REMODELING OF MYOCARDIAL COLLAGEN IN DOMESTIC CATTLE DURING PREGNANCY AND POSTPARTUM

by

Valerie Metlege

Submitted in partial fulfillment of the requirements
for the degree of Master of Applied Science

at

Dalhousie University
Halifax, Nova Scotia
August 2024

Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

Table of Contents

List of Tables

List of Figures

Abstract

Pregnancy is a volume overload state accompanied by functional cardiac hypertrophy. Interestingly, cardiac remodeling in pregnancy is distinct in its absence of myocardial fibrosis, which is a common characteristic of pathological cardiac hypertrophy. Collagen-specific remodeling of the myocardium can be discerned by decellularization – the removal of cellular components whilst preserving the extracellular matrix (ECM). Unfortunately, decellularization techniques, such as freeze-thawing and detergents, have the potential to damage the structural-mechanical integrity of the ECM, particularly the collagen network. The objectives of this study were (i) to characterize myocardial remodeling throughout pregnancy & post-partum in the maternal bovine, and (ii) to characterize myocardial collagen remodeling throughout pregnancy & postpartum by selecting a decellularization technique that preserves collagen structure and mechanics.

Fresh hearts from pregnant and previously pregnant cows were dissected, excising the left ventricle. Slices were obtained from the middle-third of the left ventricular free wall. Uniaxial tensile testing was performed to measure tissue extensibility, ultimate strength, and high-strain stiffness. Collagen denaturation temperature, load-decay half-time, and immature crosslink index were measured using hydrothermal isometric tension testing with sodium borohydride stabilization. Myocardial collagen content was quantified using the Sircol Collagen Assay. Samples were paired to assess the effects of freeze-thawing on collagen thermomechanical properties, and to assess the effects of Tergitol 15S9 decellularization on myocardial collagen.

This study has demonstrated mechanical adaptations of the maternal myocardium during pregnancy – significant increases in UTS and extensibility. Accompanying those transformations were reductions in myocardial collagen thermal stability and mature crosslinking. Together, myocardial remodeling in pregnancy is a functional process allowing the myocardium to expand to accommodate the blood volume overload state, without compromising structural-mechanical integrity. Importantly, this study suggests that myocardial remodeling leads to a permanent loosening of the myocardial collagen network in postpartum, with a persistent increase in extensibility and the replacement of mature with immature collagen crosslinks.

Additionally, freeze-thawing was found to reduce myocardial collagen thermal stability, but only in tissue from pregnant cows, whereas Tergitol treatment reduced myocardial thermal stability only in tissue from previously pregnant cows. Hence, the slackened collagen network in postpartum may result in protection from the deleterious effects of ice expansion during freeze-thawing, but increased permeability & susceptibility to the effects of Tergitol. Finally, this work suggests that Tergitol alone is not an effective decellularization agent, as cell nuclei were not completely removed following treatment.

This work has shown *for the first time*, pregnancy-induced collagen remodeling in the maternal bovine myocardium. Nevertheless, the long-term implications of myocardial collagen remodeling in pregnancy remain to be elucidated, and may offer insight into prevention and management of cardiac disease in parous women.

List of Abbreviations Used

ANOVA	Analysis of Variance
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared
CD	Circular Dichroism
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane
CHP	Collagen Hybridizing Peptide
CSA	Cross Sectional Area
DSC	Differential Scanning Calorimetry
ECM	Extracellular Matrix
EP	Early Pregnant
H&E	Hematoxylin & Eosin
HIT	Hydrothermal Isometric Tension
LP	Late Pregnant
LOX	Lysyl Oxidase
MMP	Matrix Metalloproteinase
PLA	Polylactic Acid
PP	Previously Pregnant
qPCR	Quantitative Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
SHG	Second Harmonic Generation
STE	Speckle Tracking Echocardiography
TEM	Transmission Electron Microscope
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
UTS	Ultimate Tensile Strength

Acknowledgments

This work would not have been possible without the contributions of many people. First and foremost, I would like to thank my supervisor Dr. Sarah Wells for her support. Her dedication to my learning, sharp attention to detail, and enthusiasm towards this project, contributed not only to the successful completion of this thesis, but to my personal growth as a researcher. I would also like to thank the members of my supervisory committee, Drs. J. Michael Lee and Geoffrey Maksym, for their invaluable feedback, advice, and words of encouragement throughout this process. I would like to thank Dr. Samuel Veres for serving as my external examiner for the defense. I would like to thank Darren Cole for his tireless help in keeping the lab running. I would like to thank Meghan Martin for all her guidance and assistance, and, most importantly, for being a friend. I would like to thank Lynn Thomas from Dal HistoCORE for embedding, cutting, and staining my samples for H&E. I would like to thank Dr. John Frampton for allowing me to use his lab space and equipment. I would like to thank Dr. Hessam Yaghoobi Koopaee for his assistance with using the mechanical tester and 3D printing the scaffolds. Thank you to the Natural Sciences and Engineering Research Council of Canada for their funding contributions towards this project.

I would also like to acknowledge the support of my friends and family throughout this process. I would especially like to thank my dad for always believing in me and being there for me to share a smile, laugh, music, conversation, or our personal favourite – coffee and walk. Finally, I would like to thank my mom who is able to understand everything I am feeling and know exactly how to help with just a single word – ***hi***.

Chapter 1 Introduction

1.1 The Myocardium

Myocardial Function

The myocardium is the muscular layer of the cardiac wall, responsible for the heart's pumping action. The myocardium autonomically contracts and relaxes 100,000 times per day, capable of minute adjustments in strength and rate [1]. During cardiac systole, the myocardium contracts causing the ejection of blood from the ventricles into circulation; during cardiac diastole, the myocardium relaxes allowing for the heart to fill with blood [1]. The rhythmic contraction and relaxation of the myocardium is effectuated by its cellular and extracellular components [2].

Myocardial Structure & Composition

The functional cells of the myocardium are the cardiomyocytes - 2-3 billion contractile cells occupying 70% of the myocardial volume [3]. Following birth, cardiomyocytes expand, rather than multiply, to allow for heart growth [1]. Cardiomyocytes are interconnected via intercalated discs, which are specialized gap junctions that allow for rapid transmission of electrical signals between neighbouring cells to elicit a coordinated contraction [3]. The contractile unit of the cardiomyocytes is the sarcomere, which is composed of thick and thin filaments, giving a striated appearance. It is the sliding of filaments that results in a muscle contraction [4]. Hence, the sarcomere length ranges from 2.2μm to 1.5 μm during the cardiac cycle, lengthening during diastole and shortening during systole [5].

Cardiomyocytes are enmeshed in a network of extracellular matrix (ECM) proteins, including elastin, proteoglycans, and collagen (Fig. 1.1) [6]. The ECM serves as a scaffold to influence the structure and function of the myocardium. ECM proteins, primarily collagen fibres, are interwoven around and between the cardiomyocytes [7], [8]. This network of collagen connects layers of cardiomyocytes together, and also interconnects adjacent cardiomyocytes [9]. ECM-cardiomyocyte adhesion is mediated through integrin proteins, which are transmembrane receptors located circumferentially along the

cardiomyocyte in regions between adjacent sarcomeres [9]. In this way, the ECM is linked to the contractile unit of the cardiomyocyte. Cardiomyocytes, together with ECM proteins, have an important role in determining myocardial mechanics [1].

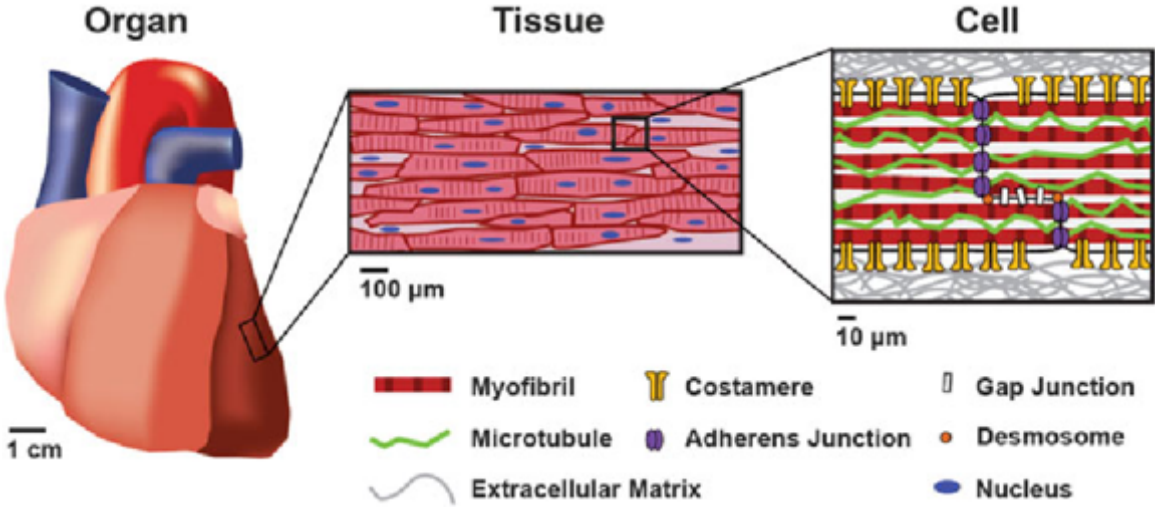

Figure 1.1. The myocardium is composed of cardiomyocytes enmeshed in dynamic meshwork of proteins (such as elastin, proteoglycans, glycoproteins, and collagens) that both provide structural support. Each cardiomyocyte contains repeating contractile units called sarcomeres, which give a striated appearance to the cell [10]. Reproduced with permission from Springer Nature.

ECM proteins are dynamic and serve a multitude of functions in addition to providing structural support, such as assisting in cell signalling via release of growth factors [11]. Cells attached to the ECM can sense changes in cardiac loading conditions and respond by remodeling of the ECM [12]. As cardiomyocytes are terminally differentiated cells with negligible regenerative capacity, ECM remodeling is a necessary response to myocardial injury [1]. Alterations in the abundance and organization of proteins within the ECM can result in major changes to the heart's mechanical properties, which has important implications for the management and prevention of cardiac diseases [11]. In particular, collagen has been implicated in both physiological [2], [7], [13]–[16] and pathological [17]–[21] myocardial adaptations.

1.2 Collagen

Structural Properties of Collagen

Collagen is the major structural protein of the ECM. Structurally, the collagen molecule consists of three polypeptide α-chains, left-handed coils rich in glycine and

proline amino acids [22]. A right-handed superhelix, stabilized by hydrogen bonds, is formed from the three α-chains, and this rod-like structure contributes to the mechanical stiffness and strength of the molecule. Indeed, collagen assists in maintaining tissue shape by providing a binding scaffold to cellular and extracellular components [22].

Collagen molecules are secreted from fibroblast cells and undergo a series of post-translational modifications [22]. These modifications include the hydroxylation of proline and lysine residues. Hydroxylation of proline increases the thermal stability of the superhelix through the formation of hydrogen bonds, whereas hydroxylation of lysine produces hydroxylysine residues, which provide sites for the formation of intermolecular cross-links [22].

The thermal stability of the collagen superhelix has been described by the polymer-in-a-box model (Fig. 1.2) [23]. At a certain temperature called 'denaturation temperature', the hydrogen bonds between alpha chains, in a region of the collagen molecule called the 'thermally labile domain' are disrupted, causing the molecule to unfold (Fig 1.2A) [23]. Thermal stability is affected by the packing of collagen molecules, with tightly packed molecules constraining the thermally labile domain (Fig 1.2B), which increases the denaturation temperature [23]. During conditions of collagen turnover or remodeling, the spacing between molecules is thought to increase, reducing thermal stability and leading to lower denaturation temperatures [23].

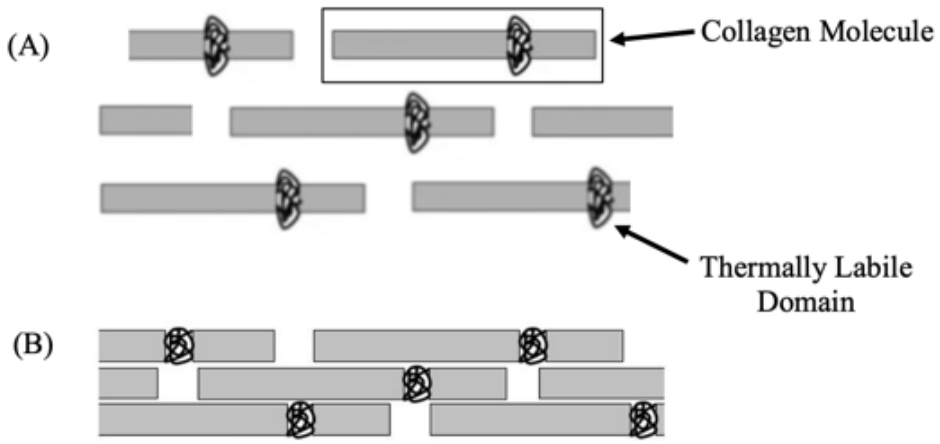

Figure 1.2 According to the polymer-in-a-box model (A) increased spacing between collagen molecules leaves the thermally labile domain unconstrained resulting in lower thermal stability, but (B) tighter packing of collagen molecules constrain the thermally labile domain and increases thermal stability [23]. Reproduced with permission from Elsevier.

Cross-links form between lysine residues on adjacent collagen molecules, in a reaction catalyzed by the enzyme lysyl oxidase (LOX) (Fig. 1.3). Divalent 'immature' cross-links may further react with a third collagen molecule, producing a trivalent 'mature' cross-link [22]. Cross-links enhance the connectivity within the collagen network, which delays molecular slippage under conditions of tensile strain; this results in increased stiffness and strength [22]. Alterations in collagen content, cross-links and density, affect mechanics.

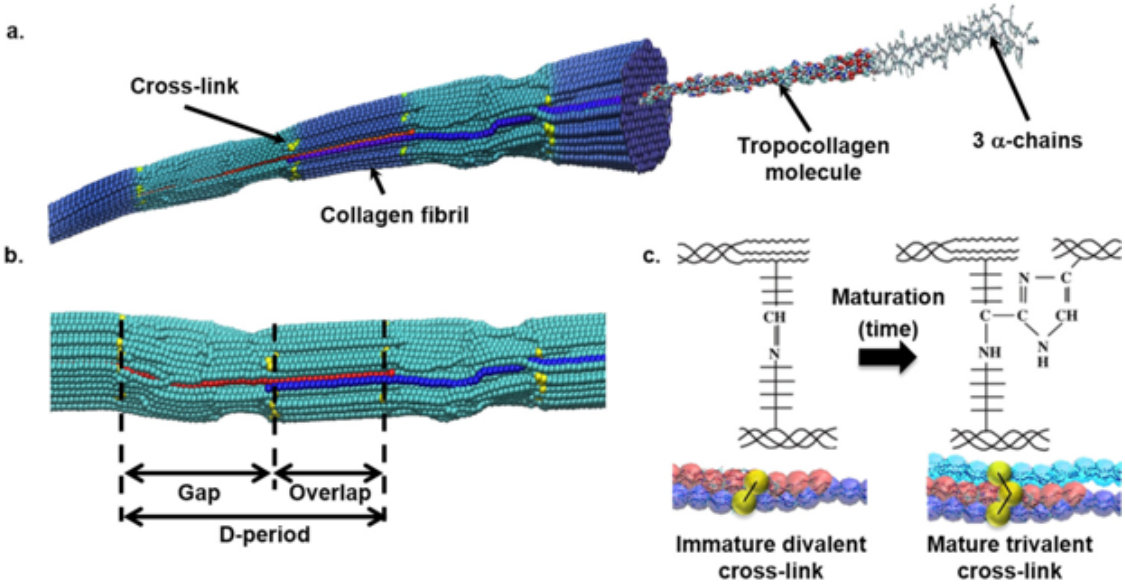

Figure 1.3 The collagen molecule, known as the 'superhelix', consists of 3 coiled α-chains stabilized by hydrogen bonds. Molecules self-assemble into collagen fibrils, with immature divalent crosslinks forming between adjacent molecules. As the tissue matures, immature crosslinks are enzymatically converted into mature trivalent crosslinks [5]. Reproduced with permission from Elsevier.

Collagen molecules 'self-assemble' into fibrils in a quarter-staggered arrangement, resulting in repeated regions of overlaps about 67 nm, called D-bands; fibrils further bundle to form collagen fibers, which often assume a wavy morphology termed 'crimp' [22]. The structure and arrangement of collagen result in both intrinsic and form birefringence, an optical phenomenon whereby light entering a material is refracted into two rays which may travel at different velocities [24], [25]. Intrinsic birefringence is derived from the anisotropic molecular structure of collagen [26]. Form birefringence is derived from the anisotropic arrangement of fibres in tissue, and accounts for 80-95% of collagen birefringence [26]. Birefringence is observed under polarized light microscopy, showing alternating light and dark transverse bands throughout collagen fibres, representing crimp

[27]. When collagen fibres have been straightened and crimp removed, the intensity of birefringence is reduced [27]. Birefringence intensity is also dependent on collagen fiber density and arrangement [26], [27].

Mechanical Properties of Collagen

Collagen mechanical properties are highly dependent on its structural properties. Collagen is stiff and strong when fibres are engaged, but in most soft tissues, fibres are crimped at low-to-moderate stresses [16]. Collagen crimp increases the extensibility of the fibers. As reflected in the 'toe' region of a typical stress-strain curve, collagen does not bear loads until the crimp has been straightened [28]. The toe region allows collagen to sustain large reversible deformations without rupture [15]. In general, highly aligned collagen fibres result in a stiffer tissue, whereas disoriented fibers contribute to a more extensible tissue [29]

Cross-links have significant effects on the mechanical properties of collagen fibrils, by delaying molecular slippage under conditions of tensile loading [14], [30]–[34]. As a result, collagen cross-links increase stiffness, strength, and toughness of fibrils [30]. Increasing cross-link density will increase fibril stiffness and strength through increases in the connectivity of the collagen network [30]. Crosslink maturity also affects collagen mechanical properties. A collagen fibril containing a density of 40% *mature* cross-links will have an ultimate stress of 0.75 GPa, whereas a fibril containing a density of 40% *immature* cross-links will have an ultimate stress of 0.50 GPa [30]. This differential behaviour is due to the enhanced intermolecular connectivity of mature trivalent cross-links compared to immature divalent cross-links.

There are several types of collagens, and the composition of the collagen network dictates tissue mechanical properties. In the cardiac ECM, collagen type I is the most common isoform and constitutes 85% of its volume [29]. Collagen I is characterized by more stiff, well-structured fibres, important for preventing overstretching of tissue by resisting tension. On the contrary, collagen type III, accounting for 11% of the cardiac ECM, is more compliant and is thought to be important for providing distensibility [35]. For example, reduced tissue compliance may be attributable to increases in total collagen content and in the Col I/Col III ratio [29]. Therefore, collagen remodelling, via alterations

in collagen types, will have significant effects on cardiac passive mechanics, and resulting function.

Role of Collagen in Ventricular Function and Disease

Collagen has a critical role in maintaining myocardial function. Collagen is aligned in parallel with cardiomyocytes, attaching via integrin proteins within the intercalated disc region of the cells [9]. During diastole, collagen fibers first uncrimp, then resist tension to allow the myocardium to expand without overstretching cardiomyocytes [36]. Studies on left ventricular endomyocardial biopsies have found that increased collagen crosslinking, assessed via colorimetry, was associated with increased myocardial stiffness and decreased ejection fraction [21]. A stiffened myocardium will be unable to sufficiently stretch to accommodate the blood, resulting in lower end diastolic volumes [21]. By contrast, rat hearts treated with collagenase exhibited increased left ventricular volumes at all pressures compared to control hearts (0.265 +/- 0.061 mL vs 0.203 +/- 0.061 mL at 5 mmHg) [37]. Indeed, collagen is an important contributor to the passive tension of the myocardium during diastole, particularly at larger deformations [13].

Further, collagen surrounds and interconnects adjacent bundles of cardiomyocytes, transmitting force and ensuring a proper cellular alignment to coordinate contractions [38]. Animal studies have found that myocardial collagen degradation reduces systolic force development, even when cardiomyocyte contractility is unchanged [39]. Human studies on left ventricular endomyocardial biopsies have found that myocardial collagen content is reduced by 28% in systolic heart failure patients, compared to healthy controls [40]. Further, myocardial protein expression of matrix metalloproteinases (MMPs), enzymes that degrade collagen, is significantly increased in patients with systolic heart failure, which is significantly negatively correlated with ejection fraction [40]–[42]. Thus, remodeling of the myocardial collagen matrix can also affect systolic performance, by reducing force generation capacity and weakening the myocardium [39].

Collagen Remodeling in Pathological Volume Overload

Volume overload refers to a state of increased blood volume, necessitating adaptation of the ventricle [43]–[46]. Volume overload raises end-diastolic ventricular wall

stress, which leads to eccentric cardiac hypertrophy: chamber enlargement disproportionate to myocardial mass [46]. Collagen remodelling serves initially as a *compensatory* response to pathological volume overload [43]–[46]. In eccentric cardiac hypertrophy, there is rapid collagen degradation, which increases chamber diameter and compliance, which precedes cardiomyocyte elongation [46]. Twelve hours following induction of volume overload, via aortocaval fistula, in rats, left ventricular end-diastolic diameter is increased by 20% and collagen volume fraction is decreased by 75%, but there is no change in cardiomyocyte length [46]. After 8 weeks, collagen volume fraction remains at 30%, whereas cardiomyocyte length increases just 9% compared to controls [46]. Stretch of cardiac fibroblasts is a mechanical stimulus that increases collagen degradation and may potentiate this rapid response to volume overload [47].

Mechanically, after 8 weeks of volume overload, induced via arteriovenous fistula, there is an increase in extensibility and a decrease in ventricular stiffness in rats, seen as a right-ward shift and decreased slope of the pressure-volume curve (Fig. 1.4) [45]. As the left ventricle is anisotropic, mechanical adaptations are more pronounced in the direction of the collagen fibers - the circumferential direction - compared to the longitudinal direction [44]. Taken together, collagen degradation during the acute stages of volume overload, increases the diameter and decreases the stiffness of the ventricle, to increase end-diastolic volume [46].

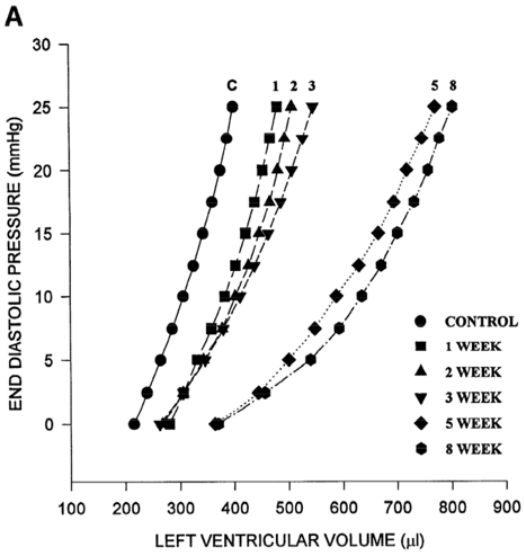

Figure 1.4 Left ventricular pressure-volume curves from rats showing mechanical adaptations to pathological volume-overload [45]. Reproduced with permission from The American Physiological Society.

Unfortunately, as volume overload progresses, collagen degradation contributes to *decompensated* ventricular failure due to reduced force transduction [48]. Further, ventricular wall thickness does not increase proportionally to diameter, increasing wall stress as per the Law of Laplace [48]. Therefore, chronic volume overload imposes a mechanical disadvantage on the ventricle, resulting in systolic failure. In sum, collagen remodeling has both compensatory and adverse effects in pathological volume overload.

1.3 Cardiac Adaptations to Pregnancy

Amazingly, volume overload in pregnancy is a physiological state, as the cardiovascular system is able to compensate for the increased blood volume without major adverse effects on the mother. [49]. Indeed, a 45% increase in blood volume is observed in the maternal vasculature, with expansion of plasma volume and increased number of blood cells to supply blood to the placenta [50]. There is an increase in peripheral vasodilation, resulting in a 25-30% decrease in vascular resistance [50]. Heart rate also increases, reaching its maximal in the third trimester [50]. These hemodynamic changes in the maternal vasculature begin early in pregnancy, with a 20% increase in cardiac output by eight weeks' gestation [51]. By term, volume expansion, decreased total peripheral resistance, and increased heart rate, contribute to a 40 % increase in cardiac output [51]. Pregnancy is a unique time to study cardiac remodeling, particularly of the collagen network, to elucidate the mechanism by which the maternal cardiovascular system adapts to volume overload for survival of both mother and child.

Ventricular Remodeling in Pregnancy

Pregnancy induces a functional cardiac hypertrophy, as the thickness and diameter of the ventricular wall are proportionately increased [52]. In human pregnancy, the left ventricle has been found to sustain a 15-25% increase in thickness beginning at 12 weeks of gestation [50]. By the third trimester, left ventricular mass is increased by 50% [52]. Cardiac hypertrophy in pregnancy occurs via a 12.4% increase in length of cardiomyocytes, enhancing the working capacity of the ventricles by reducing or maintaining wall stress

[53], [54]. Additionally, left ventricular end-diastolic volume is increased 10.5% during pregnancy, and is significantly positively correlated with cardiac output [54]. Therefore, hypertrophy of the left ventricle serves an important function for cardiac adaptation to volume overload in pregnancy – increasing cardiac output.

Studies on systolic function in pregnancy have been largely based on ejection fraction, a load-dependent parameter not entirely reflective of myocardial contractility, which has resulted in discrepant results [54]–[56]. As such, systolic function has been reported as slightly enhanced, slightly depressed, and unchanged [54]–[56]. Another parameter of systolic function, myocardial strain, can be assessed using speckle-tracking echocardiography (STE) to measure myocardial deformation in the longitudinal, circumferential, and radial dimensions [57]. STE studies show that longitudinal and circumferential strain is significantly reduced in pregnancy, beginning at the first trimester. Conversely, radial strain is significantly increased, balancing the changes in the other directions to preserve ejection fraction [57].

Diastolic function, which can be measured in terms of diastolic flow velocity, is dependent on myocardial relaxation and passive properties – size and stiffness [58]. In normotensive human pregnancies, left ventricular diastolic function is enhanced during the first trimester, with increased filling velocities, and then progressively returns to baseline throughout the remainder of gestation [57], [59]–[61].

In sum, left ventricular function is largely unaltered during pregnancy and increases in cardiac output are attributable to cardiac hypertrophy (increases end-diastolic volume), increased heart rate and decreased systemic vascular resistance [57].

Passive Mechanical Properties of Myocardial Tissue in Pregnancy

In addition to growth of the left ventricle, the passive mechanical properties of the myocardium are also altered and contribute to adaptation to volume overload in pregnancy, suggesting that collagen is being remodeled [62]. In late pregnancy, myocardial stiffness is decreased by 39% in the ventricles of pregnant rats compared to non-pregnant controls, developing lower stresses in response to extension [62]. Functionally, a less stiff and more extensible myocardium would increase the filling capacity of the left ventricle, accommodating for pregnancy-induced volume overload [63].

Ventricular Collagen Remodeling in Pregnancy

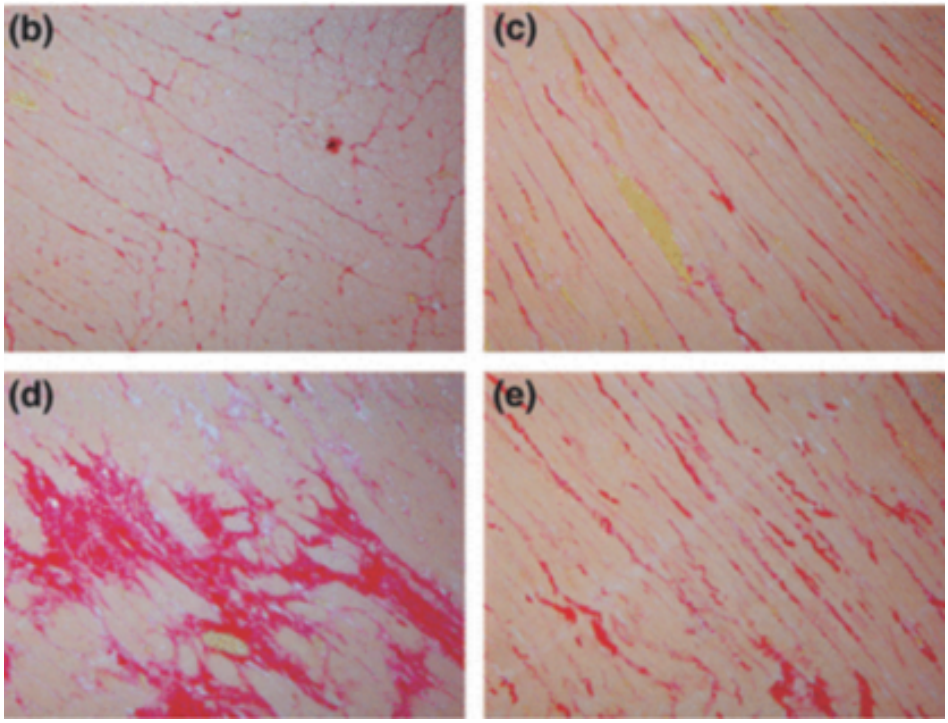

Figure 1.5 Picrosirius red stain of left ventricular myocardium exhibiting collagen content from (b) non-pregnant rats, (c) late pregnant rats, (d) non-pregnant rats treated with angiotensin II, (e) pregnant rats treated with angiotensin II [64]. Reproduced with permission from Wiley.

Importantly, the heart does not develop fibrosis during pregnancy, indicating that collagen content and crosslinks do not accumulate within the myocardium, which prevents stiffening of the ventricular wall that accompanies many forms of hypertrophy [53], [64]–[69]. Collagen content has been assessed using picrosirius red stain [64], [66]–[69] and Masson's trichrome stain [53], [65] to quantify the percent area occupied with collagen. Collagen content is unchanged at mid-pregnancy [53], [68], late pregnancy [53], [64]–[66], [68], and postpartum [65], [67]–[69]. In fact, pregnancy protects against the development of myocardial fibrosis (Fig. 1.5, downregulating fibrosis-related genes, which are highly expressed in pathological hypertrophy induced by angiotensin II [64]. This protection is significant, as the renin-angiotensin system is upregulated during pregnancy [70].

Quantitative polymerase chain reaction (qPCR) has been used to separately measure collagens I and III gene expression in the left ventricle during pregnancy [64], [71]–[74]. At mid-pregnancy, gene expression of both collagens I and III have been

reported as five-fold upregulated compared to baseline [71], [72]. At late pregnancy, both collagen isoforms have been reported as either upregulated [71], [72] or unchanged [64], [73]. Only one study reported that collagen III gene expression is downregulated (0.493-fold-changed) in late pregnancy [74]. In postpartum, collagen I has been reported as upregulated [72] or unchanged [71], [73], whereas collagen III has been reported as upregulated [72], [73] or unchanged [71], [74]. Regardless of these discrepant findings, changes in collagen gene expression are not always reflected at the protein level, due to post-transcriptional modulations, and are thus not fully indicative of tissue remodelling.

At the protein level, western blots have been used to measure collagens I and III isoforms in the left ventricle during pregnancy [71], [73]. One study on rats (n=10 per group) found that, compared to baseline, collagen I is 50% downregulated at mid-pregnancy, late pregnancy, and postpartum; whereas collagen III is 50% upregulated at mid-pregnancy and late pregnancy, returning to baseline in postpartum [71]. By contrast, another study on mice (n=4 per group) reported that levels of both collagen isoforms were unchanged in late pregnancy and postpartum [73]. Inconsistent findings may be due to sample size and/or different animal models. Nonetheless, a decrease in the col I/col III ratio within the left ventricle [71], may underlie the decreased passive stiffness seen in late pregnancy [62]. Taken together, although pregnancy does not result in myocardial fibrosis, studies on changes in collagen expression throughout gestation have been inconclusive.

The absence of fibrosis in pregnancy-induced cardiac hypertrophy suggests that collagen remodeling is tightly regulated throughout gestation. Collagen remodeling can be indirectly studied by evaluating the expression of MMPs [72]–[75] and their endogenous inhibitors - TIMPs [76]. At the transcriptional level, gene expression of cardiac MMPs (MMP1, MMP2, MMP9) is unchanged from mid-pregnancy to postpartum, whilst TIMP1 expression is significantly increased [72], [73].

By contrast, at the protein level in late pregnancy, left ventricular expression of MMP-1, MMP-2, & MMP-9 is decreased by 30%, 35%, and 50% respectively [75]; this is associated with significantly increased expression of TIMP-4 and TIMP-1, with all values returning to baseline values at postpartum [74], [75]. Taken together, it appears that collagen degradation is suppressed in late pregnancy, and then returns to baseline or is upregulated in postpartum.

The full extent of myocardial collagen remodeling in pregnancy-induced volume overload and its effects on myocardial mechanical function remains unclear. Collagen content is not the only determinant of tissue biomechanical properties. Alterations in collagen crosslinks and crimp also have substantial effects [15], [29], [30], but have not been studied in the myocardium during pregnancy. Further, collagen remodeling in early pregnancy – a period of major hemodynamic changes - has not been characterized. However, collagen remodeling during pregnancy has been studied in other cardiovascular tissue – heart valves, pericardium, & aorta – which must also adapt to accommodate volume overload.

Valvular Remodeling in Pregnancy

Pregnancy has consequences on other aspects of cardiac structure and mechanics, such as heart valves [77]. Heart valves experience increases in leaflet surface area with pregnancy, accompanying the enlargement of ventricular chambers and valve orifices [78], [79]. As per the Law of Laplace, a rise in leaflet surface area increases the stresses on the valves. When stress exceeds the strength of a material, it results in deformation and/or failure. However, despite substantial increases in leaflet surface area, the valves do not typically experience failure during pregnancy [79]. The adaptation of heart valves to the hemodynamic changes in pregnancy is believed to be owed to an extensive physiological remodeling process [77]–[81].

The mechanical properties of bovine heart valves appear to follow a biphasic pattern during pregnancy; biaxial tension tests on bovine valve leaflets have shown that, in the mitral, aortic, and pulmonary valves, there is a sharp drop in extensibility in early pregnancy, followed by a linear increase through the remainder of gestation [77]–[79]. However, the tricuspid valve exhibited no changes in extensibility, likely due to a diminished remodeling as it experiences relatively lower loading conditions [78], [79]. Although one may expect that the same mechanisms underly the adaptations of all valves, unique loading conditions stimulate unique remodeling.

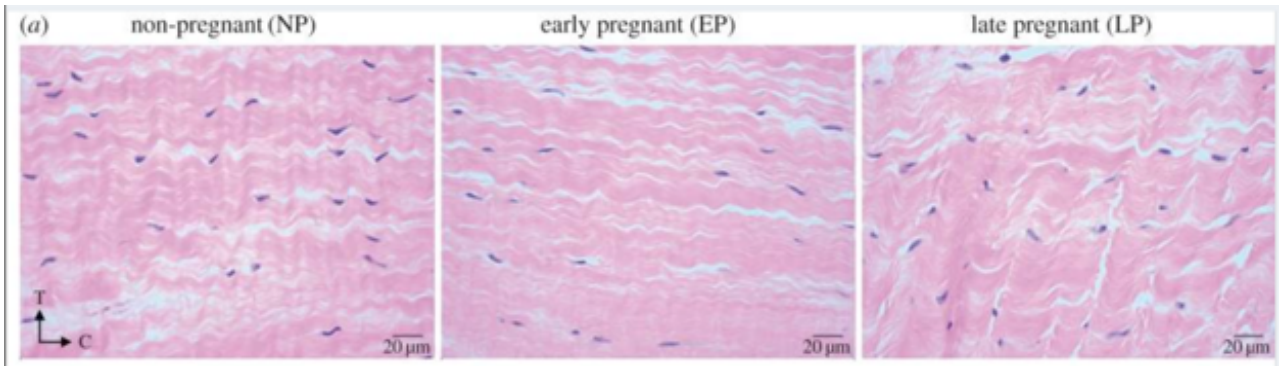

Figure 1.6 Histological images of mitral valve leaflets obtained from non-pregnant, early pregnant, and late pregnant bovine hearts [82]. Reproduced with permission from Royal Society.

Pregnancy-induced remodeling of the collagen network has also been studied and compared amongst the four valves. Valvular collagen content is increased between 8-26% during pregnancy [81]. Interestingly, the increases in collagen content throughout pregnancy coincide with increases in mature collagen crosslinks, as load decay half-time was increased between 58-125% [78], [79]. Therefore, pregnancy seems to be a unique stimulus for rapid collagen maturation [78], [79].

The structure of collagen within the valve leaflets is also altered during pregnancy, with mechanical consequences. Histological analysis of the bovine mitral valve shows that in early pregnancy, collagen fibres appear less crimped, whereas by late pregnancy, collagen architecture has recovered to resemble its non-pregnant morphology more-closely, but has a longer wavelength (Fig.1.6) [82]. As such, collagen crimp length increased by 216% (aortic), 186% (mitral), 42% (pulmonary), and 61% (tricuspid) during pregnancy [78], [79].

An innovative study proposed a model (Fig.1.7) by which, in early pregnancy, the mitral valve undergoes plastic deformation, with collagen fibers losing their crimp, as the tissue stretches to expand. Collagen remodels through the remainder of pregnancy, with new fibers being added in series, to recover crimp and homeostasis [82]. This model accounts for the initial decrease in extensibility – collagen fibers are straightened and lose their crimp – followed by the linear increase in extensibility, as new collagen is laid down.

