tendons are collagenous connective tissues responsible for transferring the load from muscle to bone [1,2]. The tendon itself does not generate contractile forces but responds to the movement and forces from the muscle; therefore, to transfer load effectively, muscles and tendons work together as one unit [3]. They aid in locomotion by positioning limbs and facilitating movement at the joints [34]. Tendons are unidirectional fibre composites; their collagen fibres are arranged in a parallel fashion [2]. This arrangement allows tendons to resist tension while allowing some compliance with the musculoskeletal system [4]. The need for tendons to resist tension while also having some degree of flexibility is very contradicting. To meet this combination of properties, tendons are comprised of a complex hierarchical system. This hierarchical structure is made up of seven levels from smallest to largest: tropocollagen, microfibril, sub-fibril, fibril, fibre, fascicle, and tendon (Figure 1.1). While the structure has several levels, there are three main groupings. Tropocollagen to fibrils are found at the nanoscale level, fibres and fascicles are on the microscale level, and the whole tendon is considered the macroscale level [5]. This arrangement gives the tissue extra extensibility, allowing the tendon to easily transfer load [3,6].

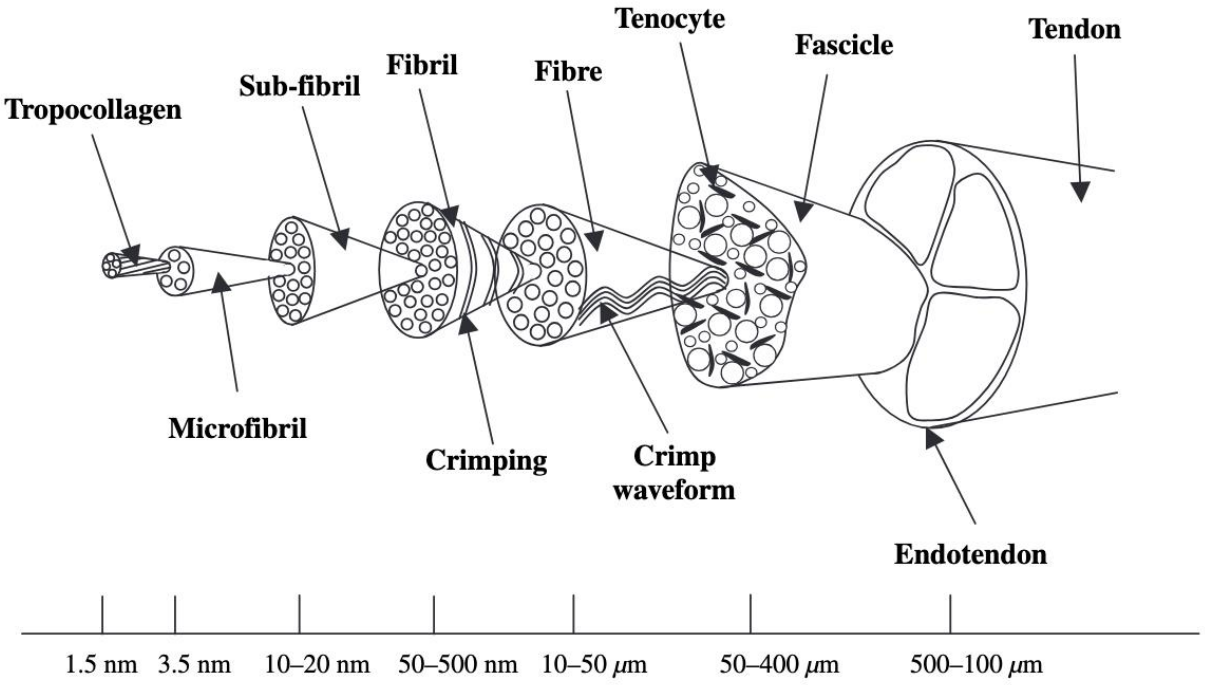

Figure 1.1: Schematic of the structural hierarchy of tendon. [3]

Along with the specialized arrangement of the tissues, many properties of tendon are due to its smallest unit, type I collagen, which is expertly arranged and gives the tendon high uniaxial strength [7,8]. There are 28 different types of human collagen, many of which make up approximately 60-85% of the dry weight of tendon [6]. Type I collagen is the most predominant in tendons, forming 95% of collagen matter [6,8]. This type of collagen provides tensile strength and a framework for the attachment of cells and extracellular biomolecules. Type I collagen molecules are triple helical structures that are covalently bound and positioned in a precise quarter-staggered arrangement to form collagen fibrils [9,10]. Collagen fibrils are a fundamental structural unit within the human body. They are long and thin nanoscale cable-like structures that, in tendon, are packed together in a parallel fashion to form fascicles [9,11]. Fascicles are incredibly important for the mechanical properties of the tendon; these units slide against each other

increasing the extensibility of the tendon [4]. The interfascicular matrix binds together adjacent fascicles to form tendon [12].

Tendons are commonly split into two categories: positional tendons and energy storing tendons, each having a specific set of functions [7]. While, for the most part, the two tendon types are share the same structural hierarchy, there are some distinct differences at the nanoscale level.

1.1.2 Tendon Types

1.1.2.1 Positional Tendons

Positional tendons function under low stress and assist in low resistance movement [11,13]. These tendons have a stiffer matrix that is associated with lower water content, lower glycosaminoglycans (GAGs) content, and lower cellularity [14]. Having a stiff matrix allows positional tendons to move and position the body in a precise manner [15]. A great example of positional tendons within the human body are the digital flexor tendons of the hand. Having a stiffer matrix and being relatively inextensible makes them the perfect candidates for completing controlled and precise fingertip movements that give our hands their dexterity [11,15,16]. This level of control within our movements is a combination of the tendon properties and the physiological properties of the neural activation system [15]. Therefore, the characteristics of positional tendons are well suited for this function.

Within many research studies, the Common Digital Extensor Tendon (CDET) found within bovine, ovine, and equine models is heavily used to demonstrate the effects of strain on positional tendons. The human anterior tibialis tendon and CDET are both purely positional and have a maximum strain of 2 to 3 % [13,16–19]. This comparison between human tendons and animal

models gives researchers the opportunity to study the mechanical and physical properties of positional tendons using animal models.

1.1.2.2 Energy Storing Tendons

Energy storing tendons function under high stress and are responsible for improving the efficiency of locomotion by storing and releasing energy [13]. Energy storing tendons have a matrix that is extensible and less stiff than their positional counterparts [14]. They also tend to have a greater water content, higher concentration of GAGs, and greater cellularity [9,16,20,21]. These differences are often attributed to the interfascicular matrix. Additionally, this type of tendon often has thinner collagen fibrils and has a slower rate of collagen turnover when compared to positional tendons [9]. These properties allow energy storing tendons to perform their job within the body.

Energy storing tendons are required to withstand larger tensile stresses *in vivo* than positional tendons. Therefore, they must allow more elongation and have greater fatigue resistance. Elongation of these tendons is facilitated by a greater level of sliding between the fascicles [7,9]. This sliding allows the tendons to experience much higher strains and gives the tendons the extra extensibility needed to perform well under high stress. Additionally, their extensibility and elongation properties allow them to store energy [7]. When in motion, energy storing tendons increase the efficiency of locomotion by storing energy during deceleration. This energy is then released to help power acceleration, acting as a spring, to allow for activities such as running or jumping to be less taxing on the body [11]. The properties of energy storing tendons are heavily suited to their function. One of the most notable energy storing tendons is the human Achilles tendon. This tendon must be able to withstand highly repetitive large forces being applied daily [11]. Maximum strains of up to 11% and 16% have been recorded for the human

Achilles tendon and equine superficial digital flexor tendon (SDFT), respectively [19,22]. When running, the Achilles tendon endures tension that exceeds 12 times the weight of the body [11]. Therefore, the properties of these tendons are critical to allow this type of motion. The SDFT is commonly used as a model for the Achilles tendon and not only because of their close strain range. The sliding capabilities of the fascicles within both the Achilles tendon and bovine SDFT are very similar, meaning that they can achieve similar levels of extensibility [6,23,24]. Therefore, within research, the SDFT is an excellent comparison to the Achilles tendon.

Energy storing tendons fail at a higher strain than positional tendons, and they are able to recover more quickly after loading events; this is likely due to their ability to resist fatigue [13,19,25]. However, the fascicles within energy storing tendons fail at lower strain rates than fascicles within positional tendons. This is due to their design focusing on maximizing energy storage. This can lead to some issues because when the fascicles within these tendons are specialized to maximize energy storage, compromises must be made [26]. These tendons must experience high strain to store the energy required to compensate for large, heavy movements such as running. As speed increases, energy consumption increases, meaning that at top speeds, a compromise must be made between storing energy to compensate for the heavy movement and keeping the stress and strain on the tendon below the elastic limit to prevent damage. This limit is easily exceeded with repetitive loading, which means that there are very low safety margins within energy storing tendons, leading to the possibility of damage to the tendon structure [25–27].

Within tendon structure and function, compromises are made to perfect overall performance. To achieve the differences in function between positional and energy storing tendons, there must be differences in their structure. As shown above, energy storing tendons

have distinct mechanical properties that allow them to be more extensible and improve energy storage, something that positional tendons have little need for.

1.1.3 Crosslinking

Crosslinks contribute heavily to the overall mechanical performance of both positional and energy storing tendons. Crosslinks are formed one of two ways, either enzymatically or non-enzymatically through advanced glycation end-products (AGE)s. Enzymatic crosslinks are formed through a deamination process with lysyl oxidase. Within the enzymatically formed crosslinks, there are two major types of crosslinks that are present: thermally labile and thermally stable crosslinks. Thermally labile crosslinks are primarily divalent aldimine crosslinks. These crosslinks are considered immature and are stable at a physiological level, but they are easily cleaved by heat or acidic conditions [28]. These crosslinks were primarily found within previously studied positional tendon models. Thermally stable crosslinks are primarily divalent ketoamine or trivalent crosslinks, either pyridinolines or pyrroles crosslinks [28]. The divalent ketoamine crosslinks are also considered immature but not susceptible to heat or acidic conditions, making them thermally stable. The trivalent crosslinks are mature and are also stable in heat or acidic conditions. These thermally stable crosslinks were primarily found within energy storing tendons of previously studied models. Without the presence of enzymatic crosslinks, tendons would be able to withstand very little force [28].

AGE-formed crosslinks occur after tendon maturation. These crosslinks are formed from the nonenzymatic reaction of glucose and an aldehyde, such as glyoxal or malondialdehyde [28,29]. This reaction crosslinks neighbouring collagen molecules together, typically occurring due to diabetes mellitus or the process of aging. This act of glycation produces many of the mechanical

changes that occur with aging. These crosslinks are unstable, and when attached to the same area as the enzymatic crosslink, they can cause deleterious effects. This leads to an increase in failure stress, meaning that the threshold that the energy level needs to reach is higher and this prevents the tendon from being able to absorb elastic energy [29]. Additionally, AGEs inhibit intermolecular sliding, which leads to the inhibition of their failure mechanisms. Overall, the presence of AGEs prevents the tendon from performing its basic functions.

1.1.4 Diameters

The size of collagen fibrils has been suggested to be linked to their overall tensile strength; therefore, the diameters of fibrils within a tendon and their abundance may be an indicator of the strength of the tendon itself [26,30]. There have been differences between the mean fibril diameters, peak fibril diameters, modality, and the spread of diameters found between tendon types. These differences likely contribute to the differing functions of these tendon types. Fibril diameters within positional tendons have been found to be larger than those in energy storing tendons, likely contributing to their greater strength and stiffness [30]. While the smaller fibril diameters of energy storing tendons likely contribute to their increased elasticity. Therefore, understanding the diversity of diameters present within a tendon may give some insight into its mechanical properties.

1.1.5 Collagenous Material

While tendons are primarily composed of type I collagen, many different types of collagens are present within tendon, and in total, they make up approximately 60-85% of its dry weight [6,16,18,31]. As stated above, up to 95% of the collagen found in tendons is type I collagen. The remaining percentage is comprised of collagen types III, V, VI, XII, and XIV [32–34]. Additionally, there are slight differences in collagen content between positional and energy storing tendons, but there is not a large understanding of how this affects their mechanics [35].

Type III collagen plays a vital role in regulating the size of type I collagen fibrils and often increases in abundance when the tendon is injured [7]. This type of collagen comprises up to 10% of the collagen within the tendon [36]. Type III collagen is often found in the interfascicular matrix and likely helps with fatigue resistance and aids in keeping the matrix healthy [7,35]. The other types of collagens within tendons do not make up a significant percentage of the collagen content. Several non-fibrillar collagens are present within tendon; the most notable is type VI collagen. This type of collagen is localized within the pericellular matrix, and it is suggested that it functions in the development of the structure and function of the extracellular matrix [36,37].

1.1.6 Non-Collagenous Components

There are also many components within tendons that are not collagenous. A few notable ones are proteoglycans and elastin. Proteoglycans are a large group of proteins. They form approximately 1-5% of a tendon's dry weight and are the most abundant non-fibrous protein [36]. Within tendons, small leucine-rich proteoglycans (SLRPs) play an important role in collagen fibrillogenesis and tendon development [6,18,36]. Additionally, they interact with collagen fibrils to help regulate the structure and biomechanics of the tendon. It has been found that the presence of SLRPs aids in

healing tendon injuries and overall helps the tendon's mechanical properties [38]. They interact with collagen fibrils, providing hydration and swelling pressure, which helps the tendon to withstand compressional forces [39]. Several types of proteoglycans are found within tendons; however, the differences in their distribution and functional traits have not been well studied [36]. Within tendons, they consist primarily of decorin, lubricin, and fibromodulin.

SLRPs have small side chains called glycosaminoglycans (GAGs). These side chains have been suggested to connect neighbouring fibrils, contributing to their mechanical strength [40]. Some of the viscoelastic properties of tendons have been suggested to involve the movement of water to act in rehydrating the tendon. Tendons lose water when under tension; therefore, remaining hydrated is important for their mechanical properties. GAGs have been suggested to aid in this mechanism due to their hydrophilic properties.

Elastin is a fibrous protein that is resistant to fatigue and is highly extensible [36]. Often, elastin is found within tissues that are subjected to high levels of cyclic loading, including heart valves, arteries, lungs, and skin [41–43]. Within tendons, elastin forms approximately 1-10% of the dry weight [36]. Previous studies have shown that tendons, specifically the interfascicular matrix, are rich in elastin [7,18,36,44,45]. However, elastin is also found within fascicles and around the cells of the tendon. Energy storing tendons need to be more elastic and extensible than positional tendons, resulting in them having a higher concentration of elastin [35,46].

1.2 Animal Models

Commonly, rats, mice, cows, sheep, and horses are used as animal models within biomedical research [47,48]. Mice and rats are typically used due to their availability and the ease at which they can be utilized for specific molecular studies. Whereas larger animals may provide better models when looking at biomechanics and when comparing musculoskeletal tissue [47].

Specifically, equine models are one of the most analogous models for human overuse injury's such as tendinopathy due to their superficial digital flexor tendon being comparable to the human Achilles tendon [47]; however, obtaining equine tendons can prove to be difficult. Due to this, researchers have worked toward validating tendons within other animal models to be used in their place, such as bovine tendon [2,47,49]

1.2.1 Bovine

Bovine tendons have been found to have similar biomechanical characteristics as human tendon [4,9,41,50,51]. Specifically, the bovine SDFT and CDET have been considered analogous to human Achilles and anterior tibialis tendons. Due to this, the bovine model has been widely used in tendon research and is a well-studied model.

The bovine common digital extensor tendon (CDET) and the superficial digital flexor tendon (SDFT) are the positional and energy storing pairs used in this study (Figure 1.2). This is due to this pairing being a universal model and will allow for comparison to previous studies.

Figure 1.2: Bovine positional and energy storing tendons. The tendon pairs used, common digital extensor and superficial digital flexor tendons are indicated by the red arrows. (A) Dissection photo of the three positional tendons: medial digital extensor common digital extensor, and lateral digital extensor. (B) Dissection photo of one of the energy storing tendons: the superficial digital flexor. (C) The chosen tendon pairs removed from the forelimb.

1.2.2 Ovine

Unlike bovine, ovine models have not undergone a comparison to human tendons. However, ovine models display very similar weight distribution and gaits to equine models during locomotion [52]. This would suggest that their tendons may experience similar loading and may display similar structural and mechanical properties. Ovine models have been previously used in tendon research, specifically in cases where an equine model would often be used [52]. An ovine model has been typically used to replace equine models due to them being more accessible to source and requiring fewer regulations. However, common ovine tendon pairs have not gone

through a rigorous comparison between either equine or bovine tendons to see if they perform similarly under various testing techniques.

The ovine common digital extensor tendon (CDET) and the superficial digital flexor tendon (SDFT) are the positional and energy storing pairs used within this study (Figure 1.3). This tendon pair is the most similar to the bovine model being used and will also allow for comparison to previous studies as this is a commonly used tendon pair within this model.

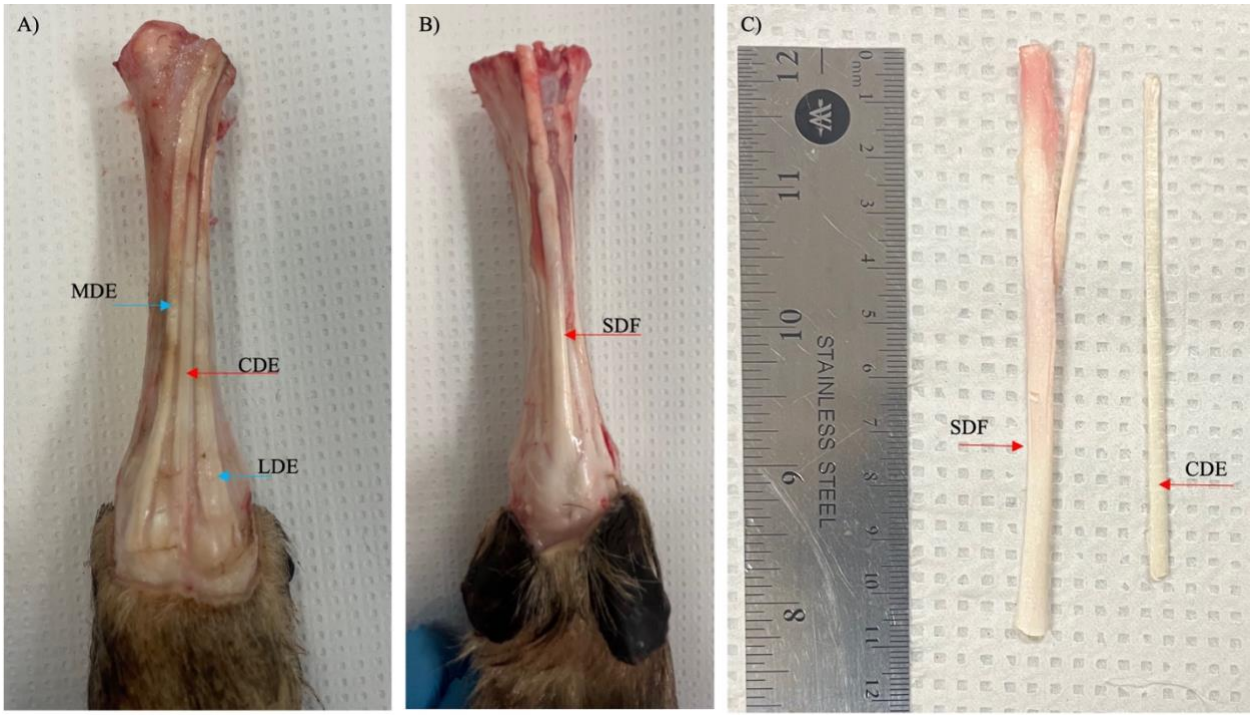

Figure 1.3: Ovine positional and energy storing tendons. The tendon pairs used, common digital extensor and superficial digital flexor tendons are indicated by the red arrows. (A) Dissection photo of the three positional tendons: medial digital extensor common digital extensor, and lateral digital extensor. (B) Dissection photo of one of the energy storing tendons: the superficial digital flexor. (C) The chosen tendon pairs removed from the forelimb.

1.2.3 Rat

Rat tendons have been widely used as a model for human tendon research, even within studies that address tendinopathy [53,54]. Rats have been found to have similar physiology and anatomy to humans. For example, their limb anatomy is very similar, with rats having many of the same muscles and tendons as a human limb [55]. However, rat tendons have also not been proven to be a comparable model for human tendons in terms of structure or mechanical properties.

Furthermore, rats have been shown to not contain all of the levels of hierarchy within their tendons that we see in bovine, ovine, and human models. Rat Achilles tendons do not contain fascicles; fibre is the largest subunit within the rat Achilles tendon [53]. Fascicles bundle collagen fibres together, taking the long rope like structures and grouping them together to give them extra support and strength. Furthermore, the fascicles within the tendon are defined by the interfascicular matrix; therefore, the tendons lacking fascicles would also lack this key feature [12]. The interfascicular matrix aids in locomotion by facilitating sliding between fascicles, which allows them to have better fatigue resistance. The rat Achilles tendon lacking this feature may indicate that they may not behave similarly to tendons that do have fascicles within their hierarchy.

However, regardless of their slightly different anatomy, the rat model has still been included in this study as they are a widely used model. The rat tibialis anterior and the Achilles tendons of the hind limb (Figure 1.4) are the chosen positional and energy storing tendons for this model. They are the most similar tendon structures within the rat limb anatomy compared to the bovine model.

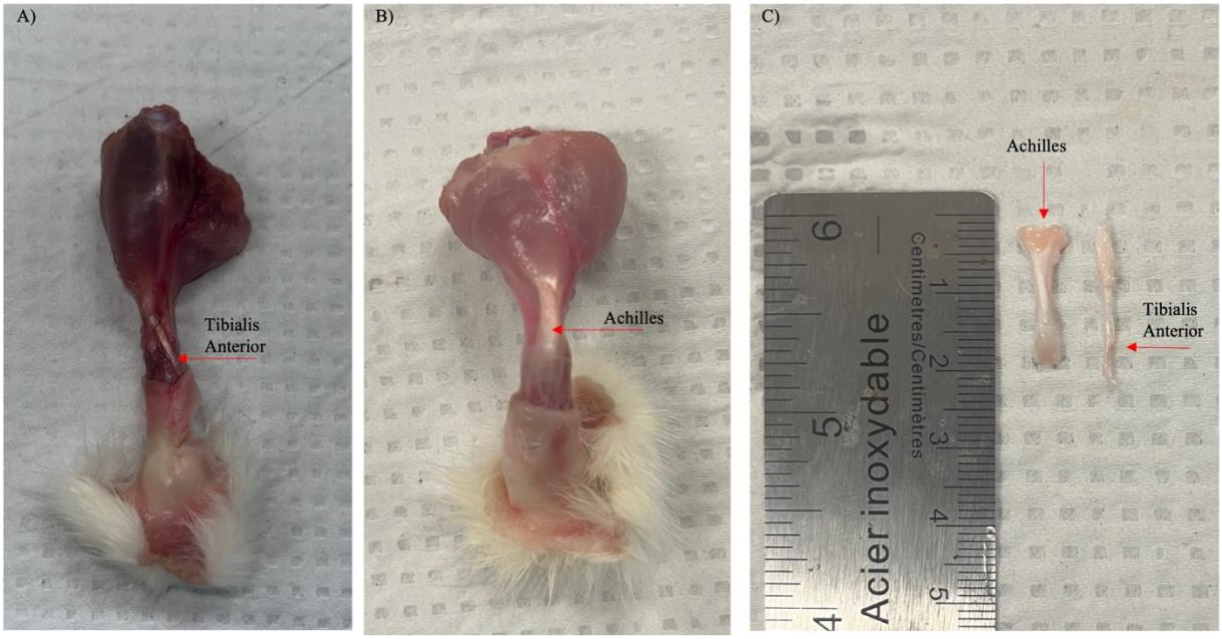

Figure 1.4: Rat positional and energy storing tendons. The tendon pairs used, tibialis anterior and Achilles tendons are indicated by the red arrows. (A) Dissection photo of one of the positional tendons: tibialis anterior (B) Dissection photo of one of the energy storing tendons: Achilles tendon. (C) The chosen tendon pairs removed from the hindlimb.

1.2.4 Comparison

As the use of human tissue in research is often constrained by the availability of tissue or the ethical concerns associated with their use, animal models are often used as an analogous model to produce similar results [2]. In the case of tendinopathy, animal models cannot precisely replicate the clinical features displayed in human tendons due to vast differences in weight distribution and locomotion in quadrupeds [49]. Additionally, tendinopathy often develops over time; it is a slow process that could take years to form and tends to occur in biologically older tissues [32]. Tendinopathy must, therefore, be induced in animal models and does not exactly replicate human tendinopathy. However, these models can come close to emulating similar clinical features.

1.3 Outcomes of Previous Literature

Recently, a study by Herod et al.[9] investigated the relationship between the nanoscale structure of collagen fibrils and their relationship to the overall mechanics of the tendons. It is well known that the specialized function of energy storing tendons must be due to differences within the structure of these tendons compared to positional tendons. It is clear that they are functionally different; however, there is much to be learned about how their specialized mechanics relate to their structure [9]. A lot of research has gone into understanding and characterizing the interfascicular matrix, but the nanoscale level of the tendon needs to be more thoroughly researched. Therefore, Herod et al.[9] set out to further understand how the structure of the nanoscale level of energy storing tendons aided in their specialized mechanics. This research began with the idea that the large differences in stiffness and strength between positional and energy storing tendons were the result of differences within the intermolecular cross-linking within collagen fibrils.

Furthermore, Herod et al.[9] pointed out the lack of research within tendon studies to validate animal models. Most studies only utilize one animal model for their research and often do not compare to models outside of that. The model most often used is rat tail tendon; on occasion, other models are used, such as the bovine tail tendon, rat patellar tendon, or the bovine CDET and SDFT. While, for the most part, these models all contain the same basic structures, the comparison of their physical and mechanical properties has not been performed. Some review articles have pointed out a few differences between various animal models, but a direct comparison has not consistently been performed [49,51,55].

Herod et al. [9] also found that the collagen fibrils within energy storing tendons were covered in thin filaments that formed a thick webbing of matrix (Figure 1.5) [9]. These filaments

ran across the collagen fibrils, laterally connecting them. This structure was not found within positional tendons. The fibrils within these tendons were free of any matrix like substance. This difference in structure between the two types of tendons may influence their function and allow for differences in their mechanical properties. While this filamentous webbing structure may play an important role within tendon structure and function, it remains poorly characterized, with its composition unknown and very few reports of its existence appearing within the literature. Leaving a gap within our knowledge of how the structure of energy storing tendons allows for its specialized function.

Herod et al.[9] further suggested possible mechanisms for this filamentous webbing matrix and why it is present within energy storing tendons. Energy storing tendons have better fatigue resistance and behave more elastically than positional tendons [4,7,12]. Additionally, they have better recovery rates and can withstand more force. The filamentous webbing matrix found within energy storing tendons may allow for these functional differences between the two tendon groups. The collagen fibrils within the energy storing tendons appeared to be bundled together by this filamentous webbing structure [9]. Laterally connecting the collagen fibrils with the filamentous webbing could possibly increase the bending stiffness of the collagen fibres. This would increase the resistance of collagen fibrils to buckling; the filamentous webbing structure would allow the tendons to be better at withstanding damage by increasing their overall strength.

