OVERGROWTH AND THE *DROSOPHILA* HEART

PROBING THE IMPACT OF OBESITY AND OVERGROWTH ON HEART FUNCTION USING A *DROSOPHILA* MODEL

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A Thesis
Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements
For the Degree Doctor of Philosophy

McMaster University DOCTOR OF PHILOSOPHY (2023) Hamilton, Ontario (Biology)

TITLE: Probing the impact of obesity and overgrowth on heart function using a *Drosophila* model

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NUMBER OF PAGES: i-xiv, 1-129

Lay abstract

The cardiac extracellular matrix (ECM) is a protein scaffold that supports heart function. Cardiovascular disease often involves increased levels of ECM proteins, a condition called fibrosis, which causes increased tissue stiffness and functional impairment. There is no cure for fibrosis and developing treatments requires an understanding of how the ECM responds to disease. I developed a dietary obesity model and a genetically triggered overgrowth model to examine how the ECM responds to disease states. I found that obesity causes ECM reorganization and functional defects, but that overgrowth models scale their hearts remarkably well with increased body size. Overgrowth models were found to have elevated levels of matrix crosslinking enzymes, which contributed to a stiffer matrix in these individuals. This was rescued by inhibition of crosslinking. Overall, this thesis reveals a connection between cardiac ECM organization, tissue elasticity, and heart function, and how these are altered in disease.

Abstract

The cardiac extracellular matrix (ECM) is a dynamic protein scaffold that is required to support cardiac function. Regular remodelling of the matrix involves protein turnover and deposition and is a highly regulated process. In disease states the normal balance of the ECM is disrupted and aberrant protein deposition and crosslinking can occur. This process, termed fibrosis, causes stiffening of the cardiac ECM, which in turn impairs organ function. Fibrosis is a hallmark of cardiovascular disease, is a progressive condition that can contribute to adverse clinical outcomes, and currently has no available treatments. One of the leading causes of cardiovascular disease is obesity and fibrosis is known to occur in this context. In order to investigate the development of fibrotic remodelling in the context of obesity I have developed a dietary obesity model in the fruit fly *Drosophila melanogaster*. Additionally, I developed a genetic overgrowth model as increased cardiac load is also known to trigger fibrotic remodelling. Dietary obesity models reveal altered ECM organization, as well as impaired cardiac contractility, while overgrowth models demonstrate a remarkable ability to appropriately scale heart morphology with increased body size. The overgrowth model does have extremely elevated expression levels of the crosslinking enzyme LOXL2, suggesting a major contributor to impaired function is increased crosslinking rather than altered protein deposition. However, inhibition of crosslinking caused only minor ECM organizational defects but was able to rescue the elasticity of the overgrowth model. Overall, this thesis raises intriguing questions for treatment of cardiovascular disease, where tissue dynamics are often overlooked in a clinical setting.

Acknowledgements

I must begin by thanking my supervisor, Roger Jacobs. Thank you for being so understanding when things went wrong (again and again), for your limitless vocabulary, for being available for as many meetings as necessary. After everything these last few years you have definitely earned your retirement!

Thank you to my committee members André Bédard and Juliet Daniel for their helpful feedback in meetings. Thank you to my external, Elizabeth Rideout, for her willingness to participate. Thank you also to Marie Elliot for agreeing to chair my exam despite her incredibly busy schedule.

Thank you to João Firmino of McMaster CALM for assistance with developing an OCT protocol and for creating the macro I use to make kymographs from videos. Your help has been much appreciated!

To all Jacobs lab members, past and present. Thank you to Danielle for sticking through this with me. Six years is a long time but we did it. To Saumya, I know the analysis made your eyes bleed but look how pretty the figures are now. To Mackenzie, your processing speed is unparalleled. I couldn't have dissected as frantically if I hadn't had you powering through the analysis. To Katie, who graduated almost 4 years ago but I still see multiple times a week. Thank you for keeping me sane when it felt like this would never be over.

To other Katie, thank you for always being ready for a Friday at 5 and for making stats less scary. I promise one day I will sit down and learn what all the words mean. Or maybe just some of them.

To all of Team 2023, but especially to Andrea, thank you for being in this with me. Writing can be a lonely endeavour, but this group has made all the difference. And to all the other departmental friends who have gone before and will come after thank you for always being willing to help with a bit of lunchtime procrastination.

To Mihaela, thank you for being you. You were the first person I met outside of my lab all those years ago and you are the one I have probably had to seek out the most since then. I will forever be grateful for your kindness and patience, especially all the times when things were hitting several fans simultaneously.

Thank you to all the Biology department administrative staff and everyone in the prep lab. You all work so hard and have been such a support through so many things.

One last big thank you to all of my friends and family. I'm sorry for talking your ears off about all the crazy things I've been up to, I promise I will stop talking about virgin collections for at least a little while.

And finally to Shane. Just thank you. I couldn't have done it without you.

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List of Abbreviations

AI – arrhythmicity index

AM – alary muscle

ANOVA – analysis of variance

AFM – atomic force microscopy

ATP – adenosine triphosphate

BAPN – β-aminoproprionitrile

cDNA – complementary DNA

CI – confidence interval

CVD – cardiovascular disease

DTT – dithiothreitol

ECM – extracellular matrix

EGTA – egtazic acid

ER – endoplasmic reticulum

gDNA – genomic DNA

GFP – green fluorescent protein

HFD – high fat diet

HSD – high sucrose diet

Hz – Hertz

IL-6 – interleukin 6

L3 – third larval instar

LOX – lysyl oxidase

LOXL1 – lysyl oxidase like 1

LOXL2 – Lysyl oxidase like 2

Lsd-2 – lipid storage droplet 2

MMPs – matrix metalloproteinases

NF-κβ – nuclear factor κβ

OCT – optical coherence tomography

PBS – phosphate buffered saline

PBST – phosphate buffered saline with 0.3% Triton-X-100

PC – pericardial cell

Phm – phantom

Prc – Pericardin

Pxn – Peroxidasin

RNA – ribonucleic acid

ROI – region of interest

ROS – reactive oxygen species

RT – room temperature

RT-qPCR – quantitative reverse transcriptase polymerase chain reaction

SEM – standard error of the mean

SPARC – secreted protein acidic and rich in cysteine

TAG – triacylglyceride

TG - triglyceride

TIF – tag image file format

TIMP – tissue inhibitor of metalloproteinases

TGF-β – transforming growth factor β

TNF-α – tumour necrosis factor α

TWOMBLI – The Workflow Of Matrix BioLogy Informatics

vkgGFP - y^1w^{1118} ; *vkg-GFP* ($vkg^{CC00791}$)

yw - y^1w^{1118}

Declaration of Academic Achievement

I performed the majority of the research presented here. All experimental design, setup, and final processing and interpreting of data was performed by me. Some experiments had assistance in data analysis, all methods for analysis were originally designed and tested by me before being demonstrated to those assisting. Details of the assistance received follows. All manuscript preparation was done by me, with oversight by Roger Jacobs.

Chapter 2: All high sucrose diet experiments were performed by Saumya Naik. I developed methods for quantifying matrix organization, Saumya Naik performed the analysis. Katie Pelletier performed the statistical analysis for the Collagen-IV matrix organization experiment, and for the live imaging experiment.

Chapter 3: Roger Jacobs performed the fibre alignment analysis.

Chapter 4: I developed the protocol for the swelling assay, Mackenzie L. Orlando then performed initial tests under my supervision. She also assisted with analysis of live imaging and fibre thickness. Roger Jacobs performed the fibre alignment analysis.

I am indebted to all those involved in this project.

1. Introduction

1.1 The extracellular matrix, fibrosis, and disease

The study of disease often focuses primarily on cellular contributors, including complex conditions such as cancer and cardiovascular disease. However, there is a significant non-cellular component to many disease processes (Bondareva et al. 2009; Brashear et al. 2022; Cox and Erler 2011; Frangogiannis 2017; Garrett et al. 2019). Much remains to be discovered about the importance of these non-cellular components to disease development and progression.

The extracellular matrix (ECM) is a protein network that surrounds cells and acts as a support structure for tissues. It has been known to contribute to cellular behaviour for over 100 years (Lewis 1922), and was originally thought to be an inert scaffold. Over time it has been revealed that the ECM is critical for a variety of processes, including modulation of tension, acting as a reservoir for signalling molecules, including growth factors and enzymes, as well as being a main contributor to the local microenvironment (Mouw, Ou, and Weaver 2014; Streuli 1999; Winkler et al. 2020).

The ECM is a complex structure, with a tissue-specific composition. Any given ECM may comprise 100-200 different proteins, with the matrisome potentially encoding up to 4% of the human proteome (Naba et al. 2012). The specificity of the makeup of the ECM allows for the creation of an optimal environment for tissue function. The role of cartilage is much different than the role of a muscle, and each ECM will have specific features that act to maintain tissue function. The ECM is also not a static structure, with continual breakdown and deposition of matrix components occurring in a process called remodelling (Bonnans, Chou, and Werb 2014; Cox and Erler 2011; Streuli 1999). The overwhelming complexity of the ECM and the need for tight control over its characteristics in order to maintain tissue homeostasis means that matrix dysregulation is linked to the development of a variety of diseases (Cox and Erler 2011; Hughes and Jacobs 2017).

Aberrant or excessive remodelling of the ECM is termed fibrosis, and is characterized by increased deposition, as well as increased levels of crosslinking between matrix proteins (Henderson, Rieder, and Wynn 2020; Pehrsson et al. 2021). Fibrosis is a form of pathological remodelling that is known to affect many tissues, including the lung, liver, kidney, and heart

(Aydin and Akcali 2018; Isaka 2018; Philp et al. 2018; Park et al. 2019). Fibrotic remodelling leads to increased tissue stiffness, can lead to severe organ dysfunction and death, and is associated with increased levels of metastasis in some cancers (Bondareva et al. 2009; Cox et al. 2013; Winkler et al. 2020). Despite its known contribution to disease progression and poor clinical outcomes, there are no treatments available that can stop or reverse fibrosis (Pehrsson et al. 2021; Leask 2010). Without an understanding of the normal functioning of the ECM and how it responds to specific disease insults, we will remain unable to develop therapeutics that can target fibrosis and improve patient outcomes.

This thesis aims to examine the interplay between the cardiac ECM and obesity and overgrowth. Obesity is known to trigger fibrotic remodelling due to systemic inflammation, while overgrowth conditions cause cardiac overload, which is also known to trigger remodelling. By maximizing the stress on the cardiovascular system, I aim to characterize the response of the ECM to insult, as well as correlate ECM alterations with functional consequences. I will also manipulate crosslinking levels in order to examine the importance of crosslinking to the cardiac ECM. Overall, I aim to examine how the ECM responds to increased stress in order to advance our understanding of *in vivo* matrix dynamics to help identify potential therapeutic targets for future study.

1.2 The extracellular matrix (ECM)

The extracellular matrix (ECM) is a protein scaffold found surrounding all tissues of the body. It plays a wide variety of roles in supporting and maintaining organ function, including modulation of tension, sequestration of growth factors, cell adhesion, migration, differentiation, and survival (Bonnans, Chou, and Werb 2014; Mouw, Ou, and Weaver 2014). For example, ECM stiffness has previously been shown to regulate glial cell migration during development (Kim et al. 2014). The ECM has also been implicated in organ shaping, with gradients of ECM tension across a tissue surface leading to specific patterns of elongation (Crest et al. 2017).

The ECM contains two separate domains, the basement membrane and the interstitial matrix. The basement membrane is an ancient structure that is present in all metazoans and is thought to be required for the advent of multicellular life (Fidler et al. 2017). It is directly adjacent to the

cell surface, is composed of a similar "toolkit" of highly conserved proteins, and is the main connection between cells and their environment (Pastor-Pareja 2020). The interstitial matrix is composed primarily of fibrillar collagens (Jourdan-LeSaux, Zhang, and Lindsey 2010). It is found further from the cell surface, and encases a tissue in a support scaffold (Bonnans, Chou, and Werb 2014). Basement membranes are critical for tissue morphogenesis and organization through their direct connection to cells. The interstitial matrix acts to reinforce this role by providing additional structural support (Morrissey and Sherwood 2015; Pompili et al. 2021).

The ECM is composed of an array of proteins, glycoproteins, and proteoglycans. Due to the varying functional demands experienced by different tissues, they will have different ECM compositions (Naba et al. 2012). For example, chondroitin sulfate proteoglycans are typically found in tissues that experience a great deal of mechanical strain, such as cartilage, tendons, and major arteries (Cox and Erler 2011; Mouw, Ou, and Weaver 2014). ECMs in different tissues have been found to have 10-30% tissue-specific components, some of which are known to have a role in tissue-specific physiology (Naba et al. 2012). The ECM is also able to adapt to changing environmental stimuli or disease states by altering the exact composition of proteins within the matrix (Mouw, Ou, and Weaver 2014). For example, immune system activation that leads to inflammation is known to upregulate the expression of ECM proteins, as well as enzymes that degrade the ECM. This causes changes in the characteristics of the ECM in response to physiological stimuli (Travers et al. 2016; Mouw, Ou, and Weaver 2014).

1.3 ECM synthesis and assembly

The basement membrane is composed of highly conserved components, including Laminin, Collagen-IV, Nidogen, and Perlecan (Pastor-Pareja 2020). The basement membrane is able to self-assemble. This process occurs in a specific order, beginning with the assembly of the Laminin sheet at the tissue surface (Hollfelder, Frasch, and Reim 2014). Membrane bound receptors such as Integrins recruit Laminin, tethering it to the cell surface where it forms a network. This Laminin network is then able to recruit Collagen IV, which forms a second network (Hughes and Jacobs 2017). The two networks are bridged and stabilized by the linker proteins Nidogen and Perlecan (Figure 1.1). Matricellular proteins are also found within the ECM, but do not contribute directly to its structure (Bornstein 2009). An example of this is

SPARC (secreted protein acidic and rich in cysteine), which binds to Collagen IV and is required for its assembly into the basement membrane but does not directly contribute to the structural properties of the ECM (Duncan et al. 2020).

ECM assembly is important for the shaping of organs, determining tissue polarity, and the migration of cells developmentally (Sánchez-Sánchez et al. 2017; Kim et al. 2014; Crest et al. 2017). The basement membrane is assembled from the cell surface outward, and most basement membrane proteins have a developmental requirement due to their involvement in the aforementioned processes (Miner et al. 2004; Pöschl et al. 2004; Urbano et al. 2009). Interestingly, some of these proteins are expressed non-cell autonomously. For example, in many mammalian tissues, myofibroblasts secrete elevated levels of the ECM proteins that are found surrounding the myocytes (Aydin and Akcali 2018; Horowitz and Thannickal 2019; Talman and Ruskoaho 2016). In *Drosophila,* the fat body, an organ analogous to the mammalian liver, is the main producer of Collagen-IV and Pericardin, a heart specific Collagen (Drechsler et al. 2013; Wilmes et al. 2018; José Carlos Pastor-Pareja and Xu 2011). While the protein composition of the ECM is one of the important factors in determining tissue characteristics, post-translational modifications such as crosslinking and glycosylation also play an important role.

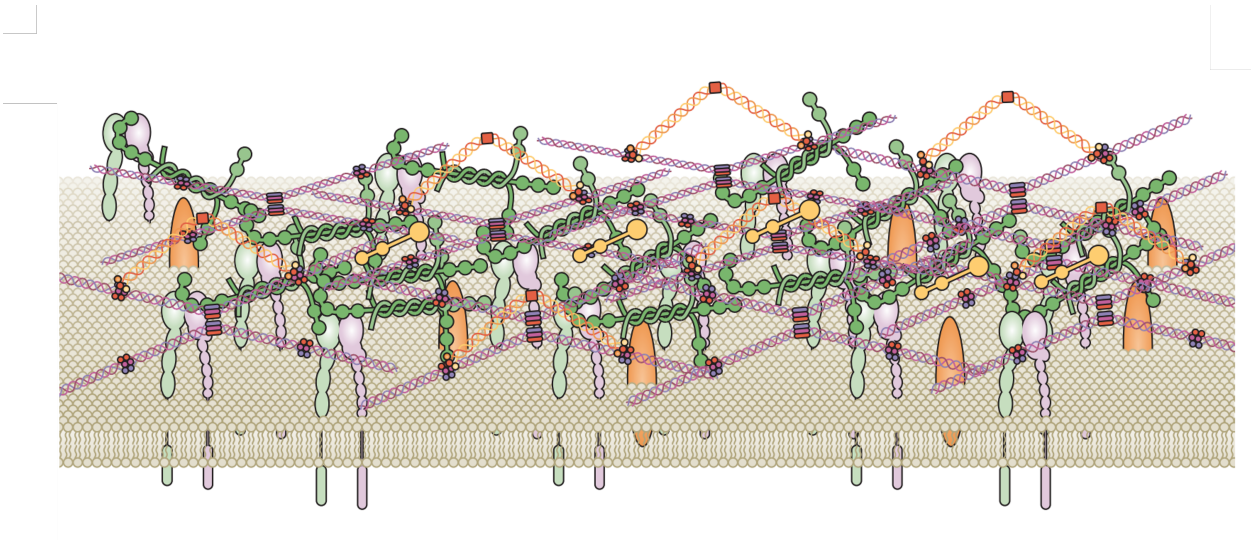

Figure 1.1: The extracellular matrix
Structure of the ECM, showing Integrin (embedded in cell membrane, light green and light purple) binding to Laminin (dark green). Laminin forms an initial sheet over the cell membrane, where it is then able to recruit Collagens (purple and orange). Nidogen (yellow) acts to hold the Laminin and Collagen matrices together. Once Collagen is recruited to the matrix, it forms a complex, cross-linked network (adapted from Mouw et al).

1.4 ECM crosslinking

The protein composition of the ECM is one way to control the biophysical properties of the matrix. Another is by the amount of covalent crosslinking between these proteins (Pehrsson et al. 2021; Gaar, Naffa, and Brimble 2020). Crosslinking of the ECM provides structural support, stabilizes the ECM network, and contributes to overall tissue stiffness (Pehrsson et al. 2021; Brashear et al. 2022). The proteins of the ECM are held together by crosslinks that can be formed enzymatically or non-enzymatically (Cox and Erler 2011). Enzymatic reactions can be catalyzed by several molecules, including Lysyl oxidases, Transglutaminases, and Peroxidasin (Figure 1.2). Lysyl oxidase (LOX) oxidizes lysine and hydroxylysine residues in Collagen peptides, allowing for the formation of a crosslink (Pehrsson et al. 2021). In mice, LOX null mutants are lethal, with pups dying shortly after birth due to cardiovascular complications (Mäki et al. 2002; 2005; Molnar et al. 2005). This demonstrates the importance of crosslinking to the structural integrity of major organs. Transglutaminases are mainly important in wound healing, while Peroxidasin (Pxn) catalyzes sulfilimine crosslinks between Collagen-IV fibres in the basement membrane. Collagen-IV is stabilized by LOX-mediated crosslinks but Peroxidasin lends extra stabilization by catalyzing links between the Collagen-IV specific NC1 domain (Pehrsson et al. 2021). Fibrillar Collagens do not possess an NC1 domain, and are not thought to be acted upon by Pxn.

Expression of crosslinking enzymes like LOX are increased in response to injury (González-Santamaría et al. 2016; Brashear et al. 2022; Sivakumar et al. 2008). They are also upregulated in some metastatic cancers (Bondareva et al. 2009; Cox et al. 2013). Upregulation of crosslinking enzymes leads to the development of a stiffer local environment. In cancer, this can promote metastasis. In other organs, it can compromise organ function due to reduced elasticity.

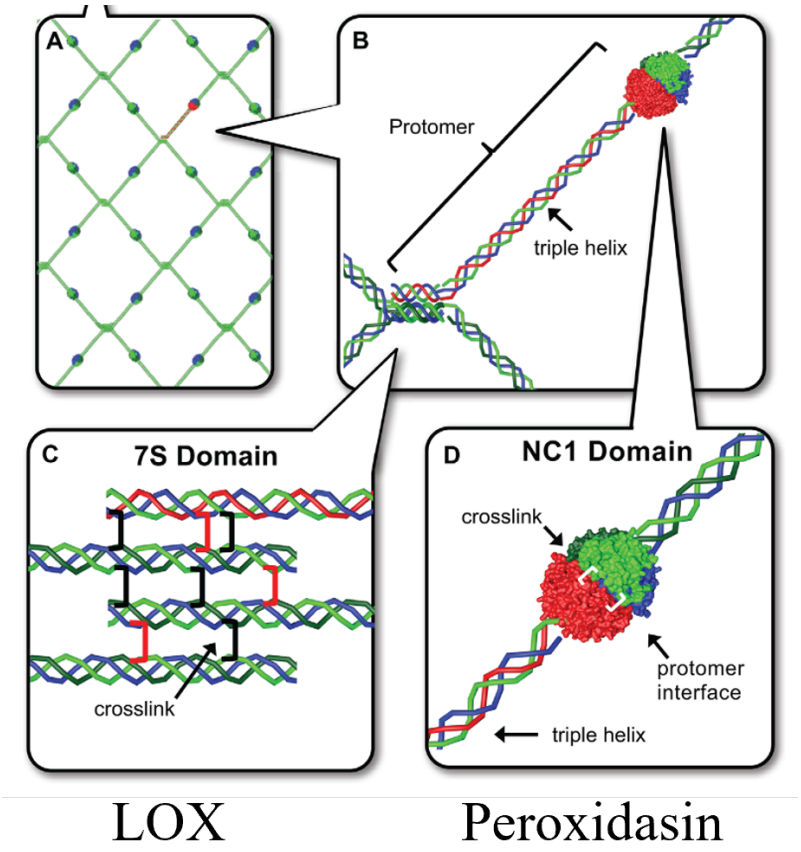

Figure 1.2: Types of ECM crosslinks
ECM crosslinking refers to covalent bonds formed between two or more protomers of the same protein. Collagen IV protomers are covalently crosslinked in two different ways, one catalyzed by LOX and the other by Peroxidasin. LOX also crosslinks fibrillar Collagens in the above manner (adapted from Brown et al).

1.5 ECM regulation

The importance of the ECM to appropriate tissue function means that it is subject to a high degree of regulation (Bonnans, Chou, and Werb 2014; Cox and Erler 2011). Remodelling of the ECM refers to the process of protein breakdown and deposition. Remodelling is a normal part of development and growth and is required to allow the ECM to respond appropriately to environmental stimuli (Cox and Erler 2011; Mouw, Ou, and Weaver 2014). The matrix metalloproteinases (MMPs) are responsible for degradation of the matrix (Hughes et al. 2020). The rate of degradation is kept in check by the tissue inhibitors of metalloproteinases (TIMPs). The balance of MMP and TIMP activity is a factor controlling the rate of matrix turnover. Matrix degradation is important in order to control the amount of protein within the ECM but also functions to release molecules like growth factors and enzymes that are contained within the matrix (Bonnans, Chou, and Werb 2014). Together, the levels of breakdown and deposition in the ECM will determine the biophysical properties of a tissue and gradients of signalling molecules, which then influence cell behaviour. This is illustrated by the differentiation of stem cells placed on matrices of various rigidity. Stem cells that are placed on a rigid structure will differentiate into bone, while those placed on a softer matrix will differentiate into muscle (Lu et al. 2011). Regulation of the characteristics of the ECM can therefore have a dramatic effect on cell behaviour and tissue function.

There are multiple factors that contribute to the rate of ECM remodelling. ECM deposition may be increased when gene expression is increased, or when ECM breakdown is prevented. Breakdown may be prevented by the balance of MMPs to TIMPs or by other factors, including the susceptibility of the matrix to degradation. Increased levels of crosslinking between ECM proteins act to insolubilize the matrix, and make it more resistant to degradation (van der Slot-Verhoeven et al. 2005).

1.6 ECM-related disease and fibrosis

As a critical structure for maintaining tissue function, the ECM is subject to a high degree of regulation. When ECM homeostasis is not maintained, changes to the biophysical properties of a tissue result, which can impact organ function (Cox and Erler 2011). ECM dysfunction is linked to the development of multiple diseases, including cancer, osteoarthritis, muscular dystrophy, Ehlers-Danlos syndrome, Marfan syndrome, and fibrosis (Bonnans, Chou, and Werb 2014; Bateman, Boot-Handford, and Lamandé 2009; Naba et al. 2012; Brashear et al. 2022; Mohassel, Foley, and Bönnemann 2018).

ECM dysregulation can lead to excessive matrix breakdown or deposition. Excessive deposition is termed fibrosis, or fibrotic remodelling, and is the root cause of many disorders with ECM involvement. Fibrosis is a pathological form of remodelling that is characterized by increased protein deposition as well as increased matrix crosslinking (Meschiari et al. 2017). It is known to occur in a wide variety of tissues, including the lung, liver, kidney, and heart (Philp et al. 2018; Isaka 2018; Aydin and Akcali 2018; Travers et al. 2016). The onset of fibrosis is often due to dysregulation of tissue repair mechanisms (Theocharis, Manou, and Karamanos 2019). When tissue repair is triggered, an inflammatory response is activated (Gonzalez et al. 2016). Inflammation is a known cause of fibrotic remodelling and will be described in greater detail in the context of cardiovascular disease below. In brief, the inflammatory response causes infiltration of a tissue by immune cells (ex. macrophages) which activate signalling cascades that upregulate expression of matrix proteins, leading to increased ECM accumulation within a tissue (Wynn 2008).

When excessive ECM is deposited and crosslinking is increased, there is an overall increase in tissue stiffness. This can severely compromise organ function. For example, in Duchenne muscular dystrophy there is excessive deposition of matrix components, causing muscle stiffness that leads to contracture. One of the most affected muscles is the diaphragm, which eventually becomes so stiff it is non-functional. This results in respiratory failure (Brashear et al. 2022). A lack of ECM degradation can also mean that reservoirs of growth factors or other signalling molecules are not being released. This can cause changes in the cell signalling and behaviour of cells in the local environment (Hynes 2009).

An important consideration in the treatment of fibrosis is the activation of additional fibrotic remodelling simply due to the presence of fibrotic tissue (Pehrsson et al. 2021). This creates a progressive condition where fibrosis will continue to worsen over time, further impairing function. The effect of ECM dysregulation and the development of fibrosis is described below for metastatic cancer, COVID-19, and cardiovascular disease.

The ECM is an important part of the local microenvironment. It is well documented that fibrosis contributes to an increased risk of cancer, an improved ability of cancer cells to metastasize, and an improved ability of cancer cells to become established at secondary locations (Cox et al. 2013). Increased ECM stiffness creates an environment that promotes cell migration. ECM levels can be predictive of disease prognosis and levels of crosslinking enzymes correlate with metastatic risk (Bondareva et al. 2009; Cox et al. 2013; Winkler et al. 2020).

Pulmonary fibrosis has had a great deal of attention recently as it is a side effect of COVID-19 (Hama Amin et al. 2022). Inflammation caused by conditions such as COVID-19 can cause increased matrix deposition in the lungs, leading to impaired lung function (Nguyen et al. 2021). In general, pulmonary fibrosis leads to a decline in lung function due to increased stiffness of the tissue (Upagupta et al. 2018). Alveolar collapse is also associated with fibrotic remodelling in the lung, which further compromises organ function (Lutz et al. 2015).

Essentially all cases of cardiovascular disease (CVD) exhibit fibrotic remodelling (Travers et al. 2016). Unlike other muscles, the heart must beat continuously to maintain life. This unique structural demand necessitates a matrix that is elastic enough to withstand the strain of repeated contractions, but also has enough structural integrity to pump blood to the extremities. This balance of properties requires a highly specialized ECM. When increased matrix deposition occurs in the heart, it leads to an increase in stiffness, which impairs cardiac function. This can ultimately lead to heart failure (Travers et al. 2016; Jourdan-LeSaux, Zhang, and Lindsey 2010; Fan et al. 2012). The study of fibrosis as it pertains to cardiovascular disease is of particular importance due to the requirement of the heart for sustaining life, and the increasing prevalence of CVD. The leading cause of death worldwide is CVD (World Health Organization 2020), and fibrosis contributes to adverse outcomes, making it absolutely critical to understand the interplay between the two and how the development of fibrosis can be triggered in a variety of situations.

1.7 The cardiac ECM and disease

ECM dysfunction leading to the development of fibrosis is well-characterized in the context of cardiovascular disease. In fact, fibrosis is so common in this context that it is considered a hallmark of cardiovascular disease (Travers et al. 2016). The fibrotic response in the heart is initially an adaptive mechanism, as it acts to create an ECM scar that will maintain organ integrity. Upon injury, areas that experience cell death are at risk of rupture as the heart continues to beat to circulate blood to the body (Jourdan-LeSaux, Zhang, and Lindsey 2010; Travers et al. 2016). In an attempt to reinforce these areas and prevent complete organ failure the response of the tissue is to fill gaps left by necrotic cells with ECM proteins. This is initially an adaptive response but develops into pathological remodelling over time.