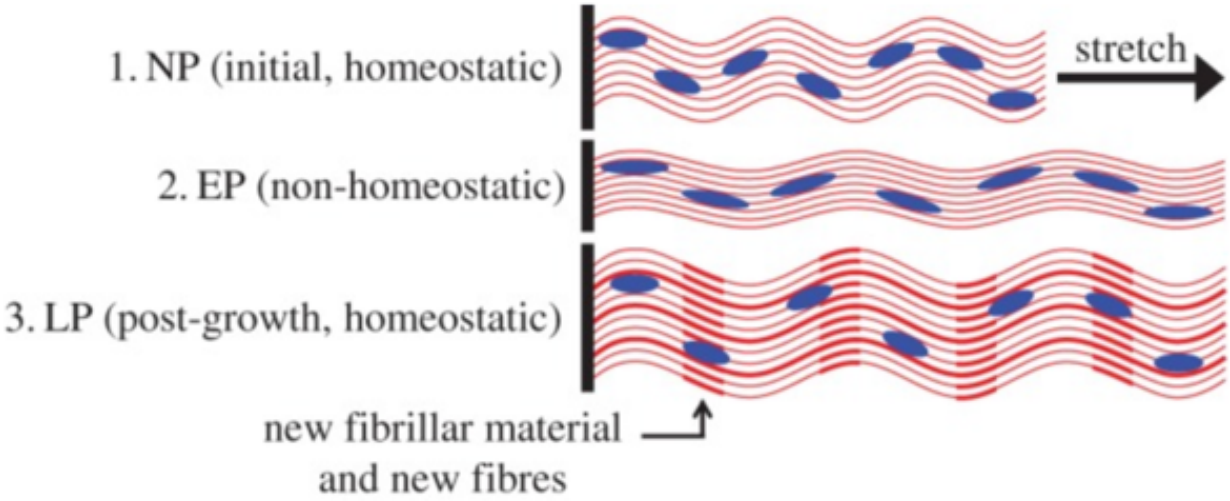

Figure 1.7 Model of valvular growth proposed by Rego et al., whereby plastic deformation is followed by serial addition of collagen allowing partial recovery of collagen crimp [82]. Reproduced with permission from Royal Society.

Pericardial Remodeling in Pregnancy

Further studies have evaluated adaptations in the pericardium during pregnancy. It provides mechanical protection to the heart due to its tough outer layer composed primarily of collagen. The outer layer is semi-rigid, which prevents overfilling and overexpansion of the heart [83]. During pregnancy, the pericardium, which is also expanded, must adapt to restrain the hypertrophied heart [84]. The adaptation of pericardial mechanical properties is due to collagen remodeling.

Collagen remodeling in the pericardium, during pregnancy, shows similarities to the structural changes seen in the valves. In early pregnancy, there is a loss of crimp and organization of the collagen bundles [84]. By late pregnancy, the collagen architecture is somewhat recovered, exhibiting crimp and laminar organization comparable to the pericardia from nonpregnant cows (Fig.1.8) [84]. Further, as seen in the mitral, aortic, and pulmonary valves, there is an increase in total and in mature collagen crosslinking in the pericardium during pregnancy (suggesting that immature collagen was being rapidly converted to mature collagen), which is strongly correlated with a decrease in extensibility [84]. However, unlike in heart valves, total pericardial collagen content and crimp *length* are unchanged during pregnancy [84]. Taken together, it appears that the pericardium experiences collagen remodeling during pregnancy, increasing collagen crosslinks so as to maintain functional integrity.

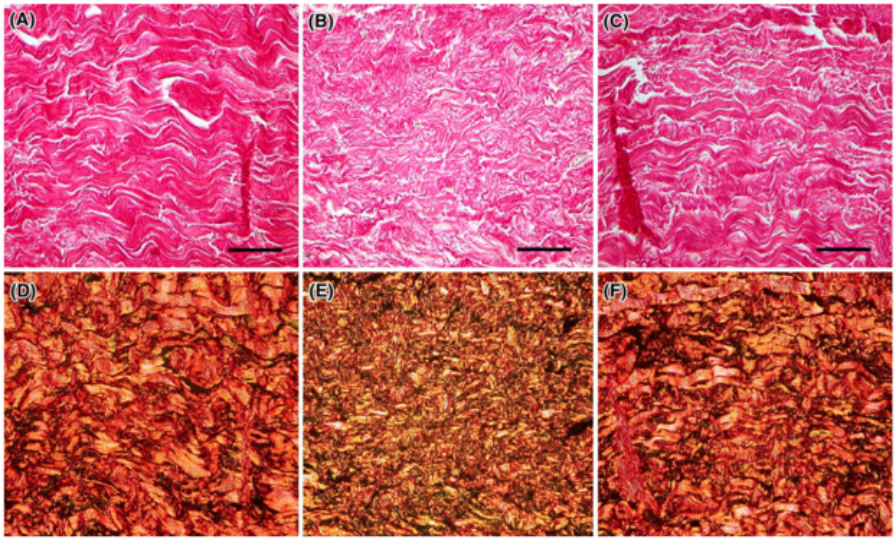

Figure 1.8 H&E (top) and picrosirius red (bottom) stains from non-pregnant (A,D), early pregnant (B,E), and late pregnant (C,F) bovine pericardium [84]. Reproduced with permission from Springer Nature.

Aortic Remodeling in Pregnancy

The aorta is a large artery that receives oxygenated blood from the left ventricle [85]. The ECM of the aorta is rich in elastin, which confers elasticity, and collagen, which confers tensile strength [85]. The elasticity of the aorta confers a Windkessel effect; during ventricular systole, the aorta expands and stores 50% of the stroke volume. During ventricular diastole, the aorta recoils to dispel the stored blood forward in the systemic circulation, ensuring a continuous flow of blood throughout the body [85]. Therefore, increased stroke volume, which increases wall stress, should trigger aortic remodeling to accommodate for volume overload in pregnancy.

Indeed, in the bovine cardiovascular system, there is a 35% increase in aortic circumference, which is accompanied by wall thinning and a 53% increase in elastin content [86]. Thus, just as the hypertrophic ventricle allows for an increased end-diastolic volume during pregnancy, the remodeled aorta can also store and eject the increased stroke volume [51], [86]. As in the pericardium, aortic collagen content was unchanged during pregnancy, though there was a trend towards a lower denaturation temperature [86]. Interestingly, the changes in aortic dimensions and elastin content were not reversed postpartum, the long-term implications of which have not been elucidated [86].

The mechanical adaptations of the bovine aorta in pregnancy [87] does not follow the pattern observed in the pericardia [84] and heart valves [79]. Unlike the pericardia and

heart valves, which must preserve structural integrity in response to volume overload, the aorta must also increase distensibility. As such, in early pregnancy, aortic extensibility is increased 13% and the elbow strain, which represents the load at which collagen fibers become straightened and engaged, is increased 15% [87]. This is reflected by a rightward shift in the stress-strain curve [87]. In late pregnancy, aortic extensibility and elbow strain recover to non-pregnant values [87]. Therefore, changes in aortic mechanical properties suggest that collagen is rapidly remodeling to allow for increased blood volume in early pregnancy. Aortic circumference gradually increases through gestation, which allows collagen to reassume its pre-pregnancy properties near term.

Reversibility of Remodelling

It is established that the maternal cardiovascular system undergoes remodelling during pregnancy; it is less clear, however, whether pre-pregnancy structural and mechanical properties are fully recovered postpartum. The question of the reversibility of remodelling is relevant, as it may help explain cardiovascular phenomenon observed later in the mother's life: cardiovascular health during pregnancy is predictive for risk of cardiovascular events [88].

Rodent studies suggest that cardiac remodeling is reversible. For example, investigations using a rat model identified that heart weight and cardiomyocyte length, were reverted to basal values by 7 days postpartum [62]. Reversibility of cardiac hypertrophy has also been observed in a mouse model of pregnancy [65]. Mechanically, myocardial stiffness returns to pre-pregnant values by 7 days postpartum, in rats [62]. However, these studies were limited by their time intervals, animal models, and biomechanical testing. Seven days may not be an appropriate length of time to obtain true postpartum measurements. Further, samples should be compared to nonpregnant animals to ensure a proper baseline condition. Many studies use a rat model, due to the short gestation time of 21 days. However, rodents differ from humans in terms of both gestation time and heart size.

A bovine model, which has previously shown unrecoverable changes in the aorta, including a 0.9℃ reduction in collagen denaturation temperature [86], may provide a more accurate representation of remodeling in pregnant human myocardium, as their gestation

time is the same length [86]. Larger specimens facilitate mechanical testing. In addition, studies on myocardial adaptation to pregnancy often measured only size and did not evaluate the more intricate structure of the collagen matrix.

Gross observation from bovine hearts suggests that profound, and perhaps unrecoverable, changes occur in the myocardium during pregnancy. Compared to hearts from (never pregnant) heifers, hearts removed from (previously pregnant) cows appear to be enlarged when empty, with an accompanying change in texture, becoming flaccid. There is a statistically significant difference in heart mass and volume between heifers (2.12 ± 0.12 kg; 2.26 ± 0.13L), and cows (3.07± 0.3 kg; 3.11± 0.26L) [86]. Thus, myocardial remodeling may be irreversible, which could underlie the link between pregnancy-related cardiovascular complications and future cardiac health.

1.4 Tissue Decellularization

1.4.1 Purposes of Tissue Decellularization

Tissue decellularization is a technique to eliminate cells and their genetic materials from tissue, whilst retaining the extracellular matrix (ECM) [89]. There are many applications for tissue decellularization, particularly in relation to xenogeneic transplantation. The removal of cellular material permits implantation without provoking immune recognition to antigens, inflammation, and ultimately rejection [89]. Another important application for tissue decellularization is for distinguishing between the contributions of cells and the extracellular matrix to mechanical properties [90]. To study collagen remodelling and its contribution to the passive mechanical properties in myocardium with volume overload in pregnancy, it may be useful to remove the cellular components.

Decellularization Techniques

Decellularization techniques have been developed due to endeavors in tissue engineering. In general, decellularization is achieved by disrupting the membrane to cause cell lysis, so that the cellular materials are released and rinsed from the tissue. These techniques involve combinations of physical, chemical, and biological methods that extract

cells from the cardiac ECM. Unfortunately, the process of decellularization has the potential to damage the structural-mechanical integrity of the ECM, particularly the collagen network. The ideal protocol will optimize cell removal, whilst preserving the structural and mechanical integrity of the natural ECM [90]. The methods used to characterize the effects of decellularization on collagen are discussed in more detail below.

Characterization of Decellularized Tissue

Studies have characterized the structural effects of decellularization at the molecular, fibrillar, and fiber level of collagen. At the molecular level, the collagen hybridizing peptide (CHP), circular dichroism (CD) spectroscopy, and attenuated total reflectance – Fourier transform infrared (AT-FTIR) spectroscopy have been utilized to assess the collagen triple helix. CHP is a synthetic peptide that mimics the triple helix structure and hybridizes with unfolded collagen strands [91]. Fluorescent labelling of CHP permits identification of denatured collagen [91], [92]. CD spectroscopy and FTIR-ATR spectroscopy measure the absorbance differences and vibrational patterns, respectively, of the collagen triple helix [93][94]. Observation of these spectra and their peaks enables identification of the presence or absence of collagen secondary structure features [93].

Differential scanning calorimetry (DSC) and hydrothermal isometric tension (HIT) testing are techniques that exploit collagen thermal properties for analysis of its molecular structure. DSC measures the heat change associated with thermal denaturation under a constant heating rate [95]. Denaturation is an endothermic process, requiring heat energy to break hydrogen bonds within collagen molecules. DSC measures the amount of energy required to denature collagen molecules and identifies the temperature at which it occurs [95]. Similarly, HIT testing is conducted by heating collagen whilst under isometric tension. In this test, the denaturation temperature refers to the temperature at which the load increases, due to the contraction of collagen upon breaking of the hydrogen bonds [95].

Electron microscopes are imaging modalities used to assess collagen fibril and fiber structure. The transmission electron microscope (TEM) emits electrons through thin samples to visualize collagen fibril inner structure, including D-banding periodicity [96]. The scanning electron microscope (SEM) utilizes electron beams to obtain high resolution images of a sample's surface [96]. It can show topographical and morphological

information of collagen fibers [96]. These microscopes have high resolution and magnification, but the utilization of electron beams may alter or damage samples [96].

Second harmonic generation (SHG) microscopy is a powerful imaging tool that utilizes the intrinsic properties of collagen and therefore does not harm samples [97], [98]. SHG is used to visualize collagen fibrillar and fiber structure, by passing photons from a laser through tissue [97]. Due to the non-centrosymmetric structure of collagen molecules, the photons are combined and converted to an SHG signal [97]. The intensity of the SHG signals depends on the concentration and organization of fibrillar collagen [98]; SHG can also be used to assess fiber orientation pre- and post-decellularization, by quantifying the coherence, which is the phase relationship between the SHG signal and the laser [97]. Coherence values range from 0, indicating absence of a dominant fiber orientation, to 1, indicating a dominant fiber orientation [97], [98]. Hence, SHG microscopy provides information on collagen fibrils and fibers without damaging tissue.

These techniques have all been employed to assess collagen structural features before and after decellularization. With that, the effects of decellularization on collagen are reviewed in detail below.

Effects of Freeze-Thawing on Collagen

Freeze-thawing is a physical method of inducing cell lysis that is often used as an initial step in tissue decellularization protocols [89]. However, studies suggest that this process is damaging to the collagen, due to the formation of extracellular ice [99], [100]. Indeed, freeze-thawing has been shown to result in a 1.4-1.6°C decrease in collagen thermal denaturation temperature, measured via DSC [100]. As collagen denaturation temperature is affected by packing and hydration levels, the expansion of extracellular ice likely disrupts molecular packing. Further, structural changes induced by freeze-thawing are exhibited at the collagen fibril level [99], [100] in posterior tibial tendons, there is a significant decrease in the number of collagen fibrils, whereas mean collagen fibril diameter is over 30% greater following freeze-thawing, and the intrafibrillar gap is increased by over 300% (Fig.1.9) [99]. Additionally, freeze-thawing has significant effects on tissue biomechanical properties, with the structural changes manifesting in 30% and

34% reductions in ultimate stress and ultimate strain, respectively [99]. Therefore, freeze-thawing damages the structural-mechanical integrity of collagen.

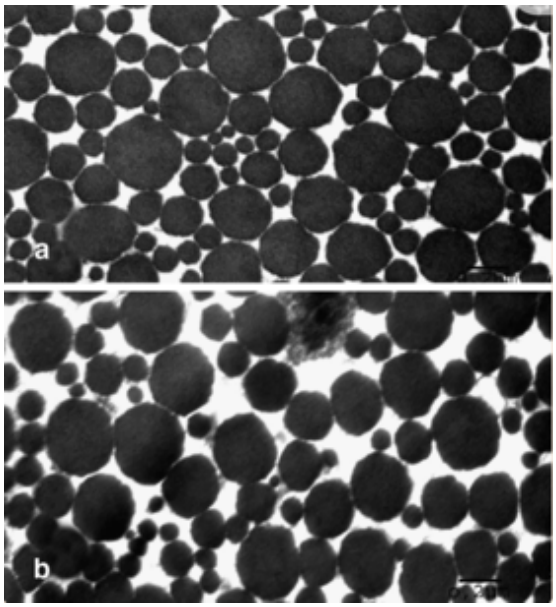

Figure 1.9 Cross-sectional TEM of fresh (a), and freeze-thawed (b) human posterior tibial tendons [99]. Reproduced with permission from Springer Nature.

Effects of Detergents on Collagen

Another common technique for decellularization is treatment with detergents. Detergents react chemically with the cell membrane to form pores and lyse the cell. Commonly used detergents are sodium dodecyl sulfate (SDS), 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), sodium deoxycholate, and Triton X-100, and their respective effects on collagen structure and mechanics were reviewed and summarized in table 1.

Briefly, SDS damages collagen at all levels of the structural hierarchy, including by unfolding the triple helix[91], [101]–[104]. In addition, the mechanical properties are compromised due to loss of collagen, following SDS treatment [102], [105]. The detergents CHAPS and sodium deoxycholate are not as damaging as SDS, with the triple helix of collagen remaining largely folded [91]. However, these detergents are disruptive to collagen at the fibrillar and fiber levels, as observed with electron microscopy[102], [104]–[107]. Further, tensile strength is not affected by these changes, whereas other mechanical properties have not been fully assessed [102], [104]–[106]. Therefore, these detergents were deemed suboptimal for tissue decellularization that preserves collagen properties.

Decellularization using Triton X-100 showed discrepant results, due to the concentrations and exposure times. At high concentrations and long exposure times, Triton X-100 has damaging effects on collagen at all hierarchical levels [91], [101]–[103]. For example, at concentrations of 3%, Triton X-100 was shown to denature collagen as evidenced by CF-CHP [91]. At concentrations of 1-2% and exposure times greater than 24 hours, Triton X-100 causes disorientation and clumping of fibers [101]–[103].

However, at lower concentrations, 0.1-1%, Triton X-100 treatment showed preservation of the collagen network [108]–[112]. Protocols involving 0.1-1% Triton X-100, protease inhibitors, endonucleases, and alternating hyper/hypotonic shock, are highly established in tissue decellularization [108]–[112]. A summary of the findings is reported in Table 1.1. This method has been used on cardiac tissue such as pericardia [108], [110]and valves [111], [113]. Studies show that the molecular structure of collagen is preserved, as evidenced by DSC and HIT [108], [110]–[112]. Fibrillar structure is also maintained, with the characteristic D-banding pattern appreciated on TEM [110], [111]. Observations on collagen fiber-level structure, and mechanical properties are inconsistent. Overall, collagen crimp appears to be maintained, but there may be slight disorganisation and clumping of the fibers [108], [112]. Similarly, the results of mechanical testing are discrepant with some studies showing preservation of mechanical integrity [108]–[110], whilst other show a loss of tensile strength [111], [112]. Although Triton X-100 has been used for tissue decellularization, it has been found to produce endocrine-sensitive by-products [114]. Therefore, a new detergent has been introduced as a replacement – Tergitol [112], [115]–[118].

Studies using Tergitol can be put in two categories - fresh and frozen tissue. When the decellularization was performed on freeze-thawed tissue, results were inconsistent, showing both preservation and disruption of structural-mechanical integrity [116]–[118]. On the contrary, when Tergitol-based decellularization was performed on fresh tissue, collagen structure at all hierarchical levels was consistently preserved, as were mechanical properties [112], [115]. Taken together, this literature review (Table 1.1) suggests that the detergent Tergitol can be used for tissue decellularization without disrupting collagen structural-mechanical properties.

Table 1.1. Results of literature search on the effects of decellularization detergents on collagen structure and mechanics.

Detergent	SDS	CHAPS	Sodium Deoxycholate	Triton X-100	Tergitol
Molecular Structure					
CF-CHP	X	✓	✓		
CD Spectroscopy	X	✓	X		
FTIR-AR	X	✓	X	✓	✓
DSC/HIT	X	✓	X	✓	✓
Fibrillar Structure					
SHG Intensity	X	X	X		✓
TEM	X	X	X	✓	✓
FTIR-AR				✓	✓
Fiber Structure					
SHG Coherence				~	✓
SEM	X	X	X	✓	✓
Histology	X	✓	✓	✓	✓
Mechanical Properties					
Ultimate Tensile Strength	X	✓	✓	~	✓
Stiffness	X	✓	✓	~	✓

Chapter 2 Rationale, Objectives, & Hypotheses

Pregnancy-induced alterations in structural-mechanical properties have been studied in several cardiac structures. However, the mechanical changes that occur in the myocardium remain unknown. It is probable that as seen in the heart valves and pericardia, functional adaptation in the myocardium will be accompanied by remodeling of its collagen network. The purpose of this study is to analyse the collagen architecture and mechanical properties of the myocardium as it adapts to the major hemodynamic changes in pregnancy and postpartum.

2.1 Characterization of Myocardial Remodeling in Pregnancy & Postpartum

Objectives:

The primary objective of this research was to characterize myocardial remodeling throughout gestation and post-pregnancy in a bovine model. Specifically, the aims were:

(i) To examine the mechanical properties of myocardium from pregnant and previously pregnant bovine.
(ii) To assess content, helical stability and crosslinking of myocardial collagen from pregnant and previously pregnant bovine.

Hypotheses:

It was hypothesized that the myocardium would remodel to accommodate the volume overload state of pregnancy and that the changes would not be fully reversed in the myocardium of previously pregnancy bovine. In particular:

(i) There will be an increase in myocardial UTS in pregnancy, which is reversed in postpartum.
(ii) There will be an increase in extensibility and a decrease in stiffness modulus throughout pregnancy, which are not fully reversed in post-pregnancy.

(iii) There will be no difference in myocardial collagen content between pregnancy and postpartum.

(iv) There will be a decrease in collagen denaturation temperature & mature crosslinks, and an increase in immature cross-link index throughout pregnancy, which are not fully reversed in post-pregnancy.

Rationale:

In pregnancy, myocardial UTS must increase to sustain elevations in wall stress [68]. Further, extensibility must increase, and stiffness modulus must decrease to maintain the diastolic function of the ventricle. Early increases in extensibility have been observed in the pregnant bovine aorta [87] and in left ventricular pressure-volume curves during acute volume overload [45]. Further, the left ventricles of pregnant rats have a decreased stiffness [62], and acute volume-overload in the rat decreases ventricular stiffness by 257% after 8 weeks [45]. Extensibility recovers to pre-pregnant values in the aorta [87] and Young's modulus recovers in the rat left ventricle [62]. However, gross observation of bovine hearts reveals flaccid tissue in the previously pregnant animals [86] and multiparous women have significantly elevated left ventricular end-diastolic volume index and cardiac output compared to nulliparous women [119]. These findings suggest that mechanical changes in the pregnant myocardium are not fully recovered, and are augmented with subsequent pregnancies.

Previous studies have found that myocardial collagen content is unchanged throughout pregnancy and postpartum [53], [64], [73]. Nonetheless, myocardial growth and collagen turnover during pregnancy are expected to decrease the packing of collagen molecules, which reduces thermal stability [23]. Given that newly synthesized collagen is laid down in its immature form [22], it is expected that the ratio of immature to mature crosslinks will increase. Based on findings that multiparous women have significantly increased left ventricular mass indexes compared to nulliparous women [119], and that hearts from previously pregnant cows remain flaccid [86], it appears that structural changes to the myocardium are not fully recovered, and are augmented with subsequent pregnancies.

2.2 Selecting & Characterizing a Decellularization Technique

Objectives:

The second objective was to study myocardial collagen remodeling in gestation and post-pregnancy, using a decellularization technique for bovine myocardium, that seeks to preserve collagen structure and mechanics. In particular, this research aimed:

(i) To assess the effects of freeze-thawing on the helical stability and connectivity of the myocardial collagen network.

(ii) To conduct a literature search to characterize the effects of detergents on collagen structural and mechanical properties.

(iii) To assess the effects of Tergitol on myocardial mechanical properties.

(iv) To assess the effects of the detergent Tergitol on the helical stability and connectivity of the collagen network.

Hypotheses:

Decellularization using the reagent Tergitol on fresh tissue, will preserve the thermomechanical and mechanical properties of myocardial collagen. Specifically:

(i) Freese-thawed tissue will exhibit decreased denaturation temperature and halftime of load decay, compared to fresh tissue.

(ii) Tergitol will have no effect on myocardial UTS, extensibility, nor stiffness.

(iii) Tergitol will have no effect on the collagen denaturation temperature nor halftime of load decay.

Rationale:

Previous studies have found that freeze-thawing has induced structural changes to collagen fibrils, such as increased fibril diameter and intrafibrillar gaps [99], [100]. Thermal testing using DSC has revealed a significant decrease in denaturation temperature post freeze-thaw [100] . Therefore, it is hypothesized that the thermomechanical properties will similarly be affected, with decreases in denaturation temperature and half-time of load decay, as compared to fresh tissue.

Tergitol has been used to effectively decellularize cardiac tissue, including myocardium [112], [115], [117], [118]. Characterization of the effects of this detergent on collagen has been employed via DSC, SHG, FTIR-ATR, electron microscopy, and uniaxial tensile testing, with favourable results. The effects of Tergitol on myocardium, have previously been characterized with SHG, suggesting that there are no effects on fibrillar nor fiber structure of collagen [117], [118]. Therefore, it is hypothesized that decellularization of bovine myocardium with this detergent with also have no effects on the thermomechanical and mechanical properties of collagen.

Chapter 3 Experimental Methodology

3.1 Tissue Collection and Dissection

The tissue harvesting protocol was approved by the University Committee on Laboratory Animals at Dalhousie University. Hearts were harvested and obtained from Curtmar Meats, a local abattoir in Shubenacadie, Nova Scotia, from pregnant and previously pregnant cows, fresh from slaughter. The gestational age of the pregnant cows was determined by measuring the crump-to-rump length (CRL) of the fetus and using the following equation [120]:

$$\text{Gestational Day} = 8.4 + 0.87(\text{CRL}) + 5.46(\text{CRL})^{1/2} \qquad 3.1$$

The hearts were transported back to the laboratory in a chilled cooler and were then freshly dissected. Myocardial samples were prepared, as described by Ghaemi et al [121]. Briefly, hearts were rinsed to clear the remaining blood. The base and apex of the heart were excised by making transverse slices with a large kitchen knife (Fig 3.1A). Subsequently, the right side of the heart was excised by cutting through the sagittal plane (Fig 3.1B) and the left ventricle was unfolded by making a longitudinal incision through the interventricular septum (Fig 3.1C). Finally, a large block was cut from the left ventricle (Fig 3.1D).

Myocardial slices were prepared by placing the endocardial surface of the left ventricular block against the gauge plate of CUSIMAX meat cutter. Tissue slices of 1-2 mm thickness were sliced from the middle third of the left ventricular free wall (Fig 3.2) [121]. Tissue slices were laid flat against a cutting board and, using a surgical scalpel, rectangular myocardial strips, 5 x 20mm, were cut along the circumferential direction of the tissue. The orientation of muscle fibres on each slice can be visualized with the naked eye and therefore, care was taken to cut along the direction of the muscle (Fig 3.2).

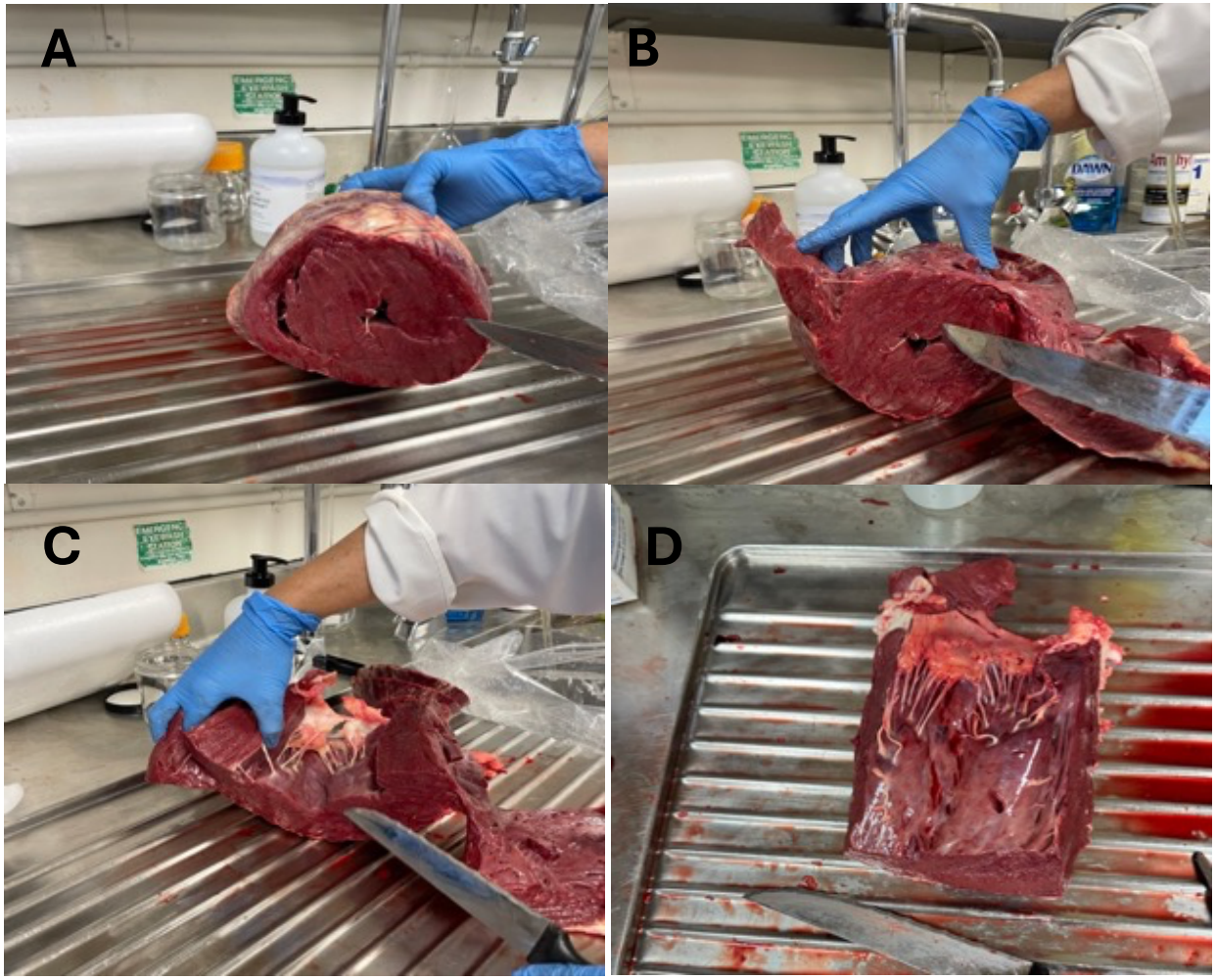

Figure 3.1A. Using a cutting board and large kitchen knife, transverse slices were made to excise the apex and base of the heart. **B.** An incision was made through the sagittal plan, permitting excision of the right side of the heart. **C.** A longitudinal incision was made through the interventricular septum so as to unfold the left ventricle**. D.** A large block was cut from the left ventricle, which was then placed on the meat slicer [121].

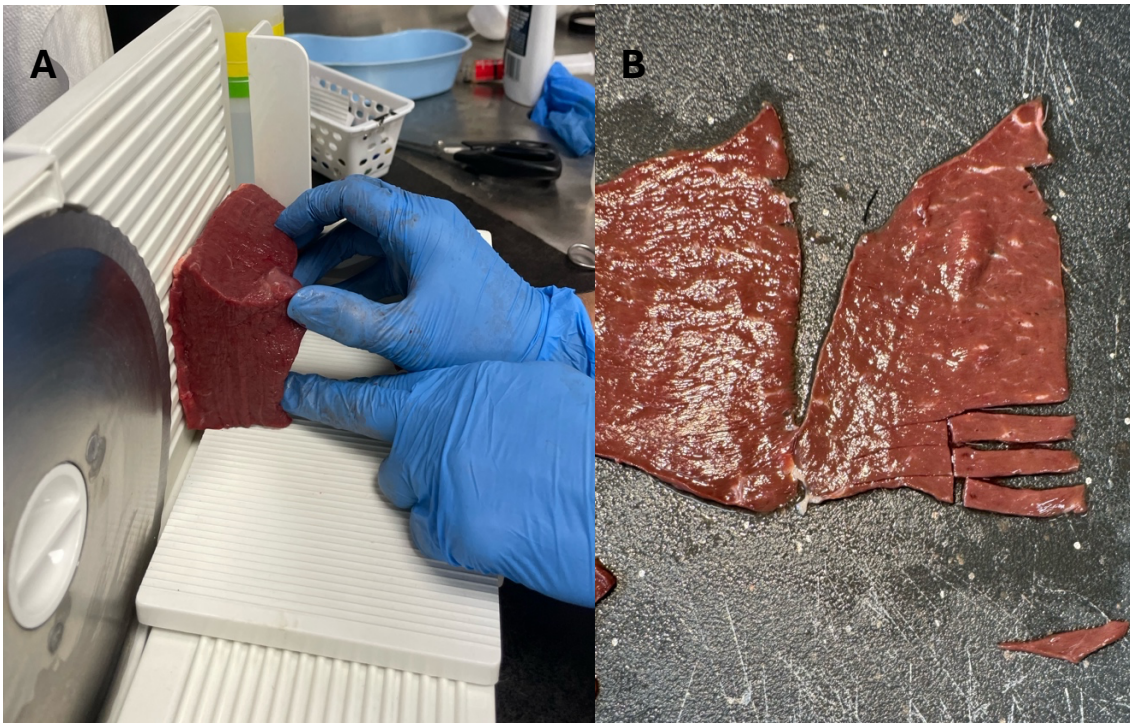

Figure 3.2 A. With the endocardial surface laid against the gauge, tissue slices were taken with a meat cutter. **B.** Rectangular strips of bovine myocardium, 1-2 mm in thickness, were prepared.

3.2 Mechanical Testing

Setup and Failure Tests

Mechanical tests were performed using Series F + IntelliMESUR® Advanced Tension / Compression Force Tester in Dr. John Frampton's lab. Testing was performed at room temperature with a humidifier to provide moisture to the samples. Paired native and Tergitol-treated myocardial samples (prepared as described in Section 3.5) from 8 pregnant and 3 previously pregnant cows were subjected to mechanical testing. The cross-sectional area (CSA) of each tissue strip was calculated prior to testing, using digital calipers to measure the width and length of the samples. The calipers were opened wider than the sample and then closed around the sample, such that the jaws were touching but not compressing the tissue.

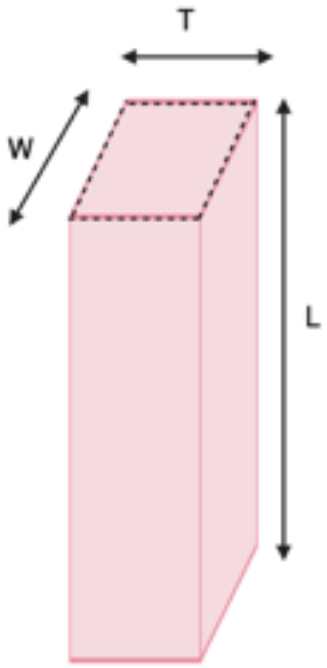

Figure 3.3 Cross-sectional area was calculated by measuring the width (W) and thickness (T) of rectangular-shaped myocardial strips. Measurements for each dimension were taken, using digital callipers, at three equidistant regions across the specimen, with the mean value being used for calculation of the CSA. Created with BioRENDER.

Measurements were taking at three locations across the width and three locations across the length of the sample. The average width and thickness values were used to calculate the CSA. As the samples were rectangular, the CSA was defined as:

$$CSA = w\ x\ t \qquad 3.2$$

To prevent slippage of the tissue from between the grips, a custom scaffold was designed, and 3D printed in polylactic acid (PLA) filament (Fig 3.4). This scaffold both increased the friction between the tissue and grips to prevent slippage, and ensured that the initial gauge length for all samples was 10 mm. Tissue strips were superglued to the scaffold prior to mechanical testing (Fig. 3.4). Then, the ends of the tissue-scaffold were clipped into the jaw grips of the force tester and the scaffold was cut as shown in Figure 3.5. During tensile testing, the top grip, which was connected to a motor, moved upwards, transmitting load along the tissue.

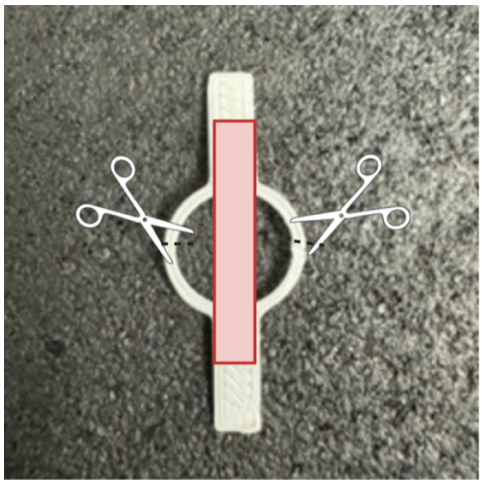

Figure 3.4 A scaffold was 3D printed. Myocardial strips were positioned such that the tissue went across the centre of the scaffold and the ends of the tissue were superglued to the arms of the scaffold. After clipping the tissue-scaffold into the jaw grips, the scaffold was cut, as shown. Created with BioRender.

Uniaxial stress-to-failure tests were conducted along the circumferential direction, which was aligned with the muscle fibers with a 10 N load cell, as described by Faggioli et al [117]. This allowed determination of the ultimate tensile strength, the extensibility, and the stiffness modulus of the myocardial strips (Fig 3.5). Tissue strips were stretched at a rate of 0.3 mm/second to failure. Tensile force, displacement and time data were recorded at a 40Hz sampling frequency. The data were exported to Excel for analysis.

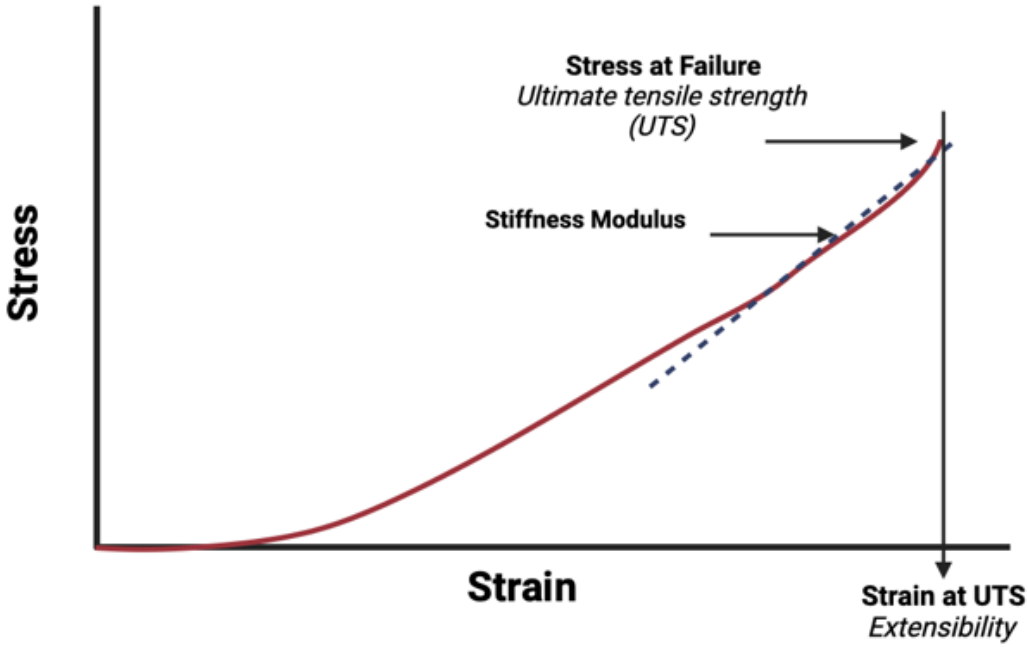

Figure 3.5 Example stress-strain curve of myocardial strip stretched to failure. Created with BioRENDER.

Data Analysis

Tensile force (F) and displacement data were exported into Microsoft Excel, and were converted to stress (σ) and strain (ε), values respectively. Stress-strain curves were plotted for each sample.

$$\sigma = \frac{F}{CSA} \tag{3.3}$$

$$\varepsilon = \frac{Displacement}{Initial\ Gauge\ Length} \tag{3.4}$$

The UTS was defined as the maximum stress achieved during the test and extensibility was defined as the strain value at the UTS (Fig. 3.5). The stiffness modulus was calculated for each sample using linear regression of the final 40% of ε, the portion where collagen is contributing to the load [117]. From this curve, ultimate tensile strength (UTS), extensibility, and stiffness modulus were derived (Fig. 3.5).