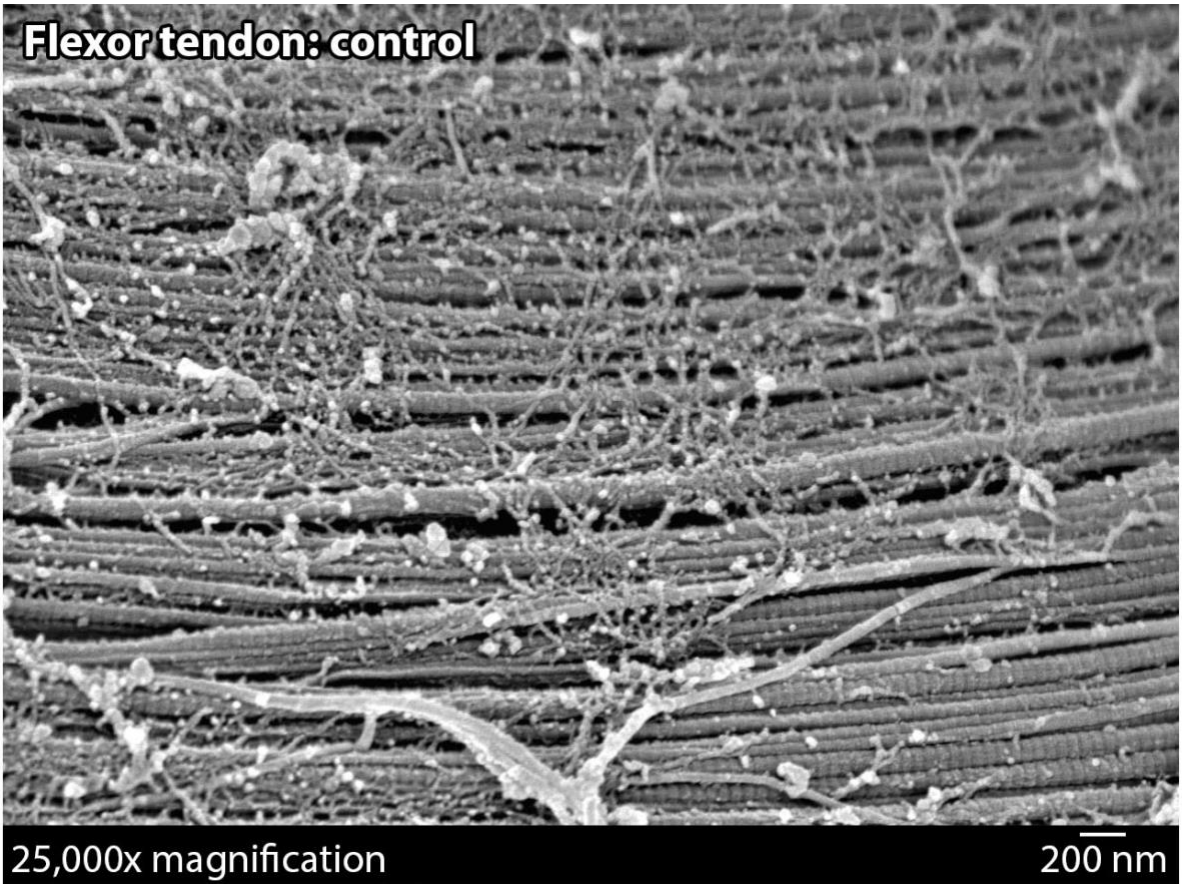

Figure 1.5: Collagen fibrils of energy storing tendons. A thick webbing of matrix coated the collagen fibrils, laterally connecting them. This webbing of matrix was not observed in positional tendons.[9]

1.4 Objectives

The lack of research in the comparison of commonly used animal models leads to the question of how universal are common tendon structures. Do the differences typically found between tendon types hold true in different models? Do the different tendon types perform the same across models? These questions lead to the first objective of this thesis.

The ill-defined filamentous webbing structure found by Herod et al.[9] raises questions not only about the webbing itself but also about how this feature may appear in different models. The first question is whether this filamentous webbing structure is primarily found in energy

storing tendons. The second question is whether this webbing feature is found universally across animal models. Leading to the second objective of this thesis.

Objective 1: To assess common tendon features to see if they are universal between tendon types and across animal models.

Objective 2: To assess the abundance of the ill-defined filamentous structure between tendon types and to see if this feature is universal across animal models.

Chapter 2 – Hydrothermal Isometric Tension Testing

2.1 Background

2.1.1 Hydrothermal Isometric Tension Testing

Understanding the thermal properties of collagen can tell us a lot about its load-bearing characteristics and basic nanoscale properties [9,56–58]. Assessing these properties within common positional and energy storing tendon pairs, both amongst themselves and across animal models, allows us to understand the types of crosslinks present within the tendons and may give some insight into the differences and similarities of the crosslinks amongst these groupings. This will expand our understanding of the properties of these models and may allow us to gain a better understanding of how these models can be used within research and how they generally compare to each other at the nanoscale.

The thermal properties were assessed using Hydrothermal Isometric Tension (HIT) testing; during this process, the tendons are placed in a beaker of distilled, deionized water (ddH2O) that is heated at a constant rate [59]. When heated to around 60-65°C, the triple helices of the collagen molecules, formed by two α-1 chains and one α-2 chain, will begin to unwind [60]. This occurs as these α-chains are susceptible to heat. When heated, the thermal energy within the tendon increases, causing the α-chains to move and take up more space, increasing their conformational freedom [61]. This, along with the steady rise in temperature, causes the intermolecular hydrogen bonds of the triple helix structure of collagen molecules to begin to rupture [60,62]. Thus, leading to the denaturation of the quasi-crystalline macrostructure of the tendons; this action causes an entropic drive that leads the tendon to randomly coil in on itself [63,64]. Typically, this contraction causes the α-chains to fold in on themselves; however, in this

case, due to the samples being placed in isometric constraints keeping the tendons under tension, these contractions will translate into a measurable force [56,57] (Figure 2.1). This contraction produces a continuous increase in load as the tension between the isometric constraints continues to be produced. The temperature that corresponds with the first distinctive positive increase in the slope of the force vs temperature curve is known as the denaturation temperature (T_d), typically occurring around 60-65°C. The T_d is an indicator of molecular stability; the higher the T_d, the more molecularly stable the tendon is compared to those with a lower T_d [57,64].

As the temperature of the ddH2O continues to rise, the α-chains within the tendons continue to be disrupted meaning, more α-chains are denaturing. The more α-chains that become denatured, the higher the tension within the tendon, which translates to a larger force being applied to the load cells. Some of the tendons will begin to drop off in load as the thermally labile crosslinks are destroyed [56]. This distinctive negative slope gives us the second measurable parameter: temperature at maximum force (T_{Fmax}). This typically occurs around 80°C and is an indication of the types of crosslinks present. A higher T_{Fmax} indicates that more thermally stable crosslinks are present than thermally labile crosslinks [9]. When the thermally labile crosslinks are destroyed by the heat experienced by the tendon, the connection between collagen fibrils is broken, causing the tendon to lose the tension required to produce a force on the load cells. This leads to drop off in load, indicating the temperature at maximum force. Some samples do not have a T_{Fmax} because they continue to contract and increase the force on the load cells up to 90°C, where this portion of testing ends. These tendons are primarily composed of thermally stable crosslinks that can withstand this heating process and are considered to have "survived 90°C".

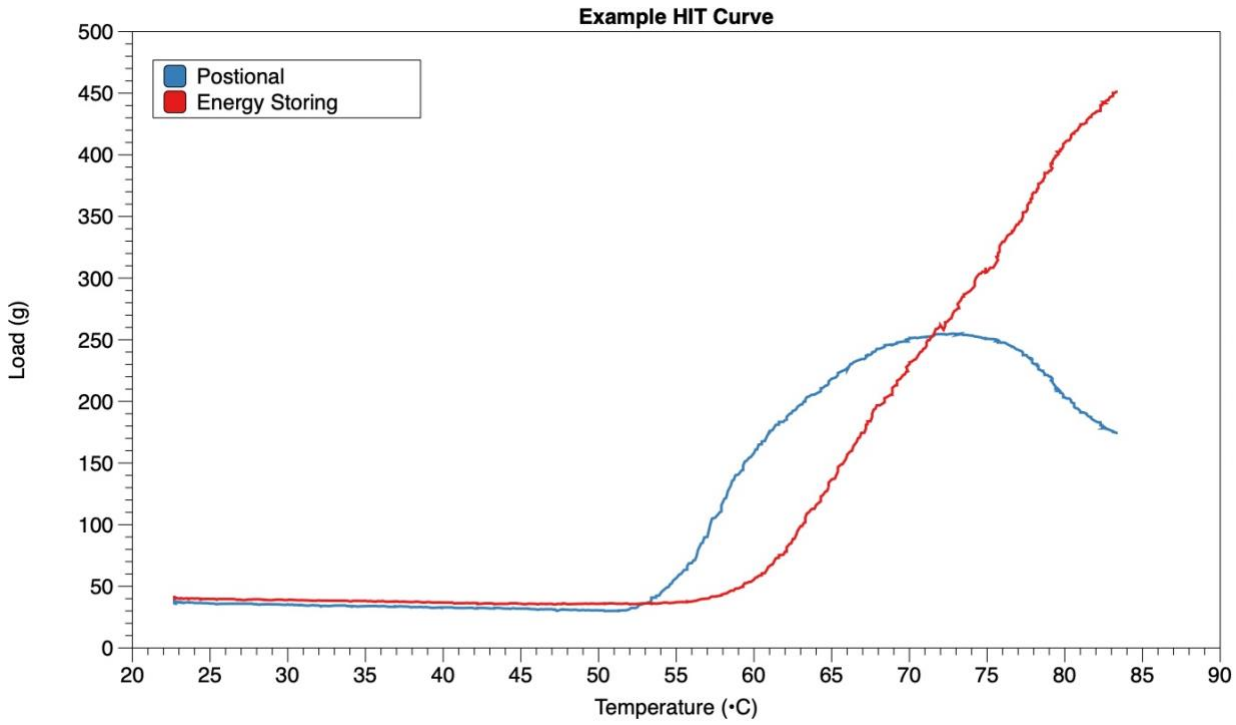

Figure 2.1: Example plot of a typical HIT bovine data; the blue line illustrates the response of a typical positional tendon while the red line illustrates the response of a typical energy storing tendon. The denaturation temperature typically occurs around 55°C – 65°C while the temperature at maximum force can occur anywhere after that, typically it is around 70°C-80°C.

2.1.2 Sodium Borohydride

The third measured parameter within HIT occurs after it switches into the isothermal portion of testing. This testing occurs after the temperature of the ddH2O reaches 90°C; the temperature is then maintained at 90°C for a 5-hour period [56]. Throughout this segment, the continuous heating of the tendon causes a gradual hydrolysis of the peptide bonds within the α-chains of collagen molecules, leading to a decay in load [59,65]. During this time, the force on the load cells is plotted against time, allowing us to measure the half-time of load decay ($t_{1/2}$) by taking the natural logarithm of the load, dividing it by the maximum load and plotting it against time (Figure 2.2).

A line of best fit is then applied to a 3,000-second data interval between 2,000 seconds and 10,000 seconds. The slope of the line is then measured to calculate the $t_{1/2}$ using the Maxwell Decay equation. The $t_{1/2}$ is an indicator of the relative density of crosslinks within a tendon [9,59,66,67]. The presence of thermally stable crosslinks slows this rate of load decay, meaning that the higher the $t_{1/2}$, the greater the relative density of thermally stable crosslinks is compared to those with a lower $t_{1/2}$.

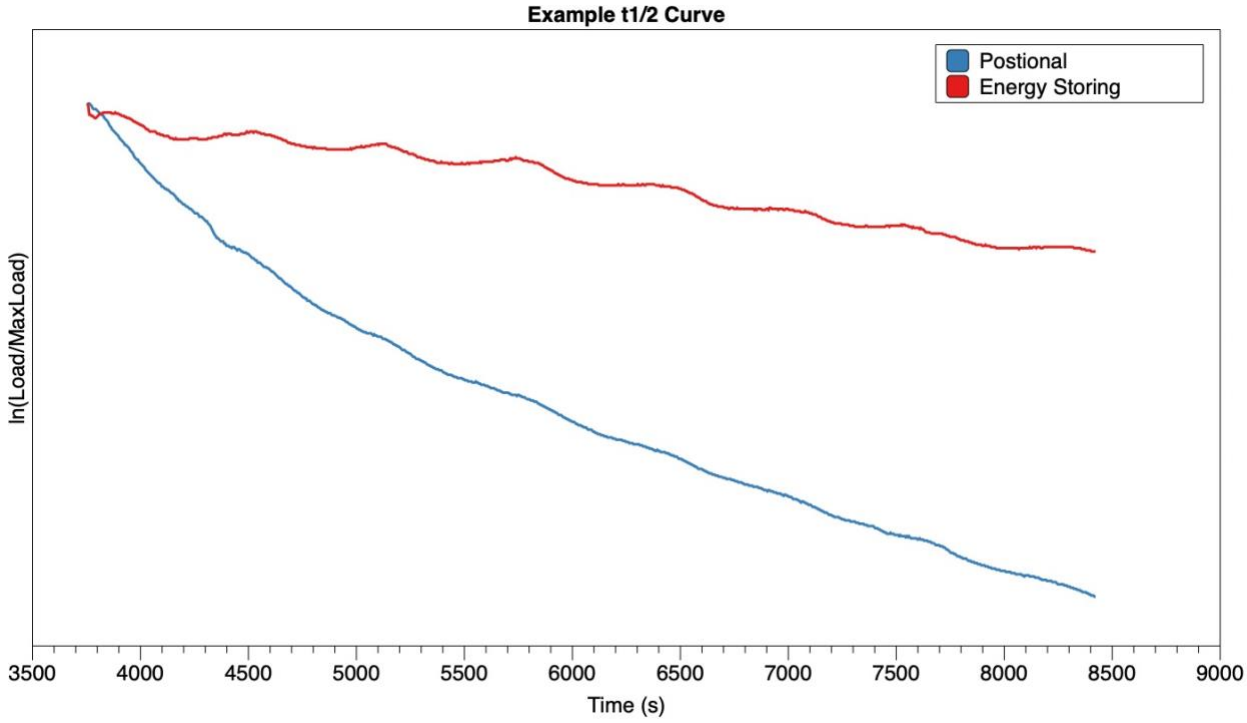

Figure 2.2: Example plot of the isothermal portion of HIT testing. The blue line represents typical bovine positional tendons, and the red line represents typical bovine energy storing tendons. The slope of the line (k) is taken from a 3000-second period and then used in calculating the half-time of load decay using this equation: $t_{1/2} = \frac{\ln(\frac{1}{2})}{-k}$

Tendons that do not survive 90°C do not have a $t_{1/2}$ due to them dropping off in load prior to the isothermal segment of testing. Therefore, to get their $t_{1/2}$, the tendons are treated with a sodium borohydride reduction. Within the thermally labile crosslinks, this treatment reduces the thermally labile carbon-nitrogen double bond to a single bond, converting the bond to a thermally stable form [56]. This allows the tendon to survive 90°C, and therefore, their $t_{1/2}$ can now be assessed.

2.1.3 Hypotheses and Rational

The objective of this section was to characterize the thermal stability of the crosslinks and to quantify the relative abundance of the total crosslinking present within the positional and energy storing tendons of bovine, ovine, and rat models.

Hypothesis 1: The energy storing tendons, SDFT and Achilles tendon, will have a greater denaturation temperature than the positional tendons, CDET and tibialis anterior tendon. These results will reflect those of other studies that suggest that the energy storing tendons have greater molecular stability [4,9,68].

Hypothesis 2: The energy storing tendons, SDFT and Achilles tendon, will have a greater temperature at max force than the positional tendons, CDET and tibialis anterior tendon. These results will reflect those of other studies that suggest that the energy storing tendons have a higher density of thermally stable intermolecular crosslinks [9,69]. Tendons that have a greater density of thermally stable crosslinks will display a continuous increase in force on the load cells and will be more likely to survive 90°C.

Hypothesis 3: In the absence of any existing data suggesting a difference, the denaturation and temperature at maximum force across animal models will be similar for each given tendon type. The performance of both thermally stable and thermally labile crosslinks will be the consistent across animal models suggesting that they have similar densities of thermally stable crosslinks.

Hypothesis 4: Following a sodium borohydride reduction, the energy storing tendons, SDFT and Achilles, will have a greater half-time of load decay than the positional tendons, CDET and tibialis anterior. The more gradual the load decay or the greater the half-time of load decay is the greater the intermolecular network integrity is within the tendon [63]. This would suggest that the energy storing tendons have a higher total density of intermolecular crosslinks.

Hypothesis 5: In the absence of any existing data suggesting a difference, the half-time of load decay across all three animal models will be the same for each given tendon type, suggesting that density of total number of crosslinks will be consistent across all three animal models for each given tendon type.

2.2 Methods

2.2.1 Sample Sourcing and Storage

2.2.1.1 Bovine

Bovine tendons were dissected from the forelimbs of adult steers ranging in age from 24 to 36 months old. Bovine forelimbs were sourced from Reid's Meats. This abattoir is a provincially inspected facility that processes beef cattle, from veal to adult, as well as cull animals (exhausted

dairy cows) as old as 10 years of age. Reid's has allowed us to source the samples immediately after the animal has been slaughtered. Through this process we can record the known sex and approximate age of the animal.

The steers were culled from 7:00 am – 11:00 am. Forelimbs were then brought back to the lab where dissection takes place. There, the CDET and the SDFT are separated from the forelimb. The tendons were wrapped in gauze soaked in phosphate buffered saline (PBS) and then placed in a labelled bag. The samples were then placed in a cooler to be transported back to Saint Mary's University. The tendons were placed in a -86°C freezer about an hour after the last tendon has been collected.

2.2.1.2 Ovine

Ovine tendons were dissected from the forelimbs of adult ewes ranging in age from 24 to 60 months old. Ovine forelimbs were sourced from Northumberlambs (Northumberland Lamb Marketing Co-op Ltd). This abattoir is a provincially inspected facility that processes sheep, from lamb to mutton. Northumberlambs has allowed us to source the samples immediately after the animal has been slaughtered, allowing us to record the known sex and approximate age of the samples.

The ewes were culled from 7:00 am – 11:00 am. Forelimbs were transported in a cooler to return them to the lab, where dissection of the tendons can commence in little more than an hour from when the last animal was slaughtered. The CDET and the SDFT were separated from the forelimb. They were then wrapped in gauze that has been soaked in PBS and placed in a labelled bag. The tendons were placed in a -86°C freezer immediately after dissection.

2.2.1.3 Rat

Rat tendons were sourced from the hindlimbs of adult Sprague Dawley rats, age 3 months. The freshly killed rats were provided by Dalhousie University, which had sourced the rats for other research purposes, but had not had any interventions during life.

The rats were culled at 11:00am. Hindlimbs were transported intact in a cooler to return them to the lab, where they are immediately placed in a -86°C freezer. Dissection of the tendons takes place when they are needed for an experiment. The Achilles tendon and tibialis anterior tendon were separated from the hindlimb. Immediately after dissection, the tendons are prepared for the experiment.

2.2.1.4 Storage

Samples were stored according to our tissue storage protocols. The samples were wrapped in gauzed soaked with PBS and stored in freezer bags. They were then stored at -86 °C. When needed the tissue was removed from the freezer and allowed to thaw at room temperature.

2.2.2 Untreated Samples

Tendons were retrieved from storage at -86°C and allowed to thaw at room temperature. A total of 30 tendons were tested, five tendon pairs from each of the three animal models. The ovine and bovine tendons were bisected longitudinally to an approximate size of 12mm x 1mm x 1mm; this does not affect the HIT response [66]. While the rat tendons were dissected from the hindlimb and kept whole due to their small size, the size of the positional tendons were ~ 20mm x 1mm x 0.5mm, and the energy storing were ~16mm x 1mm x 0.5mm.

HIT analysis was carried out using a custom-built apparatus [57,59,66] (Figure 2.3). The tendons were placed longitudinally into grips to constrain the sample isometrically. These grips were then mounted between an adjustable and fixed support. Attached to the adjustable support is a load cell that records the force produced throughout the experiment. This apparatus is placed into a 4L beaker filled with distilled, deionized water. This beaker sits on top of a hot plate. A tensile preload of 60 g was applied to the bovine and ovine samples, while a preload of 10 g was applied to the rat samples. After the preload was applied, the tendons were left to relax for 10 minutes. The water is then gradually heated to 90°C; the temperature is first increased at a rate of 1.5 ± 0.1°C /min to 75°C and is then decreased to a rate of 0.7 ± 0.2°C /min to 90°C[66]. The rate that the temperature rises is decreased at 75°C to prevent temperature overshoot. The temperature is monitored using a centrally located thermistor probe interfaced with a conditioning amplifier [56]. Once the temperature reaches 90°C, it is then maintained at that temperature for 5 hours. The time, temperature, and force were recorded throughout the testing using LabVIEW (2010 Edition, National Instruments).

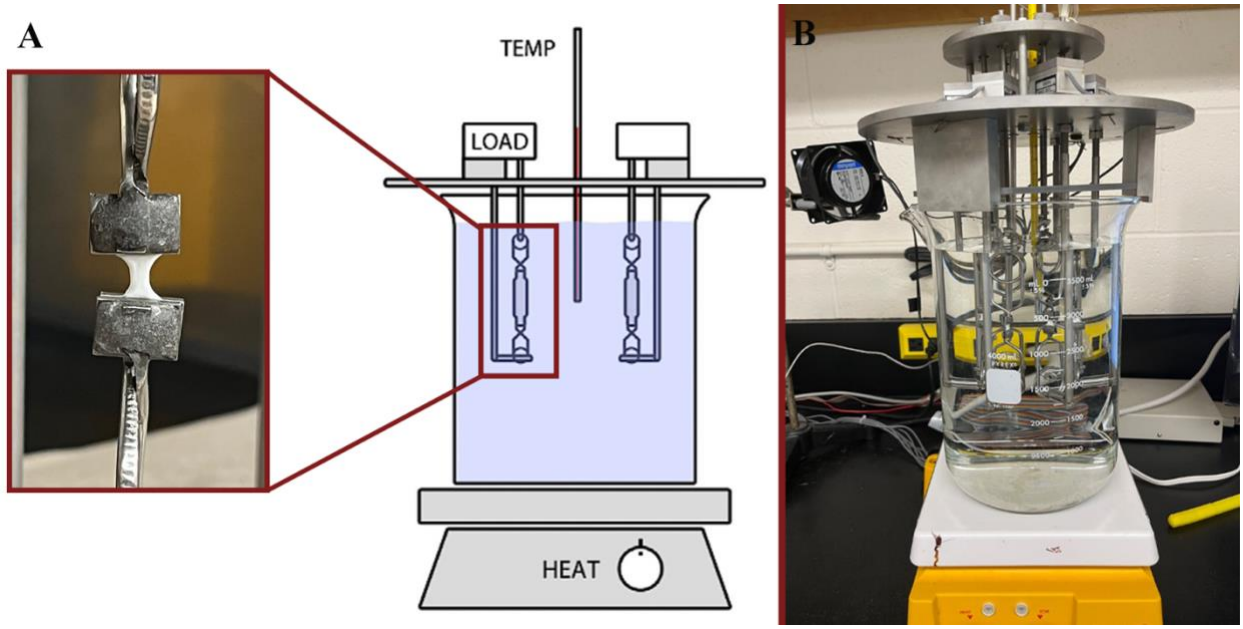

Figure 2.3: Hydrothermal Isometric Tension testing apparatus. (A) Depiction of a simplified model of the apparatus with a close up of the isometrically constrained tendon. (B) The actual HIT set up. Modified with permissions from Veres et al. [66]

After testing, the data was analyzed in Microsoft Excel (Version 16.76, Microsoft, USA). The denaturation temperature (T_d), the temperature at maximum force (T_{Fmax}), and the half-time of load decay ($t_{1/2}$) were determined for each tendon. The denaturation temperature and the temperature at maximum force occur prior to the isothermal segment of testing. The denaturation temperature is the corresponding temperature at the initial onset of continuous load that is indicated by a distinctive increase in the slope of the force vs temperature curve. The temperature at maximum force occurs at the temperature corresponding with a continuous drop-off in load indicated by a distinctive decrease in the slope of the force vs temperature curve before the water temperature reaches 90°C. The half-time of load decay is taken from the 5-hour isothermal segment. This segment is fit to a Maxwell Decay. To find the half-time of load decay,

the natural logarithm of the load is divided by the maximum load and plotted against time [59]. A line of best fit is then applied to a 3000-second interval between 2,000 and 10,000 seconds from the start of the isotherm. The slope of this line is the half-time of load decay.

2.2.3 Treated Samples

Tendons were retrieved from storage at -86°C and allowed to thaw at room temperature. A total of 30 tendons were tested, five tendon pairs from each of the three animal models. The ovine and bovine tendons were bisected longitudinally to an approximate size of 12mm x 1mm x 1mm. While the rat tendons were dissected from the hindlimb and kept whole due to their small size, the size of the positional tendons were ~ 20mm x 1mm x 0.5mm and the energy storing were ~16mm x 1mm x 0.5mm.

Samples were placed into control and treatment groups. The control group underwent four 15-minute washes in 5mL of borate buffer solution (Fisher Scientific Company and Sigma-Aldrich) (pH = 9.0) with constant agitation at 4°C followed by one 15-minute agitated rinse in distilled, deionized water [56]. The treatment group underwent four 15-minute washes in 5mL of borate buffer solution containing 0.1 mg/mL of $NaBH_4$ (Sigma-Aldrich) (pH = 9.0) with constant agitation at 4°C followed by one 15-minute agitated rinse in distilled, deionized water. HIT testing was then performed, as described in section 2.2.1, to determine the samples $t_{1/2}$.

To ensure the efficacy of the $NaBH_4$ reduction, three trial runs were performed. The trials used samples of the bovine common digital extensor tendon, known to contain predominately thermally labile crosslinks, to validate the treatment. These trials confirmed that the treatment was working as intended, with results showing stabilization of the thermally labile crosslinks. This was consistent with results from this method used in previous studies [9].

2.2.4 Statistical Analysis

Statistical analysis was conducted using JMP (Version 17.1.0, SAS Institute, USA). For the untreated samples, first, a two-way ANOVA was run to assess if there were any interactions between the data for T_d, T_{Fmax}, and $t_{1/2}$. Then, a one-way ANOVA was used to assess statistical differences between tendon types and across animal models for T_d, T_{Fmax}, and $t_{1/2}$. For T_{Fmax} and $t_{1/2}$, the data was rank transformed prior to the analysis to improve normality. A Tukey Kramer HSD test was performed on the T_d data. A Wilcoxon test between tendon types and a Kruskal-Wallis test across models, as well as, a non-parametric comparison using the Wilcoxon method were performed on the T_{Fmax} and $t_{1/2}$ data.

The data for the treated samples was rank transformed prior to the analysis to improve normality. Statistical differences between tendon types and across animal models were assessed for $t_{1/2}$ by first using one-way ANOVA, then a Wilcoxon test between tendon types and a Kruskal-Wallis test across models were performed, followed by a non-parametric comparison using the Wilcoxon method.

2.3 Results

2.3.1 Denaturation Temperature

To assess the molecular stability of the collagen molecules between the two tendon types, both within and across animal models, the T_d was evaluated. There were five samples per tendon type for each model, totalling 10 samples per animal and 30 in total.

For T_d within each model, t-test results showed that there were significant differences between the positional and energy storing tendons within all three models: bovine, ovine, and rat

(Figure 2.4, Figure 2.5, Figure 2.6, Figure 2.7). The bovine displayed a significant factor of p = 0.0025, the ovine a significant factor of p = 0.0001, and for the rat the significant factor was p = 0.0029. For the bovine and ovine, the positional tendons had a lower denaturation temperature than their energy storing tendons, while the rat displayed the opposite.

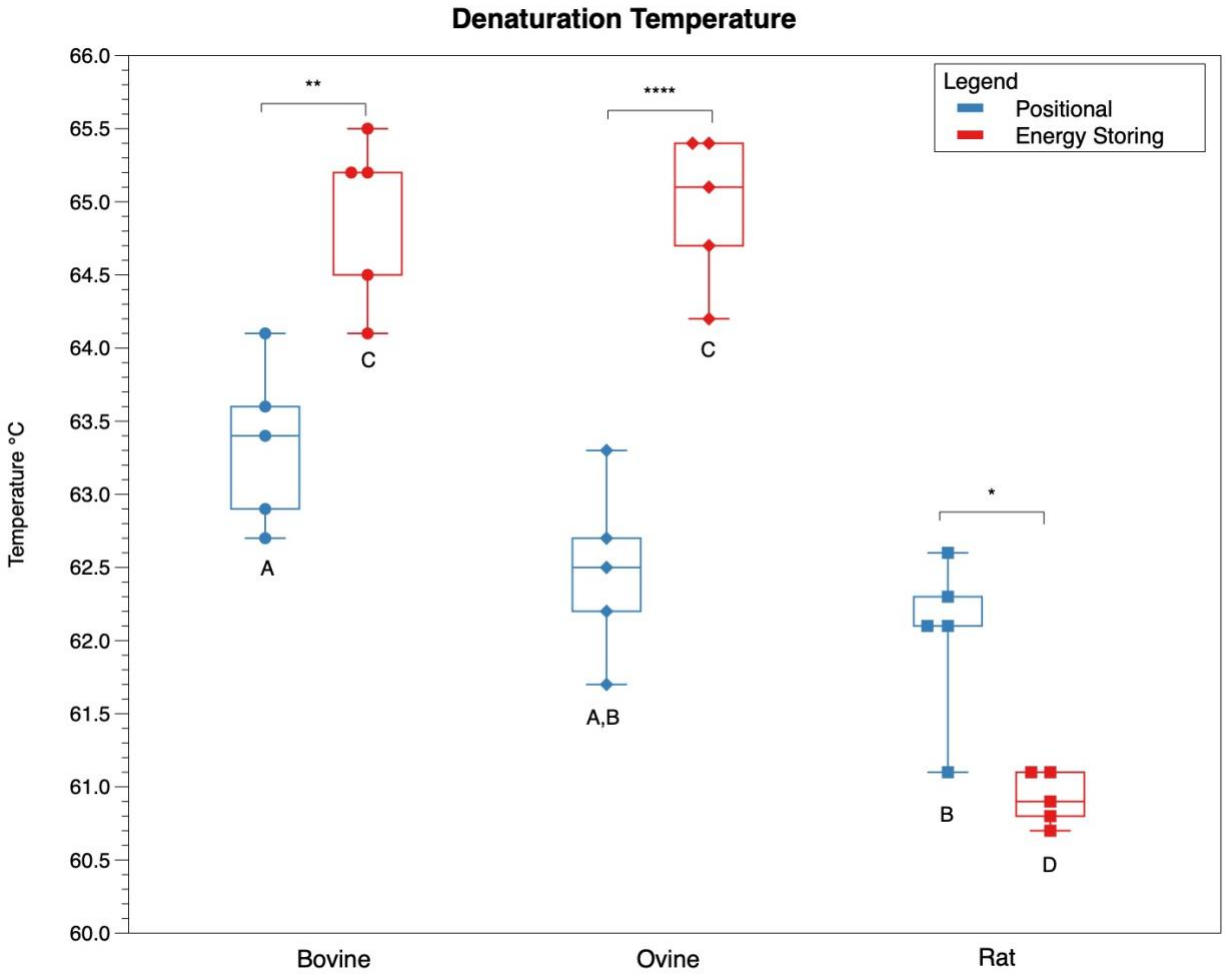

Figure 2.4: Box plots of the denaturation temperature for all 30 samples. There were significant differences found between the positional and energy storing tendons of all three species: bovine, ovine, and rat. Additionally, across species the bovine positional tendons were found to be significantly different from the rat positional tendons (p = 0.0096). The bovine and ovine energy storing tendons were found to be significantly different from the rat energy storing tendons (p = 0.0001; p = 0.0001). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Across models, ANOVA results showed that there were significant differences in the T_d of the positional tendons between the bovine and rat models (p = 0.0096) while there were no significant differences between the positional tendons of the ovine and the other two models. Additionally, there were significant differences in the T_d of the energy storing tendons between both bovine and rat models (p = 0.0001) as well as the ovine and rat models (p = 0.0001). While the bovine and ovine models displayed a similar result.

