

**Seasonal temperature induced heart-collagen remodeling response in the
rainbow darter (*Etheostoma caeruleum*)**

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Acclimation to temperature changes in fish has been shown to prompt a cardiac remodeling response, with collagen protein playing a key role, although the mechanism of this response remains unclear. Currently, it is believed to be a seasonal adaptation to shifting temperatures, with studies indicating that microRNA-29b (*miR-29b*), an epigenetic non-coding RNA, targets collagen mRNA in the heart. To further explore these questions, this thesis characterizes the remodeling response in a wild population of rainbow darters (*Etheostoma caeruleum*) to examine seasonal effects in a natural environment, which moves beyond the current lab-only studies. Rainbow darter heart tissue was collected on-site at three season timepoints (Spring, Summer, Fall 2023) from the Grand River, Grand Valley, ON. Water temperature loggers were deployed to monitor the fluctuating river temperatures throughout the study. Gene expression of *miR-29b*, and the three collagen type I protein transcripts (*col1a1*, *col1a2*, & *col1a3*) was measured in heart tissue through RT-qPCR. Hearts were embedded for histological analysis to visualize heart morphology and quantify the collagen protein content through picro-sirius red staining. Additionally, western blot analysis was performed to measure collagen type I abundance for each season. Temperature loggers revealed substantial daily water temperature fluctuations, with differing fluctuation profiles dependent on season. Results from RT-qPCR revealed seasonal differences in expression of *col1a1*, and *col1a2*, and western blot revealed a season effect driving a trend in collagen type I protein differences, suggesting the presence of this remodeling response in a non-model species. Although only a sex-specific difference was found in *miR-29b* expression and no significant regression was found between *miR-29b* and any of the collagen type I mRNA, further investigation into its role in this remodeling *in vivo* will be required. The lack of a compact myocardium layer was determined from cardiac tissue sections, which previously had not been investigated in the rainbow darter and evidence of cardiac hypertrophy at

acclimatization to colder temperatures was demonstrated by higher relative heart mass compared to body mass in spring and fall, compared to summer. Understanding the impacts of temperature fluctuations and extreme weather events on local fish populations is increasingly crucial. With limited studies of cardiac collagen remodeling in natural environments, it is important that there be continued focus on bringing elements of natural seasonality into the context of lab studies to further investigate this remodeling response as seasonal plasticity and flexibility. This thesis contributes to a more comprehensive understanding of seasonal effects in a natural environment.

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List of Abbreviations

ECM – Extracellular matrix

TGF- β 1 – Transforming growth factor- β 1

MMPs - Matrix metalloproteinases

TIMPs - Tissue inhibitors of metalloproteinases

miR – microRNA

HrSI – Heart somatic index

GSI – Gonad somatic index

Chapter 1

1.1 Introduction

1.1.1 Temperature

Predicted temperature increases of $\sim 5^{\circ}\text{C}$ across the globe will increase surface water temperatures and increase daily and seasonal temperature fluctuations (IPCC, 2021; Poesch et al., 2016). As ectotherms, fish species are vulnerable to environmental temperature changes, as they are unable to produce sufficient heat internally to self-regulate body temperature and, therefore, often must conform to the temperature of their surrounding environment if they cannot seek refuge elsewhere (Fry, 1947; Johnston & Gillis, 2022; Schulte et al., 2011). Due to the close relationship between temperature and biochemical processes, significant cooling or warming will decrease or increase, respectively, biochemical reaction speeds, and the physiological impacts of these reaction speed changes are important areas of study (Hochachka & Somero, 1984). As a critical organ, the heart has been the focus of many studies to determine the impacts of climate change on fish physiology and their current abilities to overcome temperature challenges through plastic and flexible cardiac responses (Graham & Farrell, 1989; Johnson et al., 2014; Keen et al., 2016; Klaiman et al., 2011, 2014), (see (Keen et al., 2017) for a review). The heart has also been investigated as the first level of limitation for tolerance to temperature changes (Pörtner et al., 2004).

1.1.2 The Heart

Current research into climate change impacts on fish is focusing on the role of the heart in thermal adaptation and plasticity. As it has been found to be both a critical and limiting organ for thermal tolerance, understanding the mechanisms and limitations of this organ is essential to determining the effects of the changing climate on fishes. Additionally, understanding how heart plasticity overcomes acute and chronic temperature challenges will provide insight into the risks

posed by the predicted increase in thermal fluctuations. The heart's role to supply oxygen and nutrients to the body and remove carbon dioxide and waste products from cells through the circulatory system is a key reason why it acts as a limiting factor (Keen et al., 2017). Temperature has profound effects on the heart function of fish due to them being ectotherms, with temperature being considered the main abiotic factor that impacts the physiology of teleost fish (Johnston & Gillis, 2022; Jørgensen et al., 2014). The heart plays a vital role in fish physiological thermal plasticity and acclimation as it is responsible for blood circulation to the tissues of the body that will experience changing oxygen demands as temperatures change (Farrell et al., 2009; Jørgensen et al., 2014). Some fish have been shown to use different physiological strategies to overcome the effects of temperature on their bodies and preserve their normal activity levels (Anttila et al., 2014; Cortemeglia & Beitinger, 2005; Elliott & Elliott, 2010; Klaiman et al., 2011; Rodnick et al., 2004; Sundin et al., 2019). Among these fish are rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), and Atlantic salmon (*Salmo salar*), common model organisms for cardiac research (Anttila et al., 2014; Cortemeglia & Beitinger, 2005; Johnston & Gillis, 2022; Klaiman et al., 2011).

Some strategies used by these fish to preserve heart function during cold acclimation include lengthening diastolic time (heart filling time) to increase stroke volume (Shiels et al., 2002; Vornanen et al., 2005), increasing calcium (Ca^{2+}) sensitivity in the myofilament contraction machinery (Klaiman et al., 2014), and increasing overall heart size through hypertrophy (Graham & Farrell, 1989; Klaiman et al., 2011). Cardiac hypertrophy has been widely demonstrated in response to cold acclimation (Farrell, 1987; Farrell et al., 1988; Goolish, 1987; Graham & Farrell, 1989) and it is thought to help compensate for increased blood viscosity at colder temperatures, which causes more stress on the heart (Graham & Farrell, 1989). The fish myocardium can be divided into two main layers: the compact myocardium (outer layer) and the spongy myocardium (internal layer), both of

which respond differently during cold acclimation cardiac hypertrophy (Keen et al., 2016; Klaiman et al., 2011; Pieperhoff et al., 2009). Klaiman et al. (2011) and Keen et al. (2016) found that the compact myocardium reduced in size relative to the spongy myocardium during cold temperature hypertrophy. The compact layer relies on direct coronary circulation with O₂ rich blood from the gills (Farrell & Jones, 1992), whereas the spongy layer receives O₂ from the venous blood that has entered the heart (Davie & Farrell, 1991). It has been proposed that the reduced size of the compact layer relative to spongy layer during hypertrophy may be due to less reliance on coronary circulation given the higher carrying capacity of O₂ in water and reduced general metabolic activity at colder temperatures (Farrell & Clutterham, 2003). Additionally, it may allow for higher cardiac tissue compliance, which may be needed during simultaneous tissue remodeling through collagen deposition, which makes the tissue stiffer (Johnson et al., 2014; Klaiman et al., 2014). Further details on this remodeling will be discussed later. At warmer acclimations, it has been proposed that increased compact layer thickness could help with increased activity levels at those temperatures (Farrell et al., 1988) or to offset the negative effects of warm temperature on the pressure generating ability of the heart, as seen in trout above 15°C (Farrell et al., 1996; Klaiman et al., 2011). Interestingly, an estimated 2/3rds of fishes lack this compact myocardium layer (Santer, 1985), as it is often reserved for highly active, athletic fishes (Farrell, 1996). It is likely that fishes with a compact layer require the more reliable coronary circulation (Farrell et al., 2009). Recently, in a study investigating the pumpkinseed sunfish (*Lepomis gibbosus*), Procopio et al. (2024) proposed that the epicardium (outermost layer of the heart) may respond in a similar way to the compact layer during hypertrophy in fish without this compact layer. However, their findings revealed that despite cold acclimation hypertrophy, there were no significant changes in epicardium thickness (Procopio et al., 2024).

There are a few different thoughts as to why these fish compensate for temperature changes in these ways. The first is lower temperatures and slowed biochemical processes impact the generation of contractile force within the heart muscle, so the fish compensate by increasing contractile machinery to maintain the same contraction force as in their preferred temperature range (Graham & Farrell, 1989; Klaiman et al., 2011). The second is the effects of blood viscosity, as previously discussed. At colder temperatures, the fluid of the blood is more viscous, and therefore, more difficult for the heart to pump (Clark & Rodnick, 1999; Graham & Farrell, 1989; Klaiman et al., 2011). There is evidence that erythrocytes pack more closely together at colder temperatures due to the temperature impacts on the phospholipid membranes of cells, which contributes to the increase in blood viscosity (Farrell, 1984; Graham et al., 1985; Keen et al., 2017). In addition to these strategies, significant cardiac tissue remodeling with the protein collagen has the ability to alter the contractile strength of the heart, and is currently being studied to determine its benefits for maintaining proper cardiac function (Johnson et al., 2014; Johnston & Gillis, 2022; Jørgensen et al., 2014; Keen et al., 2016; Klaiman et al., 2011; Kong et al., 2014).