When injury to the heart tissue occurs, it typically results in cell death. Necrotic cells release reactive oxygen species (ROS) which causes immune cells to infiltrate and begin the process of clearing necrotic debris and reinforcement of the heart tissue. Both injury and oxidative stress activate various signalling pathways, including TGF-β, which activates tissue resident fibroblasts to become myofibroblasts. Myofibroblasts are mobile, and secrete higher levels of ECM proteins than fibroblasts (Fan et al. 2012). The presence of myofibroblasts leads to increased ECM secretion and deposition in the heart, disrupting the balance of remodelling. Increased levels of crosslinking enzymes are also present following tissue injury, and can contribute further to the development of fibrotic scars (Cavalera, Wang, and Frangogiannis 2014; Cox and Erler 2011).

While the development of an ECM scar preserves life in the immediate case of injury, it replaces contractile cardiomyocytes with non-contractile ECM. This leads to impaired organ function long term, including the development of arrythmia, atrial fibrillation, left ventricular hypertrophy, and diastolic dysfunction (Mahajan, Lau, and Sanders 2015). Additionally, the fibrotic response tends to continue long after damage is repaired. The majority of myofibroblasts will clear following resolution, but some persist and continue to secrete high levels of ECM proteins. It has also been found that the presence of fibrosis is enough to stimulate further fibrotic remodelling (Bonnans, Chou, and Werb 2014; Pehrsson et al. 2021). Taken together, this demonstrates that cardiac fibrosis is progressive, affects appropriate functioning of the heart long term, and can ultimately lead to heart failure (Travers et al. 2016).

1.8 Cardiovascular disease and obesity

Cardiovascular disease encompasses a range of conditions. Two of the main triggers for the onset of cardiovascular disease are aging and obesity (Poirier et al. 2006; Sessions et al. 2017). Both of these factors are correspondingly high, and approximately 1 in 12 Canadian adults is living with heart disease (Canada 2017). Rates of obesity have reached epidemic levels, with a staggering total of over 60% of Canadian adults who are either overweight or obese (Government of Canada 2019). This is an enormous percentage of the population who are at an increased risk of developing heart disease at some point in their lifetime.

Fibrotic remodelling in the heart is typically driven by inflammation. In cases of injury due to cardiovascular disease, this inflammation begins with cell death of cardiomyocytes, which signals to the heart tissue that repair is needed to maintain organ integrity (Travers et al. 2016; Meschiari et al. 2017; Jourdan-LeSaux, Zhang, and Lindsey 2010). Obesity also induces fibrotic remodelling in the heart, even in the absence of tissue injury (Nishida and Otsu 2017; Anthony et al. 2019; Cavalera, Wang, and Frangogiannis 2014). Hypertrophy of adipocytes occurs in the context of obesity. This has two main effects on whole body metabolism: first, there is an increase in hypoxia within the expanding adipose tissue (Mahajan, Lau, and Sanders 2015). This leads to production of proinflammatory factors, including IL-6 and ROS (Nishida and Otsu 2017). Additionally, the adipose tissue is unable to store infinite levels of fatty acids, causing higher circulating levels of triglycerides in obese individuals. These circulating triglycerides cause activation of factors such as NF-κβ, which in turn induces the expression of proinflammatory cytokines such as TNF-α and IL-6 (Nishida and Otsu 2017). Lipid accumulation within the heart itself can also cause increased endoplasmic reticulum (ER) stress and even apoptosis of cardiomyocytes, which furthers the inflammatory response.

Overall, obesity causes an increase in systemic inflammation. This has the same effect on the heart as inflammation caused due to an acute tissue injury, including the infiltration of immune cells such as macrophages, release of pro-inflammatory cytokines, and the activation of tissue resident fibroblasts (Fan et al. 2012; Mack 2018). This ultimately leads to the onset of cardiac fibrosis as a result of obesity. Obesity is also correlated with the development of hypertension (Hall et al. 2015). Cardiac fibrosis has been shown to occur in response to hypertension (Berk, Fujiwara, and Lehoux 2007; Díez 2007). This fibrosis is considered to be reactive, and is caused

by altered tension within the chambers of the heart (Hara, Takeda, and Komuro 2017). In order to maintain cardiac function, myocytes hypertrophy, oxidative stress increases in the system, and excess ECM is laid down in order to maintain cardiac output.

1.9 Modeling ECM dynamics and disease

In order to effectively treat the millions of individuals experiencing the effects of fibrosis, it is necessary to prevent and reverse this pathological remodelling. However, there are no therapeutics that are able to reverse fibrosis (Leask 2010). There are two anti-fibrotic drugs currently approved for the treatment of pulmonary fibrosis, but they are only effective for slowing the rate of fibrosis (Pehrsson et al. 2021). Both of these drugs have been shown to limit TGF-β mediated Collagen synthesis, and one has been shown to limit proliferation of fibroblasts and prevent TGF-β mediated fibroblast activation (Raghu et al. 1999; Flaherty et al. 2019; Wollin et al. 2015). This is able to slow the progression of fibrotic remodelling but is unable to prevent it fully as there are many other profibrotic factors that participate in this process (Wollin et al. 2015). The complexity of ECM regulation and the variety of contributors in disease states means that treatments targeting only one pathway are unable to halt disease progression.

The ability to treat conditions like fibrosis necessitates an understanding of the regulation of the ECM and the process of remodelling. However, the ECM is a complex system consisting of large gene families, a great deal of genetic redundancy, and tissue specific composition and regulation (Naba 2023). An ECM can have hundreds of structural and matricellular proteins that together regulate tissue characteristics. This is a staggering level of complexity. Additionally, ECM proteins are notoriously difficult to work with owing to their large size, insolubility, levels of crosslinking, a lack of available antibodies, and the biochemical intractability of the ECM as a whole (Naba et al. 2012). These characteristics have historically made *in vivo* ECM studies difficult, relegating a great deal of ECM research to *in vitro* models where some of the complexity can be controlled (Howard et al. 2019; Naba 2023). However, this leaves cells growing in a two-dimensional environment instead of their natural three-dimensions (Frantz, Stewart, and Weaver 2010). *In vitro* work also relies on culture materials that are orders of magnitude more stiff than many tissues in the body (Travers et al. 2016). This can cause spontaneous activation of the fibroblast to myofibroblast transition. *In vitro* environments also

lack the remodelling capacity of an *in vivo* ECM, where changing matrix characteristics are key regulators of cell behaviour and survival (Frantz, Stewart, and Weaver 2010). Additionally, while there are potentially hundreds of ECM proteins within a tissue at a given time, the majority of these proteins are present at low levels. This leads to under-sampling of ECM proteins and generates an incomplete picture to guide the creation of *in vitro* models (Naba 2023; Hu, Ling, and Ren 2022). Cardiac organoid models have begun to appear in recent years, but are currently limited by their reliance on synthetic matrices, lack contribution from surrounding tissue types such as nerves and immune cells, and are only able to recapitulate early developmental stages of heart formation (Zhu et al. 2022). Genetic model systems provide a powerful solution to many of the shortcomings of *in vitro* and organoid models.

1.10 *Drosophila* as a model

A genetic model system that has begun to be used extensively for ECM research is *Drosophila melanogaster. Drosophila* has low levels of genetic redundancy, making it possible to manipulate entire gene families. For example, *Drosophila* possesses only 2 MMPs and 1 TIMP while humans have 23 MMPs and 4 TIMPs (Page-McCaw et al. 2003). Additionally, there are *Drosophila* homologues for approximately 65% of human disease causing genes (Ugur, Chen, and Bellen 2016). There are also highly conserved developmental and metabolic pathways in *Drosophila.* The mechanisms controlling fat and glucose metabolism are conserved between *Drosophila* and vertebrates*,* leading to the use of *Drosophila* as a model for metabolic studies (Diop and Bodmer 2012; Wat et al. 2020). For heart research specifically, *Drosophila* is the simplest model system that possesses a heart (Diop and Bodmer 2012).

1.11 *Drosophila* hearts and ECM

The *Drosophila* heart, or dorsal vessel, is a linear, tube-like structure (Figure 1.3). The dorsal vessel is composed of 52 pairs of contractile cardiomyocytes that enclose a lumen. It is flanked by pericardial cells, which act as detoxifying nephrocytes (kidneys), and is suspended within the body cavity by 7 pairs of alary muscles (Hughes and Jacobs, 2017). While it is morphologically different from the more complex vertebrate heart, the same developmental pathways are

employed in its formation. The mammalian heart initially forms during development as a simple tube. The initial mammalian heart tube and the *Drosophila* dorsal vessel form using the same signalling pathways, with the mammalian heart subsequently undergoing folding to generate the more complex adult structure (Ahmad 2017). The *Drosophila* heart has no stem cells so has no replacement of cells after damage (Hughes and Jacobs 2017). While the mammalian heart does have some stem cells, they do not contribute meaningfully to cardiomyocyte replacement (van Berlo et al. 2014). Following injury the mammalian heart relies on repair rather than replacement of cells (Vujic, Natarajan, and Lee 2020). Studying repair mechanisms in the *Drosophila* heart may therefore provide insights into how mammalian hearts function following injury.

Larval development in *Drosophila* involves drastic morphological changes that necessitate ECM remodelling (Bogatan et al, 2015). Over the course of larval development the length of the larva increases over 5 fold, and the heart accommodates this change in size without any cell division (Hughes et al. 2020). This system presents an intriguing model for the study of ECM dynamics and remodelling as it pertains to both development and disease. The *Drosophila* ECM has previously been shown to be of critical importance for various developmental processes (Lewellyn, Cetera, and Horne-Badovinac 2013; Kim et al. 2014; Hollfelder, Frasch, and Reim 2014). It is composed of the same core toolkit of ECM proteins as mammalians systems, but with far less genetic redundancy. Notable differences include the lack of the linker protein Fibronectin in *Drosophila* (and all invertebrate species), as well as the unique Collagen IV-like protein Pericardin. Pericardin is a heart-specific Collagen found in *Drosophila.*

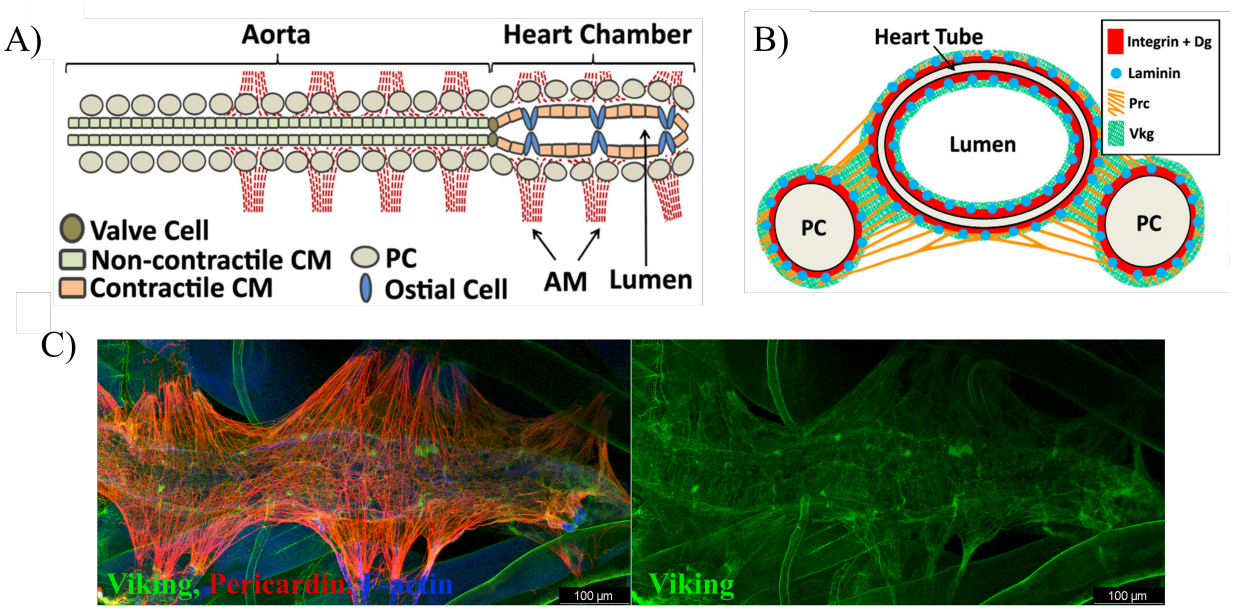

Figure 3: The *Drosophila* heart
The *Drosophila* heart (A) is a linear vessel composed of 52 pairs of cardiomyocytes, and is flanked by pericardial cells (PC). 7 pairs of alary muscles (AM) suspend the heart within the body cavity. (Adapted from Hughes and Jacobs, 2017). A cross-section of the dorsal vessel (B) shows the ECM surrounding the heart tube and PCs (Adapted from Hughes and Jacobs, 2017). The *Drosophila* cardiac ECM imaged using confocal microscopy (C) shows Pericardin in red and Collagen-IV (Viking) in green.

1.12 Aim of research

Obesity is known to cause cardiac fibrosis, which over time leads to functional impairment. There are currently no treatments available for prevention and reversal of fibrosis, leaving afflicted individuals with deteriorating heart function. The ability to treat fibrosis necessitates an understanding of ECM regulation and remodelling. This will elucidate possible therapeutic targets. However, these studies are often hampered by the complexity of *in vivo* mammalian models. To this end, I have employed the genetic model *Drosophila melanogaster* to develop both a genetic overgrowth and a dietary obesity model that will induce cardiac fibrosis. I will assess these two models for overall health, cardiac function, and ECM dynamics to establish how the ECM responds to conditions of obesity and overgrowth. This will increase our understanding of the disease process in obese humans and will identify promising therapeutic targets for follow-up study.

1.13 Genetic overload model

My genetic model for larval overgrowth locks larvae in the growth phase of development by manipulation of the hormones required for progressing to pupation (Figure 1.4). The developmental transitions in *Drosophila* are controlled by steroid hormones called ecdysteroids. The major ecdysteroid controlling moulting and pupation is ecdysone (Niwa and Niwa 2014). An ecdysone pulse occurs in the third instar stage that initiates the transition to pupation. This ecdysone pulse is triggered by the critical weight checkpoint (Thummel 2001; Yamanaka, Rewitz, and O'Connor 2013).

This checkpoint interprets both external and internal factors and commits the larva to pupation, regardless of nutrient conditions afterwards. One of the key inputs in passing the critical weight checkpoint is nutritional status, as pupae are unable to feed and larvae must accumulate enough resources to survive this period (Yamanaka, Rewitz, and O'Connor 2013). When starvation occurs prior to attaining the critical weight checkpoint, it delays the onset of pupation. If starvation occurs after the checkpoint, pupation occurs on a normal schedule (Mirth, Truman, and Riddiford 2005). The prothoracic gland acts as a sensor for this transition. In early third instar, the transcription factor *snail* causes a round of endoreplication within a portion of the prothoracic gland called the ring gland (Zeng et al. 2020). The DNA content of the ring gland

cells acts as a growth sensor, and the larva is committed to pupation after a specific DNA content is obtained. This leads to the ecdysone pulse that triggers the critical weight checkpoint.

When *snail* is knocked down in the prothoracic gland, the critical weight checkpoint is never attained. In *Drosophila* and other holometabolous insects, growth and maturation are separate developmental stages. The growth phase encompasses larval life stages, while maturation occurs during pupation (Rewitz, Yamanaka, and O'Connor 2013). This means that an arrest during larval stages produces a larva that continues to grow indefinitely (Zeng et al. 2020). These larvae attain enormous sizes (Figure 1.4) and are referred to here as giant larvae. By locking larvae in the growth phase of their lifecycle I aim to examine their ability to tolerate overgrowth, and specifically how the heart tolerates a larger-than-average body size.

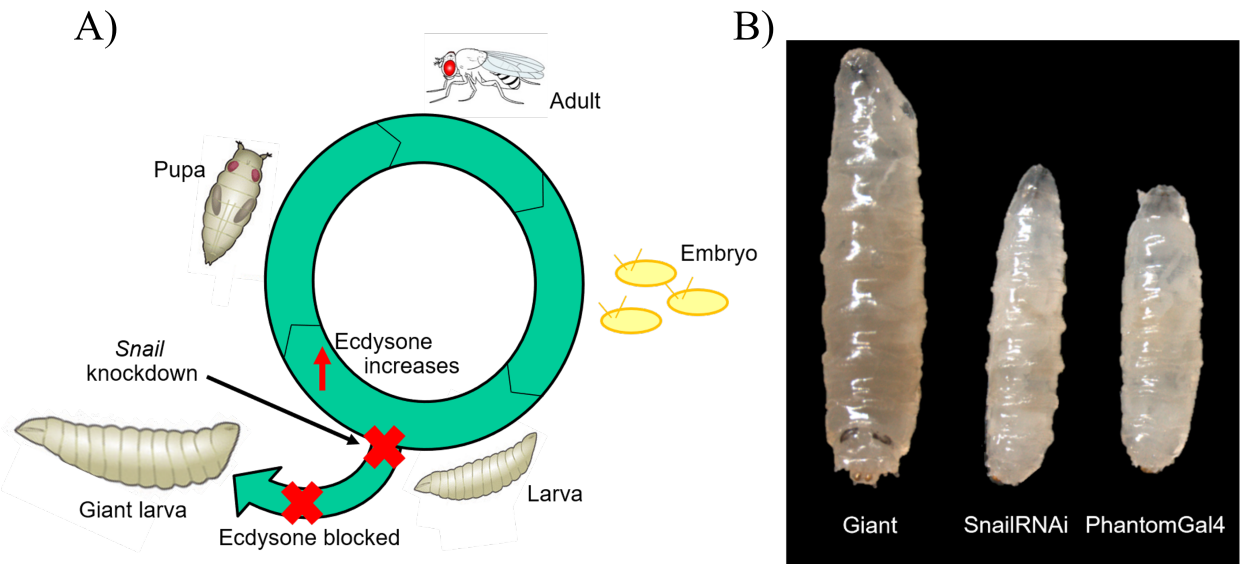

Figure 4: Genetic overgrowth model
Knockdown of the transcription factor *snail* in *Drosophila* larvae causes a developmental arrest and locks larvae in a growth phase (A). These larvae continue to grow indefinitely, and attain enormously increased body sizes, earning them the name giant larvae (B).

1.14 Dietary obesity model

To determine whether the genetic overgrowth model described above is a model for an obesity phenotype or simply a model of overgrowth, an obesity comparison is needed. I therefore generated a dietary obesity model, where I supplemented the larval diet with different percentages of fat (coconut oil). Coconut oil has previously been shown to be an effective fat supplement for *Drosophila* dietary studies due to its high content of saturated fatty acids, which are commonly associated with metabolic syndrome (Diop, Birse, and Bodmer 2017). An additional high sucrose diet was also included as this has been demonstrated previously to induce diabetes-like symptoms in adults *Drosophila* (Palanker Musselman et al. 2011; Na et al. 2013). These dietary treatments will allow me to examine the effects of diet-induced metabolic changes on the larval *Drosophila* heart, with specific focus on functional changes and the organization of the cardiac ECM. I will also be able to examine dose-dependent and sex-specific phenotypes. Previous studies performed in adult *Drosophila* have revealed cardiac dysfunction as a result of high fat diet (HFD) feeding. HFD feeding in adults has been found to cause increased triglyceride levels, lead to the development of fat deposits that physically interfere with the heart, and perturb expression of key genes involved in metabolism, such as the lipase *brummer* (Guida et al. 2019; Hardy et al. 2015; Blumrich et al. 2021). Cardiac responses to HFD have not been studied before in the context of larval growth or obesity. Previous studies have also not examined the role of the ECM in the development of cardiovascular dysfunction. Additionally, the HFD treatments employed in previous studies are typically transient, while feeding larvae from hatching creates a chronic exposure. High sucrose diets (HSD) have been found to induce insulin-resistance, ECM accumulation around the heart, and cardiac dysfunction (Na et al. 2013).

1.15 Research questions

This study aims to characterize how the heart responds to the stresses of growth and obesity by combining characterization of ECM morphology, heart physiology, gene expression, and tissue biomechanics. Overall, I aim to generate a comprehensive picture of how each part plays into overall organ function, and how changes to one aspect affect others.

Therefore, I will employ my models to answer the following questions:

1) How obesity and overgrowth affect *Drosophila* larvae, with particular focus on the cardiac ECM and heart function.
2) How *Drosophila* larvae tolerate body overgrowth.
3) The importance of enzymatic modifications to cardiac function and ECM structure in a model that lacks fibrillar Collagens.

These objectives will address the following hypothesis:
Both overgrowth and dietary manipulations will affect cardiac ECM organization, the biophysical properties of the heart, and overall heart function.

Chapter 2: Cardiac function and ECM morphology are altered with high fat diets in *Drosophila*

doi: https://doi.org/10.1101/2023.08.08.552539

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Abstract

Cardiovascular disease is characterized by aberrant and excessive extracellular matrix (ECM) remodelling, termed fibrosis. Fibrotic remodelling is typically triggered by inflammation, which occurs systemically in obesity. Despite the contribution of fibrosis to adverse clinical outcomes and disease progression, there are no available treatments for this condition. Developing therapeutics for chronic conditions requires an understanding of *in vivo* ECM regulation, and how the ECM responds to a systemic challenge. We have therefore developed a *Drosophila* model for obesity via high fat diet feeding and evaluated the response of the cardiac ECM to this metabolic challenge. We found that this model displays a striking disorganization of the cardiac ECM, with corresponding deficits in heart function. Our study shows that different genotypes tolerate varying levels of high fat diets, and that some genotypes may require a different percentage of fat supplementation for achieving an optimal obesity phenotype.

Introduction

Cardiovascular disease (CVD) is a leading cause of death world-wide. In recent years, there has been an increase in age-adjusted mortality resulting from CVD (Sidney et al. 2022). Obesity is one of the main risk factors for the development of CVD, and an increased incidence of obesity has led to a corresponding increase in CVD rates (Poirier et al. 2006). One aspect of CVD that is often overlooked is the contribution of the extracellular matrix (ECM). The ECM is a protein scaffold that surrounds tissues within the body and acts to support their function by modulation of tension, distribution of forces through the tissue, sequestration of growth factors, and, of importance in the heart, mediation of electrical conduction (Travers et al. 2016; Bonnans, Chou, and Werb 2014; Cox and Erler 2011). The importance of the ECM is demonstrated by its intimate link to a wide variety of disease states, including cancer and CVD.

The ECM is composed of two main compartments, the interstitial matrix and the basement membrane. The interstitial matrix is composed primarily of fibrillar Collagen and forms a support scaffold, while the basement membrane is found close to the cell surface and acts as a barrier to the surroundings (Bonnans, Chou, and Werb 2014; Walker and Spinale 1999). The ECM is not a static structure, and undergoes constant turnover, called remodelling. This process is a finely-tuned balance of matrix synthesis and deposition, as well as matrix breakdown (Cox and Erler 2011; Hughes and Jacobs 2017). The matrix metalloproteinases (MMPs) are mainly responsible for ECM breakdown, and their level of activity in the tissue is regulated by their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Hughes et al. 2020). The ratio of matrix deposition to breakdown is an important contributor to the biophysical properties of a tissue (Cox and Erler 2011; Mouw, Ou, and Weaver 2014).

The basement membrane is made up of highly conserved core components. It consists of a Laminin sheet that is anchored to the cell surface by Integrins. A second sheet of Collagen-IV is then anchored to the Laminin sheet, primarily through cross-linking proteins like Nidogen (Hughes and Jacobs 2017; Howard et al. 2019). The basement membrane is an ancient structure that is found in all metazoans and is thought to have facilitated the development of multicellularity (Fidler et al. 2017). The majority of metazoans also possess the fibrillar collagens that make up the interstitial matrix. One notable exception is the fruit fly, *Drosophila*

melanogaster. *Drosophila* lacks the common fibrillar collagens, making it an intriguing model for the study of basement membrane dynamics.

Due to its critical involvement in supporting and maintaining tissue function, ECM homeostasis is tightly controlled (Bonnans, Chou, and Werb 2014). One of the results of ECM dysregulation is fibrotic remodelling, or fibrosis. Fibrosis refers to increased deposition of matrix components, as well as increased levels of crosslinking between these proteins (Travers et al. 2016; Meschiari et al. 2017). In a healthy ECM there is a balance of protein breakdown and deposition that acts to maintain tissue characteristics. With fibrosis, this balance is disrupted in favour of increased protein deposition. Increased levels of crosslinking insolubilize the matrix and make it more resistant to degradation, further disrupting the normal balance of remodelling.

In the heart specifically, fibrosis can have catastrophic consequences due to the replacement of highly specialized, contractile cardiomyocytes with non-contractile ECM proteins. This compromises the ability of the heart to contract effectively, can disrupt the connections between cells that are crucial for conduction of nerve impulses, and leads to maladaptive cardiac remodelling that can progress to heart failure (Travers et al. 2016). Cardiac fibrosis is initially adaptive, with activated fibroblasts laying down additional ECM proteins in order to replace necrotic cells and prevent cardiac rupture (Travers et al. 2016). However, as these cells persist in the tissue, fibrosis becomes progressive and heart function is further compromised (Jourdan-LeSaux, Zhang, and Lindsey 2010).

Despite being a prevalent component of many diseases, fibrosis has no available treatments (Pehrsson et al. 2021). Essentially every case of CVD exhibits some level of fibrotic remodelling where it is also known to worsen surgical outcomes (Travers et al. 2016; El Hajj et al. 2018). Fibrotic remodelling of the heart is also known to occur in the context of obesity, one of the leading comorbidities of heart disease (Cavalera, Wang, and Frangogiannis 2014). This necessitates a deeper understanding of how the cardiac ECM responds to obesity specifically, which could allow for more targeted treatment of disease symptoms.

In order to identify the specific effects of obesity on the cardiac ECM, we have employed *Drosophila melanogaster. Drosophila* is a powerful tool for performing this research due to its lack of genetic redundancy, simple heart tube that is not required to support life, and a similar ECM to mammals, including the presence of highly conserved basement membrane proteins (Hughes and Jacobs 2017; José C. Pastor-Pareja 2020b; Rotstein and Paululat 2016). The *Drosophila* heart, or dorsal vessel, is a linear, tube-like structure that follows the same developmental pathways as the early mammalian heart. The *Drosophila* heart has no stem cells so has no replacement of cells after damage (Hughes and Jacobs 2017). While the mammalian heart does have some stem cells they do not contribute meaningfully to cardiomyocyte replacement (van Berlo et al. 2014). Following injury the mammalian heart relies on repair rather than replacement of cells (Vujic, Natarajan, and Lee 2020). Studying repair mechanisms in the *Drosophila* heart may therefore provide insights into how mammalian hearts function following injury.

The *Drosophila* cardiac ECM is composed of the same core components as other basement membranes. It contains both a Laminin sheet and Collagen-IV, as well as the heart-specific collagen Pericardin. Pericardin is a Collagen-IV like protein that organizes similarly to fibrillar collagens in mammals (Chartier et al. 2002; Sessions et al. 2017). Thus, the *Drosophila* cardiac ECM is composed of two Collagen matrices, one of Collagen-IV and one of the more fibrillar appearing Pericardin. Pericardin has previously been shown to be critical for the maintenance of the *Drosophila* heart (Chartier et al. 2002; Drechsler et al. 2013).

Diet has been used previously to induce obesity-like phenotypes in *Drosophila,* including methods that supplement a standard *Drosophila* diet with coconut oil as a source of fat and excess calories (Birse et al. 2010; Diop, Birse, and Bodmer 2017). The present study utilized a dosage series of high fat diets (HFDs) to determine what affect dietary supplementation had on the *Drosophila* heart. Previous studies in adult *Drosophila* have revealed cardiac dysfunction, increased triglyceride levels, and altered metabolism as a result of short-term HFD feeding (Guida et al. 2019; Birse et al. 2010; Diop, Birse, and Bodmer 2017). The present study aimed to expand on these results by feeding larval *Drosophila* a HFD from hatching. Larvae were allowed to feed until late third instar in order to maximize the duration of dietary treatment, producing a

chronic high fat diet model. In *Drosophila* growth and maturation are distinct stages, with growth occurring exclusively during larval stages (Rewitz, Yamanaka, and O'Connor 2013). By performing these experiments during a growth phase of the life cycle we are able to administer the HFD chronically, circumvent egg-laying and other adult behaviours, and maximally stress the heart as it must grow and adapt to HFD conditions. Utilizing larval stages also eliminates the confound of aging, which naturally leads to ECM accumulation (Sessions et al. 2017; Hinderer and Schenke-Layland 2019). Additionally, studies conducted on adult *Drosophila* heart function typically examine only females due to their larger body size (Birse et al. 2010; Guida et al. 2019; Walls et al. 2020). Focusing on the larval life stage allows for analysis of both female and male larvae as size dimorphisms are more limited during the growth stage. This allows us to examine any sex-specific effects of HFD treatments more easily than in adult *Drosophila*.