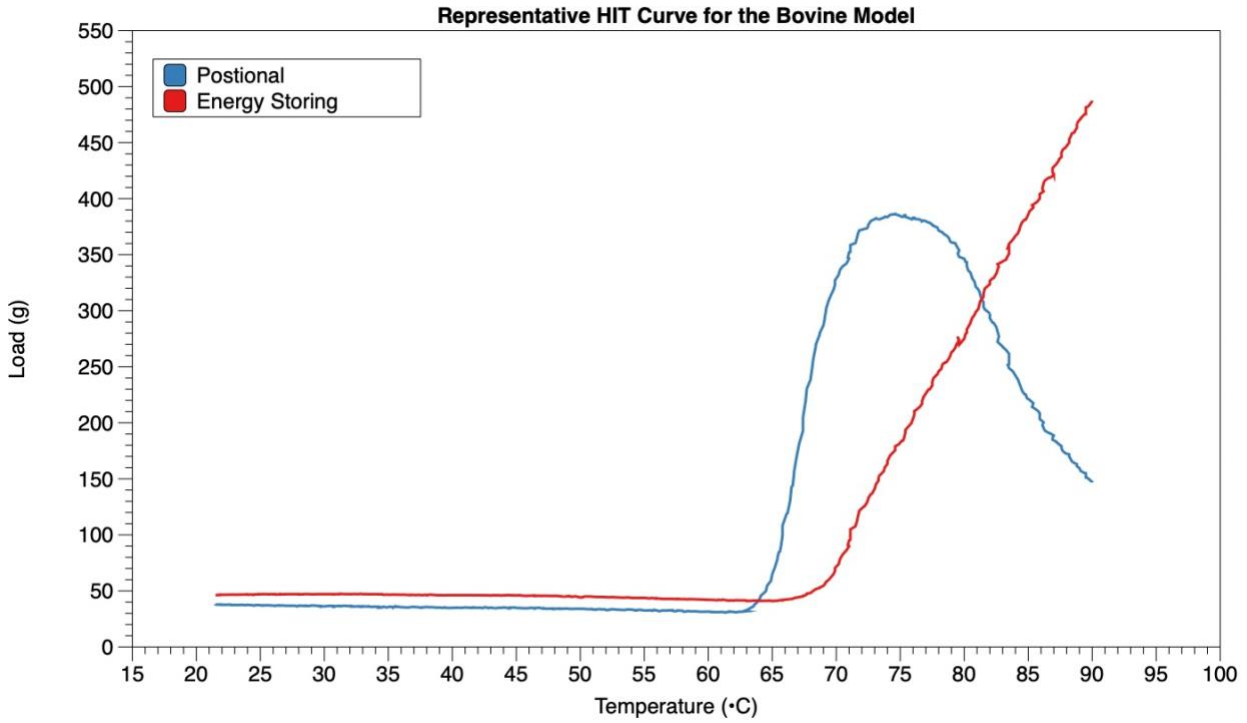

Figure 2.5: HIT Curve displaying the representative outcome for the positional and energy storing tendon pairs of the Bovine model.

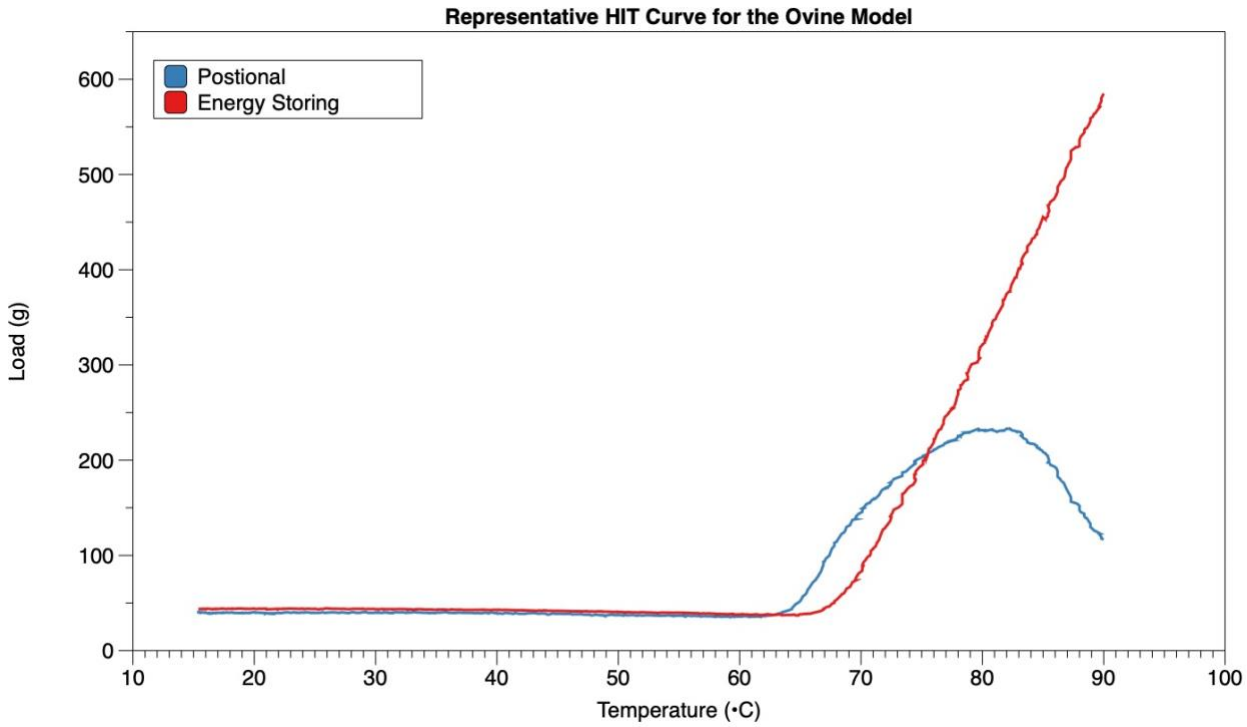

Figure 2.6: HIT Curve displaying the representative outcome for the positional and energy storing tendon pairs of the Ovine model.

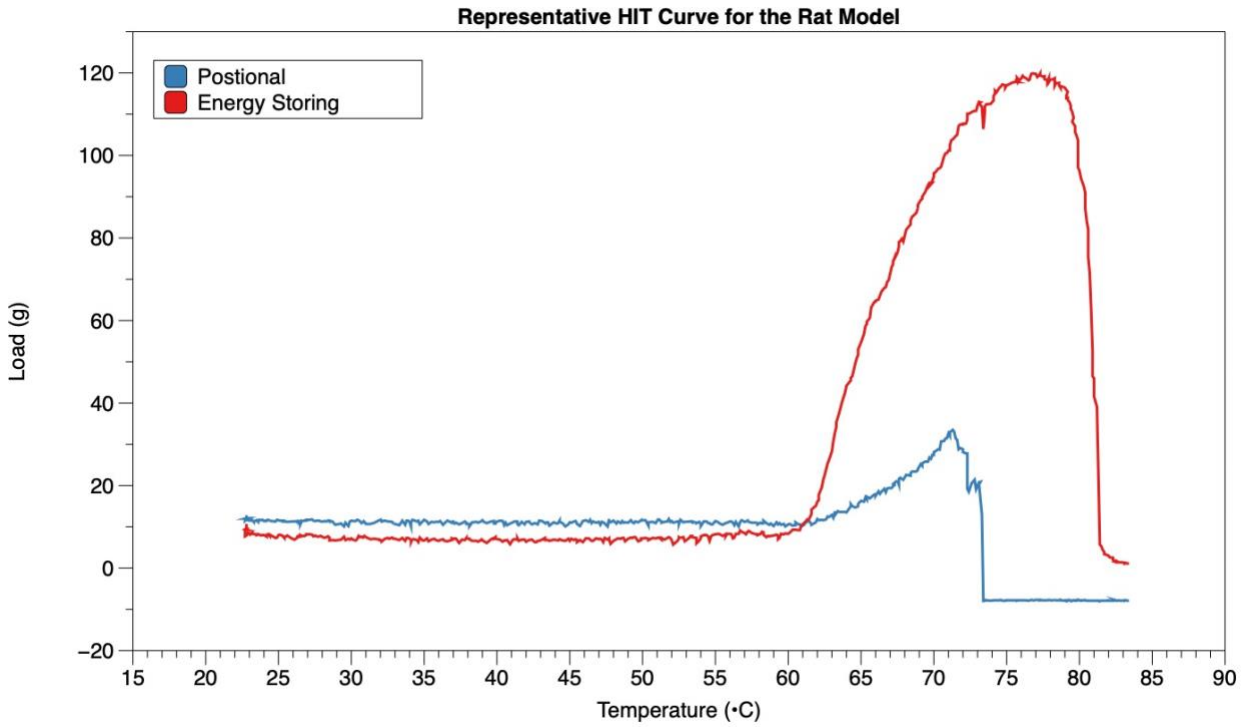

Figure 2.7: HIT Curve displaying the representative outcome for the positional and energy storing tendon pairs of the Rat model.

2.3.2 Temperature at Maximum Force

To compare the amount of thermally stable crosslinks both within and across the three animal models the T_{Fmax} was assessed. There were five samples per tendon type for each model, totalling 10 samples per animal and 30 in total.

For T_{Fmax} within each model, t-test results showed that there were significant differences found between the positional and energy storing tendons within the bovine and ovine models but there was no difference found between the two tendon types within the rat model (Figure 2.8). The bovine model displayed a significant difference of $p = 0.0367$ between the two tendon types while the ovine model displayed a significant difference of $p = 0.0216$.

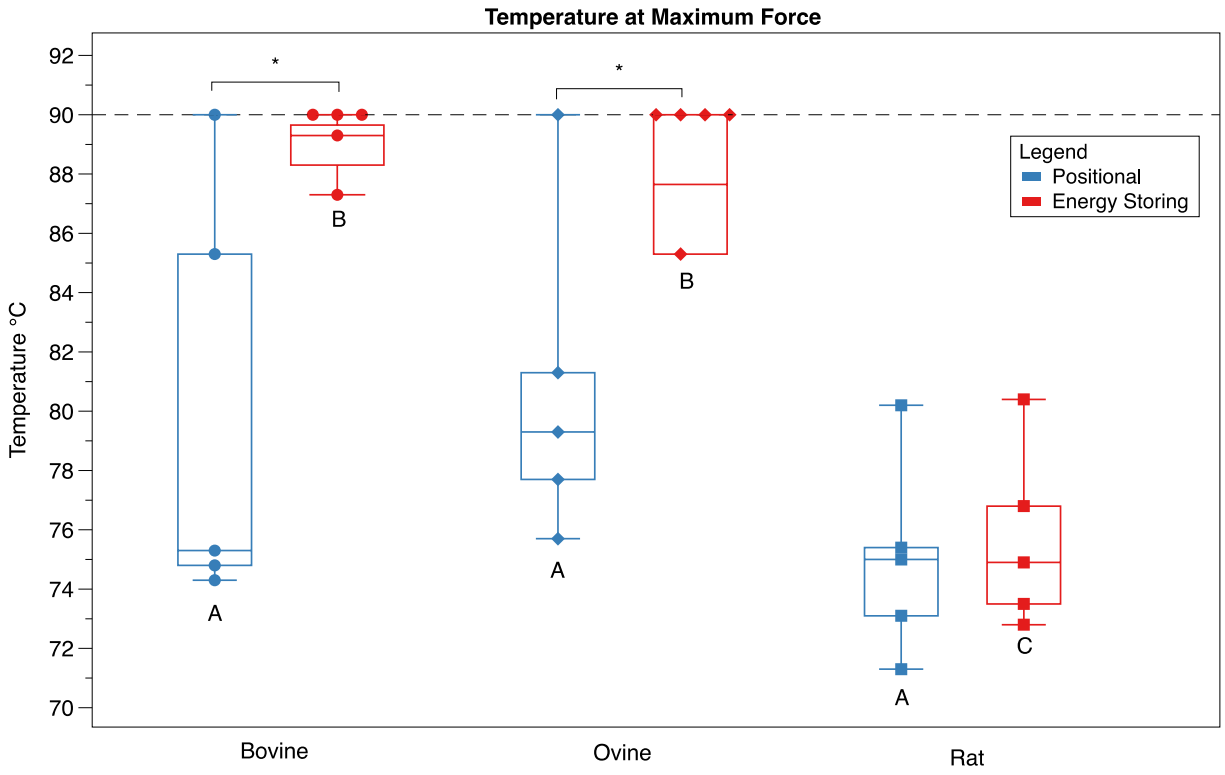

Figure 2.8: Box plots of the temperature at maximum force for all 30 samples. The dotted line at 90°C represents the end of the temperature ramp, heating did not continue past 90°C. The bovine and ovine models showed a significant difference in T_{Fmax} between their positional and energy storing tendons. Across models, both the bovine and ovine energy storing tendons displayed a significant difference in T_{Fmax} when compared to the rat model ($p = 0.0112$; $p = 0.0122$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Across models, ANOVA results for the T_{Fmax} between the positional tendons displayed that there were no significant differences between any of the three models. While the results for the T_{Fmax} between energy storing tendons showed that there were significant differences between the bovine and rat models ($p = 0.0112$) as well as the ovine and rat models ($p = 0.0122$). While there were no differences found between the T_{Fmax} of the bovine and ovine energy storing tendons.

2.3.3 Half-Time of Load Decay

To assess total overall crosslinking within the tendon's, HIT analysis was used on tendons that were treated with $NaBH_4$, the controls for this section were the untreated samples from sections 2.3.1 and 2.2.2. The same number of samples were used; 5 of each tendon type for each animal, totalling 30 samples.

For $t_{1/2}$ within each model, the control and treatment group were assessed for each tendon type; positional and energy storing. Within the bovine positional tendons there was a significant difference between the control and treatment groups (p =0.0216) but there was not a significant difference found between the two groups within the bovine energy storing tendons (Figure 2.9, Figure 2.10). For the ovine positional tendons, there was also a significant difference found between the control and treatment group (p = 0.0122) (Figure 2.9, Figure 2.11), and again this difference was not observed in the ovine energy storing tendons. Lastly, for the rat, there were significant differences found between the control and treatment groups for both the positional (p = 0.0122) and energy storing tendons (p = 0.0122) (Figure 2.9, Figure 2.12). Between the tendon types within the treatment groups, there were significant differences found between the tendon pairs in the bovine and ovine groups (p = 0.0122, p = 0.0122).

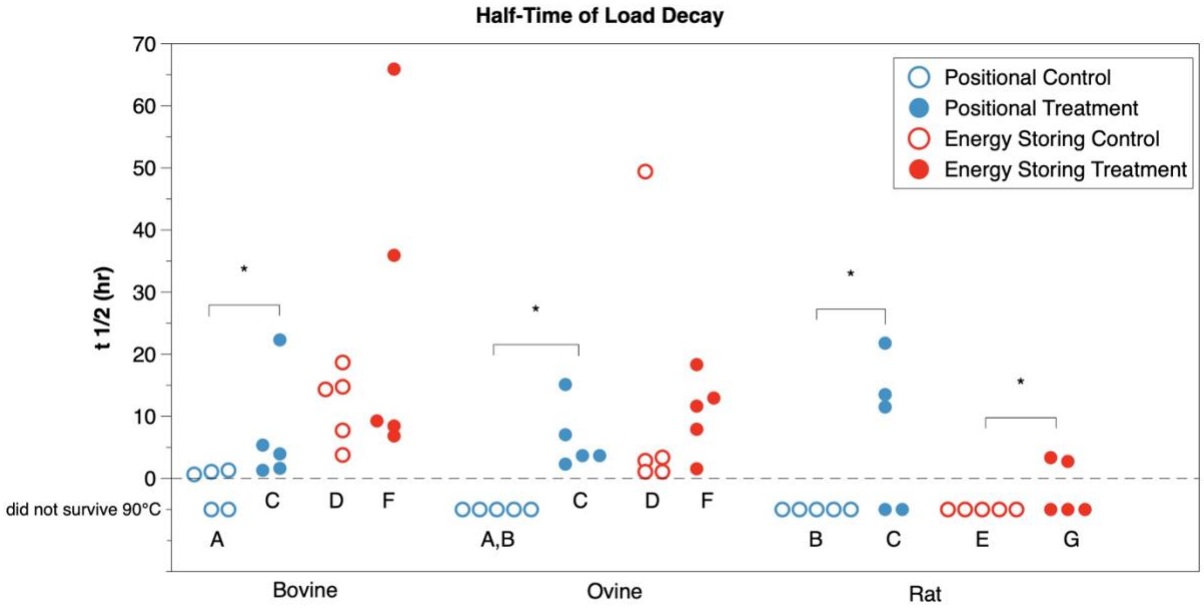

Figure 2.9: Plot of the half-time of load decay for both the control and treatment groups. All points below the dashed line at zero represent the tendons that did not make it the isothermal portion of testing, they did not survive 90°C. For all three models there was a significant difference in the $t_{1/2}$ of their positional tendons between the control and treatment group. Additionally, there was a significant difference found between the control and treatment group within the rat energy storing tendon, this difference was not found in the bovine or ovine energy storing tendons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Across models there were significant differences found within the treatment group between the energy storing tendons of both bovine and rat models ($p = 0.0122$), as well as the ovine and rat models ($p = 0.0367$). There were no significant differences found between the positional tendons within the treatment group. For the control group, there was a significant difference found between the positional tendons of the ovine and rat model ($p = 0.0112$) but there were not any other differences found between the positional tendons in the control group. Additionally, there were significant differences found between the energy storing tendons of the

bovine and rat models (p = 0.0112) as well as the ovine and rat models (p = 0.0112), in the control group.

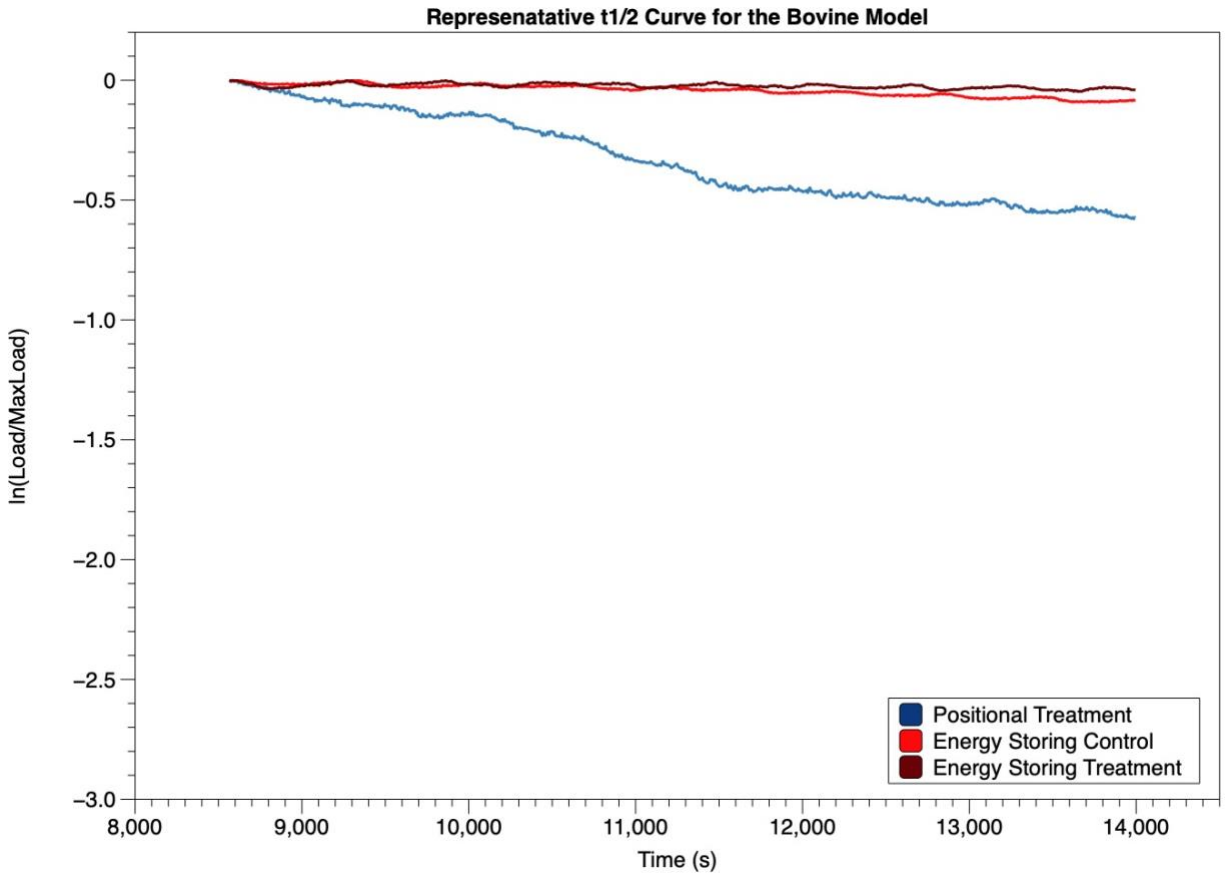

Figure 2.10: Plot of the isothermal portion of HIT testing displaying the representative outcome for the positional and energy storing tendon pairs of the Bovine model.

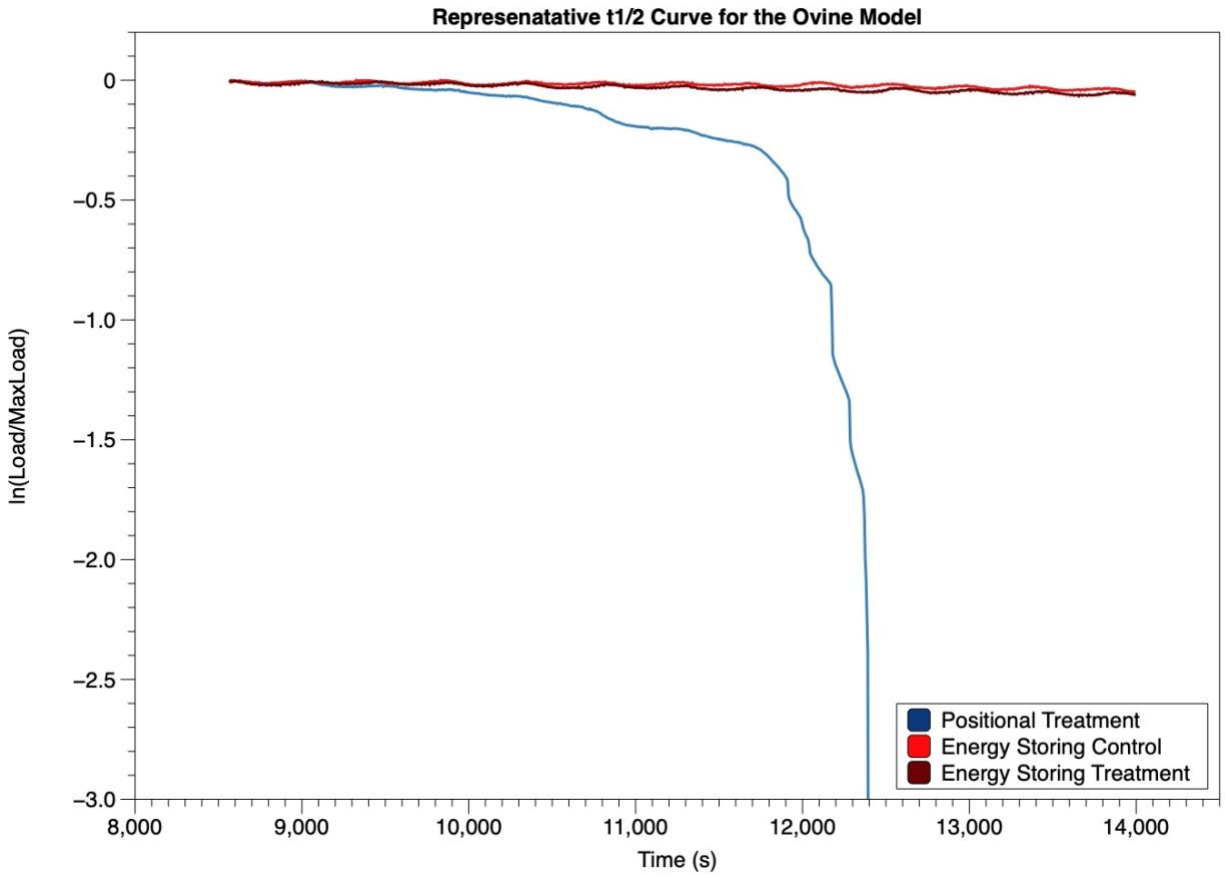

Figure 2.11: Plot of the isothermal portion of HIT testing displaying the representative outcome for the positional and energy storing tendon pairs of the Ovine model.

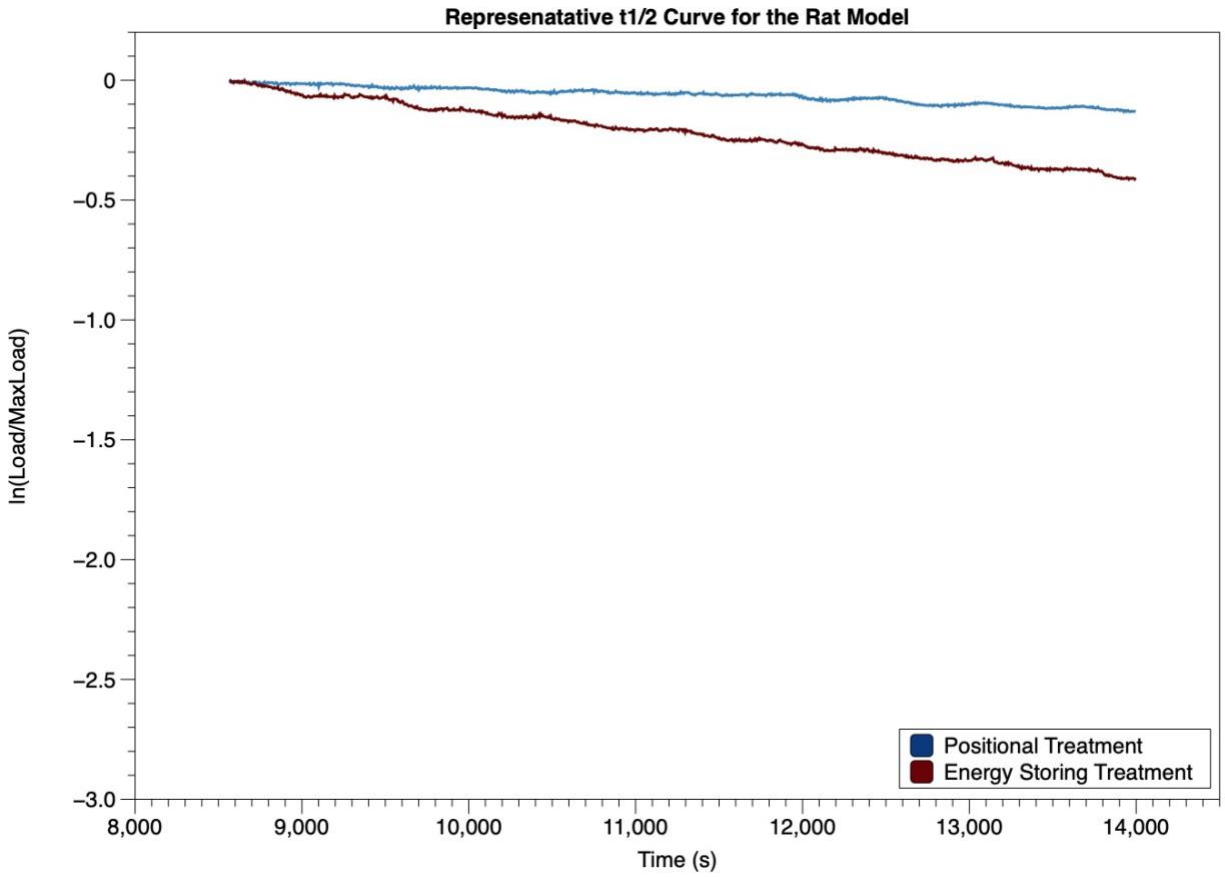

Figure 2.12: Plot of the isothermal portion of HIT testing displaying the representative outcome for the positional and energy storing tendon pairs of the Rat model.