1.1.3 Collagen

Collagen is an abundant fibrous protein found in the extracellular matrix (ECM) that provides structure to the tissue, regulates cell adhesion, supports cell communication and migration, and directs tissue development (Frantz et al., 2010). There are several collagen types throughout the body, but one of significant interest in the fish heart is collagen type I. Collagen type I is the most abundant collagen in vertebrate connective tissues, including the fish heart (Eghbali & Weber, 1990; Frantz et al., 2010). It is made from three subunit monomers: COL1a1, COL1a2, and COL1a3, in contrast to mammals which only have the COL1a1 and COL1a2 subunits (Pöschl et al., 1988; Saito et al., 2001). The COL1a3 subunit in fish has been found to be more similar to the mammalian COL1a1 subunit

than COL1a2 (Saito et al., 2001). During protein folding, these three subunits combine to make the full collagen type I protein in fish. (Saito et al., 2001).

Collagen deposition in the ECM is controlled by fibroblast cells. These cells make up about 2/3rd of the total cells in the heart tissue of fish, but are only about 1/3rd the total tissue volume due to their small size compared to the contraction-producing myocytes (Chang et al., 2016). Through a series of protein pathways, collagen content is controlled by balancing the deposition and removal of the protein from the heart tissue (Fomovsky et al., 2010; Johnston & Gillis, 2022). It has been proposed by Johnston & Gillis (2022) that collagen deposition in the fish heart initiates by stress on the cardiac cells from temperature changes (Farrell, 1984; Graham et al., 1985; Keen et al., 2017), which leads to release of transforming growth factor- β 1 (TGF- β 1) (Katsumi et al., 2004; MacKenna et al., 1998) and activation of G-coupled protein pathways (Johnston & Gillis, 2020; Katsumi et al., 2004). This results in a cascade of signals that influence the expression of proteins involved in collagen deposition, namely decreases in matrix metalloproteinases (MMPs; degrade collagen), increases in tissue inhibitors of metalloproteinases (TIMPs; inhibit MMPs), and increases in the collagen mRNA transcripts themselves (Johnston & Gillis, 2018; Kolosova et al., 2011; Pramod & Shivakumar, 2014; Reed et al., 1994; Sinfield et al., 2013; Visse & Nagase, 2003), which leads to an increase in collagen protein in the tissue (Johnston & Gillis, 2017). For an extensive look at the proposed mechanisms of temperature-induced collagen deposition in the fish heart, see Johnston & Gillis (2022).

In the heart, collagen increases the stiffness of the cardiac muscle tissue, strengthening the propagation of contraction force (Collier et al., 2012), however, too much collagen causes fibrosis, which results in lower contractile strength and force conductivity (Gomes et al., 2013; Jalil et al., 1989; Kong et al., 2014). Fibrosis in this way typically leads to hindered diastolic performance in the

heart, which is due to the inability of the heart to fully relax due to the tissue stiffness, and therefore, the heart will not be able to fill effectively before its next contraction (Breisch et al., 1984; Lorell & Carabello, 2000; Messerli et al., 2017). In mammals, fibrosis of the heart is caused by damage to the cardiac muscle, which then gets replaced by fibrous collagen tissue (Hinderer & Schenke-Layland, 2019; Murtha et al., 2017). This process is considered to be permanent in mammals and is often a precursor to heart failure (Hinderer & Schenke-Layland, 2019; Wynn, 2007). However, fish have been shown to remove collagen from their heart tissue in response to temperature changes, most likely through an opposing response pathway to the one described in the previous paragraph, where upregulation of MMPs would degrade collagen in the tissue (Johnston & Gillis, 2022; Keen et al., 2018). Currently, the *col1a3* transcript is of most interest from the collagen type I protein, as it is fish-specific and has been demonstrated to respond the most to temperature out of the three subunits (Keen et al., 2016).

1.1.4 Heart-Collagen Temperature Remodeling Response

A key area of current research is determining how fish remodel their hearts by altering the collagen content to overcome the challenges posed by environmental temperature changes. Past studies have shown that temperature acclimation will induce collagen remodeling in the hearts of rainbow trout, zebrafish, and Atlantic salmon (Johnson et al., 2014; Jørgensen et al., 2014; Keen et al., 2016; Klaiman et al., 2011) (Fig. 1.1). In rainbow trout, it has been demonstrated that cold acclimation increases collagen type I protein in the heart and warm acclimation decreases collagen type I (Keen et al., 2016; Klaiman et al., 2011). Interestingly, studies on zebrafish and Atlantic salmon have found the opposite heart-collagen response to temperature, with cold acclimation decreasing collagen type I in zebrafish (Johnson et al., 2014; Shaftoe et al., 2023) and warm acclimation increasing collagen type I in Atlantic salmon (Jørgensen et al., 2014). These changes are

thought to be part of a plastic seasonal response to temperature changes impacting cardiac and body functions (Johnston & Gillis, 2022). To further support this hypothesis, it has recently been demonstrated in zebrafish that the cold acclimation response is reversible in the same cohort of fish (Shaftoe et al., 2023). These findings demonstrate that fishes are reacting differently to similar thermal stimuli, meaning there must be additional factors beyond the direction of a temperature change that drive the heart-collagen remodeling response.

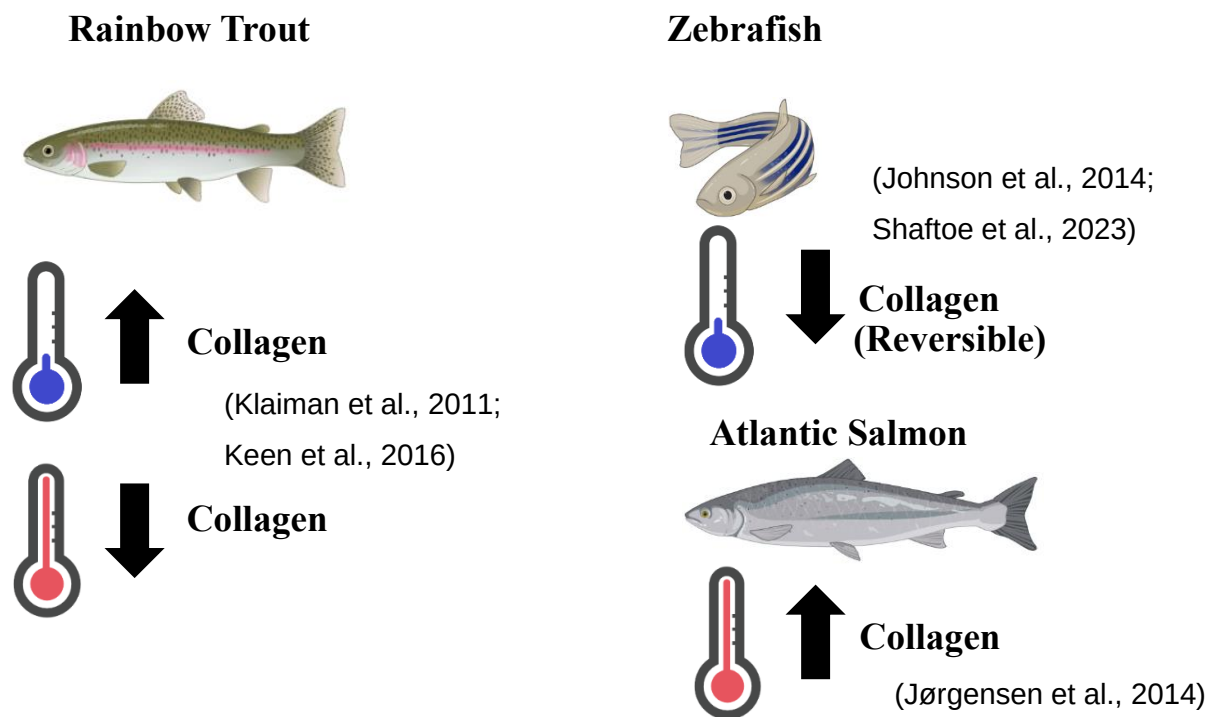


Figure 1.1 Heart collagen remodeling in rainbow trout, zebrafish, and Atlantic salmon.

Heart-collagen responses induced by cold and warm acclimation in three major organisms studied: rainbow trout (Keen et al., 2016; Klaiman et al., 2011), zebrafish (Johnson et al., 2014; Shaftoe et al., 2023), and Atlantic salmon (Jørgensen et al., 2014). Zebrafish cold acclimation response was demonstrated to be reversible (Shaftoe et al., 2023).

The reason for these opposing responses is not yet fully understood, although there are a few hypotheses at present. One suggests that the response could be highly species-specific (Keen et al., 2016). It is possible that many life history traits could impact the needs of a species when responding to temperature changes. For example, rainbow trout are much larger and live in more temperate environments compared to the much smaller tropical zebrafish (Klaiman et al., 2011; López-Olmeda & Sánchez-Vázquez, 2011). However, the Atlantic salmon shares a similar size and temperate environment to rainbow trout, yet they have opposing responses (Jørgensen et al., 2014). Zebrafish and Atlantic salmon also have significant size differences and live in different climates, yet they share similar heart-collagen responses. Therefore, it's possible other species-specific factors are driving these responses.

Another suggestion related to size are differences in blood pressure between species (Keen et al., 2017). Zebrafish, being much smaller fish than rainbow trout, have peak ventricular pressures of 3 mmHg, while trout of about 750g have a blood pressure around 50 mmHg (Clark & Rodnick, 1999; Hu et al., 2001; Keen et al., 2017). The hypothesis is that the lower pressure in zebrafish results in less pressure overall to fill the ventricle during ventricle filling, meaning that increasing collagen content in the heart (which increases heart stiffness) could make it more difficult for the zebrafish to fill their hearts at cold temperatures (Keen et al., 2017). However, this comparison still does not account for the opposing responses seen between rainbow trout and Atlantic salmon.