3.3 Thermomechanical Testing

Hydrothermal Isometric Tension (HIT) Testing

Myocardial strips were subjected to denaturation temperature tests (DTT) and hydrothermal isometric tension (HIT) testing, as described by Lee et al [122]. HIT testing is a thermomechanical experiment for determination of collagen thermal stability and crosslinking. Myocardial strips are held between two grips under isometric tension. The apparatus is submerged in a water bath, whilst time, tensile load and temperature are continuously recorded in a LabVIEW^{TM} 7.0 (National Instruments) VI.

During the initial segment of the test, the temperature of the water bath is increased from room temperature to 90°C, enabling determination of the denaturation temperature. The denaturation temperature is the temperature at which the hydrogen bonds within the collagen molecules are disrupted, resulting in unfolding of the collagen superhelix. A force is generated upon denaturation of the collagen molecules, as the sample is under isometric constraint. The thermally labile immature crosslinks are destroyed as the temperature rises to 90°C, such that only the thermally stable mature crosslink remain bearing tension in the tissue [123].

During the isothermal segment of the experiment, the temperature is held at 90°C for three hours. As the temperature is held at 90°C, peptide bonds are hydrolyzed causing chain slippage under isometric tension and the load to decay. Mature collagen crosslinks delay molecular slippage, resulting in a slower load decay. Therefore, the load decay halftime is an indicator of thermally stable collagen crosslinking [123].

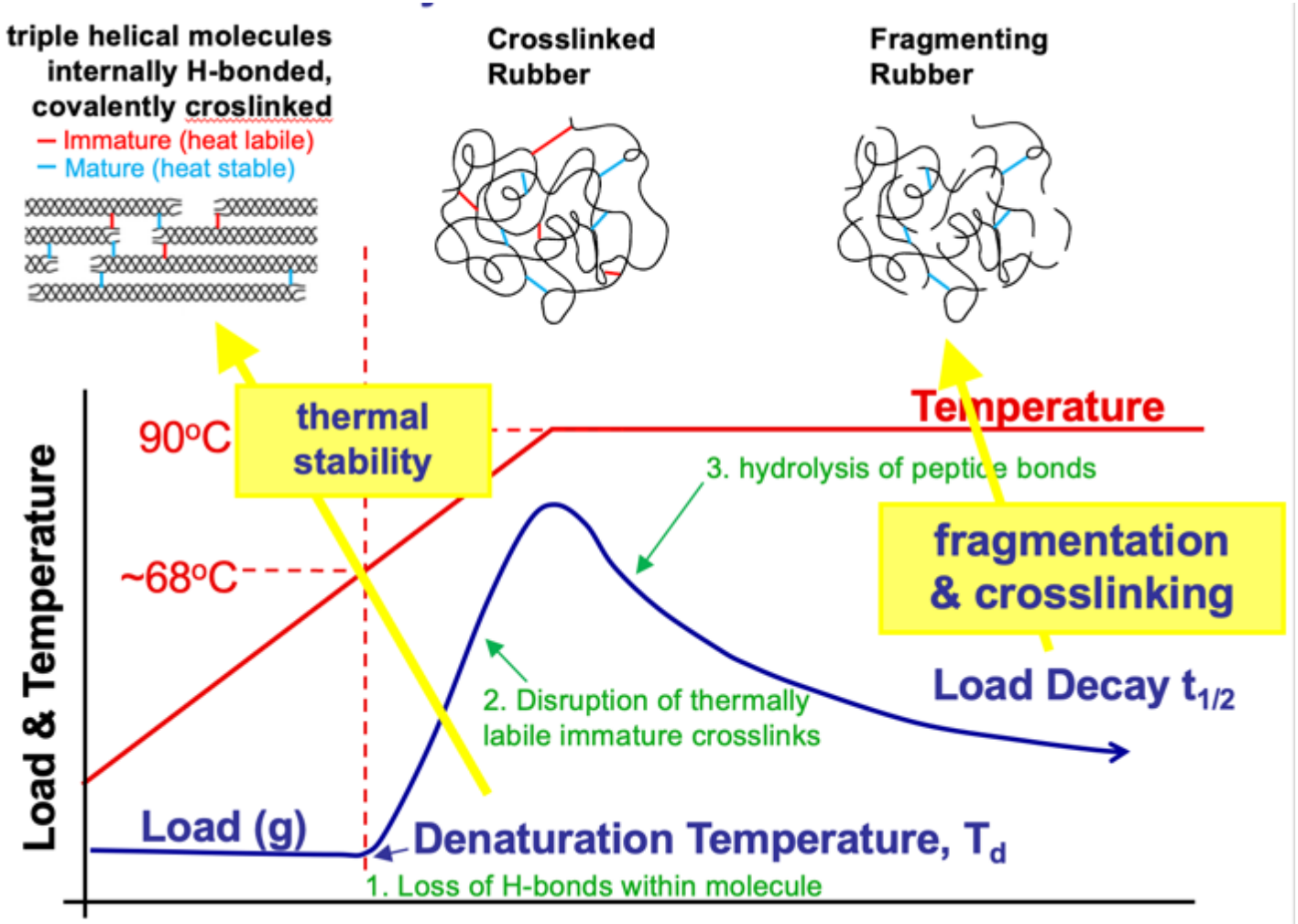

Figure 3.6 Typical HIT/DTT curve showing an inflection in load at the denaturation temperature where hydrogen bonds are broken within the molecule ('crosslinked rubber'), and load decay as the peptide backbone of the tissue is broken ('fragmented rubber'). The additional curve in green depicts the effect of NabH4 stabilization on immature crosslinks, slowing the rate of load decay. Image by Dr. Sarah Wells, October 2023.

Sodium Borohydride ($NaBH_4$) Stabilization

Sodium borohydride ($NaBH_4$) treatment is used to chemically stabilize thermally labile immature crosslinks via a reduction reaction [124]. Briefly, control samples were washed, for a duration of 15 minutes, with borate buffer (pH=9.0 ± 0.1), four consecutive times. Treatment samples were washed, for a duration of 15 minutes, with borate buffer containing 0.1mg/mL of sodium borohydride ($NaBH_4$), four consecutive times [77]. Reactions were performed under constant agitation at 4°C. Finally, all samples underwent a 15-minute rinse in PBS.

HIT Apparatus

The HIT testing apparatus [122] (Fig.3.7), is a custom-built stainless steel base plate with 6 strain-gauged cantilever load cells (Vishay Micro-measurements). Hooks are attached to each load cell, with the top hook being adjusted to apply tension to the tissue and the bottom hook remaining stationary. This apparatus is suspended in a 4L water bath

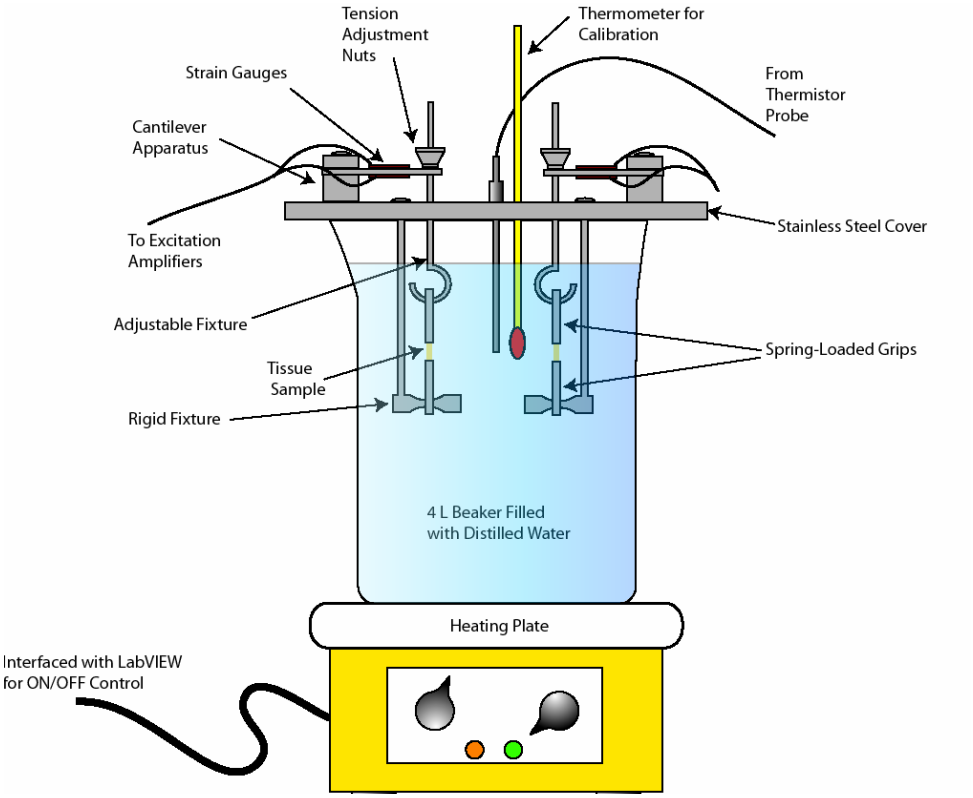

Figure 3.7 The HIT testing apparatus is a custom-built machine used for thermomechanical testing of tissue. Image by Dr. Peter Massaro, March 2007.

and heated on a heating plate. A thermistor probe is placed in the middle of the water bath for temperature monitoring. The apparatus is interfaced with a PC, with which load, temperature, and time data are recorded in a LabVIEW^{TM} 7.0 (National Instruments) VI, which was originally developed by Christopher Pereira. Conditioning amplifiers (A-tech Instruments Ltd., Scarborough, Ont.) were used to relay load and temperature data from the load cells and thermistor probe, respectively.

HIT Test Protocol

HIT tests were performed as described by Lee et al [122]. Briefly, a 4L beaker, filled with room temperature deionized H_2O was placed on the heating plate (Fig 3.7). On the HIT apparatus, Control and $NaBH_4$ treated myocardial strips were attached between grips and placed on the top load cell hook, with the bottom grips hanging freely. The samples were submerged in the water bath. The load cell amplifiers were zeroed and then the bottom grips were placed through the bottom stationary hooks. Finally, an initial preload of 30g was applied to each sample. The heating plate was turned on and the test was carried out. Samples were heated from room temperature to 90°C, at a rate of 1°C/min. Then, the temperature was held at 90°C for 3 hours, with load, temperature, and time data being recorded every ten seconds.

Data Analysis

Following HIT testing, the data were saved to an external hard-drive and then uploaded to an Excel spreadsheet for analysis. For the denaturation segment of the procedure, the temperature vs load data were plotted. The denaturation temperature was determined manually and was defined as the lowest load that was reached during this testing segment prior to an ultimately increasing sequence (see Figures 3.6 and 4.6).

The isothermal segment was plotted as load vs time data. The load decay, which is indicative of hydrolysis of the peptide bonds within the collagen molecules and chain slippage under isometric tension, is treated as a Maxwell element [125]. Therefore, the load decay was defined as the ratio of the load at time, L(t), to the maximum load, L(0), reached at (or shortly after) the start of the isotherm.

$$\frac{L(t)}{L(0)} = e^{-kt} \quad 3.4$$

For determination of k, the natural logarithm of this relationship was taken and plotted against time, with -k now representing the slope of the curve.

$$\ln\left(\frac{L(t)}{L(0)}\right) = -kt \quad 3.5$$

k was determined in Excel using linear regression of the natural logarithm of load vs time to the first 2000-3000 seconds of the isothermal segment for each sample. The below equation represents the load decay half-time,

$$\frac{1}{2} = e^{-kt_{1/2}} \quad 3.6$$

Finally, the load decay half-time can be calculated as:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \quad 3.7$$

The load decay halftime was calculated for control and $NaBH_4$-treated samples, and the ratio was calculated as the index of immature collagen crosslinks [123].

$$\frac{t_{1/2 NaBH4}}{t_{1/2 control}} \quad 3.8$$

3.4 Sircol™ Collagen Assay

Soluble and insoluble collagen content was determined using the Sircol™ Colorimetric Collagen Assay, following the manufacturer's protocol [126]. The wet weight of each tissue slice was recorded prior to testing. The tissue was placed in low protein binding 1.5mL microcentrifuge tubes and digested in an acid wash, consisting of 0.1 mg/ml pepsin in 0.5M acetic acid, overnight at 4°C under agitation. Acid-pepsin digestion is used to solubilize newly synthesized 'immature' collagen, by cleaving the molecule at non-helical regions called telopeptides [127]. In mature collagen, crosslinks form between neighbouring telopeptides, blocking cleavage access, and making this collagen insoluble [128].

The following day, the microcentrifuge tubes were centrifuged for ten minutes at a speed of 10,000 rpm. A pipette was used to transfer 15µl of supernatant (soluble collagen) and 85 µl of deionized water into a new set of microcentrifuge tubes. The remaining tissue

residues (insoluble collagen) were transferred to 2mL screw-capped digestion tubes and Fragmentation Reagent (50µl/mg tissue residue) was added to each tube. The screw-capped tubes were heated at 65°C for three hours, with the tubes being vortexed every thirty minutes. This procedure serves to solubilize the remaining collagen. Following this step, 100 µl of supernatant from each screw-capped tube was transferred to microcentrifuge tubes and the insoluble collagen assay was carried out as described below.

Collagen reference standards and blanks were prepared into microcentrifuge tubes. One mL of Sircol dye reagent was added test samples, reference standards and blanks; this dye contains Sirius Red, picric acid & surfactants, and binds to basic groups of soluble collagen molecules. Addition of the Sircol dye to the tubes results in staining and precipitation of collagen. The tubes were placed on a mechanical shaker for thirty minutes at room temperature, resulting in a pellet forming at the bottom of each tube. The collagen pellets were isolated and washed by centrifuging the tubes for ten minutes at 13,000 rpm. The supernatant was drained into a waste beaker, with the assistance of cotton swabs, taking great care to preserve the pellet. Unbound dye was further removed with the addition of 750 µl of cold Acid-Salt Wash to each tube and centrifugation at 13,000 rpm for ten minutes. The supernatant was drained from the tubes. Finally, Alkali Reagent, (250 µl for soluble and 1mL for insoluble), which consisted of 0.5M sodium hydroxide, was added to each tube; this reagent serves to unbind Sircol dye from the collagen pellet.

A vortex mixer was used to fully dissolve the pellet and 125 µl of liquid from each tube was transferred into a 96 micro-well plate. A microplate reader was used to measure the absorbance of the collagen standards and test samples at 555nm against water. The absorbance values were exported to Excel. The absorbance values of the standards were plotted against their known concentrations to produce a calibration curve. The curve was compared to the reference in the Sicrol™ Assay Manual to ascertain the accuracy of the measurements. Linear regression was used to obtain the equation of the absorbance curve.

$$y = mx + b \tag{3.9}$$

Where y represents the absorbance value at 555 nm and x represents the µg of collagen. This equation can be used to determine the mass of collagen in each test sample, by rearranging such that

$$x = \frac{y-b}{m} \tag{3.10}$$

Where x represents the mass in µg of collagen in each tube. This mass was divided by the volume of supernatant used, yielding the concentration in µg/ µL of collagen in each tube. The concentration was multiplied by the total extract volume, to calculate the total mass of collagen, in µg, in each tissue sample. Finally, the collagen content of each tissue sample was obtained by dividing the collagen mass by the wet mass of the tissue.

3.5 Fresh vs Frozen Tissue

A subset of experiments examined the effects of freeze-thawing on collagen molecular stability. Briefly, paired samples were obtained from bovine myocardium, as described in Section 3.1, and grouped into 'frozen' and 'fresh' categories. Frozen tissue was wrapped in PBS-soaked Kim-Wipes and stored in freezer bags at -80°C for five days. Prior to testing, the plastic bags containing the frozen samples were placed into a bucket of 37°C water and thawed. Fresh and freeze-thawed myocardial tissue were subjected to thermomechanical tests as described in section 3.3.

3.6 Tissue Decellularization

Following a literature review of decellularization detergents (see section 1.4), Tergitol was selected as it had been shown to have minimal effects on collagen structure and mechanics (Table 1.1). A tissue decellularization protocol (Fig. 3.8) was developed based on previously published work [115], [117], [118]. Briefly, myocardial slices, prepared as described in section 3.1, were initially washed in phosphate buffered saline (PBS) for 15 minutes to rinse any remaining blood. Myocardial slices were placed into Falcon tubes and washed overnight in a solution of 1% protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) in PBS at 4°C under agitation. This solution served to inhibit endogenous proteases within the tissue from degrading the proteins [118]. To prevent contamination, 1% gentamicin (Sigma Aldrich, St. Louis, MO, USA), and 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA), were also added [118].

The overnight solution was switched to 1% protease inhibitor cocktail and 1% Tergitol 15S9 (cat. no. 15S9, Sigma Aldrich, St. Louis, MO, USA), in sterile PBS for 8

hours at 4°C under agitation. Tergitol is a non-ionic detergent, consisting of a hydrophilic head group and hydrophobic tails [129]. In solution, Tergitol molecules preferentially aggregate and arrange into micelles, such that the head groups are exposed to water, and the tail groups are positioned in the centre of the micelle isolated from the water [129]. As head groups are attracted to phospholipids, micelles insert themselves within cell membranes eventually causing cell rupture [129]. The Tergitol solution was switched to a hypotonic wash of ten-times-diluted PBS for 3 hours at room temperature, under agitation. When cells are placed in hypotonic solution, there is a net flux of water into the cell leading to swelling and lysis. A final detergent wash was done by washing the slices in 1% Tergitol in sterile PBS solution for 16 hours at room temperature, under agitation.

Finally, the myocardial slices were rinsed with sterile PBS for 72 hours, with solution changes every 24 hours. This was done at room temperature, under constant agitation. These washes were used to remove cellular debris from the tissue. Control and Tergitol-treated tissues were subjected to mechanical, thermomechanical and biochemical testing, as described in sections 3.2, 3.3 and 3.4 respectively.

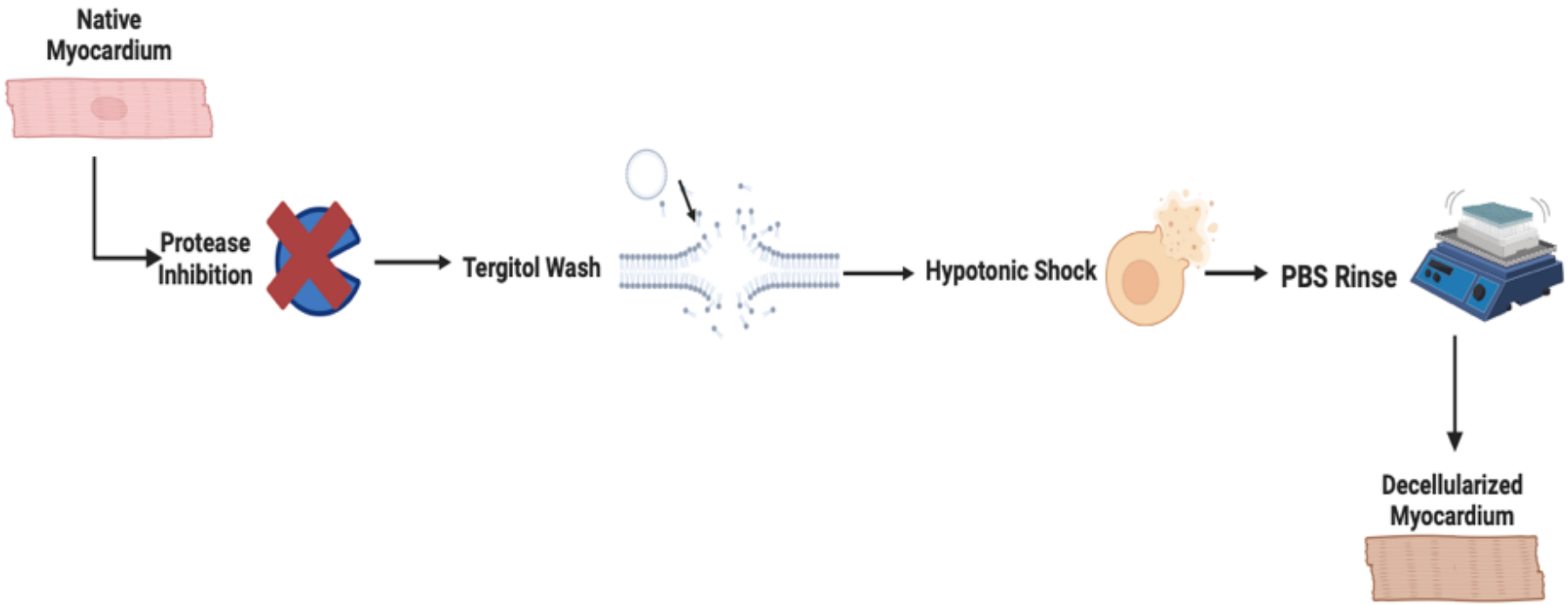

Figure 3.8 Overview of protocol utilized to decellularized bovine myocardium. Created with BioRENDER.

3.7 Histology

Myocardial strips, control and Tergitol-treated, taken from three previously pregnancy cows were studied histologically. Tissue samples were fixed in 4% paraformaldehyde solution for one week. Then, tissue was enclosed into tissue processing cassettes and placed in 70% ethanol solution for dehydration. Samples were sent to Dalhousie HistoCORE for embedding in paraffin wax, cutting into 5 μm sections, and staining with hematoxylin & eosin (H&E). H&E-stained slides were visualized under a Nikon Eclipse 600 light microscope, with and without polarized light, and birefringence filters.

3.8 Statistical Analysis

Statistical testing was performed in R (v.4.0.2, The R Foundation for Statistical Computing, Vienna, Austria). Comparisons between the means of pooled pregnant (early and late) and previously pregnant were made using T-tests. Statistical significance was defined as $p<0.05$, whereas statistical trends were defined as $p<0.1$. The pregnant group was further subdivided into 'early' and 'late', defined as gestational ages <150 days, and >150 days respectively. Comparisons between early, late, and previously pregnant groups were done using analysis of variance (ANOVA), with post hoc Tukey. In cases where the sample sizes were low, non-parametric statistical methods were used, the Kruskal Wallis test followed by post hoc Dunn's test. Two-way repeated measures ANOVA was performed to analyses the effects of both pregnancy state and Tergitol or freeze-thawing treatment on collagen content and thermomechanical properties. This was followed by one way ANOVA analysis for within group comparisons.

Chapter 4 Results

4.1 Myocardial Mechanics

Myocardial mechanical adaptations to gestation are not fully reversed post-pregnancy.

(i) Changes in post-pregnancy

Uniaxial tensile testing was conducted on myocardium from eight pregnant and three previously pregnant animals, showing differences in mechanical properties (Fig 4.1). The results are summarized in Table 4.1. When the data from all pregnant animals were pooled, the differences did not reach significance, but did indicate statistical trends in mechanical properties, namely that the myocardium is stronger (Fig 4.2A) and stiffer (Fig 4.4A) during pregnancy compared to post pregnancy.

Table 4.1. Summary of uniaxial tensile testing on pregnant vs previously pregnant cows myocardium, reported as mean ± SD. Myocardial samples were obtained from 8 pregnant and 3 previously pregnant cows. The ultimate tensile stress (UTS) was defined as the maximal stress prior to failure and the extensibility was defined as the strain at the UTS. Analysis was performed with t-tests to identify differences in mechanical properties between the myocardium of pregnant and previously pregnant cows. ^{t}denotes a trend between pregnancy states, $p<0.1$.

condition	UTS (MPa)	Extensibility	Stiffness Modulus (MPa)
Pregnant n=8	0.15 ± 0.06^{t}	1.15 ± 0.47	0.19 ± 0.08^{t}
Previously Pregnant n=3	0.10 ± 0.02	0.89 ± 0.22	0.13 ± 0.03

(ii) Changes during gestation

The pregnant animals were further subdivided into early (EP; gestational age<150 days), late (LP; gestational age>150 days) pregnant groups, revealing that interesting biphasic changes occur throughout gestation and post pregnancy (Fig. 4.1). UTS increased from early to late pregnancy ($p=0.036$), then decreased post-pregnancy, returning to EP values (Fig 4.2B). Similarly, there was an increase in extensibility throughout gestation ($p=0.014$), that partially recovered post-pregnancy (Fig 4.3B). These findings are

indicative of an adaptive response towards the volume overload state of pregnancy, allowing for the myocardium to sustain the increases in wall stress and filling capacity of the left ventricle.

Unlike the findings from Table 4.1, which suggested that myocardial stiffness is reduced in post-pregnancy (Fig 4.4A), when comparisons were made between EP, LP, and PP (Table 4.2), there were no differences in stiffness between groups (Fig 4.4B). The disappearance of this statistical trend may be due to the lower sample size numbers after subdividing the pregnant animals. These results may suggest that myocardial stiffness is unchanged throughout gestation, but reduced in postpartum.

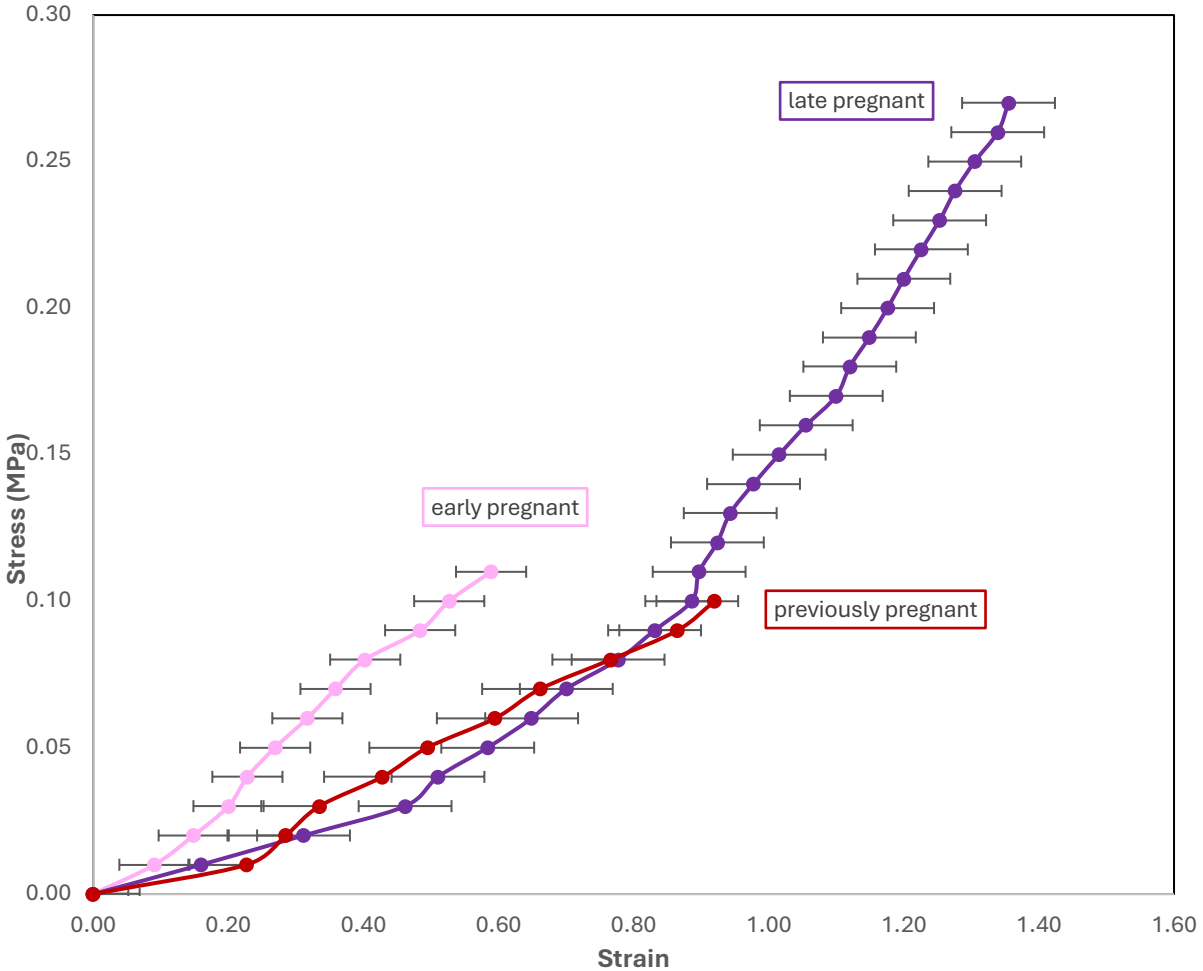

Figure 4.1 Average stress-strain relationships of myocardium taken from early (n=2; gestation age<150 days; pink), late (n=6; gestation age>150days; purple), and previously pregnant (n=3; red) cows, at 0.05 MPa intervals. Myocardial strips were stretched to rupture at a strain rate of 0.3mm/s. Error bars represent SE.

Although not used for statistical analysis, uniaxial tensile testing was performed on one heifer (e.g. never pregnant animal); the results are reported in Table 4.2. The myocardium from the heifer was similar in tensile strength to those of the early pregnant and previously pregnant animals, whereas myocardial extensibility of the heifer was similar to the values from early pregnant animals only. Further, myocardial stiffness in the heifer was lower than the values from the pregnant animals. Taken together, the data in Table 4.2 suggest that pregnancy results in reversible increases in myocardial UTS & stiffness, and a permanent increase in myocardial extensibility.

Table 4.2. Summary of uniaxial tensile testing at difference stages of gestation in bovine myocardium, reported as mean ± SD. Myocardial samples were obtained from 2 early pregnant (gestational age<150 days), 6 late pregnant (gestational age >150 days), and 3 previously pregnant cows. Analysis was performed with the Kruskal Wallis test, a nonparametric test to identify differences between groups. This was followed by a post-hoc Dunn's test. Different superscripts denote a difference between groups $p<0.1$.

Tissue	UTS (MPa)	Extensibility	Stiffness Modulus (MPa)
Never Pregnant n=1 (not used for analysis)	0.08	0.60	0.12
Early pregnant n=2	0.09 ± 0.00^{a}	0.56 ± 0.03^{a}	0.16 ± 0.01
Late pregnant n=6	0.17 ± 0.05^{b}	1.35 ± 0.33^{b}	0.20 ± 0.09
Previously Pregnant n=3	0.10 ± 0.02^{a}	0.89 ± 0.22^{ab}	0.13 ± 0.03

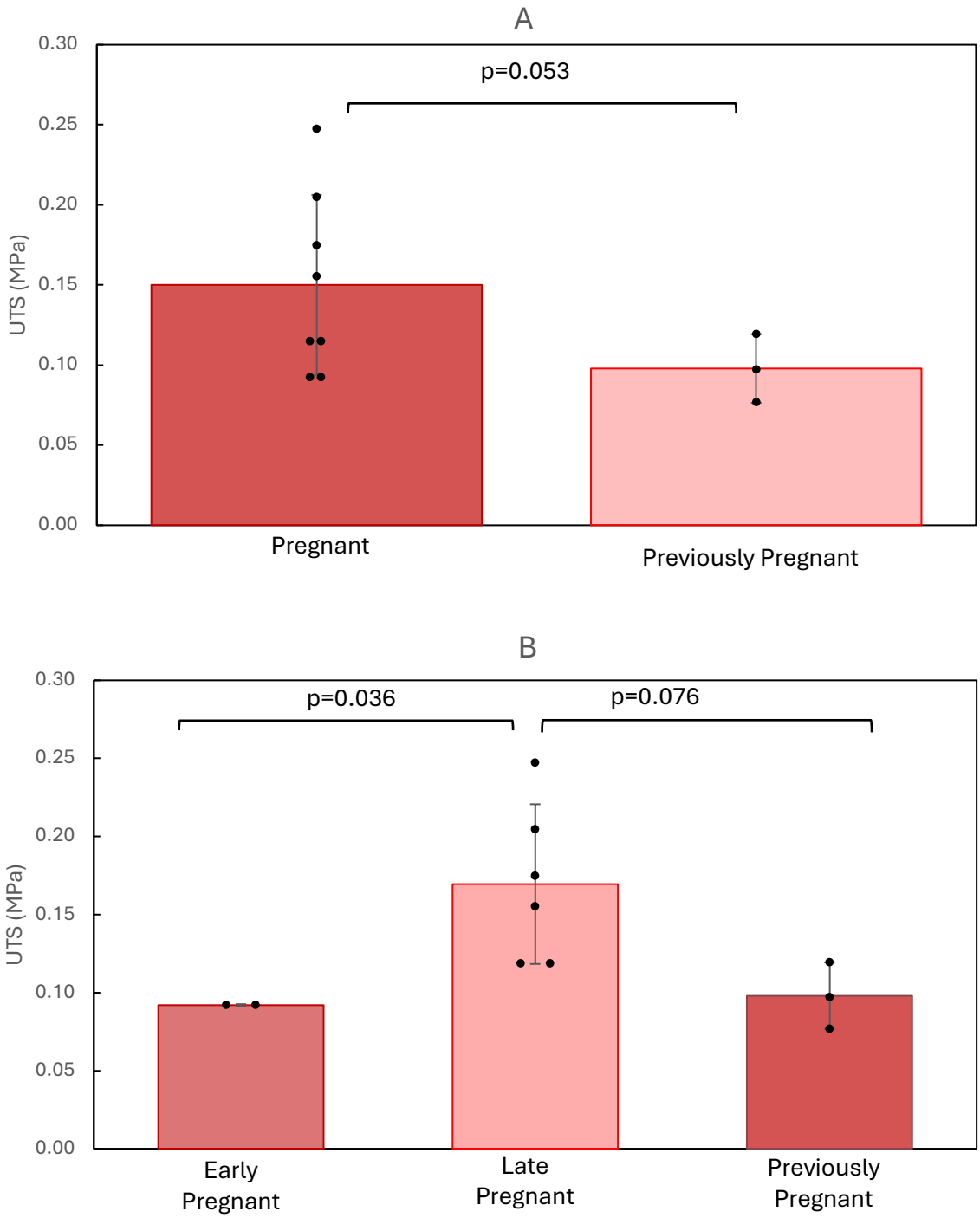

Figure 4.2A. Mean myocardial UTS in pregnant and previously pregnant cows, with error bars representing SD. The UTS was defined as the maximal stress prior to failure. Comparisons between groups were made using t-tests. **B.** Mean myocardial UTS in early pregnant (gestational age<150 days), late pregnant (gestational age >150 days), and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using the Kruskal Wallis test, with a post-hoc Dunn's analysis. p values<0.1 are shown.

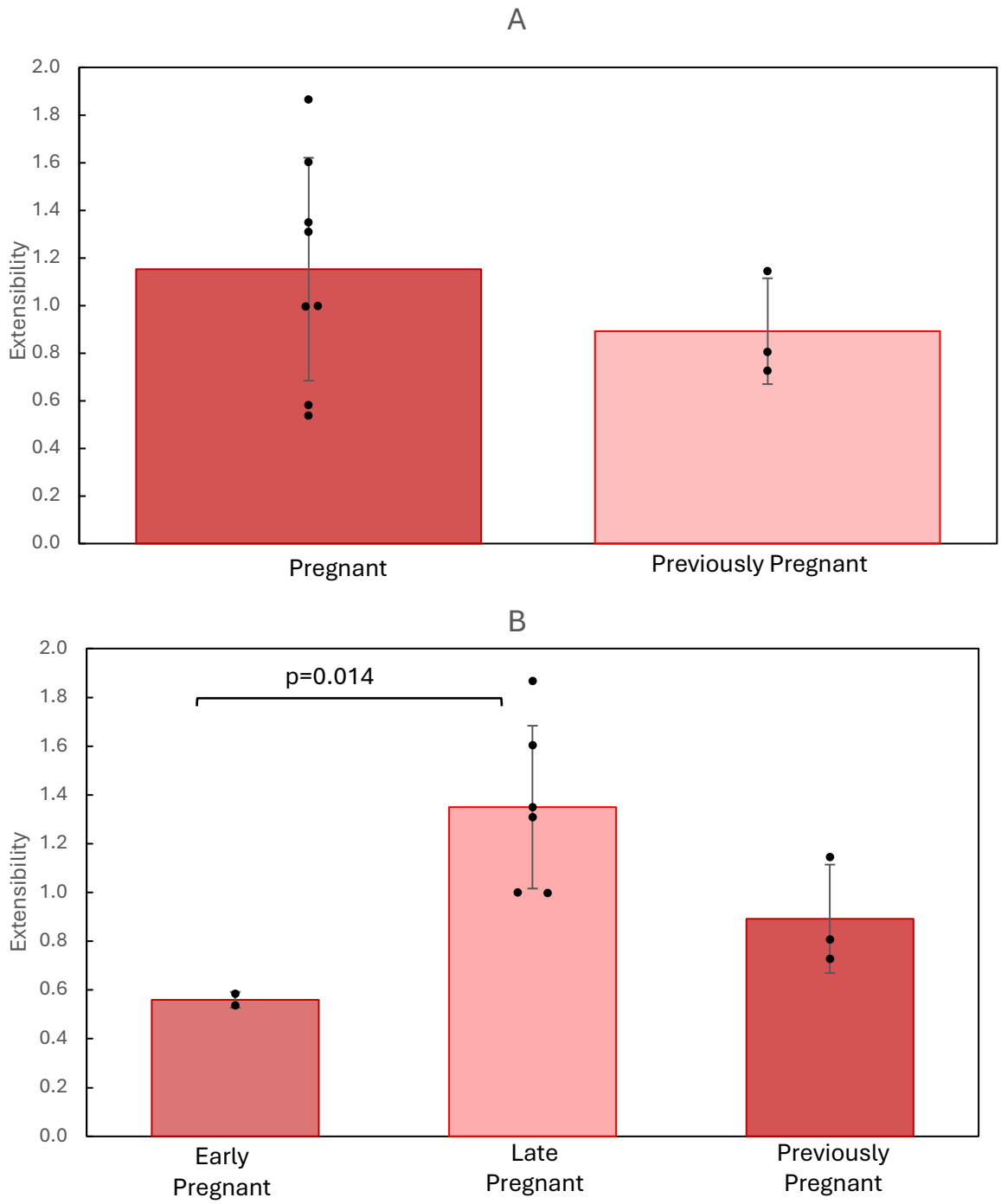

Figure 4.3 A. Mean myocardial extensibility in pregnant and previously pregnant cows, with error bars representing SD. Extensibility was defined as the strain at the UTS. Comparisons between groups were made using t-tests. **B.** Mean myocardial extensibility in early pregnant (gestational age<150 days), late pregnant (gestational age >150 days), and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using the Kruskal Wallis test, with a post-hoc Dunn's analysis. p values <0.1 are shown.