Additionally, a high sucrose diet was employed in this study to determine if the type of nutrient providing the excess calories affected the heart specifically. High sucrose diets have been shown to induce a diabetes-like phenotype in *Drosophila*, exhibiting insulin resistance, cardiac arrhythmias, and accumulation of Pericardin (Na et al. 2013). This dietary treatment allows for a distinction to be made between excess caloric intake or inducing an obesity phenotype as the cause of cardiac dysfunction.

Here, we describe the effects of chronic HFD feeding on the larval *Drosophila* cardiac ECM. We observed that HFD feeding results in several hallmarks of obesity and causes changes to matrix organization of both Pericardin and Collagen-IV. Pericardin organization was severely perturbed, with the protein network revealing an anterior-posterior fibre alignment phenotype that was rarely observed in controls. The Collagen-IV matrix demonstrated a clumping phenotype, with a dose-dependent level of clumping within the matrix. We also observed functional impairment of the heart, namely an inability to contract fully at systole. This could be due to the rearrangement of the cardiac ECM and altered tension through the dorsal vessel. Overall, our results suggest that HFD feeding in *Drosophila* larvae induces an obesity-like phenotype that affects the organization of the cardiac ECM and leads to cardiovascular impairment.

Methods

Drosophila strains and dietary treatments

y^1w^{1118} and y^1w^{1118} *; vkg-GFP* ($vkg^{CC00791}$) lines were used for these experiments (Buszczak et al. 2007). Flies were maintained at room temperature and fed one of 6 different dietary treatments – regular fly food (control), 1.0M sucrose, 10%, 20%, 30%, and 40% coconut oil supplemented (high fat diet). In all dietary treatments the protein source was scaled to match the volume of food. 1.0M sucrose is approximately calorically equivalent to 20% HFD.

All treatments were supplements made to ordinary lab food. Ordinary lab food consists of 3.6L of water, 300g sucrose (0.2M), 150g yeast, 24g KNa tartrate, 3g dipotassium hydrogen orthobasic, 1.5g NaCl, 1.5g $CaCl_2$, 1.5g $MgCl_2$, 1.5g ferric sulfur, and 54g of agar. Fly food is autoclaved, cooled to 55^0C, then 22mL of 10% tegosept and 15mL of acid mix is added before dispensing. Coconut oil supplements were by volume, sucrose by molarity.

Triglyceride assay

Triglyceride levels were measured using a serum triglyceride determination kit (Sigma Aldrich, TR0100) (Wat et al. 2020). 5 intact third instar larvae were flash frozen in liquid nitrogen and stored at -80^0C before sample preparation. Frozen larvae were ground with a manual homogenizer in 0.1% Tween in PBS. 20µl of buffer per larva was used. Samples were heat treated at 70^0C for 10 minutes, then centrifuged at maximum speed for 3 minutes. 10µL of each sample was loaded into a 96 well plate in triplicate. 10µL of a glycerol standard at 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.315mg/mL, 0.156mg/mL, and 0mg/mL were also loaded. 250µL of free glycerol reagent was added to each well, incubated at 37^0C, and absorbance was read at 540nm. 50µL of triglyceride reagent was then added, incubated for 10 minutes at 37^0C, and absorbance read at 540nm. The change in glycerol levels after addition of the triglyceride reagent was calculated to determine the level of stored triglycerides in the sample. A Bradford assay was then conducted on the same samples and the level of stored triglycerides was divided by the amount of protein in the sample to control for body size.

Dissections

Heart:

Dissections were performed by fixing larvae dorsal down to a surface using pins (Brent, Werner, and McCabe 2009). Larvae were bathed in PBS and an incision was made at the ventral midline. The cuticle was pinned back and the gut and fat bodies were removed to reveal the heart. Dissections were performed at third instar, after the onset of wandering behaviour.

Fat body:

Above process was followed but only the gut was removed to expose the fat bodies.

Immunohistochemistry

Heart:

Dissections were fixed for 20 minutes without shaking at room temperature in 4% paraformaldehyde in PBS. Specimens were then washed 3x10 minutes in PBST (0.3% Triton-X-100), before blocking for 30 minutes with NGS (1:15). Primary antibodies were incubated overnight at 4^0C with shaking. After incubation with primary 3x10 minute washes in PBST were performed before adding secondary antibodies for one hour at room temperature. Phalloidin was added at the same time as secondary antibodies. Specimens were then washed 3x10 minutes in PBST, with a final wash in PBS to remove detergent. 50% glycerol was added for at least 3 hours, then 70% glycerol overnight. The primary antibody used was mouse anti-Prc (Pericardin, EC11, DSHB, 1:30 dilution). Secondary antibodies used were Alexa Fluor 488 anti-mouse and Alexa Fluor 647 anti-mouse (1:150 dilution). Alexa Fluor 546 and 647 Phalloidin (Thermofisher Scientific) were also used (1:75 dilution).

Fat body:

Dissections were fixed for 30 minutes at room temperature in 4% paraformaldehyde. Specimens were washed 2x5 minutes in PBST, then incubated in 493/503 BODIPY (1:1000) for 30 minutes. Specimens were then washed 2x5 minutes, placed in 70% glycerol, and immediately mounted for imaging.

Imaging

A Leica SP5 confocal microscope was used to obtain image stacks. 1µm intervals between frames were used for heart dissections, 0.5µm intervals were used for fat bodies. Fat bodies were imaged from the surface to a depth of 30µm. Hearts were imaged from the ventral face of the cardiac ECM to the dorsal edge of the heart tube. Images were processed using Leica software (LAS AF), ImageJ, and ZEN blue.

Image quantification and statistics

Pericardin linearity was measured by blind score, with a scale of 1 being a normal meshy matrix, 2 having some linearity but not the majority, and 3 being majority or completely linear.
Pericardin matrix to heart tube ratio was obtained by tracing the outline of the matrix and the heart tube, obtaining areas for each, and generating a ratio.
The percentage of the Collagen-IV matrix that was occupied by clumps was obtained by tracing the total area of the matrix and then tracing the area of each clump within the matrix. The sum of clump areas was then expressed as a percentage of the total matrix area.
Lipid droplet diameter was measured using the line tool in ZEN 3.4 (blue edition).
All measurements from confocal images were performed on unedited images. For publication only images have had brightness and colour balance adjusted using Photoshop CS6.
Statistical analysis of larval health (mass, triglyceride levels, lipid droplet size), Pericardin organization, and diastole/systole were performed using Graphpad Prism (v.9.5.1). One-way analysis of variance (ANOVA) with a Dunnett's multiple comparisons test was performed. Graphs are plotted with SEM.
For Collagen-IV matrix organization statistics we fit a linear model with the terms sex, diet, and their interaction using the R programming language. Significance of the terms was tested by two-way ANOVA and differences in group means using the emmeans(v1.7.2) package, with 95% confidence intervals reported for all statistics. Sucrose and the equivalent calorie HFD were compared to controls separately to determine if sucrose had a comparable effect to HFD feeding. Because sucrose was not different from controls it was excluded from HFD comparisons. Diastolic and systolic volumes for HFD treatments were fit using a linear model with the term diet to estimate the slope of the line as 95% confidence intervals.

OCT imaging

Optical coherence tomography (OCT) was used to visualize the heart beating *in vivo* in real time in late third instar larvae. Larvae were adhered to a microscope slide dorsal side up before being placed under the OCT camera. B scans were taken in 3D acquisition mode using a Thorlabs OCT Telesto series TEL221PS system at the widest point of the heart chamber with the following parameters: X size 1257 pixels, 1.03mm, Y size 0, 400 frames, Z field of view 1.2mm. This gives a 20 second video with 20 frames per second. Image stacks were then exported as TIFs and processed in ImageJ (Abràmoff, Magalhaes, and Ram 2004). The cross-sectional area was measured at both diastole and systole. Diastolic and systolic volumes across HFD groups were compared using a model estimate with 95% confidence intervals.

Results

Viability of larvae on high fat and high sucrose diets

To determine the dilution series of HFD treatments larvae were reared from hatching on food containing 10%-50% coconut oil. 50% had no larvae survive to late L3 (data not shown) so 10%-40% dosages were used for all experiments. 1M sucrose was selected based on previous studies (Palanker Musselman et al. 2011) and is calorically comparable to 20% HFD supplementation. Larvae did not survive on a 5M diet which would have been equivalent in calories to the 40% HFD treatment (data not shown).

Health of larvae was assessed by measuring larval mass, triglyceride levels, and lipid droplet diameter. All assays separated female and male larvae in order to isolate possible sex specific effects. Assays were also conducted on both y^1w^{1118} and y^1w^{1118}*; vkg-GFP* (hereafter *vkgGFP*) so both Collagens in the cardiac ECM could be examined. *VkgGFP* is a protein trap that labels the *viking* subunit of Collagen-IV with a GFP tag, so endogenous Collagen-IV is fluorescently tagged (Buszczak et al. 2007). Larval mass was mildly elevated in 10% and 20% female y^1w^{1118} high fat diet treatment groups, and 10%-30% in males (elevation between 13.8-14.8% in females, and 15.1-20.6% in males) (Figure 1A). A modest increase or unchanged body size was not unexpected as the critical weight checkpoint triggers the transition from larval development to pupation (Zeng et al. 2020). An increase in weight could therefore trigger early pupation rather than continued increase in larval size. The high sucrose diet resulted in larvae that were significantly smaller than controls and the calorically equivalent HFD treatment (females 43.6% mass of controls, males 54%) (Figure 1A). This is consistent with previous studies that demonstrate high sucrose inducing diabetes-like phenotypes as a result of impaired insulin signalling (Na et al. 2013).

In *vkgGFP* larvae 20% and 30% HFD females were smaller than the control genotype (control=2.267mg+/-0.0335mg, 20%=1.931mg+/-0.0468, 30%=1.959mg+/-0.0895). This suggests that *vkgGFP* does not tolerate the HFD treatment as well as the y^1w^{1118} genotype (Figure S1).

Triglyceride levels were measured and y^1w^{1118} larvae exhibited a dose dependent increase in triglyceride levels compared to controls (Figure 1B). The overall trend was similar for both female and male larvae but female larvae exhibited a more significant change. *vkgGFP* larvae did not demonstrate an elevation in triglyceride level but controls of this genotype had triglyceride levels over 4 times higher than y^1w^{1118} controls (Figure S2). Oregon R triglyceride levels were intermediate between y^1w^{1118} and *vkgGFP,* suggesting that triglyceride level can vary markedly with genotype (Figure S2). The higher baseline triglyceride level in *vkgGFP* individuals may contribute to their reduced ability to tolerate HFD feeding when compared to y^1w^{1118}. This may also indicate that there is a limit to the level of triglycerides that larvae are able to process effectively, and *vkgGFP* does not have a significant increase in triglyceride levels with HFD feeding because they are already close to this limit at baseline.

Lipid droplet diameter in y^1w^{1118} individuals showed a dose dependent effect with HFD feeding (Figure 1 C). All HFD had increased lipid droplet diameter (Figure 1D). The high sucrose diet also exhibited significantly increased droplet diameter.

Overall this shows that high fat diets are able to induce obesity-like phenotypes, while high sucrose diets have some characteristics of obesity but are significantly smaller than controls and HFD treatments. Previous studies have shown that this is due to changes in insulin signalling that induces a diabetes-like phenotype in these individuals (Palanker Musselman et al. 2011).

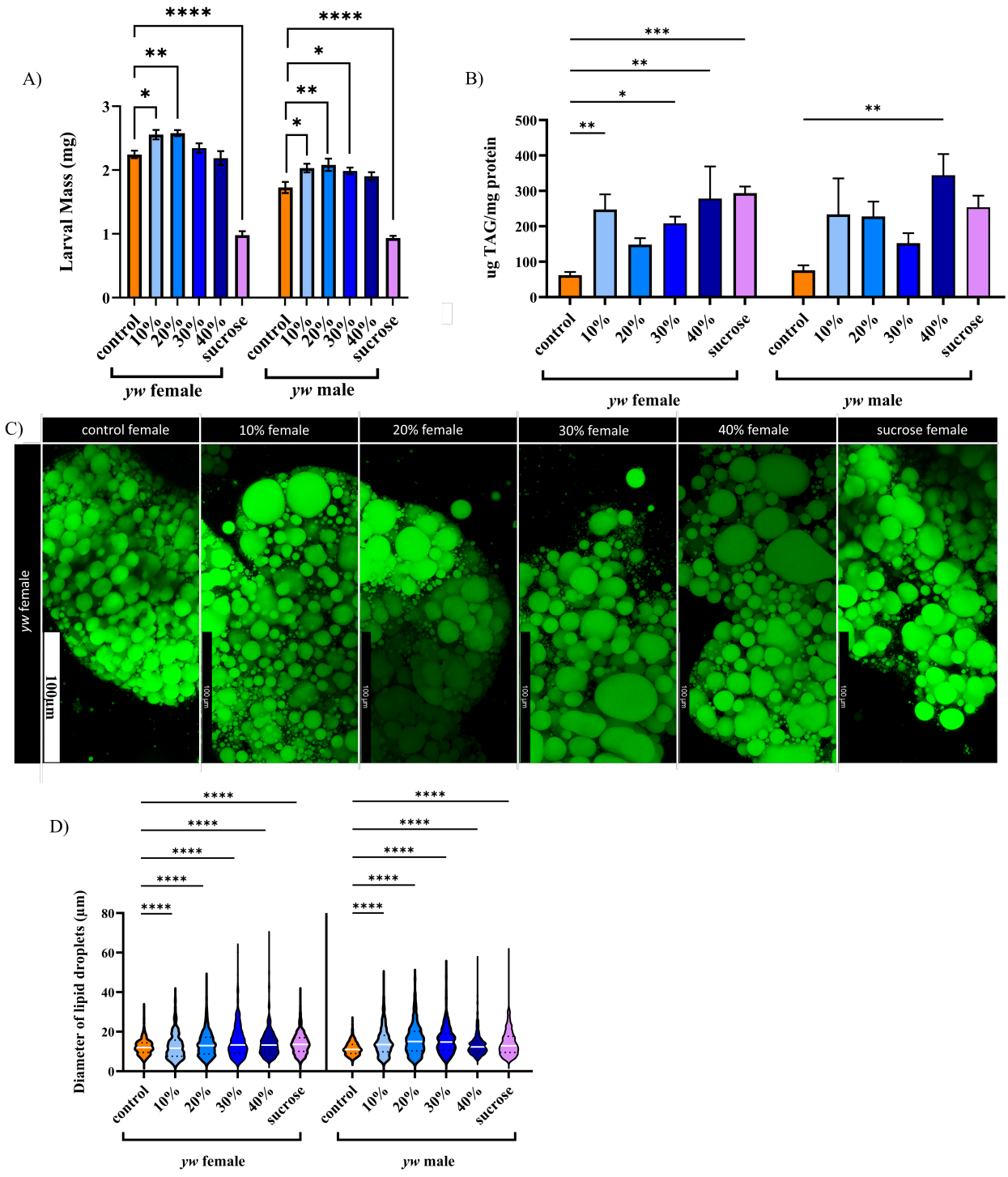
A)
Larval Mass (mg)
control
10%
20%
30%
40%
sucrose
yw female
yw male
B)
ug TAG/mg protein
yw female
yw male
C)
control female
10% female
20% female
30% female
40% female
sucrose female
yw female
100µm
D)
Diameter of lipid droplets (µm)
yw female
yw male

Figure 1: Larvae fed a high fat diet show a dose dependent increase in markers of obesity
Larval mass is not lowered by high fat diet feeding, in contrast to high sucrose (A). HFD results in a dose dependent increase in triglyceride levels in y^1w^{1118} larvae, especially females (B). Lipid droplets were visualized with BODIPY 493/503 (C) and reveal that both HFD feeding and a high sucrose diet result in a dose-dependent increase in lipid droplet size (D). Error bars in A and B are SEM. White lines in D represent the median, dotted lines represent quartiles. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$

Pericardin fibres have abnormal organization with dietary treatment

Having established that HFD feeding results in several hallmarks of obesity, we investigated the effect of HFD feeding on the cardiac ECM. The fibrous, heart specific collagen Pericardin showed marked changes in its organization with all dietary treatments. A normal Pericardin matrix is an organized meshwork that forms a honey-comb like pattern and extends away from the heart tube (Figure 2A, A'). With dietary treatments, the matrix takes on an anterior-posterior linearity phenotype (Figure 2B-F') that is rarely observed in controls. Both y^1w^{1118} and *vkgGFP* larvae demonstrate this phenotype with all dietary treatments (Figure 3A). Interestingly, in *vkgGFP* specifically, there is a slight improvement in the percentage of the population that is affected at 40% feeding (Figure 4F). This was likely due to a survivor bias in this group. Percent survival of the y^1w^{1118} genotype was relatively unaffected at 30% and 40% HFD but was significantly reduced in *vkgGFP,* especially at 40%. *vkgGFP* appears to be less able to tolerate the highest HFD treatments than y^1w^{1118} larvae.

The Pericardin matrix also appears to not extend away from the heart tube with high fat diet feeding (Figure 2B-F'). This could be due to altered tension due to changes in matrix organization. The ratio of the area of the Pericardin matrix to the area of the heart tube revealed a clear dose-dependent downward trend in the female *vkgGFP* larvae (Figure 3C). A similar trend was observed in the female y^1w^{1118} larvae but was not statistically significant (Figure 3B).

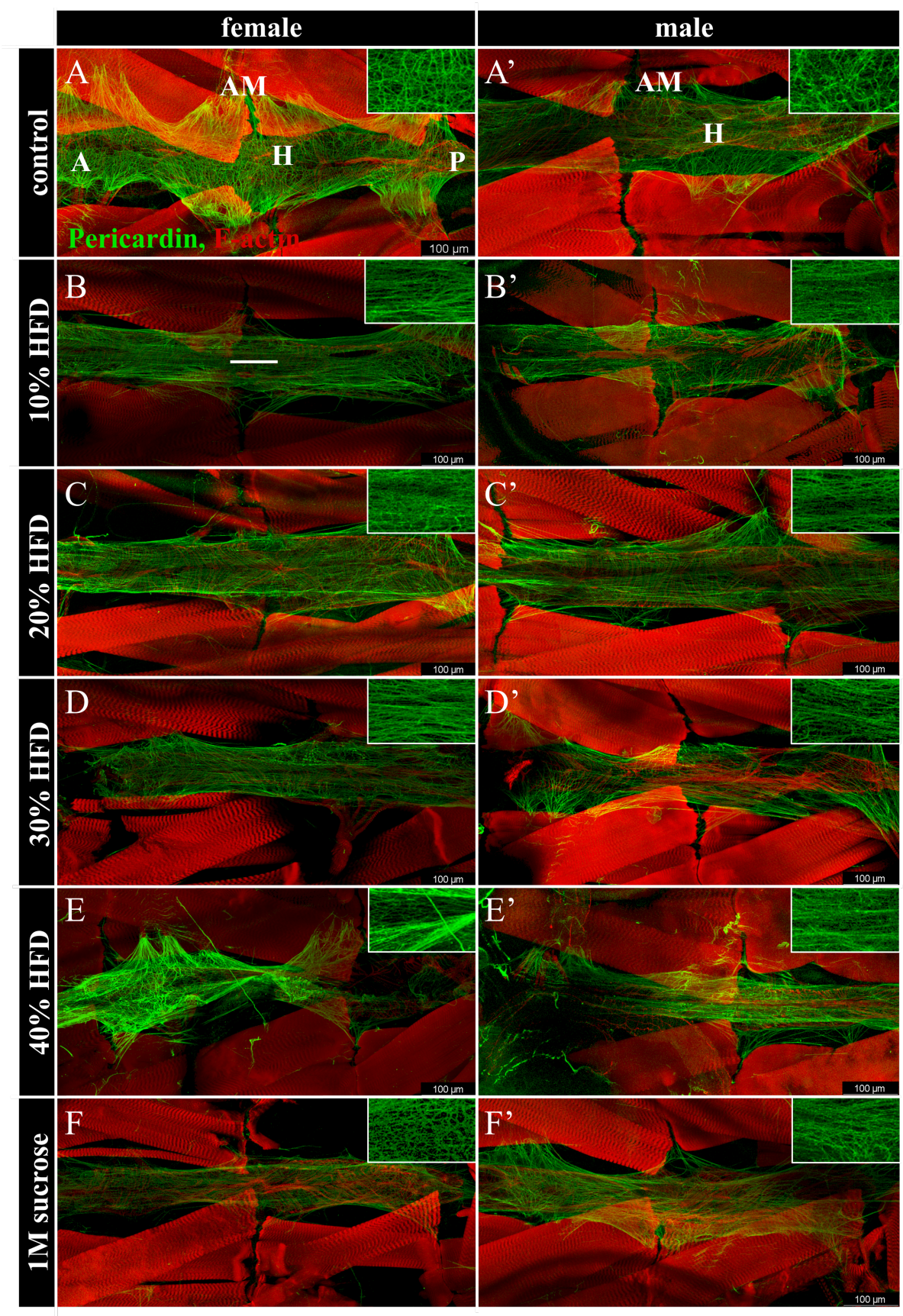
female
male
control
10% HFD
20% HFD
30% HFD
40% HFD
1M sucrose
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
AM
H
A
P
Pericardin, F-actin
100 µm

Figure 2: Pericardin fibre organization is perturbed in y^1w^{1118} dietary treatments
Controls demonstrate a normal, organized meshwork (A-A') while dietary treatments show a change in matrix organization, with matrix fibres becoming oriented anterior-posterior (B-F'). The cardiac ECM is visualized by immunolabelling Pericardin (green) and F-actin (red). The F-actin label in the background is the body wall muscles. In panel A, H labels the heart tube, AM labels alary muscles, A is anterior, P is posterior. All images are oriented anterior to the left. White line in panel B follows direction of anterior-posterior orientation of Pericardin fibres.

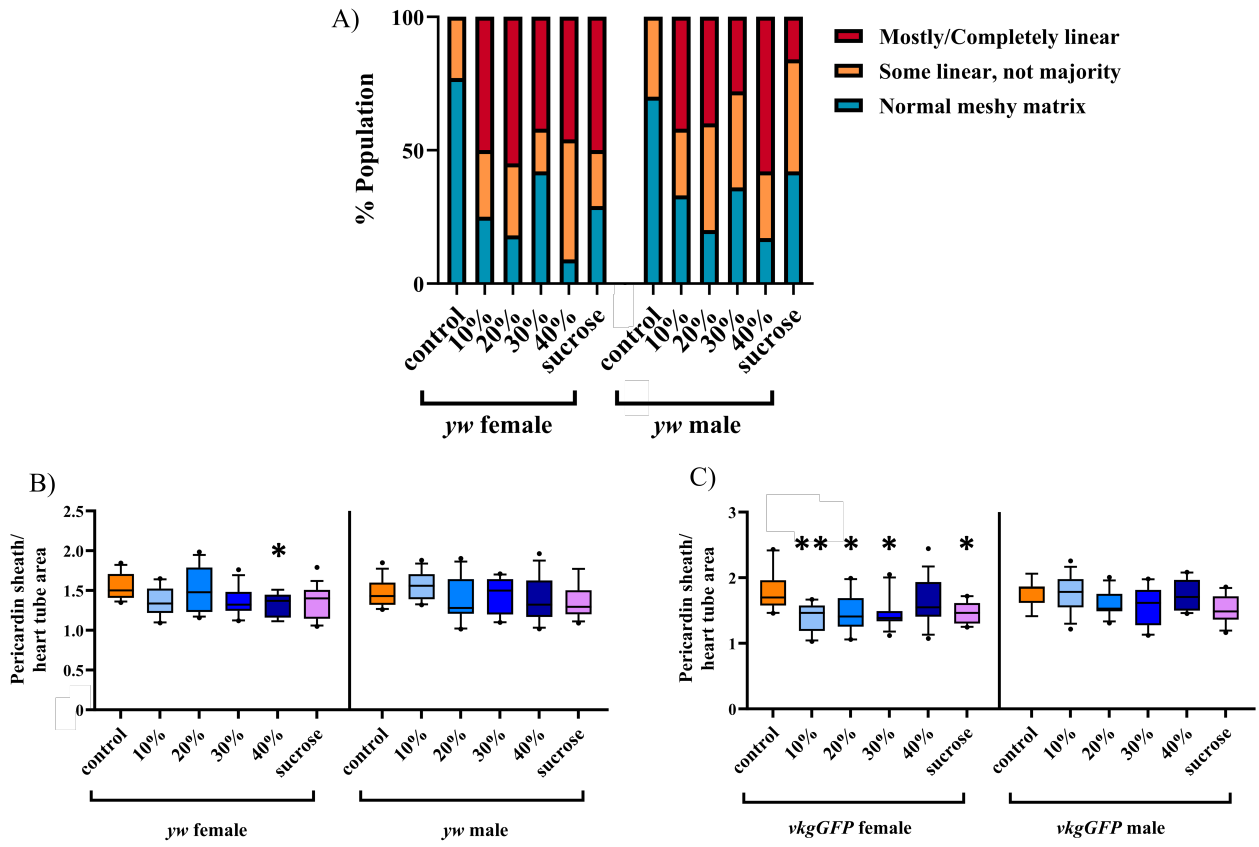

Figure 3: Matrix organization shows severe rearrangement in dietary treatments
Dietary treatments demonstrate a linearity phenotype that is more common and more severe than controls (A). In females, there is a downward trend in the size of the Pericardin matrix relative to the heart tube (B, not statistically significant, and C) but not in males. *=p<0.05, **=p<0.01

Collagen-IV distribution is altered with dietary treatment

The Collagen-IV matrix was visualized using the *vkgGFP* line. The distribution of this matrix is normally sheet-like, covering the entire surface of the heart (Figure 4A'). The Collagen-IV matrix showed no changes in the high sucrose diet but showed a clumping phenotype in the high fat diet treatments (Figure 4B', C', D', E'). The area of the Collagen-IV matrix occupied by clumps was quantified and revealed no significant difference between the controls and the high sucrose diet (Figure 5A). The 20% HFD had significantly elevated levels of clumping in the Collagen-IV matrix in contrast to the calorically equivalent high sucrose diet, with 2.29% more clumping in the HFD treatment (95% CI: -3.74, -0.834, $p<0.001$). The high sucrose diet and the controls were not significantly different, with the high sucrose diet having 1.32% more clumping than controls (95% CI: -2.79, 1.47, $p=0.08$). Given that the sucrose diet did not have a significant difference in clumping compared to the controls it was excluded from the HFD comparison. The HFD treatments demonstrated a significant dose-dependent effect on clumping phenotype ($DF=2$, $F=20$, $p<0.001$) (Figure 5B). While it was found that while diet has an effect on the clumping phenotype, there was no significant sex effect ($DF=1$, $F=1.9891$, $p=0.164$).

A different fibre organization defect was observed in the high sucrose dietary treatment. This group was 1.32% (95% CI: 2.79, 0.15, $p=0.08$) more likely to have holes or gaps within the normally sheet-like Collagen-IV matrix as compared to the control treatment. This phenotype is not as strong as the clumping phenotype observed in HFD groups. No other treatment group exhibited this phenotype (Figure S3). This suggests that the defects in Collagen-IV deposition and remodelling may vary with treatment but this could also be due to reduced growth and altered physiology in the high sucrose group, as major signalling pathways like insulin are known to be affected with this treatment.