To complicate matters further, studies on rainbow trout have shown that there is a sex-specific response in heart-collagen remodeling. The responses discussed earlier have been seen in male trout, but no significant results were found for females exposed to the same treatment (Klaiman et al., 2011). This has led to studies in zebrafish that focus predominantly on males given that they

appear to be the sex that responds to these temperature differences (Johnson et al., 2014). This means that sex is an important trait to account for when examining this response in other species.

At the genetic level, it has been demonstrated that the expression of the three collagen type I subunit mRNA transcripts respond differently to these temperature treatments. In rainbow trout, it was determined that the *col1a3* subunit mRNA showed the greatest response to temperature, increasing expression at cold acclimation and decreasing expression at warm acclimation (Keen et al., 2016, 2018). Both rainbow trout *col1a1* and *col1a2* mRNA expression decreased with cold and warm acclimation, indicating that other mechanisms are likely involved (Keen et al., 2016, 2018). Given that the *col1a3* subunit is fish-specific, it is possible that the presence of this subunit and its interactions with temperature are reasons why fish are able to remodel their hearts in this way and mammals cannot.

One element that is consistent across these studies is that they were all performed in a controlled laboratory setting. To imitate seasonal temperature differences, static temperatures were used for the duration of the multi-week trials to represent the average temperatures of warmer or colder seasons, with photoperiod often being adjusted as well to further mimic a seasonal change (Johnson et al., 2014; Jørgensen et al., 2014; Keen et al., 2016; Klaiman et al., 2011; Shaftoe et al., 2023). While these conditions are beneficial to limit confounding factors that could influence results, there are few studies examining how this proposed seasonal plastic response works in a natural population. One of the only studies that has currently investigated this examined the pumpkinseed sunfish in two different populations: one in Canada that was subject to high seasonality and one in Spain that was subject to low seasonality (Procopio et al., 2024). Their results demonstrated an increase in heart tissue collagen during warmer seasons and lower collagen in cooler seasons in both populations (Procopio et al., 2024). In a broad sense, this would make their collagen response

comparable to that of the zebrafish and Atlantic salmon, where colder temperatures decrease heart collagen, and warmer temperatures increase collagen. This study also observed the absence of a compact myocardium layer in the pumpkinseed sunfish (Procopio et al., 2024). Therefore, rather than measuring the compact myocardium layer thickness, they measured epicardium thickness. However, they observed no changes in epicardium thickness across their season groups despite observing cardiac hypertrophy at cold acclimatization (Procopio et al., 2024). Combining all of these species observations together, it is evident that there is a wide range of collagen responses to temperature and season, and significant further investigation is required to determine why these differences exist (Fig. 1.2).

While these heart-collagen remodeling temperature responses have been characterized, there is still limited information on how these responses are regulated. It is also unknown how fast these responses occur, how fish respond under less consistent temperature changes, and how reversible these effects are, although evidence of reversibility has now been demonstrated in the zebrafish (Keen et al., 2016, 2017; Shaftoe et al., 2023). One proposed mechanism of regulation is microRNA (miRNA), a small non-coding RNA that can inhibit mRNA expression (Bartel, 2004; Johnston & Gillis, 2022). In addition, it is thought that this heart response could be seasonal, yet examinations on how fish hearts respond to seasonal temperature changes has not been explored extensively (Keen et al., 2016).

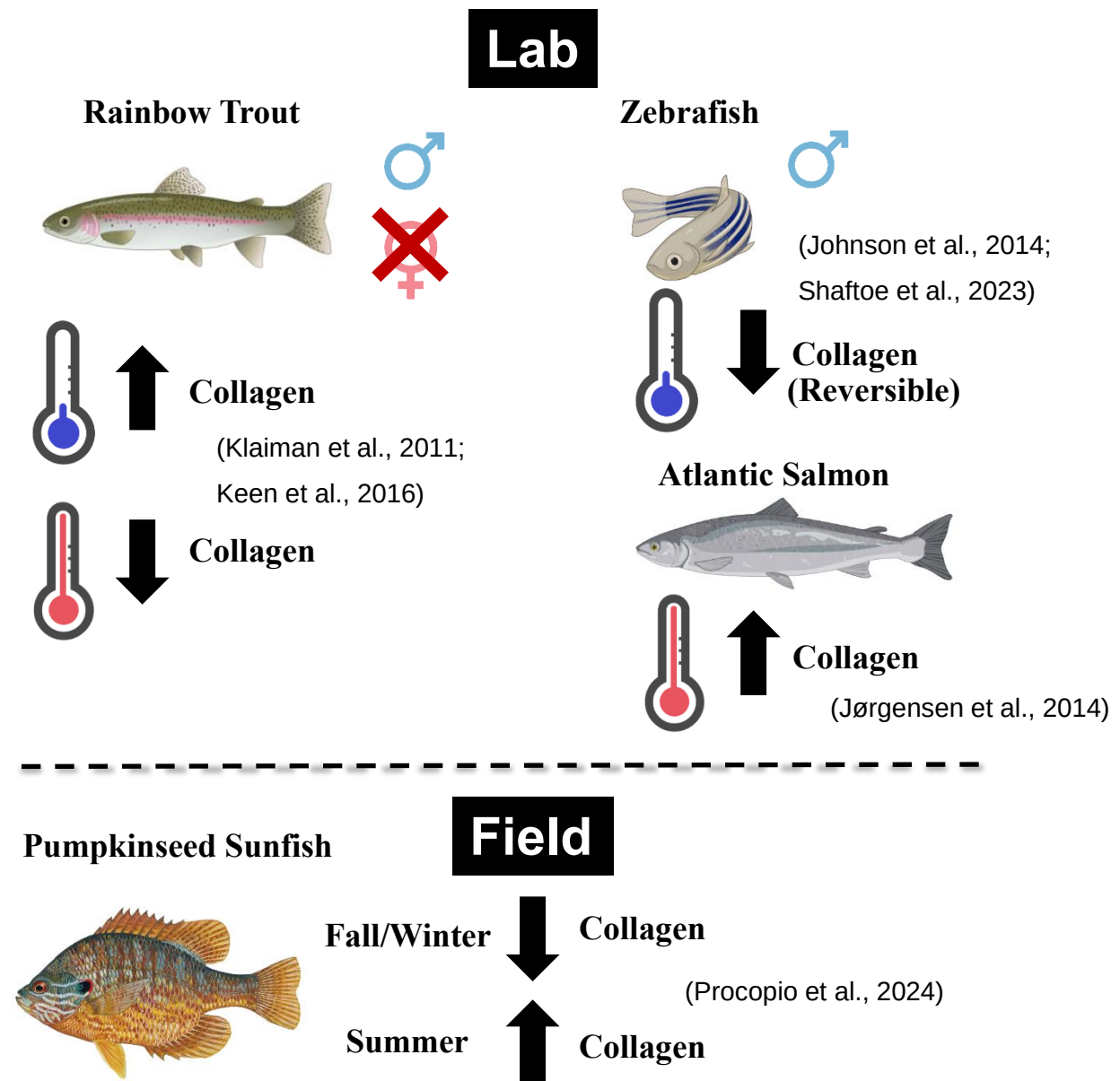


Figure 1.2 Lab vs. Field: Heart collagen remodeling in rainbow trout, zebrafish, Atlantic salmon, and pumpkinseed sunfish.

Heart-collagen responses induced by cold and warm acclimation in three major organisms studied (rainbow trout (Keen et al., 2016; Klaiman et al., 2011), zebrafish (Johnson et al., 2014; Shaftoe et al., 2023), and Atlantic salmon (Jørgensen et al., 2014)) and a field study on the pumpkinseed sunfish (Procopio et al., 2024). Zebrafish cold acclimation response was demonstrated to be reversible (Shaftoe et al., 2023). Some sex-specific responses were observed in rainbow trout and zebrafish.

1.1.5 MicroRNA

MicroRNAs (miRNAs) are an emerging focus for understanding regulatory pathways of protein expression. MicroRNAs are small, non-coding RNAs that block protein production from mRNA by either targeting mRNA for degradation or blocking ribosome binding (Bartel, 2004; Griffiths-Jones et al., 2006). In mammals, it has been determined that miRNA-29 is involved in regulating collagen deposition and removal (Abonnenc et al., 2013; van Rooij et al., 2008). Johnston et al., 2019 investigated the role of miRNA-29 in fish cardiac fibroblast cells, looking particularly at miRNA-29b because it is a highly conserved sequence across vertebrate species, with the nucleotide sequence being 100% identical in both rainbow trout and zebrafish compared to humans (Johnston et al., 2019). Additionally, the seed sequence, the region where there are perfect complementary nucleotide pairs between a microRNA and its target mRNA (Eichhorn et al., 2014), was determined to be conserved across many vertebrates at lengths of 7 or 8 nucleotides, depending on the collagen transcript (Johnston et al., 2019). A key target of miRNA-29b in mammals is the mammalian *col1a1* mRNA transcript (Kriegel et al., 2012). Since the COL1a1 isoform in mammals is most similar to the fish-specific COL1a3 isoform, this indicates that miRNA-29b may target the *col1a3* transcript in addition to *col1a1* in fish (Johnston et al., 2019; Saito et al., 2001). By overexpressing miRNA-29b in rainbow trout cardiac fibroblast cell culture, Johnston et al., 2019 observed a 54% decrease in *col1a3* expression and 84% decrease in collagen type I protein (Johnston et al., 2019). While *col1a1* and *col1a3* are both targets of miRNA-29b in trout, bioinformatic analysis showed that this miRNA likely had a higher affinity to binding to *col1a3* as opposed to *col1a1* (Johnston et al., 2019). Therefore, it was proposed that the significant decrease in expression of *col1a3*, and no significant change in either *col1a1* or *col1a2*, indicates that the decrease in collagen type 1 observed was due to the limited amount of the COL1a3 peptide present, preventing a significant amount of intact collagen type I triple helix from forming (Johnston et al., 2019).