2.4 Discussion

2.4.1 Denaturation Temperature

The HIT results for T_d indicate that there is a significant difference in the molecular stability between the positional tendons and the energy storing tendons in all three models. The positional tendons of the bovine and ovine models displayed a lower T_d than their energy storing tendons, indicating that their energy storing tendons have greater molecular stability. This result was expected as previously, the positional tendons of the bovine model have been shown to have a lower T_d than their energy storing counterparts [9]. The rat tendons displayed a contrasting result;

their positional tendons had a higher T_d than their energy storing tendons, indicating that their positional tendons are more molecularly stable.

Additionally, across models, a significant difference was observed between the positional tendons of the bovine and ovine models versus the rat model and between the energy storing tendons of the bovine model versus the rat model. These differences indicate that the bovine and ovine tendons behaved very similarly and thus have a similar level of molecular stability in their two respective tendon groups. In contrast, the rat tendons displayed a different level of molecular stability than the other two models.

It has been stated that the thermal stability of collagen molecules increases as their conformational freedom is restricted, which means that tendons that have a higher T_d likely have a tighter packing of collagen molecules [59,61]. This may indicate that the packing of collagen molecules is greater in the energy storing tendons of the bovine and ovine models and in the positional tendons of the rat model relative to their respective tendon pair. As well, across models, this may suggest that the collagen molecules within the positional tendons of the bovine and ovine models, as well as in the bovine energy storing tendon, are more tightly packed than those in the rat model.

Together, these findings prove the first hypothesis incorrect: while the ovine and bovine energy storing tendons have a higher denaturation temperature than their positional tendons, this is not true of the rat tendons. Suggesting that a higher level of molecular stability is not a characteristic of all energy storing tendons and is model-specific.

2.4.2 Temperature at Maximum Force

The HIT results for the temperature at maximum force indicate that there is a significant difference in the amount of thermally stable crosslinks between positional and energy storing tendons in both the bovine and ovine models. This difference is seen in the T_{Fmax} with a higher temperature indicating the presence of more thermally stable crosslinks when compared to those with lower T_{Fmax}. In these two groups, their energy storing tendons had a higher T_{Fmax} than their positional tendons, indicating that they were more thermally stable. While there was a difference found between the two tendon types in the bovine and ovine models, this difference was not seen in the rat model, with their positional and energy storing tendons showing a similar T_{Fmax}. This suggests that there is not much of a difference in the amount of thermally stable crosslinks between the positional and energy storing tendons in the rat model.

Across tendon types, there was no difference found between the positional tendons of any of the three models. However, between the energy storing tendons, significant differences were found between the bovine and rat models and the ovine and rat models. While the bovine and ovine energy storing tendons showed a similar result. This indicated that the bovine and ovine energy storing tendons have a similar level of thermally stable crosslinks, while it appears that the rat energy storing tendons have more thermally labile crosslinks in comparison.

This suggests that the collagen molecules in the positional tendons of the bovine and ovine models, along with both tendon types in the rat model, are joined mainly by immature aldimine crosslinks, which are thermally labile [28,70,71]. While the collagen molecules within the energy storing tendons of the bovine and ovine models are joined mainly by thermally stable crosslinks, either immature keto-amine crosslinks or mature trivalent crosslinks. The types of

crosslinks present in the bovine and ovine positional tendons are chemically distinct from their energy storing tendons; this same distinction is not observed in the rat model.

These findings prove the second hypothesis incorrect: the bovine and ovine models showed the expected result with their energy storing having a higher T_{Fmax} when compared to their positional tendons. However, the rat tendons did not show this difference. The positional and energy storing rat tendons displayed a similar T_{Fmax}. This suggests that having more thermally stable crosslinks is not a characteristic of all energy storing tendons and may only be attributed to larger mammals. Additionally, these results prove the third hypothesis incorrect: both the rat positional and energy storing tendons displayed differing results than the bovine and ovine positional and energy storing tendons.

2.4.3 Half-time of Load Decay

The results for the half-time of load decay indicate that there is a significant difference in the density of total crosslinks between the positional and energy storing tendons in all three groups. For all three models, there was a significant difference between the control and treatment groups of their positional tendons, with the treatment groups having a higher $t_{1/2}$. This, along with many of these tendons not surviving 90°C, indicates that many of the crosslinks present in the positional tendons were thermally labile. The significant difference in the $t_{1/2}$ between the control and treatment groups within the positional tendons was due to the sodium borohydride reduction, reducing the thermally labile crosslinks into thermally stable crosslinks.

Within the bovine and ovine groups, there was no difference observed between the control and treatment groups of the energy storing tendons indicating that within these two groups, there were not many thermally labile crosslinks, if any, to reduce into thermally stable

crosslinks. Therefore, within the bovine and ovine models, their energy storing tendons had a greater amount of thermally stable crosslinks than their positional counterparts. There was a significant difference in the $t_{1/2}$ between the control and treatment groups within the energy storing tendons of the rat model. This difference, much like that in the positional tendons, indicated that prior to the sodium borohydride reduction, the tendons had a higher amount of thermally labile crosslinks present, and the treatment reduced those crosslinks to be thermally stable. Previous analysis of the rat Achilles tendon showed similar results, with the tendons displaying comparable T_d and $t_{1/2}$ results [72]. These results were not compared directly to any other tendons within the study but do give confidence to the results found in the current study.

Within the treatment groups, there was a significant difference between the positional and energy storing tendons for both the bovine and ovine models. For both the bovine and ovine models their positional tendons had a lower $t_{1/2}$ than their energy storing tendons. While there was not a significant difference between the treated tendon pairs in the rat model, this model displayed the opposite with their positional tendons appearing to have a higher $t_{1/2}$. This suggests that the energy storing tendons of the bovine and ovine models have a higher relative density of total crosslinks present than their positional tendons.

Above, it has been stated that the T_d may be attributed to the packing of collagen molecules, which suggests that the energy storing tendons of the bovine and ovine model are more tightly packed than their positional tendons [59,61]. This corresponds with the $t_{1/2}$ data, where the positional tendons of the bovine and ovine models have a lower $t_{1/2}$ than their energy storing tendons, suggesting that they have a lower overall density of crosslinks present. The packing of the collagen molecules within the tendons of these models is likely, at least in part, attributed to the overall crosslinks present. In the rat tendons, their positional tendons had a higher T_d, but

both their positional and energy storing tendons displayed a similar $t_{1/2}$. This indicates that while the overall density of their crosslinks may be similar, their positional tendons have more tightly packed collagen molecules.

Additionally, it has been previously found that within the bovine and equine model, the positional tendons had fewer crosslinks and lower glycosaminoglycans content [14,33,68]. The difference in stiffness between the two tendon types, positional and energy storing, has often been attributed to these structural variations. As the density of crosslinks within a tendon increases so does the overall stiffness leading to a decrease in that tendon's susceptibility to damage [68]. The positional and energy storing rat tendons behaved very similarly when $t_{1/2}$ was assessed, suggesting that it is likely that these known differences may not be present between the two tendon types in rats and may contribute to differences in their mechanical properties when compared to other models.

These results prove the fourth and fifth hypotheses wrong: while the ovine and bovine tendons behaved as expected, the rat tendons did not. The rat energy storing tendons did not have a significantly different $t_{1/2}$ than the positional tendons indicating that they have a similar density of overall crosslinks. This is not consistent with the stated hypothesis.

2.5 Conclusion

Overall, these results show that the bovine and ovine models behave similarly when undergoing thermal mechanical testing. Given structural and functional similarities, both the bovine and ovine models appear to be suitable for investigating the differences in structure and function between positional and energy storing tendons. Additionally, these results suggest that rats may not make good models for this same comparison.

Chapter 3 – Transmission Electron Microscopy

3.1 Background

3.1.1 TEM

Transmission electron microscopes (TEM) use a beam of electrons that travels down a high vacuum tunnel via a series of lenses and is transmitted through a thin sample to create an image [73,74]. This beam provides a detailed visualization of the sample, allowing users to assess their samples at the nanoscale. TEMs can produce much higher resolution images than traditional light microscopes and scanning electron microscopes, allowing users to view their samples clearly at 2,000,000 X with 0.1 nm of resolution [75,76].

3.1.2 TEM Limitations

TEMs have some limitations that need to be considered when viewing biological samples. The resin used in the embedding process can cause noise which may lead to a decrease in the quality of the final image [74]. The thicker the resin layer the larger this noise becomes, meaning that thin samples are necessary for high quality images. As well, not only is there a possibility of limitations due to the resin used to embed the sample, but also in the fixing agents that are used. Biological samples need to be fixed before being embedded in resin and this fixing process can cause changes in the structure of the tissue [77]. For collagen-based tissues, fixing agents can affect the diameter of the collagen fibrils [77,78]. Different fixative solutions including glutaraldehyde and Spurr's resin, glutaraldehyde + osmium tetroxide and Spurr's resin, and paraformaldehyde and LR White resin, all produced samples with different fibril diameters in the human corneal buttons; on average 40 nm, 30 nm, and 55 nm respectively. The percent difference in diameter between the smallest and largest value is 58.8%, which is quite a large

difference for an accurate comparison to be made. Therefore, it is critical that all the samples that are being assessed are prepared similarly and that researchers describe their preparation methods thoroughly, so the fibril diameters can be compared. Furthermore, researchers should be aware that while it is suitable to compare samples that are similarly prepared, that the structural details may not be 100% representative of the fibrils in their native physiological, fully hydrated state.

Lastly, the preparation methods for TEM requires the tissue to undergo a dehydration step. Under physiological conditions, collagen fibrils are well hydrated, as it is essential for their functions. Therefore, removing this hydration would have some effect on the collagen fibrils themselves. It has been previously found that the length and the diameter of collagen fibrils decrease when the tendon is dehydrated [79–81]. The change in diameter has been reported in a bovine skin model to be 42.5% difference, with the diameters changing from 57 nm to 37 nm when dehydrated [79]. Additionally, within a bovine tail model, there were also 47.2% [81] and 47.4% [82] differences in diameter between hydrated and dehydrated collagen fibrils reported when assessed with atomic force microscopy (AFM) [81,82]. When dehydrated, the percent change of diameter is influenced by the initial fibril diameter; respectively, larger fibrils tend to experience a more significant change than smaller fibrils [83]. This decrease in diameter was attributed to not only the lack of hydration but also the decreases in the intermolecular spacing that occurred with dehydration [79]. The tropocollagen within the collagen fibrils form water-mediated hydrogen bonds that separate the triple helices, therefore, when the water is removed, these bonds are broken leading the tropocollagen to closely pack together ultimately causing a decrease in diameter [79]. Overall, adding in an extra consideration, as it is not as simple as just using an average percent diameter change to estimate the size of the fibrils in a hydrated physiological environment.

In combination, these limitations, need to be noted and taken into consideration when using this technique. However, despite these limitations, this method is still considered one of the main techniques for fibrils diameter analysis [84,85]. Not only is the resolution of TEMs well suited for viewing collagen fibrils and giving a clear image of the edges of the fibrils but the preparation techniques allow for cross sections of the tendon to be taken without damaging the structure of the tendon [75,76,84]. This ability allows for the area of the cut end of the collagen fibrils to be assessed giving a more accurate assessment of diameter. Overall, TEMs allow for a clearer assessment of the diameters of collagen fibrils when compared to other methods.

3.1.3 Fibril Diameter

The mechanical properties of a tendon are determined by several factors such as the types of crosslinks present and the abundance of GAGs [30,86]. While tendons are made of many different components, collagen makes up approximately 60-85% of its dry weight, and therefore, plays a large role in the overall mechanics of the tendon itself. There have been differences in collagen fibril diameters found between the superficial digital flexor tendon (SDFT) and common digital extensor tendon (CDET). The diameter of collagen fibrils is generally smaller in the SDFT than in the CDET, when studied in a bovine model using a scanning electron microscope (SEM) the average diameters were found to be 80 ± 7 nm and 134 ± 5 nm, respectively [9]. However, the mean fibril diameters of the same tendon pairs, under AFM were found to be 144 ± 16 nm and 247 ± 29 nm, respectively, showing the same trend [11]. Not only do these functionally distinct tendons have different fibril diameters, but the diversity of fibril size also varied between the two tendon types as well. Under SEM, the positional tendons (CDET) typically displayed a mixture

of small (70 nm) and large (160 nm) collagen fibrils, while the energy storing tendons were composed of mostly small fibrils (35–75 nm) [9].

It has been suggested that the differences in the diameter of collagen fibrils, as well as the distribution of diameters, between these two tendon types may cause differences in the tensile strength of the tissue itself with the average diameter of the collagen fibrils and the tensile strength being shown to have a positive correlation suggesting that an increase in the size of the fibril diameter may come with an increase in the intrafibrillar covalent crosslinks, and therefore, increasing the tensile strength of the tendon [30,79,87]. Fibrils with smaller diameters are thought to be able to withstand creep loading more efficiently due to the increase in surface area [30,79]. This increase in surface area increases the probability of interfibrillar non-covalent crosslinks being present. These non-covalent bonds are responsible for holding the collagen structure together and are often created by hydrophobic amino acid residues, typically occurring during fibrillogenesis [88]. Given the differences in fibril diameters previously found within each tendon type, the suspected influence that small or large fibril diameters have on how the tendons perform correlates with the overall functions of the two tendon types.

This chapter aims to assess the fibril diameters of positional and energy storing tendons in bovine, ovine, and rat models. Understanding the similarities and differences in the fibril diameter between the two tendon types may help us to better understand their mechanical properties, both within each individual model and how they compare against models.

3.1.4 Hypotheses and Rational

The objective of this section was to assess the fibril diameters within the positional and energy storing tendons of bovine, ovine, and rat models.

Hypothesis 1: Fibril diameter within the energy storing tendons, SDFT and Achilles, will be much smaller than those within the positional tendons, CDET and tibialis anterior. This will reflect the results of previous studies that have shown energy-storing tendons having a smaller average diameter than positional tendons [11,89].

Hypothesis 2: Fibril diameters within positional tendons will show a bimodal distribution, having two distinct peaks, while the energy storing tendons will display a unimodal distribution [30]. Positional tendons often display a wider variety of fibril diameters than positional tendons and that is expected to be reflected in these results [11].

Hypothesis 3: The fibril diameters across models will be similar for each given tendon type. The average size and distribution of fibrils will be consistent across models for both the positional and energy storing tendons suggesting that these features are universal across tendon types.

3.2 Methods

3.2.1 Sample Preparation

Samples were obtained and stored according to the same protocols in Chapter 2 (2.2.1). For each model, bovine, ovine, and rat 10 samples were used, 5 of each tendon type, totalling 30 samples. Each tendon pair was obtained from a different animal, 5 different animals were used for each model. Once samples were ready to be used, they were taken out of the -86°C freezer and left to

thaw at room temperature. The samples were then cut into 8mm x 4mm long segments and placed into a 12-well plate containing PBS. The samples were then rinsed in PBS for ten minutes on a shaker table. This was repeated three times. Then a 2.5% glutaraldehyde solution was made using 10 mL of 10% glutaraldehyde (Electron Microscopy Sciences), 15 mL of 2.0M sodium cacodylate buffer (Electron Microscopy Sciences), and 15 mL of ddH_2O. The samples were then placed into a 10 mL falcon tube containing 6 mL of the 2.5% glutaraldehyde solution. These falcon tubes were then placed on a shaker table and left for 1-hour, they were then placed in the fridge and left overnight.

The next day the samples were taken to the Electron Microscope Core Facility at Dalhousie University, where the rest of the preparation took place. There the glutaraldehyde fixed samples were first rinsed in in 0.1 M sodium cacodylate buffer for 10 minutes, this was repeated three times. The samples then underwent secondary fixation in 1% osmium tetroxide (Alfa Division) for two hours and then rinsed in ddH2O. They were then placed in 0.25% uranyl acetate (Fisher Scientific Company) at 4°C overnight. After that they were dehydrated in a graduated series of acetone: 50%, 70%, 95%, and 100%. They were then infiltrated with Epon Araldite Resin (Electron Microscopy Sciences), first with a 3:1 ratio of acetone (Fisher Scientific Company) to resin for 3 hours, then a 1:3 ratio or acetone to resin overnight, and lastly in 100% resin two times for 3 hours each. The samples were then embedded in the Epon Araldite Resin and placed in a 60°C oven overnight. Thin sections were then cut using a Reichert – Jung Ultracut E Ultramicrotome with a diamond knife, approximately 100 nm thick, and placed on 300 mesh copper grids. The grids were then stained with 2% aqueous uranyl acetate for 10 minutes, rinsed twice with ddH_2O, and then stained with lead citrate (TAAB Laboratories). They were then rinsed one final time with ddH_2O and left to air dry. The samples were then viewed

using a JEOL JEM 1230 Transmission Electron Microscope at 80kV and images captured using a Hamamatsu ORCA-HR digital camera.

3.2.2 Diameter Assessment

Images were captured at 40,000 X magnification. For each tendon, four grid meshes were assessed with all fibrils within the field of view being analyzed. The fibril diameters were evaluated using Image J (Verison 1.53, NIH, USA). In Image J, the largest possible circle that did not extend outside the fibrils visible perimeter was fit to each fibril and the area of the circle recorded (Figure 3.1), from there the diameters were found in Microsoft Excel using the equation: $D = \left(\sqrt{\frac{A}{\pi}}\right)2$ (Version 16.76, Microsoft, USA).

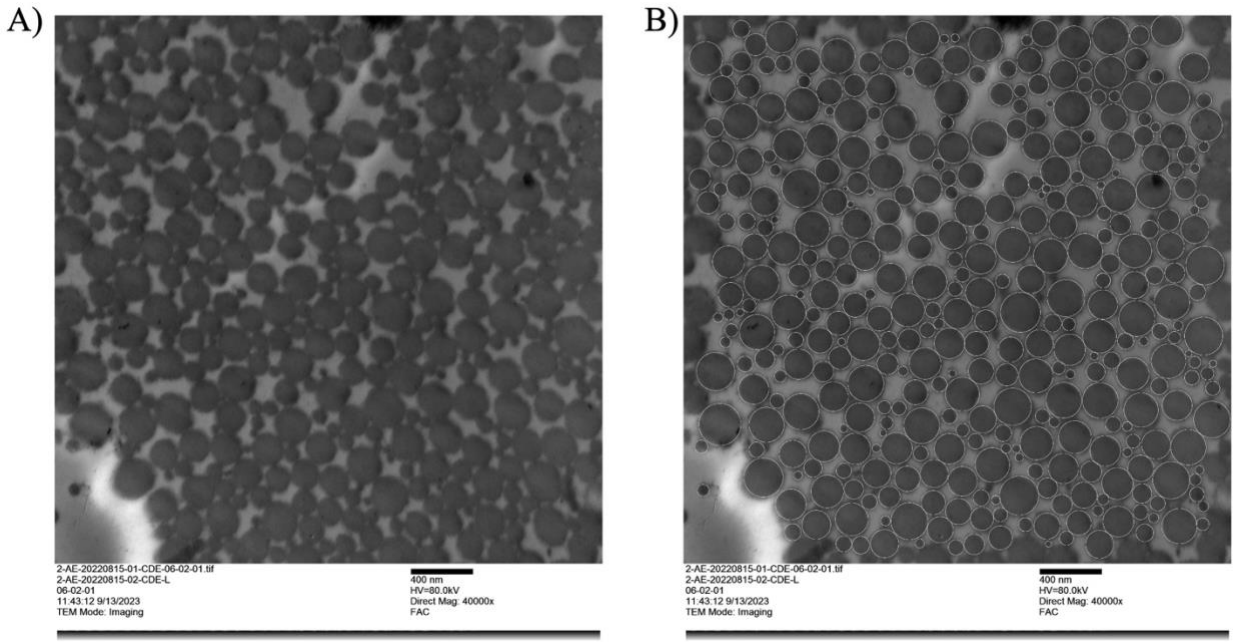

Figure 3.1: Example of TEM fibril diameter assessment in Image J. (A) Field of view of the tendon prior to fibril assessment (B) Field of view of the tendon after fibril assessment. A circle was fit to the outmost edges of each fibril and the area of the fibril was recorded. Diameter was derived from the area.

The overall sample size was quite large for this method of analysis. The number of fibrils that were assessed per tendon ranged from 192 to 959. Grouped together as model and tendon type the number of fibrils per group ranged from 5500 to 11824, totaling 50,556 fibril diameters assessed (Figure 3.1).

3.2.3 Statistical Analysis

Statistical analysis for mean fibril diameter was conducted using JMP (Version 17.1.0, SAS Institute, USA). First, the data were rank transformed, and a two-way ANOVA was run to assess any possible statistical differences within the data. Next, a one-way ANOVA was used to assess statistical differences between tendon types and across animal models for fibril diameter. This was performed by running the analysis on the data for the tendon type but separating it by animal and then doing the opposite. For this test, the data was not ranked. However, a Wilcoxon test between tendon types and a Kruskal-Wallis test across models were performed on the data, along with a nonparametric comparison for each pair using the Wilcoxon method.

Statistical analysis for modality and comparison of modes was conducted using R (Version 2023.06.2+561). First the modality of the data were assessed. The data were separated by animal and tendon types and a dip test was performed on each of these data sets to assess if the distribution was different from a typical unimodal distribution. Then LaPlace's Demon package was used to assess if the distributions were similar to a bimodal distribution. Once modality was determined, the modes of each data set were found by creating a histogram and using a locate modes function.

Then a two-way ANOVA was run on the modes to assess if there were any significance between any of the groups. A one-way ANOVA was then used to determine where those

significant interactions were occurring, both between tendon types and across models. A Tukey Kramer HSD test was also performed on the modes of each group. Lastly, the full width at half the maximum was also assessed in R (Version 2023.06.2+561). This was found by determining the maximum y-value of the distribution, taking half of that value, and then finding the lowest and highest x-values corresponding with this half maximum y-value. The lowest x-value is then subtracted from the highest x-value giving the full width at half the maximum.

3.3 Results

3.3.1 Mean Fibril Diameter

Within each model, ANOVA results showed that there were significant differences between the positional and energy storing tendons within all three models (Figure 3.2, Table 3.1). The positional tendons displayed a greater mean fibril diameter then the energy storing tendons, in all three models.

Between models, there were significant differences found between the positional tendons of the bovine and ovine models (p = <0.0001) and the ovine and rat models (p = <0.0001). With the ovine model having a greater mean fibril diameter than the other two models. Additionally, there was a significant difference found between the energy storing tendons of all three models. The significant factor between the bovine and ovine was p = <0.0001, between the bovine and rat was p = 0.0003, and between the ovine and rat was p = <0.0001. The ovine energy storing tendon had a greater mean fibril diameter than the other two models. While the difference between the bovine and rat energy storing tendons is attributed to the data not being normally distributed and having to be rank transformed for statistical analysis, rather than a larger differences in their mean fibril diameters.

Table 3.1: Mean Fibril Diameter

Animal	Positional	Energy Storing	*p*-value
Bovine	132 nm ± 60 [n = 7818]	77 nm ± 34 [n = 9988]	$p < 0.0001$
Ovine	138 nm ± 54 [n = 5500]	117 nm ± 49 [n = 7136]	$p < 0.0001$
Rat	126 nm ± 44 [n = 8290]	78 nm ± 38 [n = 11824]	$p < 0.0001$

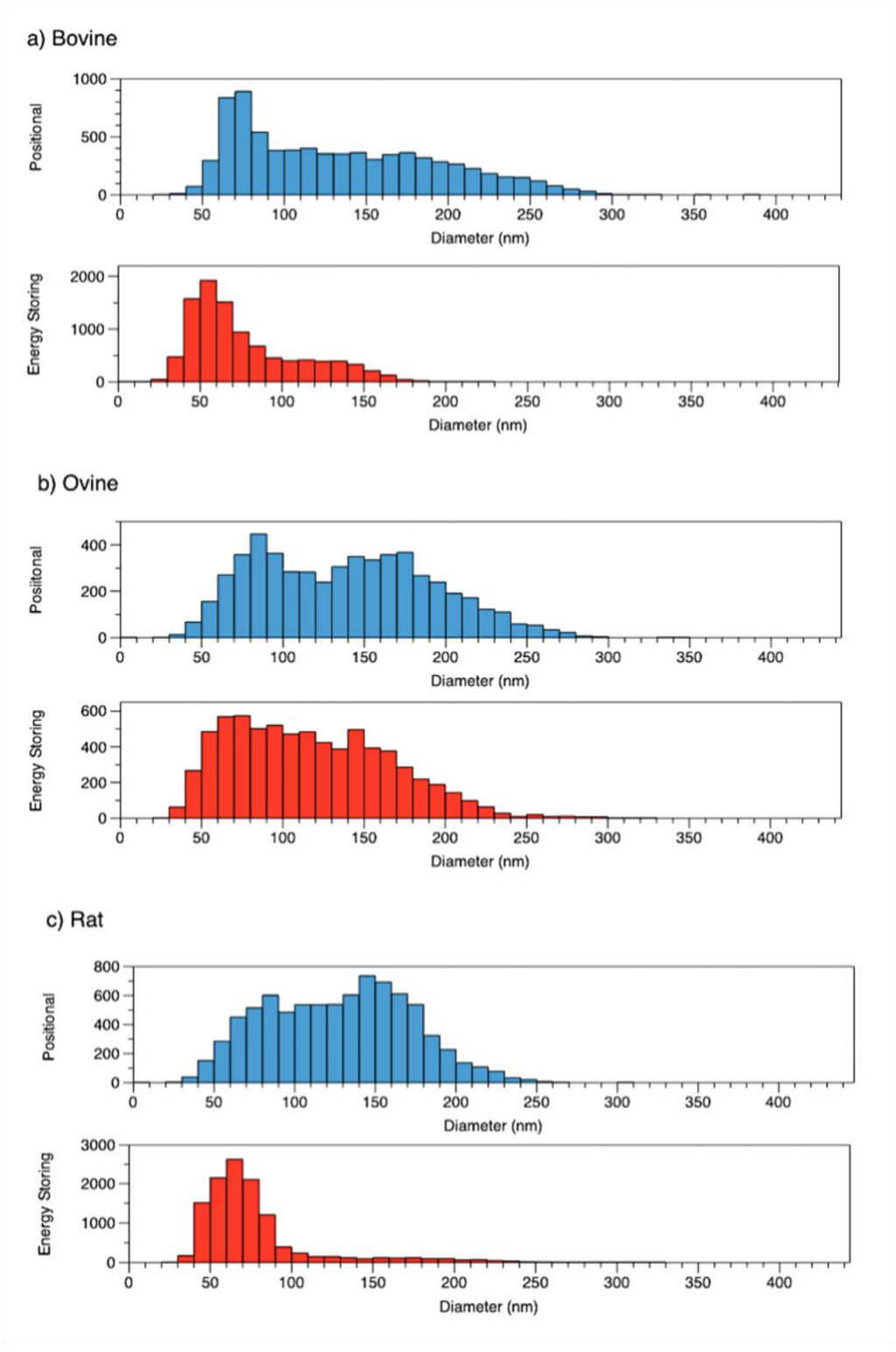

Figure 3.2: Ridge plot of the fibril diameters of the positional (blue) and energy storing (red) tendons for the (a) bovine, (b) ovine, and (c) rat models.

3.3.2. Modality

Both tendon types in the bovine and ovine models, along with the rat positional tendons, displayed a bimodal distribution (Figure 3.3). While the rat energy storing tendon displayed a unimodal distribution. Between models, there were no significant differences found in any of the three models. Across models, the positional tendons of all three pairs had peaks around 70 – 85 nm and around 150 – 170 nm (Table 3.2). These were not considered significantly different from each other. For the energy storing tendons, the bovine and ovine tendons had peaks around 55 – 66 nm and 126 – 145 nm. The rat energy storing tendons had a peak at 67 nm. There were no significant differences found between the energy storing tendons.