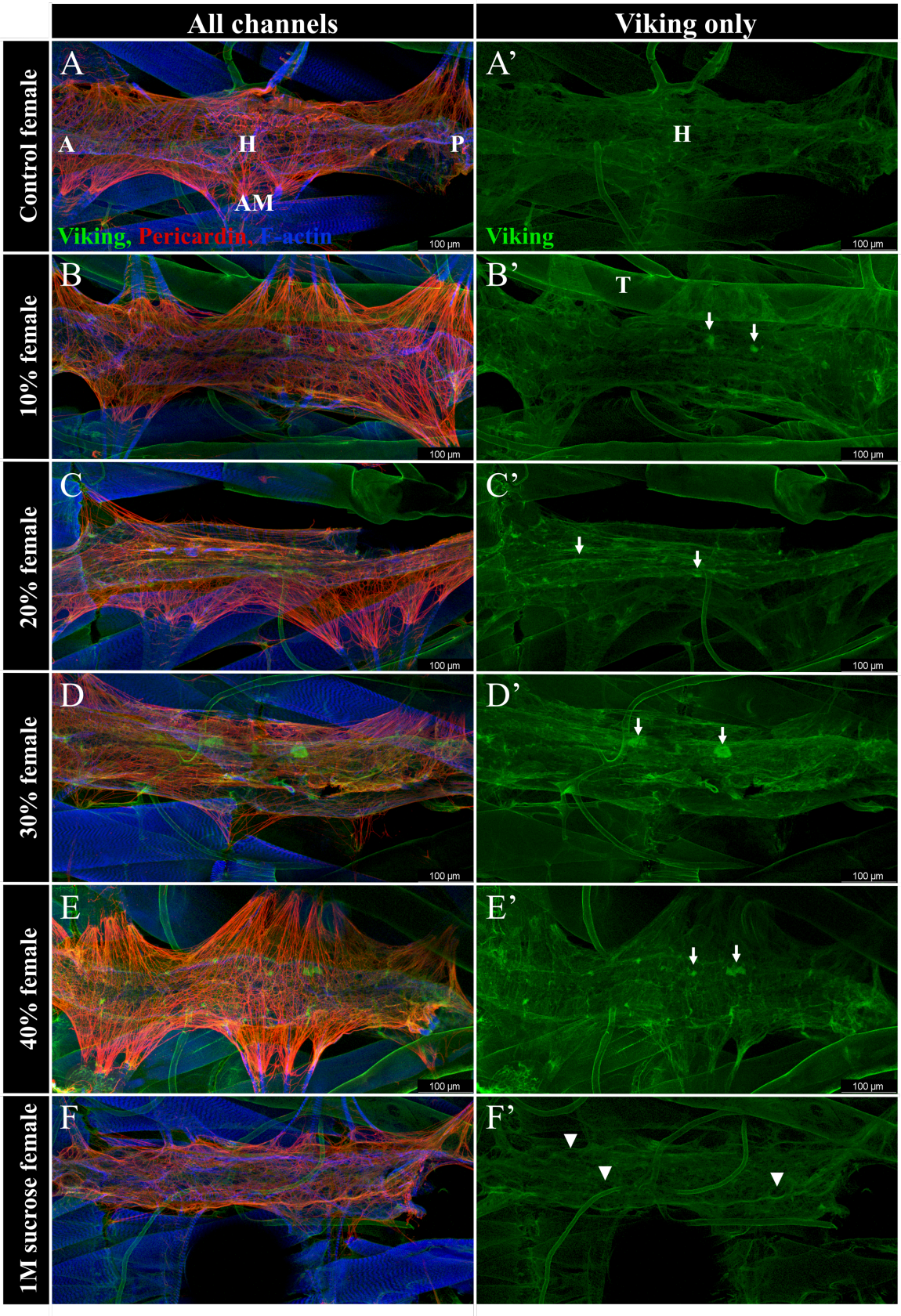
All channels
Viking only
Control female
10% female
20% female
30% female
40% female
1M sucrose female
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
A
H
P
AM
Viking, Pericardin, F-actin
Viking
T
100 µm

Figure 4: Collagen-IV distribution is abnormal in *vkgGFP* dietary treatments
In control individuals the Collagen-IV matrix shows a regular, sheet-like distribution across the surface of the heart (A'). HFD treated larvae experience elevated levels of clumping within the Collagen-IV matrix, indicated by white arrows, that is not found in the high sucrose diet treatment (B',C', D', E'). Holes are observed in the high sucrose Collagen-IV matrix instead, indicated by white arrowhead (F'). The cardiac ECM is visualized by endogenous *vkgGFP* fluorescence (green), and immunolabelling Pericardin (red) and F-actin (blue). In panel A, H labels the heart tube, AM labels alary muscles, A is anterior, P is posterior. *vkgGFP* is also found in the trachea, indicated by T in panel B'. All images are oriented anterior to the left.

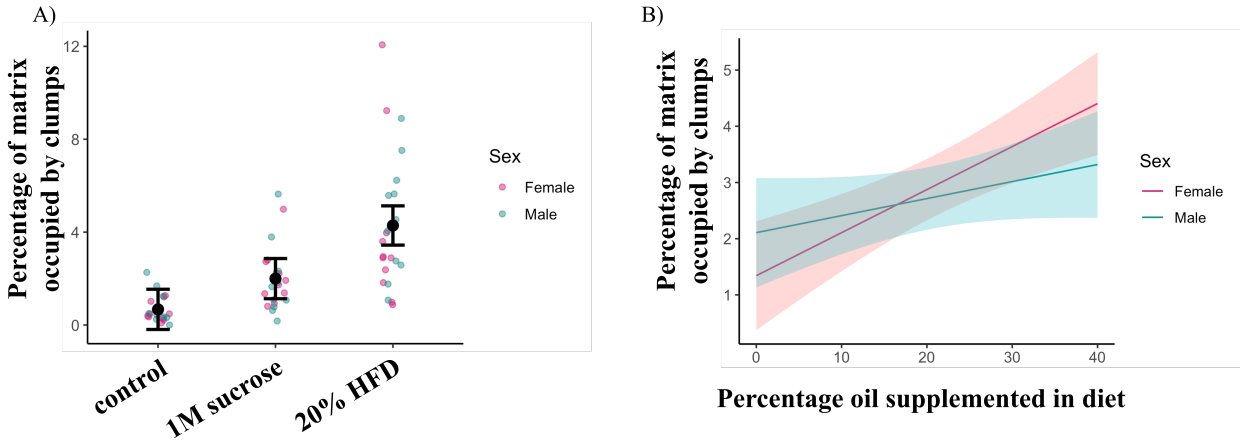

Figure 5: Collagen-IV shows a dose-dependent relationship with clumping defect
High sucrose diet is compared to 20% HFD as they are calorically equivalent. High sucrose diet does not have increased Collagen-IV clumping compared to controls while the calorically comparable HFD does (A). HFD feeding causes a dose-dependent increase in the amount of clumping within the matrix (B). This trend is more pronounced in female larvae. Error bars in A represent 95% confidence intervals. Graph in B is a model estimate with 95% confidence intervals.

Live imaging reveals impair ability of the heart to contract with HFD feeding

If the cardiac ECM of larvae raised on a HFD reveal perturbations in the organization of both cardiac Collagens how then might this affect cardiac physiology? We performed live imaging to determine if these ECM perturbations have functional consequences using optical coherence tomography (OCT). Movies of beating hearts were used to measure the cross-sectional area of the lumen at both diastole and systole (Figure 6A) and it was found that the high sucrose diet generates larvae with much smaller hearts than the controls (Figure 6C, D). This is likely due to the smaller body size of these individuals compared to their control counterparts. The high fat diet treatments on the other hand had similar diastolic areas across all treatment groups but showed a dose-dependent increase in systolic area (Figure 6E). This indicates an inability of the heart to contract fully with increasing dietary fat, suggesting these larvae have impaired heart function similar to mammalian cases of CVD. However, heart rate was unaffected and arrhythmicity index was only elevated in y^1w^{1118} high sucrose diet males (Figure S4). Live imaging also revealed that the hearts of HFD treated larvae were more likely to be abnormally shaped (Figure 6B). The majority of control hearts were close to round or oval and contracted evenly on all sides. In the high fat diet treated individuals there were irregularly shaped hearts (Figure 6B), as well as contraction that mainly occurred in one plane instead of uniformly around the circumference of the heart.

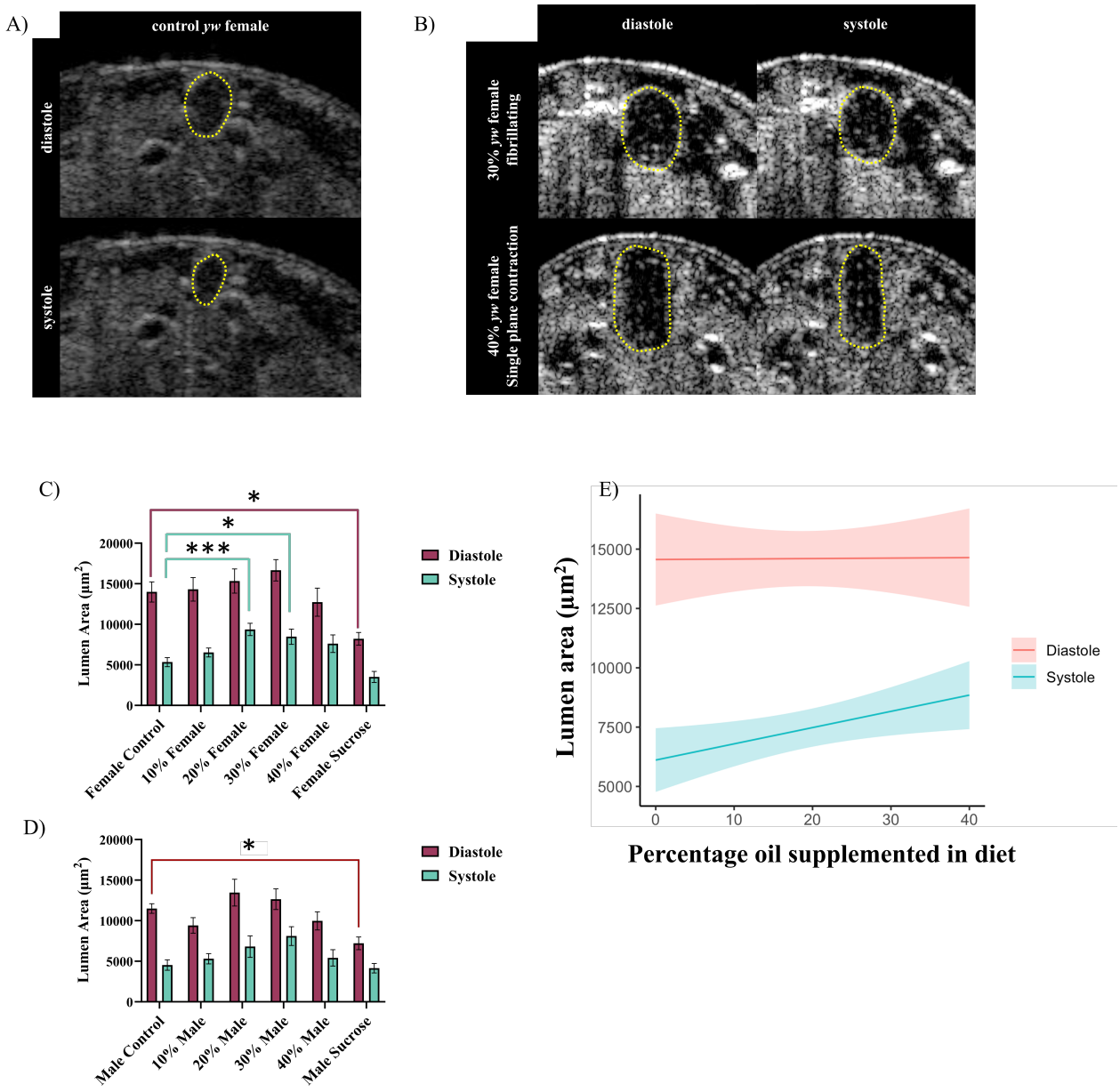

Figure 6: Optical coherence tomography reveals impaired contraction with HFD treatments

OCT can be used to visualize the heart beating in cross-section, revealing the area inside the lumen at both diastole and systole (A). Control hearts show round or oval hearts that contract evenly along the perimeter, while HFD treatments cause abnormal heart shape and can also cause an inability to contract evenly on all sides (B). Lumen cross-sectional area in y^1w^{1118} females shows a change in diastole only in high sucrose individuals (C), but does show increases in systolic volume at higher concentrations of HFD. A similar trend is observed in males (D). This demonstrates a dose-dependent impairment of heart contractions (E). Error bars in B and C are SEM. Graph in E is a model estimate with 95% confidence intervals. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001

Discussion

A chronic high fat diet in growing *Drosophila* larvae generates dose-dependent effects on overall organismal health as well as on the form and function of the heart. HFD individuals have clear signs of obesity and CVD, while the high sucrose diet shares some markers of obesity as well as other metabolic disorders. Previous studies in adult *Drosophila* have found that HFD feeding causes increased fat storage, including ectopic deposition of triglycerides, as well as cardiac dysfunction, including decreased diastolic and systolic diameters, reduced fractional shortening (a proxy for stroke volume), and reduced heart period (Hardy et al. 2015; Guida et al. 2019). These health effects point towards the development of lipotoxic cardiomyopathy. Here, we find that larval *Drosophila* experience similar effects, and that the observed cardiac dysfunction may be as a result of altered ECM dynamics.

Genotype was found to affect the magnitude of effect of HFD feeding. y^1w^{1118} individuals tolerated the HFD more readily than *vkgGFP* individuals. This may be due to the elevated triglyceride levels found in *vkgGFP* control larvae. These larvae have comparable triglyceride levels to HFD fed y^1w^{1118} individuals, suggesting that they are close to a maximum level of tolerance of triglycerides. The increase due to HFD feeding may have caused the reduced viability seen in *vkgGFP* in contrast to y^1w^{1118} on HFD treatments. Oregon R triglyceride levels are intermediate to y^1w^{1118} and *vkgGFP,* suggesting that triglyceride levels on ordinary lab diets are a variable trait.

HFD feeding affected cardiac ECM organization, with both the Pericardin and Collagen-IV matrices developing defects. Collagen-IV phenotypes for HFD and high sucrose diets were different, with HFD treatments exhibiting a clumping phenotype while high sucrose diets possessed gaps in the matrix. The Collagen-IV clumping phenotype in the HFD feeding treatments suggest that matrix deposition may be elevated. This could be due to increased expression of Collagen-IV or to reduced breakdown (or turnover) of the matrix. Matrix breakdown is performed by the matrix metalloproteinases (MMPs) and altered levels or activity of these proteins have been shown to promote fibrotic remodelling. Altered MMP expression profiles have been demonstrated in a variety of fibrotic diseases (Garrett et al. 2019; Tian, Luo, and Liu 2022). We described previously how depletion of MMP2 by overexpression of its

inhibitor TIMP during larval growth causes accumulation of Collagen-IV, suggesting MMP2 expression is required for the maintenance of a healthy ECM (Hughes et al. 2020). Reduced MMP2 activity, either as a result of altered gene expression or due to elevated expression of TIMP, presents an intriguing possibility for the clumping observed the Collagen-IV matrix in these HFD treatment groups.

Organization of the Pericardin network was severely affected by HFD feeding. Instead of forming a honey-comb like meshwork as in controls, HFD feeding and the high sucrose diet were found to induce an anterior-posterior fibre linearity. This linearity phenotype was observed in all HFD treatments. Linearity is rarely seen in controls, suggesting that the ability to organize the matrix appropriately is affected by HFD feeding. This could be due to increased inflammatory responses due to HFD feeding. Excess triglycerides are known to cause an inflammatory response which can lead to remodelling of fibrillar Collagens in cases of CVD in mammalian systems (Cavalera, Wang, and Frangogiannis 2014; Nishida and Otsu 2017). The excess triglycerides measured in HFD fed larvae may be causing a similar inflammatory response that is affecting matrix deposition and turnover, which could in turn lead to the observed reorganization of the Pericardin matrix reported here. ECM regulation is known to be affected in disease states, with elevated levels of crosslinking enzymes, altered post-secretion activation of MMPs and TIMPs, and alterations to proteins important for appropriate Collagen deposition like its chaperone, SPARC (Hartley et al. 2016; Hughes and Jacobs 2017). Our previous study found that MMP activity was critical for the appropriate organization of the Collagen-IV matrix (Hughes et al. 2020). It is therefore feasible that Pericardin organization also requires specific post-translational modifications or enzymatic activity in order to form a normally organized network. If levels of ECM regulators like MMP and SPARC are affected by chronic inflammation, it could affect the overall organization of the Pericardin matrix, generating a matrix with less structural integrity that is prone to collapsing in on itself and appearing linear as observed here.

The typical arrangement of the ECM has Pericardin fibres extending in many directions in order to transmit tension evenly across the heart. This allows for the heart to open evenly in all directions and helps to maintain a uniform shape as the heart contracts and relaxes. Increased

linearity of fibres in HFD treated larvae also correlates with defects in the ability of the heart to contract evenly around its perimeter. Both effects suggest that HFD feeding results in an uneven distribution of tension around the heart by affecting the organization of the ECM. Additionally, live imaging by OCT revealed a dose-dependent inability of the heart to contract fully at systole. Diastolic area is unaffected, indicating this defect is in the ability of the heart to contract, not relax. A common finding in CVD in humans is an inability of the heart to contract effectively, including in cases of obesity-related CVD (Alpert, Omran, and Bostick 2016). The functional defects observed in *Drosophila* larvae with an obesity-like phenotype are consistent with those observed in cases of human disease, suggesting that a HFD feeding regime in *Drosophila* larvae can be used to model human disease.

Drosophila fed a HFD exhibit many of the same metabolic and physical symptoms as obese humans (Birse et al. 2010; Guida et al. 2019; Hardy et al. 2015). However, existing studies have predominantly focused on transient feeding of adults, and analyzed only females. Our work suggests that larval *Drosophila* provides a better model for chronic dietary treatments, and for the examination of the sex specific effects of diet. We also find that while previous studies have reported most consistent and reproducible effects of HFD treatments on 30% coconut oil supplemented diets (Birse et al. 2010; Diop, Birse, and Bodmer 2017; Guida et al. 2019), there may be variability in tolerance with different genotypes. Our results show that 30% is ideal for inducing cardiac phenotypes in y^1w^{1118}, but that a 20% diet is better for *vkgGFP*. This presents an interesting opportunity to examine the effects of genetic background on response to dietary treatment. It has previously been found that a HFD affects heart function due to increased TOR signalling and decreased expression of the lipase Brummer (Birse et al. 2010). These pathways are highly conserved and also altered in human disease. Based on our findings it is possible that different *Drosophila* genotypes have different levels of signalling of these key pathways, therefore altering their ability to respond to the lipotoxic insult of a HFD. Further study can utilize this feeding regime as a model for understanding the genetic basis of differing tolerances to HFD feeding, which may provide insight into how these processes are differentially regulated in human populations.

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Supplemental figures

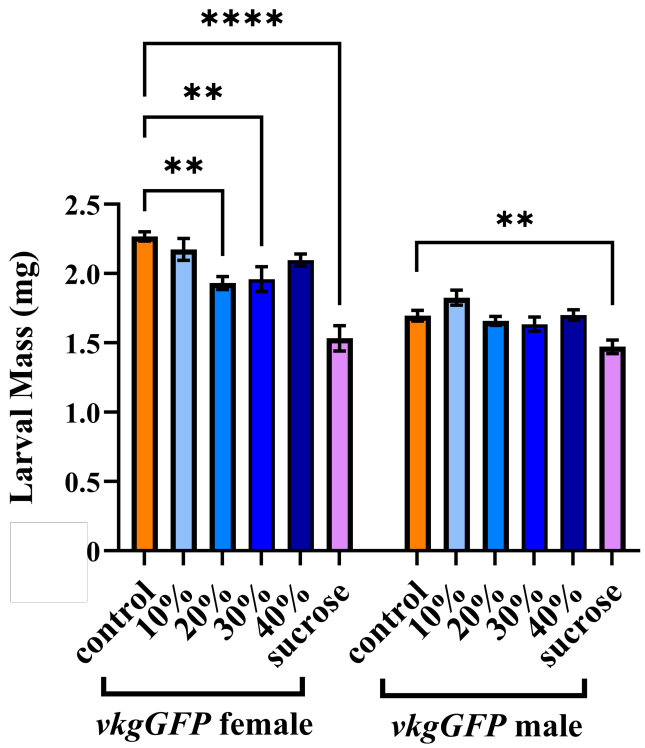

Figure S1: *vkgGFP* larval mass
vkgGFP female 20% and 30% high fat diet treatments exhibit lower mass than controls, in males no HFD treatments have significantly reduced mass compared to controls. The high sucrose diet generates smaller larvae in both males and females. Error bars are SEM. *=p<0.05, **=p<0.01, ****=p<0.0001

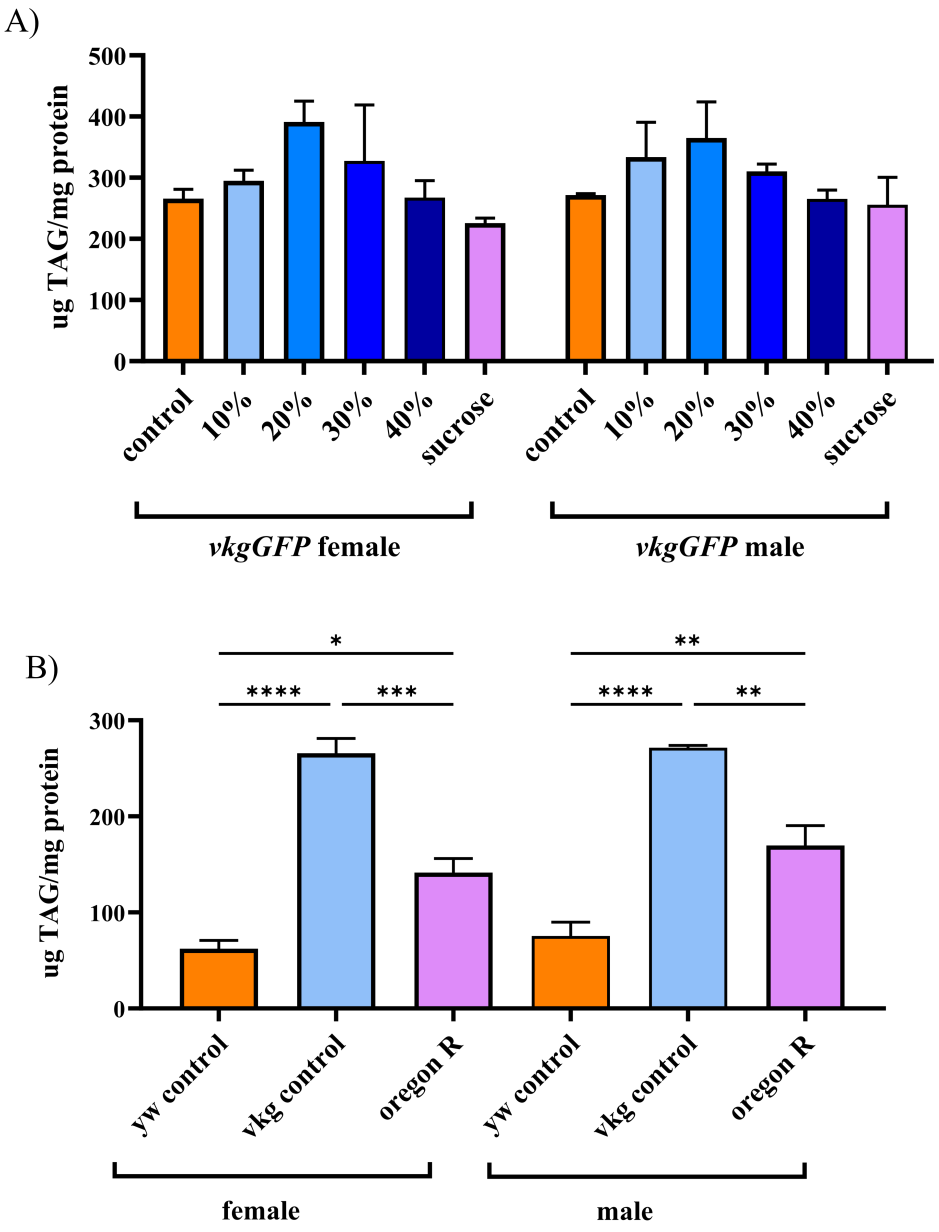

Figure S2: Baseline triglyceride levels vary with genotype
vkgGFP larvae demonstrate elevated triglyceride levels at baseline (A). HFD and high sucrose diet treatments do not have an effect on triglyceride levels in this genotype. The baseline level of triglycerides in $y^{1}w^{1118}$, *vkgGFP* and Oregon R larvae are variable (B). Both female and male larvae exhibit this variability.

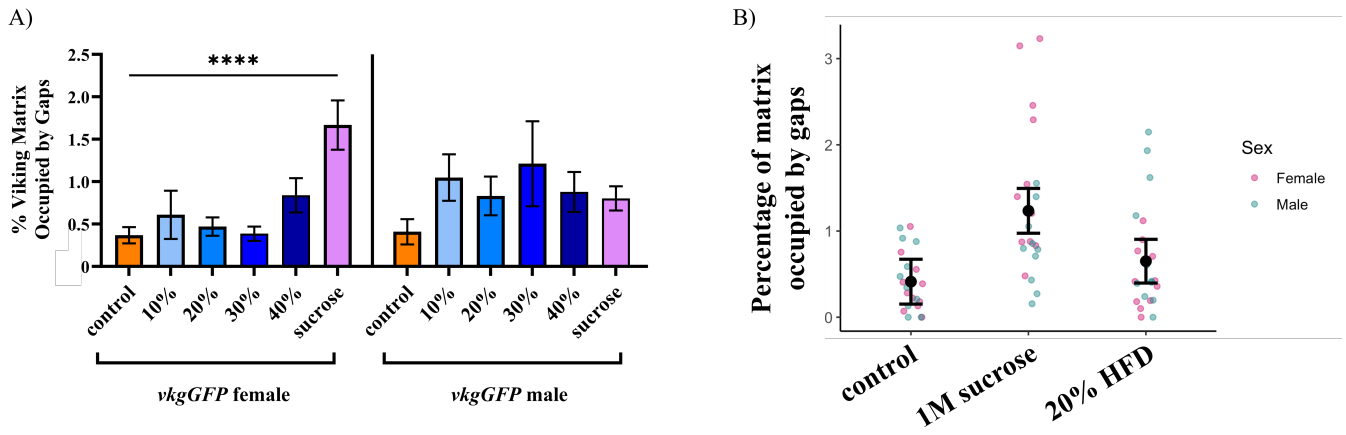

Figure S3: High sucrose diet causes gaps rather than clumps within the *vkgGFP* matrix
The high sucrose diet was found to induce the formation of gaps or holes within the Collagen-IV matrix (A). This phenotype was significant only in females. High fat diets were not found to cause this phenotype (B). Error bars in A are SEM, error bars in B are 95% confidence intervals. ****=p<0.0001

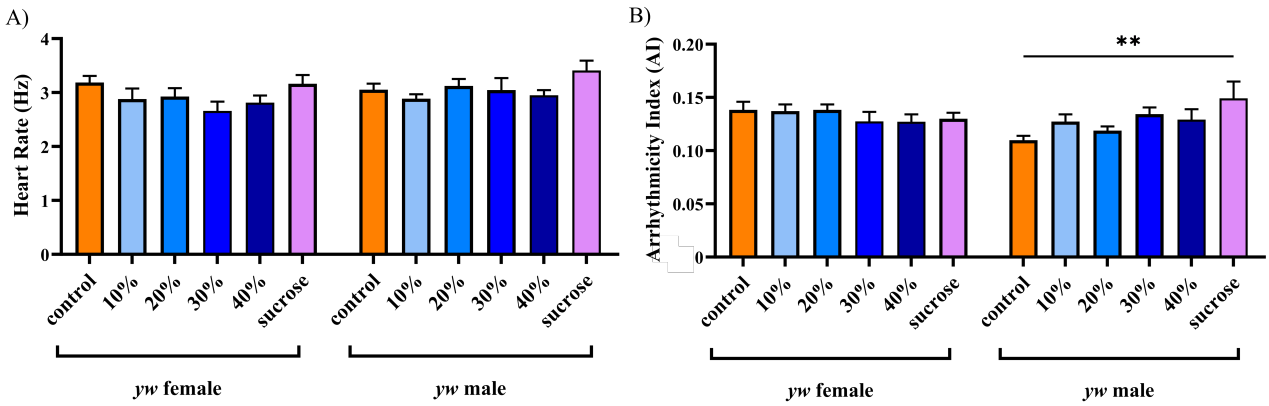

Figure S4: Cardiac functional parameters are unchanged in most dietary treatments
Heart rate was not significantly different with any dietary treatment (A). Arrhythmicity index was unaffected in all treatments except for high sucrose diet males (B). Error bars are SEM. **=$p<0.01$

Chapter 3: A *Drosophila* genetic overgrowth model shows remarkable ability to scale growth and cardiac morphology

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Abstract

The cardiac extracellular matrix (ECM) is of critical importance for overall organ biophysical characteristics and function. The ECM is commonly dysregulated in disease states, contributes to adverse outcomes, and there are no treatment options that address ECM fibrosis. Developing therapeutic targets necessitates an understanding of the regulation of ECM remodelling. Here, we utilize a larval *Drosophila* overgrowth model ("giant larvae") as a way to overload the heart and cause ECM remodelling *in vivo.* These larvae grow to immense sizes without exhibiting hallmarks of obesity. Remarkably, the organization of the ECM is unaffected by severe overload. The main effect observed is a change in Collagen fibril thickness, possibly suggesting changes to tension in the system. Gene expression revealed a dysregulated ECM, with extremely elevated expression of LOXL2, the main Collagen crosslinking enzyme. This suggests that giant larvae may be attempting to return their ECM to a balanced state by making it more resistant to remodelling. Overall, our overgrowth model presents an intriguing opportunity to examine the ability of a system to tolerate overgrowth without the metabolic inputs of obesity.

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, and rates are increasing globally (World Health Organization 2020). The most common risk factors for the development of CVD include aging, obesity, and diabetes. However, there are a wide variety of conditions that are at increased risk of developing CVD, including those with chronic kidney disease, inflammatory bowel disease, Marfan syndrome, and acromegaly. CVD is the leading cause of death in all of these conditions (Jankowski et al. 2021; Follin-Arbelet et al. 2023; Schicho, Marsche, and Storr 2015; Vanem et al. 2018; Kamenický, Maione, and Chanson 2021). In some conditions, like acromegaly, even individuals with controlled disease still have a higher risk of developing CVD (Wolters et al. 2020). This highlights the importance of studying the development and progression of cardiac dysfunction and CVD.