Beyond cell culture, studies have yet to demonstrate the expression and effect of miRNA-29b *in vivo* (Johnston & Gillis, 2022). The physiological environment within a live organism introduces additional interactions that can shape the performance of protein expression pathways, and the concentration of miRNA-29b used in cell culture by Johnston et al., 2019 is not necessarily applicable *in vivo*, depending on how high the miRNA concentrations reach in the live organism (Johnston et al., 2019). Given that there is clear evidence of miRNA-29b's involvement in the fish heart-collagen remodeling response in cell culture, future directions in exploring *in vivo* microRNA expression and its link to collagen content are warranted (Johnston & Gillis, 2022).

1.1.6 Research Organism

This study investigates the rainbow darter (*Etheostoma caeruleum*), a small benthic perch species (Fig. 1.3) native to the Grand River, Ontario (Grand River Conservation Authority, 2022; Paine, 1990). Current literature is lacking for the heart-collagen remodeling response in natural seasonal environments, aside from the pumpkinseed sunfish (Procopio et al., 2024). The rainbow darter and the Grand River have been prime candidates to study local effects of climate change and anthropogenic influences on the fish native to the region (Gauvreau et al., 2022; Robichaud & Craig, 2022; Tetreault et al., 2014). Additionally, there is currently no research on the function and structure of the rainbow darter heart, as it is currently unknown whether this fish has the ability to alter collagen content like the previous species investigated, or whether its heart tissue has a compact myocardium layer. Given the less motile nature of this species, it would be expected that there would be no compact myocardium layer present since it is mostly only present in athletic species (Farrell, 1996).



Figure 1.3 The rainbow darter.

Rainbow darter (male), distinguished by its blue banding and orange colouration. The rainbow darter is a benthic perch native to many watersheds in North America, including the Grand River, ON (Grand River Conservation Authority, 2022). Photo by Konrad P. Schmidt, The Native Fish Conservancy.

1.1.7 Research Objectives

The objective of this thesis was to characterize the collagen type I content and *miR-29b* expression in the heart of wild rainbow darters to better understand how this heart-collagen remodeling response functions in a natural environment that experiences true seasonal dynamics. The main objectives can be divided as follows:

1. To better characterize the heart-collagen remodeling response in a natural seasonal environment and the temperature conditions those fish experience.
2. To determine how *miR-29b* responds *in vivo* to seasonal changes and to characterize its interactions with the collagen type I mRNA transcripts.
3. To determine if the rainbow darter undergoes heart tissue remodeling at the protein level in response to seasonal changes.

1.1.8 Hypothesis

It was hypothesized that that the rainbow darter would undergo seasonal heart remodeling, which could be observed through changes in *col1a1*, *col1a2*, and *col1a3* expression as well as overall collagen type I protein. It was also hypothesized that *miR-29b* would be involved in this response by targeting collagen type I mRNA transcripts during seasons with lower heart tissue collagen.

Chapter 2

Methods & Results

2.1 Methods

2.1.1 Fish Collection

Rainbow Darter fish were collected from the Grand River at an access point near the township of Grand Valley, ON (43°56'32.6"N 80°19'32.8"W) at three season timepoints: Spring (May 29, 2023), Summer (August 22, 2023), and Fall (October 31, 2023). Using backpack electrofishing, fish were captured from the river and dissected on-site. Length and weight measurements were taken, and heart and gonad tissue were removed. Hearts and gonads were weighed for heart somatic index (HrSI) and gonad somatic index (GSI) measurements, respectively, and heart tissue was either stored on dry ice for gene expression/protein analysis or placed in a 2% formalin solution and kept on ice for histological analysis. Heart tissue on dry ice was later transferred into -80°C storage and 2% formalin-submerged heart tissue was transferred to 4°C (See Fig. 2.1 & Fig. 2.2 for overview).

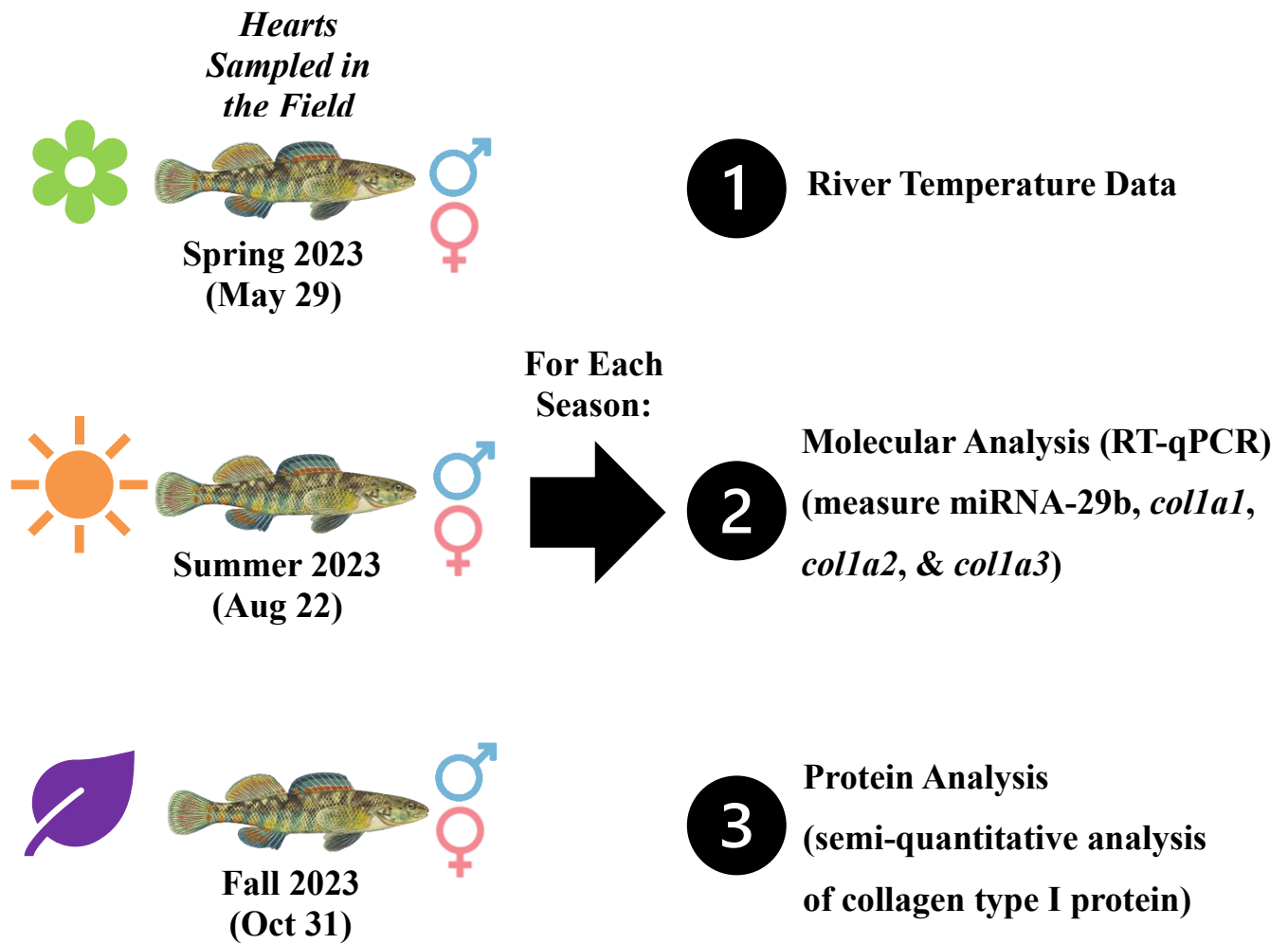


Figure 2.1 Rainbow darter sampling methods.

Overview of rainbow darter heart sampling at three season time points (2023): spring (May 29), summer (Aug 22), and fall (Oct 31). Males and females were collected in equal numbers in each season. River temperature was collected at two weeks around sampling time. Hearts were collected for either molecular analysis (RT-pPCR) or protein analysis (Histology/Western Blot).