The density of the number of fibrils at each peak was different across models (Figure 3.3). The bovine and ovine positional tendons, as well as the bovine and rat positional tendons, showed a difference with the bovine tendons having a noticeably larger peak in smaller fibril diameters. While the ovine and rat positional tendons displayed a similar density of small and larger fibrils present. In the energy storing tendons the ovine model displayed a different density of fibrils than the bovine and rat models. The bovine and rat models had a noticeably larger peak in the smaller fibril diameters, whereas the ovine energy storing tendons had a more similar density of fibrils at its two peaks.

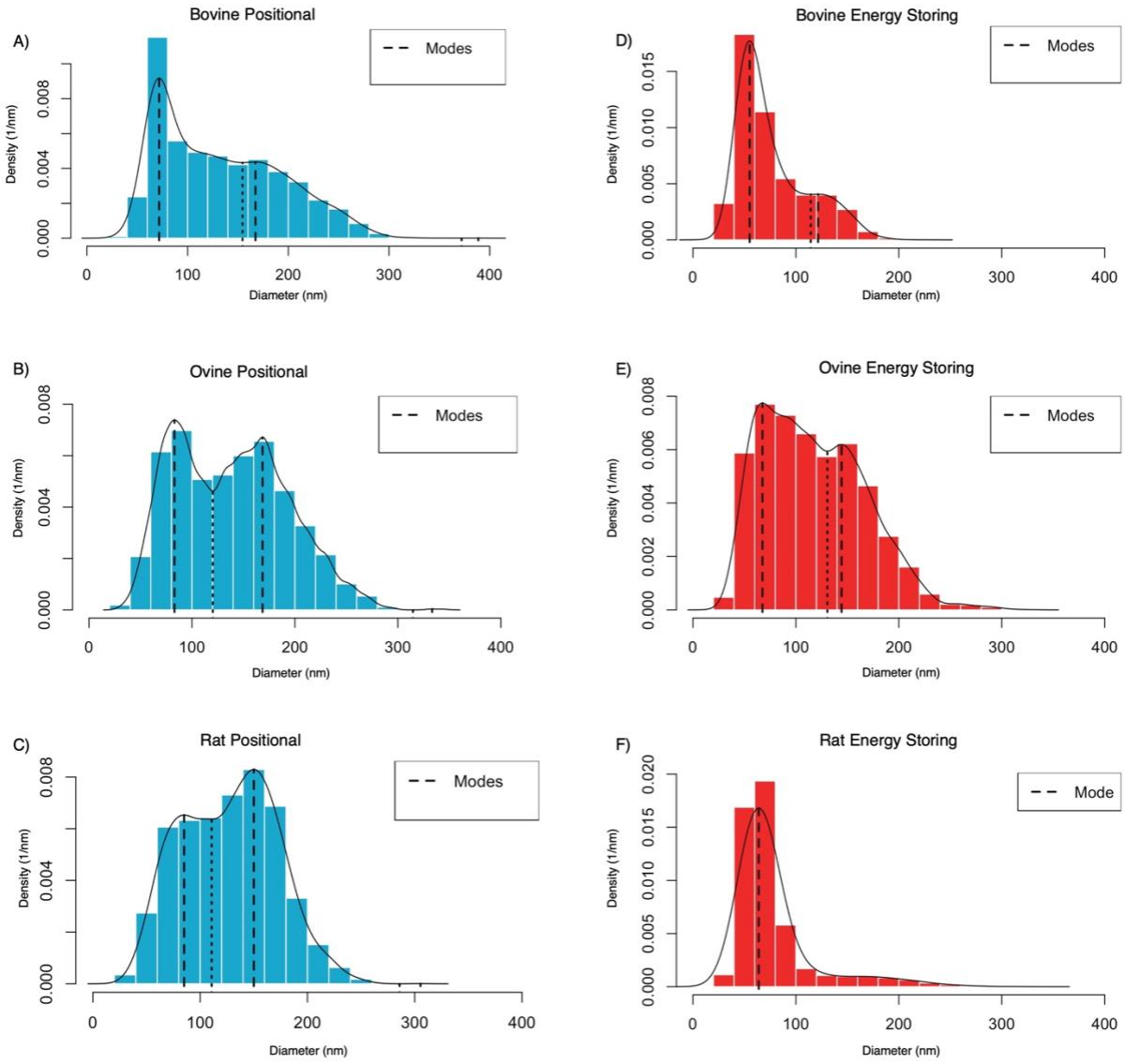

Figure 3.3: Density plots of TEM Diameters. Dotted lines indicate where the modes are located. (A) Bovine Positional (B) Ovine Positional (C) Rat Positional (D) Bovine Energy Storing (E) Ovine Energy Storing (F) Rat Energy Storing

Table 3.2: Modes of the Fibril Diameter Data

	Positional		Energy Storing	
Bovine	70 nm	170 nm	55 nm	126 nm
Ovine	84 nm	165 nm	66 nm	145 nm
Rat	84 nm	150 nm	67 nm	N/A

3.3.3. Full Width at Half the Maximum

Between the positional and energy storing tendons of the rat model there was a difference in the full width at half the maximum (FWHM), with the positional tendons having a larger FWHM than the energy storing tendons (Table 3.3, Figure 3.4). Across models, there were differences in the positional tendons of the bovine and ovine models and the bovine and rat models, with the bovine positional tendons having a lower FWHM. For the energy storing tendons, there was a difference between the ovine and bovine models and then ovine and rat models. The ovine energy storing tendons had a higher FWHM than the other two models.

Table 3.3: Full Width at Half Maximum

Animal	Positional	Energy Storing
Bovine	51	35
Ovine	149	132
Rat	127	38

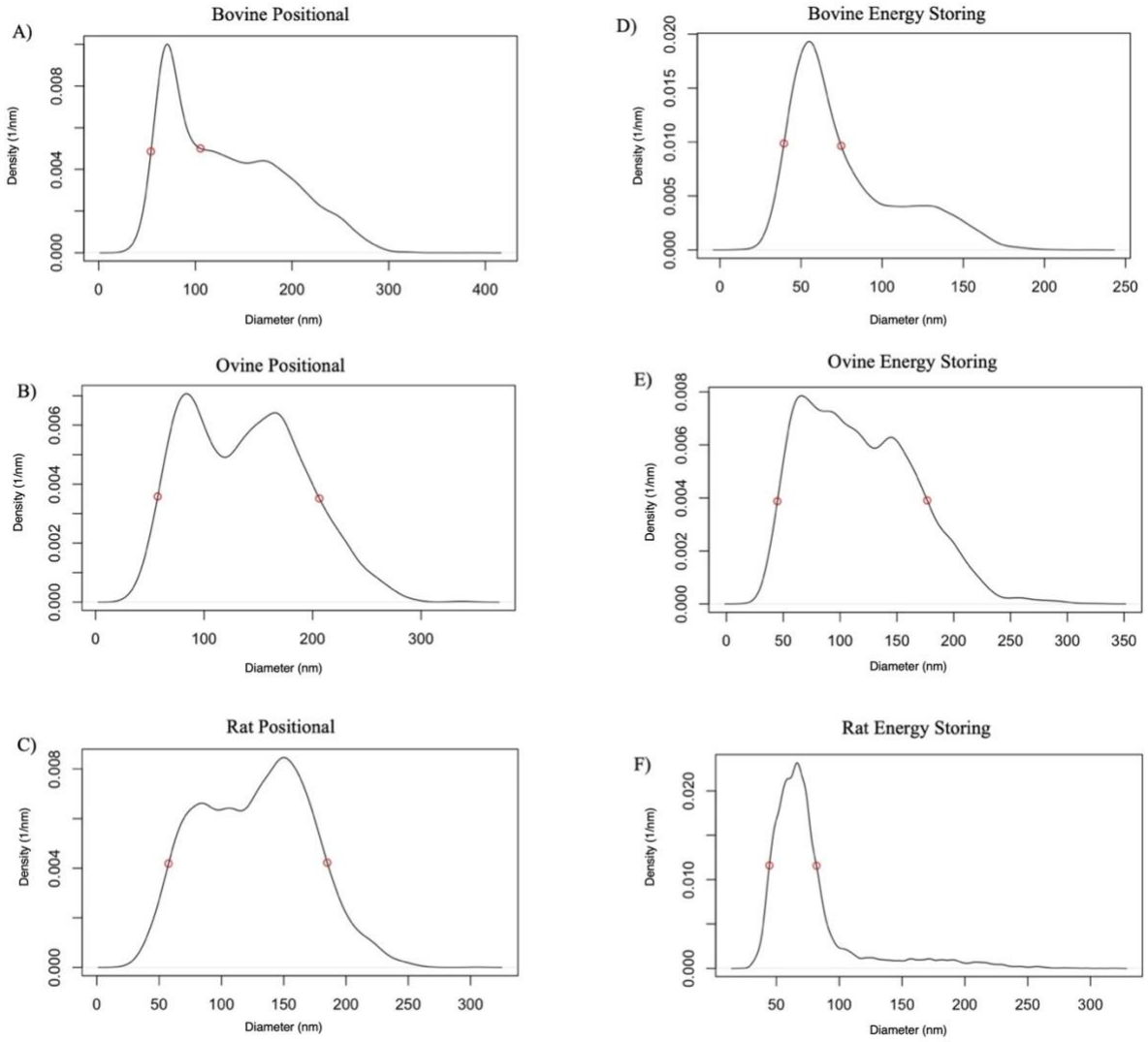

Figure 3.4: Density plots of TEM Diameters showing full width at half maximum. The red dots display x-min and x-max. (A) Bovine Positional (B) Ovine Positional (C) Rat Positional (D) Bovine Energy Storing (E) Ovine Energy Storing (F) Rat Energy Storing

3.4 Discussion

3.4.1 Mean Fibril Diameter

The results for the mean fibril diameter indicate that there were significant differences between both between the tendon types within each model and across models. Within each model there were significant differences between the tendon types in all three models. This indicates that mean fibril diameter is different for each given tendon type. Mirroring what has been found in the bovine model, previously, Herod et al.[9] found that the mean fibril diameter in the positional tendons was 134 ± 5 nm and in the energy storing tendons was 80 ± 7 nm. The bovine and rat models reflected this previous finding more closely, as their mean fibril diameters were not significantly different from these values, for each given tendon type. This close comparison is quite interesting, as Herod et al.[9] utilized a different method; using SEM and taking the diameter from the width of the fibril. The consistency between the two findings indicates that not only are the two methods possibly interchangeable but also gives added confidence in the findings themselves. Another study, using bovine positional and energy storing tendon pairs, found an overall similar result. Quigley et al.[11], using AFM, found mean fibril diameters of 247 ± 29 nm and 144 ± 16 nm, for the positional and energy storing respectively. While this is not quite as similar to the present results, the overall trend is the same, with the positional tendons having a larger mean fibril diameter than the energy storing tendons.

Other models, such as an equine model, displayed similar results to the previous bovine models: results using TEM showed the positional tendons having a larger mean fibril diameter than the energy storing; 229 ± 36 nm and 169 ± 19 nm respectively [89]. Contrastingly, an ovine model displayed differing results with the energy storing tendons having a larger mean fibril diameter than the positional tendons, 240 ± 6 nm and 187 ± 16 nm, respectively [14]. However,

these tendons were taken from the hindlimb rather than the forelimb. Overall, the results from the present study reflect the general trend of previous findings but did not reflect the findings as closely as Herod et al.[9] Regardless, the relationship between the fibril diameters of the differing tendon types being repeatedly shown indicates that the difference in mean fibril diameter may be a contributing factor to their overall distinct functions.

Across models, the results suggest that there were significant differences found between the positional tendons of the ovine and rat model, as well as the ovine and bovine model. The ovine displayed a greater mean fibril diameter than the other two models, suggesting that the ovine model has a greater distribution of larger fibrils. Additionally, the results show that there were significant differences found between the energy storing tendons between all three models, indicating that they do not have similar distributions of fibrils diameters. Comparing these results to the previously studied models, it is clear that there are differences across models. The previous models all displayed greater mean fibril diameters for both their positional and energy storing tendons than the models within this study. While the chosen method may be a contributing factor, there are still clear differences between models.

This may suggest that tendon types within different animal models may have differing capabilities. Previously, fibril diameter has been positively correlated within the tensile strength of the tendon, where an increase in fibril diameter led to an increase in tensile strength [30]. Therefore, the difference in mean fibril diameters for each given tendon type between models may suggest that the tendons will behave differently within each model, despite showing a similar trend when compared to each other within the models.

Overall, these results suggest that the compositional differences in fibril diameter between tendon types is universal across these three models and potentially may be similar

within large and small animal models. The standard deviation for both tendon types in all three models is larger than what was found by Herod et al.[9], indicating a large range in fibril diameters within each of these models. This is likely attributed to the large sample size.

These results do not disprove the first hypothesis: within all three models the positional tendons did display a greater mean fibril diameter than the energy storing tendons, within all three models. However, the results do disprove the third hypothesis: across models the mean fibril diameters were different for each given tendon type.

3.4.2 Modality

The results for modality show that both tendon types within the bovine and ovine models, and the rat positional tendons have a bimodal distribution. This indicates that these groups have a mixture of small and large fibrils present. However, the bovine positional and energy storing tendons had a greater density of smaller diameter fibrils than larger diameter fibrils, despite being bimodal. Whereas the ovine positional and energy storing tendons, along with the rat positional tendons, had a more similar density of small and large diameter fibrils. The rat energy storing tendons had a unimodal distribution, indicating that these tendons were mostly comprised of smaller diameter fibrils with only a few larger fibrils mixed in. Previous work in rat tendons, particularly rat tail tendon, showed that in the adult tissue that the collagen fibril diameters displayed a bimodal distribution, displaying a similar distribution as the tendons within this study having diameters ranging from 35 nm- 200 nm, with exception of the rat energy storing tendons[90].

Both between the tendon types within each model and across models, there was not a significant difference found between the modes. Overall, the peak for the smaller diameter fibrils

was around 70 – 84 nm for positional tendons and 55 – 67 nm for energy storing tendons. The peaks for the larger diameter fibrils ranged from 150 – 170 nm for positional tendons and 126 – 145 nm for energy storing tendons. Despite not being significantly different, the positional tendons still displayed a greater density of fibril diameters at both peaks than the energy storing tendons. This coincides with what was previously found by Herod et al.[9], where bovine positional tendons had a mixture of small and larger fibril diameters, the small peak was around 70 nm, and the larger peak was around 160 nm. They also found that the bovine energy storing tendons had a larger number of small diameter fibrils ranging from 35 – 75 nm, with only a few larger diameter fibrils present.

Overall, these results indicate that the peak fibril diameters are similar for both tendon types, within and across models. This suggests that the range and density of fibril diameter size is universal and does not differ between small and large animal models. While the rat energy storing tendon does show a slightly contrasting result, for the most part it is similar to the other tendons and models. As tendons mature their fibril diameters increase and with that so does the distribution of the fibril diameters [91]. This is important to note as the increase in diameter and distribution is coincided with an increase in mechanical strength, while this change in fibril size is not the only parameter that contributes to this increase in strength, it is still a factor. The lack of larger diameter fibrils within the rat energy storing tendons suggests that they may not display the same properties as the energy storing tendons within the other models. Additionally, the overall distributions of the fibril diameters may need to be considered when comparing tendons. The bovine tendon pairs showed a different distribution of small and large fibrils than the ovine pairs and the rat positional, this difference in distribution may indicate that they could, to some extent, display differing mechanical properties.

These results prove the second hypothesis incorrect: only the rat energy storing tendons displayed a unimodal distribution. However, all the positional tendons had a bimodal distribution and did have a wider range of fibril diameters than the energy storing tendons, which is in line with the expected result and reflects that of previous studies.

3.4.3 Full Width at Half the Maximum

The results for full width at half the maximum (FWHM) show that within the bovine and ovine model the spread of the fibril diameters is quite similar between tendon types. The rat model was the only one to show a difference between each tendon type, with the positional tendons having a larger FWHM. This indicates that for the rat model, their positional tendons had a vaster distribution of fibril diameters, this coincides with what was found in *3.4.2 Modality*. The rat model was the only one to show a difference in modality between the two tendon types, again indicating that the spread of the data were different.

Across models, there were noticeable differences between the bovine and ovine and bovine and rat positional tendons. The bovine model had a smaller FWHM than the other two groups of positional tendons, indicating that the bovine models did not have as large of a distribution of fibrils diameters. Within the energy storing tendons there were noticeable differences between the ovine and bovine models and the ovine and rat model. The ovine energy storing tendons had a greater FWHM than the other two models, indicating that the ovine model had a greater distribution in fibril diameter. This mirrors what was found in *3.4.2 Modality*, as the ovine model showed a more even density of small and large fibril diameter in both tendon types, where the other two models did not. This may suggest that the ovine tendons may be more

equipped to withstand creep loading while also providing good tensile strength, while the other two models may have tendons that are more specialized.

Additionally, FWHM has been shown to be a parameter that could possibly be used to measure collagen packing, with a smaller FWHM suggesting that the fibrils are more densely packed comparatively [92,93]. The bovine positional tendons had a smaller FWHM than the other positional tendons while the ovine energy storing displayed a larger FWHM than the other energy storing tendons. This may suggest that the bovine positional tendons were more densely packed than the other models and that the ovine energy storing were the more loosely packed compared to the other energy storing tendons. Again, this may not indicate that there are large differences within the properties of these tendons but does give some insight to how they may be functionally different.

3.5 Conclusion

Overall, these results suggest that the differences found in fibril diameter between tendon types is not different across models. This suggests that the differences observed between tendon types may be universal within mammals. While for the most part these findings were quite similar between tendon types, across models there were some differences found in mean fibril diameter and modality. These differences between the positional and energy storing tendons were more pronounced within the rat model. Again, indicating that the structure function relationship within the rat model may be different compared to those present within large animal models.

Additionally, the limitation of the fixatives used needs to be noted as the fibril diameters found in this study may not reflect those in living tissue. However, due to the same methods

being used on all samples, the overall outcome of the above results should still hold true in a living biological environment.

Chapter 4 – Scanning Electron Microscopy

4.1 Background

4.1.1 SEM

Scanning electron microscopes (SEM) produce an image by scanning the surface of a sample with a focused beam of electrons [74,75]. This allows the user to view their specimen at the nanoscale with a three-dimensional appearance. SEMs can produce usable images of samples at 200,000x with 1 nm resolution [75]. Additionally, this technique requires the sample to either be conductive itself or to have a conductive coating to prevent the pooling of electrons on top of the sample, which would distort the final image [75]. A coating of either metal, such as gold/palladium or carbon, is typically used to create this conductive surface. While this technique does allow for high resolution images on a small scale, it only captures the surface of the sample. This leads to limitations due to the microscope itself, depending on the sample and the expected outcome.

4.1.2 Ill-Defined Filamentous Webbing Structure

While assessing bovine CDETs and SDFTs Herod et al.[9] discovered an ill-defined filamentous webbing structure that was only notably found on the energy storing tendons (SDFTs). With the known specialized characteristics of these tendons, Herod et al.[9] hypothesized that this ill-defined structure may contribute to their specialized mechanics. This structure appeared to laterally connect the collagen fibrils, potentially bundling the fibrils together, allowing them to act as one unit. Giving the fibrils the opportunity to have some added strength, as acting as one "cable" rather than many would increase their resistance to fibril buckling.

4.1.3 Hypotheses and Rational

The objective of this section was to assess the presence of the filamentous webbing structure within the positional and energy storing tendons of bovine, ovine, and rat models.

Hypothesis 1: The energy storing tendons, SDFT (bovine and ovine) and Achilles tendon (rat), will have a greater abundancy of the filamentous webbing structure than the positional tendons, CDET (bovine and ovine) and tibialis anterior tendon (rat). This will reflect the results found by Herod et al.[9] that suggests that this feature is likely utilized in the specialized functions of energy storing tendons.

4.2 Methods

4.2.1 Sample Preparation

Samples were obtained and stored according to the same protocols in Chapter 2 (2.2.1). For each model, bovine, ovine, and rat 10 samples were used, 5 of each tendon type, totalling 30 samples. Once samples were ready to be used, they were taken out of the -86°C freezer and left to thaw at room temperature. The samples were then cut into 8mm x 4mm long segments and placed into a 12-well plate containing PBS. In a fume hood, a 2.5% glutaraldehyde solution was made using 10mL of 10% glutaraldehyde 30 mL of ddH2O. The samples were then placed into a new well containing 5mL of the 2.5% glutaraldehyde solution. The 12-well plate was then placed on a shaker table and left for 2-hours. After the 2-hours, the samples were transferred to a new well containing ddH2O and left on the shaker table for 15 minutes, this step was then repeated. The samples were then placed into 30% EtOH solution for 15 minutes and subsequently placed into a 70% ETOH solution for another 15 minutes. The samples were then stored in 70% EtOH overnight.

The next day, the samples were longitudinally bisected using a razor blade. These sample halves were then placed into a series of EtOH solution to dehydrate the samples (90%, 95%, and 100%) all for 15-minute wash cycles. The samples were then place into fresh 100% EtOH and taken to the Electron Microscope Core (EMC) Facility at Dalhousie University, where the samples were critical point dried on a Leica EM CPD 300. Once the samples are critically point dried, they are received back from the EMC Facility and then mounted on SEM stubs using carbon tape. The mounted samples are then sputter coated on the Leica EM ACE200 for 60 seconds on defuse, using a gold/palladium (80/20) coating.

4.2.2 SEM

The samples are then viewed on the Zeiss Scanning Electron Microscope, in the Electron Microscopy Core at Dalhousie University. Each sample is first viewed at the lowest magnification, and a series of photos are taken so that a complete picture of the whole tendon can be captured (Figure 4.1A). From these whole tendon photos, a viewing map is created. These maps were used to select 10-16 viewing sites on each tendon, two sites on each collagen fibre (Figure 4.1B).

The sites were chosen by assessing the total length of the tendon by placing the first site close to the end of the tendon and then placing the next site on the fibril $\frac{1}{3} - \frac{1}{2}$ of the length of the tendon away from the first site. Sites were staggered on subsequent fibres to ensure that the were not in lateral alignment with one another.

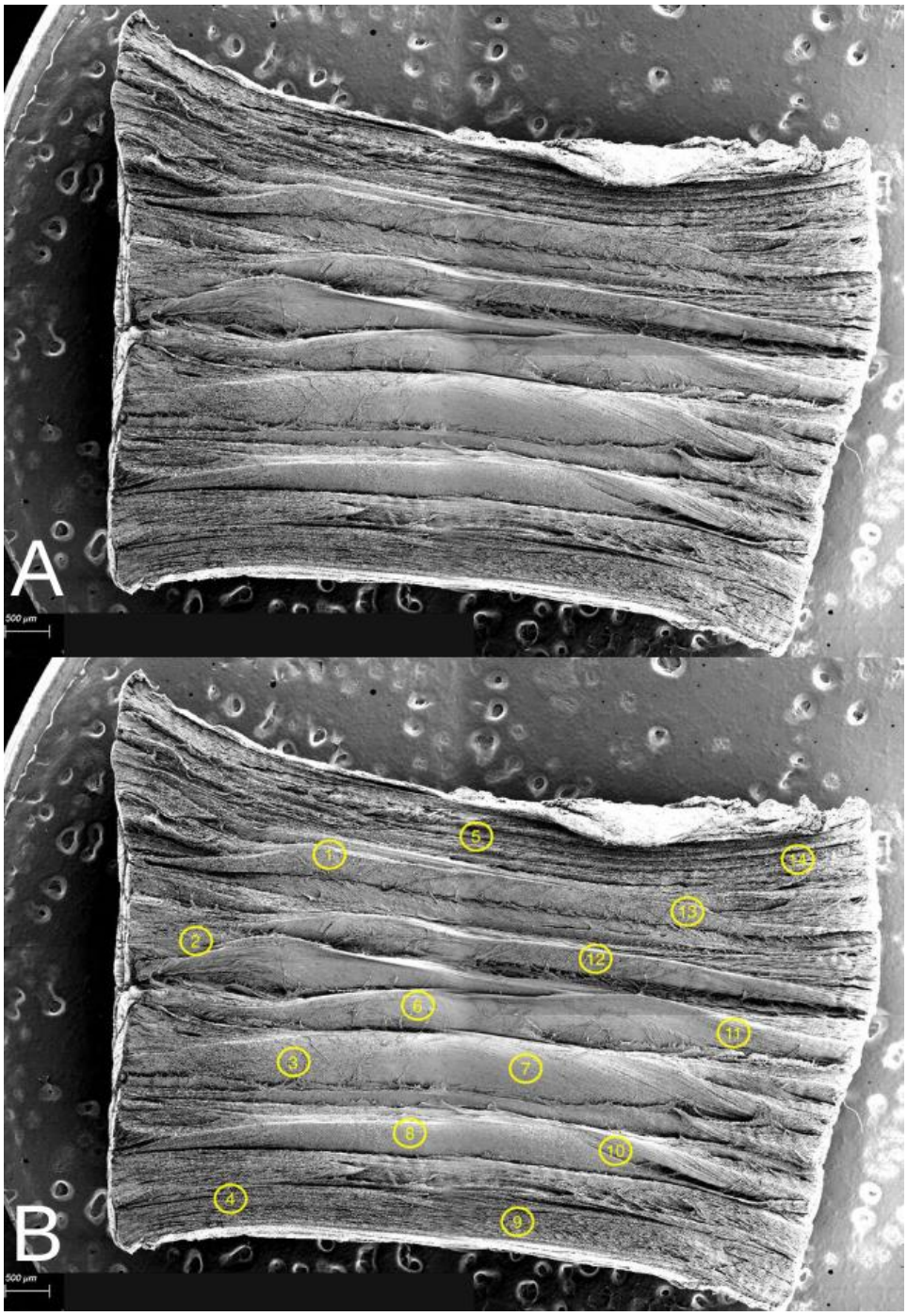

Figure 4.1: Example of the site maps created for each tendon. (A) Whole tendon photo created on Photoshop (Version 24.1.1) using 6 separate photos of the tendon, taken at 21X magnification on a scanning electron microscope. (B) Tendon site map created by choosing two sites on each fascicle.

After the sites were chosen, the samples were then viewed under the SEM again. At each site 3 – 5 areas of each site were assessed with three images being captured at each area: one at 15,000 X, one at 25,000X, and the last at 40,000 X magnification. These images were then used for the visual and grid assessments.

4.2.3 Visual Assessment

Each site was placed into one of four categories based on the abundance of webbing within the images taken at the site. These categories are abundance of webbing, partial webbing, some webbing, and no webbing (Figure 4.2, Figure 4.3). This assessment was done by eye and categorization was chosen based on the webbing present relative to all of the other images. For each site three fields of view were assessed, and one category was chosen based on those images.

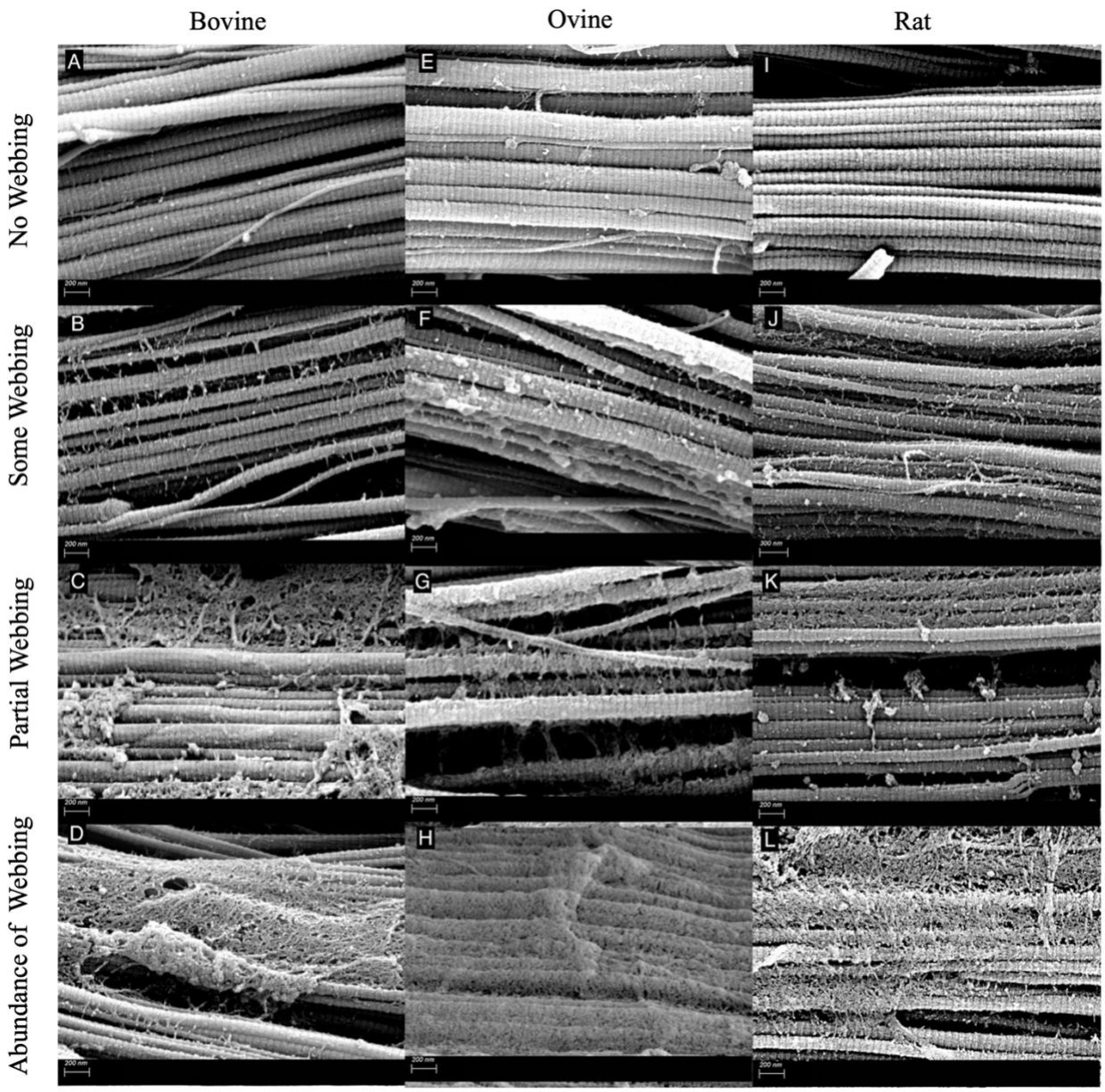

Figure 4.2: Representative images the visual assessment categories for Bovine (A-D), Ovine (E-H), and Rat (I-L) positional tendons taken on a Scanning Electron Microscope at 40,000X magnification. Images are representative of the four categories the tendon sites were placed into no webbing, some webbing, partial Webbing, and abundance of webbing respectively.