One of the often-overlooked components of CVD is the contribution of the cardiac extracellular matrix (ECM). In a healthy system, the ECM acts as a protein scaffold that supports tissue function (Li, Zhao, and Kong 2018). The ECM is predominantly made up of Collagens, as well as proteoglycans and glycoproteins that form a highly organized network around the tissue (Jourdan-LeSaux, Zhang, and Lindsey 2010). These proteins are adhered to one another by covalent crosslinks that may be formed either enzymatically or nonenzymatically (Cox and Erler 2011; Pehrsson et al. 2021). One of the main contributions of the ECM to the tissue overall is the regulation of biophysical properties like elasticity. This is of special importance in the heart, which must be elastic enough to contract continuously in order to maintain life, yet strong enough to support heart shape. In disease states however, dysregulation of the ECM leads to increased deposition of matrix proteins, particularly Collagens, which causes an increase in the stiffness of the heart itself. Over time this can have severe functional consequences, as Collagen is noncontractile and can also disrupt cell-cell connections and nerve impulses through the heart (Travers et al. 2016).

This pathological increase in matrix deposition is called fibrosis and is considered a hallmark of CVD due to its overwhelming prevalence in diseased heart tissue (Meschiari et al. 2017; Travers et al. 2016). An additional concern is the progressive nature of fibrosis. The presence of fibrotic deposits is enough to cause the formation of more fibrotic tissue, so overtime the issue will only

continue to compound and further compromise function (Bonnans, Chou, and Werb 2014; Pehrsson et al. 2021). Despite the importance of fibrosis clinically and its known contribution to disease prognosis it has no available treatments (Leask 2010).

Developing treatments for fibrosis necessitates an understanding of how the regulation of the ECM changes in disease states. Mammalian models have been limited in their ability to address these fundamental questions due to the complexity of the ECM (Diop and Bodmer 2012). The human matrisome comprises 4% of the total proteome, and any given ECM may be composed of 100-200 different proteins (Naba et al. 2012). The use of small animal models is therefore an attractive alternative for addressing broad concepts. The genetic model system *Drosophila melanogaster* has emerged in recent years for its utility in studying the cardiac ECM. *Drosophila* is the simplest model system that possesses a heart, the formation of its simple, tubular heart follows the same developmental pathways that govern formation of the human heart, and it has a low degree of genetic redundancy, making it possible to manipulate whole gene families (Diop and Bodmer 2012; Hughes and Jacobs 2017). It has previously been reported that an arrest of *Drosophila* larvae during their growth phase generates larvae that grow indefinitely and reach an immense body size (Zeng et al. 2020). This model presents an intriguing opportunity to examine how the heart and the cardiac ECM adapt and change in the face of cardiac overload, which is known to cause ECM remodelling (Frangogiannis 2017; Hutchinson, Stewart, and Lucchesi 2010).

Here, we report that this *Drosophila* overgrowth model (referred to hereafter as "giant larvae" or "giants") does not exhibit hallmarks characteristic of obesity. Giant larvae are significantly larger than their control counterparts, but do not have elevated triglyceride levels or increased lipid droplet size. These larvae also possess a remarkably conserved organization of the cardiac ECM protein Pericardin, a heart specific Collagen found in *Drosophila.* The heart is able to adapt to this increase in body size without visible defects in the matrix, and with minimal functional effects. Gene expression revealed significant upregulation of the matrix crosslinking enzyme LOXL2, suggesting that giants may compensate for an increase in body size by altering the biophysical properties of their ECM. This may also be indicative of fibrotic remodelling as fibrosis involves both increased protein deposition as well as increased crosslinking of the matrix.

Methods

Drosophila strains and dietary treatments

UAS-*snail-RNAi* (50003) was obtained from VDRC. UAS-*Dicer2* (BDSC 24644) was obtained from Bloomington stock centre. *phm22-GAL4* (on third chromosome) was obtained from Dr. Michael B. O'Connor. y^1w^{1118} was used for high fat diet treatments. UAS-*snail-RNAi,* UAS-*Dicer2* and *phm22-GAL4* were crossed to y^1w^{1118} for use as controls and are referred to here as UAS-*snail-RNAi* and *phm22GAL4*. UAS-*snail-RNAi* crossed to *phm22-GAL4* yields the giant phenotype, which is referred to here as either UAS-*snail-RNAi;phm22-GAL4* or giant larvae.

Flies were maintained on ordinary lab food. Ordinary lab food consists of 3.6L of water, 300g sucrose (0.2M), 150g yeast, 24g KNa tartrate, 3g dipotassium hydrogen orthobasic, 1.5g NaCl, 1.5g $CaCl_2$, 1.5g $MgCl_2$, 1.5g ferric sulfur, and 54g of agar. Fly food is autoclaved, cooled to 55^0C, then 22mL of 10% tegosept and 15mL of acid mix is added before dispensing. Giant larvae were allowed to grow for 14 days at 25^0C before being sacrificed for analysis, parental controls were taken at wandering third instar. For high fat diet treatment, 30% volume was supplemented with coconut oil and flies were maintained at room temperature.

Triglyceride assay

Triglyceride levels were measured using a serum triglyceride determination kit (Sigma Aldrich, TR0100) ((Wat et al. 2020). 5 intact third instar larvae were flash frozen in liquid nitrogen and stored at -80^0C before sample preparation. Frozen larvae were ground with a manual homogenizer in 0.1% Tween in PBS. 20µl of buffer per larva was used. Samples were heat treated at 70^0C for 10 minutes, then centrifuged at maximum speed for 3 minutes. 10µL of each sample was loaded into a 96 well plate in triplicate. 10µL of a glycerol standard at 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.315mg/mL, 0.156mg/mL, and 0mg/mL were also loaded. 250µL of free glycerol reagent was added to each well, incubated at 37^0C, and absorbance was read at 540nm. 50µL of triglyceride reagent was then added, incubated for 10 minutes at 37^0C, and absorbance read at 540nm. The change in glycerol levels after addition of the triglyceride reagent was calculated to determine the level of stored triglycerides in the sample. A Bradford assay was then conducted on the same samples and the level of stored triglycerides was divided by the amount of protein in the sample to control for body size.

Dissections

Heart:

Dissections were performed by fixing larvae dorsal down to a surface using pins (Brent, Werner, and McCabe 2009). Larvae were bathed in PBS and an incision was made at the ventral midline. The cuticle was pinned back and the gut and fat bodies were removed to reveal the heart. Control dissections were performed at third instar, after the onset of wandering behaviour. Giant larvae were dissected at day 14 post laying.

Fat body:

Above process was followed but only the gut was removed to expose the fat bodies.

Immunohistochemistry

Heart:

Dissections were fixed for 20 minutes without shaking at room temperature in 4% paraformaldehyde in PBS. Specimens were then washed 3x10 minutes in PBST (0.3% Triton-X-100), before blocking for 30 minutes with NGS (1:15). Primary antibodies were incubated overnight at 4^0C with shaking. After incubation with primary 3x10 minute washes in PBST were performed before adding secondary antibodies for one hour at room temperature. Phalloidin was added at the same time as secondary antibodies. Specimens were then washed 3x10 minutes in PBST, with a final wash in PBS to remove detergent. 50% glycerol was added for at least 3 hours, then 70% glycerol overnight. The primary antibody used was mouse anti-Prc (Pericardin, EC11, DSHB, 1:30 dilution). Secondary antibodies used were Alexa Fluor 488 anti-mouse and Alexa Fluor 647 anti-mouse (1:150 dilution). Alexa Fluor 546 and 647 Phalloidin (Thermofisher Scientific) were also used (1:75 dilution).

Fat body:

Dissections were fixed for 30 minutes at room temperature in 4% paraformaldehyde. Specimens were washed 2x5 minutes in PBST, then incubated in 493/503 BODIPY (1:1000) for 30 minutes. Specimens were then washed 2x5 minutes, placed in 70% glycerol, and immediately mounted for imaging.

Imaging

A Leica SP5 confocal microscope was used to obtain image stacks. 1µm intervals between frames were used for heart dissections, 0.5µm intervals were used for fat bodies. Fat bodies were imaged from the surface to a depth of 30µm. Hearts were imaged from the ventral face of the cardiac ECM to the dorsal edge of the heart tube. Images were processed using Leica software (LAS AF), ImageJ, and ZEN blue.

OCT imaging

Optical coherence tomography (OCT) was used to visualize the heart beating *in vivo* in real time in late third instar larvae. Larvae were adhered to a microscope slide dorsal side up before being placed under the OCT camera. B scans were taken in 3D acquisition mode using a Thorlabs OCT Telesto series TEL221PS system at the widest point of the heart chamber with the following parameters: X size 1257 pixels, 1.03mm, Y size 0, 400 frames, Z field of view 1.2mm. This gives a 20 second video with 20 frames per second. Image stacks were then exported as TIFs and processed in ImageJ (Abràmoff, Magalhaes, and Ram 2004). The cross-sectional area was measured at both diastole and systole. The difference between diastolic and systolic volumes was used as a proxy for stroke volume.

qPCR

RNA extraction and RT-qPCR

Total RNA was extracted from larvae using TRIzol (Invitrogen, 15596026). Wandering third instar larvae or 14 day old giant larvae were flash frozen in liquid nitrogen in groups of 5 (n=3). Samples were stored at -80 or left in liquid nitrogen until ready to use. Samples were then ground in 800µL TRIzol, and incubated at room temperature (RT) for 5 minutes. 80µL chloroform was then added, samples were shaken for 15 seconds, incubated at RT for 3 minutes, and spun at 12,000RPM for 15 minutes at 4^{O}C. After the spin, the supernatant was added to a gDNA eliminator column (Qiagen RNeasy plus kit, 74034) and spun at RT for 30 seconds at 10,000RPM. 600µL of 70% ethanol was added to the flow through, which was then transferred to the RNeasy spin column. This was spun for 15 seconds at 10,000RPM RT, flow through was discarded, 700µL of buffer RW1 was added, spun for 15 seconds at 10,000 RPM RT, flow through discarded, 500µL buffer RPE was added, and spun at 10,000RPM RT for 2 minutes. The

column was then placed in a fresh tube, 40µL RNase free water was added, incubated for 10 minutes, and spun for 1 minute at 10,000RPM RT. Samples were then used to make cDNA using the Applied Biosystems High-Capacity cDNA reverse transcription kit (ThermoFisher, 4368814).

RT-qPCR was performed (in triplicate) using the Bio-Rad cycler CFX 96 and the Luna universal qPCR master mix (NEB, M3003X). Gene expression levels were normalized to housekeeping genes EF1, Rpl32, and α-tubulin. Primers can be found in supplemental materials.

Quantification and statistics

Fibre alignment was quantified using using the Twombli plug-in in Fiji 2.14 (ImageJ2 ver2.9.0, http://imagej.net) (Abràmoff, Magalhães, and Ram 2004; Schindelin et al. 2012). Parameters were adjusted to detect fibres of 7-25 units and minimum branch length of 15 units. Masks were compared against original single channel confocal images. (Wershof et al. 2021).

Fibre thickness was measured using 63x images with 4x zoom in ImageJ (Abràmoff, Magalhaes, and Ram 2004). All fibres within a 15x15µm ROI were measured.

Lipid droplet diameter was measured using the line tool in ZEN 3.4 (blue edition).

Statistical analysis of larval health (mass, triglyceride levels, lipid droplet size), Pericardin fibre thickness, and OCT measurements were performed using Graphpad Prism (v.9.5.1). One-way analysis of variance (ANOVA) with a multiple comparisons test was performed. Graphs are plotted with SEM.

RT-qPCR results were analyzed using CFX Maestro 3.1 software (Bio-Rad, Canada; https://www.bio-rad.com/en-ca/product/cfx-maestro-software-for-cfx-real-time-pcr-instruments), which performed an ANOVA or a t-test depending on number of groups being compared.

Results

Giant larvae do not possess characteristics of obesity

In order to determine if giant larvae are a model for overgrowth or obesity we first examined some key phenotypes associated with obesity. Overall, larvae are larger in length, width, and mass compared to a wandering third instar (Figure 1A, Figure S1). Giant larvae are also less active than parental controls, and never initiate wandering behaviour. This was also observed previously (Zeng et al. 2020). Larval mass was significantly elevated, with giant larvae attaining a mass over twice that of controls (Figure 1B). Triglyceride (TG) levels however were significantly lower in both male and female giant larvae (Figure 1C). Elevated TG levels have previously been noted in response to high fat diet (HFD) treatments that result in obesity phenotypes (Birse et al. 2010; Guida et al. 2019, chapter 2 of this thesis). We investigated further by labelling lipid droplets (Figure 1D) within the fat body and quantifying their diameter. We find that lipid droplet diameter is reduced in both female and male giant larvae (Figure 1E). Taken together, these results suggest that this is a model for overgrowth rather than obesity.

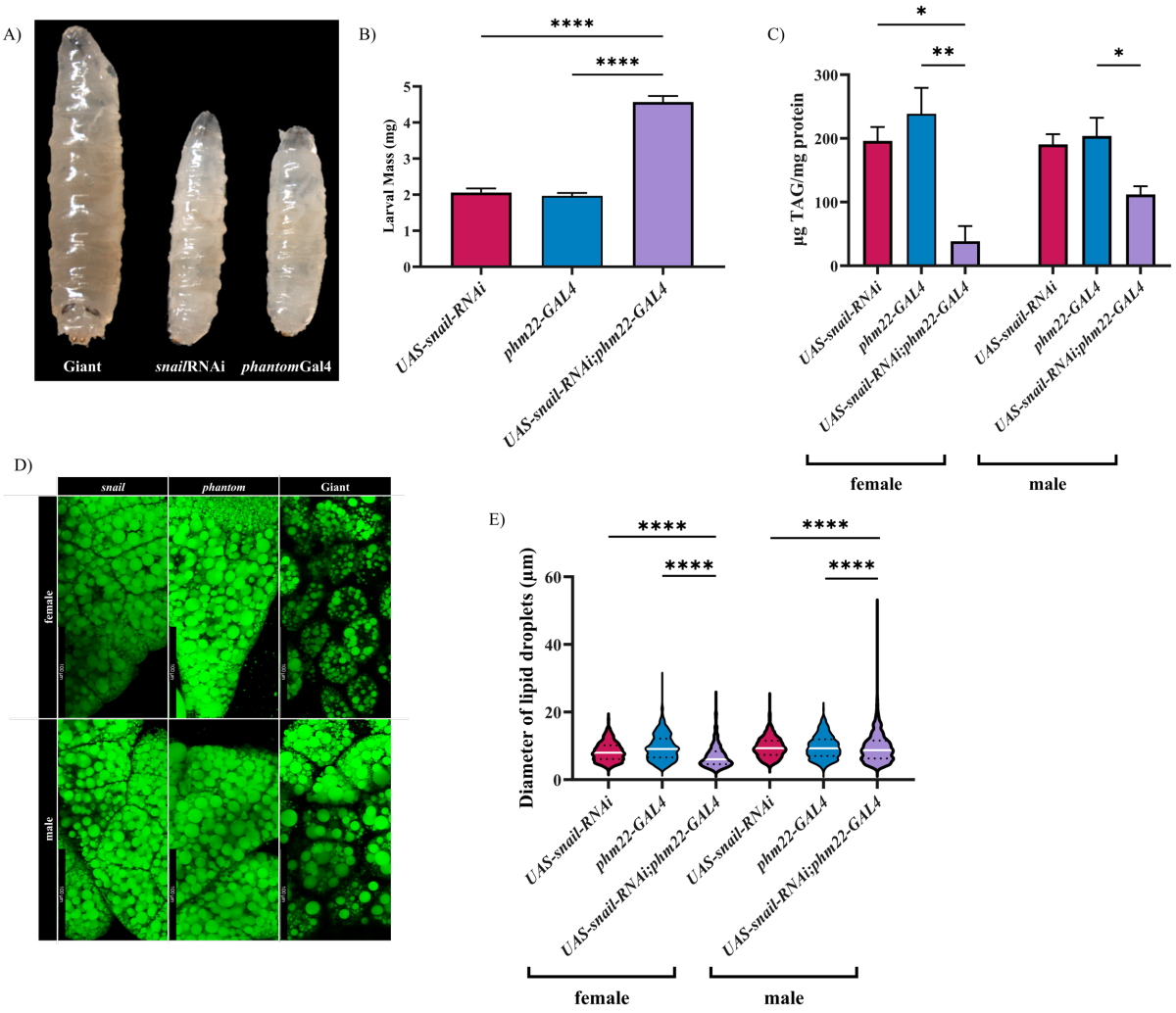

Figure 1: Giant larvae attain large sizes but do not demonstrate hallmarks of obesity
Giant larvae are substantially larger than parental controls at late third instar (A). Mass of giant larvae is significantly elevated compared to parental controls (B), but they have lower triglyceride levels (C). Fat body morphology is abnormal in giant larvae, with smaller lipid droplets contained within small sections of the fat body (D,E). Error bars in B and C are SEM. White lines in E represents the median, dotted lines represent quartiles. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 (n>15 for larval mass, n=3 for TAG, n>5 fat bodies imaged)

Giant larvae scale the cardiac ECM remarkably well despite overgrowth

We next aimed to determine how the cardiac ECM of the giant larvae adapts to the increase in body size. The cardiac ECM showed a remarkable tolerance for this overgrowth condition (Figure 2). In controls, this network has a honeycomb appearance, with fibres pulled away laterally from the heart by the alary muscles (Figure 2A-B'). The appearance of the matrix was similar in giants (Figure 2C-C'). In order to quantify the organization of the Pericardin fibrils we utilized the Fiji macro TWOMBLI to determine the degree of alignment of fibres within the matrix. Quantifying alignment of the Pericardin fibres (see methods for full description) showed no significant changes in alignment between giants and controls (Figure 3A). However, giants do possess thinner Pericardin fibrils than controls (Figure 3B). The distribution of fibril thickness in both female and male giants was skewed towards smaller fibre widths (Figure 3C, D). Fibre thickness was comparable in parental controls. This alteration in giants could be due to increased tension in the system due to a larger body size, thus stretching fibres to cover more area. Overall this reveals a truly remarkable ability of the larval *Drosophila* heart to adapt to increasing body size with minimal alterations to ECM organization.

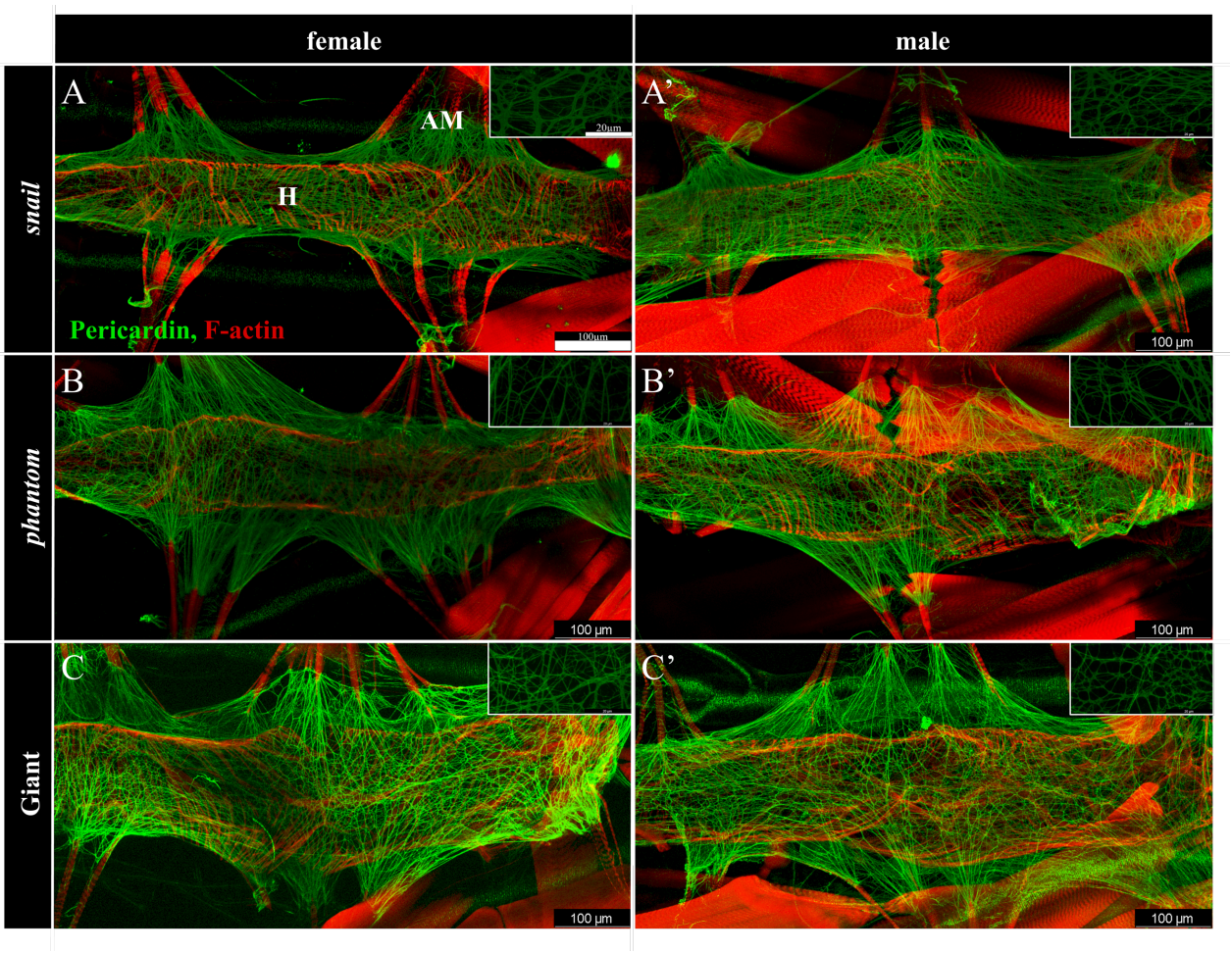

Figure 2: The organization of the cardiac ECM of giant larvae is remarkably conserved Parental controls (A&A', B&B') show the normal Pericardin network, with a honey comb organization and the matrix being pulled away from the heart tube and up the alary muscles. Giant larvae display a remarkable level of plasticity in the organization of Pericardin. Pericardin in green, F-actin labels muscles in red. All images are oriented with anterior to the left, posterior to the right. In panel A, H labels the heart tube, AM an alary muscle. Insets 63x with 4x zoom. Scale in A is 100µm, scale in A inset 20µm. n>10 for all groups.

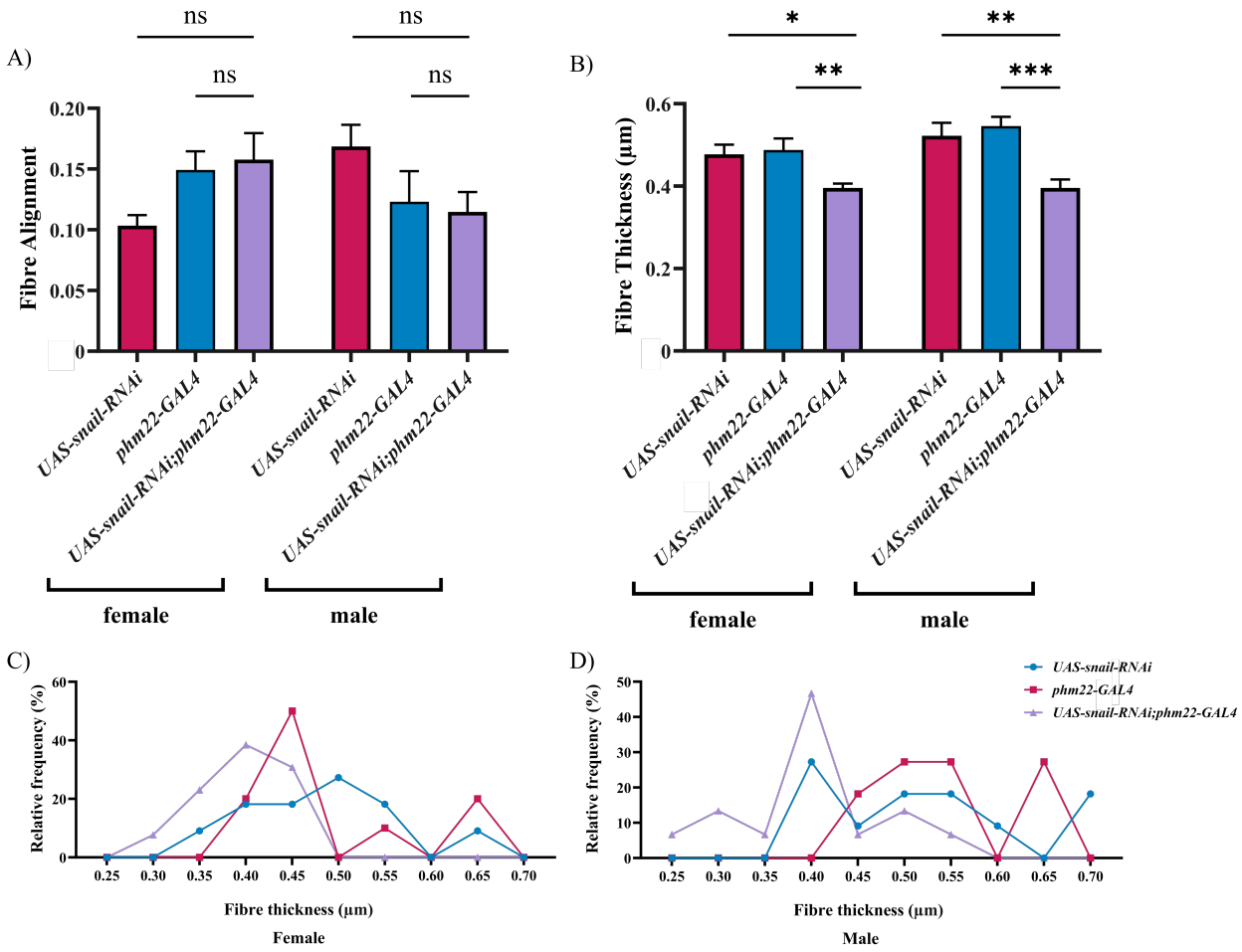

Figure 3: Pericardin matrix is not aligned differently in giant larvae but fibrils are thinner Fibre alignment scores were unchanged between controls and giants (A), but the thickness of Pericardin fibres was reduced in giants (B). The distribution of fibre thicknesses was similar in control genotypes and skewed toward thinner fibres in giants (C). Error bars in A and B are SEM. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, $n>10$ individuals for all groups.

Cardiac function does not scale with increase in body size

Live imaging of giant larval hearts was conducted using optical coherence tomography (OCT) (Figure 4A). Giant larvae were found to possess increased systolic and diastolic areas (Figure 4C). This corresponded to an increase in stroke volume (Figure 4D). Stroke volume was 1.79 fold increased in females, and 1.77 in males. However, the observed increase in mass of giant larvae is 2.22, suggesting that the increase in stroke volume may not be enough to compensate for increased body size. Additionally, the percentage of the cross-sectional area of the body cavity that the heart occupies at diastole is significantly elevated in giant larvae (Figure 4B). This suggests that the heart is growing hyperallometrically with increasing body size, but that function is not maintained, perhaps due to an inability to contract the heart as effectively at systole.