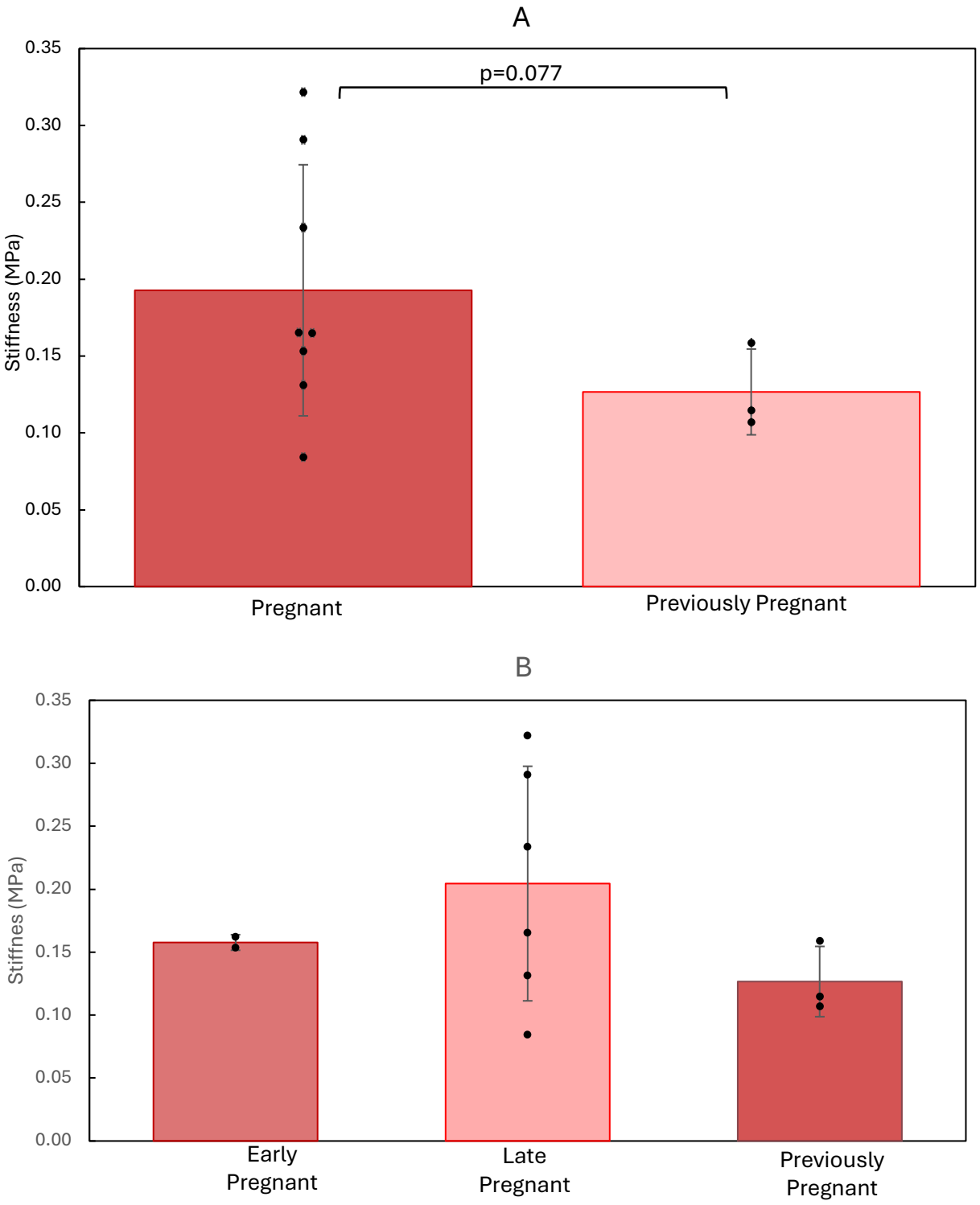

Figure 4.4 A. Mean myocardial stiffness modulus in pregnant and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using t-tests. **B.** Mean myocardial stiffness modulus in early pregnant (gestational age<150 days), late pregnant (gestational age >150 days), and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using the Kruskal Wallis test, with a post-hoc Dunn's analysis. p values<0.1 are shown.

4.2 Myocardial Collagen Content

Myocardial collagen content is unchanged between pregnancy and post-pregnancy.

Myocardial collagen content was measured using the Sircol Collagen Assay, and the data are summarized in Table 4.3. The assay showed that collagen occupied approximately 7% of the myocardial wet tissue weight, with the majority being insoluble collagen. For comparison, only 14% of the total collagen was soluble collagen, suggesting a relatively low quantity of newly deposited compared to matured collagen in the myocardium. Myocardial collagen content did not differ between pregnant and previously pregnant animals (Fig 4.5). Indeed, these findings suggest that there is no net deposition of collagen in response to pregnancy-induced volume overload. It should be noted that the myocardial samples used in this analysis were obtained from previously pregnant and late pregnant (gestational age<150 days) animals. Therefore, observations of myocardial collagen content in early pregnancy could not be made.

Table 4.3. Summary of Sircol Collagen Assay results for bovine myocardium from pregnant and previously pregnant cows, reported as mean± SD. Myocardial samples were collected from 6 pregnant and 6 previously pregnant cows. T-tests were performed to identify differences between groups; there were no significant differences.

Tissue	soluble collagen (ug/mg wet tissue)	insoluble collagen (ug/mg wet tissue)	total collagen (ug/mg wet tissue)
Late Pregnant (n=6)	10 ± 6	62 ± 31	72 ±35
Previously Pregnant (n=6)	16 ± 13	61 ± 49	76 ± 60

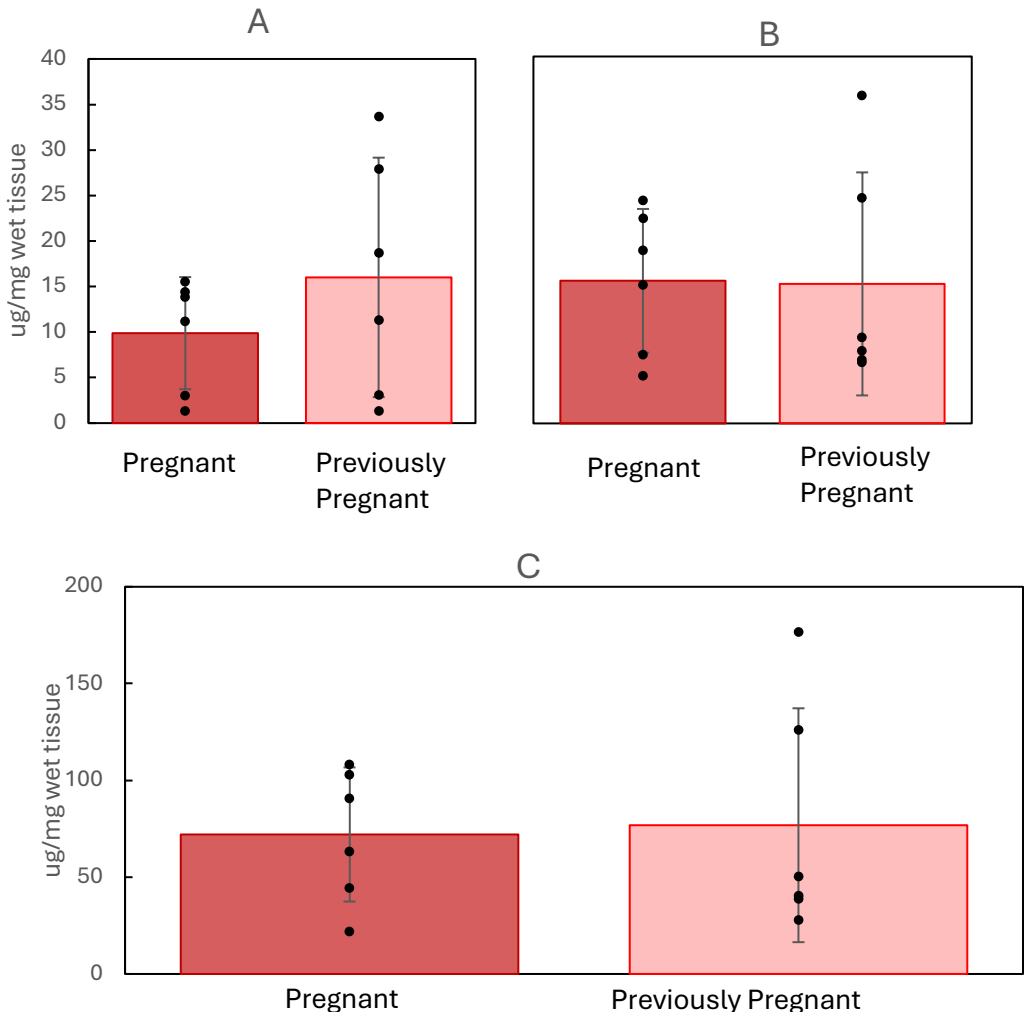

Figure 4.5 Mean soluble **(A),** insoluble **(B),** and total **(C)** collagen content in myocardium from pregnant and previously pregnant cows, with error bars representing SD.

4.3 Myocardial Collagen Thermomechanical Properties

Myocardial collagen thermal stability and mature crosslinks are reduced in post-pregnancy.

(i) Changes in post-pregnancy

Thermomechanical testing was performed on myocardial samples from twelve pregnant and five previously pregnant animals. (Table 4.4). Typically, a single curve can be derived from the entire isotherm using linear regression, as the load undergoes exponential decay. However, the behaviour of these curves was abnormal, with the initial segment of the isotherm exhibiting exponential decay, followed by either a flattening/plateau in the load, or a continued contraction. Therefore, to obtain the best the measurement of the load decay half time, linear regression was fitted to the initial linear segment of the natural logarithm curves, corresponding to the first 3000 or 2000 seconds of the isotherm (Appendix I). The data are summarized in table 4.4.

Denaturation temperature (T_d) was significantly decreased in post pregnancy compared to during gestation (p= 0.003) (Fig. 4.8A). Likewise, load decay half-time was significantly decreased in post pregnancy compared to during gestation (p=0.009) (Fig 4.9A). The index of immature crosslinks was significantly increased in post pregnancy compared to during gestation (p=0.001) (Fig 4.10A). Taken together, these findings suggest collagen turnover, resulting in decreased molecular packing and a loss of mature crosslinks in post pregnancy.

Table 4.4. Summary of results from HIT/DTT with $NaBH_4$ stabilization tests on myocardium samples from pregnant vs previously pregnant cows, reported as mean ± SD. Myocardial samples were obtained from 12 pregnant and 6 previously pregnant cows. T-test analyses were performed to identify differences in thermomechanical properties in the myocardium of pregnant and previously pregnant cows. * denotes a significant difference between pregnancy groups, p<0.05.

Tissue	T_d (°C)	$t_{1/2}$ (hours)	$\frac{t_{1/2\ NaBH4}}{t_{1/2\ control}}$
Pregnant n=12	67.1 ± 0.9	6.4 ± 2.0	1.1 ± 0.2
Previously Pregnant n=6	66.4 ± 0.6*	3.2 ± 2.6*	1.6 ± 0.3*

(ii) Changes during gestation

The animals were further subdivided into early (EP; gestational age<150 days), late (LP; gestational age>150 days), and previously (PP) pregnant groups, to examine changes throughout gestation (Fig. 4.6) The data are reported in table 4.5. There was a significant difference in denaturation temperature between groups (p=0.027), with denaturation temperature gradually decreasing from EP to PP (Fig 4.8B).

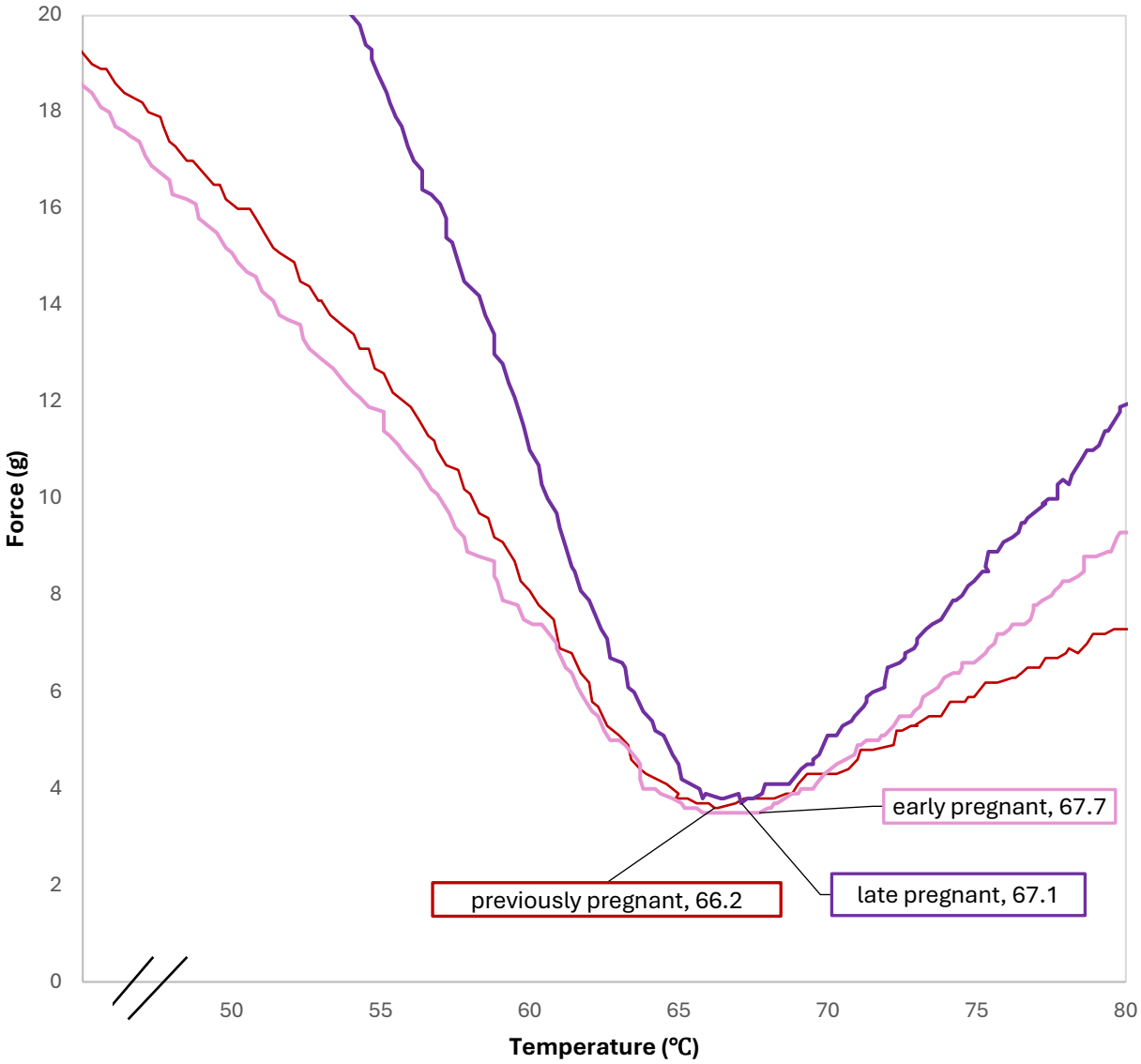

Figure 4.6 Representative denaturation curves of myocardial samples taken from early (gestation age<150 days; pink), late (gestation age>150days; purple), and previously pregnant (red) cows heated from room temperature to 90°C at a rate of 1-2°C/min.

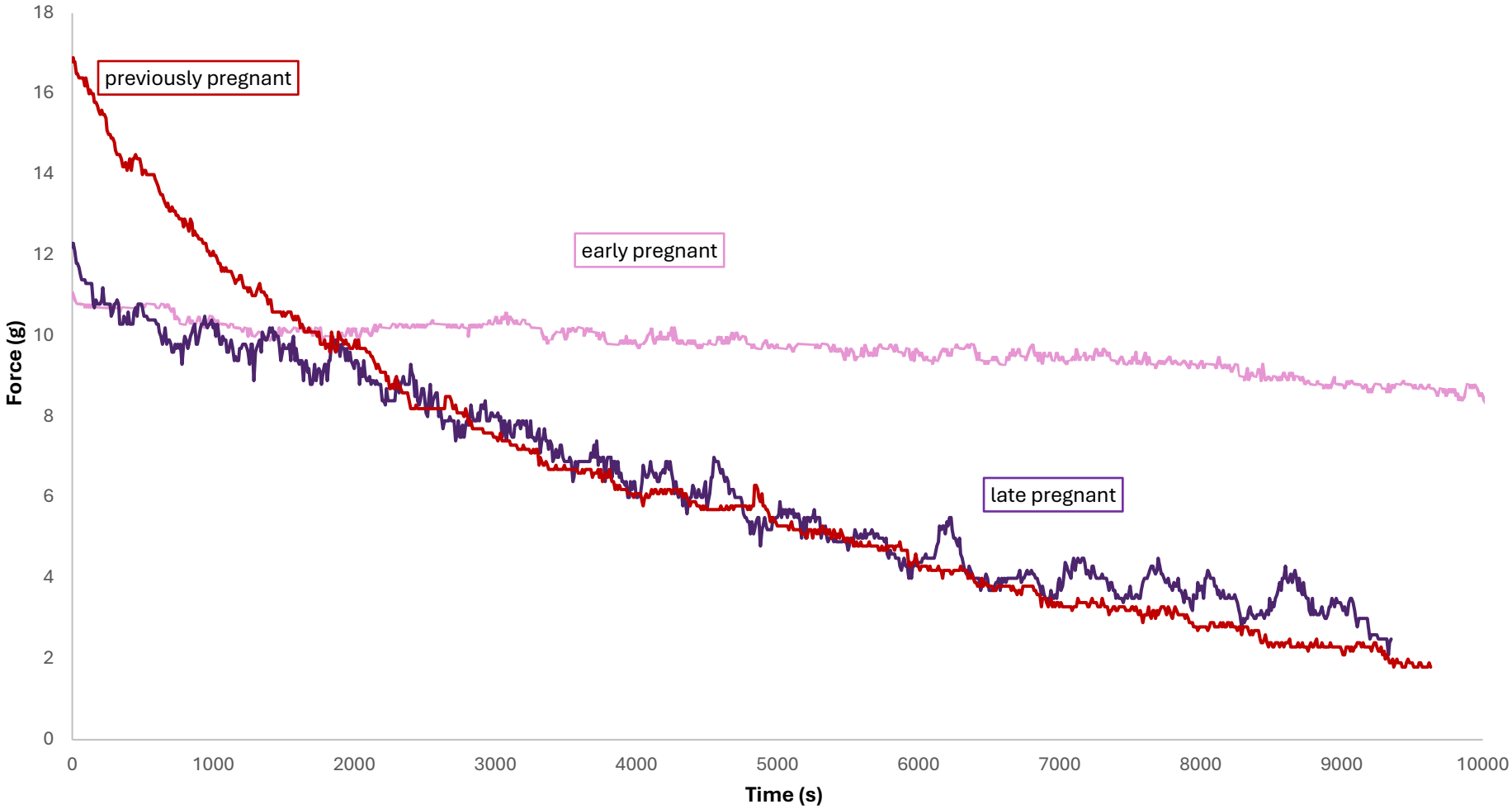

Figure 4.7 Representative isothermal (90°C) relaxation curves of myocardium taken from early (gestation age<150 days; pink), late (gestation age>150days; purple), and previously pregnant (red) cows.

Similarly, the load decay half-time was significantly different between groups (Fig. 4.7). Specifically, there was a significant reduction in load decay half-time between early pregnancy and post-pregnancy (Fig 4.9B). The index of immature crosslinks was also significantly different between groups ($p=0.005$), with a significantly increased index in post pregnancy compared to both early and late pregnancy (Fig 4.10B). These results are suggestive of a gradual reduction in collagen thermal stability and crosslinking throughout pregnancy, which reaches significance in post pregnancy. Immature collagen is then laid down in post pregnancy, increasing the immature crosslink index, and balancing collagen degradation and deposition.

Although not used for statistical analysis, HIT/DTT testing was performed on myocardial samples from one heifer (e.g. never pregnant animal); the results are reported in Table 4.5. Based on these results, one may hypothesize that changes in collagen thermomechanical properties follow a biphasic pattern, with an initial reinforcement of the myocardial network very early in pregnancy, increasing collagen molecular packing and crosslinks, followed by a reversal as the heart continues to grow.

Table 4.5. Summary of results from HIT/DTT with $NaBH_4$ stabilization tests on bovine myocardium at different stages of gestation, reported as mean ± SD. Myocardial samples were obtained from 5 early pregnant (gestational age<150 days), 7 late pregnant (gestational age >150 days), and 6 previously pregnant cows. Analysis was performed with an ANOVA test, a parametric test to identify differences between groups. This was followed by a post-hoc Tukey's test. Different subscripts denote a significant difference between pregnancy states, $p<0.05$.

Tissue	T_d (°C)	$t_{1/2}$ (hours)	$\frac{t_{1/2\ NaBH4}}{t_{1/2\ control}}$
never pregnant n=1	67.4	3.8	1.1
early pregnant n=5	68.0 ± 1.0^{a}	7.5 ± 2.1^{a}	1.0 ± 0.1^{a}
late pregnant n=7	67.1 ± 1.2^{ab}	5.6 ± 1.5^{ab}	1.2 ± 0.1^{a}
previously pregnant n=6	66.4 ± 0.6^{b}	3.2 ± 2.6^{b}	1.6 ± 0.2^{b}

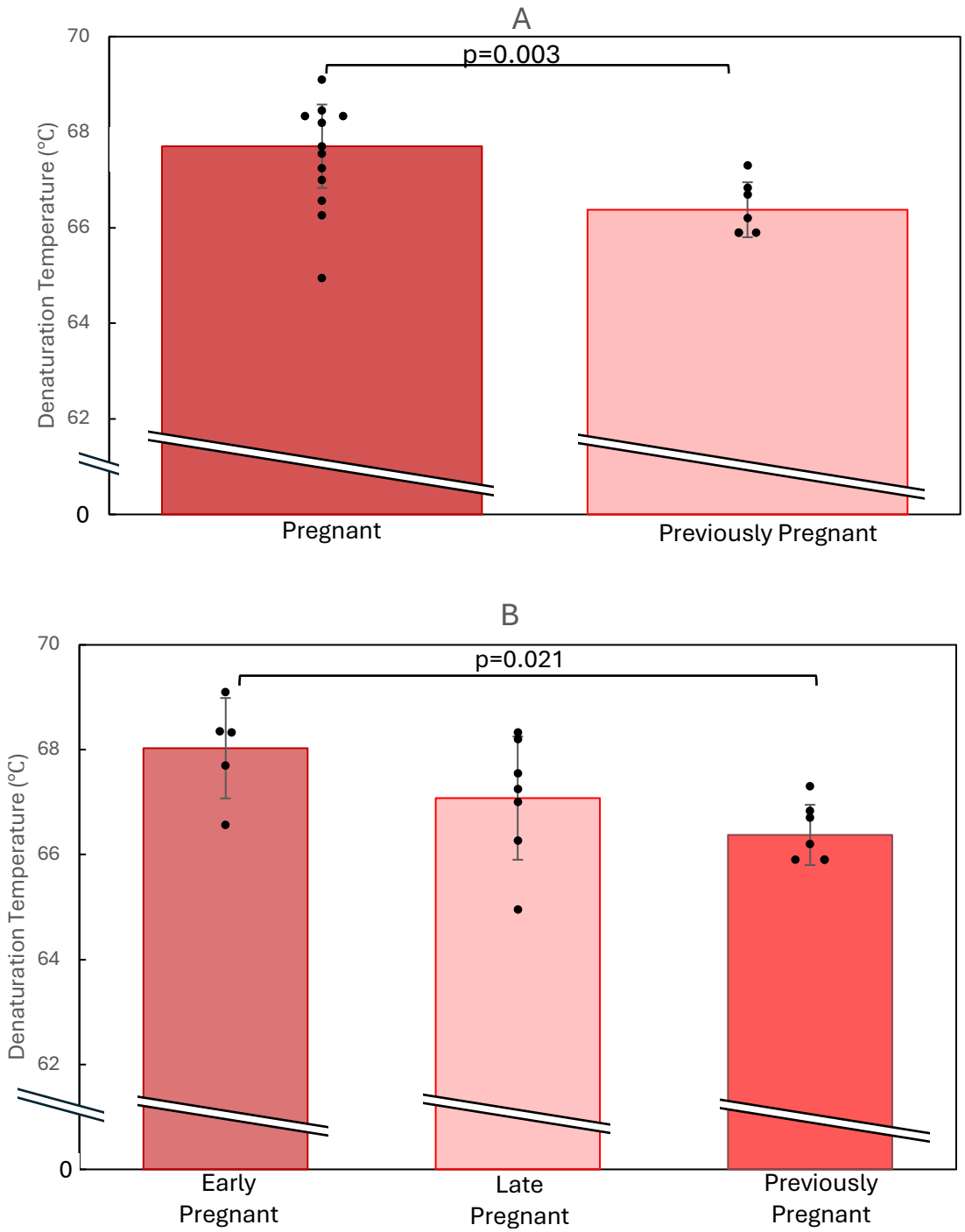

Figure 4.8 A. Mean collagen denaturation temperature in myocardium from pregnant and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using t-tests. **B.** Mean collagen denaturation temperature in myocardium from early pregnant (gestational age<150 days), late pregnant (gestational age >150 days), and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using an ANOVA test, with a post-hoc Tukey's analysis. p values<0.1 are shown.

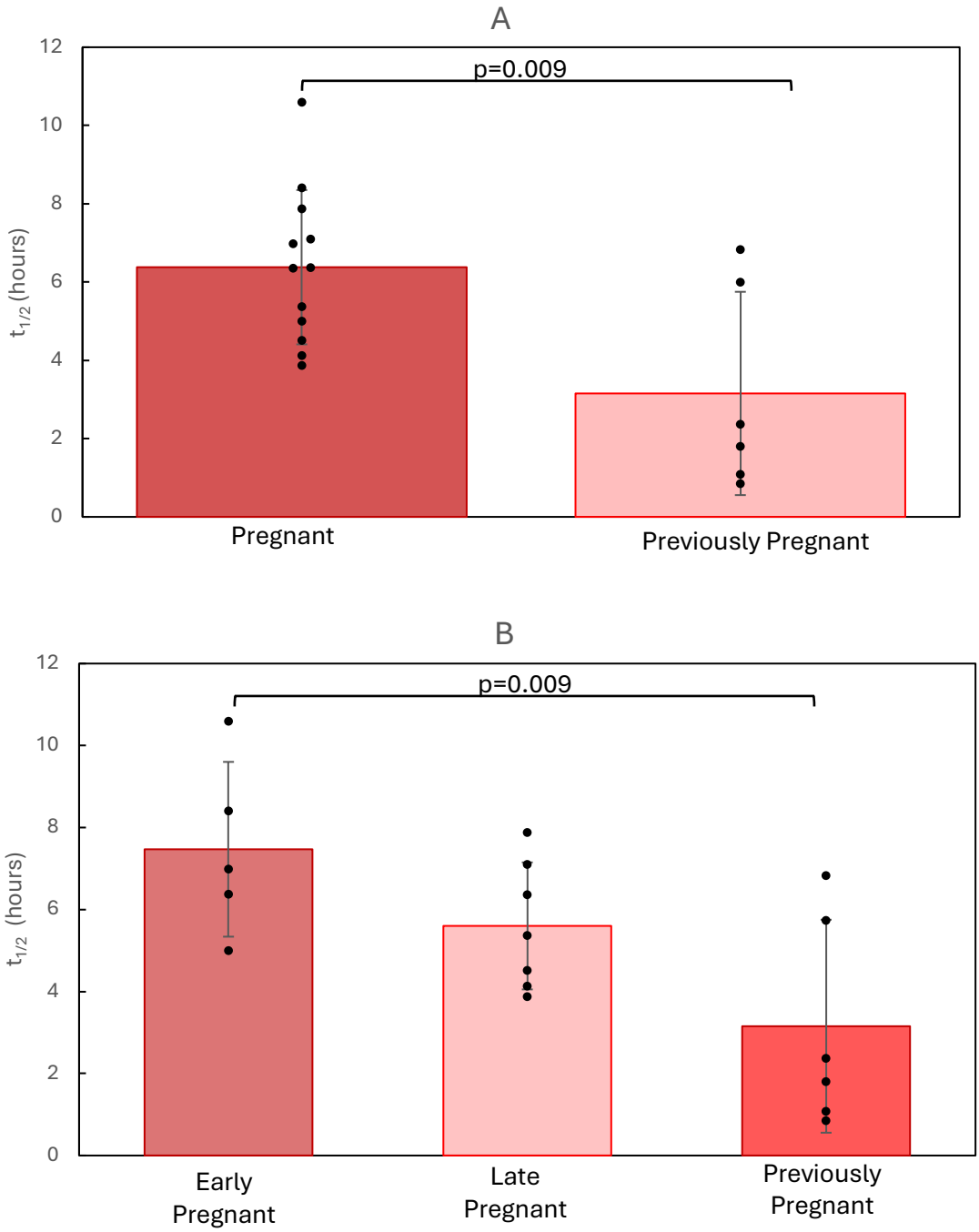

Figure 4.9 A. Mean load decay half-time in myocardium from pregnant and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using t-tests. **B.** Mean load decay half-time in myocardium from early pregnant (gestational age<150 days), late pregnant (gestational age >150 days), and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using an ANOVA test, with a post-hoc Tukey's analysis. p values<0.1 are shown.

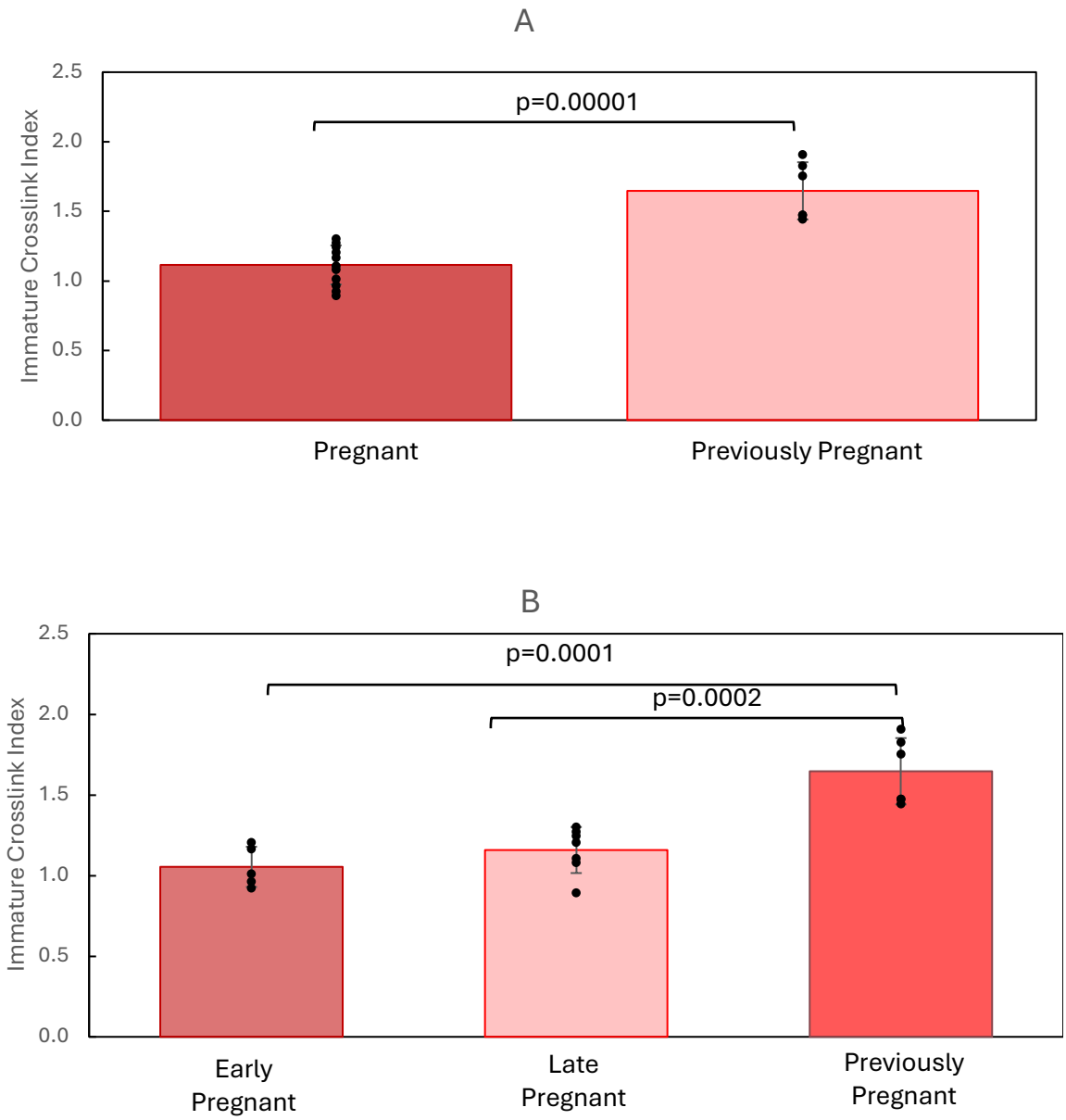

Figure 4.10 A. Mean immature crosslink index in myocardium from pregnant and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using t-tests. **B.** Mean immature crosslink index in myocardium from early pregnant (gestational age<150 days), late pregnant (gestational age >150 days), and previously pregnant cows, with error bars representing SD. Comparisons between groups were made using an ANOVA test, with a post-hoc Tukey's analysis. p values<0.1 are shown.

4.4 Effects of Freeze-Thawing

Myocardial collagen is less susceptible to freeze-thaw induced disruption post-pregnancy.

Freeze-thawing had differential effects on myocardium, depending on gestational state (Table 4.6). The myocardium from pregnant animals sustained significant reductions in denaturation temperature (p=0.022) (Fig 4.11A), load decay half-time (p=0.0177) (Fig 4.11B), and immature crosslink index (p=0.045) (Fig 4.11C), following freeze-thawing. Conversely, in the myocardium from previously pregnant animals, freeze-thawing had no effect on myocardial thermomechanical properties. Freeze-thawing is known to expand the intrafibrillar space through ice formation, reducing collagen thermal stability [100]; however, this effect was dependent on gestational state (p=0.043). These findings suggest that ice formation did not cause expansion of the myocardial intrafibrillar space in previously pregnant animals, possibly because the collagen network is already less tightly packed. In contrast, the myocardial collagen network may be more tightly packed in pregnancy, such that ice formation had a more pronounced effect on the intrafibrillar space.

Table 4.6. Summary of results from HIT/DTT with $NaBH_4$ stabilization tests on fresh vs frozen bovine myocardium, reported as mean ± SD. Paired myocardial samples were collected from 4 pregnant and 3 previously pregnant cows, with samples being either tested fresh or following freeze-thawing. Following two-way ANOVA with repeated measures, which identified a significant interaction between the effects of freezing and pregnancy state on T_d (p=0.043), $t_{1/2}$ (p=0.017) and $\frac{t_{1/2\ NaBH4}}{t_{1/2\ control}}$ (p=0.068), paired t-tests were performed for within group comparisons* denotes significant difference between groups <0.05.

Tissue	Condition	T_d (°C)	$t_{1/2}$ (hours)	$\frac{t_{1/2\ NaBH4}}{t_{1/2\ control}}$
Pregnant n=4	Fresh	67.6 ± 1.4	8.3 ± 1.7	1.1± 0.2
	Frozen	64.8 ± 0.8*	3.1 ±1.1*	0.7 ± 0.3*
Previously pregnant n=3	Fresh	66.5 ± 0.7	5.6 ± 1.5	1.6 ± 0.2
	Frozen	65.7 ± 1.6	4.8 ± 2.6	2.0 ± 0.4

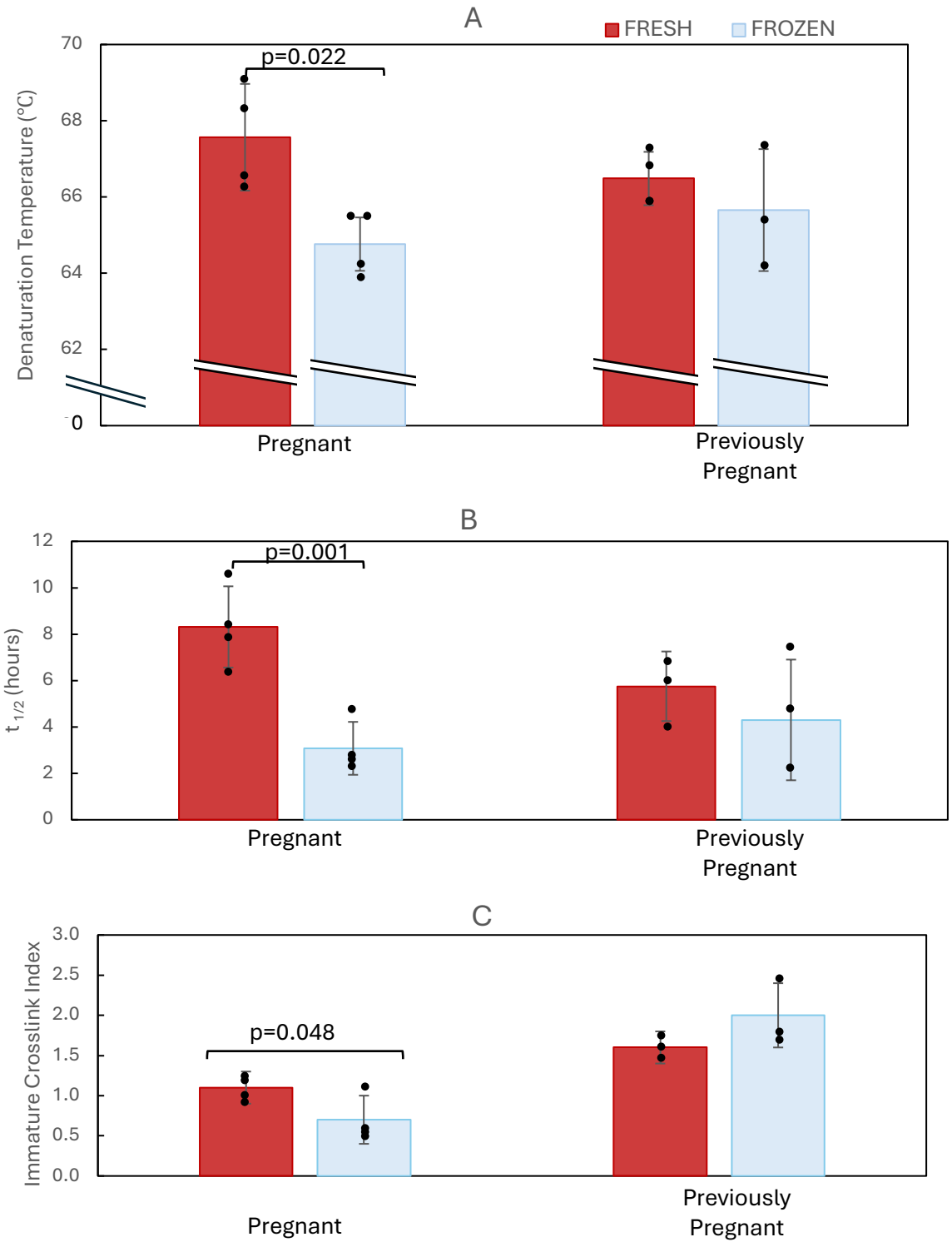

Figure 4.11 A. Mean collagen denaturation temperature in fresh and frozen myocardial samples, grouped by pregnancy state. **B.** Mean load decay half-time in fresh and frozen myocardium cows, grouped by pregnancy state. **C.** Mean immature crosslink index of fresh and frozen myocardium, grouped by pregnancy state. Error bars represent SD; Following two-way ANOVA with repeated measures, paired t-tests were used for within group comparisons. p values <0.1 are shown.