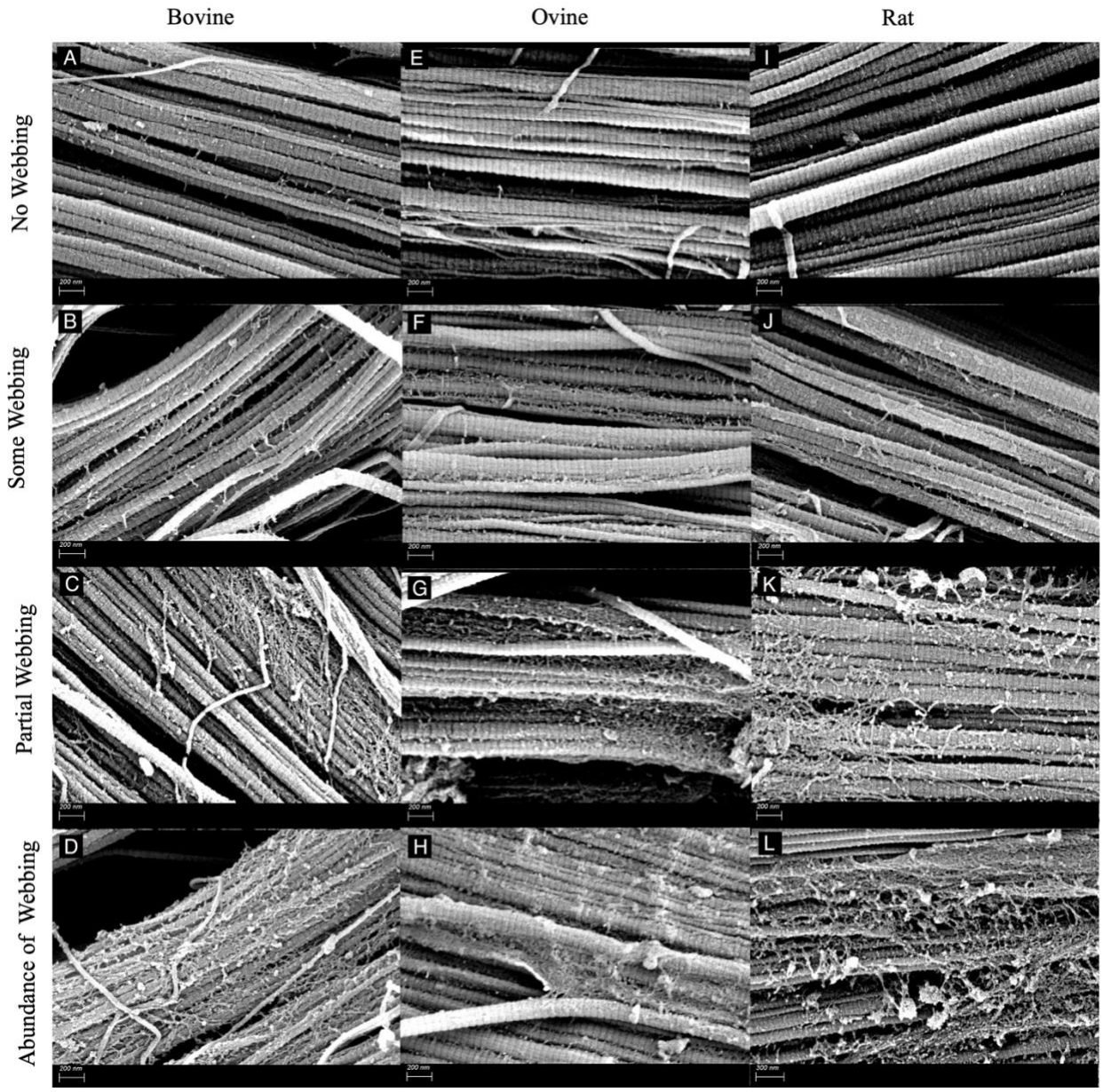

Figure 4.3: Representative images the visual assessment categories for Bovine (A-D), Ovine (E-H), and Rat (I-L) energy storing tendons taken on a Scanning Electron Microscope at 40,000X magnification. Images are representative of the four categories the tendon sites were placed into no webbing, some webbing, partial Webbing, and abundance of webbing respectively.

4.2.4 Grid Assessment

Using Image J, a grid was placed over the SEM images (Figure 4.4). The area of each grid square was 25,000 nm^2, with a grid area of approximately 17 grid squares by 11 grid squares, totalling ~ 187 grid squares per field of view. Each grid square was assessed to see if any of the webbing was found in that square, if the webbing covered 50% or more of the grid square than it was considered to have webbing present. If not, then it was ruled that there was no webbing present in that square. This binary assessment aims to result in a percentage of the field of view covered in webbing. The size of the grid went through validation by starting off with a grid area of 10,000 nm^2 and then increasing the grid area until there was a significant change to the percentage of webbing present *Appendix I – Grid Validation*.

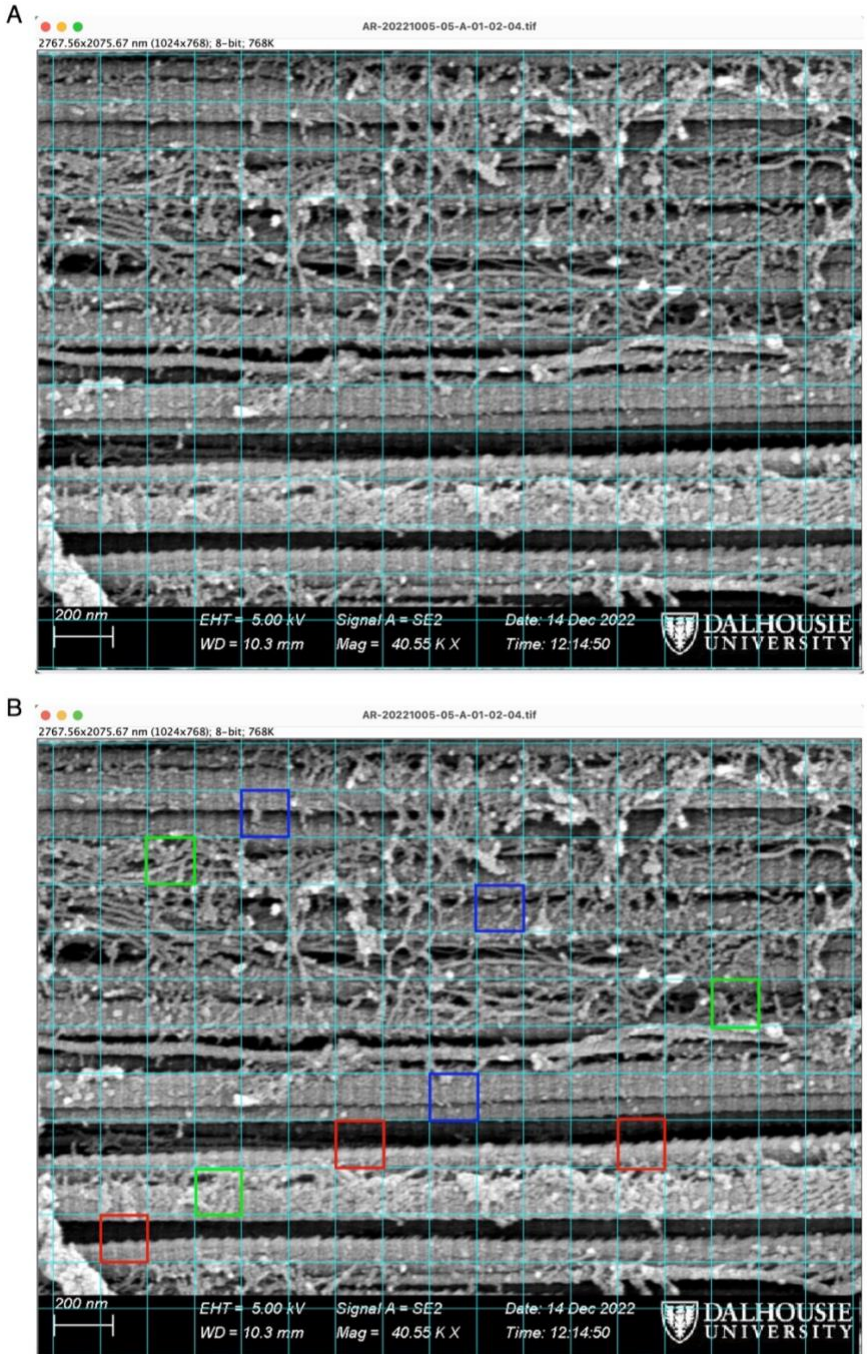

Figure 4.4: (A) Example of grid overlay for SEM grid assessment. Each grid square is 25,000 nm^2. (B) the red outlines represent a sample of the squares that would not have been included in the assessment as there was not enough tendon in the squares, the blue outlines represent a sample of the squares that would have been considered to not have any webbing in them, and the green outlines represent a sample of those that would be considered to have webbing present.

4.2.5 Statistical Analysis

Statistical analysis was conducted using JMP (Version 17.1.0, SAS Institute, USA). For the visual assessment a contingency table was used, and a Fisher's Exact test was run to assess if there were statistical differences within the table. Then, the table was broken down into each webbing category and separated so there were only two animal models in each table. Fisher's Exact tests were run to assess differences within these specific groupings.

For the grid assessment, using JMP (Version 17.1.0, SAS Institute, USA), a two-way ANOVA was run to determine if there were any differences in the percent coverage of webbing between tendon types and across models. Then a one-way ANOVA was run to see where those differences were found. The one-way ANOVA was run first to determine if there were differences between the two tendon types in all three models and then run to assess if there were differences in the tendon types across models. Additionally, a Tukey Kramer HSD test was performed on the data.

4.3 Results

4.3.1 Visual Assessment

Between tendon types there were significant differences found between the positional and energy storing tendons of bovine and ovine models (Figure 4.5). Both models displayed a significant difference in the amount of webbing between their two tendon types in the "abundance of webbing" (bovine, $p = < 0.0001$; ovine $p = < 0.0001$) and "no webbing" categories (bovine, $p =$

0.0006; ovine, $p = 0.0012$). Within the rat model there was a significant difference between the positional and energy storing tendons within the "partial webbing" category ($p = 0.0012$).

Across models, there were significant differences found between the bovine and rat positional tendons in two of the categories; abundance of webbing and partial webbing ($p = < 0.0001$, $p = 0.0489$) (Table 4.1, Figure 4.6). Between the bovine and rat energy storing tendons, there was a significant difference found within the "no webbing" category ($p = 0.0434$) (Table 4.2). As well, there were also significant differences found between the ovine and rat models. Between their positional tendons there was a significant difference found in one of the categories; abundance of webbing ($p = <0.0001$).

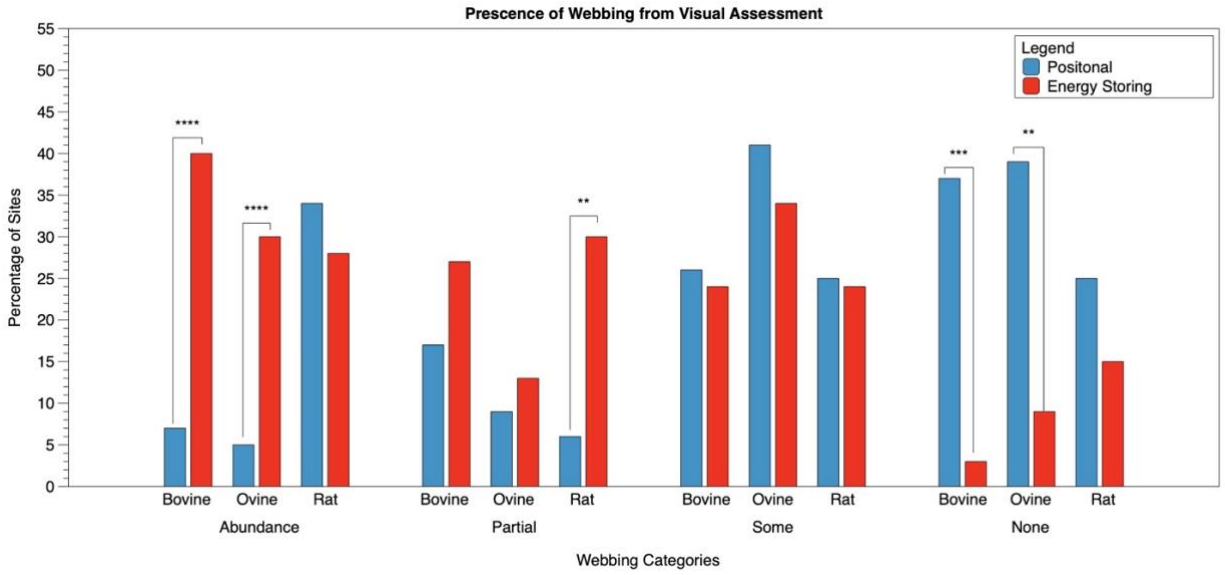

Figure 4.5: Bar graph displaying the results from the visual webbing assessment. Each graph shows the percentage of sites in each of the categories for both tendon types by animal model Bovine, Ovine, and Rat. There were significant differences found between the bovine tendons in the abundance of webbing and no webbing categories. Between the ovine tendons in the abundance of webbing and no webbing categories. Between the rat tendons in the partial webbing category. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

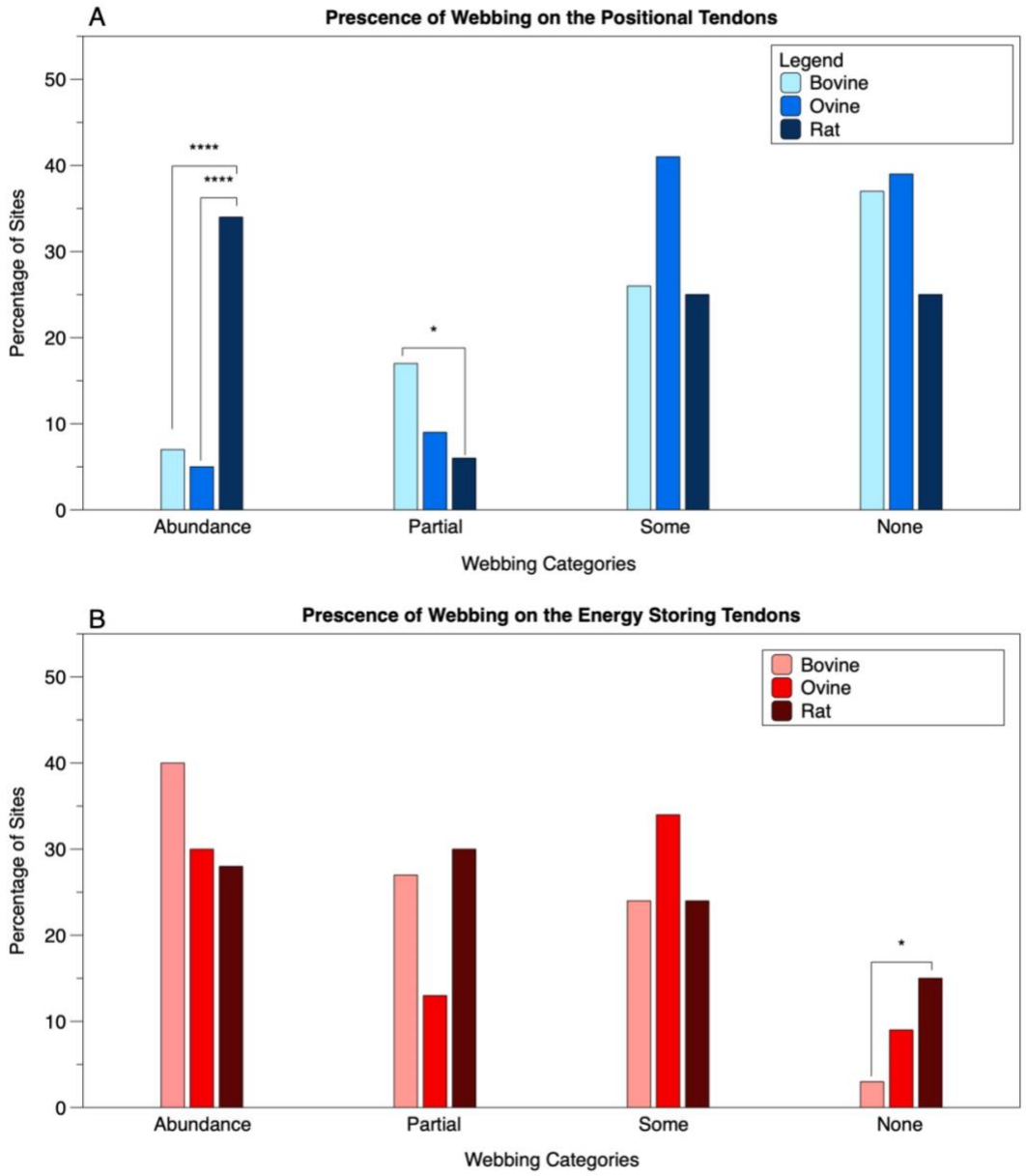

Figure 4.6: Bar graphs displaying the results from the visual webbing assessment. Each graph shows the percentage of sites in each of the categories for all three models separated by tendon type (A) Positional and (B) Energy Storing. For the positional tendons (A), there were significant differences found between the bovine and rat in the abundance of webbing and the partial webbing groups. As well, there was a difference between the ovine and rat tendons in the abundance of webbing group. For the energy storing tendons (B), there was a significant difference between the bovine and rat in the no webbing group.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Table 4.1: Presence of webbing on the positional tendons of all three animal models

Animal	Abundance	Partial	Some	None
Bovine n = 5	7% [7 sites]	17% [16 sites]	26% [25 sites]	37% [35 sites]
Ovine n = 5	5% [2 sites]	9% [4 sites]	41% [19 sites]	39% [18 sites]
Rat n = 5	34% [22 sites]	6% [4 sites]	25% [16 sites]	25% [16 sites]

Table 4.2: Presence of webbing on the energy storing tendons of all three animal models

Animal	Abundance	Partial	Some	None
Bovine n = 5	40% [25 sites]	27% [17 sites]	24% [15 sites]	3% [2 sites]
Ovine n = 5	30% [14 sites]	13% [6 sites]	34% [16 sites]	9% [4 sites]
Rat n = 5	28% [15 sites]	30% [16 sites]	24% [13 sites]	15% [8 sites]

4.3.2 Grid Assessment

Two-way ANOVA results showed that both factors of tendon type and animal model were significant. One-way ANOVA results showed that between tendon types there were significant differences between the positional and energy storing tendons of both the bovine and ovine models ($p = 0.0001$ and $p = 0.0092$, respectively) (Table 4.3, Figure 4.7). In both models the energy storing tendons displayed a greater percentage of webbing than the positional tendons. Within the rat model, there was not a significant difference found.

Across models, there was a significant difference in the percentage of webbing on the positional tendons of the ovine and the rat models ($p = 0.0455$). The rat positional tendons displayed a greater percentage of webbing present than the ovine model (Table 4.4). Between the energy storing tendons there was a difference in the percentage of webbing found on the bovine

and ovine models ($p = 0.0497$). The bovine models displayed a greater percentage of webbing present, however, the difference in the webbing between these models was not very large.

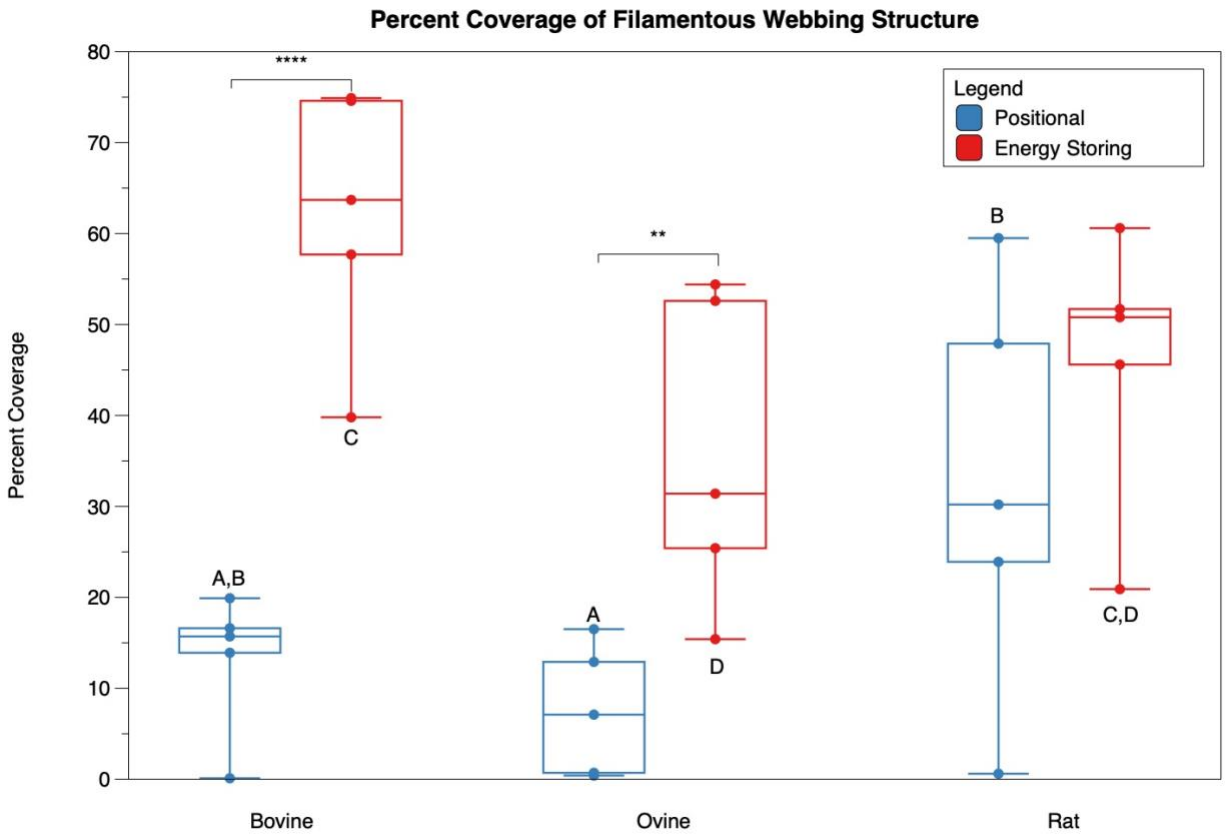

Figure 4.7: Box plots of the percentage coverage of webbing from the grid assessment. There was a significant difference found between the positional and energy storing tendons of the bovine and ovine models. Across models there was a significant difference found between the ovine and rat positional tendons ($p < 0.05$), as well as the bovine and ovine energy storing tendons ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Table 4.3: Percent coverage of filamentous webbing structure on tendons

Bovine		Ovine		Rat	
Positional	Energy Storing	Positional	Energy Storing	Positional	Energy Storing
0.0% n = 1728	74.6% n = 2288	0.4% n = 1259	15.4% n = 2486	23.9% n = 1096	51.7% n = 2578
13.9% n = 2980	57.7% n = 3277	16.5% n = 1443	31.4% n = 1597	47.9% n = 1767	50.8% n = 2313
15.7% n = 3144	39.8% n = 2320	12.9% n = 3296	54.4% n = 2032	0.6% n = 720	60.6% n = 2028
16.6% n = 2613	63.7% n = 2085	0.7% n = 2254	52.6% n = 1530	30.2% n = 1371	45.6% n = 1606
19.9% n = 1729	74.9% n = 2602	7.1% n = 1232	25.4% n = 1769	59.5% n = 1943	20.9% n = 1666

Table 4.4: Mean percent coverage of filamentous webbing structure on tendons

	Bovine		Ovine		Rat	
	Positional	Energy Storing	Positional	Energy Storing	Positional	Energy Storing
Mean	13.2%	62.1%	7.5%	35.8%	32.4%	45.9%
SD	± 0.08	± 0.14	± 0.07	± 0.17	± 0.23	± 0.15

4.4 Discussion

4.4.1 Visual Assessment

The results of this section show that the ovine and bovine model display distinct differences in the amount of webbing between their positional and energy storing tendons. The two tendon types within both models had significant differences between the abundance of webbing and the no webbing categories. Having distinct differences at the two extreme groups indicates that the overall level of webbing within these tendons is different. These differences coincide with what has been previously found that the filamentous webbing structure appears mostly on energy storing tendons [9]. Additionally, as stated previously, this may be an indicator that this ill-defined webbing may play a very important role in the overall function of energy storing tendons possibly helping them to complete their required functions by allowing them to act as one unit giving them an increase in their ability to resist fatigue damage.

The rat model did not show this same difference. Between the two tendon types, the rat model only had a significant difference within the partial webbing category. While this still indicates a slight difference between the two tendon types because it is not at either of the extreme categories and is on its own. Regardless of the reason, this small distinction does not indicate an overall difference in the level of webbing between the two tendon types in this model. Once again, the rat model displays a contrasting result to the bovine and ovine models.

Across models, there were significant differences found between the bovine and rat positional tendons in the abundance of webbing and partial webbing categories. This indicates that there was a difference in the percentage of sites that had a lot of webbing present, meaning that the rat positional tendons displayed a larger amount of webbing than the bovine positional tendons. The ovine positional and rat positional tendons also had a significant difference between

the abundance of webbing category. Again, the rat positional tendons displayed a larger amount of webbing than the ovine positional tendons. Indicating that the positional tendons within the rat and ovine models would likely behave differently, depending on the role that this filamentous webbing structure plays in tendon function.

Across models, there were also differences found between the energy storing tendons. The bovine and rat models displayed a difference between their no webbing categories, with the rat model having a higher percentage of sites with no webbing present. This difference indicates that within the rat tendons, there were more sites that did not have any webbing present than in the bovine model. Coupled with the differences found in the positional tendons between the bovine and rat models, this indicates that the two tendon types have a different level of webbing between these two modes. This suggests that any added benefit or disadvantage that this ill-defined webbing may bring a tendon would cause these tendon types to behave differently between these models. Meaning that overall, they would have slight differences in their mechanical functions.

These results prove the hypothesis incorrect, as within the rat models, there was only a slight difference found between the two tendon types.

2.4.2 Grid Assessment

The results of this section show that there were distinct differences in the percent coverage of webbing between tendon types in bovine and ovine models. The energy storing tendons of both models displayed a higher percentage of webbing present. This matches what has been previously found by Herod et al. [9], with the positional tendons having less webbing present than the energy storing tendons. Suggesting that this difference in webbing may contribute to the differing mechanical properties of the two tendon types. Notably, the rat model did not show a

significant difference between the two tendon types. Mirroring the results from the visual assessment and indicating that the differences in function between the two tendon types may be different in the rat model.

Across models, there were differences found in the positional tendons of the ovine and rat tendons. The rat tendons displayed a greater percentage of webbing present. This difference in webbing may suggest that the positional tendons of the rat model would display differing mechanical properties than the ovine positional model. Additionally, between the bovine and ovine model and the bovine and rat model, there was no differentiation in the percentage of webbing present. Indicating that the level of webbing present in the positional tendons of these models was similar. The ovine positional tendons had the lowest percentage of webbing present when compared to the other two models and had the least variation as the standard deviation was lowest for the ovine positional model. The rat positional tendons had the highest percentage of webbing present and had the highest standard deviation.