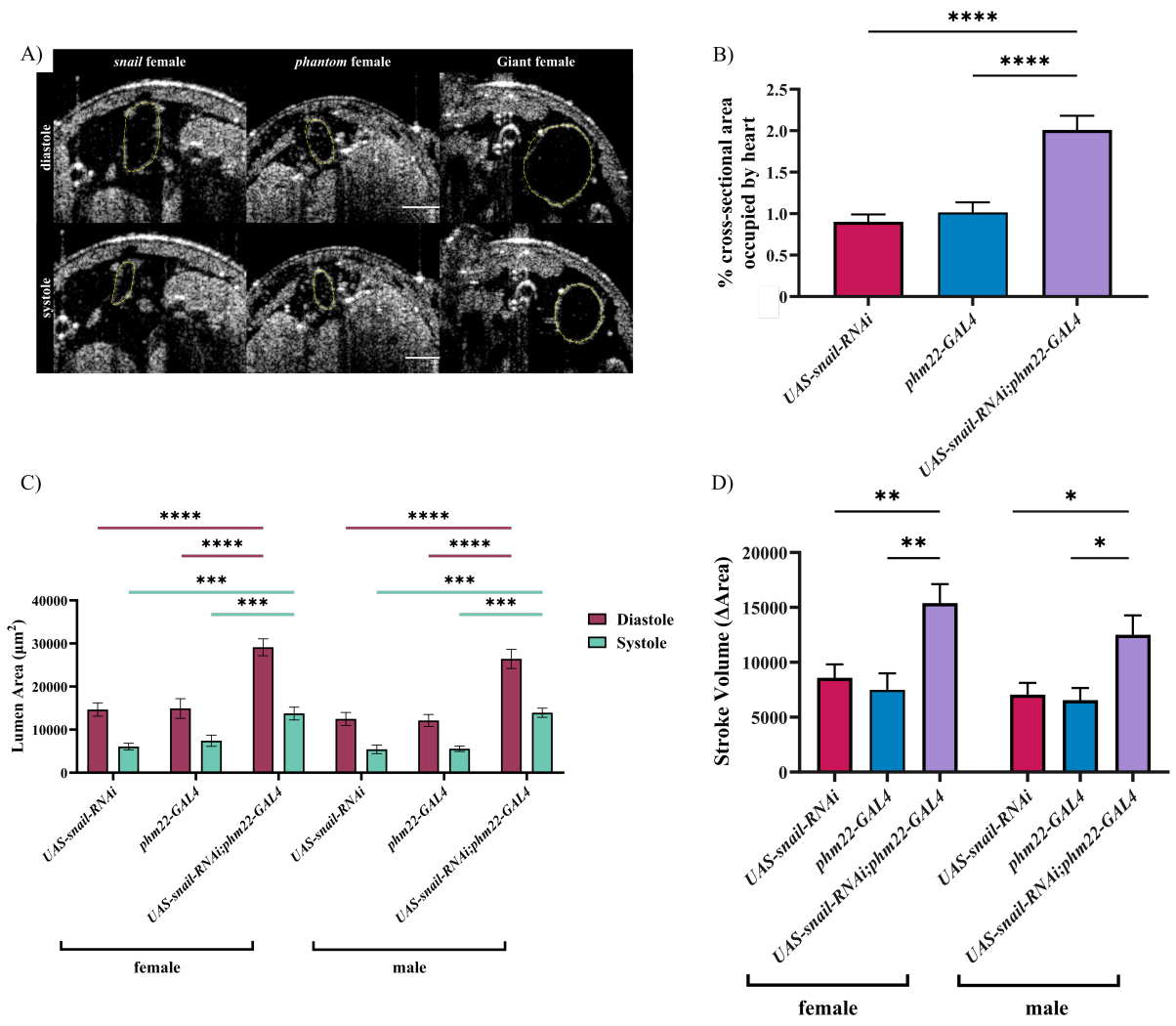

Figure 4: Functional analysis of larval hearts reveals disproportionately enlarged hearts in giant larvae

Optical coherence tomography (OCT) imaging reveals enlarged diastolic and systolic volume in giant larvae (outlined in yellow in A). The percentage of the body cavity that the heart occupies at diastole is also significantly elevated (B). Both female and male giant larvae have significantly increased diastolic and systolic volume (C). This corresponded to an increase in stroke volume (D), but this increase is smaller than the magnitude of the increase in body size. Error bars in B, C, and D are SEM. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, n>10 for all groups.

Gene expression in giant larvae is different from high fat diet treatments

Organization of the cardiac ECM was relatively conserved in giant larvae but heart growth did not increase proportionally with body size. We therefore decided to quantify gene expression of core ECM proteins, and ECM associated proteins. We also quantified expression of some genes involved in fat metabolism to determine if giants are metabolically affected by their increased body size. We compared giant larvae to wandering third instar parental controls, as well as to y^1w^{1118} high fat diet (HFD) treated wandering third instars. The parental controls revealed some variation in expression of core ECM proteins, including Pericardin, and both Collagen-IV subunits (*viking* and *cg25c*). Giant larvae tended to follow the expression pattern of one parent or the other, with the exception of giant males showing increased Pericardin expression (Figure 5A, C). Nidogen and Perlecan showed opposite expression patterns, with giant larvae upregulating *nidogen* but downregulating *perlecan*. *MMP2* and *TIMP* were both also elevated (Figure 5A, C). HFD treated individuals did not mirror these changes, exhibiting decreased *pericardin* expression, and in females increased *cg25c* (Figure 5B, D). This indicates that HFD treatments and giant larvae regulate their ECMs differently. One extremely notable finding was an enormous increase in expression of *LOXL2* in both female and male giant larvae (Figure 5A, C). Lysyl oxidase (LOX) family members are the main Collagen crosslinking enzymes. Female giant larvae showed a larger increase (~24 fold overexpression) compared to males (~8 fold overexpression). This trend was not observed in HFD treated larvae (Figure 5B,D). Overall, this suggests that ECM regulation in giant larvae is affected more severely than in HFD treatments, with opposing expression patterns for genes that perform similar roles, like Nidogen and Perlecan.

Gene expression of lipid metabolism genes *lsd-2*, *pummelig*, *seipin*, and *CG5966* revealed differences in between expression in giant larvae and HFD treatments (Figure 6). Female and male giant larvae follow similar trends, with both sexes downregulating *lsd-2*, and upregulating *pummelig*, *seipin*, and *CG5966* (Figure 6A, C). *lsd-2* is involved in preventing the mobilization of lipid stores, suggesting that giant larvae may be utilizing energy stores (Beller et al. 2010). *pummelig* mutants experience increased lipogenesis, while *CG5966* is involved in lipid catabolism. Elevated levels of these two genes suggests that lipids are being broken down for energy and that there is decreased lipogenesis in giant larvae. HFD treatments show no

differences in any of these genes in males compared to controls (Figure 6D), while females have mild downregulation of both *lsd-2* and *pummelig* (Figure 6B). *lsd-2* depletion prevents lipid storage, while *pummelig* depletion accumulates fat (Beller et al. 2010; Hehlert et al. 2019). These changes could indicate a mild alteration in the regulation of lipid storage in HFD females compared to males.

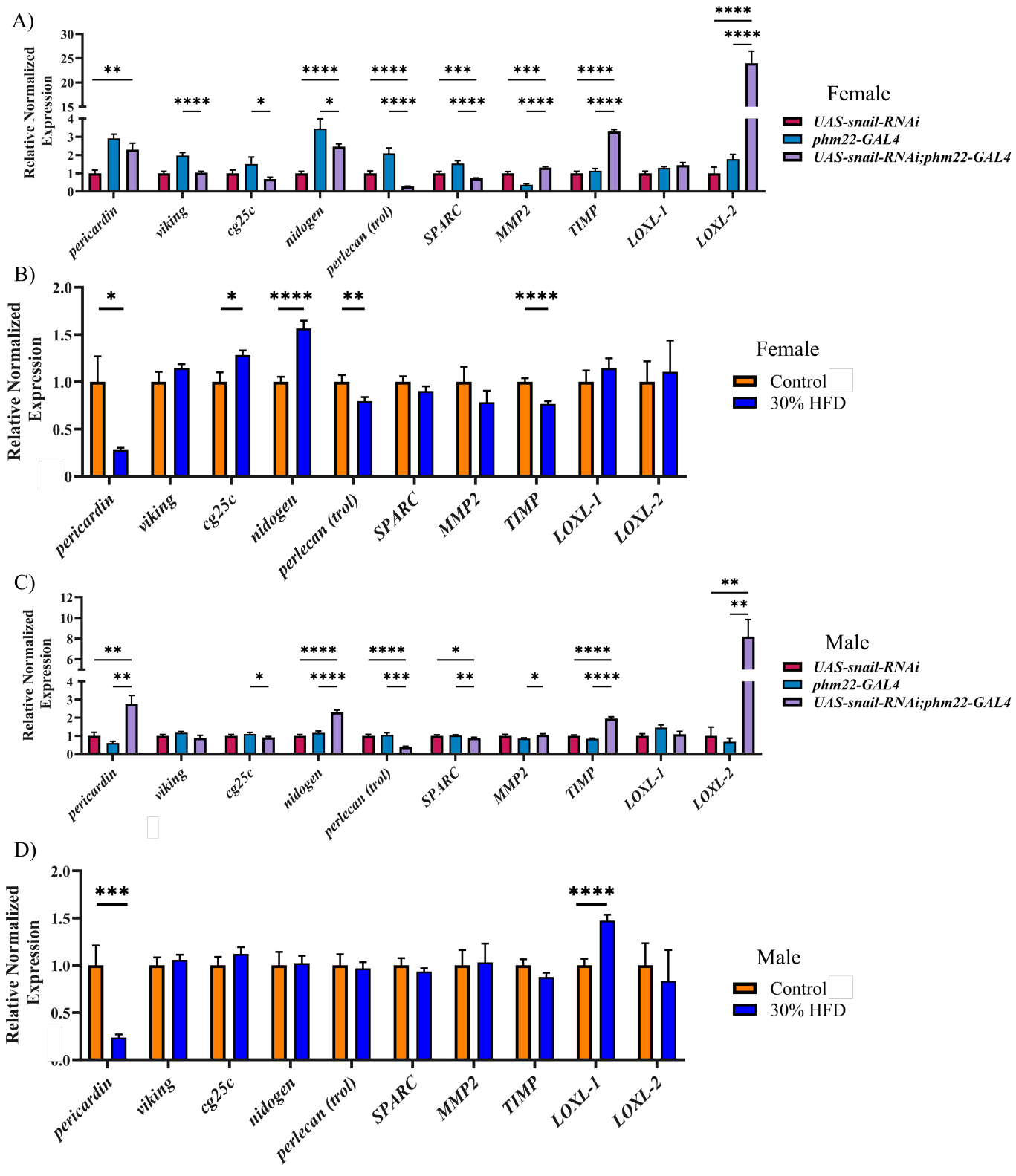
A)
Relative Normalized Expression
Female
UAS-snail-RNAi
phm22-GAL4
UAS-snail-RNAi;phm22-GAL4
pericardin
viking
cg25c
nidogen
perlecan (trol)
SPARC
MMP2
TIMP
LOXL-1
LOXL-2
B)
Relative Normalized Expression
Female
Control
30% HFD
C)
Relative Normalized Expression
Male
UAS-snail-RNAi
phm22-GAL4
UAS-snail-RNAi;phm22-GAL4
D)
Relative Normalized Expression
Male
Control
30% HFD

Figure 5: Gene expression in giant larvae shows differing pattern to high fat diet treatment
Giant larvae have many changes in gene expression of ECM components and regulators (A,C). Female giant larvae exhibit an enormous increase in expression of the crosslinking enzyme LOXL2 (A). Males have a similar increase, but also show increased Pericardin gene expression (B). This is in contrast to the high fat diet treatment where a decrease in Pericardin expression is observed in both females and males (C, D). Other ECM components are relatively unaffected in HFD treatments (B, D). Levels of LOXL2 are unchanged in HFD treatments.
Error bars are SEM. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$, n=3 biological replicates.

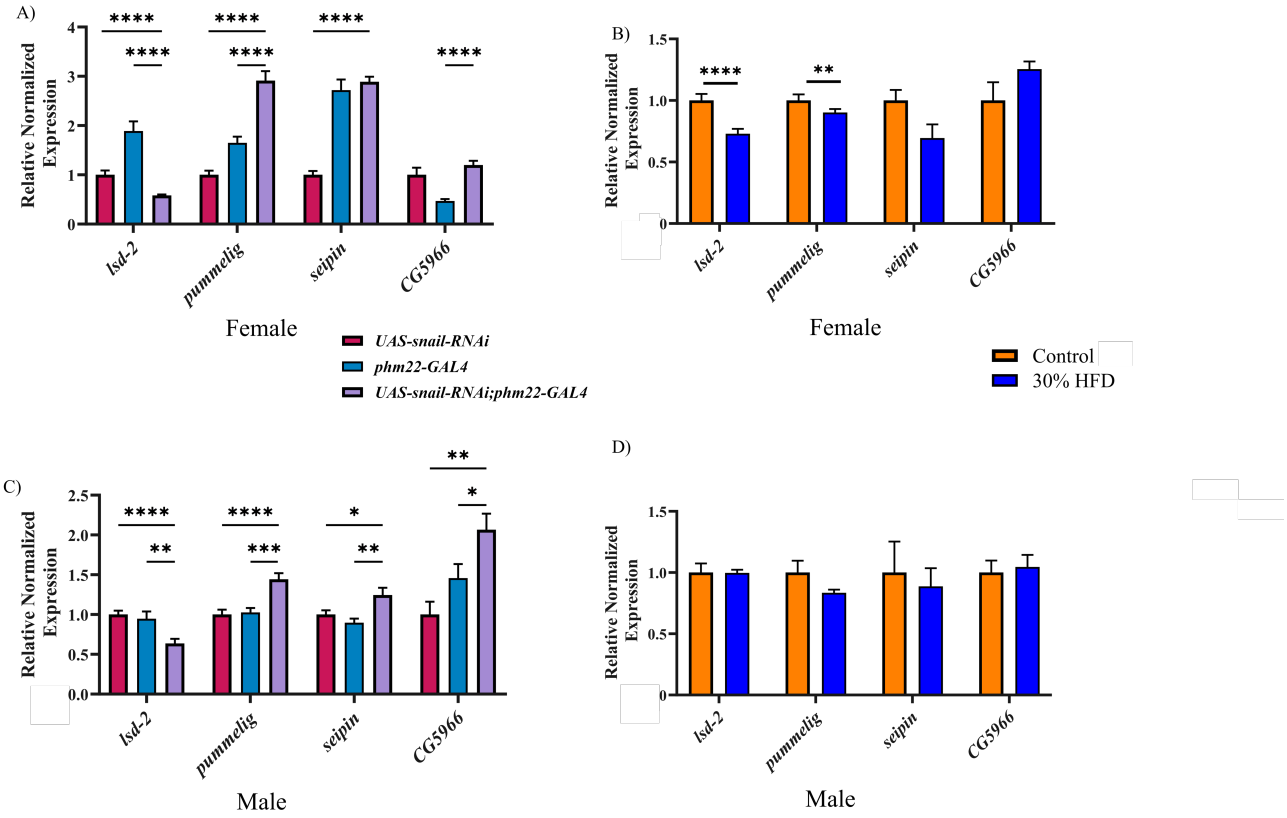

Figure 6: Giant larvae have significantly altered expression of genes involved in lipid metabolism

Giant larvae downregulate *lsd-2*, but upregulate *pummelig, seipin,* and *CG5966* (A, C). These changes in lipid metabolism are not observed in HFD treatment groups, with only small changes observed in females (B). Male HFD treatments show no differences in these genes (D). Female and male giant larvae follow similar trends. Error bars are SEM. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, n=3 biological replicates.

Discussion

Arresting *Drosophila* larvae during the growth phase of development results in larvae that are over twice the mass of a wandering third instar. Characterization of this overgrowth phenotype revealed that giant larvae grow to immense sizes without exhibiting hallmarks of obesity, including low triglyceride levels, reduced lipid droplet size, and gene expression pointing towards an increase in mobilization of stores lipids, as well as decreased lipogenesis (Beller et al. 2010; Hehlert et al. 2019). This is in contrast to previous studies that have demonstrated an obesity phenotype in response to high fat diet treatments (Birse et al. 2010; Guida et al. 2019, chapter 2 of this thesis). This indicates that giant larvae attain their size by overgrowth, not weight gain. This presents an opportunity to examine how body size can affect the heart and other organ systems in the absence of metabolic effects, which are typically present in obesity. Cardiac overload is known to cause fibrotic remodelling, making giant larvae a useful model for the study of overload in the absence of obesity (Frangogiannis 2017; Hutchinson, Stewart, and Lucchesi 2010). Additionally, acromegaly, a condition characterized by excessive growth in humans, is known to cause cardiovascular disease (Sharma et al. 2017; Wolters et al. 2020). Cardiovascular disease is the leading cause of death in patients with acromegaly, even if they have controlled disease (Wolters et al. 2020). Therefore, having a genetic model system in which to examine adaptations made by the heart to increased body size will be informative for humans with overgrowth conditions.

Amazingly, the organization of the cardiac ECM was largely unaffected by the overgrowth of giant larvae. Despite supporting a body over twice the size, the morphology of the heart was unchanged. The one notable difference was a decrease in Pericardin fibre thickness in both female and male giants. This indicates that there may be increased tension in the heart tissue that is stretching the matrix. Functional analysis further supports the presence of altered biophysical characteristics as heart dimensions and functional parameters do not scale appropriately with increasing body size. The heart was found to be disproportionately enlarged, with both diastolic and systolic dimensions increased. The heart itself also occupies a larger percentage of the cross-sectional area of the body at diastole than controls. The function of the heart is also mildly affected, with an increase in stroke volume of a smaller magnitude than the increase in body size. This functional deficit in the presence of a disproportionately enlarged heart could suggest that

matrix tension or stiffness is increased, preventing the heart from contracting effectively at systole. Overall, this data suggests that the heart has reached its maximum physiological capability and is beginning to stretch to accommodate increasing body size. While the heart is able to adapt remarkably well to this overgrowth condition, it is perhaps approaching a maximum limit.

Gene expression of important ECM components as well as ECM regulators reveals opposing expression patterns of genes with similar roles. For example, Nidogen and Perlecan are both known to play a role in stabilizing the basement membrane (Grigorian et al. 2013; Sasse et al. 2008; Wolfstetter et al. 2019; Dai et al. 2018), but Nidogen expression was upregulated and Perlecan was downregulated. Additionally, MMP2 expression was upregulated but TIMP was also upregulated. MMP2 is responsible for breakdown of the ECM during remodelling, and TIMP is the inhibitor of MMP2. Taken together, this suggests that turnover of the matrix is dysregulated in this system. Overall, gene expression reveals an ECM with severely affected remodelling compared to HFD treatments, which show comparatively minor changes in gene expression. This may explain why LOXL2 is so elevated in giant larvae. LOXL2 is responsible for matrix crosslinking, which insolubilizes the matrix and makes it more resistant to degradation (Meschiari et al. 2017; Sivakumar et al. 2008). It is possible that giant larvae are attempting to compensate for significantly altered ECM remodelling by creating a matrix that is more resistant to degradation and remodelling.

Here we have described a model for overgrowth that is free from confounding hallmarks of obesity. The *Drosophila* larva, an organism that grows 5 times in size from hatching to pupation, is able to scale its cardiac morphology well with increasing body size, even when taken beyond normal limits. The functional consequences are relatively minimal. This remarkable model presents a fascinating opportunity to determine how an organism can scale its physiology to accommodate overgrowth, which could provide insights into how interventions can be applied in a human context to improve health outcomes. The hormonally-triggered overgrowth condition acromegaly leads to increased risk factors for several diseases, even when the hormone imbalance responsible is being kept under control by treatment protocols (Wolters et al. 2020).

By studying how this *Drosophila* model is able to accommodate overgrowth so well it may illuminate novel treatment avenues for acromegaly.

Giant larvae could also be used to provide insight into how organs and body systems other than the heart are able to scale but still retain functionality. For example, the nervous system is known to be affected by metabolic syndromes like diabetes and obesity. In obesity, peripheral neuropathy is known to occur even in the absence of hyperglycemia and is associated with lower nerve density in the extremities (Callaghan et al. 2020; Lim et al. 2022). Metabolic contributors are assumed to play the predominant role in the development of obesity-associated peripheral neuropathy but there is the possibility that increased body size is enough to contribute to peripheral nervous system dysfunction. Giant larvae present an ideal model for examining this possibility as they attain a large body size without the metabolic challenges associated with obesity. Overall, there are several fascinating avenues for follow up that would shed light on how these larvae are able to scale organ size effectively that could be applied to a variety of conditions in humans.

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Supplemental methods

Primers used:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
EF1	GCGTGGGTTTGTGATCAGTT	GATCTTCTCCTTGCCCATCC
Rpl32	ATGCTAAGCTGTCGCACAAATG	GTTCGATCCGTAACCGATGT
α-tubulin	TGTCGCGTGTGAAACACTTC	AGCAGGCGTTTCCAATCTG
pericardin	GCAAGCGCAAATGGAGCTG	CCCTCGAATAGCCTCTGCC
cg25c	GATCGCGGGAGCGTTAGTC	TCACGGAGTCCTGAATCGAAC
viking	TCTAAGGCATCTCTCGGGTCT	CTTTGCAGTCGCATAGCGTTG
nidogen	ATCCATATCCTGAGGAGCAGAT	GGTGCAGGTGTAGCCAT
perlecan (trol)	CGCCGATAGTAATGATCGCAG	ACCCTAATGTTGGGAATCTCCA
SPARC	CCAGGCCTCTACGGAGTTTT	AGGTCGAGGTCCTCATCCAG
MMP2	GAAATCGGCTCCAATGTGCG	GCTCCACGTAAGATCCGTTCTG
TIMP	GAGTCCTTCGCAAATCGGATAC	GCTTCGGATGTAGCCTTGTAGG
LOXL-1	GTCTGCGCAGCCCACGGAAA	TACCGGAAGGTGGCCCAGGG
LOXL-2	CATTCACATGGCAGATGCGG	GAATCCCACTCGTCATCGCA
lsd-2	AGTCTGGCTGTCAACGGAGT	ATTGGATAGCCGTCCAACTG
pummelig	CGCAGTACATACACCAGTGC	CGCTGCGACTTGATCTTCTC
seipin	CCCGTTCACATGCAGTTCAA	GCCAACCATCAGGAGTTGC
CG5966	TCTTTCGAGAGCTTTAAGGACA	AGGGCTTGCTATCTCCAGTC

Supplemental figures

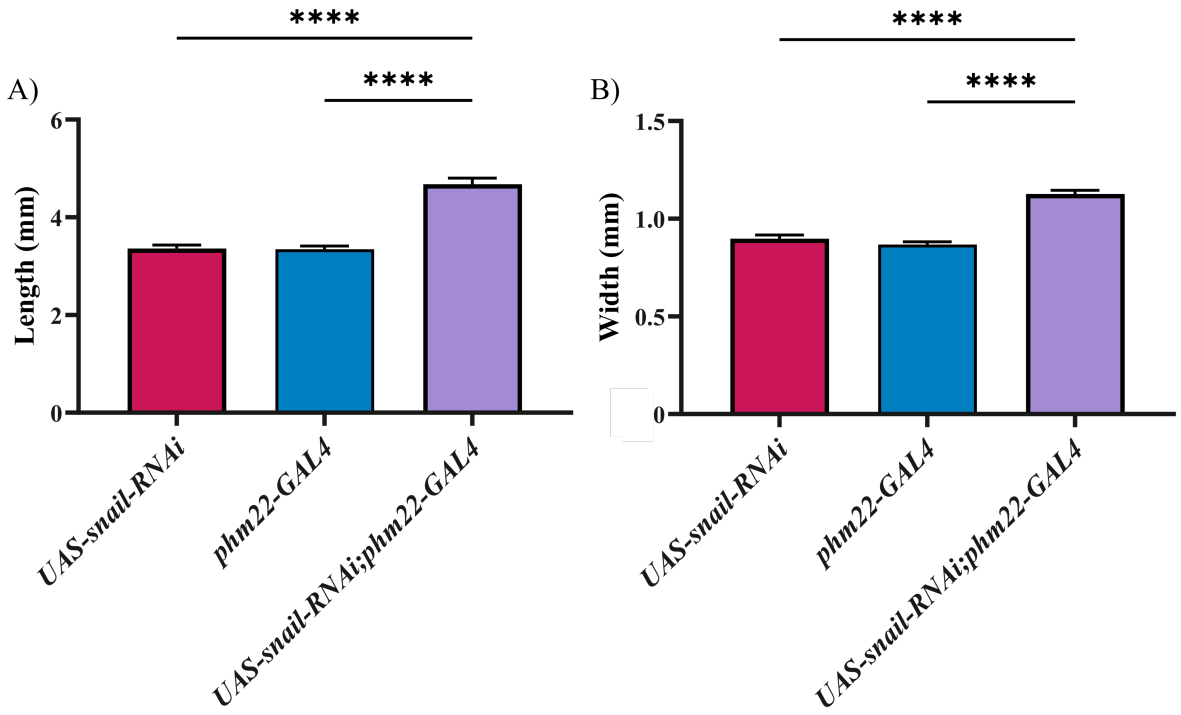

Figure S1: Giant larvae have significantly increased body measurements
Giant larvae have significantly increased body length (A) as well as width (B). Error bars are SEM. ****=p<0.0001

Chapter 4: Crosslinking by LOX maintains the extracellular matrix during tissue overgrowth

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Abstract

Many disease states are characterized by altered deposition and crosslinking of extracellular matrix (ECM) proteins. This condition is called fibrosis and has no available treatment options. Crosslinking levels have been shown to play a critical role in the function of various organs, including the heart, lungs, and skeletal muscle. This makes crosslinking enzymes an attractive target for the treatment of various diseases with a contribution of the ECM. However, results of studies that target these enzymes are mixed in their ability to resolve symptoms. Here, we utilize the *Drosophila* larva to examine the importance of crosslinking to the cardiac ECM and heart function. We also use a previously described overgrowth model with extremely elevated crosslinking levels to determine if matrix crosslinking is what allows their remarkable ability to scale the heart with increased body size. We find that LOX inhibition causes sex-specific changes in matrix organization in *yw* larvae, and that this correlates with changes to the functional parameters of the heart. LOX inhibition in a background with elevated LOX expression was able to ameliorate some of the organizational and functional changes associated with elevated levels of crosslinking. Measurements of tissue elasticity supported these results. Overall, we find that LOX plays a role in matrix organization and heart function but that it may not be as important in the *Drosophila* heart as in mammalian systems.

Introduction

The heart is a vital organ and must beat continuously throughout the life of an organism. This is uniquely demanding of a muscle and the heart is made up of highly specialized components in order to perform this role. Contractility is controlled by the cardiomyocytes, which are also specialized for high, sustained production of ATP, and for appropriate conduction of electrical impulses through the tissue (Guo and Pu 2020). There is also a non-cellular support structure that is critical for maintaining a balance of elasticity and tissue structure in the heart (Fan et al. 2012; Frangogiannis 2017). This protein scaffold is called the extracellular matrix (ECM). ECMs are found surrounding all tissues and the cardiac ECM is so vital for heart function that it is implicated in essentially all cases of cardiovascular disease (CVD) (Bonnans, Chou, and Werb 2014; Travers et al. 2016). In disease states, excessive remodelling of the ECM occurs, leading to increased protein deposition. This condition is termed fibrosis, is present in a wide variety of diseases, and is considered a hallmark of cardiovascular disease (Travers et al. 2016). Increased levels of ECM proteins cause increased stiffness of the tissue, which in the heart compromises organ contractility (Fan et al. 2012; González-Santamaría et al. 2016). The presence of fibrotic remodelling is enough to trigger additional remodelling, making fibrosis a progressive condition (Bonnans, Chou, and Werb 2014; Pehrsson et al. 2021). Despite its importance to the development and progression of CVD there are no available treatments for fibrosis.

An additional component of fibrotic remodelling is the presence of increased levels of crosslinking between matrix proteins. Fibrosis is often considered to be mainly an issue of protein deposition but levels of key collagen crosslinking enzymes are also known to be elevated in disease states (González-Santamaría et al. 2016; López et al. 2009). Increased levels of crosslinking can lead to an increase in tissue stiffness without an increase in protein levels. Crosslinking of matrix proteins also increases their resistance to degradation, perpetuating the effects of fibrotic remodelling (Song et al. 2021).

Crosslinking of the ECM can be enzymatic or non-enzymatic. Enzymatic crosslinking occurs after Collagen fibrils are assembled into the matrix (Pehrsson et al. 2021; Song et al. 2021). The main Collagen crosslinking enzymes are LOX family members. LOX and LOX-like (LOXL) proteins catalyze the formation of crosslinks by acting on lysine residues, some of which were

hydroxylated intracellularly. LOX is then able to act on these residues once the Collagen molecules have been incorporated into a Collagen fibril. LOX acts to oxidatively deaminate both lysine and hydroxylysine residues, which facilitates the formation of crosslinks across Collagen fibrils (Pehrsson et al. 2021; Song et al. 2021; Yamauchi and Sricholpech 2012). Peroxidasin, another crosslinking enzyme, catalyzes crosslinks only between nonfibrillar Collagen-IV strands. Disrupting the formation of crosslinks destabilizes the ECM, and can disrupt the interaction between cells and the ECM (Pehrsson et al. 2021).

LOX enzymes have gathered interest as potential therapeutic targets for the treatment of fibrosis. LOX is known to be upregulated in fibrosis (González-Santamaría et al. 2016; Brashear et al. 2022). β-aminoproprionitrile (BAPN) is an irreversible chemical inhibitor of LOX and has been tested for potential anti-fibrotic effects (Brashear et al. 2022). The results of these studies have been mixed, with some reporting improved performance post-treatment and others showing no improvement in levels of fibrosis (González-Santamaría et al. 2016; Brashear et al. 2022; Bondareva et al. 2009). In order to determine if BAPN or inhibition of LOX is an effective therapeutic we need to understand what structural and physiological factors are altered by changes in LOX activity.