4.5 Effects of Tergitol Treatment

Tergitol treatment was ineffective for myocardial decellularization, but disrupted the muscle fibers and collagen network.

(i) Gross appearance and histological findings

Tergitol, a chemical detergent, is a lipophilic agent that partitions into the cellular membrane, causing cell lysis [129]. Following Tergitol treatment, the myocardium was transformed from a dark red colour to a light brown (Fig. 4.12). However, staining of Tergitol-treated myocardial samples revealed that the decellularization protocol had been ineffective. H&E stains of control myocardial samples taken from previously pregnant cows displayed aligned muscle cells, with acidophilic sarcoplasm and central oval nuclei (Fig.4.13A, Fig.4.14A). Stains of Tergitol-treated myocardial samples revealed that nuclei were present in the specimens, though they appeared darkly pyknotic (white arrow) and were floating in the interstitial space within some regions (black arrow) (Fig. 4.13B). The sarcoplasm was deeply acidophilic. The muscle cells were misaligned and widely separated (Fig. 4.13B). Interestingly, muscle striations and intercalated discs were more prominent in the Tergitol-treated samples (Fig.4.14B). Although complete decellularization was not achieved, the histological findings indicate that the Tergitol indeed disrupted the membrane of cardiomyocytes, causing dispersion and swelling of muscle cells. This likely resulted in the more prominent appearance of the striations and eosin stain.

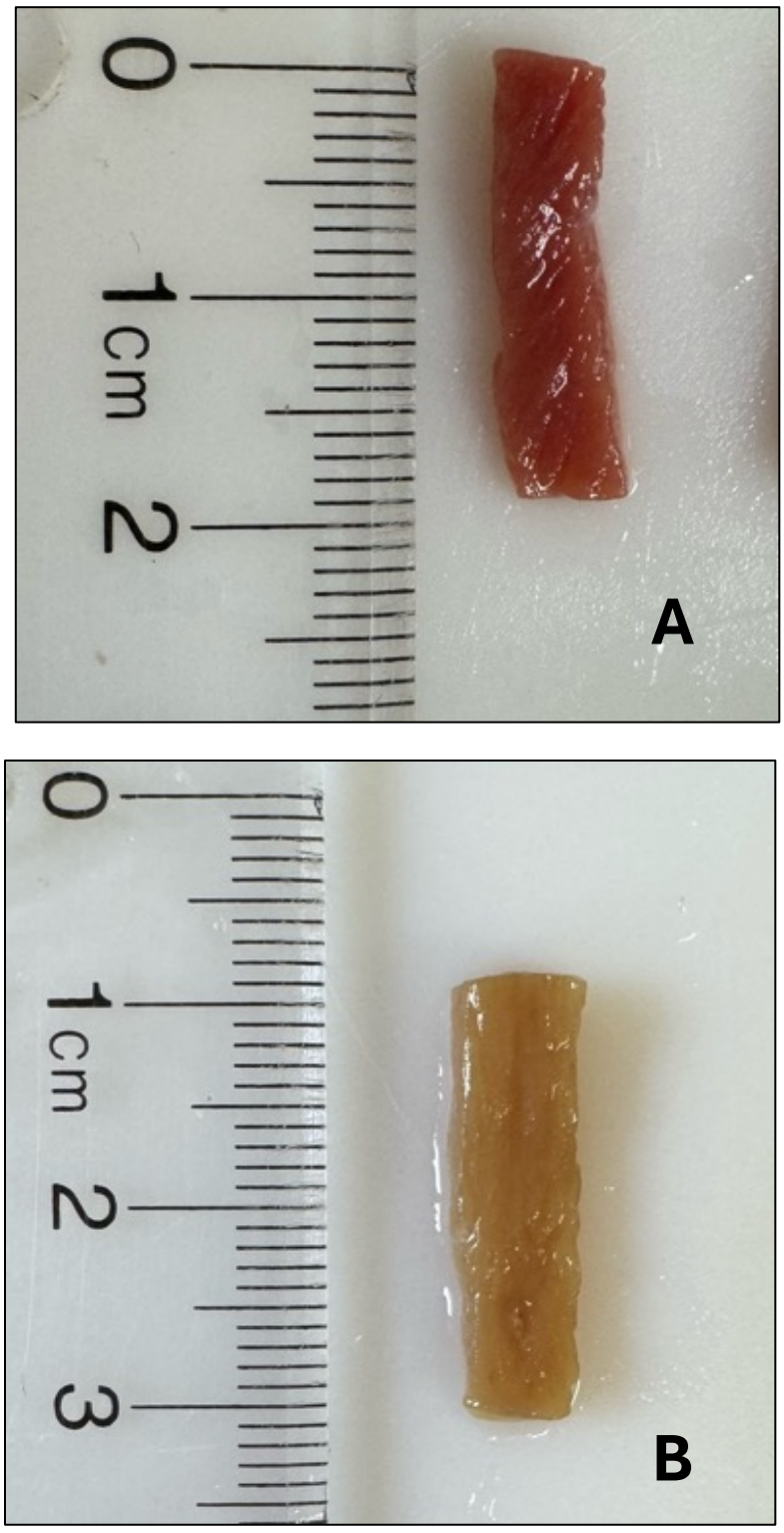

Figure 4.12 Bovine myocardial samples before **(A)** and after (**B**) Tergitol treatment.

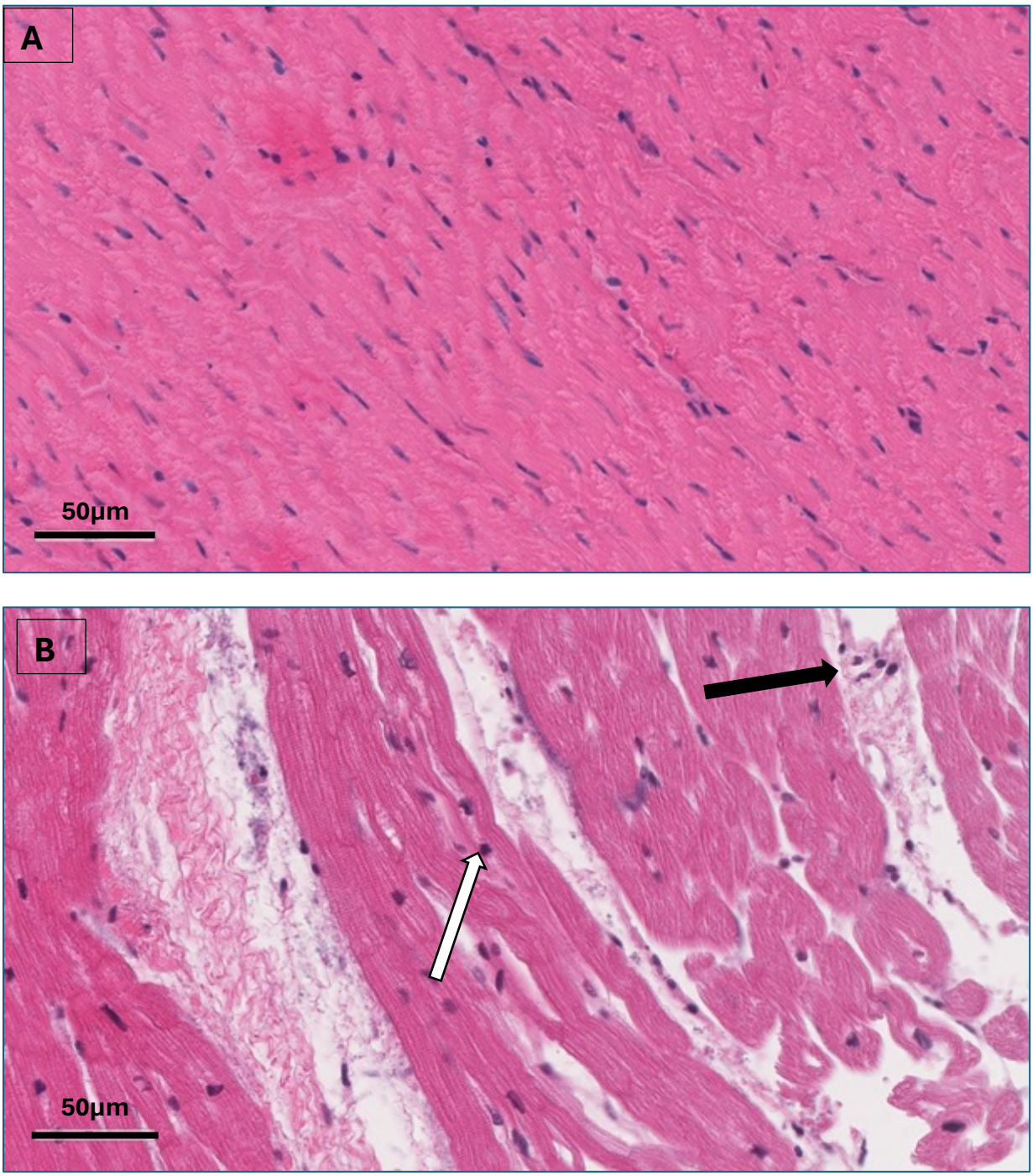

Figure 4.13. Cross-sections of (**A)** control, and (**B**) Tergitol-treated myocardium from previously pregnant bovine stained with H&E and viewed at 40X magnification. (Black arrow pointing at nuclei floating in interstitial space. White arrow pointing at pyknotic nucleus)

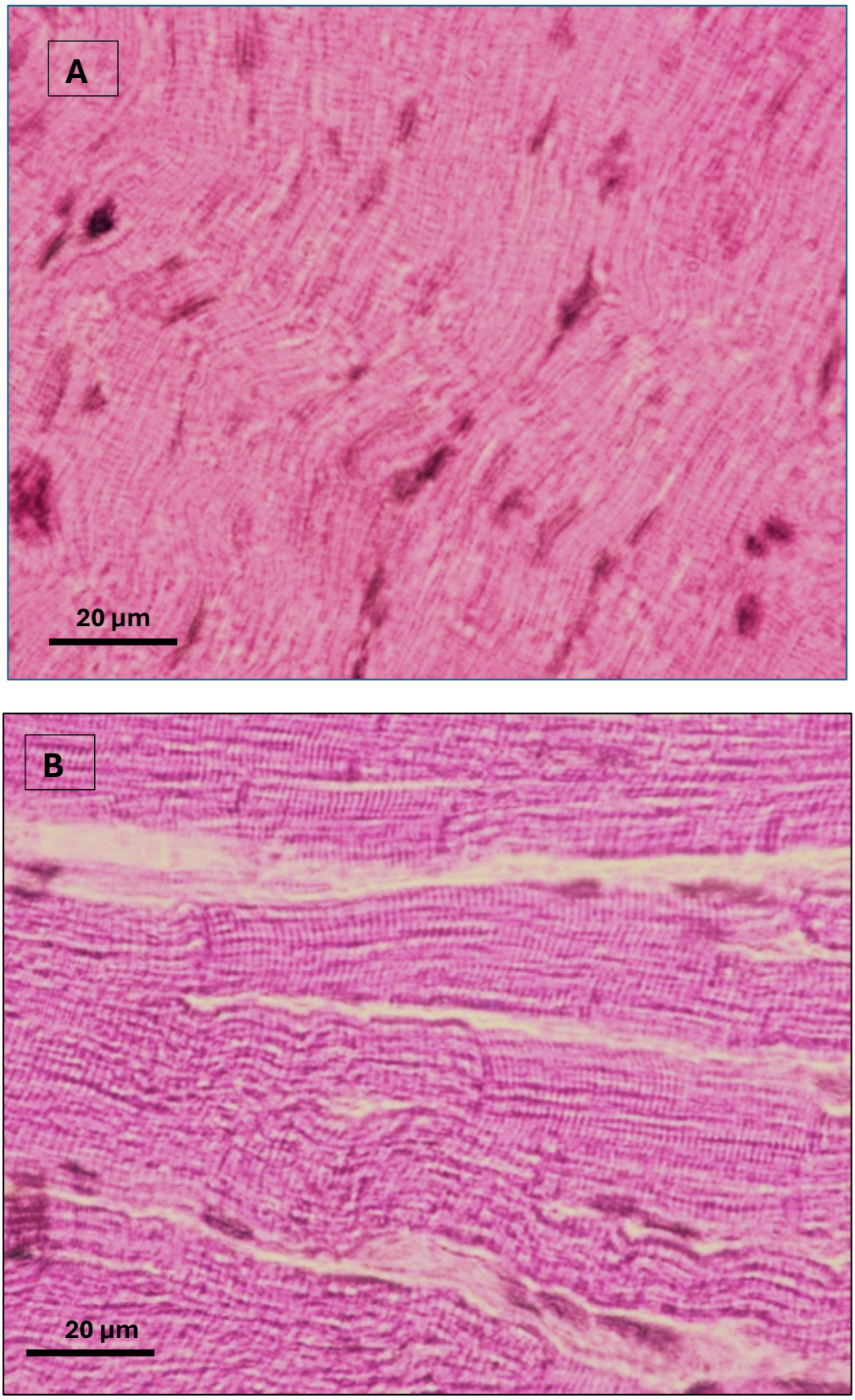

Figure 4.14. Cross-sections of (**A)** control, and (**B**) Tergitol-treated myocardium from previously pregnant bovine stained with H&E and viewed at 100X magnification.

(ii) Observations with polarized light microscopy (PLM)

Under polarized light, the control myocardial samples exhibited birefringence, arising due to the anisotropy of collagen fibers. Alternating dark and bright patterns corresponding to the collagen crimping pattern were apparent (Fig.4.15A, Fig.4.16A). Notably, this pattern was absent in the Tergitol-treated samples, with an overall reduction in birefringence (Fig.4.15B, Fig.4.16B). Further, despite the intermuscular space containing collagen fibers (as observed without PLM), there was no birefringence in these spaces, suggesting a disorganization in the collagen network or a loss of collagen crimp. This is not unexpected, as the collagen network encapsulates cardiomyocytes; with the separation of myocytes, the surrounding collagen fibers were likely dispersed as well.

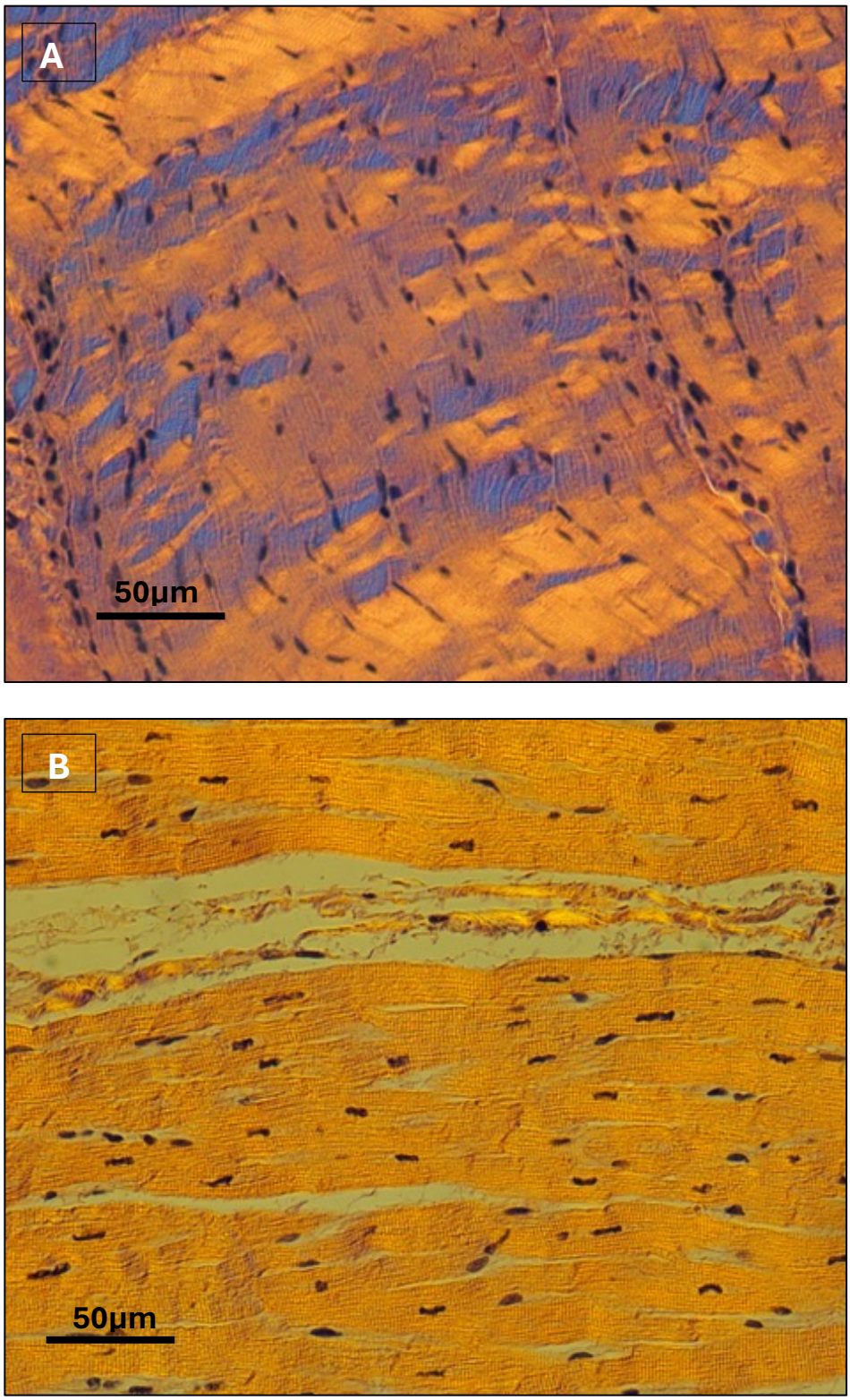

Figure 4.15. Cross-sections of (**A)** control, and (**B**) Tergitol-treated myocardium from previously pregnant bovine stained with H&E and viewed with polarized light microscopy at 40X magnification.

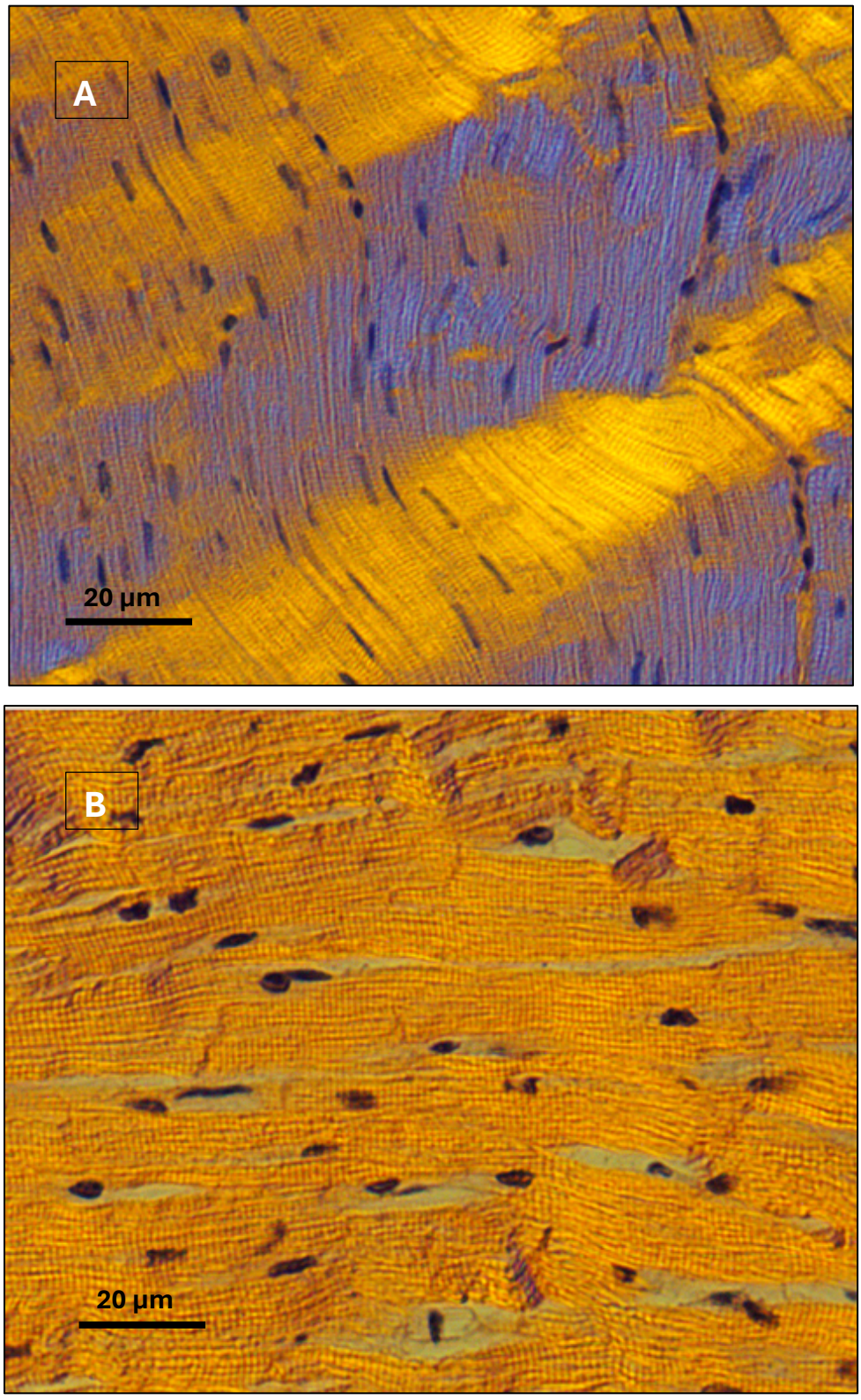

Figure 4.16. Cross-sections of (**A)** control, and (**B**) Tergitol-treated myocardium from previously pregnant bovine stained with H&E and viewed with polarized light microscopy at 100X magnification.

Tergitol does not affect myocardial mechanical properties.

The effects of Tergitol treatment on myocardial mechanical properties are summarized in Table 4.7. As expected, there were no changes in myocardial UTS (Fig 4.17A), extensibility (Fig 4.17B), nor stiffness (Fig 4.17C), following treatment with Tergitol. Further, a two-way repeated measures ANOVA found that there was no interaction between Tergitol treatment and gestational state.

Table 4.7. Summary of uniaxial tensile testing from control and Tergitol-treated bovine myocardium samples, reported as mean ± SD. Paired myocardial samples were obtained from 11 bovine. Analysis was performed with paired t-tests, which identified no significant differences between groups.

condition	UTS (MPa)	Extensibility	Stiffness Modulus (MPa)
Control n=11	0.14 ± 0.05	1.08 ± 0.42	0.17 ± 0.08
Tergitol n=11	0.15 ± 0.08	1.09 ± 0.37	0.23 ± 0.19

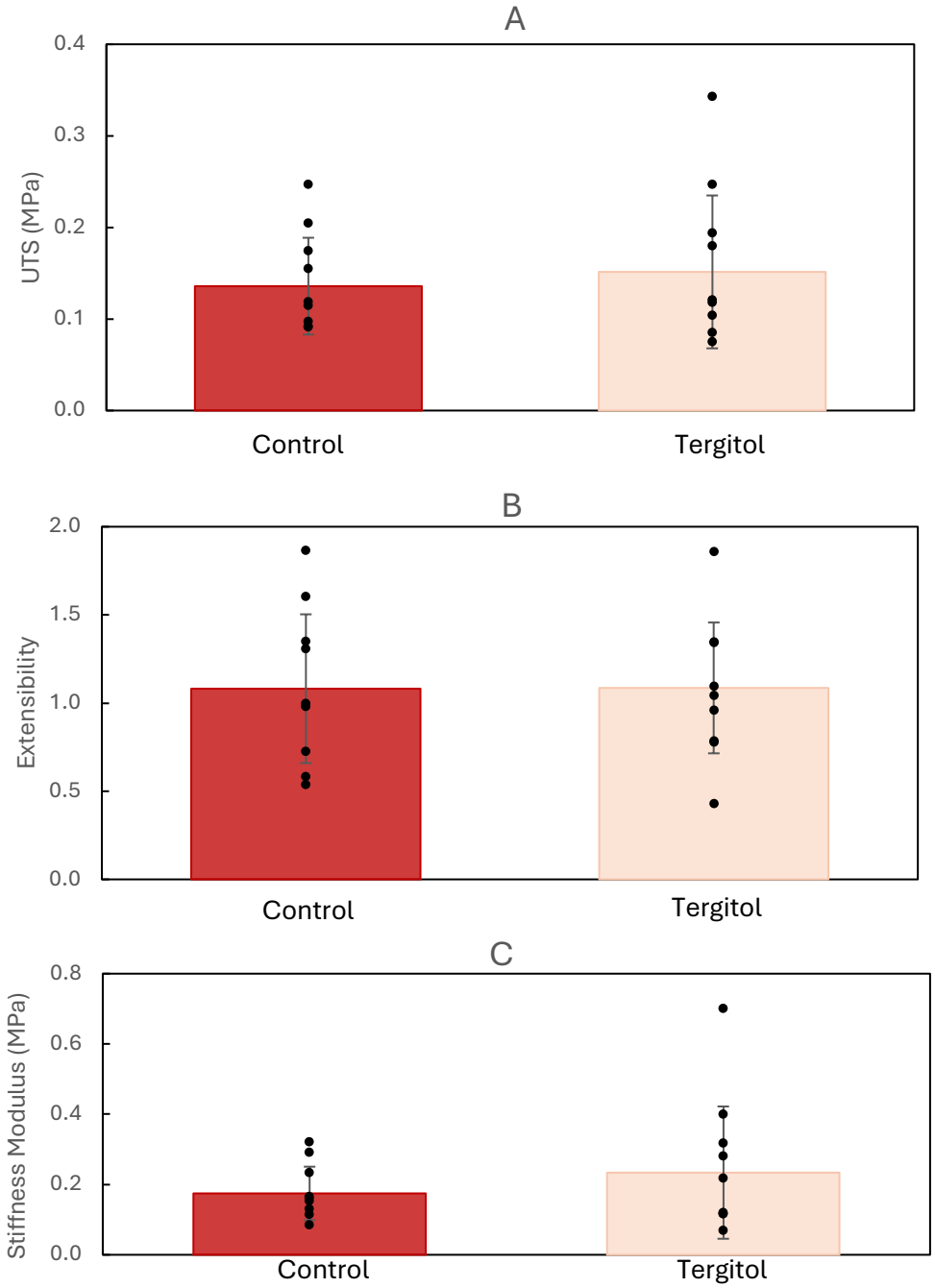

Figure 4.17. Mean myocardial (A) UTS, (B) extensibility, and (C) stiffness from control and Tergitol-treated samples.

Myocardial collagen is more susceptible to Tergitol induced disruption in post-pregnancy.

(i) Effects on collagen content

Tergitol treatment had differential effects on myocardial collagen content, depending on gestational state (Table 4.8). In the samples taken from pregnant animals, there was a significant increase in insoluble collagen content following Tergitol treatment (Fig 4.18B). This may be caused by the expected reductions in cellular mass resulting in the collagen occupying a greater percentage of the myocardial volume. Conversely, in the samples from previously pregnant animals, Tergitol treatment did not result in collagen occupying a greater percentage of the volume, but instead there was a trend towards reduced myocardial collagen content (Fig 4.18C); these findings suggest that the removal of cells was accompanied by an inadvertent loss of collagen. This suggests that the myocardial collagen network is more susceptible to chemical disruption in post-pregnancy, compared to during gestation.

Table 4.8. Summary of Sircol Collagen Assay results for bovine myocardium treated with Tergitol compared to control according to pregnancy state, reported as mean± SD. Paired myocardial samples were collected from 6 pregnant and 6 previously pregnant cows to examine the effects of Tergitol treatment on collagen content. Following a two-way repeated measures ANOVA, which identified a significant interaction between the effects of Tergitol treatment and pregnancy state on soluble ($p=0.076$), insoluble ($p<0.001$) and total ($p<0.001$) collagen content, one-way ANOVA was performed to examine differences between control and Tergitol-treated samples, according to pregnancy state. *denotes a significant difference between treatment groups, $p<0.05$. ^{t} denotes a trend between treatment groups, $p<0.1$.

Tissue	condition	soluble collagen (ug/mg wet tissue)	insoluble collagen (ug/mg wet tissue)	total collagen (ug/mg wet tissue)
Pregnant n=6	Control Tergitol	10 ± 6 17 ± 17	62 ± 31 242 ± 44*	72 ±31 253± 44*
Previously Pregnant n=6	Control Tergitol	16 ± 13 2 ± 0*	61 ± 49 29 ± 7	76 ±48 31± 7^{t}

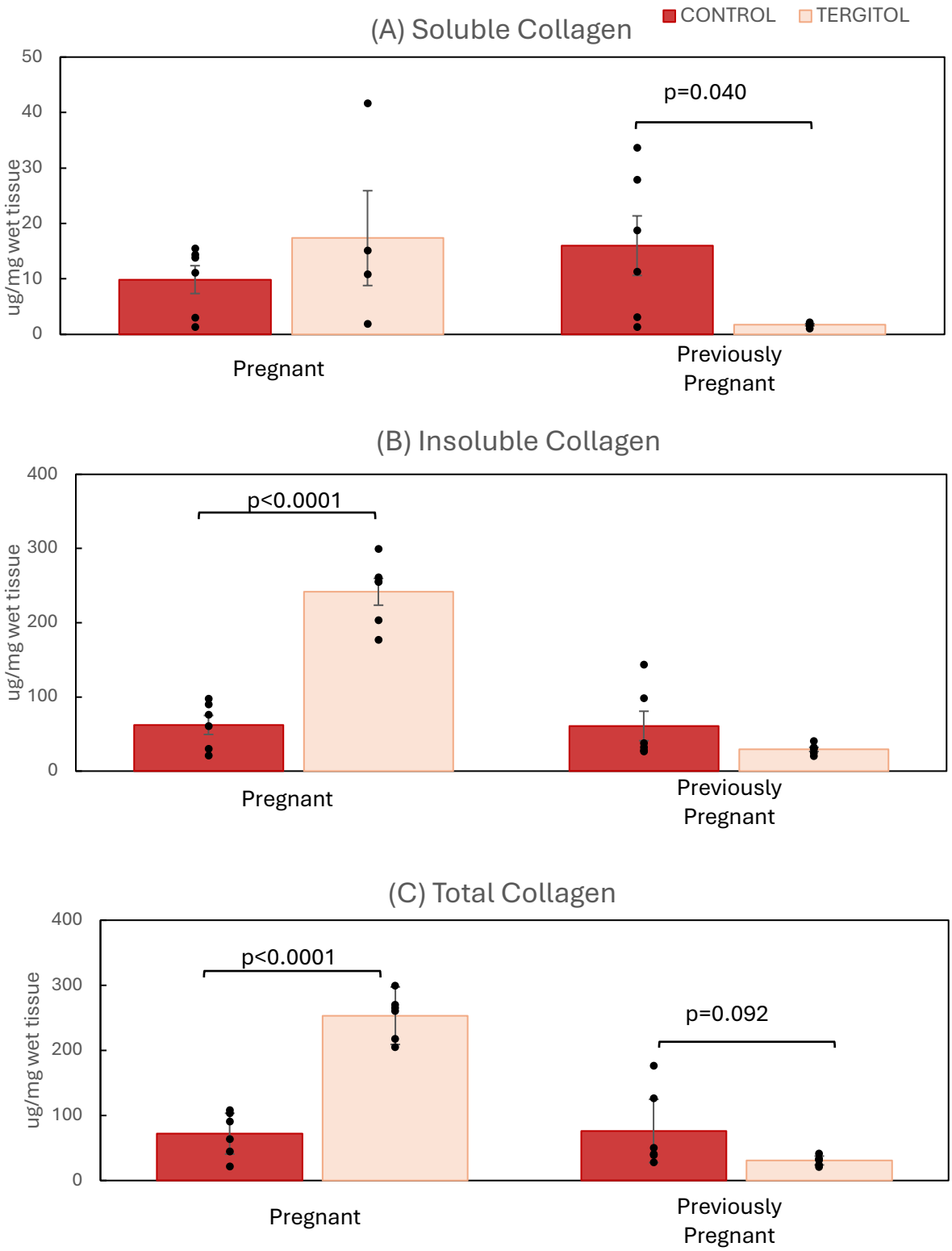

Figure 4.18. Mean soluble **(A),** insoluble **(B),** and total **(C)** collagen content in myocardium from pregnant and previously pregnant cows. Error bars represent SD; Following two-way ANOVA with repeated measures, one-way ANOVA was used for within group comparisons. p values<0.1 are shown.

(ii) Effects on collagen thermomechanical properties

Tergitol treatment also had differential effects on myocardial thermomechanical properties depending on gestational state (p=0.041) (Table 4.9). As seen with regards to changes in collagen content, the Tergitol treatment had no effect on the pregnant samples, but there was a significant reduction in the denaturation temperature of myocardial samples taken from previously pregnant animals, following Tergitol treatment (p=0.035) (Fig 4.19A). This could be due to the loosening of the collagen network in pregnancy, which was previously noted (i.e. reductions in denaturation temperature and crosslinking), allowing for a deeper penetration of Tergitol throughout the tissue. Conversely, the collagen network may be more tightly packed during pregnancy, impeding Tergitol penetration and thus protecting against any deleterious effects.

Table 4.9. Summary of results from HIT/DTT with $NaBH_4$ stabilization tests on bovine myocardium treated with Tergitol compared to control, according to pregnancy state, reported as mean ± SD. Paired myocardial samples were collected from 6 pregnant and 3 previously pregnant cows to examine the effects of Tergitol treatment on collagen thermomechanical properties. previously pregnant bovine, with samples being either tested fresh or following freeze-thawing. Following two-way ANOVA with repeated measures, which identified a significant interaction between the effects of Tergitol treatment and pregnancy state on T_d(p=0.041), paired t-test were performed for within group comparisons. *denotes a significant difference between groups, p<0.05.

Tissue	Condition	T_d (°C)	$t_{1/2}$ (hours)	$\frac{t_{1/2\ NaBH4}}{t_{1/2\ control}}$
Pregnant n=6	Control	67.8 ± 0.6	5.6 ± 1.4	1.2 ± 0.4
	Tergitol	66.7 ± 2.7	4.4 ± 1.6	1.7 ± 0.1
Previously Pregnant n=3	Control	66.2 ± 0.5	1.4 ± 0.8	3.8 ± 3.8
	Tergitol	63.5 ± 1.1*	1.6 ± 1.1	1.0 ± 0.9

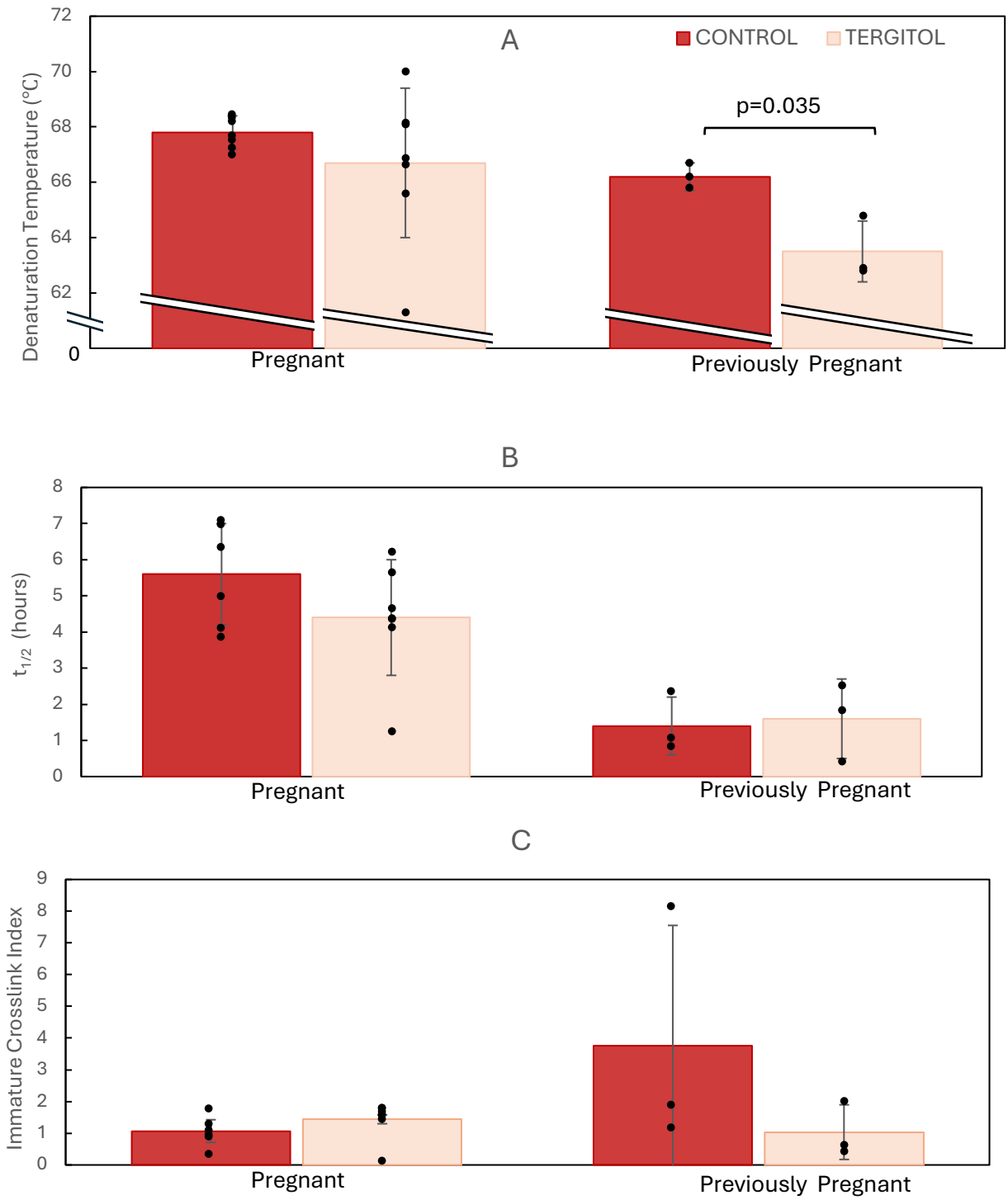

Figure 4.19. A. Mean collagen denaturation temperature in control and Tergitol-treated myocardial samples, grouped according to pregnancy state. **B.** Mean load decay half-time in control and Tergitol-treated bovine myocardium grouped by pregnancy state. **C.** Mean immature crosslink index for control and Tergitol-treated bovine myocardium group by pregnancy state. Error bars represent SD; Following two-way ANOVA with repeated measures, paired t-tests were used for within group comparisons. p values<0.1 are shown.