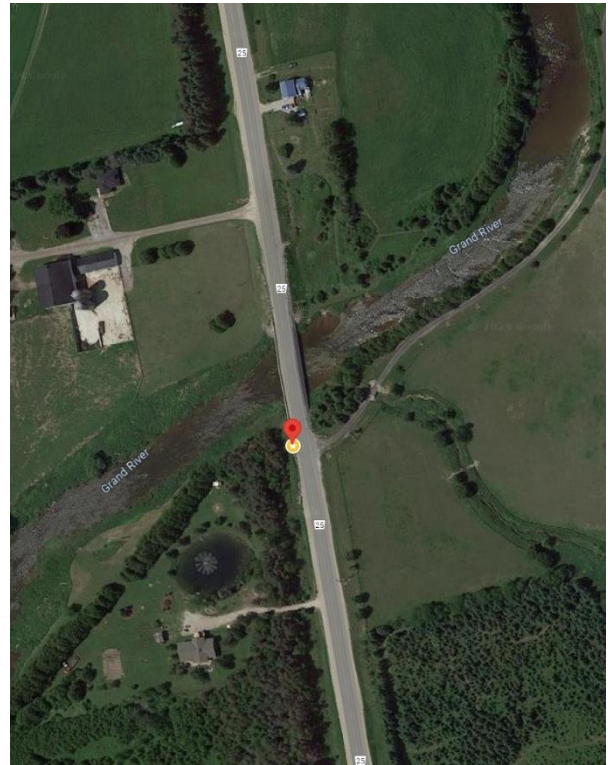
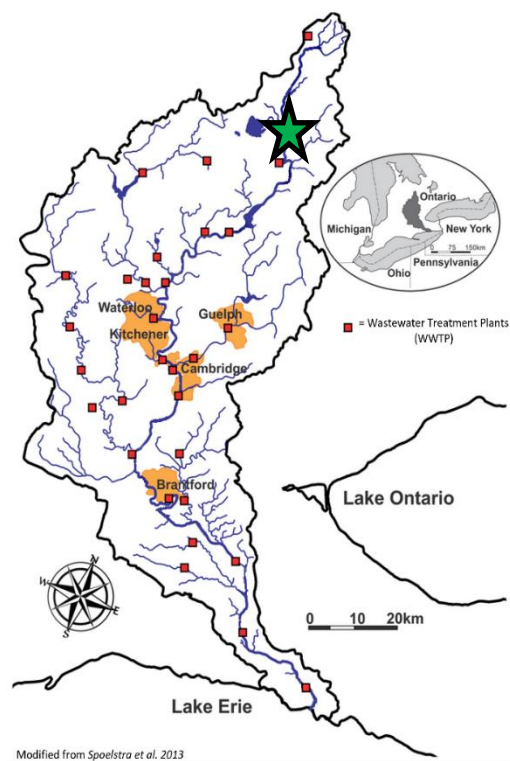


Figure 2.2 Grand River field sampling site.

Sampling site for wild rainbow darters: Grand River at Grand Valley, ON township ($43^{\circ}56'32.6''\text{N}$ $80^{\circ}19'32.8''\text{W}$). Left image shows a stylized map of the Grand River watershed in Southern Ontario; green star indicates the sampling site at the township of Grand Valley, ON. Right image shows a satellite view of the sampling site (Google Maps, 2024).

2.1.2 River Temperature

River temperature data was collected using iButton temperature loggers (iButtonLink Technology). iButtons were waterproofed using Plasti Dip, which has been demonstrated to be an effective waterproofing agent that does not significantly hinder the temperature reading ability of the devices (Roznik & Alford, 2012). Cinder blocks with two iButtons attached to each were deployed in three separate locations at the collection site. iButtons were classified as “Lower” when attached near the bottom of the blocks (~3cm from the riverbed) or as “Upper” when attached near the top of the blocks (~20cm from the riverbed). One block was placed under a nearby bridge which had high shade cover, one was placed upstream of this bridge, and the last placed downstream of the bridge to observe differences in river temperature profiles between the locations (see Fig. 2.3).

Devices were deployed for approximately 2 weeks, at a sampling rate of once every 10 minutes, around the time of sampling for each season: Spring (May 26 – June 8, 2023), Summer (August 8-22, 2023), and Fall (October 20-31, 2023). An additional set of iButtons were deployed from (August 8 – October 20, 2023) to capture the late summer to early fall window at a sampling rate of once every 2 hours. Once collected, the devices were removed from their protective coating and data was retrieved using the OneWireView iButton software. Air temperatures from the Kitchener-Waterloo, ON Environment Canada monitoring site were used to match air temperatures to collected river water temperatures (*Station Results - Kitchener/Waterloo - Historical Data - Climate - Environment and Climate Change Canada*, 2023).

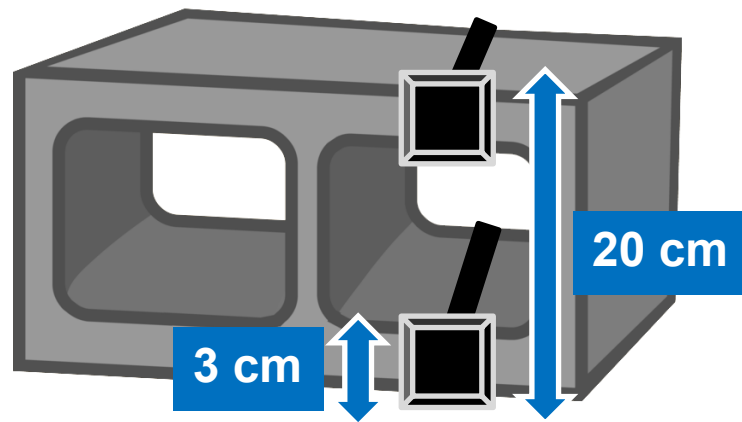
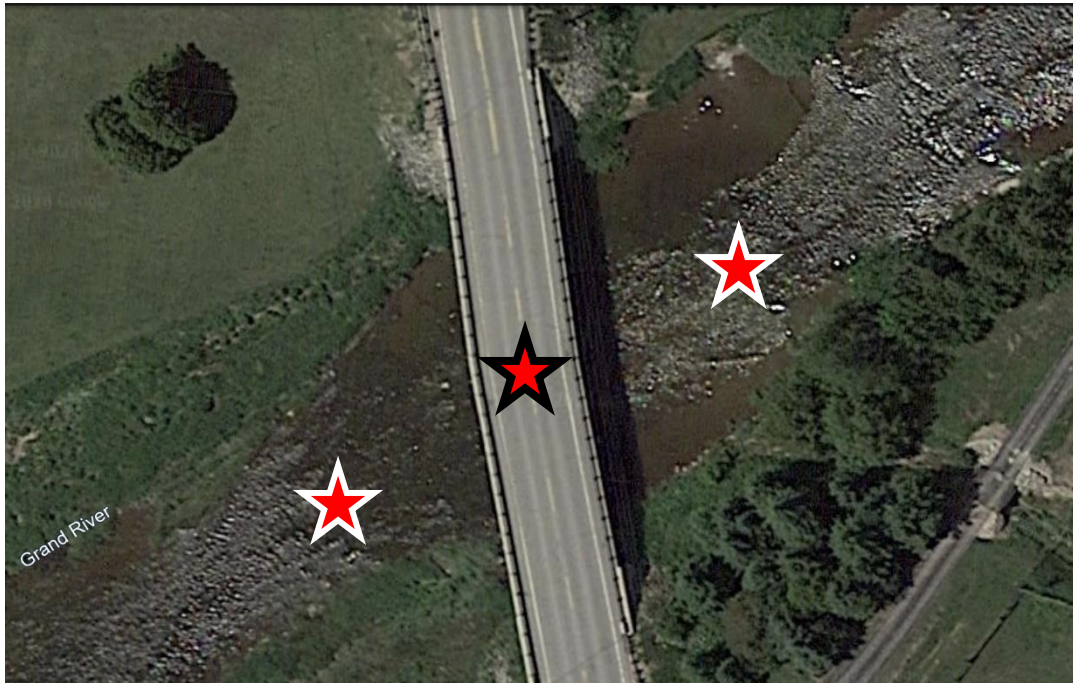


Figure 2.3 iButton river setup.

Diagram of iButton setup on cinder blocks. Two iButton were attached to each of three cinder blocks, one iButton 3cm from the riverbed and one 20cm from the riverbed. The cinder blocks were placed in three locations at the field site, one under the bridge (middle star), one upstream of the bridge (right star), and one downstream of the bridge (left star).

2.1.3 RT-qPCR

Frozen (-80°C) heart tissue with the bulbous arteriosus removed was homogenized with an OMNI Tissue Homogenizer TH (OMNI International, Kenneaw, USA) for 30 seconds in QIAzol® Lysis Reagent and purified for total RNA according to the Qiagen miRNeasy Mini Kit protocol, without deviations, which allows for the purification of very small RNAs, such as miRNA, in addition to mRNA. A volume of 18µL of nuclease-free water was used in the final elution step. Overall RNA concentration was then quantified with Qubit™ microRNA Broad Range Assay Kits (Invitrogen, by ThermoFisher Scientific). Using the known concentration of RNA, 900ng of RNA was used for cDNA synthesis for each sample using the miRNA 1st-Strand cDNA Synthesis Kit protocol (Agilent Technologies) with no deviations.

RT-qPCR was used to quantify the expression of *miR-29b*, and the three collagen type I subunits: *col1a1*, *col1a2*, *col1a3*. Primers for these subunit transcripts were designed with TaqMan probes (Integrated DNA Technologies) as their sequences are very similar, meaning the higher specificity of the probes was required to ensure accurate results (Table 2.1). TaqMan probes were designed using in-lab sequenced transcriptome of the rainbow darter (Pfeifer & Craig, unpublished) that was then BLASTed against the Arkansas darter (*Etheostoma cragini*) collagen type I transcripts that were already present in the NCBI online database. The *miR-29b* primer was based on zebrafish (*Danio rerio*), as it is a highly conserved sequence in vertebrates and was determined to have good efficiency when used with rainbow darter heart tissue (Johnston et al., 2019). Primers and probes were then reconstituted with nuclease-free water per the manufacturer instructions and stored at -20°C. All sample qPCR data was reported as relative abundances, normalized to the reference housekeeping genes *5S rRNA*, *16S rRNA*, and *18S rRNA* using the standard $2^{-\Delta\Delta C_t}$ equation (Livak & Schmittgen, 2001).