To this end, we employed a *Drosophila* larval model to examine the importance of crosslinking to cardiac ECM organization, elasticity, and heart function. We raised *yw* larvae on food containing either 5mM or 10mM BAPN from hatching to assess the impact of chronic crosslinking inhibition on the *Drosophila* heart. BAPN has been shown previously to inhibit both of the *Drosophila* LOXL genes, LOXL-1 and LOXL-2 (Molnar et al. 2005; Kim et al. 2014).

We have shown previously that locking *Drosophila* larvae in the late third instar stage of development results in enormously overgrown larvae (hereafter called giant larvae) that possess remarkably well-scaled hearts and cardiac ECMs (chapter 3 of this thesis). These larvae were also found to have extremely elevated levels of LOXL-2. This raises the possibility that increased crosslinking levels allow giant larvae to accommodate this overgrowth. If this is the case, inhibition of crosslinking by BAPN may reveal defects in heart overgrowth in this model.

We therefore employed BAPN feeding in giant larvae in order to reveal whether crosslinking levels are controlling their ability to adapt to increased body size.

Here, we report that inhibition of LOX-catalyzed crosslinking by BAPN alters fibril thickness and organization of Pericardin, a heart specific, fibre-forming Collagen in *Drosophila.* Female *yw* larvae experience an increase in fibre thickness with treatments, while male larvae reveal changes in Pericardin fibre alignment. Both sexes experience reduced diastolic diameters and reduced stroke volume, suggesting the ECM rearrangement has functional consequences.

Giant larvae were previously found to have reduced fibre thickness (chapter 3 of this thesis) and BAPN treatment restored Pericardin fibrils to a normal width in both females and males. BAPN treatment also led to improvement in the functional properties of the heart, with treated male giants experiencing a significant improvement in contraction at systole. Females showed a similar trend.

In order to generate a more complete picture of the biophysical effects of crosslinking inhibition on the cardiac ECM, we developed a novel swelling assay to assess elasticity. We find our assay is able to detect differences in elasticity between different groups, and that inhibition of crosslinking in giant larvae resulted in improved matrix elasticity. Overall, our results suggest that LOX-catalyzed crosslinking plays a role in maintaining the organization of the cardiac ECM, but that the functional effects observed here are likely due to a combination of matrix disorganization and altered elasticity.

Methods

Drosophila strains and dietary treatments

y^1w^{1118} was used as a control for these experiments. UAS-*snail-RNAi* (50003) was obtained from VDRC. UAS-*Dicer2* (BDSC 24644) was obtained from Bloomington stock centre. *phm22-GAL4* (on third chromosome) was obtained from Dr. Michael B. O'Connor. All treatments were performed at 25^0C.

All treatments were supplements made to ordinary lab food. Ordinary lab food consists of 3.6L of water, 300g sucrose (0.2M), 150g yeast, 24g KNa tartrate, 3g dipotassium hydrogen orthobasic, 1.5g NaCl, 1.5g $CaCl_2$, 1.5g $MgCl_2$, 1.5g ferric sulfur, and 54g of agar. Fly food is autoclaved, cooled to 55^0C, then 22mL of 10% tegosept and 15mL of acid mix is added before dispensing. BAPN supplementation was added at the same time as tegosept and acid mix, to a final concentration of 5mM or 10mM.

Dissections

Dissections to expose the heart were performed by fixing larvae dorsal down to a surface using pins (Brent, Werner, and McCabe 2009). Larvae were bathed in PBS and an incision was made at the ventral midline. The cuticle was pinned back and the gut and fat bodies were removed to reveal the heart. Dissections for controls were performed at third instar, after the onset of wandering behaviour. Dissections for giant larvae were performed at day 14 post laying.

Immunohistochemistry

Dissections were fixed for 20 minutes without shaking at room temperature in 4% paraformaldehyde in PBS. Specimens were then washed 3x10 minutes in PBST, before blocking for 30 minutes with NGS (1:15). Primary antibodies were incubated overnight at 4^0C with shaking. After incubation with primary 3x10 minute washes in PBST were performed before adding secondary antibodies for one hour at room temperature. Phalloidin was added at the same time as secondary antibodies. Specimens were then washed 3x10 minutes in PBST, with a final wash in PBS to remove detergent. 50% glycerol was added for at least 3 hours, then 70% glycerol overnight. The primary antibody used was mouse anti-Prc (Pericardin, EC11, DSHB, 1:30 dilution). The secondary antibody used was Alexa Fluor 488 anti-mouse (1:150 dilution). Alexa Fluor 647 Phalloidin (Thermofisher Scientific) was also used (1:75 dilution).

Imaging

A Leica SP5 confocal microscope was used to obtain image stacks. 1µm intervals between frames were used for heart dissections. Hearts were imaged from the ventral face of the cardiac ECM to the dorsal edge of the heart tube. Images were acquired at 20x to visualize the entire heart, 63x for use for fibre alignment analysis, and 63x with 4x zoom for analysis of Pericardin fibre thickness. Images were processed using Leica software (LAS AF) and ImageJ (Abràmoff, Magalhães, and Ram 2004).

OCT imaging

Optical coherence tomography (OCT) was used to visualize the heart beating *in vivo* in real time in late third instar larvae for controls and day 14 post-laying for giant larvae. Larvae were adhered to a microscope slide dorsal side up before being placed under the OCT camera. B scans were taken in 3D acquisition mode using a Thorlabs OCT Telesto series TEL221PS system at the widest point of the heart chamber with the following parameters: X size 1257 pixels, 1.03mm, Y size 0, 400 frames, Z field of view 1.2mm. This gives a 20 second video with 20 frames per second. Image stacks were then exported as TIFs and processed in ImageJ (Abràmoff, Magalhães, and Ram 2004). The cross-sectional area was measured at both diastole and systole. The difference between diastolic and systolic volumes was used as a proxy for stroke volume.

Quantification and statistics

Fibre alignment was quantified using using the Twombli plug-in in Fiji 2.14 (ImageJ2 ver2.9.0, http://imagej.net) (Abràmoff, Magalhães, and Ram 2004; Schindelin et al. 2012). Parameters were adjusted to detect fibres of 7-25 units and minimum branch length of 15 units. Masks were compared against original single channel confocal images (Wershof et al. 2021).

Fibre thickness was measured using 63x images with 4x zoom in ImageJ (Abràmoff, Magalhães, and Ram 2004). All fibres within a 15x15µm ROI were measured.

Statistical analysis of Pericardin fibre thickness and alignment, OCT measurements, and swelling comparisons were performed using Graphpad Prism (v.9.5.1). One-way analysis of variance (ANOVA) with a Dunnett's multiple comparisons test was performed for most analyses. An unpaired t-test was performed for before and after width comparison and *yw* to giant time comparison in swelling assay. Graphs are plotted with SEM.

All image analysis was performed on unadjusted confocal images. For publication only brightness and colour balance were adjusted using Photoshop CS6.

Swelling assay

Larvae were dissected to expose the heart as described previously (Brent, Werner, and McCabe 2009). Dissections were performed in muscle relaxing buffer (Xiao, Schöck, and González-Morales 2017). Relaxing buffer consists of 20mM phosphate buffer (stock solution 1M, pH 7), 5mM $MgCl_2$, 5mM EGTA, 5mM ATP, 5mM DTT, and 5 mini protease inhibitor tablets (Millipore Sigma, 11836153001), to a total volume of 50mL with distilled water. The solution was stored at -20^{0}C. When dissections were complete, larvae were left in relaxing buffer for 5 minutes. Relaxing buffer was then removed and distilled water (with food colouring for contrast) was added. A video recording was made of the heart over the first 2 minutes after addition of the water using a Leica M165 FC dissecting microscope and LAS V4.3 software. The initial size of the heart, the maximum size the heart swelled to, and the time to the maximum size were then measured from the video.

Results

The cardiac ECM reveals sex-specific organizational defects due to LOX inhibition

Inhibition of LOX family function was carried out by rearing larvae from hatching on food containing 5mM or 10mM BAPN. The heart specific Collagen Pericardin was immunolabelled to assess cardiac ECM organization. Control larvae (Figure 1A, A') possess a Pericardin network that is organized in a honeycomb-like pattern, with the matrix extending up the alary muscles, away from the heart tube. In 5mM and 10mM BAPN treated *yw* larvae (Figure 1B-C') the Pericardin matrix appears to have less space between fibrils, with some individuals revealing an aligned, anterior-posterior organization of Pericardin fibrils (inset of Figure 1C'). Quantifying Pericardin fibre thickness revealed an increase in thickness in BAPN treated female larvae, but not in males (Figure 2A). Fibre alignment scores were also calculated, with a score of 0 meaning organization was completely random and 1 indicating perfect alignment. BAPN treated male larvae demonstrated an increase in alignment compared to controls (Figure 2B). Females showed a similar trend, but it was not statistically significant (Figure 2B).

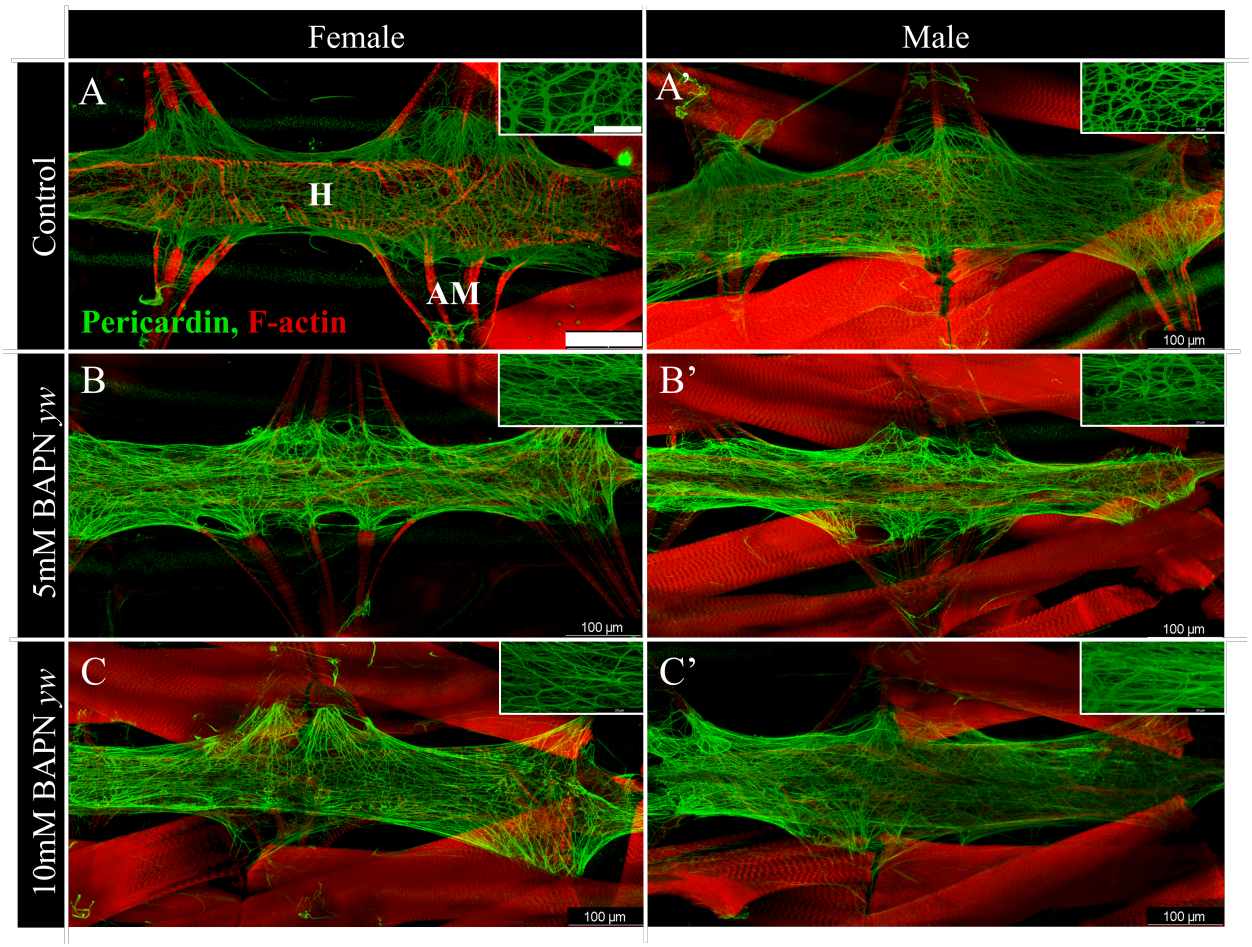

Figure 1: The cardiac ECM in BAPN fed *yw* larvae reveals reorganization
Control larvae display a cardiac ECM that is organized in a honey-comb pattern, and extends away from the heart tube up the alary muscles (A, A'). In 5mM BAPN fed *yw* larvae, the fibres appear to collapse in and the heart tube itself is thinner than in controls (B, B'). In 10mM BAPN fed *yw* larvae the matrix organization is further affected (C, C'), with some individuals displaying an anterior-posterior linear alignment of fibrils (inset of C'). Pericardin, a heart specific Collagen, is labelled in green, F-actin labels muscles in red. The red label behind the heart is body wall muscles. In panel A, H labels the heart tube, AM labels an alary muscle. The scale bar in A is 100µm, the scale bar in the inset is 20µm. In all images anterior is to the left, posterior to the right. n>10 for all groups.

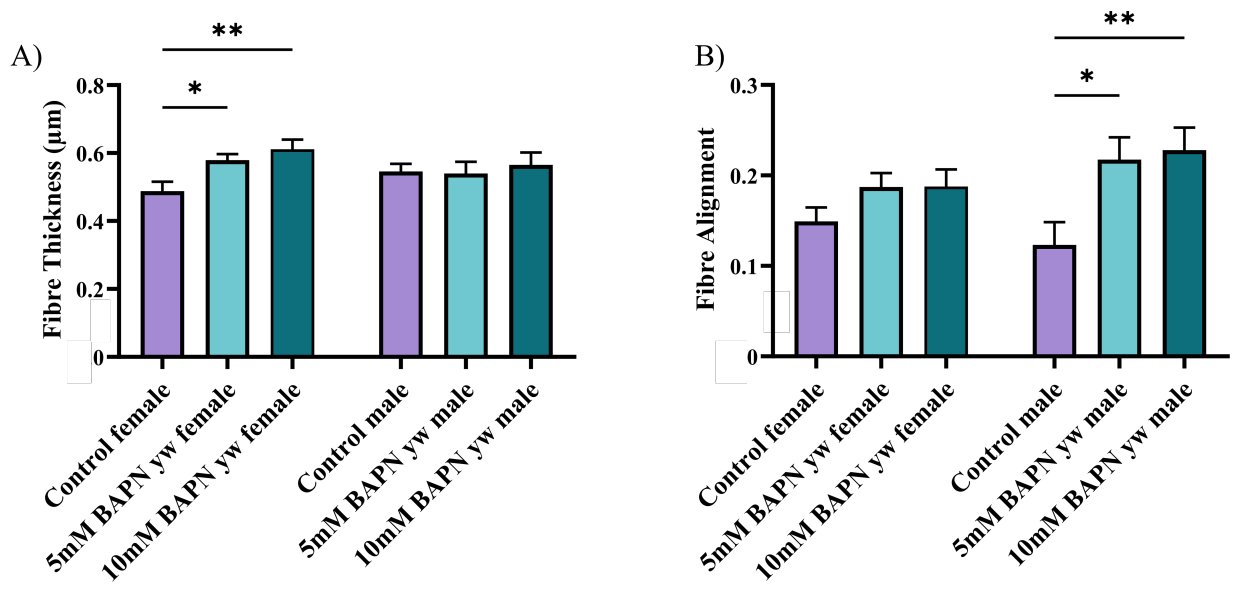

Figure 2: Female and male larvae show different matrix alterations with BAPN treatment Thickness of Pericardin fibres (A) is increased in *yw* female larvae treated with BAPN. Fibre alignment scores reveal a more aligned matrix in male larvae treated with BAPN (B). Female larvae show a similar trend but it is not statistically significant (B). Error bars are SEM. *=$p<0.05$, **=$p<0.01$, $n>10$ for all groups.

BAPN treated *yw* larvae possess smaller hearts than controls

Live imaging using optical coherence tomography (OCT) was used to visualize beating hearts in cross-section (Figure 3A). BAPN treatments correlated with increased incidence of cardiovascular defects, including fibrillation (hearts that are unable to contract fully at systole), and hearts that did not contract or expand evenly around their entire perimeter (Figure 3B). Cross-sectional area of the lumen was taken at both diastole and systole and revealed BAPN treated individuals have reduced diastolic area (Figure 3C). This trend was similar in both 5mM and 10mM groups, and was significant for both female and male larvae. The change in area between diastole and systole was used as a proxy for stroke volume and revealed significantly reduced stroke volume in all treatment groups (Figure 3D).

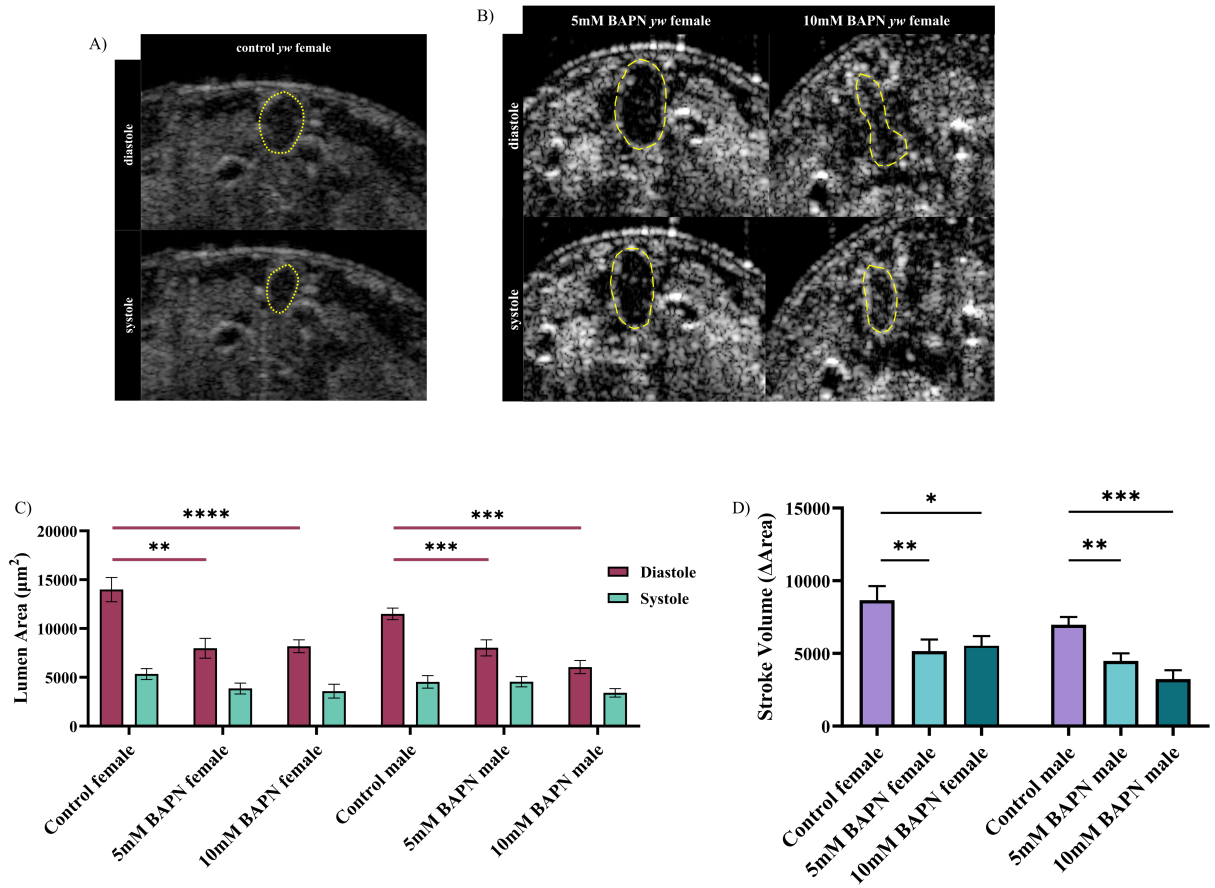

Figure 3: Live imaging reveals abnormal hearts and decreased diastolic diameter with BAPN treatment

Live imaging by OCT was used to measure the cross-sectional area of the heart at diastole and systole (A). BAPN treated larvae often had fibrillating hearts (5mM in B), or hearts that did not contract evenly around the perimeter (10mM in B). Diastolic area was significantly reduced in all BAPN treatment groups (C). Stroke volume was also significantly reduced in all treatment groups (D). Error bars are SEM. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$, $n>10$ for all groups.

Giant larval matrix characteristics are mildly perturbed by LOX inhibition

The Pericardin matrix of giant larvae scales well with overgrowth as shown previously (Figure 4A, A'). Inhibition of LOX by BAPN did not produce any major defects in the matrix (Figure 4B-C'). However, fibre thickness was increased in all BAPN treated groups (Figure 5A). It was shown previously that giant larvae have decreased fibre thickness compared to controls, here we show that BAPN treatment restored this trait. Fibre alignment analysis revealed a trend towards a more random matrix organization in BAPN treated giant larvae, with the exception of 10mM treated male giants (Figure 5B).

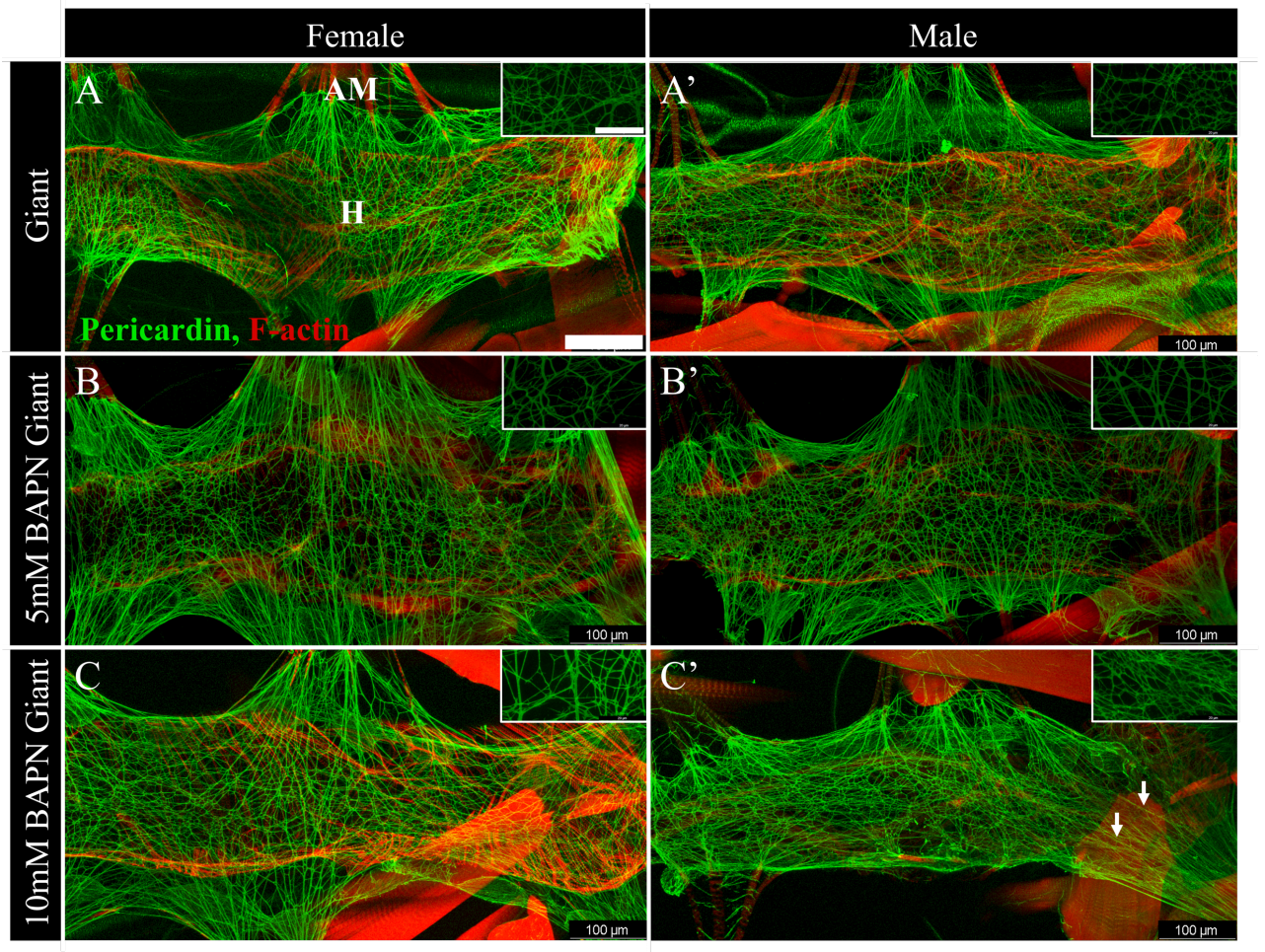

Figure 4: The cardiac ECM of BAPN treated giant larvae does not have major organizational defects

Giant larvae have conserved matrix organization, with a honey-comb appearance extending away from the heart tube (A, A'). 5mM BAPN treated giants do not exhibit any major defects in matrix organization, but appear to have increased cardiac dimensions (B, B'). 10mM BAPN treated female giants do not have any obvious defects in Pericardin matrix organization (C). 10mM BAPN treatment in male giants causes an increased level of fibre alignment, with parallel fibres highlighted by white arrows (C'). Pericardin is labelled in green, F-actin labels muscles in red. In panel A, H labels the heart tube, AM labels an alary muscle. The scale bar in A is 100µm, the scale bar in the inset is 20µm. In all images anterior is to the left, posterior to the right.

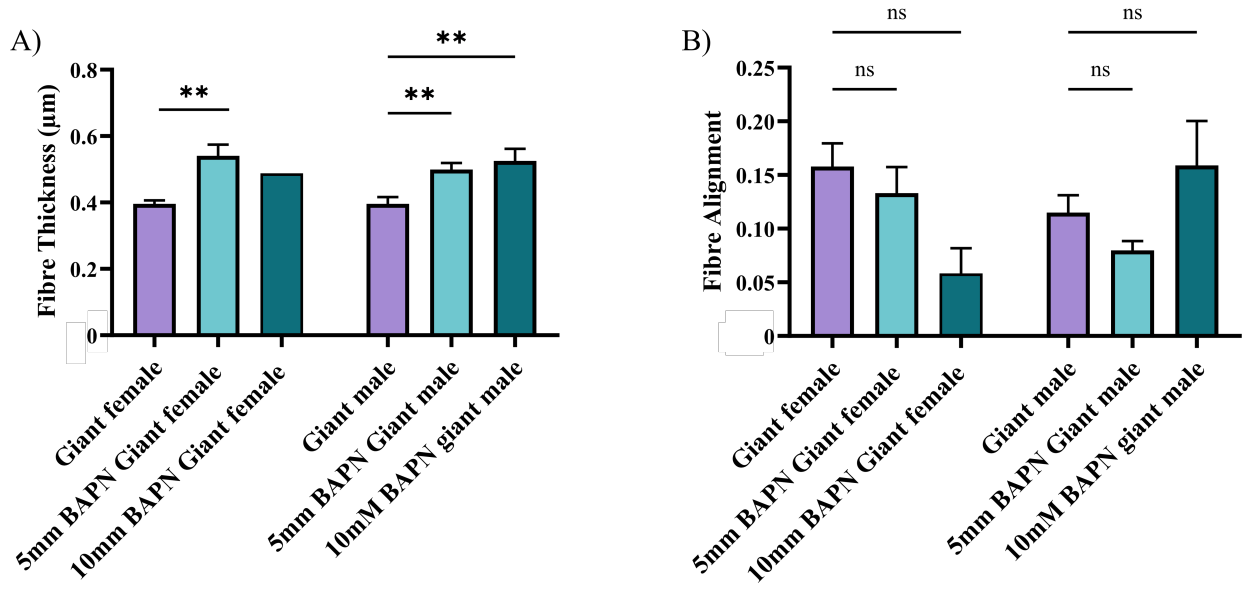

Figure 5: BAPN treatment restores Pericardin fibril thickness in giant larvae
Pericardin fibres were previously found to have reduced width in giant larvae. BAPN treatment restores fibre thickness is restored to wildtype levels in both female and male giant larvae (A). With the exception of 10mM BAPN treated male giant larvae, fibre alignment reveals a trend toward a more random matrix organization in female and male giants (B). Error bars are SEM. **=$p<0.01$

BAPN treated male giant larvae have improved systolic function

OCT imaging did not reveal any obvious defects in heart morphology in BAPN treated giants (Figure 6A, B). Intriguingly, 5mM BAPN treated male larvae had significantly reduced systolic volume compared to untreated giants (Figure 6C). Our previous work has shown an increase in stroke volume that is not proportional to the increase in body size of giant larvae (chapter 3 of this thesis). The reduction in systolic volume observed here may indicate an improvement in cardiac functional parameters. This improved ability to contract at systole showed a trend towards improved stroke volume in male giants but it was not statistically significant (Figure 6D).