Chapter 5 Discussion

This is the first study to investigate myocardial remodeling during pregnancy and postpartum in a bovine model. The myocardium accommodates the blood volume overload state by increasing UTS and extensibility, from early to late pregnancy (Table 4.1). It was discovered that, whereas UTS reverts to early pregnant values in postpartum, myocardial extensibility is increased and stiffness is reduced in previously pregnant animals (Table 4.1). Moreover, for the first time, myocardial collagen remodeling in pregnancy and postpartum was identified, using HIT/DTT. Although, myocardial collagen content is unchanged (Table 4.3), there is a significant reduction in collagen helical stability (Table 4.4), accompanied by the replacement of mature for immature crosslinks (Table 4.5), in postpartum. The loosening of the myocardial collagen networks between pregnancy and postpartum may explain the gestation-dependent effects of freeze-thawing and Tergitol treatment on tissue. The myocardium from previously pregnant cows was less susceptible to collagen thermal destabilization from extracellular ice expansion (Table 4.6), but was more vulnerable to collagen disruption following Tergitol treatment (Table 4.8; Table 4.9), compared to tissue from pregnant animals.

5.1 Myocardial Mechanics in Pregnancy & Postpartum

UTS

The current study found that myocardial UTS was significantly increased between early and late pregnancy (Fig 4.2). This mechanical adaptation was expected, as left ventricular function is reportedly unchanged during pregnancy [57], despite increases in wall stress due to blood volume overload [50], [51]. Unlike pathological states of volume overload which do not trigger cardiomyocyte elongation [43], [45], [46], significant increases in cardiomyocyte surface area and length have also been observed during pregnancy in rodents [62]. In human pregnancy, changes in left ventricular geometry have also been reported, showing increases in both mass and thickness, thus normalizing wall stress, per the Law of Laplace [52]–[54]. As ventricular wall stress is normalized, the increase in myocardial UTS may serve as an additional fail-safe mechanism. Taken together, the findings from the current and past studies indicate that the myocardium adapts

to volume overload and increased wall stress in pregnancy not only by proportional rises in left ventricular diameter and width, but also by increasing myocardial UTS.

Regarding post-pregnancy recovery, the data from previously pregnant cows indicate that myocardial UTS reverts to EP values in postpartum (Fig 4.2). Similarly, studies have found that cardiomyocyte length and left ventricular thickness and mass are recovered in the postpartum as well [62]. As UTS is a measure of the maximum stress that can be applied, it follows that with elevated load on the heart, UTS increases in parallel, and that when the load is reduced, the UTS also decreases.

Extensibility

This study reported that myocardial extensibility is significantly increased between early and late pregnancy (Fig 4.3). Extensibility refers to the strain, or normalized change in length, that can be sustained by a tissue under tension, such as during diastole [130]. Previous studies have shown that end-diastolic volume is increased during pregnancy [50], [131]. Hence, an increased myocardial extensibility may underly the increased blood storage capacity of the left ventricle during pregnancy. Previous studies on pregnancy-induced remodeling of the pericardium and heart valves observed a biphasic pattern: extensibility was reduced in early pregnancy, compared to baseline and recovered through the remainder of gestation [77], [78] [84]. In the current study, the gestational ages of the cows were all over 75 days, thus very early mechanical adaptations to pregnancy may have been overlooked. Further, in the current student, values were not compared to those of never pregnant animals and thus the initial extensibility cannot be confirmed.

Curiously, the results from the current study suggest that myocardial extensibility does not fully reverse in postpartum (Fig 4.3). Therefore, pregnancy may result in a permanent dilation of the left ventricle; this would certainly account for the flaccid hearts harvested from previously pregnant cows that have been observed in the lab.

Stiffness

In the current study, there was no difference in myocardial stiffness between early and late pregnancy (Fig 4.4B). However, the sample sizes used for mechanical testing were low (EP=2; LP=6; PP=3), which may have caused differences between groups to remain

undetected. Interestingly, there was a trend towards an increased myocardial stiffness in pregnant cows compared to previously pregnancy cows (p=0.077; Fig 4.4A). Therefore, these results suggest that myocardial stiffness may be reduced in postpartum, compared to gestation. Taken together, it may be postulated that pregnancy-induced myocardial remodeling serves to maintain a constant stiffness throughout gestation, but may result in decreased myocardial stiffness in postpartum perhaps due to reductions in collagen crosslinks (Fig 4.9).

The results of the current study differed from the published literature. Virgen-Ortiz reported that elastic modulus of the left ventricle was significantly *reduced* in LP compared to PP [62]. A possible explanation for this discrepancy is the fact that the Virgen-Ortiz study only stretched the myocardial samples to 10% of their initial length to derive the elastic modulus [62], whereas the current study stretched the samples to failure and derived the stiffness modulus from the final 40% of the strain at failure. Myocardial stiffness is derived from a number of constituents, including collagen [132]. The crimped structure of collagen means that it does not bear load until it has been straightened, hence the stress-strain curves are commonly separated into two regions [132]. Therefore, it is possible that the inconsistent findings of myocardial stiffness in pregnancy are due to different regions of the stress-strain curve being utilized for its derivation.

Another difference between the Virgen-Ortez study and the current study is the animal model. The current study utilized cows, which have larger heart sizes compared to rodents. Whereas Virgen-Ortiz suggests that decreased myocardial stiffness enhances diastolic filling during pregnancy, it is possible that due to the much larger size of cow hearts, the increased ventricular size is more highly attributable for the increased end-diastolic volume rather than changes in stiffness. [62]. Therefore, myocardial remodeling may differ between species.

5.2 Collagen Remodeling in Pregnancy & Postpartum

Collagen Content

This study found that collagen does not accumulate in the heart over gestation; this is an observation that has also been noted in the literature [64]–[69]. Myocardial collagen content was not different between late pregnant and previously pregnant cows (Fig 4.5).

The absence of increased myocardial collagen content in pregnancy may be explained by the downregulation of fibrosis-related genes [64], as well as the expression of the relaxin. Relaxin is a pregnancy hormone responsible for relaxing the muscles and connective tissues of the pelvis to prepare for delivery and has been found to inhibit collagen synthesis and suppress crosslinking [133], [134]. As relaxin receptors are expressed on the heart [134], this hormone may confer protection against the development of fibrosis in pregnancy.

It is also important to state that comparisons were not made with early pregnant nor never pregnant cows. Therefore, collagen content may have been increased or decreased at the timepoints measured compared to baseline. Nonetheless, changes in collagen content are not always indicative of remodeling; collagen remodeling also encompasses alterations in molecular packing, crosslinks, and isoforms, all of which can have substantial effects on myocardial properties. For instance, Limon-Miranda et al. found that protein expression of collagen I was downregulated, and collagen III was upregulated in the left ventricles of pregnant rats [71]. For that reason, collagen thermomechanical properties were also explored.

Thermomechanical Properties

In the current study, there were no differences in myocardial collagen denaturation temperature throughout gestation, but there was a significant reduction in postpartum (Fig. 4.8). These findings indicate that collagen helical stability is significantly reduced in the myocardium of previously pregnant cows, compared to pregnant cows (Fig 4.8). Helical stability is influenced by molecular packing, according to the polymer-in-a-box model which explains that a smaller box confines the thermally labile domain of collagen, enhancing helical stability [23]. Hence, the current study suggests that collagen molecules are less tightly packed in postpartum, possibly due to collagen turnover.

Interpretation of collagen crosslinking was less clear, due to the abnormal load-decay behaviour of certain myocardial strips during the isothermal segment of the HIT/DTT (see Appendix I); whereas some myocardial tissue displayed an exponential decay in load throughout the entirety of the isotherm, others did not; rather, the initial 2000-300 seconds of the isotherm followed the regular behaviour, with peptide bond hydrolysis

leading to a decay in the load, but further into the isotherm contractions and plateaus in load occurred, for some samples (Appendix I). This behaviour may have been due to the heterogeneous composition of myocardial tissue, with various proteins contributing to its passive properties [13]. Water uptake within tissue, may have also occurred later in the isotherm, which can increase passive tension [135]. Therefore, some process *later* in the isotherm, be it thermoelastic contraction of other proteins or water uptake, may oppose the load relaxation from peptide bond hydrolysis.

The load decay half-time was calculated from the first 2000 (for EP samples) or 3000 (for LP and PP samples) seconds of the isotherm. These results suggested that collagen mature crosslinks are significantly reduced in the myocardium of previously pregnant cows compared to pregnant cows (Fig 4.9). When gestation was subdivided into EP (<150 days) and LP (>150 days), the results indicated that mature collagen crosslinks may begin to be lost during gestation, as there is a 25% reduction in load decay half-life from EP to LP (Fig 4.9). Loss of collagen crosslinking may serve to increase ventricular dilation throughout gestation. Additionally, the index of immature collagen crosslinks was significantly increased in the myocardium from previously pregnant cows compared to pregnant cows (Fig 4.10). As collagen crosslinks are initially synthesized as immature divalent crosslinks [33], the findings from this study suggest that there is increased myocardial collagen turnover in postpartum.

These findings suggest that myocardial collagen remodeling differs from remodeling of the pericardium and heart valves, which are associated with significant increases in mature crosslinking throughout gestation, a proposed adaptation to elevated tissue stress [77]–[79], [84]. The function and composition of the myocardium, compared to the latter cardiac structures, may explain the divergent collagen remodelling processes in pregnancy. Firstly, the myocardium must expand to permit diastolic filling and increases in collagen crosslinking have been found to stiffen the myocardium, compromising this function [34]. Thus, unlike in the pericardium and heart valves, an increase in collagen crosslinks may have adverse effects on myocardial adaptation to pregnancy. Secondly, whereas heart valves and pericardia are primarily collagenous structure, 70% of the myocardial volume is occupied by cardiomyocytes [3]. During pregnancy, cardiomyocytes

lengthen [62] and therefore, remodeling of the myocardial collagen network must also balance changes of the cardiomyocytes.

The current study suggests that pregnancy does not trigger rapid collagen degradation, as helical stability (Fig. 4.8) and mature crosslinks (Fig. 4.9) are not significantly different between EP and LP, and myocardial collagen content is not significantly different between pregnancy and postpartum. Therefore, myocardial collagen remodeling in pregnancy differs from pathological volume overload, wherein collagen breakdown is rapid and precedes cardiomyocyte lengthening [46]. Further, rodent studies indicate that MMP expression is downregulated in LP [73], [75], indicating that pregnancy inhibits excessive degradation of myocardial collagen. Total (Fig. 4.10) and mature (Fig. 4.9) collagen crosslinking were significantly reduced in postpartum, which may explain the reductions in UTS (Fig. 4.2) and stiffness (Fig. 4.4) which were observed postpartum. Taken together, the current study suggests that mature collagen is slowly lost throughout gestation and is replaced with immature collagen in postpartum. This explanation also explains how collagen content is unchanged between LP and PP (Fig 4.5), as degradation and deposition are balanced, and why collagen denaturation temperature is significantly reduced in PP, as turnover reduces molecular packing.

5.3 Effects of Freeze-Thawing

Freeze-thawing is commonly used in tissue decellularization, as well as for applications such as storage and preservation [100], [136], [137]. Thus, the effects of freeze-thawing on tissue structure and stability must be elucidated. The effects of freeze-thawing on collagen stability were dependent on the cow's gestational state (Table 4.6). In the myocardium of pregnant cows, freeze-thawing resulted in significant decreases in collagen denaturation temperature (Fig. 4.11), indicating that helical stability was reduced. Further, in the myocardium of pregnant cows, the load decay half-time was significantly reduced by freeze-thawing (Fig 4.11), suggesting perhaps a mechanical disruption of the connectivity between molecules.

Conversely, myocardium from previously pregnant cows did not exhibit reduction in collagen denaturation temperature, nor load decay half-time (Fig 4.11). This was an unexpected finding, and there are several possible explanations for this phenomenon.

Firstly, collagen thermal stability was found to already be reduced in the myocardium of postpartum cows, compared to pregnant animals (Fig 4.8), reflecting a larger intermolecular space. Hence, the looser myocardial collagen network of previously pregnant cows may result in sufficient intermolecular gap space to accommodate ice expansion, protecting against any deleterious effects.

There are inconsistent findings in the literature pertaining to the effects of intermolecular gaps on ice expansion. Some studies report that larger gaps are more susceptible to ice formation, as the freezing temperature of water is elevated [137]. Conversely, other studies suggest that larger gaps are indeed more resistant to ice expansion [136]. Clearly, more information is required on the effects of intermolecular gaps on freeze-thawing resistance. Another explanation for the differential responses of the pregnant and postpartum myocardium is intracellular water content. During freezing, intracellular water diffuses into the extracellular space and expands upon forming ice [138]. Pregnancy is associated with increased water retention, [139] which may explain the increased susceptibility of myocardium from pregnancy cows to freeze-thawing disruptions to the collagen network.

5.4 Effects of Tergitol

Efficacy of Decellularization

An unexpected finding from the current study was the ineffectiveness of Tergitol for myocardial decellularization. Although previous studies were able to remove all cellular material from myocardium using Tergitol [117], [118], there were several differences between the protocols used in the current study and those in the literature. Notable, the current study omitted the use of DNAse and dimethyl sulfoxide (DMSO), instead selecting a gentler combination to minimize possible damage to the collagen network. Whereas the actions of Tergitol are to adsorb and disrupt the cellular membrane [129], DNases are enzymes that digest DNA [140]. The histological images depict pyknotic nuclei within cells (Fig 4.13B), suggesting that the process of cell lysis had been initiated. Other regions of the tissue samples showed nuclei floating within the interstitial space (Fig 4.13B). By contrast, in the control (untreated) samples, nuclei were oval-shaped and centrally located within cardiomyocytes (Fig 4.13A). Therefore, based on these stains, it appears that the

Tergitol indeed adsorbed to cell membranes, causing cellular swelling in the case of condensed nuclei within intact cells, and cell lysis in the case of the free-floating nuclei. The absence of a DNase may explain the preservation of nuclei within the Tergitol-treated tissue. Numerous studies on tissue decellularization have found that the addition of DNase treatment to protocols significantly reduces DNA content [141].

The efficacy of tissue decellularization is dependent not only on the choice of detergents, but also on the concentration used, exposure time, tissue thickness, and cell density [89], [90]. For thick tissue in particular, such as myocardium, the penetration depth of the detergent is an important parameter for an effective decellularization [142], [143]. DMSO is a chemical solvent that has been used in other Tergitol-based decellularization protocols, serving to enhance detergent penetration [115]–[118]. As DMSO was not used in the current study, it may explain the ineffectiveness of the Tergitol treatment, with the cells in the deeper levels of the tissue not being accessible to the detergent. The addition of DNase and DMSO to the decellularization process in the current study should be used to enhance the removal of genetic material in subsequent studies.

Native myocardial tissue is dark red in colour (Fig. 4.12A), due to the presence of the intracellular protein myoglobin [144]. Following decellularization, myocardial tissue, from pregnant and previously pregnant cows, appeared light brown (Fig 4.12B) suggesting the removal of myoglobin, and thus that some cells had been partially affected by the Tergitol treatment. Indeed, the histological stains, despite showing nuclei retention, displayed regions of increased intramuscular spacing and regions where muscle fibers appeared fragmented (Fig 4.13B). Further, the Tergitol-treated samples were more eosinophilic showing a more intense pink staining with H&E (Fig 4.13B; Fig 4.14B). Eosin binds to positively charged particles [145], which results in altered staining intensities between compounds with different side chain groups, such as muscle and collagen. Changes in the intensity of the eosin stain suggest that the tissue composition has been altered. Following Tergitol treatment, intensity of the eosin stain was stronger (Fig 4.13B), likely because the proteins became more concentrated. During cell death, the cytoplasm condenses, resulting in tighter packing of cytoplasmic proteins and potentially stronger eosin staining [146]. Therefore, the stronger pink colour of the Tergitol treated samples may be due to initiation of cell lysis.

Effects on Myocardial Collagen

The results of the uniaxial tensile tests indicated that Tergitol had no effect on myocardial UTS, extensibility, nor stiffness. However, previous decellularization studies, such as those using sodium deoxycholate and CHAPS, also found that, whilst tissue mechanical properties were unaltered, the collagen network had been disrupted [102], [104]–[106]. Therefore, damage sustained by collagen may not be manifested in changes to tissue mechanical properties.

The current study found that Tergitol did affect myocardial collagen, depending on the gestational status of the animal (Table 4.8; Table 4.9). In the myocardium from previously pregnant cows, the results from the collagen assay show that there was a trend towards a reduction in collagen content following Tergitol treatment (Table 4.8), suggesting that some collagen may have been degraded. Further, collagen denaturation temperature (Fig 4.19A) was significantly reduced in the myocardium of previously pregnant cows following Tergitol treatment. H&E staining of control (untreated) and Tergitol-treated myocardial samples from previously pregnant cows, revealed stronger eosin staining intensity and a loss of collagen crimp, as the alternating birefringence bands were absent (Fig 4.15B; Fig 4.16B). This may be explained by a deeper penetration of the eosin through the Tergitol-treated tissue, and a disorganization of myocardial collagen following Tergitol treatment, resulting in a disruption in fiber alignment. Taken together, the findings of the current study suggest that Tergitol treatment resulted in a reduction of thermal stability and organization of the myocardial collagen network from previously pregnant animals.

Conversely, in the myocardium from pregnant cows, collagen content was increased following Tergitol treatment, as per the results of the collagen assay (Table 4.8). As new collagen could not have been synthesized, it is likely that the cellular mass had been reduced allowing the collagen to occupy a greater percentage of the myocardial volume. Additionally, there were no changes in myocardial collagen thermomechanical properties (Fig 4.19) for pregnant cows following Tergitol treatment. The explanation for the differential effects of Tergitol on the myocardium from pregnant cows, compared to previously pregnant, may be in the structural properties of collagen. According to the current study, myocardial collagen molecular packing is increased in pregnancy (Fig 4.8), in conjunction with increases in collagen crosslinking (Fig 4.9), compared to postpartum.

Crosslinking has been shown to increase collagen molecular packing and to reduce tissue permeability [32], which is pertinent because the effects of Tergitol are mediated by its penetration through the tissue [129]. Taken together, the findings from the current study indicate that the myocardial collagen network is more densely packed in pregnancy reducing susceptibility to Tergitol-induced damage.

5.5 Clinical Implications

Pregnancy is a time of dramatic changes in the maternal cardiovascular system. As cardiac health during gestation is a significant indicator for cardiac disease in later life, it is critical to improve our understanding of myocardial remodeling underlying the hemodynamic transformations and their reversal in postpartum.

5.5.1 Long Term Effects

Cardiovascular disease is the leading cause of death worldwide [147]; hence, identification of risk factors is critical to ensure early detection and intervention, halting disease progression. Adverse pregnancy outcomes have already been identified as risk factors for the later development of cardiovascular disease [148], [149]. For example, women who suffer from hypertension during pregnancy have a four-fold higher risk for heart failure and a two-fold higher risk of ischemic heart disease, compared to women with normotensive pregnancies [149]. The current study has important implications for the management of cardiovascular health; myocardial remodeling in pregnancy may be associated with effects that persist in postpartum, including increases in extensibility and reductions in collagen helical stability and mature crosslinks. For instance, decreases in myocardial collagen crosslinks may be a predisposition for ventricular dilation in cardiomyopathy [17], [20], [32]. Further, reductions in mature collagen may contribute to the development of systolic heart failure [39]. Alternatively, collagen remodeling in pregnancy and postpartum may be protective against the development of cardiac fibrosis later in life, as mature collagen is replaced with looser immature collagen. These questions remain to be elucidated, but are critical for enhancing our comprehension of cardiac diseases in women. Although cardiac disease is diagnosed more frequently in men, women are commonly diagnosed with heart disease at older ages and more advanced stages of

disease [150]. Therefore, identification of risk factors can contribute to earlier detection and intervention.

Abnormal and/or Cumulative Remodeling

Peripartum cardiomyopathy (PPCM) is a rare and life-threatening form of heart failure that develops during the final weeks or postpartum period of pregnancy, in women with no prior cardiac history [151]. PPCM, which is characterized by a reduced left ventricular ejection fraction, is associated with a high mortality rate [152]. The causes and pathophysiology of PPCM are not well understood, but certain risk factors have been identified for the development of disease including high parity [151]–[153]. Studies have shown that the majority of women afflicted with PPCM have had three or more prior pregnancies [152]. The current study suggests that permanent changes in myocardial extensibility and myocardial collagen crosslinks occur through gestation and postpartum. It is possible that abnormal myocardial remodeling and/or the augmented effects with multiple pregnancies contribute to the development of PPCM. By improving our understanding of the pathophysiology, the treatment of PPCM can be ameliorated, as current treatment is directed towards symptoms management rather than targeting the causes of disease. Taken together, the current study may be indicative of the role of myocardial remodeling to the development of PPCM.

5.6 General Conclusions

The current study identified myocardial remodeling, in a bovine model, as the heart adapts to the hemodynamic changes in pregnancy and postpartum. Mechanically, between EP and LP, there are significant increases in the UTS and extensibility of the myocardium, which allow the left ventricle to sufficiently expand and sustain the stress of blood volume overload. Interestingly, there was no significant difference in myocardial stiffness between EP and LP; this suggests that, during pregnancy for larger animals, reductions in myocardial stiffness are not the adaptation by which the left ventricle accommodates the left ventricle. Additionally, the current study found that, unlike UTS, myocardial extensibility does not fully recover in postpartum, suggesting that pregnancy may have long lasting effects on the heart.

Amazingly, this study identified for the first time myocardial collagen remodeling in pregnancy and postpartum. Whilst, collagen content is indeed unaffected by gestation, alterations in collagen helical stability and crosslinks were observed. It appears that the collagen network loosens during pregnancy through a loss of mature crosslinks, potentially to allow ventricular dilation or maintenance of myocardial stiffness, and that immature crosslinks are added in postpartum.

This study showed that, depending on pregnancy state, freeze-thawing negatively affects the myocardial collagen network, reducing thermal stability and connectivity. Interestingly, myocardial collagen from previously pregnancy displayed no differences in thermomechanical properties following freeze-thawing. The exact reason for these differential results is unclear, but may be due to increased intermolecular gaps of the myocardial collagen network in postpartum negating the effects of ice formation.

Another important finding from the current study was that Tergitol alone is ineffective for the decellularization of myocardium. Given the histological appearance of the Tergitol-treated tissue, specifically the nuclei in the interstitial space, it is evident that an endonuclease should be incorporated into decellularization protocols to fully remove genetic material from tissue. Additionally, the Tergitol treatment had differential on myocardial collagen based on pregnancy state - in postpartum, the replacement of mature for immature collagen crosslinks may have increased myocardial permeability, resulting in losses or destabilization of collagen in the previously pregnant animals. Therefore, tissue permeability affects the penetration, and ultimately efficacy of detergents; chemical agents such as DMSO should be utilized to improve decellularization efficacy, particularly in thick tissue.

5.7 Recommendations for Future Work

The current study identified differences in myocardial properties between pregnancy and postpartum. However, only one heifer was available for analysis, and therefore establishment of true baseline myocardial mechanical and collagen properties was not possible. Further, the parity of each cow was not assessed in this study. Future studies should examine the effects of multiple pregnancies on myocardial properties to

identify whether changes are cumulative. Future studies should also perform histological analysis on native and control-treated myocardium from never-pregnant and pregnant cows, to make observations on both the effect of the Tergitol treatment and the effect of pregnancy. A DNA assay should be used to quantify the amount of nuclear material pre- and post-decellularization. The current study utilized H&E stains, which identified that nuclei remained in the myocardium following Tergitol treatment, but the relative reduction could not be assessed.

References

[1] A. Leri, M. Rota, F. S. Pasqualini, P. Goichberg, and P. Anversa, “Origin of Cardiomyocytes in the Adult Heart,” *Circ Res*, vol. 116, no. 1, pp. 150–166, Jan. 2015, doi: 10.1161/CIRCRESAHA.116.303595.

[2] J. S. Janicki and G. L. Brower, “The role of myocardial fibrillar collagen in ventricular remodeling and function,” *J Card Fail*, vol. 8, no. 6, pp. S319–S325, Dec. 2002, doi: 10.1054/jcaf.2002.129260.

[3] P. Zhou and W. T. Pu, “Recounting Cardiac Cellular Composition,” *Circ Res*, vol. 118, no. 3, pp. 368–370, Feb. 2016, doi: 10.1161/CIRCRESAHA.116.308139.

[4] R. E. Ahmed, T. Tokuyama, T. Anzai, N. Chanthra, and H. Uosaki, “Sarcomere maturation: function acquisition, molecular mechanism, and interplay with other organelles,” *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 377, no. 1864, pp. 1–16, Nov. 2022, doi: 10.1098/rstb.2021.0325.

[5] W. A. Linke, V. I. Popov, and G. H. Pollack, “Passive and active tension in single cardiac myofibrils,” *Biophys J*, vol. 67, no. 2, pp. 782–792, Aug. 1994, doi: 10.1016/S0006-3495(94)80538-7.

[6] L. R. Perreault, M. C. Daley, M. C. Watson, S. Rastogi, A. Jaiganesh, E. C. Porter, B. M. Duffy, and L. D. Black, “Characterization of cardiac fibroblast-extracellular matrix crosstalk across developmental ages provides insight into age-related changes in cardiac repair,” *Front Cell Dev Biol*, vol. 12, pp. 1–12, Feb. 2024, doi: 10.3389/fcell.2024.1279932.

[7] J. B. Caulfield and T. K. Borg, “The collagen network of the heart.,” *Lab Invest*, vol. 40, no. 3, pp. 364–72, Mar. 1979.

[8] T. F. Robinson, L. Cohen-Gould, and S. M. Factor, “Skeletal framework of mammalian heart muscle. Arrangement of inter- and pericellular connective tissue structures.,” *Lab Invest*, vol. 49, no. 4, pp. 482–98, Oct. 1983.

[9] T. K. Borg, L. D. Johnson, and P. H. Lill, “Specific attachment of collagen to cardiac myocytes: In vivo and in vitro,” *Dev Biol*, vol. 97, no. 2, pp. 417–423, Jun. 1983, doi: 10.1016/0012-1606(83)90098-2.

[10] M. B. Knight, A. Grosberg, and M. L. McCain, “In Vitro Tools for Quantifying Structure–Function Relationships in Cardiac Myocyte Cells and Tissues,” in *Cardiac Cytoarchitecture*, Cham: Springer International Publishing, 2015, pp. 15–39. doi: 10.1007/978-3-319-15263-9_2.

[11] A. C. Silva, C. Pereira, A. C. R. G. Fonseca, P. Pinto-do-Ó, and D. S. Nascimento, “Bearing My Heart: The Role of Extracellular Matrix on Cardiac Development, Homeostasis, and Injury Response,” *Front Cell Dev Biol*, vol. 8, pp. 1–18, Jan. 2021, doi: 10.3389/fcell.2020.621644.

[12] B. Yue, “Biology of the Extracellular Matrix,” *J Glaucoma*, vol. 23, pp. S20–S23, 2014, doi: 10.1097/IJG.0000000000000108.

[13] H. L. Granzier and T. C. Irving, “Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments,” *Biophys J*, vol. 68, no. 3, pp. 1027–1044, Mar. 1995, doi: 10.1016/S0006-3495(95)80278-X.

[14] S. Kato, F. G. Spinale, R. Tanaka, W. Johnson, G. Cooper, and M. R. Zile, “Inhibition of collagen cross-linking: effects on fibrillar collagen and ventricular diastolic function,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 269, no. 3, pp. H863–H868, Sep. 1995, doi: 10.1152/ajpheart.1995.269.3.H863.

[15] B. S. L. da Costa and L. C. S. Nunes, “Mechanical behavior of crimped collagen fibers under uniaxial tension,” *Mechanics of Materials*, vol. 172, p. 104404, Sep. 2022, doi: 10.1016/j.mechmat.2022.104404.

[16] D. A. MacKenna, J. H. Omens, and J. W. Covell, “Left ventricular perimysial collagen fibers uncoil rather than stretch during diastolic filling,” *Basic Res Cardiol*, vol. 91, no. 2, pp. 111–122, 1996, doi: 10.1007/BF00799683.

[17] K. T. Weber, R. Pick, M. A. Silver, G. W. Moe, J. S. Janicki, I. H. Zucker, and P. W. Armstrong, “Fibrillar collagen and remodeling of dilated canine left ventricle.,” *Circulation*, vol. 82, no. 4, pp. 1387–1401, Oct. 1990, doi: 10.1161/01.CIR.82.4.1387.

[18] B. Villari, S. E. Campbell, O. M. Hess, G. Mall, G. Vassalli, K. T. Weber, and H. P. Krayenbuehl, “Influence of collagen network on left ventricular systolic and diastolic function in aortic valve disease,” *J Am Coll Cardiol*, vol. 22, no. 5, pp. 1477–1484, Nov. 1993, doi: 10.1016/0735-1097(93)90560-N.

[19] M. M. H. Marijianowski, P. Teeling, J. Mann, and A. E. Becker, “Dilated cardiomyopathy is associated with an increase in the type I/type III collagen ratio: A quantitative assessment,” *J Am Coll Cardiol*, vol. 25, no. 6, pp. 1263–1272, May 1995, doi: 10.1016/0735-1097(94)00557-7.

[20] D. Badenhorst, “Cross-linking influences the impact of quantitative changes in myocardial collagen on cardiac stiffness and remodelling in hypertension in rats,” *Cardiovasc Res*, vol. 57, no. 3, pp. 632–641, Mar. 2003, doi: 10.1016/S0008-6363(02)00733-2.

[21] M. Kasner, D. Westermann, B. Lopez, R. Gaub, F. Escher, U. Kühl, H.-P. Schultheiss, and C. Tschöpe, “Diastolic Tissue Doppler Indexes Correlate With the Degree of Collagen Expression and Cross-Linking in Heart Failure and Normal Ejection Fraction,” *J Am Coll Cardiol*, vol. 57, no. 8, pp. 977–985, Feb. 2011, doi: 10.1016/j.jacc.2010.10.024.

[22] B. Suki, “Collagen supramolecular structures: Evolution, organization, and biogenesis,” in *Structure and Function of the Extracellular Matrix*, Elsevier, 2022, pp. 55–76. doi: 10.1016/B978-0-12-819716-5.00003-4.

[23] C. A. Miles and M. Ghelashvili, “Polymer-in-a-Box Mechanism for the Thermal Stabilization of Collagen Molecules in Fibers,” *Biophys J*, vol. 76, no. 6, pp. 3243–3252, Jun. 1999, doi: 10.1016/S0006-3495(99)77476-X.

[24] J. F. Ribeiro, E. H. M. dos Anjos, M. L. S. Mello, and B. de Campos Vidal, “Skin Collagen Fiber Molecular Order: A Pattern of Distributional Fiber Orientation as Assessed by Optical Anisotropy and Image Analysis,” *PLoS One*, vol. 8, no. 1, p. e54724, Jan. 2013, doi: 10.1371/journal.pone.0054724.

[25] M. Wolman and F. H. Kasten, “Polarized light microscopy in the study of the molecular structure of collagen and reticulin,” *Histochemistry*, vol. 85, no. 1, pp. 41–49, 1986, doi: 10.1007/BF00508652.

[26] B. de C. Vidal, “Evaluation of the carbohydrate role in the molecular order of collagen bundles: microphotometric measurements of textural birefringence.,” *Cell Mol Biol*, vol. 32, no. 5, pp. 527–35, 1986.

[27] M. Franchi, M. Fini, M. Quaranta, V. De Pasquale, M. Raspanti, G. Giavaresi, V. Ottani, and A. Ruggeri, “Crimp morphology in relaxed and stretched rat Achilles tendon,” *J Anat*, vol. 210, no. 1, pp. 1–7, Jan. 2007, doi: 10.1111/j.1469-7580.2006.00666.x.

[28] R. Avazmohammadi, J. S. Soares, D. S. Li, S. S. Raut, R. C. Gorman, and M. S. Sacks, “A Contemporary Look at Biomechanical Models of Myocardium,” *Annu Rev Biomed Eng*, vol. 21, no. 1, pp. 417–442, Jun. 2019, doi: 10.1146/annurev-bioeng-062117-121129.

[29] A. Nikolov and N. Popovski, “Extracellular Matrix in Heart Disease: Focus on Circulating Collagen Type I and III Derived Peptides as Biomarkers of Myocardial Fibrosis and Their Potential in the Prognosis of Heart Failure: A Concise Review,” *Metabolites*, vol. 12, no. 4, p. 297, Mar. 2022, doi: 10.3390/metabo12040297.

[30] B. Depalle, Z. Qin, S. J. Shefelbine, and M. J. Buehler, “Influence of cross-link structure, density and mechanical properties in the mesoscale deformation mechanisms of collagen fibrils,” *J Mech Behav Biomed Mater*, vol. 52, pp. 1–13, Dec. 2015, doi: 10.1016/j.jmbbm.2014.07.008.

[31] K. Yoshida, H. Jiang, M. Kim, J. Vink, S. Cremers, D. Paik, R. Wapner, M. Mahendroo, and K. Myers, “Quantitative Evaluation of Collagen Crosslinks and Corresponding Tensile Mechanical Properties in Mouse Cervical Tissue during Normal Pregnancy,” *PLoS One*, vol. 9, no. 11, p. e112391, Nov. 2014, doi: 10.1371/journal.pone.0112391.

[32] P. Guo, Y. Miao, Y. Jing, S. Akella, F. Wang, C. Y. Park, C. Zhang, and R. S. Chuck, “Changes in Collagen Structure and Permeability of Rat and Human Sclera After Crosslinking,” *Transl Vis Sci Technol*, vol. 9, no. 9, p. 45, Aug. 2020, doi: 10.1167/tvst.9.9.45.

[33] B. Depalle, A. G. Duarte, I. A. K. Fiedler, L. Pujo-Menjouet, M. J. Buehler, and J.-P. Berteau, “The different distribution of enzymatic collagen cross-links found in adult and children bone result in different mechanical behavior of collagen,” *Bone*, vol. 110, pp. 107–114, May 2018, doi: 10.1016/j.bone.2018.01.024.

[34] G. R. Norton, J. Tsotetsi, B. Trifunovic, C. Hartford, G. P. Candy, and A. J. Woodiwiss, “Myocardial Stiffness Is Attributed to Alterations in Cross-Linked Collagen Rather Than Total Collagen or Phenotypes in Spontaneously Hypertensive Rats,” *Circulation*, vol. 96, no. 6, pp. 1991–1998, Sep. 1997, doi: 10.1161/01.CIR.96.6.1991.

[35] I. Medugorac, “Characterization of intramuscular collagen in the mammalian left ventricle,” *Basic Res Cardiol*, vol. 77, no. 6, pp. 589–598, Nov. 1982, doi: 10.1007/BF01908312.

[36] G. M. Fomovsky, S. Thomopoulos, and J. W. Holmes, “Contribution of extracellular matrix to the mechanical properties of the heart,” *J Mol Cell Cardiol*, vol. 48, no. 3, pp. 490–496, Mar. 2010, doi: 10.1016/j.yjmcc.2009.08.003.

[37] D. A. MacKenna, J. H. Omens, A. D. McCulloch, and J. W. Covell, “Contribution of collagen matrix to passive left ventricular mechanics in isolated rat hearts,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 266, no. 3, pp. H1007–H1018, Mar. 1994, doi: 10.1152/ajpheart.1994.266.3.H1007.

[38] D. L. Mann and F. G. Spinale, “Activation of Matrix Metalloproteinases in the Failing Human Heart,” *Circulation*, vol. 98, no. 17, pp. 1699–1702, Oct. 1998, doi: 10.1161/01.CIR.98.17.1699.

[39] C. F. Baicu, J. D. Stroud, V. A. Livesay, E. Hapke, J. Holder, F. G. Spinale, and M. R. Zile, “Changes in extracellular collagen matrix alter myocardial systolic performance,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 284, no. 1, pp. H122–H132, Jan. 2003, doi: 10.1152/ajpheart.00233.2002.

[40] B. López, A. González, R. Querejeta, M. Larman, and J. Díez, “Alterations in the Pattern of Collagen Deposition May Contribute to the Deterioration of Systolic Function in Hypertensive Patients With Heart Failure,” *J Am Coll Cardiol*, vol. 48, no. 1, pp. 89–96, Jul. 2006, doi: 10.1016/j.jacc.2006.01.077.

[41] R. T. Lee, “Matrix Metalloproteinase Inhibition and the Prevention of Heart Failure,” *Trends Cardiovasc Med*, vol. 11, no. 5, pp. 202–205, Jul. 2001, doi: 10.1016/S1050-1738(01)00113-X.

[42] W. Motz and B. E. Strauer, “Left ventricular function and collagen content after regression of hypertensive hypertrophy.,” *Hypertension*, vol. 13, no. 1, pp. 43–50, Jan. 1989, doi: 10.1161/01.HYP.13.1.43.