RT-qPCR reactions for *5S rRNA*, *16S rRNA*, *18S rRNA*, and *miR-29b* were in 10 μ L wells (5 μ L Biorad SYBR Green Master Mix (Cat# 1725272), 0.8 μ L of 3.125 μ M forward primer, 0.8 μ L of 3.125 μ M reverse primer, 2.4 μ L nuclease-free water, 1 μ L sample/standard). In a CFX96 Touch Real-Time PCR Detection System (Biorad), reactions were initially exposed to 95°C for 30 sec for polymerase activation and DNA denaturation, then 40 cycles of 10 sec at 95°C for DNA denaturation, followed by 20 sec at 60°C (65°C for *miR-29b*) for annealing/extension. Afterwards, a melt curve analysis was performed to ensure that only the target transcripts were being amplified. For *col1a1*, *col1a2*, and *col1a3*, reactions were in 10 μ L wells (5 μ L PrimeTime, 0.8 μ L of 3.125 μ M forward primer, 0.8 μ L of 3.125 μ M probe, 0.8 μ L of 3.125 μ M reverse primer, 0.6 μ L nuclease-free water, 2 μ L samples/standard). Thermocycling protocol in the CFX96 Touch Real-Time PCR Detection System (Biorad) was an initial exposure at 95°C for 3 min for polymerase activation, then 40 cycles of 15 sec at 95°C for denaturation, followed by 1 min at 60°C for annealing/extension. Afterwards, a melt curve analysis was performed to ensure only the target transcripts were being amplified.

Standard curves were made for each target gene using a 4x, 16x, 64x, 256x dilution series (for *5S rRNA*, *16S rRNA*, *18S rRNA*, and *miR-29b*) or a 4x, 8x, 16x, 32x, 64x, 128x, 265x dilution series (*col1a1*, *col1a2*, and *col1a3*), each made with a pooled sample from all groups and nuclease-free water. Efficiencies between 90-110% were accepted and R^2 was > 0.95 for all standard curves. For *miR-29b*, a temperature gradient was performed (55.0°C, 55.7°C, 57.0°C, 59.0°C, 61.4°C, 63.3°C, 64.5°C, and 65.0°C), because the standard 60°C annealing temperature was inefficient, demonstrating an ideal annealing temperature of 65°C. Samples were diluted to 5x using nuclease-free water to ensure they would fall on the standard curve.

Table 2.1 mRNA and miRNA primer for target genes for RT-qPCR.

Forward, reverse, and probe (when relevant) primer sequences used for RT-qPCR. Efficiency (%), amplicon length (bp) and annealing temperature (°C) are reported. Amplicon length of miR-29b-3p not able to be determined due to the Universal Reverse Primer (Qiagen) sequence being proprietary and undisclosed.

Primer	Sequence (5'-3')	Efficiency (%)	Amplicon (bp)	Annealing Temp (°C)
5S	F: CCTGAACACGCCCCGATCTC R: TAGGCGGTCTCCCATCCAAG	103.4	80	60
16S RBD	F: AACCCGTCTCTGTGGCAAAA R: TCCGGGAGGCTGAACTTCTA	107.4	112	60
18S dre	F: ATGGCCGTTCTTAGTTGGTG R: GAACGCCACTTGTCCCTCTA	100.3	130	60
miR-29b-3p	F: TAGCACCATTGAAATCAGTGTT R: Universal Reverse Primer (Qiagen, Cat# 218073)	105.7	N/A	65
col1a1	F: GCAAGACAGTCATCGACTACAA PRB: /56-FAM/ATCGCTCCT/ZEN/ATGGACGTTGGTGC/3IABkFQ/ R: GTCCAATTTCAAGGCCAAACTC	99.2	111	60
col1a2	F: GGTGGATATGATGTCTCTGGATATG PRB: /56-FAM/AGAGCCAAG/ZEN/GACTACGAGGTCGAT/3IABkFQ/ R: GATCTGAGTGTTGAGGGACTTG	102.0	108	60
col1a3	F: CCTCAGCAAGAACATCAGAGAG PRB: /56-FAM/AGGAAGCCC/ZEN/ATCTGGTTCA GCGAG/3IABkFQ/ R: CTGCCATACTGGAAGTGAAT	100.2	81	60

2.1.4 Collagen Type I Gene Sequence Alignment

To determine the potential for miR-29b to bind to and interact with the collagen type I mRNA transcripts in the rainbow darter, an alignment of the collagen type I transcripts was performed using MEGA11 (Molecular Evolutionary Genetics Analysis) alignment software. This alignment built off the alignment performed by (Johnston et al., 2019), where the conservation of the miR-29b seed sequence was investigated. Comparing to the 3'UTRs of *col1a1*, *col1a2*, and *col1a3* that (Johnston et al., 2019) determined for rainbow trout, the following species (and genome assembly identifiers) were used in an alignment with the respective transcripts in the rainbow darter: *Danio rerio*: GRCz11 (GCF_000002035.6); *Salmo salar*: ICSASG_v2 (GCF_000233375.1); *Salvelinus alpinus*: ASM291031v2 (GCF_002910315.2). Additionally, the Arkansas darter (*Etheostoma cragini*; CSU_Ecrag_1.0 (GCF_013103735.1)) was added to the alignment given its close taxonomical relationship to the rainbow darter. Rainbow darter collagen type I transcript 3'UTRs were determined by aligning the full gene sequence to the coding sequence of the same gene in the closely related Arkansas darter. This 3'UTR was then used in the final alignment with the other species to determine in the miR-29b binding site is conserved in the rainbow darter and, therefore, predicted to be influenced by it.

2.1.5 Histology Protocol & Heart Morphology Measurements

Heart tissue placed in 2% formalin solution after dissection was transferred to 4°C for 24h with constant agitation. Afterwards, hearts were moved to 70% EtOH at 4°C for long term storage. Tissue was processed using the Thermo Scientific Excelsior AS automated tissue processor standard protocol. For processing, tissue was submerged in EtOH until the processing run was scheduled to start, then decreasing concentrations of EtOH were applied to the tissue in a total of six 30-minute intervals. Afterwards, three rounds of 30 minutes of 100% xylene were applied to the tissue, which

was finally followed by three 80-minute intervals of wax infiltration. Processed hearts were then embedded in paraffin wax in a Shandon Tissue Processor. To ensure the same orientation of the tissue, a small amount of tissue dye (Thermo Scientific) was used on the end of the bulbous arteriosus. Using a Leica RM2235 microtome, hearts were sectioned at 5µm, sections flattened in a warm water bath with 0.1% Leica Surgipath® Sta-On® Tissue Section Adhesive and adhered to slides with a Mayer's albumin coating to ensure the tissue remained attached to the slides during staining. Slides were then deparaffinized and stained with the Picro-Sirius Red Stain Kit (Cardiac Muscle) (Abcam #ab245887), which stains collagen fibers a deep red and the surrounding tissue yellow (*Picro-Sirius Red Stain Kit (Cardiac Muscle) (Ab245887) | Abcam, Canada, n.d.*).

Images were taken using a Leica DM1000 LED microscope and Leica LAS V4.4 imaging software at 50X magnification. These images were then opened in ImageJ where they were separated into Hue, Saturation, and Brightness components. In the Hue component, deep red colours become very dark black or bright white. Pixel density of bright white pixels was quantified using the hue value range of 221-256 to denote the red collagen fibres (Rich & Whittaker, 2005; Shaftoe et al., 2023).

HrSI for each individual fish was determined by dividing heart mass by total body mass, with gonad weight removed from total body mass to account for differences in the reproductive cycle: $[(\text{heart mass (g)})/(\text{total mass (g)} - \text{gonad mass (g)})]*100$. GSI was similarly calculated by dividing gonad mass by total body mass: $[(\text{gonad mass (g)})/\text{total mass (g)}]*100$.

Epicardium thickness in each sample was measured from the same tissue sections in images at 400X magnification in three fields of view (see Appendix Fig. A.1, Fig A.2, & Fig. A.3). In each field, two measurements of epicardium thickness were made, for a total of six measurements for each sample. The mean of the six measurements was then determined to represent the epicardium thickness

for that sample. Samples were only grouped by season, as there were too few samples to separate by sex.

2.1.6 Western Blot Protein Analysis

Frozen (-80°C) collected heart tissue with the bulbous arteriosus removed was homogenized for protein with Lysis buffer (50mM Tris, 1% Triton X-100, 150mM NaCl, pH 8.0) on ice, kept at 4°C with agitation for 2 hours, and then centrifuged to retrieve the supernatant. A Bicinchoninic acid (BCA) assay for total protein content was then performed on all samples. Sexes for the three seasons were combined into the three season groups because too few heart samples were available to examine the sexes independently in each season. Using a concentration of 20µg of protein and 4X Laemmli (BioRad), samples were run through gel electrophoresis for ~30 minutes to separate the protein by weight. Pre-cast gels (BioRad Mini-PROTEAN®TGX Stain-Free™ Precast Gels) were used in a BioRad Mini-PROTEAN® Tetra System gel electrophoresis unit. Finished gels were imaged in a BioRad Chemidoc™ MP Imaging System using ImageLab (BioRad) software. Protein from imaged gels were then transferred to membranes using a BioRad Trans-Blot® Turbo™ Transfer System at 2.5 A, 25V for 3 min and pre-made transfer blot sandwiches (BioRad Trans-Blot®Turbo™ Transfer Pack). Membranes were blocked for 1 hour with 5% skim milk powder and then washed in 2 washes of 1X TBST for 5 min each. Following the washes, membranes were incubated with the primary antibody, rabbit anti-salmon collagen type I (Cedarlane), at a concentration of 1:1000, diluted with 5% skim milk powder, overnight at 4°C with constant agitation. The next day, the membranes were washed in 1X TBST once for 20 minutes, then 4 more times at 5 minutes each. Membranes were then incubated with the horseradish peroxidase (HRP) linked-secondary goat anti-rabbit antibody (Invitrogen), at a concentration of 1:1000, diluted with 5% skim milk powder, for 1 hour at room temperature. Afterwards, membranes were washed again with 1X TBST for 20 minutes and then 4

more times for 5 minutes each. To visualize the bound secondary antibodies, membranes were put in BioRad Clarity™ Western ECL Substrate and subsequently imaged in the Chemidoc using ImageLab. On ImageLab, bands present at 150kDa, the protein mass for collagen type I, were analyzed and normalized to protein content on the gel images.