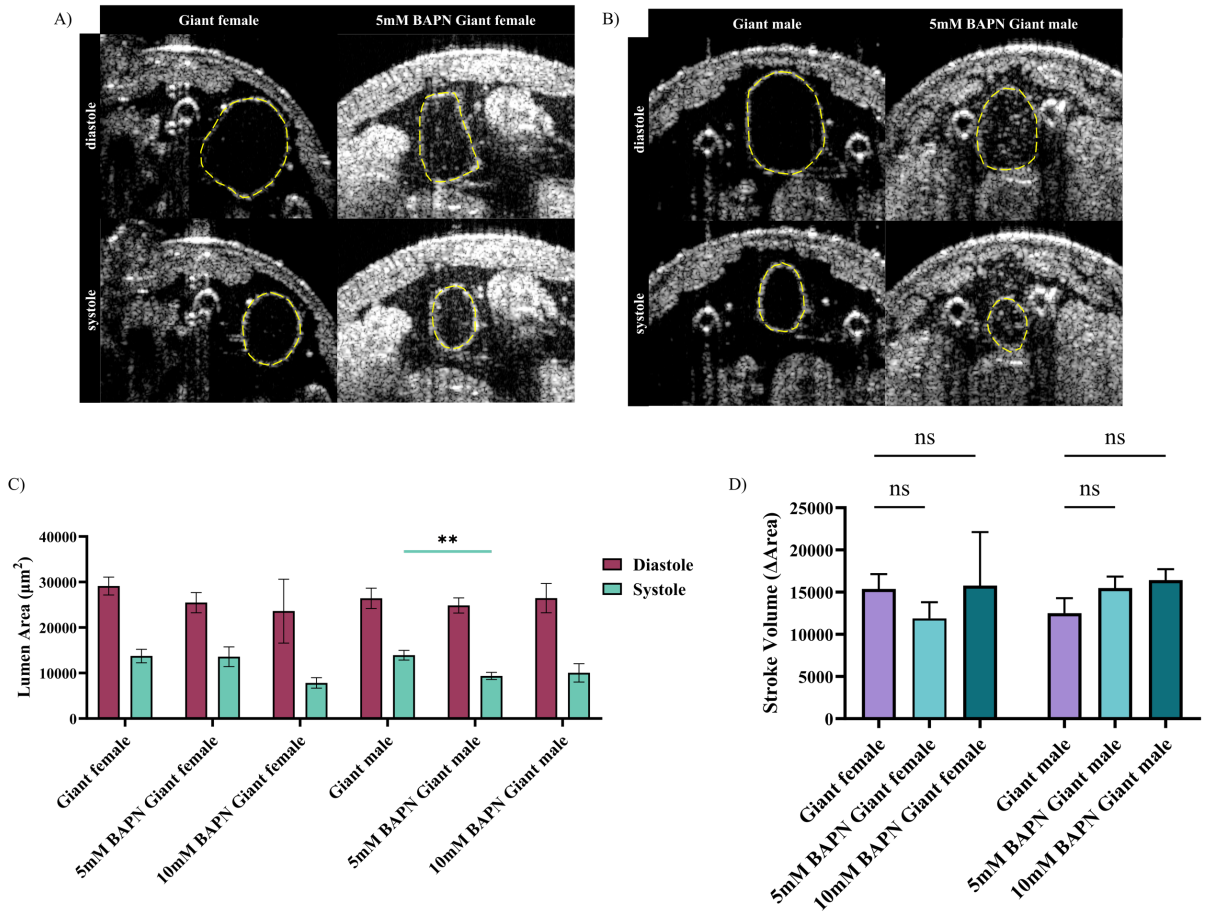

Figure 6: Live imaging reveals improved contractility in male giants treated with BAPN
OCT imaging (A, B) was used to visualize the cross-sectional area of the heart at both diastole and systole. Giant larvae treated with BAPN trend towards improved cardiac performance, with 5mM BAPN treated males revealing significantly decreased systolic volume (C). There is also a trend toward increased stroke volume in BAPN treated giant larvae (D). Error bars are SEM. **=$p<0.01$

A novel swelling assay detects changes in elasticity of the cardiac ECM

To further probe the biophysical characteristics of the cardiac ECM, we developed a novel swelling assay to assess tissue elasticity. In this assay, we bathe the larva in muscle relaxing buffer to remove the contribution of the cardiomyocytes to heart tension. After this treatment, a hypotonic solution (distilled water) is added and the change in the width of the heart can be measured over time (Figure 7A, B). When no muscle relaxing buffer is used, hearts do not swell (data not shown), indicating our relaxation protocol functions appropriately. Controls reveal significantly increased widths in *yw* larvae after completion of the assay (Figure 7C), indicating that the elasticity of the matrix can be measured indirectly by this method. The fold change of the width of the heart over time reaches a plateau within the first minute after adding the hypotonic solution (Figure 7D, representative individual). Using this assay, we measured the elasticity of the heart in both *yw* and giant larvae and found that giant larvae take significantly more time to swell to their maximum width than *yw* (Figure 7E). Inhibition of LOX by BAPN treatment in *yw* larvae does not alter time to maximum width (Figure 7F), but a 5mM treatment does reduce time to maximum width in giant larvae (Figure 7G).

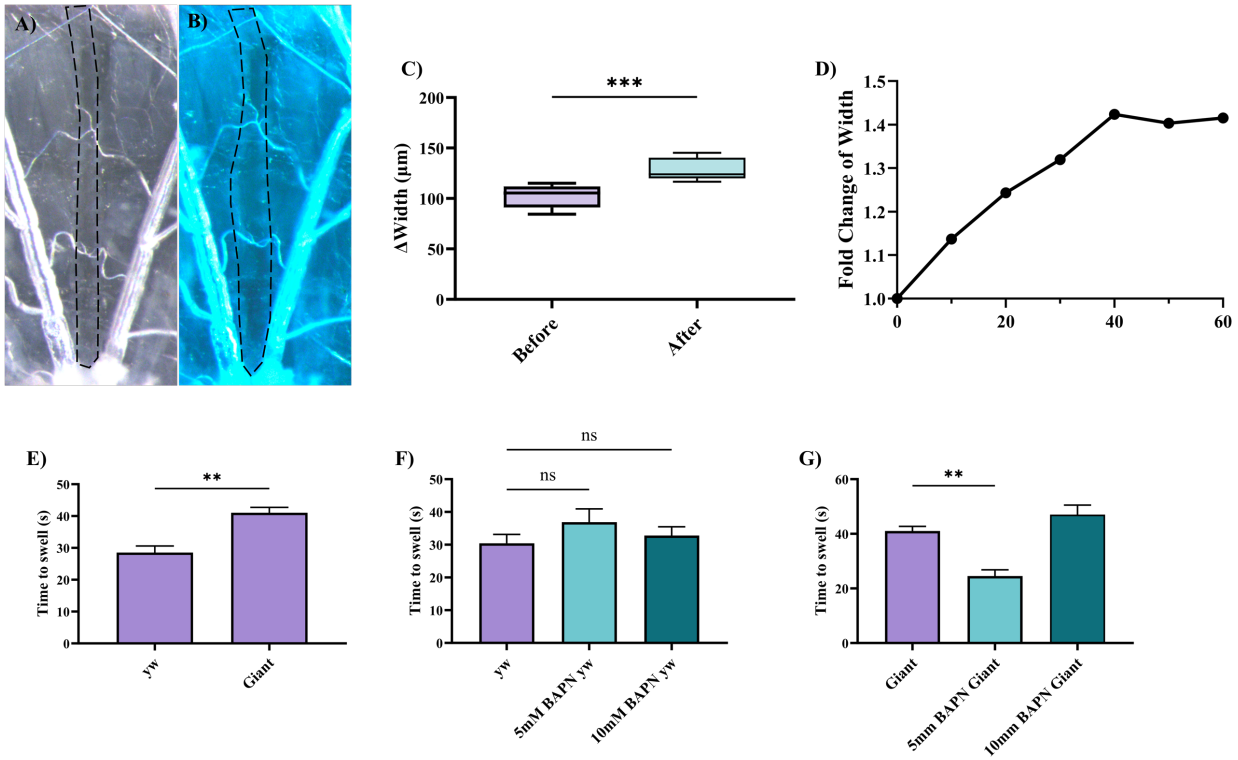

Figure 7: A novel swelling assay detects changes in elasticity in giant larvae that can be ameliorated by 5mM BAPN treatment

Hearts exposed to a hypotonic solution after a relaxation protocol are able to swell (A before, B after). The change in width of the heart of *yw* controls after swelling is significant (C). The fold change in width plateaus within 60 seconds of hypotonic solution addition (D, shows a representative individual). Giant larvae take significantly longer to reach the maximum fold change compared to *yw* larvae (E). BAPN inhibition does not alter the time to reach maximum heart width in *yw* larvae (F), but the 5mM treatment does reduce the time to reach maximum in giant larvae. All swelling experiments shown here were performed on female larvae. Hearts in panels A and B are outlined in black. Error bars in C, E, F, and G are SEM. **=$p<0.01$, ***=$p<0.001$, $n\geq4$ for all groups.

Discussion

LOX inhibition by BAPN dosing was found to have sex-specific effects on Pericardin organization in *yw* larvae that correlated with functional defects observed in these groups. Female larvae experienced an increase in Pericardin fibril thickness, while male larvae had more aligned matrix fibrils compared to controls. Female larvae did show a similar trend to male larvae but the magnitude was lower and the effect was not statistically significant. This suggests that male larvae may be more sensitive to changes in crosslinking levels. Functional analysis revealed heart defects, with BAPN treated larvae showing fibrillation and uneven contractions, neither of which are observed in controls. This suggests that despite the organizational changes to the cardiac ECM appearing relatively minor, the biophysical properties of the tissue have been affected to a greater degree than what can be observed with the naked eye. Changes in elasticity cannot be observed, which has led to this metric being overlooked in many studies.

Our previous study (see chapter 3 of this thesis) discovered that a *Drosophila* overgrowth model had extremely elevated expression levels of LOXL2. This model also scales its cardiac morphology exceptionally well despite accommodating a body size over twice that of controls. To determine if altered crosslinking levels were part of the compensatory mechanism being employed by giant larvae, we inhibited LOX using BAPN in these larvae as well. Crosslinking inhibition was able to restore fibre thickness in giant larvae to control levels, suggesting that some of the tension leading to stretched fibrils was eliminated. Additionally, male giants demonstrated improved systolic parameters, suggesting their hearts were able to contract more effectively following inhibition of crosslinking. This may indicate that contraction defects were due to an increase in matrix stiffness. Female giants showed a similar trend, but to a lesser extent. This again suggests that male larvae are more sensitive to the inhibition of crosslinking compared to females. In giants, this may be due to the significantly higher expression of LOX in female giants (25 fold overexpression) compared to male giants (9 fold overexpression) (chapter 3 of this thesis). Inhibition of LOX by BAPN in *Drosophila* is never able to eliminate 100% of LOX activity (Molnar et al. 2005), so the activity level present in female giants may still be enough to have a physiological effect.

The results from both of our models suggest that the effect of crosslinking inhibition may be on biophysical characteristics of the cardiac ECM, like elasticity. In order to measure elasticity,

traditional assays such as atomic force microscopy (AFM) require placing a probe in direct contact with the surface of the tissue. However, AFM was developed to be used at a nano scale and the *Drosophila* heart exists on a micro scale, which forces the device to the limits of its capabilities, and is not adaptable to *in situ* tissue preparations like those required here (Andrews 2017). Previous studies have used an alternative approach to measuring the strength of the ECM in organs like the *Drosophila* ovary that can be observed easily *in vitro* and lack the tension input of musculature (Crest et al. 2017). This assay utilizes a hypotonic solution and measures time to bursting of the ovary to determine matrix strength. We adapted this approach to measure elasticity of the cardiac ECM indirectly by submerging the heart in a hypotonic solution and quantifying the degree of swelling that occurs. To adapt this protocol to the more complex heart, we first used a muscle relaxing buffer to neutralize the contribution of cardiac muscle to the elasticity of the heart tube. In controls, this results in a significant increase in the width of the heart within one minute of addition of the hypotonic solution. Giant larvae were found to take significantly more time than controls to reach their maximum width, and this could be reversed by treatment with 5mM BAPN. This indicates that our assay is sensitive enough to detect differences in elasticity between different groups, and that the increased expression of LOXL-2 in giant larvae has an effect on the biophysical properties of their cardiac ECM.

Our results suggest that LOX plays a role in regulating biophysical characteristics of the heart like elasticity, but has a surprisingly minor role in regulating matrix organization. LOX null mice exhibit lethal cardiovascular complications (Mäki et al. 2002; 2005), indicating a major role of LOX in establishing a functional heart in mammals. Larvae in this study show some changes to matrix organization and function, but not at the same level as complications observed in mammalian systems. A previous study in *Drosophila* found that hydroxylation of Pericardin is not required for its synthesis or incorporation into the cardiac ECM (Wilmes et al. 2018). This suggests that hydroxylysine is dispensable for generation of the Pericardin matrix. LOX can catalyze crosslinks on either hydroxylysine or lysine residues, but the oxidized hydroxylysine is more reactive and forms crosslinks between Collagen molecules more effectively (Trackman 2016). Pericardin crosslinking by LOX may therefore predominantly occur on lysine residues, which do not form crosslinks as readily. Therefore, Pericardin crosslinking by LOX may not be the predominant method for stabilizing the cardiac ECM in *Drosophila.*

This raises the question of what compensatory role other crosslinking enzymes are playing in the *Drosophila* heart. Peroxidasin is an enzyme that catalyzes specific crosslinks between the NC1 domains of Collagen-IV molecules (Pehrsson et al. 2021). The cardiac ECM of *Drosophila* contains both Collagen-IV and Pericardin, a Collagen-IV like protein that contains an NC1 domain (Chartier et al. 2002). It is therefore possible that the Pericardin matrix has additional stabilization from Peroxidasin mediated crosslinks, which prevent severe matrix defects in our treated larvae. Peroxidasin does not stabilize fibrillar Collagens, which play an important structural role in mammalian hearts (Pehrsson et al. 2021; Jourdan-LeSaux, Zhang, and Lindsey 2010). This could explain the difference we observe between the importance of LOX-mediated crosslinking in mammals and *Drosophila.* It would be intriguing to extend the results of this study to examine the role of Peroxidasin in maintaining the organization of both the Pericardin and Collagen-IV matrices to determine if this enzyme functions redundantly with LOX in *Drosophila.*

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5. Conclusions and next steps

5.1 Major findings

Overall, this thesis demonstrates the relationship between the cardiac ECM, and the maintenance of appropriate cardiac structure and function. The complexity of this relationship is also revealed by the variety of differences observed in the matrix due to different stressors. The effect of a HFD treatment was markedly different than that of overgrowth, which reinforces the idea that regulation of ECM turnover and organization is complex. This may necessitate targeting treatment regimes to each specific case of ECM dysregulation and fibrosis. As shown here, matrix alterations and their functional consequences vary with the cause of defect. In human cases of fibrosis it may therefore be necessary to investigate the gene expression that is behind aberrant remodelling and develop treatments that address specific gene expression profiles. The ability to treat fibrosis in order to improve health outcomes will therefore rely on foundational research performed in model systems like *Drosophila melanogaster,* where the complexity of this system can be untangled more simply than in mammalian models.

This thesis advances our knowledge of how diet can affect the cardiac ECM, how overgrowth can be accommodated in specific systems, and the importance of crosslinking by Lysyl oxidase to overall matrix dynamics. The non-protein contributions to the matrix have historically been overlooked due to difficulties in measuring tissue characteristics like tension and elasticity. However, these factors are some of the most important for determining overall cardiac functionality. The ability to examine the relationship between gene expression, protein organization, and matrix biophysics and how these factors respond to environmental stimuli will allow for a much broader understanding of the role of the ECM in cardiac function, and how we can intervene in cases of disease.

5.2 A larval obesity model demonstrates the importance of acknowledging the contribution of genotype to organism response to manipulations

High fat diet (HFD) treatments were found to induce obesity phenotypes, with different genotypes revealing an optimal supplementation modality. Previous studies have reported most consistent obesity phenotypes with 30% HFD supplementation (Diop, Birse, and Bodmer 2017; Guida et al. 2019). While this was true of *yw* larvae, a chronic HFD of greater than 20% fat supplementation was not tolerated well by the *vkg-GFP* strain. Quantifying triglyceride levels in these larvae revealed an elevated baseline compared to *yw* or Oregon R strains. This suggests that basal triglyceride level varies significantly with genotype, which may affect the optimal feeding regime of a specific genotype. Previous studies have primarily focused on transient HFD feeding in adults. Future studies on larval *Drosophila* being treated with a chronic HFD will require strain-specific determination of optimal dietary dosage.

This thesis demonstrates the first time a chronic HFD has been administered to *Drosophila* larvae. Previous studies have predominantly examined the effects of transient HFD treatments in adult *Drosophila.* The larval *Drosophila* model is attractive for several reasons. HFD feeding by coconut oil supplementation leaves a greasy film on vials that adult flies are likely to stick to. This is not a concern with larvae. It is also well-documented that aging leads to a natural accumulation of ECM proteins (Hinderer and Schenke-Layland 2019; Sessions et al. 2017). Studies focusing on the ECM in adults must contend with this confounding variable. Larvae present an attractive alternative as growth and maturation are separate in *Drosophila* and larvae are strictly a growth phase of development (Rewitz, Yamanaka, and O'Connor 2013). Sex-specific studies are also more straightforward in larvae. The size dimorphism in adults has led to heart specific studies being conducted primarily on female adults as they are larger and easier to perform live imaging studies on. In larvae it is no more difficult to perform live imaging or dissections on either sex, making larvae a better model for examining sex specific differences of the heart or the cardiac ECM.

The organization of both the Pericardin and Collagen-IV matrices were perturbed by HFD feeding, suggesting a connection between lipotoxicity and ECM organization. The cardiac ECM is established during embryogenesis (Drechsler et al. 2013), so the phenotypes reported here are changes to the matrix as it grows and remodels, and not as a result of the matrix being

established improperly. Matrix organization is unable to be maintained during growth, suggesting that lipotoxicity due to a HFD is able to alter the post-translational modifications to ECM proteins.

An intriguing extension of this work would be to determine when and to what extent the matrix disorganization reported here occurs in larvae. Due to their small size, the first and second larval instars were not included in our analysis. The first and second larval instars are too small for dissection, preventing immunolabelling of the cardiac ECM. This leaves an open question of whether matrix disorganization occurs gradually with growth, if the observed defects are predominantly due to increasing larval size, and what role the molts between instar stages play in this process. The advent of new imaging techniques and endogenously fluorescent ECM proteins may allow for live imaging of these early larval stages which could help to determine more specifically when and how these changes occur.

5.3 Giant larvae are an overgrowth model without the metabolic changes associated with obesity

Locking larvae in the growth phase of development generates the giant larvae described here. These larvae reach immense sizes without exhibiting hallmarks of obesity like increased triglyceride levels and larger lipid droplets. The heart also scales remarkably well with increasing body size, with minimal defects observed in heart function or the cardiac ECM. The ability of the heart to scale and accommodate this size increase suggests that giant larvae have the potential to be a fascinating model for the tolerance of overgrowth, both as a whole organism and in other tissues and organs. Giant larvae may be an ideal model for research focused on how a system is able to scale effectively, and how the ECM is able to adapt to rigorous physiological challenges.

Obesity in humans is associated with a variety of health effects, including increased risk of cardiovascular disease, diabetes, and even some types of cancer (Jung 1997). A primary focus of studies on the interplay between obesity and the development of other health conditions is how the metabolic component plays into the development of disease. For example, conditions like peripheral neuropathy are more common in obese individuals, even in the absence of hyperglycemia (Callaghan et al. 2020; Lim et al. 2022). This is correlated with a lower neuronal

density in the extremities of these individuals, which is assumed to be due to metabolic inputs (Callaghan et al. 2020). However, it is possible that a combination of increased size as well as changes to metabolism are responsible for this difference. Evaluation of the input of body size alone on neuronal structure and density could be performed on giant larvae to determine how they are able to adapt their nervous system to increased size. This could then be compared to the observed traits in obese humans to determine if there is a change in neuronal density independent of metabolic dysfunction.

Giant larvae were also found to have extremely elevated expression of LOXL-2, one of the main Collagen crosslinking enzymes. One of the products of the reaction catalyzed by LOX family enzymes is hydrogen peroxide (Lucero et al. 2008). Oxidative stress is known to be caused by increased levels of reactive oxygen species (ROS), and mouse models have shown that increase levels of LOX correlate with an increase in ROS and oxidative stress that can be blocked by inhibition of LOX by BAPN (Martínez-Revelles et al. 2017). Giant larvae are lethargic and their cuticle appears darker as they age, which may suggest they are experiencing hypoxia. ROS levels are elevated with hypoxia as well. It is possible that the movement and colouration of giant larvae is affected by hypoxia or by increased ROS due to the increase in LOX expression. Supplementing giant larvae with oxygen could be used to determine whether they are experiencing hypoxia-induced defects or if these phenotypes can be attributed to increased activity of crosslinking enzymes.

5.4 Matrix elasticity can be measured indirectly by a novel assay and does not always correlate with observable ECM organization

The observed differences in matrix organization and heart function in HFD treatments and giant larvae led us to explore the effects of post-translational modifications on the larval *Drosophila* heart. The elevated LOX levels in giant larvae raised the intriguing question of whether overgrowth of the heart was possible due to compensation from altered crosslinking levels. We therefore sought to determine how alterations in crosslinking levels impact *yw* control larvae, as well as giant larvae themselves. We find that LOX inhibition by BAPN causes sex specific differences to Pericardin matrix organization in *yw* larvae. The functional manifestation of these organizational differences is similar, with both female and male larvae experiencing decreased

diastolic diameters that translate into reduced stroke volume. This suggests that female and male larvae may undergo differential remodelling of the ECM in response to environmental stimuli in order to achieve the same functional goal.

Giant larvae revealed similar changes to matrix characteristics between females and males, but male larvae experienced heightened responses to BAPN treatment. This corresponded with an improvement in systolic contraction in giant males treated with BAPN, suggesting that functional defects in untreated giants (stroke volume not proportional to body size) could be due to altered levels of crosslinking.

Both genotypes treated with BAPN showed defects in cardiac function that did not appear to be explained by the magnitude of the ECM organizational changes observed. Elasticity of the matrix is highly affected by LOX-mediated crosslinking but assays that have traditionally been used to measure elasticity are not amenable to all *in vivo* applications (Andrews 2017). The development of a novel swelling assay allowed indirect measurements of biophysical characteristics, when traditional methods require direct contact between probe and sample. The simple assay described here is a relatively high-throughput method to examine tissue elasticity without long wait times for protocol optimization. A similar technique has been applied previously in the *Drosophila* ovary (Crest et al. 2017). This technique functions on simple organs where the main contributor to tension or elasticity is the ECM. It also requires that the organ be removed from the body, limiting its application substantially. Our alterations will allow it to be applied to tissues with muscular input or more complex arrangements of tissue layers. This will allow for assessment of tension *in vivo,* in a variety of organs that have previously been impossible to probe. Our use of this assay demonstrates that elasticity of the matrix is affected in giant larvae, and that this defect can be ameliorated by LOX inhibition. This demonstrates that our assay is sensitive enough to detect elastic differences in tissues and reveals that matrix organization does not always predict tissue biophysical characteristics.

An intriguing future direction of this work would examine the role of Peroxidasin-mediated crosslinking on the cardiac ECM. Peroxidasin (Pxn) catalyzes the formation of sulfilimine crosslinks in the NC1 domain of Collagen-IV (Pehrsson et al. 2021). Both Collagen-IV and Pericardin possess this domain and would therefore be targets for Pxn-mediated crosslinking. These crosslinks may be able to stabilize the matrix enough to prevent catastrophic cardiac

effects, even with reduced LOX-mediated crosslinking. It is possible that the *Drosophila* cardiac ECM requires the input of both LOX and Pxn crosslinks for stability and the maintenance of function. Further examination of this relationship would be a fascinating look into the complexity of post-translational modification of Collagen that is required for stabilizing the cardiac ECM.

5.5 Concluding thoughts

This thesis has explored the connection between organization of the cardiac ECM and its relation to heart function. It has also begun to address the idea that Collagen integration into the matrix is only the first step in determining the functional characteristics of the heart. Tissue elasticity as a result of post-translational modifications to matrix proteins was found to explain defects in cardiac function that did not reflect the magnitude of the observed changes in matrix organization. Overall, this suggests that a holistic approach to assessing the response of the heart, and possibly other tissues, to matrix alterations will better describe observed functional effects. This is an exciting avenue for future study, as it will allow for improved assessment of the onset and progression of adverse ECM remodelling and fibrosis, which will eventually allow for the development of therapeutics targeted specifically to the cause of remodelling in each individual.

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Appendix 1

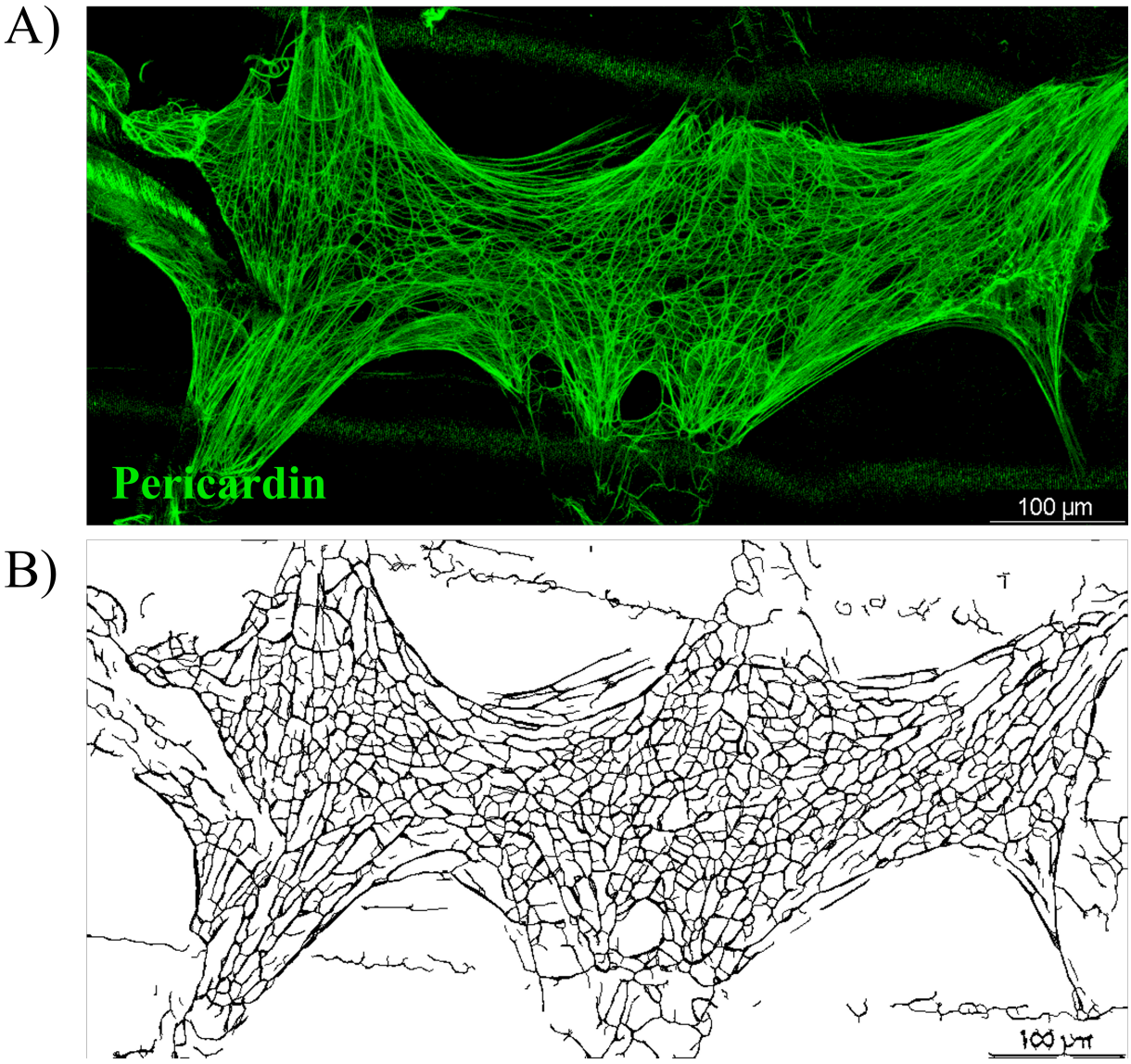

Figure A1: Mask created for fibre alignment using TWOMBLI macro
Images of the Pericardin network (A, in green) were run through the TWOMBLI workflow to generate masks (B) of the ECM for analysis of fibre alignment.