[43] B. A. Carabello, K. Nakano, W. Corin, R. Biederman, and J. F. Spann, “Left ventricular function in experimental volume overload hypertrophy,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 256, no. 4, pp. H974–H981, Apr. 1989, doi: 10.1152/ajpheart.1989.256.4.H974.

[44] R. C. Childers, A. J. Trask, J. Liu, P. A. Lucchesi, and K. J. Gooch, “Paired Pressure–Volume Loop Analysis and Biaxial Mechanical Testing Characterize Differences in Left Ventricular Tissue Stiffness of Volume Overload and Angiotensin-Induced Pressure Overload Hearts,” *J Biomech Eng*, vol. 143, no. 8, pp. 1–10, Aug. 2021, doi: 10.1115/1.4050541.

[45] G. L. Brower, J. R. Henegar, and J. S. Janicki, “Temporal evaluation of left ventricular remodeling and function in rats with chronic volume overload,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 271, no. 5, pp. H2071–H2078, Nov. 1996, doi: 10.1152/ajpheart.1996.271.5.H2071.

[46] T. D. Ryan, E. C. Rothstein, I. Aban, J. A. Tallaj, A. Husain, P. A. Lucchesi, and L. J. Dell’Italia, “Left Ventricular Eccentric Remodeling and Matrix Loss Are Mediated by Bradykinin and Precede Cardiomyocyte Elongation in Rats With Volume Overload,” *J Am Coll Cardiol*, vol. 49, no. 7, pp. 811–821, Feb. 2007, doi: 10.1016/j.jacc.2006.06.083.

[47] S. C. Tyagi, K. Lewis, D. Pikes, A. Marcello, V. S. Mujumdar, L. M. Smiley, and C. K. Moore, “Stretch-induced membrane type matrix metalloproteinase and tissue plasminogen activator in cardiac fibroblast cells,” *J Cell Physiol*, vol. 176, no. 2, pp. 374–382, Aug. 1998, doi: 10.1002/(SICI)1097-4652(199808)176:2<374::AID-JCP16>3.0.CO;2-3.

[48] G. L. Brower and J. S. Janicki, “Contribution of ventricular remodeling to pathogenesis of heart failure in rats,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 280, no. 2, pp. H674–H683, Feb. 2001, doi: 10.1152/ajpheart.2001.280.2.H674.

[49] A. Khalil, “Physiology of pregnancy,” in *MRCOG Part One*, A. Fiander and B. Thilaganathan, Eds., 2nd ed.RCOG Press, 2015, pp. 439–448. doi: 10.1017/CBO9781107587519.031.

[50] M. Sanghavi and J. D. Rutherford, “Cardiovascular Physiology of Pregnancy,” *Circulation*, vol. 130, no. 12, pp. 1003–1008, Sep. 2014, doi: 10.1161/CIRCULATIONAHA.114.009029.

[51] P. Soma-Pillay, C. Nelson-Piercy, H. Tolppanen, and A. Mebazaa, “Physiological changes in pregnancy,” *Cardiovasc J Afr*, vol. 27, no. 2, pp. 89–94, May 2016, doi: 10.5830/CVJA-2016-021.

[52] K. Melchiorre, R. Sharma, and B. Thilaganathan, “Cardiac structure and function in normal pregnancy,” *Curr Opin Obstet Gynecol*, vol. 24, no. 6, pp. 413–421, Dec. 2012, doi: 10.1097/GCO.0b013e328359826f.

[53] E. Chung, F. Yeung, and L. A. Leinwand, “Akt and MAPK signaling mediate pregnancy-induced cardiac adaptation,” *J Appl Physiol*, vol. 112, no. 9, pp. 1564–1575, May 2012, doi: 10.1152/japplphysiol.00027.2012.

[54] T. Geva, M. B. Mauer, L. Strikera, B. Kirshon, and J. M. Pivarnik, “Effects of physiologic load of pregnancy on left ventricular contractility and remodeling,” *Am Heart J*, vol. 133, no. 1, pp. 53–59, Jan. 1997, doi: 10.1016/S0002-8703(97)70247-3.

[55] M. E. Estensen, J. O. Beitnes, G. Grindheim, L. Aaberge, O. A. Smiseth, T. Henriksen, and S. Aakhus, “Altered maternal left ventricular contractility and function during normal pregnancy,” *Ultrasound in Obstetrics & Gynecology*, vol. 41, no. 6, pp. 659–666, Jun. 2013, doi: 10.1002/uog.12296.

[56] O. Savu, R. Jurcuţ, S. Giuşcă, T. van Mieghem, I. Gussi, B. A. Popescu, C. Ginghină, F. Rademakers, J. Deprest, and J.-U. Voigt, “Morphological and Functional Adaptation of the Maternal Heart During Pregnancy,” *Circ Cardiovasc Imaging*, vol. 5, no. 3, pp. 289–297, May 2012, doi: 10.1161/CIRCIMAGING.111.970012.

[57] S. P. Sengupta, M. Bansal, L. Hofstra, P. P. Sengupta, and J. Narula, “Gestational changes in left ventricular myocardial contractile function: new insights from two-dimensional speckle tracking echocardiography,” *Int J Cardiovasc Imaging*, vol. 33, no. 1, pp. 69–82, Jan. 2017, doi: 10.1007/s10554-016-0977-y.

[58] M. R. Zile, C. F. Baicu, and W. H. Gaasch, “Diastolic Heart Failure — Abnormalities in Active Relaxation and Passive Stiffness of the Left Ventricle,” *New England Journal of Medicine*, vol. 350, no. 19, pp. 1953–1959, May 2004, doi: 10.1056/NEJMoa032566.

[59] C. Iacobaeus, E. Andolf, M. Thorsell, K. Bremme, E. Östlund, and T. Kahan, “Cardiac function, myocardial mechano-energetic efficiency, and ventricular–arterial coupling in normal pregnancy,” *J Hypertens*, vol. 36, no. 4, pp. 857–866, Apr. 2018, doi: 10.1097/HJH.0000000000001612.

[60] M. Hieda, J.-K. Yoo, D. Sun, Y. Okada, R. S. Parker, M. A. Roberts-Reeves, B. Adams-Huet, D. B. Nelson, B. D. Levine, and Q. Fu, “Time course of changes in maternal left ventricular function during subsequent pregnancy in women with a history of gestational hypertensive disorders,” *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 315, no. 4, pp. R587–R594, Oct. 2018, doi: 10.1152/ajpregu.00040.2018.

[61] W. Y. Fok, L. Y. Chan, J. T. Wong, C. M. Yu, and T. K. Lau, “Left ventricular diastolic function during normal pregnancy: assessment by spectral tissue Doppler imaging,” *Ultrasound in Obstetrics and Gynecology*, vol. 28, no. 6, pp. 789–793, Nov. 2006, doi: 10.1002/uog.3849.

[62] A. Virgen-Ortiz, J. L. Marin, A. Elizalde, E. Castro, E. Stefani, L. Toro, and J. Muñiz, “Passive mechanical properties of cardiac tissues in heart hypertrophy during pregnancy,” *The Journal of Physiological Sciences*, vol. 59, no. 5, pp. 391–396, Sep. 2009, doi: 10.1007/s12576-009-0047-5.

[63] V. P. Novak, F. C. P. Yin, and J. D. Humphrey, “Regional mechanical properties of passive myocardium,” *J Biomech*, vol. 27, no. 4, pp. 403–412, Apr. 1994, doi: 10.1016/0021-9290(94)90016-7.

[64] M. B. Aljabri, N. T. Songstad, T. Lund, M. C. Serrano, T. V. Andreasen, S. Al-Saad, S. Lindal, V. Sitras, G. Acharya, and K. Ytrehus, “Pregnancy protects against antiangiogenic and fibrogenic effects of angiotensin II in rat hearts,” *Acta Physiologica*, vol. 201, no. 4, pp. 445–456, Apr. 2011, doi: 10.1111/j.1748-1716.2010.02234.x.

[65] S. Umar, R. Nadadur, A. Iorga, M. Amjedi, H. Matori, and M. Eghbali, “Cardiac structural and hemodynamic changes associated with physiological heart hypertrophy of pregnancy are reversed postpartum,” *J Appl Physiol*, vol. 113, no. 8, pp. 1253–1259, Oct. 2012, doi: 10.1152/japplphysiol.00549.2012.

[66] K. Lemmens, K. Doggen, and G. W. De Keulenaer, “Activation of the neuregulin/ErbB system during physiological ventricular remodeling in pregnancy,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 300, no. 3, pp. H931–H942, Mar. 2011, doi: 10.1152/ajpheart.00385.2010.

[67] Y. Yang *et al.*, “Cardiac Remodeling During Pregnancy With Metabolic Syndrome,” *Circulation*, vol. 143, no. 7, pp. 699–712, Feb. 2021, doi: 10.1161/CIRCULATIONAHA.120.051264.

[68] K. L. Fulghum, J. B. Smith, J. Chariker, L. F. Garrett, K. R. Brittian, P. K. Lorkiewicz, L. A. McNally, S. Uchida, S. P. Jones, B. G. Hill, and H. E. Collins, “Metabolic signatures of pregnancy-induced cardiac growth,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 323, no. 1, pp. H146–H164, Jul. 2022, doi: 10.1152/ajpheart.00105.2022.

[69] C. Che, K. Dudick, and R. Shoemaker, “Cardiac hypertrophy with obesity is augmented after pregnancy in C57BL/6 mice,” *Biol Sex Differ*, vol. 10, no. 1, p. 59, Dec. 2019, doi: 10.1186/s13293-019-0269-z.

[70] M. A. Brown, E. D. M. Gallery, M. R. Ross, and R. P. Esber, “Sodium excretion in normal and hypertensive pregnancy: A prospective study,” *Am J Obstet Gynecol*, vol. 159, no. 2, pp. 297–307, Aug. 1988, doi: 10.1016/S0002-9378(88)80071-1.

[71] S. Limon-Miranda, D. G. Salazar-Enriquez, J. Muñiz, M. V. Ramirez-Archila, E. A. Sanchez-Pastor, F. Andrade, J. G. Soñanez-Organis, E. F. Moran-Palacio, and A. Virgen-Ortiz, "Pregnancy Differentially Regulates the Collagens Types I and III in Left Ventricle from Rat Heart," *Biomed Res Int*, vol. 2014, pp. 1–5, 2014, doi: 10.1155/2014/984785.

[72] M. E. Parrott, E. Aljrbi, D. L. Biederman, R. N. Montalvo, J. L. Barth, and H. A. LaVoie, "Maternal cardiac messenger RNA expression of extracellular matrix proteins in mice during pregnancy and the postpartum period," *Exp Biol Med*, vol. 243, no. 15–16, pp. 1220–1232, Nov. 2018, doi: 10.1177/1535370218818457.

[73] A. A. Thurstin, A. N. Egeli, E. C. Goldsmith, F. G. Spinale, and H. A. LaVoie, "Tissue inhibitor of metalloproteinase-4 deletion in mice impacts maternal cardiac function during pregnancy and postpartum," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 324, no. 1, pp. H85–H99, Jan. 2023, doi: 10.1152/ajpheart.00408.2022.

[74] E. Chung, J. Heimiller, and L. A. Leinwand, "Distinct Cardiac Transcriptional Profiles Defining Pregnancy and Exercise," *PLoS One*, vol. 7, no. 7, p. e42297, Jul. 2012, doi: 10.1371/journal.pone.0042297.

[75] A. Virgen-Ortiz, S. Limón-Miranda, D. G. Salazar-Enríquez, V. Melnikov, E. A. Sánchez-Pastor, and E. M. Castro-Rodríguez, "Matrix Metalloproteinases System and Types of Fibrosis in Rat Heart during Late Pregnancy and Postpartum," *Medicina (B Aires)*, vol. 55, no. 5, p. 199, May 2019, doi: 10.3390/medicina55050199.

[76] V. Arpino, M. Brock, and S. E. Gill, "The role of TIMPs in regulation of extracellular matrix proteolysis," *Matrix Biology*, vol. 44–46, pp. 247–254, May 2015, doi: 10.1016/j.matbio.2015.03.005.

[77] S. M. Wells, C. M. Pierlot, and A. D. Moeller, "Physiological remodeling of the mitral valve during pregnancy," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 303, no. 7, pp. H878–H892, Oct. 2012, doi: 10.1152/ajpheart.00845.2011.

[78] C. M. Pierlot, A. D. Moeller, J. M. Lee, and S. M. Wells, "Biaxial Creep Resistance and Structural Remodeling of the Aortic and Mitral Valves in Pregnancy," *Ann Biomed Eng*, vol. 43, no. 8, pp. 1772–1785, Aug. 2015, doi: 10.1007/s10439-014-1230-2.

[79] C. M. Pierlot, A. D. Moeller, J. M. Lee, and S. M. Wells, "Pregnancy-induced remodeling of heart valves," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 309, no. 9, pp. H1565–H1578, Nov. 2015, doi: 10.1152/ajpheart.00816.2014.

[80] B. Scott and S. Wells, “Remodelling of bovine mitral valve chordae tendinae during pregnancy,” *Canadian Journal of Cardiology*, vol. 32, no. 10, pp. S217–S218, Oct. 2016, doi: 10.1016/j.cjca.2016.07.344.

[81] C. M. Pierlot, J. M. Lee, R. Amini, M. S. Sacks, and S. M. Wells, “Pregnancy-Induced Remodeling of Collagen Architecture and Content in the Mitral Valve,” *Ann Biomed Eng*, vol. 42, no. 10, pp. 2058–2071, Oct. 2014, doi: 10.1007/s10439-014-1077-6.

[82] B. V. Rego, S. M. Wells, C.-H. Lee, and M. S. Sacks, “Mitral valve leaflet remodelling during pregnancy: insights into cell-mediated recovery of tissue homeostasis,” *J R Soc Interface*, vol. 13, no. 125, p. 20160709, Dec. 2016, doi: 10.1098/rsif.2016.0709.

[83] M. Jaworska-Wilczynska, P. Trzaskoma, A. A. Szczepankiewicz, and T. Hryniewiecki, “Pericardium: structure and function in health and disease,” *Folia Histochem Cytobiol*, vol. 54, no. 3, pp. 121–125, Oct. 2016, doi: 10.5603/FHC.a2016.0014.

[84] J. A. E. Doane, J. M. Lee, and S. M. Wells, “Structural-Mechanical Changes in the Pericardium During Pregnancy,” *Cardiovasc Eng Technol*, vol. 4, no. 1, pp. 39–52, Mar. 2013, doi: 10.1007/s13239-012-0116-4.

[85] G. G. Belz, “Elastic properties and Windkessel function of the human aorta,” *Cardiovasc Drugs Ther*, vol. 9, no. 1, pp. 73–83, Feb. 1995, doi: 10.1007/BF00877747.

[86] S. Wells and M. Martin, “Remodelling of bovine descending aorta and aortic valve during pregnancy are not reversed postpartum ,” *Canadian Journal of Cardiology*, vol. 38, no. 10, p. S112, Oct. 2022, doi: 10.1016/j.cjca.2022.08.035.

[87] D. R. DeBay, “Pregnancy-Induced Vascular Remodelling: Changes in the Structure and Mechanical Properties of the Bovine Thoracic Aorta,” M.A.Sc. Dissertation, Dalhousie University, Halifax, 2007.

[88] L. C. Poon *et al.*, “Hypertensive disorders of pregnancy and long-term cardiovascular health: FIGO Best Practice Advice,” *International Journal of Gynecology & Obstetrics*, vol. 160, no. S1, pp. 22–34, Jan. 2023, doi: 10.1002/ijgo.14540.

[89] D. Moffat, K. Ye, and S. Jin, “Decellularization for the retention of tissue niches,” *J Tissue Eng*, vol. 13, p. 204173142211011, Jan. 2022, doi: 10.1177/20417314221101151.

[90] K. M. Whitehead, H. K. L. Hendricks, S. N. Cakir, and L. E. de Castro Brás, “ECM roles and biomechanics in cardiac tissue decellularization,” *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 323, no. 3, pp. H585–H596, Sep. 2022, doi: 10.1152/ajpheart.00372.2022.

[91] J. Hwang, B. H. San, N. J. Turner, L. J. White, D. M. Faulk, S. F. Badylak, Y. Li, and S. M. Yu, “Molecular assessment of collagen denaturation in decellularized tissues using a collagen hybridizing peptide,” *Acta Biomater*, vol. 53, pp. 268–278, Apr. 2017, doi: 10.1016/j.actbio.2017.01.079.

[92] J. L. Zitnay, Y. Li, Z. Qin, B. H. San, B. Depalle, S. P. Reese, M. J. Buehler, S. M. Yu, and J. A. Weiss, “Molecular level detection and localization of mechanical damage in collagen enabled by collagen hybridizing peptides,” *Nat Commun*, vol. 8, no. 1, p. 14913, Mar. 2017, doi: 10.1038/ncomms14913.

[93] K. E. Drzewiecki, D. R. Grisham, A. S. Parmar, V. Nanda, and D. I. Shreiber, “Circular Dichroism Spectroscopy of Collagen Fibrillogenesis: A New Use for an Old Technique,” *Biophys J*, vol. 111, no. 11, pp. 2377–2386, Dec. 2016, doi: 10.1016/j.bpj.2016.10.023.

[94] Y. A. Nashchekina, A. A. Starostina, N. A. Trusova, M. Y. Sirotkina, A. I. Lihachev, and A. V Nashchekin, “Molecular and fibrillar structure collagen analysis by FTIR spectroscopy,” *J Phys Conf Ser*, vol. 1697, no. 1, p. 012053, Dec. 2020, doi: 10.1088/1742-6596/1697/1/012053.

[95] F. Iranmanesh and T. L. Willett, “A linear systems model of the hydrothermal isometric tension test for assessing collagenous tissue quality,” *J Mech Behav Biomed Mater*, vol. 125, p. 104916, Jan. 2022, doi: 10.1016/j.jmbbm.2021.104916.

[96] R. E. Gordon, “Electron Microscopy: A Brief History and Review of Current Clinical Application,” 2014, pp. 119–135. doi: 10.1007/978-1-4939-1050-2_7.

[97] R. M. Williams, W. R. Zipfel, and W. W. Webb, “Interpreting Second-Harmonic Generation Images of Collagen I Fibrils,” *Biophys J*, vol. 88, no. 2, pp. 1377–1386, Feb. 2005, doi: 10.1529/biophysj.104.047308.

[98] C. G. Fuentes-Corona, J. Licea-Rodriguez, R. Younger, R. Rangel-Rojo, E. O. Potma, and I. Rocha-Mendoza, “Second harmonic generation signal from type I collagen fibers grown in vitro,” *Biomed Opt Express*, vol. 10, no. 12, p. 6449, Dec. 2019, doi: 10.1364/BOE.10.006449.

[99] S. Giannini, R. Buda, F. Di Caprio, P. Agati, A. Bigi, V. De Pasquale, and A. Ruggeri, “Effects of freezing on the biomechanical and structural properties of human posterior tibial tendons,” *Int Orthop*, vol. 32, no. 2, pp. 145–151, Apr. 2008, doi: 10.1007/s00264-006-0297-2.

[100] A. Ozcelikkale and B. Han, “Thermal Destabilization of Collagen Matrix Hierarchical Structure by Freeze/Thaw,” *PLoS One*, vol. 11, no. 1, p. e0146660, Jan. 2016, doi: 10.1371/journal.pone.0146660.

[101] L. J. White, A. J. Taylor, D. M. Faulk, T. J. Keane, L. T. Saldin, J. E. Reing, I. T. Swinehart, N. J. Turner, B. D. Ratner, and S. F. . Badylak, "The impact of detergents on the tissue decellularization process: A ToF-SIMS study," *Acta Biomater*, vol. 50, pp. 207–219, Mar. 2017, doi: 10.1016/j.actbio.2016.12.033.

[102] L. Laker, P. M. Dohmen, and F. E. Smit, "Synergy in a detergent combination results in superior decellularized bovine pericardial extracellular matrix scaffolds," *J Biomed Mater Res B Appl Biomater*, vol. 108, no. 6, pp. 2571–2578, Aug. 2020, doi: 10.1002/jbm.b.34588.

[103] Y. Hashimoto, S. Hattori, S. Sasaki, T. Honda, T. Kimura, S. Funamoto, H. Kobayashi, and A. Kishida, "Ultrastructural analysis of the decellularized cornea after interlamellar keratoplasty and microkeratome-assisted anterior lamellar keratoplasty in a rabbit model," *Sci Rep*, vol. 6, no. 1, p. 27734, Jun. 2016, doi: 10.1038/srep27734.

[104] M. Bertanha, A. Moroz, R. G. Jaldin, R. A. M. Silva, J. C. Rinaldi, M. A. Golim, S. L. Felisbino, M. A. C. Domingues, M. L. Sobreira, P. P. Reis, and E. Deffune, "Morphofunctional characterization of decellularized vena cava as tissue engineering scaffolds," *Exp Cell Res*, vol. 326, no. 1, pp. 103–111, Aug. 2014, doi: 10.1016/j.yexcr.2014.05.023.

[105] T. H. Petersen, E. A. Calle, M. B. Colehour, and L. E. Niklason, "Matrix Composition and Mechanics of Decellularized Lung Scaffolds," *Cells Tissues Organs*, vol. 195, no. 3, pp. 222–231, 2012, doi: 10.1159/000324896.

[106] A. Daugs, B. Hutzler, M. Meinke, C. Schmitz, N. Lehmann, A. Markhoff, and O. Bloch, "Detergent-Based Decellularization of Bovine Carotid Arteries for Vascular Tissue Engineering," *Ann Biomed Eng*, vol. 45, no. 11, pp. 2683–2692, Nov. 2017, doi: 10.1007/s10439-017-1892-7.

[107] D. M. Faulk, C. A. Carruthers, H. J. Warner, C. R. Kramer, J. E. Reing, L. Zhang, A. D'Amore, and S. F. Badylak, "The effect of detergents on the basement membrane complex of a biologic scaffold material," *Acta Biomater*, vol. 10, no. 1, pp. 183–193, Jan. 2014, doi: 10.1016/j.actbio.2013.09.006.

[108] S. Zouhair, E. Dal Sasso, S. R. Tuladhar, C. Fidalgo, L. Vedovelli, A. Filippi, G. Borile, A. Bagno, M. Marchesan, G. De Rossi, D. Gregori, W. F. Wolkers, F. Romanato, S. Korossis, G. Gerosa, and L. Iop, "A Comprehensive Comparison of Bovine and Porcine Decellularized Pericardia: New Insights for Surgical Applications," *Biomolecules*, vol. 10, no. 3, p. 371, Feb. 2020, doi: 10.3390/biom10030371.

[109] G. J. Wilson, H. Yeger, P. Klement, J. M. Lee, and D. W. Courtman, "Acellular matrix allograft small caliber vascular prostheses.," *ASAIO Trans*, vol. 36, no. 3, pp. M340-3, 1990.

[110] D. W. Courtman, C. A. Pereira, V. Kashef, D. McComb, J. M. Lee, and G. J. Wilson, “Development of a pericardial acellular matrix biomaterial: Biochemical and mechanical effects of cell extraction,” *J Biomed Mater Res*, vol. 28, no. 6, pp. 655–666, Jun. 1994, doi: 10.1002/jbm.820280602.

[111] D. W. Courtman, C. A. Pereira, S. Omar, S. E. Langdon, J. M. Lee, and G. J. Wilson, “Biomechanical and ultrastructural comparison of cryopreservation and a novel cellular extraction of porcine aortic valve leaflets,” *J Biomed Mater Res*, vol. 29, no. 12, pp. 1507–1516, Dec. 1995, doi: 10.1002/jbm.820291207.

[112] T. Palmosi, A. M. Tolomeo, C. Cirillo, D. Sandrin, M. Sciro, S. Negrisolo, M. Todesco, F. Caicci, M. Santoro, E. Dal Lago, M. Marchesan, M. Modesti, A. Bagno, F. Romanato, P. Grumati, A. Fabozzo, and G. Gerosa, “Small intestinal submucosa-derived extracellular matrix as a heterotopic scaffold for cardiovascular applications,” *Front Bioeng Biotechnol*, vol. 10, pp. 1–23, Dec. 2022, doi: 10.3389/fbioe.2022.1042434.

[113] G. J. Wilson, D. W. Courtman, P. Klement, J. Michael Lee, and H. Yeger, “Acellular matrix: A biomaterials approach for coronary artery bypass and heart valve replacement,” *Ann Thorac Surg*, vol. 60, pp. S353–S358, Aug. 1995, doi: 10.1016/0003-4975(95)98967-Y.

[114] H.-J. Chen, D.-H. Tseng, and S.-L. Huang, “Biodegradation of octylphenol polyethoxylate surfactant Triton X-100 by selected microorganisms,” *Bioresour Technol*, vol. 96, no. 13, pp. 1483–1491, Sep. 2005, doi: 10.1016/j.biortech.2004.11.013.

[115] M. Todesco, S. J. Imran, T. M. Fortunato, D. Sandrin, G. Borile, F. Romanato, M. Casarin, G. Giuggioli, F. Conte, M. Marchesan, G. Gerosa, and A. Bagno, “A New Detergent for the Effective Decellularization of Bovine and Porcine Pericardia,” *Biomimetics*, vol. 7, no. 3, p. 104, Aug. 2022, doi: 10.3390/biomimetics7030104.

[116] M. Casarin, T. M. Fortunato, S. J. Imran, M. Todesco, D. Sandrin, M. Marchesan, G. Gerosa, F. Romanato, A. Bagno, F. Dal Moro, and A. Morlacco, “Preliminary In Vitro Assessment of Decellularized Porcine Descending Aorta for Clinical Purposes,” *J Funct Biomater*, vol. 14, no. 3, p. 141, Mar. 2023, doi: 10.3390/jfb14030141.

[117] M. Faggioli, A. Moro, S. Butt, M. Todesco, D. Sandrin, G. Borile, A. Bagno, A. Fabozzo, F. Romanato, M. Marchesan, S. Imran, and G. Gerosa, “A New Decellularization Protocol of Porcine Aortic Valves Using Tergitol to Characterize the Scaffold with the Biocompatibility Profile Using Human Bone Marrow Mesenchymal Stem Cells,” *Polymers (Basel)*, vol. 14, no. 6, p. 1226, Mar. 2022, doi: 10.3390/polym14061226.

[118] S. Tondato, A. Moro, S. Butt, M. Todesco, D. Sandrin, G. Borile, M. Marchesan, A. Fabozzo, A. Bagno, F. Romanato, S. J. Imran, and G. Gerosa, "Tergitol Based Decellularization Protocol Improves the Prerequisites for Pulmonary Xenografts: Characterization and Biocompatibility Assessment," *Polymers (Basel)*, vol. 15, no. 4, p. 819, Feb. 2023, doi: 10.3390/polym15040819.

[119] M. Yılmaz and H. Korkmaz, "Recurrent births (multiparity) lead to permanent changes in cardiac structure," *Journal of Obstetrics and Gynaecology Research*, vol. 48, no. 4, pp. 946–955, Apr. 2022, doi: 10.1111/jog.15172.

[120] C. E. Rexroad, L. E. Casida, and W. J. Tyler, "Crown-Rump Length of Fetuses in Purebred Holstein-Friesian Cows," *J Dairy Sci*, vol. 57, no. 3, pp. 346–347, Mar. 1974, doi: 10.3168/jds.S0022-0302(74)84891-5.

[121] H. Ghaemi, K. Behdinan, and A. D. Spence, "In vitro technique in estimation of passive mechanical properties of bovine heart," *Med Eng Phys*, vol. 31, no. 1, pp. 76–82, Jan. 2009, doi: 10.1016/j.medengphy.2008.04.008.

[122] J. M. Lee, C. A. Pereira, D. Abdulla, W. A. Naimark, and I. Crawford, "A multi-sample denaturation temperature tester for collagenous biomaterials," *Med Eng Phys*, vol. 17, no. 2, pp. 115–121, Mar. 1995, doi: 10.1016/1350-4533(95)91882-H.

[123] M. Le Lous, J.-C. Allain, L. Cohen-solal, and P. Maroteaux, "Hydrothermal Isometric Tension Curves from Different Connective Tissues. Role of Collagen Genetic Types and Noncollagenous Components," *Connect Tissue Res*, vol. 11, no. 2–3, pp. 199–206, Jan. 1983, doi: 10.3109/03008208309004856.

[124] M. L. Tanzer, "Collagen crosslinks: Stabilization by borohydride reduction," *Biochimica et Biophysica Acta (BBA) - Protein Structure*, vol. 133, no. 3, pp. 584–587, Apr. 1967, doi: 10.1016/0005-2795(67)90564-8.

[125] M. Le Lous, J.-C. Allain, L. Cohen-Solal, and P. Maroteaux, "The Rate of Collagen Maturation in Rat and Human Skin," *Connect Tissue Res*, vol. 9, no. 4, pp. 253–262, Jan. 1982, doi: 10.3109/03008208209160271.

[126] *Sircol™ - (Original) Soluble Collagen Assay kit*. Belfast: Bicolor Ltd., 2023.

[127] J. Qian, Y. Okada, T. Ogura, K. Tanaka, S. Hattori, S. Ito, J. Satoh, T. Takita, and K. Yasukawa, "Kinetic Analysis of the Digestion of Bovine Type I Collagen Telopeptides with Porcine Pepsin," *J Food Sci*, vol. 81, no. 1, pp. 27–34, Jan. 2016, doi: 10.1111/1750-3841.13179.

[128] J. P. Orgel, T. J. Wess, and A. Miller, "The in situ conformation and axial location of the intermolecular cross-linked non-helical telopeptides of type I collagen," *Structure*, vol. 8, no. 2, pp. 137–142, Feb. 2000, doi: 10.1016/S0969-2126(00)00089-7.

[129] R. Khan, M. A. Inam, S. Khan, A. N. Jiménez, D. R. Park, and I. T. Yeom, “The Influence of Ionic and Nonionic Surfactants on the Colloidal Stability and Removal of CuO Nanoparticles from Water by Chemical Coagulation,” *Int J Environ Res Public Health*, vol. 16, no. 7, p. 1260, Apr. 2019, doi: 10.3390/ijerph16071260.

[130] P. Page, “Current concepts in muscle stretching for exercise and rehabilitation.,” *Int J Sports Phys Ther*, vol. 7, no. 1, pp. 109–19, Feb. 2012.

[131] K. Melchiorre, R. Sharma, A. Khalil, and B. Thilaganathan, “Maternal Cardiovascular Function in Normal Pregnancy,” *Hypertension*, vol. 67, no. 4, pp. 754–762, Apr. 2016, doi: 10.1161/HYPERTENSIONAHA.115.06667.

[132] H. L. Granzier and T. C. Irving, “Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments,” *Biophys J*, vol. 68, no. 3, pp. 1027–1044, Mar. 1995, doi: 10.1016/S0006-3495(95)80278-X.

[133] C. Samuel, J. Coghlan, and J. Bateman, “Effects of relaxin, pregnancy and parturition on collagen metabolism in the rat pubic symphysis,” *Journal of Endocrinology*, vol. 159, no. 1, pp. 117–125, Oct. 1998, doi: 10.1677/joe.0.1590117.

[134] X. Du, “Increased myocardial collagen and ventricular diastolic dysfunction in relaxin deficient mice: a gender-specific phenotype,” *Cardiovasc Res*, vol. 57, no. 2, pp. 395–404, Feb. 2003, doi: 10.1016/S0008-6363(02)00663-6.

[135] D. A. Sleboda, E. S. Wold, and T. J. Roberts, “Passive muscle tension increases in proportion to intramuscular fluid volume,” *Journal of Experimental Biology*, vol. 222, no. 21, pp. 1–5, Jan. 2019, doi: 10.1242/jeb.209668.

[136] I. Netinger Grubeša, M. Vračević, V. Ducman, B. Marković, I. Szenti, and Á. Kukovecz, “Influence of the Size and Type of Pores on Brick Resistance to Freeze-Thaw Cycles,” *Materials*, vol. 13, no. 17, p. 3717, Aug. 2020, doi: 10.3390/ma13173717.

[137] Y. Lu, S. Liu, E. Alonso, L. Wang, L. Xu, and Z. Li, “Volume changes and mechanical degradation of a compacted expansive soil under freeze-thaw cycles,” *Cold Reg Sci Technol*, vol. 157, pp. 206–214, Jan. 2019, doi: 10.1016/j.coldregions.2018.10.008.

[138] M. Toner, E. G. Cravalho, and M. Karel, “Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells,” *J Appl Phys*, vol. 67, no. 3, pp. 1582–1593, Feb. 1990, doi: 10.1063/1.345670.

[139] J. M. Davison, “Edema in pregnancy.,” *Kidney Int Suppl*, vol. 59, pp. S90-6, Jun. 1997.

[140] A. Varela-Ramirez, J. Abendroth, A. A. Mejia, I. Q. Phan, D. D. Lorimer, T. E. Edwards, and R. J. Aguilera, "Structure of acid deoxyribonuclease," *Nucleic Acids Res*, vol. 45, no. 10, pp. 6217–6227, Jun. 2017, doi: 10.1093/nar/gkx222.

[141] F. Koenig, M. Kilzer, C. Hagl, and N. Thierfelder, "Successful decellularization of thick-walled tissue: Highlighting pitfalls and the need for a multifactorial approach," *Int J Artif Organs*, vol. 42, no. 1, pp. 17–24, Jan. 2019, doi: 10.1177/0391398818805624.

[142] M. Shah, P. KC, and G. Zhang, "In Vivo Assessment of Decellularized Porcine Myocardial Slice as an Acellular Cardiac Patch," *ACS Appl Mater Interfaces*, vol. 11, no. 27, pp. 23893–23900, Jul. 2019, doi: 10.1021/acsami.9b06453.

[143] G. Basara, S. G. Ozcebe, B. W. Ellis, and P. Zorlutuna, "Tunable Human Myocardium Derived Decellularized Extracellular Matrix for 3D Bioprinting and Cardiac Tissue Engineering," *Gels*, vol. 7, no. 2, p. 70, Jun. 2021, doi: 10.3390/gels7020070.

[144] S. P. Suman and P. Joseph, "Myoglobin Chemistry and Meat Color," *Annu Rev Food Sci Technol*, vol. 4, no. 1, pp. 79–99, Feb. 2013, doi: 10.1146/annurev-food-030212-182623.

[145] H.-J. Shin, E.-K. Lee, J.-H. Lee, D. Lee, C.-S. Chang, Y.-S. Kim, and S. R. Paik, "Eosin interaction of α-synuclein leading to protein self-oligomerization," *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, vol. 1481, no. 1, pp. 139–146, Aug. 2000, doi: 10.1016/S0167-4838(00)00106-0.

[146] N. J. Ernest, C. W. Habela, and H. Sontheimer, "Cytoplasmic condensation is both necessary and sufficient to induce apoptotic cell death," *J Cell Sci*, vol. 121, no. 3, pp. 290–297, Feb. 2008, doi: 10.1242/jcs.017343.

[147] G. A. Roth *et al.*, "Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019," *J Am Coll Cardiol*, vol. 76, no. 25, pp. 2982–3021, Dec. 2020, doi: 10.1016/j.jacc.2020.11.010.

[148] K. Khosla, S. Heimberger, K. M. Nieman, A. Tung, S. Shahul, A. C. Staff, and S. Rana, "Long-Term Cardiovascular Disease Risk in Women After Hypertensive Disorders of Pregnancy: Recent Advances in Hypertension," *Hypertension*, vol. 78, no. 4, pp. 927–935, Oct. 2021, doi: 10.1161/HYPERTENSIONAHA.121.16506.

[149] L. J. Leon, F. P. McCarthy, K. Direk, A. Gonzalez-Izquierdo, D. Prieto-Merino, J. P. Casas, and L. Chappell, "Preeclampsia and Cardiovascular Disease in a Large UK Pregnancy Cohort of Linked Electronic Health Records," *Circulation*, vol. 140, no. 13, pp. 1050–1060, Sep. 2019, doi: 10.1161/CIRCULATIONAHA.118.038080.

[150] A. Cannata, P. Manca, V. Nuzzi, C. Gregorio, J. Artico, P. Gentile, C. Pio Loco, F. Ramani, G. Barbati, M. Merlo, and G. Sinagra, “Sex-Specific Prognostic Implications in Dilated Cardiomyopathy After Left Ventricular Reverse Remodeling,” *J Clin Med*, vol. 9, no. 8, p. 2426, Jul. 2020, doi: 10.3390/jcm9082426.

[151] J. Petryka-Mazurkiewicz, K. Kryczka, Ł. Mazurkiewicz, B. Miłosz-Wieczorek, M. Śpiewak, M. Marczak, J. Henzel, J. Grzybowski, M. Demkow, and Z. Dzielińska, “Cardiovascular Magnetic Resonance in Peripartum Cardiomyopathy: Comparison with Idiopathic Dilated Cardiomyopathy,” *Diagnostics*, vol. 11, no. 10, p. 1752, Sep. 2021, doi: 10.3390/diagnostics11101752.

[152] J. G. Demakis and S. H. Rahimtoola, “Peripartum Cardiomyopathy,” *Circulation*, vol. 44, no. 5, pp. 964–968, Nov. 1971, doi: 10.1161/01.CIR.44.5.964.

[153] K. Otani *et al.*, “Deficiency of Cardiac Natriuretic Peptide Signaling Promotes Peripartum Cardiomyopathy-Like Remodeling in the Mouse Heart,” *Circulation*, vol. 141, no. 7, pp. 571–588, Feb. 2020, doi: 10.1161/CIRCULATIONAHA.119.039761.