Between the energy storing tendons, there was only a significant difference between the bovine and ovine models, with the bovine model displaying a greater percentage of webbing. There was no difference found between the bovine and rat model and the ovine and rat model. Overall, the ovine model has the lowest percentage of webbing present in the energy storing tendons but had the most variation. The bovine energy storing model had the highest percentage of webbing present compared to the other two models and had the lowest standard deviation. This suggests that the energy storing tendons of the bovine and rat model and the ovine and rat model would likely all have the same benefit that this webbing structure may bring the tendon. The difference in the percentage of webbing between the bovine and ovine models may suggest

that their energy storing tendons may behave differently under mechanical strain. However, the role that this webbing structure plays in tendon function is unknown.

This assessment of the percent coverage of webbing proves the hypothesis incorrect, as only the bovine and ovine models showed a significant difference in the level of webbing between the two tendon types.

4.4.3 Possible Protein Composition of the Filamentous Webbing Structure

The composition of a structure dictates a lot about its mechanics; gaining an understanding of the composition of this structure is very important to help us understand its function. There are many proteins that are found within a tendon, each with a unique set of skills [36]. While not assessed in this study, understanding the protein structure of this ill-defined filamentous webbing structure is key to understanding its overall functions. The composition of the filamentous webbing structure could be any one of these proteins, or it could be several of them working together. The likelihood that a protein can be found within this webbing structure depends on its normal function, properties, and abundance within the tendon.

The distribution of proteins within positional and energy storing tendons are different, specifically the distribution between elastin and proteoglycans [36]. Apart from collagen, these are the two leading groups of proteins found within the tendon. However, the understanding of the distribution of proteins within tendons has only been studied recently, and therefore, the assessment of the tendon proteome is not quite extensive [7].

4.4.3.1 Collagen

Tendon is mostly made up of collagen which accounts for approximately 60-85% of its dry weight [6,16,18,31]. There are 28 different types of collagens, but type I collagen is responsible for 95% of the collagen found within tendon. The rest is comprised of collagen types III, V, VI, XII, and XIV [32–34]. Between positional and energy storing tendons there is a difference in their collagen content but how this affects their overall mechanics is not well known [35]. Therefore, it is quite unsurprising to find collagen within many areas of the tendon, and it is likely that within the filamentous webbing structure, there is some form of collagen.

Basing the likelihood of certain collagens being present within the filamentous webbing structure on their abundance would mean that type I collagen would be the prime candidate. However, the webbing structure does not seem to have similar properties and does not have the distinctive D-banding that is viewed in this type of collagen (Figure 4.8). The next suspect would be type III collagen, which comprises up to 10% of the collagen within the tendon [36]. Type III collagen aids in regulating the size of type I collagen fibrils and the percentage of type III collagen often increases when an injury occurs [7]. This type of collagen likely helps the interfascicular matrix with fatigue resistance and aids in keeping the matrix healthy.

There are other types of collagens present within tendon that do not make up a significant percentage of the collagen content. Likely, part of the filamentous webbing structure is comprised of non-fibril forming collagen due to the inconsistent structure that the webbing forms. Several non-fibrillar collagens are present within tendon. The most notable is type VI collagen. This type of collagen is localized within the pericellular matrix, and it is suggested that it functions in the development of the structure and function of the extracellular matrix [36,37]. Herod et al.[9] , who discovered this webbing structure, suggests that the material appears to look

similar to images of type VI collagen (Figure 4.9). While physical characteristics do not describe the properties of a structure, often physical characteristics are comparable between structures that have similar functions.

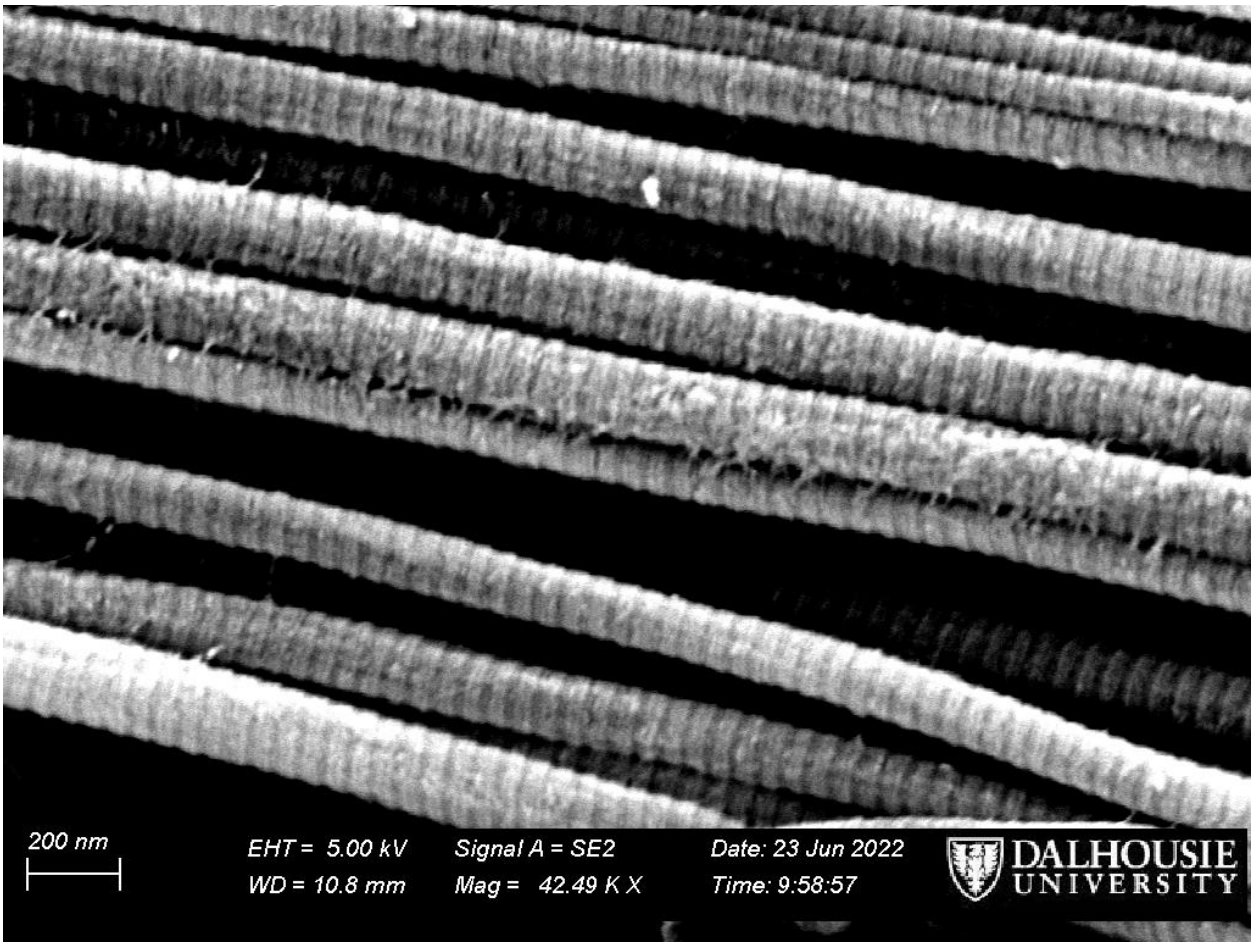

Figure 4.8: Image of an ovine positional tendon displaying the D-banding found on type I collagen.

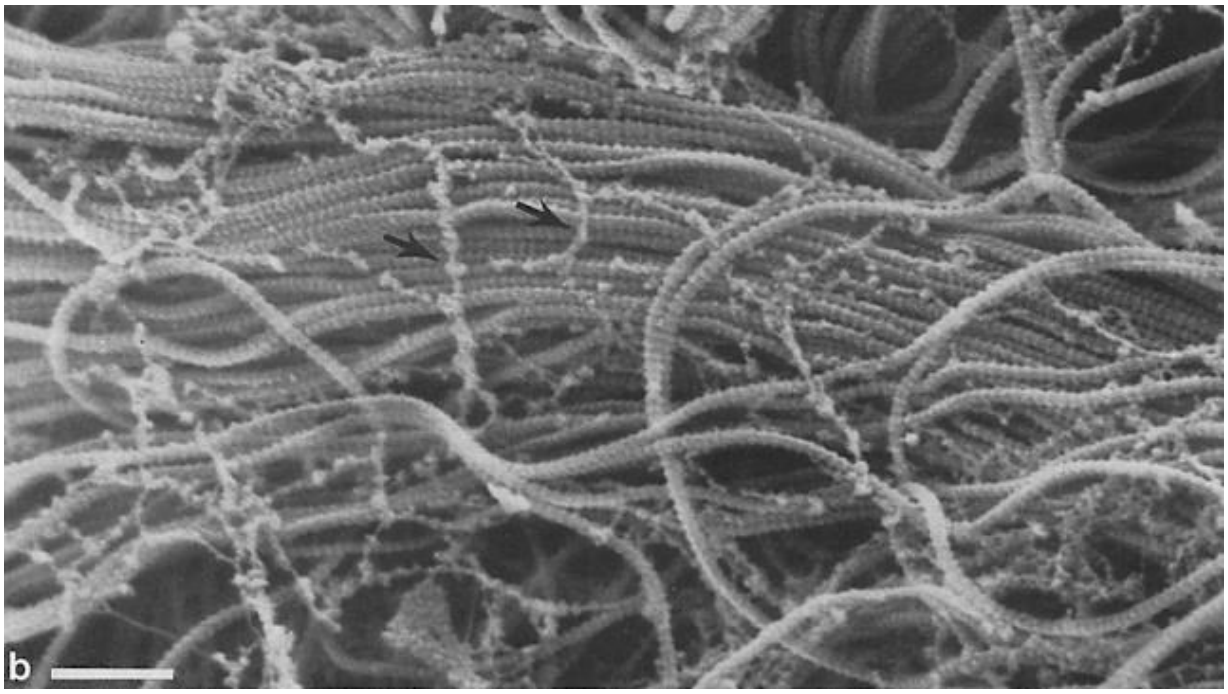

Figure 4.9: Type VI collagen network indicated by arrows. Image is of the collagen network in human skin. Scale bar is 500nm. [94]

4.4.3.2 Non-Collagenous Proteins

Glycoproteins play an important role in the development of the tendons as well as in their overall structure and functions [95]. Additionally, they aid with cell-cell interactions by acting as membrane proteins. Approximately 1-5% of the dry weight of tendon tissue is composed of a type of glycoprotein, with proteoglycans making up the majority of that percentage [95].

Proteoglycans are the most abundant group of non-fibrous proteins [36]. They play a role in aiding in regulating tendon structure, healing tendon injuries, and improving mechanical properties [6,18,36,38]. Additionally, they interact with collagen fibrils, providing hydration and swelling pressure, which helps the tendon to withstand compressional forces [39]. Several types of proteoglycans are found within tendons. However, the differences in their distribution and functional traits have not been well studied [36].

Decorin is the most abundant proteoglycan, comprising 80% of the proteoglycan content within tendon [6]. Decorin consists of a core protein that attaches single side chains made of chondroitin or dermatan. The core protein binds to collagen fibrils and can interact with other decorin molecules to form an interfibrillar bridge, attaching adjacent collagen fibrils [16,36]. Decorin plays a large role in the regulation of collagen fibril organization, which affects the structure and function of the tendon[38]. This proteoglycan has been found to increase the tensile strength of collagen, and those found with decorin depletions often display fragile skin along with abnormal tendon phenotypes [96]. Suggesting that this proteoglycan is often a key factor in not only the organization of collagen fibrils but possibly the organization and creation of matrix within tendons.

Many studies have shown that the interfascicular matrix is rich with lubricin and that it is more abundant within energy storing tendons than within positional tendons [36,97,98]. This proteoglycan is a heavily glycosylated protein, which means that it attaches carbohydrates through enzymatic reactions [99]. Lubricin is also found within joints, where it acts as a boundary layer to allow the joints to move and slide with ease. Additionally, lubricin has high viscoelastic properties that aid in the extensibility of the tendon [99,100]. The properties of lubricin and the functions that it provides would be an asset to the filamentous webbing structure, making it a likely candidate.

Lastly, one of the glycoproteins found within tendon is fibronectin. Fibronectin functions as a link between the extracellular matrix components, such as collagens and proteoglycans, and cell surface receptors [7,36,101]. It also plays a key role in matrix organization and assembly. Fibronectin is often found with type III collagen within connective tissues [101,102]. While there is not much information on the distribution or role of fibronectin within tendon, what is known

about this glycoprotein suggests that it is a good candidate for a possible protein within the filamentous webbing structure, specifically due to its interactions linking collagens with cell surface receptors and how it aids in matrix assembly.

4.4.3.4 Elastin

The role of elastin is incredibly important for tendon mechanics. Elastin aids in tendon recoil and allows energy storing tendons to save and return energy to aid in locomotion [41,46]. Elastin provides the extracellular matrix of many tissues within extensibility and resilience, allowing them to be repetitively loaded and often with relatively large forces [41]. Additionally, within ligaments, elastin aids in the resistance of shear forces and deformation with repeated loading [36]. While it has not been established, it is suggested that elastin also plays a similar role within tendon. With the importance of elastin within tendon structure and how it aids in mechanics, particularly within energy storing tendons, it is not a far stretch to suggest that it is also a part of this uncharacterized webbing structure. Finding elastin within a region of the tendon provides great insight into the mechanics of that region [103]. Given the suggested mechanics of the filamentous webbing structure, elastin is a great candidate to possibly be a part of the composition of this structure.

While the above is not a complete description of all the possible candidate proteins, these are likely the leading contenders. The overall structure of a protein and its role translates into the larger tissue it helps to build. Therefore, the protein structure can possibly be predicted if the overall function of the tissue is known. However, the function of this webbing structure is currently unknown and making predicting its protein composition difficult. While we know what proteins are found in tendons, their overall distribution is still under investigation. Therefore,

future work needs to assess the protein composition of this webbing structure, possibly taking these candidate proteins into consideration.

4.5 Conclusion

In both assessments of the presence of webbing, it was found that there was a difference in the level of webbing between the positional and energy storing tendons of the bovine and ovine model. This mirrors what has been previously found and may indicate that this filamentous webbing structure plays a role in the mechanical properties of the tendon. The difference in levels of webbing found between the tendon types may indicate that this webbing structure increases the ability of energy storing tendons to withstand damage from cyclic loading by laterally connecting the collagen fibrils [9]. Additionally, this difference could possibly be explained by the higher cellularity within energy storing tendons [16,19,89]. The greater abundance of webbing viewed within the energy storing tendons may be a result of the higher cellularity found within those tendons. This same difference was only found in the visual assessment for the rat model. However, while the visual assessment did indicate a difference in the "partial webbing" category for the rat model, this difference could have been due to a number of factors. Regardless, all three models did display some difference between the positional and energy storing tendons. Additionally, both assessment methods used displayed a similar result.

Across models, different results were found by both methods for the positional tendons. The visual assessment displayed a difference between the bovine and rat model and the ovine and rat model. In contrast, the grid assessment only displayed a difference between the ovine and rat models. The results for the level of webbing within the energy storing tendons was also different between the two methods of assessment. The visual assessment displaying a difference between the bovine and rat models, while the grid assessment showed a difference between the

bovine and ovine models. These differing results suggest that the two methods may not be interchangeable. Additionally, the differing result from the two methods does not give a clear picture of the abundancy of the webbing structure across models. It is likely that there is a difference in the results due to the grid method being more thorough than the visual assessment. However, neither method can account for density properly, which must be taken into consideration.

These results show that the bovine and ovine models distinctly followed the pattern of their energy storing tendons having more webbing present than their positional tendons. The rat model did follow this pattern in one of the methods, but it was not as clear cut. Additionally, for each tendon type, the bovine and ovine tendons displayed the most similar results, with only one difference being found between the percent coverage of webbing in the energy storing tendons. At the same time, the rat model was found to have differing levels of webbing than the other two models within both tendon types. Overall, these results suggest that a greater amount of webbing present in energy storing tendons may be a universal feature across larger animal models and could contribute to the mechanical properties of their tendons.

Chapter 5 – Conclusion

5.1 Between Tendon Types

Understanding how the two tendon types behave in each of these models may give us some insight into the differing functions of the tendon types themselves and if these functions are specific to certain animal models. The roles that the positional and energy storing tendons play are critical to the animal's overall locomotion; therefore, understanding their relationship with each other can help us have a broader picture of the mechanical properties of the model being used.

The current study found that overall, there was a significant difference between the positional and energy storing tendons within the bovine and ovine models in all testing methods used. This indicates that by the standards of the assessments used within this study, the bovine and ovine models have the same relationship between their tendon types. These assessments covered chemical compositions, including thermal stability and crosslinking, along with physical composition, such as diameter and presence of webbing. These two aspects are key components of a tendon's overall structure and contribute largely to how the tendon functions; therefore, the results found within these assessments do tell us a lot about the properties of the tendons.

The rat tendon pairs did display similar results to the other two models within some of these methods, including mean fibril diameter and the visual filamentous webbing structure assessment. However, for all of the other methods of testing, the differing results indicate that the rat tendon pair does not display the same distinction in their properties.

Overall, within each model, these results suggest that the common positional and energy storing tendon pairs of larger animal models will have similar differences within their properties. Indicating that the difference between the tendon types within large animal models may be a

universal feature. While smaller animal models, such as the rat model used in this study, may not display this same relationship between the two tendon types.

5.2 Across Animal Models

Often, positional and energy storing pairs are chosen and used based on their supposed functions, and these functions may not be the same for each of the tendon types across models. Therefore, it is not only important to understand the relationship between the tendon pairs within an animal model but also how they compare directly to other models.

Within the positional tendons, the bovine and ovine tendons displayed a similar result in all assessments except for the mean fibril diameters and the full width at half the maximum. The rat positional tendons only displayed similar results to the bovine model in all assessments except for T_d, full width at half the maximum, and the visual assessment for webbing. The rat also displayed similar results to the ovine model in all assessments except $t_{1/2}$, mean fibril diameter, and the grid assessment for webbing. Overall, the assessments for the positional tendons in these models suggest that all three models displayed reasonably similar results, with the rat having similar results to both the bovine and ovine models in 5 out of the 8 assessments and the bovine and ovine models being similar in 6 out of 8. Positional tendons are less specialized than their energy storing counterparts and do not have the same trade-offs that have been made in the energy storing tendons. Therefore, the similarity between the positional tendons within these models was expected.

For the energy storing tendons, the bovine and ovine models displayed similar results in all assessments except for the mean fibril diameter, modality, full width at half the maximum, and visual assessment of the filamentous webbing structure. Between the bovine and rat models,

they displayed similar results in all assessments except T_d, T_{Fmax}, $t_{1/2}$, mean fibril diameter, and the visual assessment of the filamentous webbing structure. Lastly, between the ovine and rat model, their energy storing tendons displayed similar results for all assessments except T_d, T_{Fmax}, $t_{1/2}$, mean fibril diameter, modality, and full width at half the maximum. The bovine and ovine models had similar results in 4 out of 8 of the assessments, the bovine and rat in 3 of 8, and the ovine and rat in 2 of 8. Overall, the energy storing tendons of the bovine and ovine models did display similar results when compared to the rat model but did differ in some areas.
The energy storing tendons of these models displayed slightly different results than their positional tendons, with the energy storing tendons being less similar to each other. This is not surprising as energy storing tendons do bear a lot of load during locomotion, and the sizing of the animals themselves may play a prominent role in the overall function of this tendon type.

As seen in the comparison between both positional and energy storing tendons across these three models, the rat model does not display the same results as the bovine and ovine models. This indicates that the structure and functions of the tendon pair within the rat model are not the same as the other two, meaning that results found in the rat model would not be comparable to the bovine or ovine model. This could be due to several reasons, such as the difference in loading that is experienced by the rat tendons due to both the weight of the animal and the types of movement that it participates in [47]. Rodents often experience short bursts of movement rather than the heavier and more lengthy movements seen in larger animal models. Therefore, the role that their tendons play in movement is different, and this difference would likely appear in the properties of the tendons themselves. As for the bovine and ovine energy storing tendons, they did display quite similar results in half of the testing methods, specifically in the thermal assessments and the grid assessment for webbing. This indicates that the bovine

and ovine models are likely to yield similar results when it comes to a direct comparison between their energy storing tendons and may suggest that the ovine model may be a good option when it comes to tendon research.

5.3 Limitations

5.3.1 Sex of Models

When using choosing a large animal model within tendon research males are often used. This is due to female models having the possibility of having been previously pregnant. Pregnancy is characterized by hormone changes that often lead to the altering of musculoskeletal properties, particularly being noted in the uterus and cervix [104–106]. However, these changes in musculoskeletal properties may also affect tendon tissue. Bey et al.[105] found that the stiffness of the human patellar tendon was not affected during pregnancy as there were no results that would suggest that tendons were more compliant due to changes that occur throughout pregnancy. While Waugh et al.[104] found that there were changes in collagen organization in the human Achilles tendon during pregnancy that seemed to result in a slight decrease in stiffness. However, this change in stiffness did not seem to affect the tensile strength of the tissue as that remained unchanged. Additionally, it has been found that there was an increase in laxity of the ACL as pregnancy progressed but between the third trimester and postpartum this laxity then decreased, suggesting that after pregnancy the ligament began to return to normal function [104,107].

In the current study, steers were used for the bovine model, and therefore provide consistency across samples. However, the samples from the ovine model were all from ewes as that was what was available from the abattoir. Given farming practices, it is likely that most, if not all, of the ewes produced lambs at some point. For the rat model, the sexes of the individuals

were unknown, however, due to the animals having no previous interventions, the possibility of pregnancy causing limitations was not an issue.

Previous studies have shown contradicting result on whether pregnancy alters the structure and function of tendons. However, these studies do show that if there is a change, it is not large and overall does not affect the mechanics. Regardless, due to this area not being extensively studied within animal models, the sex of the models used within research must be taken into consideration and may be a limitation within this study.

5.3.2 Acquiring Samples

The samples for all three models were obtained from different places all being varying distances away from Saint Mary's University. Therefore, there were differences in the time that the animal was culled to when the tendon was place in the freezer. These differences in time of death could possibly be a limitation within the study as it could not be controlled for.

Previous studies have shown that while controlling for time of death is important, it may not be a large contributor to possible issues within this particular study. Chang et al.[108] found that the fibril diameter within mouse tendon did vary based on the time of day that the animal was culled. Using Zeitgeber time, it was found that there were differences in fibril diameter when comparing diameters taken from 3 hours into light verse 3 hours into darkness, with there being larger diameters at night. However, due to the small window of time that the models within this study were culled in, this change in diameter based on time of day is likely not a factor. Additionally, studies that look at circadian rhythm in terms of tendon variation often group the morning hours together as there is not a large difference in the results during that time period [109,110].

5.3.2.1 Bovine

The bovine samples were obtained from Reid's Meats that is located 70 km away from Saint Mary's. The steers were culled from 7:00 am – 11:00 am. Typically, the forelimbs are retrieved from the abattoir around 11:00 am, placed in a cooler with ice, and then the tendons are dissected from the forelimb back at Saint Mary's. Directly after dissection the tendons are wrapped in PBS soaked gauze and placed in the -86°C freezer. Total time from culling to freezer ranged from 2 – 5 hours.

5.3.2.2 Ovine

The ovine samples are obtained from Northumberlambs that is located 104 km from Saint Mary's University. The ewes were culled from 7:00 am – 11:00 am. Typically, the forelimbs are retrieved from the abattoir around 12:00 pm, placed in a cooler with ice, and then the tendons are dissected from the forelimb back at Saint Mary's. Directly after dissection the tendons are wrapped in PBS soaked gauze and placed in the -86°C freezer. Total time from culling to freezer ranged from 3 – 6 hours.

5.3.2.3 Rat

The rat samples were obtained from Dalhousie University that is located 0.7 km from Saint Mary's University. The rats were culled at 11:00 am. The hindlimbs were removed, placed on ice, and then brought to Saint Mary's where they were put directly into the -86°C freezer. Total time from culling to freezer was approximately 1 hour.

5.4 Significance

When it comes to choosing an animal model for your research, it is important to know the baseline for how the animal functions, and in the case of tendinopathy research, it is essential to know how the research can be related to other models, such as human models. Therefore, expanding the known comparisons of animal models is very important. In this study, we aimed to gain a more extensive understanding of how common tendon pairs in the bovine, ovine, and rat models compare to each other by putting them through well-known testing methods.

Additionally, we aimed to confirm the existence of the ill-defined filamentous webbing structure that was first discovered by Herod et al. [9]. Within the body, often, structure dictates functions and gaining a better understanding of this structure may tell us a lot about the specialized mechanics it may give a tendon. Confirming the exitance of this ill-defined structure is the first step in growing our knowledge of how it may affect tendon function.

Overall, the results of this study suggest that small and large animal models are not interchangeable within tendon research. This research has found that anatomically analogous tendons of the rat model appear to be significantly different from high-load tendons in large animal models. This should be understood when analyzing the outcomes from such studies and considered when choosing models for future work.

5.5 Future Work

Future studies should aim to assess the properties of these tendons under mechanical strain to understand further how they may compare to each other in a physiological environment. While each of the individual assessments used in this study does broaden our understanding of tendon function, they are only a small piece of the puzzle. Testing these models under different strain

rates would allow for a more complete understanding of the differences and similarities in their properties. As well, expanding the number and variety of animal models used in comparisons, such as the ones in this study, would help to broaden our knowledge of tendon structure and function and to gain a clearer picture of the possible differences between small and large animal models.

Additionally, future work needs to evaluate the filamentous webbing structure, to assess how this webbing may affect the overall properties of the tendon and as well as, to gain an understanding of the composition of the webbing itself. The overall composition of this webbing structure may be able to tell us a lot about its functions as the protein structure of tissue often dictates its properties and could give us some more insight into the specialized mechanics of energy storing tendons.

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Appendix I – Grid Validation

To validate the accuracy of the method for the grid assessment, a series of grid square areas were evaluated. These areas ranged from 10,000 nm^2 to 45,000 nm^2. The smallest area was chosen based off the number of total grid square it would provide, with it being over 1000 grid squares. From there the grid areas were increase at an interval of 5,000 nm^2 up to 45,000 nm^2, giving six different grid areas for validation (Figure 7.1). A total of 6 tendons were assess one positional and one energy storing from all three animal models. These the abundance of webbing was assessed at all six grid areas for each of the tendons, giving a percent coverage for a positional and energy storing tendon for each model. The images for each model that were chosen needed to have at least some webbing present, to allow for the evaluation of the change in percent coverage as the grid area increased. Overall, a grid area of 25,000 nm^2 was chosen as it did not display a large difference in percent coverage from the smallest grid area of 10,000 nm^2. Allowing this method to remain accurate while having the largest possible grid squares.

Table 7.1: Example of the method of grid validation the rat positional model.

Area of Grid square	Number of total grid squares	Grid Squares with webbing	Grid Squares without webbing	Grid squares not included	Percent coverage of webbing
10,000 nm^2	1036	711	105	220	87.1%
15,000 nm^2	600	501	89	10	84.9%
20,000 nm^2	442	378	58	5	86.7%
25,000 nm^2	345	294	46	5	86.5%
30,000 nm^2	294	233	53	8	81.5%
35,000 nm^2	260	211	42	7	83.4%
40,000 nm^2	216	168	41	7	80.4%

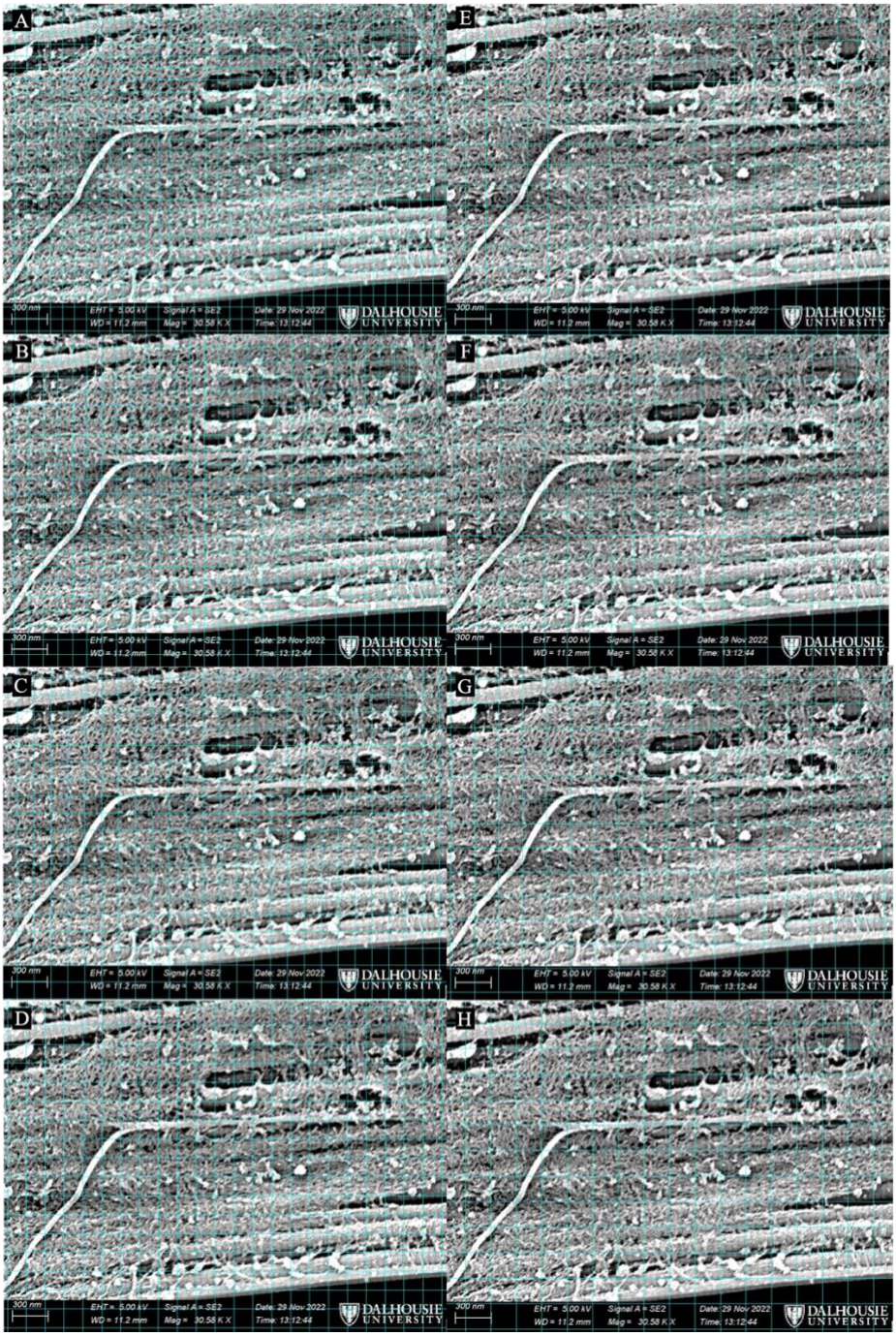

Figure 7.1: Representative images of the method for grid validation in the rat positional model. Sites were assessed for percent coverage of webbing with (A) 10,000 nm^2 grid squares (B) 15,000 nm^2 grid squares (C) 20,000 nm^2 grid squares (D) 25,000 nm^2 grid squares (E) 30,000 nm^2 grid squares (F) 35,000 nm^2 grid squares (G) 40,000 nm^2 grid squares (H) 45,000 nm^2 grid squares.