2.1.7 Statistical Analysis

Data was statistically analyzed with GraphPad Prism 10.2.1 Software. RT-pPCR data and HrSI were analyzed with Two-way ANOVAs to determine the effects of season, sex, and their interaction on gene expression (See Appendix Table A.1). Follow-up Tukey post hoc tests were performed when appropriate to determine the specific differences between the groups. For western blot protein data analysis, sexes were combined to examine season effects due to a lower available sample count. One-way ANOVAs were performed to determine the effect of season on protein abundance, epicardium thickness, and GSI (see Appendix Table A.2), followed by a Tukey post hoc test to determine specific differences between the groups. All ANOVA alpha values were set to 5% ($\alpha = 0.05$), where p values were considered significant when $p < \alpha$. The assumptions of normality of the residuals and homoskedasticity for the Two-way ANOVA were tested with the Shapiro-Wilk normality test and Spearman's test for heteroskedasticity, respectively. The assumptions of normality of the residuals and equality of variances were tested with the Shapiro-Wilk normality test and Bartlett's test, respectively. Linear regression analysis was performed on comparisons between *miR-29b* and the three collagen type I mRNA, and additionally with total means collagen type I content determine through western blot. When needed, data was transformed to pass assumption tests. All data presented in graphical figures show untransformed data.

2.2 Results

2.2.1 River water temperature profiles differ by season

Water temperature in the Grand River, ON at the Grand Valley, ON sampling site showed differing temperature profiles at each of the three season timepoints (Fig. 2.4). All six iButtons showed extremely similar temperature readings that they were effectively the same regardless of location, shade cover, or depth, so all six iButton readings were averaged to determine the river water temperature at each timepoint. Air temperature denoted by the red lines and described here was provided by Environment Canada at the Kitchener-Waterloo, ON weather station (*Station Results - Kitchener/Waterloo - Historical Data - Climate - Environment and Climate Change Canada, 2023*).

In the spring (Fig. 2.4), water temperatures averaged 21.0°C over the two weeks, with a peak of 28.0°C on May 31 at 5PM & June 2 at 4PM and a minimum of 13.5°C on May 26 at 6AM. This season saw the largest temperature swings with average fluctuations of 6.8°C. The largest temperature fluctuation was observed on May 29 with a change of 8.5°C from maximum to minimum throughout the day, and a minimum fluctuation of 5.0°C on June 3 & 5. Spring air temperatures averaged 17.0°C across the two weeks and had much larger variation than the water. With a maximum recorded temperature of 31.5°C on June 2 at 2PM and a minimum of 1.5°C on May 26 at 4AM, the air temperature experienced a difference of 30°C across two weeks. Average daily air temperature fluctuations were 17.7°C, with a maximum fluctuation of 21.8°C in one day on May 30 and a minimum fluctuation of 12.4°C in one day on June 3 & 6.

Summer river water temperatures (Fig. 2.5) were similar to those of spring but with smaller fluctuations. Average water temperature was 20.5°C across the two weeks, with a maximum of 24.1°C observed on August 16 at 4PM and minimum of 15.5°C observed on August 19 at 7AM. Water temperature fluctuations averaged at 3.86°C, reaching a 5.7°C daily fluctuation on August 19

and a minimum of a 1.5°C daily fluctuation on August 15. Air temperatures followed a similar pattern, showing a similar profile to those in the spring, but with lower overall fluctuations. Average air temperature was 18.8°C over the two weeks, reaching a maximum of 27.6°C on August 9 at 2PM and a minimum of 9.2°C on August 19 at 6AM. Fluctuations were considerably lower than spring, averaging at 9.9°C across the two weeks, with a maximum daily fluctuation of 14.4°C on August 9 and a minimum daily fluctuation of 3.4°C on August 15.

Fall river water temperatures (Fig. 2.6) were much more stable than either of the previous two seasons. As expected, the two-week water temperature average was considerably lower than spring or summer at 10.4°C, with a maximum of 14.1°C on October 27 at 9PM and a minimum of 5.5°C on October 23 at 9AM. Water fluctuations were very limited with many days showing little to no daily fluctuations. The average fluctuations across the sampling time were 2.02°C, with a maximum daily fluctuation of 3.0°C observed on October 23, 24, & 28 and a minimum daily fluctuation of 1.0°C observed on October 20 & 26. Fall air temperatures showed more variability than the water and was not as closely linked with the water temperature as in other seasons. The two-week average air temperature was 9.4°C, reaching a maximum of 21.7°C on October 27 at 3PM and a minimum of -3.8°C on October 23 at 7AM. Air temperatures reached below freezing for no longer than one day on October 23 and October 31, and water temperatures never reached below 0°C. Air daily fluctuations were smaller than previous seasons, with a two-week average of 7.38°C. Daily fluctuations reached as high as 16.6°C on October 23 right after the temperature was below freezing, and as low as 2.3°C on October 29.

Finally, the longer-term river water temperature monitoring from early August to mid-October (Fig. 2.7) shows trends consistent with the summer temperatures in Figure 2.5, with the exception of the clear drop-off starting around October 7. It is worth noting that the temperature at

this drop-off decreased from $\sim 19^{\circ}\text{C}$ to $\sim 9^{\circ}\text{C}$ from October 6 to October 10 with minimal fluctuations during that time. Overall, this 2.5 month time period experienced an average water temperature of 17.7°C , with a maximum of 26.5°C on September 6 at 3PM and a minimum of 8.4°C on October 12 at 8AM. Unsurprisingly, since most of this time frame captures summer, the average fluctuations were very close to those of the summer two-week time frame, at 3.28°C . Maximum daily fluctuations reached 5.67°C on August 19 and were as low as 0.67°C on October 15.

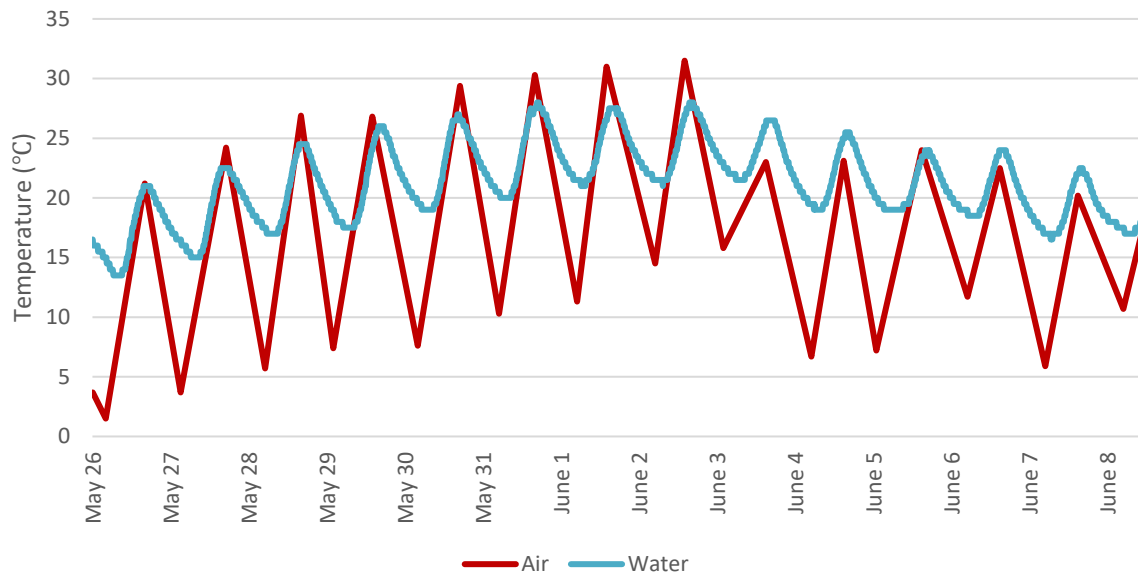


Figure 2.4 May-June (spring) river temperature.

Spring season, 2023 (late May-early June) temperature profile of the Grand River at the Grand Valley, ON sampling site two weeks around the sampling date. Since all locations recorded very similar temperatures, water data was averaged from six iButtons on cinder blocks placed in three river locations at the sampling site (under bridge, upstream of bridge, and downstream of bridge), where each cinder block had an iButton 3cm and 20cm from the riverbed. Air temperature denoted in red, provided by Environment Canada Kitchener-Waterloo weather station.