Appendix I

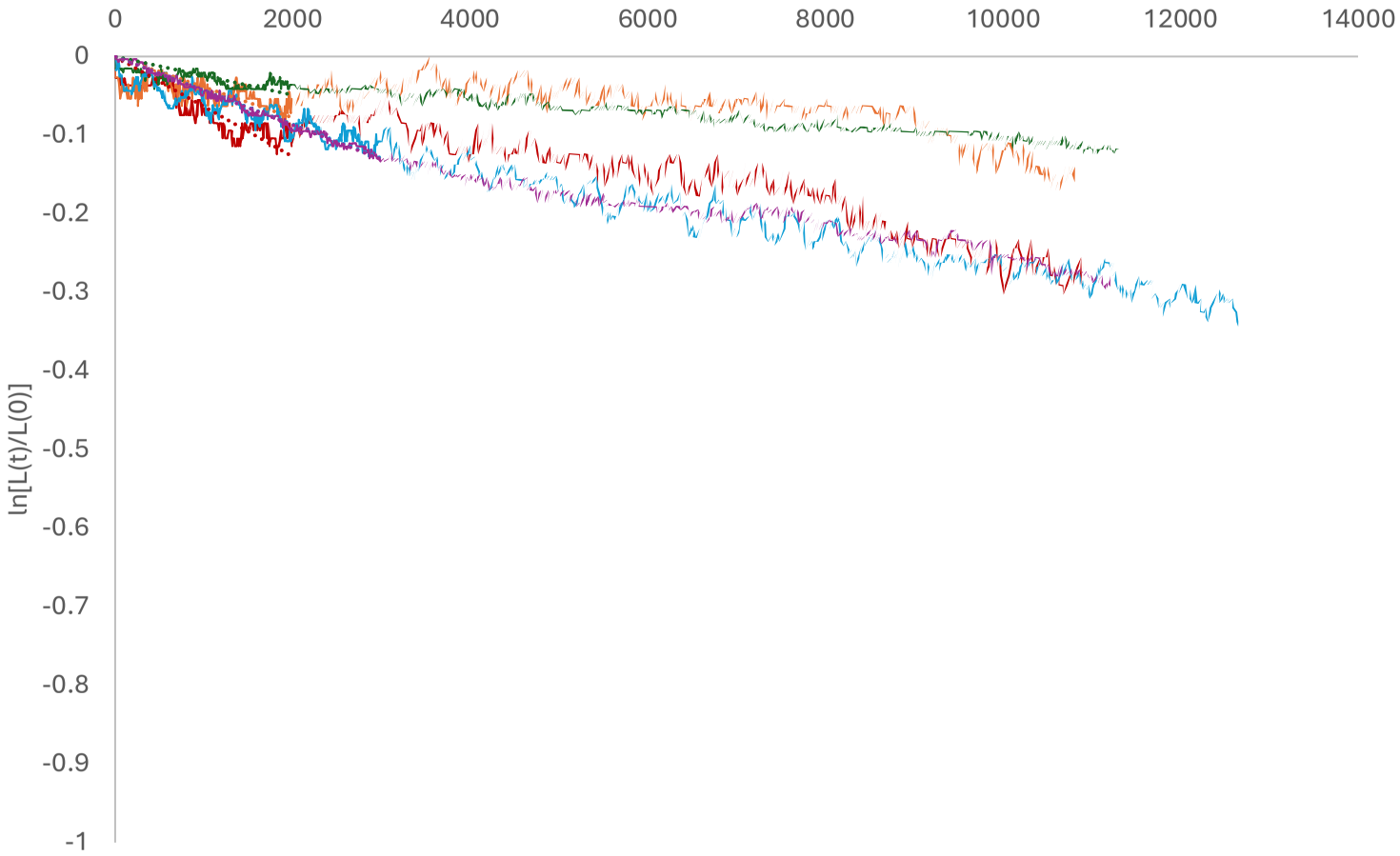

Figure 1. Representative curves of the natural logarithm of L(t)/L(0) vs time, as measured during the 90°C isotherm, of myocardial samples during early pregnancy (gestation day<150; n=5).

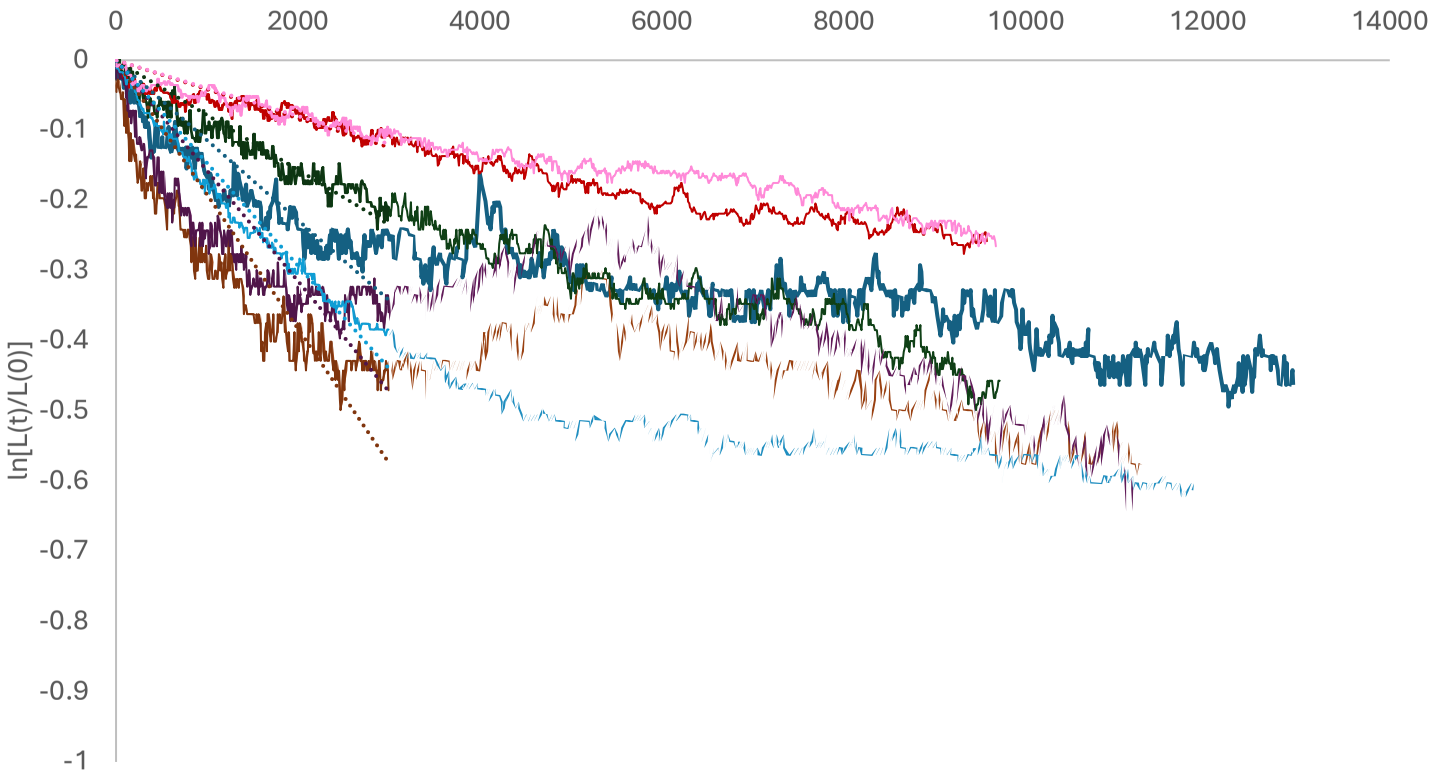

Figure 2. Representative curves of the natural logarithm of L(t)/L(0) vs time, as measured during the 90°C isotherm, of myocardial samples during late pregnancy (gestation day>150; n=7).

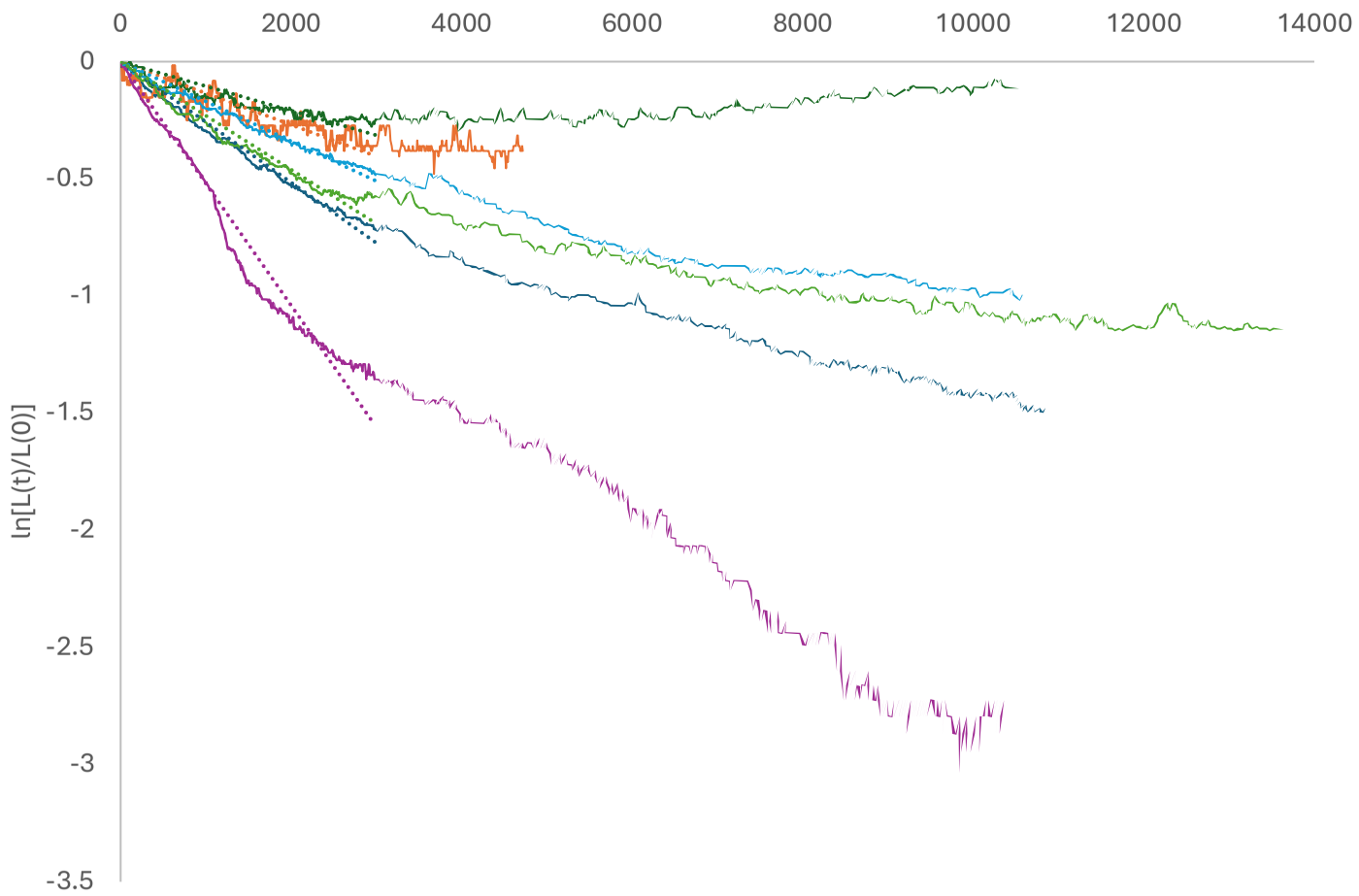

Figure 3. Representative curves of the natural logarithm of L(t)/L(0) vs time, as measured during the 90°C isotherm, of myocardial samples from previously pregnant bovine (n=6).

Appendix II

Print

SPRINGER NATURE LICENSE TERMS AND CONDITIONS

Aug 27, 2024

This Agreement between Valerie Metlege ("You") and Springer Nature ("Springer Nature") consists of your license details and the te opyright Clearance Center.

License Number	5852980507951
License date	Aug 20, 2024
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Springer eBook
Licensed Content Title	In Vitro Tools for Quantifying Structure–Function Relationships in Cardiac Myocyte Cells and Tissues
Licensed Content Author	Meghan B. Knight, Anna Grosberg, Megan L. McCain
Licensed Content Date	Jan 1, 2015
Type of Use	Thesis/Dissertation
Requestor type	non-commercial (non-profit)
Format	electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title of new work	Remodeling of Myocardial Collagen in the Bovine during Pregnancy and Postpartum
Institution name	Dalhousie University
Expected presentation date	Aug 2024
Order reference number	1
Portions	figure 2.1
The Requesting Person / Organization to Appear on the License	Valerie Metlege
Requestor Location	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Billing Type	Invoice
Billing Address	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Total	**0.00 CAD**

Print

Terms and Conditions

Springer Nature Customer Service Centre GmbH Terms and Conditions

The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightsLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or create derivative works using the Licensed Material for the purpose(s) specified in your RightsLink Licence Details only. Licenses are granted for the specific use requested in the order and for no other use, subject to these Terms and Conditions. You acknowledge and agree that the rights granted to you under this License do not include the right to modify, edit, translate, include in collective works, or create derivative works of the Licensed Material in whole or in part unless expressly stated in your RightsLink Licence Details. You may use the Licensed Material only as permitted under this Agreement and will not reproduce, distribute, display, perform, or otherwise use or exploit any Licensed Material in any way, in whole or in part, except as expressly permitted by this License.

1. 2. You may only use the Licensed Content in the manner and to the extent permitted by these Terms and Conditions, by your RightsLink Licence Details and by any applicable laws.

1. 3. A separate license may be required for any additional use of the Licensed Material, e.g. where a license has been purchased for print use only, separate permission must be obtained for electronic re-use. Similarly, a License is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the License.

1. 4. Any content within the Licensed Material that is owned by third parties is expressly excluded from the License.

1. 5. Rights for additional reuses such as custom editions, computer/mobile applications, film or TV reuses and/or any other derivative rights requests require additional permission and may be subject to an additional fee. Please apply to journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

2. Reservation of Rights

Licensor reserves all rights not expressly granted to you under this License. You acknowledge and agree that nothing in this License limits or restricts Licensor's rights in or use of the Licensed Material in any way. Neither this License, nor any act, omission, or statement by Licensor or you, conveys any ownership right to you in any Licensed Material, or to any element or portion thereof. As between Licensor and you, Licensor owns and retains all right, title, and interest in and to the Licensed Material subject to the license granted in Section 1.1. Your permission to use the Licensed Material is expressly conditioned on you not impairing Licensor's or the applicable copyright owner's rights in the Licensed Material in any way.

3. Restrictions on use

3. 1. Minor editing privileges are allowed for adaptations for stylistic purposes or formatting purposes provided such alterations do not alter the original meaning or intention of the Licensed Material and the new figure(s) are still accurate and representative of the Licensed Material. Any other changes including but not limited to, cropping, adapting, and/or omitting material that affect the meaning, intention or moral rights of the author(s) are strictly prohibited.

3. 2. You must not use any Licensed Material as part of any design or trademark.

3. 3. Licensed Material may be used in Open Access Publications (OAP), but any such reuse must include a clear acknowledgment of this permission visible at the same time as the figures/tables/illustration or abstract and which must indicate that the Licensed Material is not part of the governing OA license but has been reproduced with permission. This may be indicated according to any standard referencing system but must include at a minimum 'Book/Journal title, Author, Journal Name (if applicable), Volume (if applicable), Publisher, Year, reproduced with permission from SNCSC'.

4. STM Permission Guidelines

4. 1. An alternative scope of license may apply to signatories of the STM Permissions Guidelines ("STM PG") as amended from time to time and made available at https://www.stm-assoc.org/intellectual-property/permissions/permissions-guidelines/.

4. 2. For content reuse requests that qualify for permission under the STM PG, and which may be updated from time to time, the STM PG supersede the terms and conditions contained in this License.

4. 3. If a License has been granted under the STM PG, but the STM PG no longer apply at the time of publication, further permission must be sought from the Rightsholder. Contact journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

5. Duration of License

5. 1. Unless otherwise indicated on your License, a License is valid from the date of purchase ("License Date") until the end of the relevant period in the below table:

Reuse in a medical communications project	Reuse up to distribution or time period indicated in License
Reuse in a dissertation/thesis	Lifetime of thesis
Reuse in a journal/magazine	Lifetime of journal/magazine
Reuse in a book/textbook	Lifetime of edition
Reuse on a website	1 year unless otherwise specified in the License
Reuse in a presentation/slide kit/poster	Lifetime of presentation/slide kit/poster. Note: publication whether electronic or in print of presentation/slide kit/poster may require further permission.
Reuse in conference proceedings	Lifetime of conference proceedings
Reuse in an annual report	Lifetime of annual report
Reuse in training/CME materials	Reuse up to distribution or time period indicated in License
Reuse in newsmedia	Lifetime of newsmedia
Reuse in coursepack/classroom materials	Reuse up to distribution and/or time period indicated in license

6. Acknowledgement

6. 1. The Licensor's permission must be acknowledged next to the Licensed Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract and must be hyperlinked to the journal/book's homepage.

6. 2. Acknowledgement may be provided according to any standard referencing system and at a minimum should include "Author, Article/Book Title, Journal name/Book imprint, volume, page number, year, Springer Nature".

7. Reuse in a dissertation or thesis

7. 1. Where 'reuse in a dissertation/thesis' has been selected, the following terms apply: Print rights of the Version of Record are provided for; electronic rights for use only on institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/) and only up to what is required by the awarding institution.

7. 2. For theses published under an ISBN or ISSN, separate permission is required. Please contact journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

7. 3. Authors must properly cite the published manuscript in their thesis according to current citation standards and include the following acknowledgement: '*Reproduced with permission from Springer Nature*'.

8. License Fee

You must pay the fee set forth in the License Agreement (the "License Fees"). All amounts payable by you under this License are exclusive of any sales, use, withholding, value added or similar taxes, government fees or levies or other assessments. Collection and/or remittance of such taxes to the relevant tax authority shall be the responsibility of the party who has the legal obligation to do so.

9. Warranty

9. 1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. **You are solely responsible for ensuring that the material you wish to license is original to the Licensor and does not carry the copyright of another entity or third party (as credited in the published version).** If the credit line on any part of the Licensed Material indicates that it was reprinted or adapted with permission from another source, then you should seek additional permission from that source to reuse the material.

9. 2. EXCEPT FOR THE EXPRESS WARRANTY STATED HEREIN AND TO THE EXTENT PERMITTED BY APPLICABLE LAW, LICENSOR PROVIDES THE LICENSED MATERIAL "AS IS" AND MAKES NO OTHER REPRESENTATION OR WARRANTY. LICENSOR EXPRESSLY DISCLAIMS ANY LIABILITY FOR ANY CLAIM ARISING FROM OR OUT OF THE CONTENT, INCLUDING BUT NOT LIMITED TO ANY ERRORS, INACCURACIES, OMISSIONS, OR DEFECTS CONTAINED THEREIN, AND ANY IMPLIED OR EXPRESS WARRANTY AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, PUNITIVE, OR EXEMPLARY DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE LICENSED MATERIAL REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION APPLIES NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

10. Termination and Cancellation

10. 1. The License and all rights granted hereunder will continue until the end of the applicable period shown in Clause 5.1 above. Thereafter, this license will be terminated and all rights granted hereunder will cease.

10. 2. Licensor reserves the right to terminate the License in the event that payment is not received in full or if you breach the terms of this License.

11. General

11. 1. The License and the rights and obligations of the parties hereto shall be construed, interpreted and determined in accordance with the laws of the Federal Republic of Germany without reference to the stipulations of the CISG (United Nations Convention on Contracts for the International Sale of Goods) or to Germany´s choice-of-law principle.

11. 2. The parties acknowledge and agree that any controversies and disputes arising out of this License shall be decided exclusively by the courts of or having jurisdiction for Heidelberg, Germany, as far as legally permissible.

11. 3. This License is solely for Licensor's and Licensee's benefit. It is not for the benefit of any other person or entity.

Print

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Aug 27, 2024

This Agreement between Valerie Metlege ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	5854181128700
License date	Aug 22, 2024
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Acta Physiologica
Licensed Content Title	Pregnancy protects against antiangiogenic and fibrogenic effects of angiotensin II in rat hearts
Licensed Content Author	K. Ytrehus, G. Acharya, V. Sitras, et al
Licensed Content Date	Feb 1, 2011
Licensed Content Volume	201
Licensed Content Issue	4
Licensed Content Pages	12
Type of Use	Dissertation/Thesis
Requestor type	University/Academic
Format	Electronic
Portion	Figure/table
Number of figures/tables	1
Will you be translating?	No
Title of new work	Remodeling of Myocardial Collagen in the Bovine during Pregnancy and Postpartum
Institution name	Dalhousie University

Expected presentation date	Aug 2024
Order reference number	3
Portions	Figure 1b,c,d,e
The Requesting Person / Organization to Appear on the License	Valerie Metlege
Requestor Location	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Publisher Tax ID	EU826007151
Total	**0.00 CAD**

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order,** is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.
- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner.**For STM Signatory Publishers clearing permission under the terms of the STM Permissions Guidelines only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts,** You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.
- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto
- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.
- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Print

ELSEVIER LICENSE TERMS AND CONDITIONS

Aug 27, 2024

This Agreement between Valerie Metlege ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	5854190273143
License date	Aug 22, 2024
Licensed Content Publisher	Elsevier
Licensed Content Publication	Bone
Licensed Content Title	The different distribution of enzymatic collagen cross-links found in adult and children bone result in different mechanical behavior of collagen
Licensed Content Author	Baptiste Depalle,Andre G. Duarte,Imke A.K. Fiedler,Laurent Pujo-Menjouet,Markus J. Buehler,Jean-Philippe Berteau
Licensed Content Date	May 1, 2018
Licensed Content Volume	110
Licensed Content Issue	n/a
Licensed Content Pages	8
Start Page	107
End Page	114
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of new work	Remodeling of Myocardial Collagen in the Bovine during Pregnancy and Postpartum

Institution name	Dalhousie University
Expected presentation date	Aug 2024
Order reference number	5
Portions	figure 1
The Requesting Person / Organization to Appear on the License	Valerie Metlege
Requestor Location	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Publisher Tax ID	GB 494 6272 12
Total	**0.00 CAD**
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your RightsLink account and that are available at any time at https://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given. The material may not be reproduced or used in any other way, including use in combination with an artificial intelligence tool (including to train an algorithm, test, process, analyse, generate output and/or develop any form of artificial intelligence tool), or to create any derivative work and/or service (including resulting from the use of artificial intelligence tools).
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors:** the following clauses are applicable in addition to the above:
Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions
You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.
Terms & Conditions applicable to all Open Access articles published with Elsevier:
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

Licensed Content Publisher	The American Physiological Society
Licensed Content Publication	Am J Physiol- Heart and Circulatory Physiology
Licensed Content Title	Temporal evaluation of left ventricular remodeling and function in rats with chronic volume overload
Licensed Content Author	G. L. Brower, J. R. Henegar, J. S. Janicki
Licensed Content Date	Nov 1, 1996
Licensed Content Volume	271
Licensed Content Issue	5
Type of Use	Thesis/Dissertation
Requestor type	non-profit academic/educational
Readers being charged a fee for this work	no
Format	electronic
Portion	figures/tables/images
Number of figures/tables/images	1
Will you be translating?	no
World Rights	no
Order reference number	2
Title of new work	Remodeling of Myocardial Collagen in the Bovine during Pregnancy and Postpartum
Institution name	Dalhousie University
Expected presentation date	Aug 2024
Order reference number	2
Portions	Left ventricular pressure-volume curves from rats showing mechanical adaptations to pathological volume-overload
The Requesting Person / Organization to Appear on the License	Valerie Metlege
Requestor Location	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Billing Type	Invoice
Billing Address	Miss. Valerie Metlege 15 Houda Court

[redacted]

	Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Billing Type	Invoice
Billing Address	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Total	**0.00 CAD**

Terms and Conditions

Original Authors of Published Works: To see a full list of original authors rights concerning their own published work http://www.the-aps.org/publications/authorinfo/copyright.htm

Content reuse rights awarded by the APS may be exercised immediately upon issuance of this license, provided full disclosure and complete and accurate details of the proposed reuse have been made; no license is finally granted unless and until full payment is received either by the publisher or by CCC as provided in CCC's Billing and Payment Terms and Conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these Terms and Conditions or any of CCC's Billing and Payment Terms and Conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of the license, may constitute copyright infringement and the Publisher reserves the right to take action to protect its copyright of its materials.

The APS makes no representations or warranties with respect to the licensed material. You hereby indemnify and agree to hold harmless the publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

This license is personal to you /your organization and may not be sublicensed, assigned, or transferred by you /your organization to another person /organization without the publisher's permission.This license may not be amended except in writing signed by both parties, or in the case of the publisher, by CCC on the publisher's behalf.

The APS reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these Terms and Conditions and (iii) CCC's Billing and Payment Terms and Conditions.

v1.0

Questions? E-mail us at customercare@copyright.com.

Print

ELSEVIER LICENSE TERMS AND CONDITIONS

Aug 27, 2024

This Agreement between Valerie Metlege ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	5856490046453
License date	Aug 26, 2024
Licensed Content Publisher	Elsevier
Licensed Content Publication	Biophysical Journal
Licensed Content Title	Polymer-in-a-Box Mechanism for the Thermal Stabilization of Collagen Molecules in Fibers
Licensed Content Author	Christopher A. Miles,Michael Ghelashvili
Licensed Content Date	Jun 1, 1999
Licensed Content Volume	76
Licensed Content Issue	6
Licensed Content Pages	10
Start Page	3243
End Page	3252
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of new work	Remodeling of Myocardial Collagen in the Bovine during Pregnancy and Postpartum
Institution name	Dalhousie University
Expected presentation date	Aug 2024
Order reference number	6
Portions	figure 1
The Requesting Person / Organization to Appear on the License	Valerie Metlege

Requestor Location	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Publisher Tax ID	GB 494 6272 12
Total	**0.00 CAD**
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your RightsLink account and that are available at any time at https://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given. The material may not be reproduced or used in any other way, including use in combination with an artificial intelligence tool (including to train an algorithm, test, process, analyse, generate output and/or develop any form of artificial intelligence tool), or to create any derivative work and/or service (including resulting from the use of artificial intelligence tools).
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors:** the following clauses are applicable in addition to the above:
Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement

- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions
You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.
Terms & Conditions applicable to all Open Access articles published with Elsevier:
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.10

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Aug 27, 2024

This Agreement between Valerie Metlege ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	5856491328225
License date	Aug 26, 2024
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Cardiovascular Engineering and Technology
Licensed Content Title	Structural-Mechanical Changes in the Pericardium During Pregnancy
Licensed Content Author	Jennifer A. E. Doane et al
Licensed Content Date	Jan 11, 2013
Type of Use	Thesis/Dissertation
Requestor type	non-commercial (non-profit)
Format	electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title of new work	Remodeling of Myocardial Collagen in the Bovine during Pregnancy and Postpartum
Institution name	Dalhousie University
Expected presentation date	Aug 2024
Order reference number	9
Portions	figure 4
The Requesting Person / Organization to Appear on the License	Valerie Metlege
Requestor Location	Miss. Valerie Metlege 15 Houda Court

	Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Billing Type	Invoice
Billing Address	Miss. Valerie Metlege 15 Houda Court Halifax, NS B3m 4s3 Canada Attn: Valerie Metlege
Total	**0.00 CAD**

Terms and Conditions

Springer Nature Customer Service Centre GmbH Terms and Conditions

The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightsLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or create derivative works using the Licensed Material for the purpose(s) specified in your RightsLink Licence Details only. Licenses are granted for the specific use requested in the order and for no other use, subject to these Terms and Conditions. You acknowledge and agree that the rights granted to you under this License do not include the right to modify, edit, translate, include in collective works, or create derivative works of the Licensed Material in whole or in part unless expressly stated in your RightsLink Licence Details. You may use the Licensed Material only as permitted under this Agreement and will not reproduce, distribute, display, perform, or otherwise use or exploit any Licensed Material in any way, in whole or in part, except as expressly permitted by this License.

1. 2. You may only use the Licensed Content in the manner and to the extent permitted by these Terms and Conditions, by your RightsLink Licence Details and by any applicable laws.

1. 3. A separate license may be required for any additional use of the Licensed Material, e.g. where a license has been purchased for print use only, separate permission must be obtained for electronic re-use. Similarly, a License is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the License.

1. 4. Any content within the Licensed Material that is owned by third parties is expressly excluded from the License.

1. 5. Rights for additional reuses such as custom editions, computer/mobile applications, film or TV reuses and/or any other derivative rights requests require additional permission and may be subject to an additional fee. Please apply to journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

2. Reservation of Rights

Licensor reserves all rights not expressly granted to you under this License. You acknowledge and agree that nothing in this License limits or restricts Licensor's rights in or use of the Licensed Material in any way. Neither this License, nor any act, omission, or statement by Licensor or you, conveys any ownership right to you in any Licensed Material, or to any element or portion thereof. As between Licensor and you, Licensor owns and retains all right, title, and interest in and to the Licensed Material subject to the license granted in Section 1.1. Your permission to use the Licensed Material is expressly conditioned on you not impairing Licensor's or the applicable copyright owner's rights in the Licensed Material in any way.

3. Restrictions on use

3. 1. Minor editing privileges are allowed for adaptations for stylistic purposes or formatting purposes provided such alterations do not alter the original meaning or intention of the Licensed Material and the new figure(s) are still accurate and representative of the Licensed Material. Any other changes including but not limited to, cropping, adapting, and/or omitting material that affect the meaning, intention or moral rights of the author(s) are strictly prohibited.

3. 2. You must not use any Licensed Material as part of any design or trademark.

3. 3. Licensed Material may be used in Open Access Publications (OAP), but any such reuse must include a clear acknowledgment of this permission visible at the same time as the figures/tables/illustration or abstract and which must indicate that the Licensed Material is not part of the governing OA license but has been reproduced with permission. This may be indicated according to any standard referencing system but must include at a minimum 'Book/Journal title, Author, Journal Name (if applicable), Volume (if applicable), Publisher, Year, reproduced with permission from SNCSC'.

4. STM Permission Guidelines

4. 1. An alternative scope of license may apply to signatories of the STM Permissions Guidelines ("STM PG") as amended from time to time and made available at https://www.stm-assoc.org/intellectual-property/permissions/permissions-guidelines/.

4. 2. For content reuse requests that qualify for permission under the STM PG, and which may be updated from time to time, the STM PG supersede the terms and conditions contained in this License.

4. 3. If a License has been granted under the STM PG, but the STM PG no longer apply at the time of publication, further permission must be sought from the Rightsholder. Contact journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

5. Duration of License

5. Duration of License

5. 1. Unless otherwise indicated on your License, a License is valid from the date of purchase ("License Date") until the end of the relevant period in the below table:

Reuse in a medical communications project	Reuse up to distribution or time period indicated in License
Reuse in a dissertation/thesis	Lifetime of thesis
Reuse in a journal/magazine	Lifetime of journal/magazine
Reuse in a book/textbook	Lifetime of edition
Reuse on a website	1 year unless otherwise specified in the License
Reuse in a presentation/slide kit/poster	Lifetime of presentation/slide kit/poster. Note: publication whether electronic or in print of presentation/slide kit/poster may require further permission.
Reuse in conference proceedings	Lifetime of conference proceedings
Reuse in an annual report	Lifetime of annual report
Reuse in training/CME materials	Reuse up to distribution or time period indicated in License
Reuse in newsmedia	Lifetime of newsmedia
Reuse in coursepack/classroom materials	Reuse up to distribution and/or time period indicated in license

6. Acknowledgement

6. 1. The Licensor's permission must be acknowledged next to the Licensed Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract and must be hyperlinked to the journal/book's homepage.

6. 2. Acknowledgement may be provided according to any standard referencing system and at a minimum should include "Author, Article/Book Title, Journal name/Book imprint, volume, page number, year, Springer Nature".

7. Reuse in a dissertation or thesis

7. 1. Where 'reuse in a dissertation/thesis' has been selected, the following terms apply: Print rights of the Version of Record are provided for; electronic rights for use only on institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/) and only up to what is required by the awarding institution.

7. 2. For theses published under an ISBN or ISSN, separate permission is required. Please contact journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

7. 3. Authors must properly cite the published manuscript in their thesis according to current citation standards and include the following acknowledgement: '*Reproduced with permission from Springer Nature*'.

8. License Fee

You must pay the fee set forth in the License Agreement (the "License Fees"). All amounts payable by you under this License are exclusive of any sales, use, withholding, value added or similar taxes, government fees or levies or other assessments. Collection and/or remittance of such taxes to the relevant tax authority shall be the responsibility of the party who has the legal obligation to do so.

9. Warranty

9. 1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. **You are solely responsible for ensuring that the material you wish to license is original to the Licensor and does not carry the copyright of another entity or third party (as credited in the published version).** If the credit line on any part of the Licensed Material indicates that it was reprinted or adapted with permission from another source, then you should seek additional permission from that source to reuse the material.

9. 2. EXCEPT FOR THE EXPRESS WARRANTY STATED HEREIN AND TO THE EXTENT PERMITTED BY APPLICABLE LAW, LICENSOR PROVIDES THE LICENSED MATERIAL "AS IS" AND MAKES NO OTHER REPRESENTATION OR WARRANTY. LICENSOR EXPRESSLY DISCLAIMS ANY LIABILITY FOR ANY CLAIM ARISING FROM OR OUT OF THE CONTENT, INCLUDING BUT NOT LIMITED TO ANY ERRORS, INACCURACIES, OMISSIONS, OR DEFECTS CONTAINED THEREIN, AND ANY IMPLIED OR EXPRESS WARRANTY AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, PUNITIVE, OR EXEMPLARY DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE LICENSED MATERIAL REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION APPLIES NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

10. Termination and Cancellation

10. 1. The License and all rights granted hereunder will continue until the end of the applicable period shown in Clause 5.1 above. Thereafter, this license will be terminated and all rights granted hereunder will cease.

10. 2. Licensor reserves the right to terminate the License in the event that payment is not received in full or if you breach the terms of this License.

11. General

11. 1. The License and the rights and obligations of the parties hereto shall be construed, interpreted and determined in accordance with the laws of the Federal Republic of Germany without reference to the stipulations of the CISG (United Nations Convention on Contracts for the International Sale of Goods) or to Germany´s choice-of-law principle.

11. 2. The parties acknowledge and agree that any controversies and disputes arising out of this License shall be decided exclusively by the courts of or having jurisdiction for Heidelberg, Germany, as far as legally permissible.

11. 3. This License is solely for Licensor's and Licensee's benefit. It is not for the benefit of any other person or entity.

CCC Marketplace

This is a License Agreement between Valerie Metlege ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Permissions General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Permissions General Terms and Conditions below.

Order Date	26-Aug-2024	Type of Use	Republish in a thesis/dissertation
Order License ID	1519245-1	Publisher	ROYAL SOCIETY
ISSN	1742-5689	Portion	Chart/graph/table/figure

LICENSED CONTENT

Publication Title	Journal of the Royal Society interface	Rightsholder	The Royal Society (U.K.)
Article Title	Mitral valve leaflet remodelling during pregnancy: insights into cell-mediated recovery of tissue homeostasis.	Publication Type	Journal
		Start Page	20160709
Author / Editor	Royal Society (Great Britain)	Issue	125
Date	01/01/2004	Volume	13
Language	English	URL	https://royalsociety.org/journals/
Country	United Kingdom of Great Britain and Northern Ireland		

REQUEST DETAILS

Portion Type	Chart/graph/table/figure	Distribution	Canada
Number of Charts / Graphs / Tables / Figures Requested	2	Translation	Original language of publication
		Copies for the Disabled?	No
Format (select all that apply)	Electronic	Minor Editing Privileges?	No
Who Will Republish the Content?	Academic institution	Incidental Promotional Use?	No
Duration of Use	Life of current edition	Currency	CAD
Lifetime Unit Quantity	Up to 499		
Rights Requested	Main product		

NEW WORK DETAILS

NEW WORK DETAILS

Title	REMODELING OF MYOCARDIAL COLLAGEN IN THE BOVINE DURING PREGNANCY AND POSTPARTUM	**Institution Name**	Dalhousie University
		Expected Presentation Date	2024-08-31
Instructor Name	Valerie Metlege		

ADDITIONAL DETAILS

Order Reference Number	8	**The Requesting Person / Organization to Appear on the License**	Valerie Metlege

REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	figure 10, figure 11	**Title of the Article / Chapter the Portion Is From**	Mitral valve leaflet remodelling during pregnancy: insights into cell-mediated recovery of tissue homeostasis.
Editor of Portion(s)	Rego, Bruno V; Wells, Sarah M; Lee, Chung-Hao; Sacks, Michael S	**Author of Portion(s)**	Rego, Bruno V; Wells, Sarah M; Lee, Chung-Hao; Sacks, Michael S
Volume / Edition	13	**Issue, if Republishing an Article From a Serial**	125
Page or Page Range of Portion	20160709	**Publication Date of Portion**	2016-12-01

RIGHTSHOLDER TERMS AND CONDITIONS

Out of Copyright - any journal requested with a Publication date older than 70 years.

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,
- 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.

CCC | Marketplace

This is a License Agreement between Valerie Metlege ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Permissions General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Permissions General Terms and Conditions below.

Order Date	26-Aug-2024	**Type of Use**	Republish in a thesis/dissertation
Order License ID	1519246-1	**Publisher**	SPRINGER-VERLAG
ISSN	0341-2695	**Portion**	Chart/graph/table/figure

LICENSED CONTENT

Publication Title	International orthopaedics	**Rightsholder**	Springer Nature BV
Article Title	Effects of freezing on the biomechanical and structural properties of human posterior tibial tendons.	**Publication Type**	Journal
		Start Page	145
		End Page	151
Author / Editor	INTERNATIONAL SOCIETY OF ORTHOPAEDIC SURGERY AND T	**Issue**	2
Date	01/01/1977	**Volume**	32
Language	English, English, French, French		
Country	Germany		

REQUEST DETAILS

Portion Type	Chart/graph/table/figure	**Distribution**	Canada
Number of Charts / Graphs / Tables / Figures Requested	1	**Translation**	Original language of publication
		Copies for the Disabled?	No
Format (select all that apply)	Electronic	**Minor Editing Privileges?**	No
Who Will Republish the Content?	Not-for-profit entity	**Incidental Promotional Use?**	No
Duration of Use	Life of current edition	**Currency**	CAD
Lifetime Unit Quantity	Up to 499		
Rights Requested	Main product		

NEW WORK DETAILS

Title	REMODELING OF MYOCARDIAL COLLAGEN IN THE BOVINE DURING PREGNANCY AND POSTPARTUM	**Institution Name**	Dalhousie University
		Expected Presentation Date	2024-08-31
Instructor Name	Valerie Metlege		

ADDITIONAL DETAILS

The Requesting Person / Organization to Appear on the License	Valerie Metlege

REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	Figure 3	**Title of the Article / Chapter the Portion Is From**	Effects of freezing on the biomechanical and structural properties of human posterior tibial tendons.
Editor of Portion(s)	Sandro, Giannini; Roberto, Buda; Francesco, Caprio; Patrizia, Agati; Adriana, Bigi; Viviana, Pasquale; Alessandro, Ruggeri	**Author of Portion(s)**	Sandro, Giannini; Roberto, Buda; Francesco, Caprio; Patrizia, Agati; Adriana, Bigi; Viviana, Pasquale; Alessandro, Ruggeri
Volume / Edition	32		
Page or Page Range of Portion	145-151	**Publication Date of Portion**	2008-04-01

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

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 t

Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.

"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 a 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 t 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 th 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,
- 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.

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.