Appendix II – Size Comparison of Bovine Tendons to Rat Tendons

To further investigate the differing results found between the rat tendons and the other two models, particularly in HIT, the bovine tendon pairs were cut down to be the same size as the rat tendons and then run on HIT to see the results (Figure 8.1).

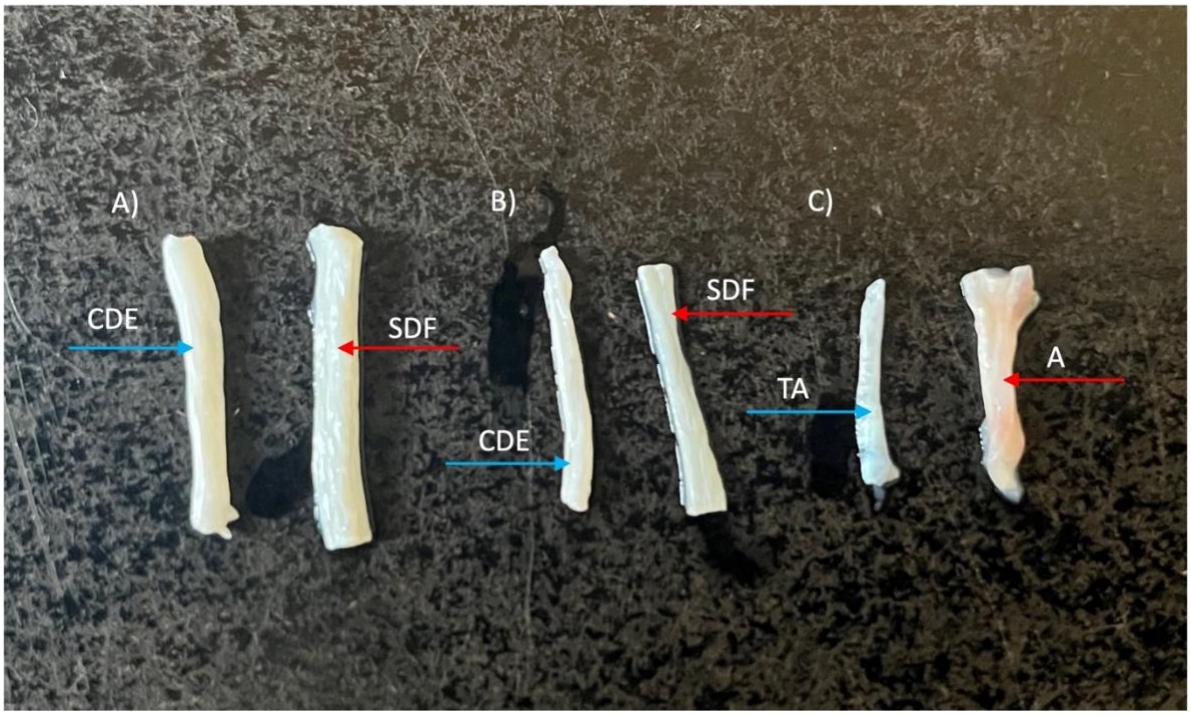

Figure 8.1: Size comparison of bovine tendon to rat tendon. A) Typical size of bovine tendon pair for HIT B) Bovine tendon pair cut to be the size of the rat tendons. C) Typical size of rat tendon pair.

The rat-sized bovine tendons had very similar results to the regular-sized bovine tendons, in both the positional and energy storing tendons (Figure 8.2, Figure 8.3). This indicates that the smaller size of the rat tendons was not a factor in the differences that were found between the rat model and the other two models.

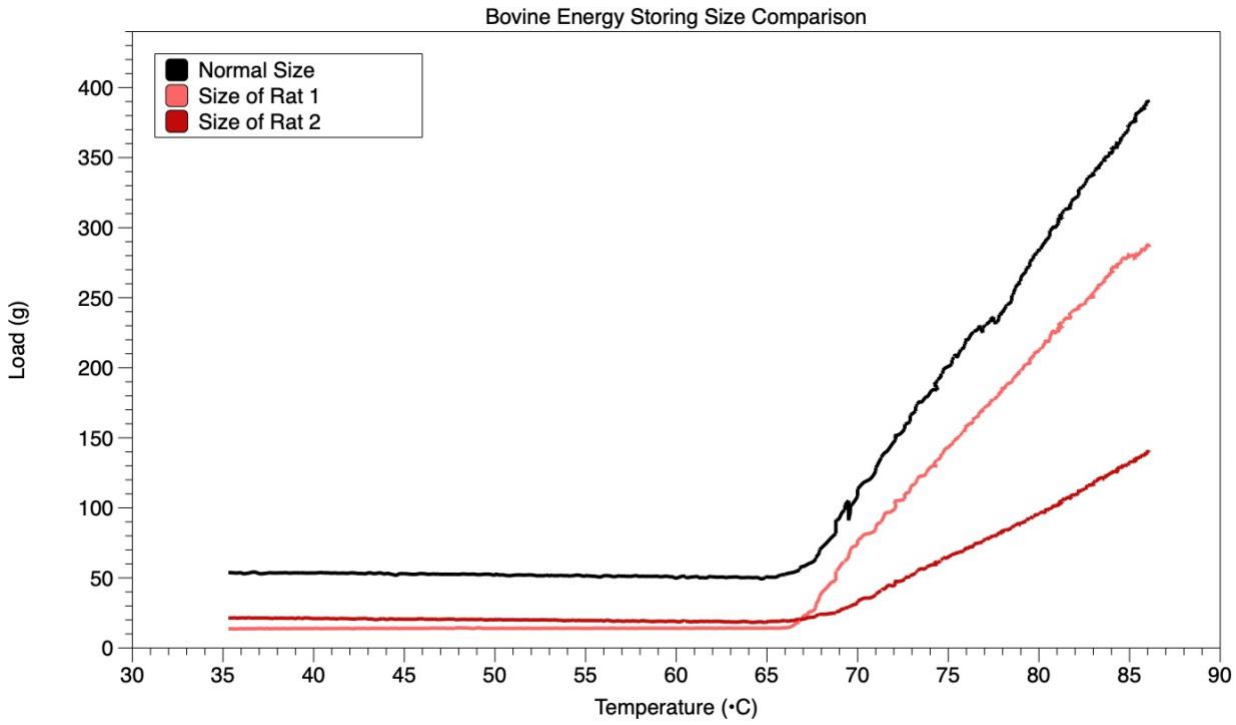

Figure 8.2: HIT cure for the size comparison of the bovine energy storing tendon to the rat energy storing tendon.

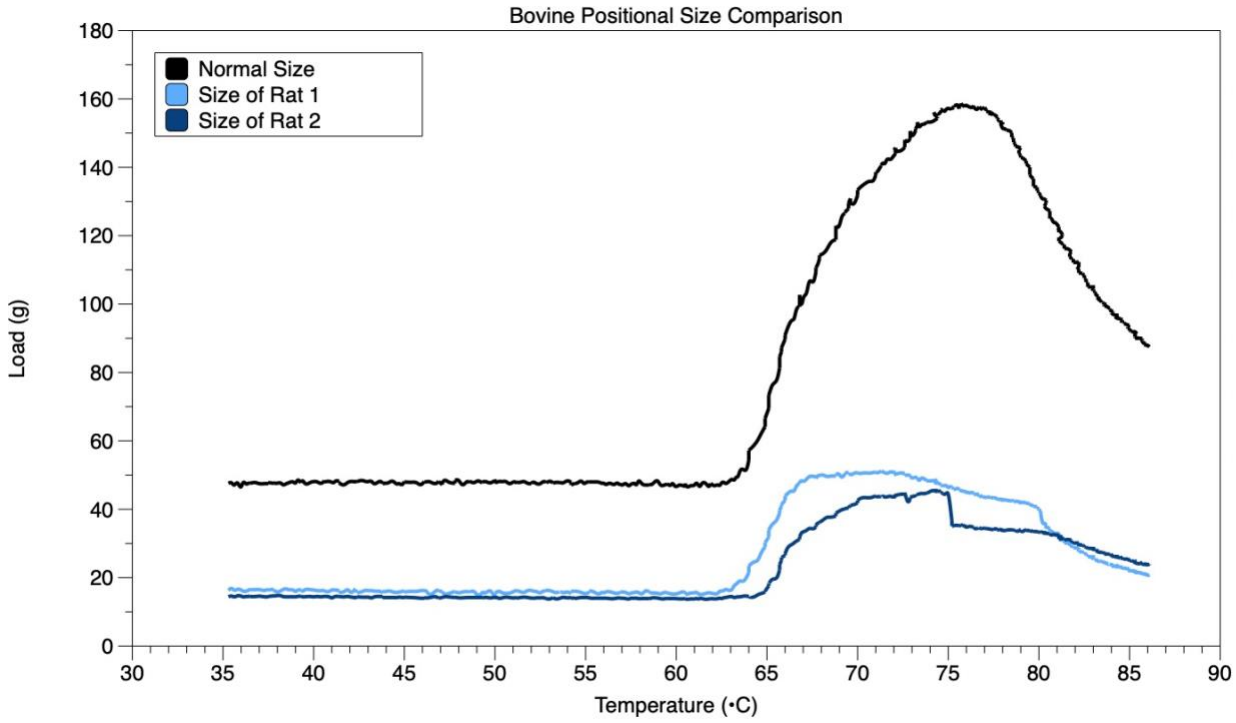

Figure 8.3: HIT cure for the size comparison of the bovine positional tendon to the rat positional storing tendon.

Appendix III – Example of Webbing Structure

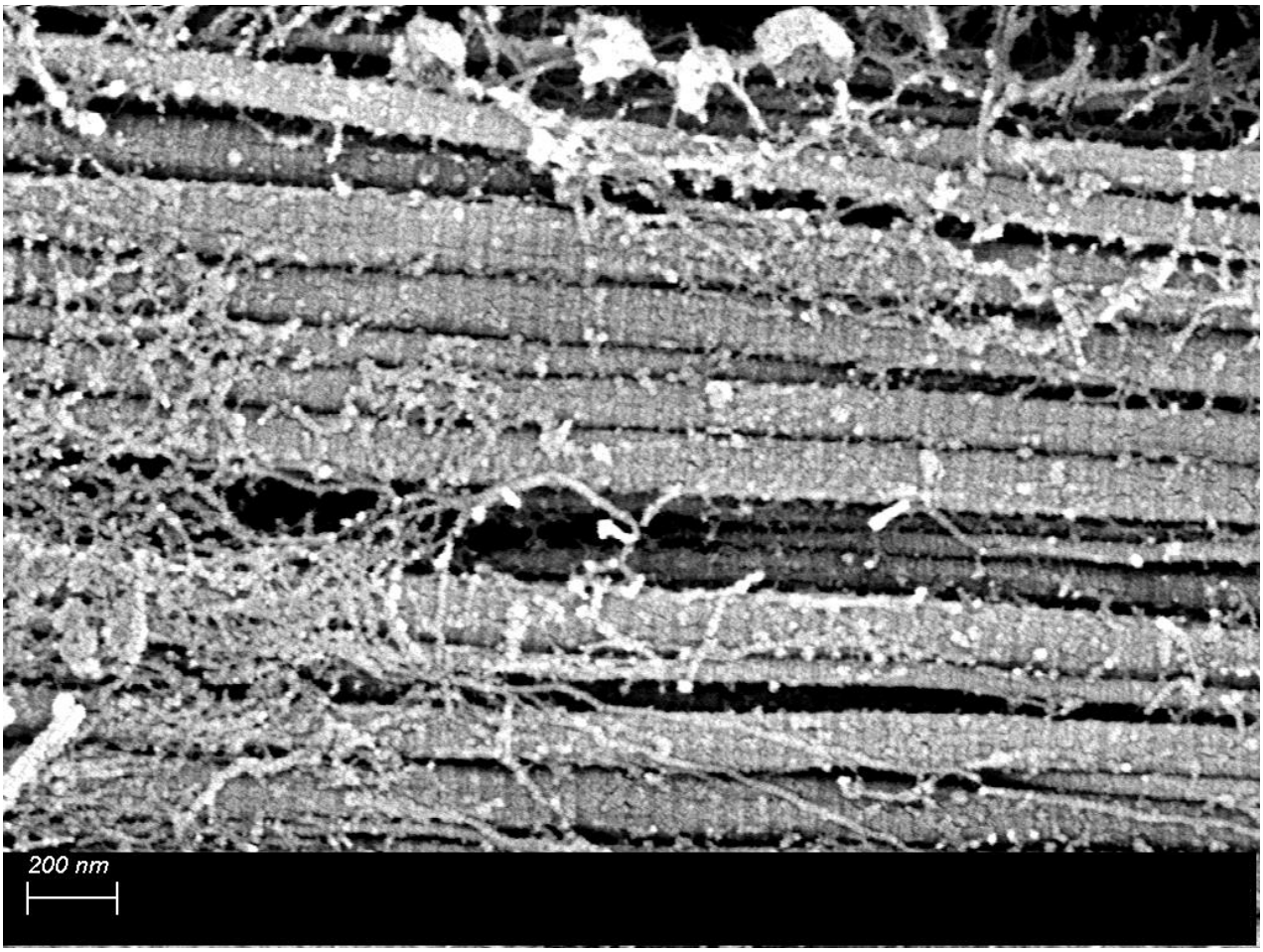

Figure 9.1: Example photo of filamentous webbing structure within a Rat energy storing tendon at 40,000 X magnification.

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E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) Books and Records; Right to Audit. As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) ***Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets).*** For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) **Posting e-reserves, course management systems, e-coursepacks for text-based content,** which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii)

permit “publishing ventures” where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

- a proper copyright notice, identifying the Rightsholder in whose name CCC has granted permission,
- a statement to the effect that such copy was made pursuant to permission,
- a statement identifying the class to which the material applies and notifying the reader that the material has been made available electronically solely for use in the class, and
- a statement to the effect that the material may not be further distributed to any person outside the class, whether by copying or by transmission and whether electronically or in paper form, and User must also ensure that such cover page or other means will print out in the event that the person accessing the material chooses to print out the material or any part thereof.

G) any permission granted shall expire at the end of the class and, absent some other form of authorization, User is thereupon required to delete the applicable material from any electronic storage or to block electronic access to the applicable material.

iv) Uses of separate portions of a Work, even if they are to be included in the same course material or the same university or college class, require separate permissions under the electronic course content pay-per-use Service. Unless otherwise provided in the Order Confirmation, any grant of rights to User is limited to use completed no later than the end of the academic term (or analogous period) as to which any particular permission is granted.

v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User’s ordinary business hours, upon two days’ prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

c) ***Pay-Per-Use Permissions for Certain Reproductions (Academic photocopies for library reserves and interlibrary loan reporting) (Non-academic internal/external business uses and commercial document delivery).*** The License expressly excludes the uses listed in Section (c)(i)-(v) below (which must be subject to separate license from the

applicable Rightsholder) for: academic photocopies for library reserves and interlibrary loan reporting; and non-academic internal/external business uses and commercial document delivery.

i) electronic storage of any reproduction (whether in plain-text, PDF, or any other format) other than on a transitory basis;

ii) the input of Works or reproductions thereof into any computerized database;

iii) reproduction of an entire Work (cover-to-cover copying) except where the Work is a single article;

iv) reproduction for resale to anyone other than a specific customer of User;

v) republication in any different form. Please obtain authorizations for these uses through other CCC services or directly from the rightsholder.

Any license granted is further limited as set forth in any restrictions included in the Order Confirmation and/or in these Terms.

d) ***Electronic Reproductions in Online Environments (Non-Academic-email, intranet, internet and extranet).*** For "electronic reproductions", which generally includes e-mail use (including instant messaging or other electronic transmission to a defined group of recipients) or posting on an intranet, extranet or Intranet site (including any display or performance incidental thereto), the following additional terms apply:

i) Unless otherwise set forth in the Order Confirmation, the License is limited to use completed within 30 days for any use on the Internet, 60 days for any use on an intranet or extranet and one year for any other use, all as measured from the "republication date" as identified in the Order Confirmation, if any, and otherwise from the date of the Order Confirmation.

ii) User may not make or permit any alterations to the Work, unless expressly set forth in the Order Confirmation (after request by User and approval by Rightsholder); provided, however, that a Work consisting of photographs or other still images not embedded in text may, if necessary, be resized, reformatted or have its resolution modified without additional express permission, and a Work consisting of audiovisual content may, if necessary, be "clipped" or reformatted for purposes of time or content management or ease of delivery (provided that any such resizing, reformatting, resolution modification or "clipping" does not alter the underlying editorial content or meaning of the Work used, and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular License described in the Order Confirmation and the Terms.

15) **Miscellaneous.**

a) User acknowledges that CCC may, from time to time, make changes or additions to the Service or to the Terms, and that Rightsholder may make changes or additions to the Rightsholder Terms. Such updated Terms will replace the prior terms and conditions in the order workflow and shall be effective as to any subsequent Licenses but shall not apply to Licenses already granted and paid for under a prior set of terms.

b) Use of User-related information collected through the Service is governed by CCC's privacy policy, available online at www.copyright.com/about/privacy-policy/.

c) The License is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the License or any rights granted thereunder; provided, however, that, where applicable, User may assign such License in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in any new material which includes the Work(s) licensed under this Service.

d) No amendment or waiver of any Terms is binding unless set forth in writing and signed by the appropriate parties, including, where applicable, the Rightsholder. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by or on behalf of the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the License described in the Order Confirmation, which terms are in any way inconsistent with any Terms set forth in the Order Confirmation, and/or in CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

e) The License described in the Order Confirmation shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or

proceeding arising out of, in connection with, or related to such License shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.

Last updated October 2022

Marketplace

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Publication Title	Journal of orthopaedic research	Publication Type	e-Journal
		Start Page	1907
Article Title	Cross-link stabilization does not affect the response of collagen molecules, fibrils, or tendons to tensile overload.	End Page	1913
		Issue	12
		Volume	31
		URL	http://www.journals.elsevierhealth.com/periodicals/ortres
Author/Editor	ORTHOPAEDIC RESEARCH SOCIETY		
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NEW WORK DETAILS

Title	Benchmarking Native Collagen: Evaluation of structural differences between tendon type and across animal models	**Institution Name**	Saint Mary's University
		Expected Presentation Date	2023-12-20
Instructor Name	Samuel Veres		

ADDITIONAL DETAILS

The Requesting Person / Organization to Appear on the License	Amanda Lee

REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	Figure 1	**Title of the Article / Chapter the Portion Is From**	Cross-link stabilization does not affect the response of collagen molecules, fibrils, or tendons to tensile overload.
Editor of Portion(s)	Veres, Samuel P.; Harrison, Julia M.; Lee, J. Michael	**Author of Portion(s)**	Veres, Samuel P.; Harrison, Julia M.; Lee, J. Michael
Volume / Edition	31		
Page or Page Range of Portion	1907-1913	**Publication Date of Portion**	2013-12-01

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Marketplace Permissions General Terms and Conditions

The following terms and conditions (“General Terms”), together with any applicable Publisher Terms and Conditions, govern User’s use of Works pursuant to the Licenses granted by Copyright Clearance Center, Inc. (“CCC”) on behalf of the applicable Rightsholders of such Works through CCC’s applicable Marketplace transactional licensing services (each, a “Service”).

1) **Definitions.** For purposes of these General Terms, the following definitions apply:

“License” is the licensed use the User obtains via the Marketplace platform in a particular licensing transaction, as set forth in the Order Confirmation.

“Order Confirmation” is the confirmation CCC provides to the User at the conclusion of each Marketplace transaction. “Order Confirmation Terms” are additional terms set forth on specific Order Confirmations not set forth in the General Terms that can include terms applicable to a particular CCC transactional licensing service and/or any Rightsholder-specific terms.

"Rightsholder(s)" are the holders of copyright rights in the Works for which a User obtains licenses via the Marketplace platform, which are displayed on specific Order Confirmations.

"Terms" means the terms and conditions set forth in these General Terms and any additional Order Confirmation Terms collectively.

"User" or "you" is the person or entity making the use granted under the relevant License. Where the person accepting the Terms on behalf of a User is a freelancer or other third party who the User authorized to accept the General Terms on the User's behalf, such person shall be deemed jointly a User for purposes of such Terms.

"Work(s)" are the copyright protected works described in relevant Order Confirmations.

2) **Description of Service.** CCC's Marketplace enables Users to obtain Licenses to use one or more Works in accordance with all relevant Terms. CCC grants Licenses as an agent on behalf of the copyright rightsholder identified in the relevant Order Confirmation.

3) **Applicability of Terms.** The Terms govern User's use of Works in connection with the relevant License. In the event of any conflict between General Terms and Order Confirmation Terms, the latter shall govern. User acknowledges that Rightsholders have complete discretion whether to grant any permission, and whether to place any limitations on any grant, and that CCC has no right to supersede or to modify any such discretionary act by a Rightsholder.

4) **Representations; Acceptance.** By using the Service, User represents and warrants that User has been duly authorized by the User to accept, and hereby does accept, all Terms.

5) **Scope of License; Limitations and Obligations.** All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The License provides only those rights expressly set forth in the terms and conveys no other rights in any Works

6) **General Payment Terms.** User may pay at time of checkout by credit card or choose to be invoiced. If the User chooses to be invoiced, the User shall: (i) remit payments in the manner identified on specific invoices, (ii) unless otherwise specifically stated in an Order Confirmation or separate written agreement, Users shall remit payments upon receipt of the relevant invoice from CCC, either by delivery or notification of availability of the invoice via the Marketplace platform, and (iii) if the User does not pay the invoice within 30 days of receipt, the User may incur a service charge of 1.5% per month or the maximum rate allowed by applicable law, whichever is less. While User may exercise the rights in the License immediately upon receiving the Order Confirmation, the License is automatically revoked and is null and void, as if it had never been issued, if CCC does not receive complete payment on a timely basis.

7) **General Limits on Use.** Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) involves only the rights set forth in the Terms and does not include subsequent or additional uses, (ii) is non-exclusive and non-transferable, and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Terms. Upon completion of the licensed use as set forth in the Order Confirmation, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work. User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is unlawful, including without limitation if such use would violate applicable sanctions laws or regulations, would be defamatory, violate the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit, or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. Any unlawful use will render any licenses hereunder null and void. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

8) **Third Party Materials.** In the event that the material for which a License is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) that are identified in such material as having been used by permission (or a similar indicator), User is responsible for identifying, and seeking separate licenses (under this Service, if available, or otherwise) for any of such third party materials; without a separate license, User may not use such third party materials via the License.

9) **Copyright Notice.** Use of proper copyright notice for a Work is required as a condition of any License granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as

follows: "Used with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc." Such notice must be provided in a reasonably legible font size and must be placed either on a cover page or in another location that any person, upon gaining access to the material which is the subject of a permission, shall see, or in the case of republication Licenses, immediately adjacent to the Work as used (for example, as part of a by-line or footnote) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

10) **Indemnity.** User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs, and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein and in the Order Confirmation, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy, or other tangible or intangible property.

11) **Limitation of Liability.** UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL, OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OR BOTH OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for the relevant License. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors, and assigns.

12) **Limited Warranties.** THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS." CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS, OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

13) **Effect of Breach.** Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the License set forth in the Order Confirmation and/or the Terms, shall be a material breach of such License. Any breach not cured within 10 days of written notice thereof shall result in immediate termination of such License without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

14) **Additional Terms for Specific Products and Services.** If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

a) ***Print Uses of Academic Course Content and Materials (photocopies for academic coursepacks or classroom handouts).*** For photocopies for academic coursepacks or classroom handouts the following additional terms apply:

i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

ii) No License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied) (ii) permit "publishing ventures" where any particular anthology would be systematically marketed at multiple institutions.

iii) Subject to any Publisher Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the academic pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) Books and Records; Right to Audit. As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) ***Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets).*** For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) **Posting e-reserves, course management systems, e-coursepacks for text-based content,** which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with

Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit "publishing ventures" where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

- a proper copyright notice, identifying the Rightsholder in whose name CCC has granted permission,
- a statement to the effect that such copy was made pursuant to permission,
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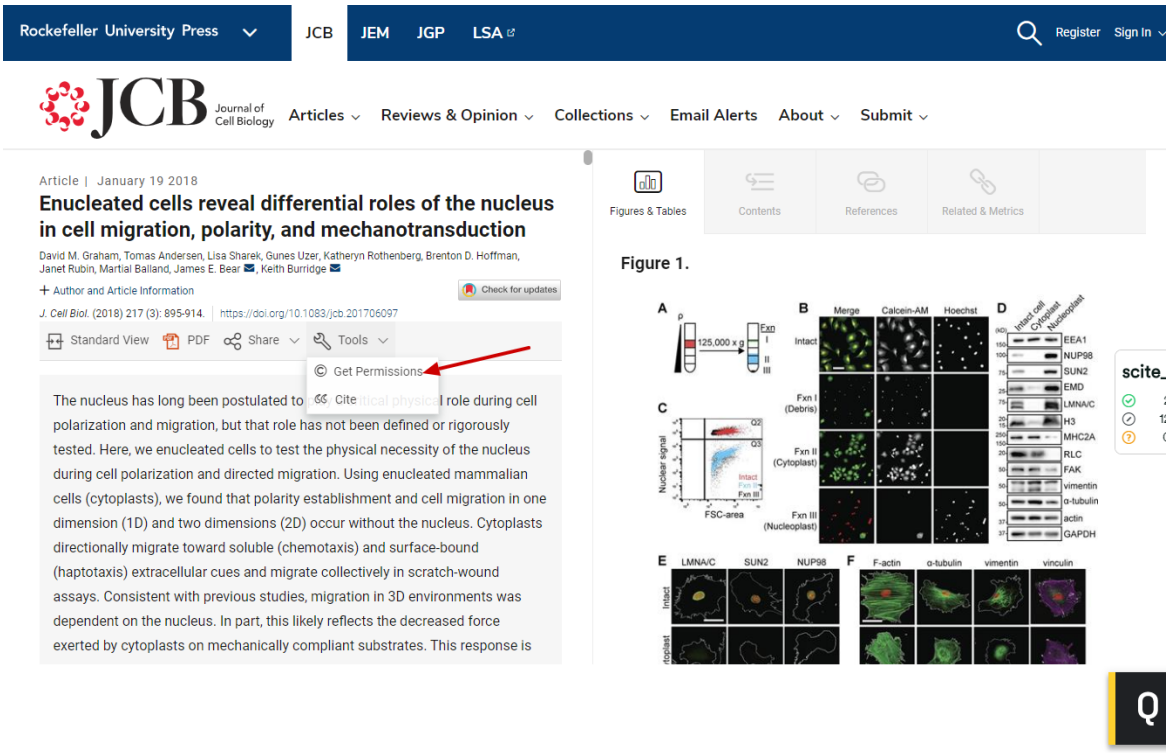

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