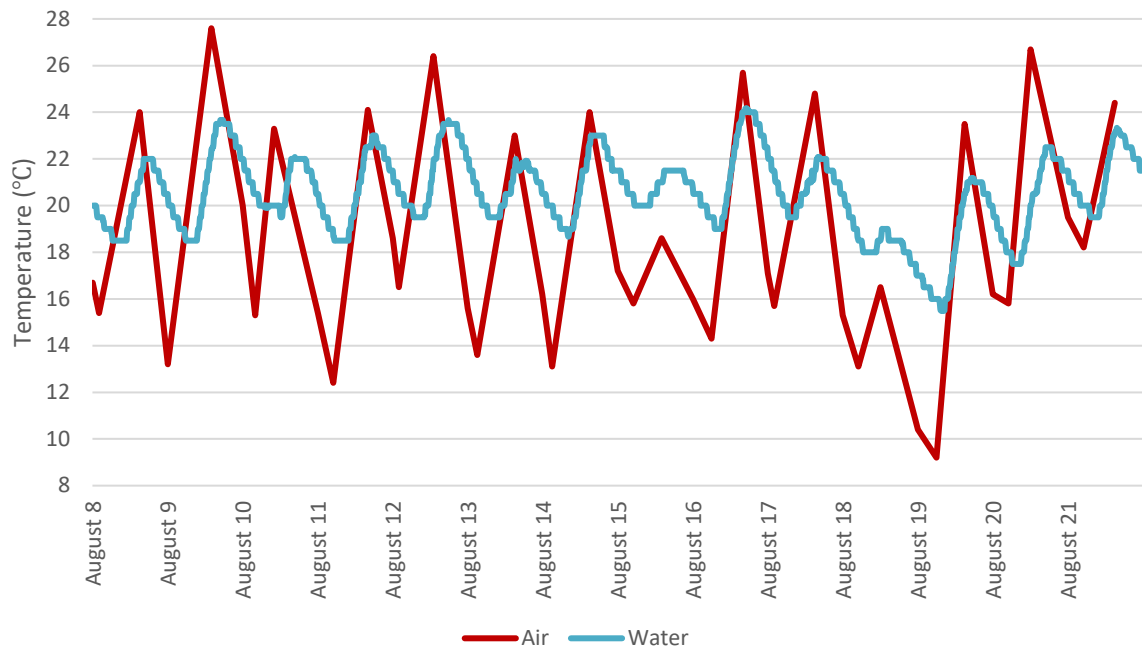


Figure 2.5 August (summer) river temperature.

Summer season, 2023 (mid-August) temperature profile of the Grand River at the Grand Valley, ON sampling site two weeks around the sampling date. Since all locations recorded very similar temperatures, water data was averaged from six iButtons on cinder blocks placed in three river locations at the sampling site (under bridge, upstream of bridge, and downstream of bridge), where each cinder block had an iButton 3cm and 20cm from the riverbed. Air temperature denoted in red, provided by Environment Canada Kitchener-Waterloo weather station.

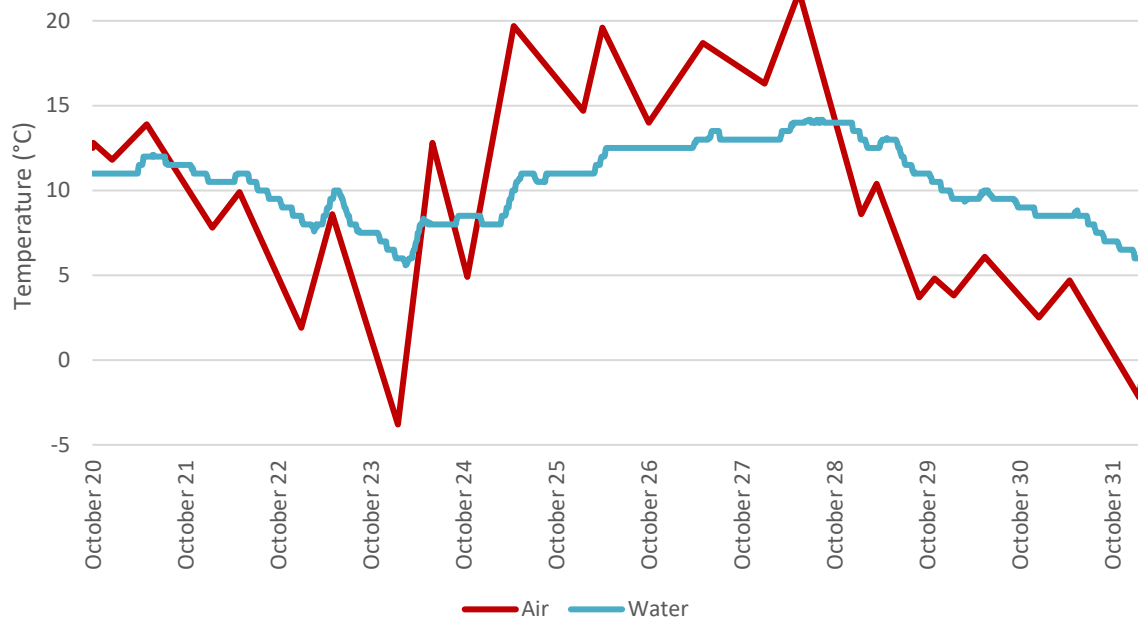


Figure 2.6 October (fall) river temperature.

Fall season, 2023 (late October) temperature profile of the Grand River at the Grand Valley, ON sampling site two weeks around the sampling date. Since all locations recorded very similar temperatures, water data was averaged from six iButtons on cinder blocks placed in three river locations at the sampling site (under bridge, upstream of bridge, and downstream of bridge), where each cinder block had an iButton 3cm and 20cm from the riverbed. Air temperature denoted in red, provided by Environment Canada Kitchener-Waterloo weather station.

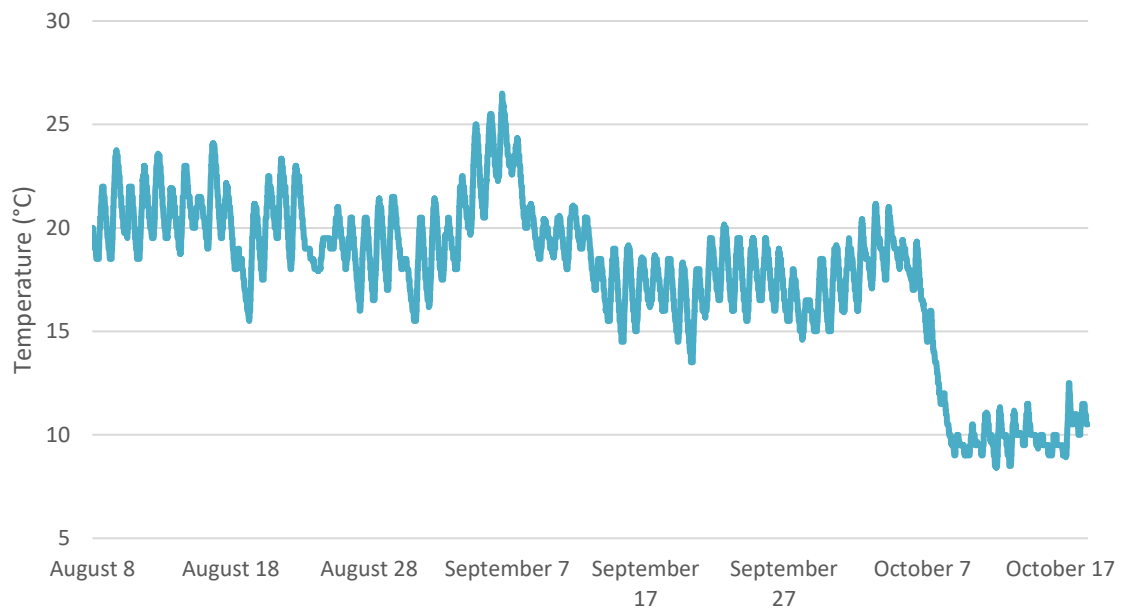


Figure 2.7 August to October river temperature.

Mid-summer to early fall, 2023 (August to October) temperature profile of the Grand River at the Grand Valley, ON sampling site two weeks around the sampling date. Since all locations recorded very similar temperatures, water data was averaged from six iButtons on cinder blocks placed in three river locations at the sampling site (under bridge, upstream of bridge, and downstream of bridge), where each cinder block had an iButton 3cm and 20cm from the riverbed.

2.2.2 Collagen type I gene expression changes with season

A significant season effect was found in heart *col1a1* expression (Fig 2.8A: Two-way ANOVA, $F_{2,46} = 8.972$, $p = 0.0005$). No significant sex effect was found (Two-way ANOVA, $F_{1,46} = 0.2600$, $p = 0.6126$). Additionally, no interactive effect between season and sex was observed (Two-way ANOVA, $F_{2,46} = 0.01072$, $p = 0.9893$). Tukey post hoc test found heart *col1a1* expression was significantly different in the summer season compared to both the spring and fall seasons, with a 2.3-fold increase in the spring compared to summer (Tukey HSD, $p = 0.0003$) and a 1.4-fold increase in fall compared to summer (Tukey HSD, $p = 0.04$). Fall sees a slight decrease in mean expression but was not found to be statistically significant (Tukey HSD, $p = 0.1409$).

For *col1a2*, a significant season effect was observed (Fig 2.8B: Two-Way ANOVA, $F_{2,46} = 4.460$, $p = 0.0170$) and no sex effect was found (Two-Way ANOVA, $F_{1,46} = 0.07156$, $p = 0.7903$) like was seen in *col1a1*. No significant interactive effect between season and sex was observed (Two-Way ANOVA, $F_{2,46} = 2.353$, $p = 0.1064$). Tukey post hoc testing showed a significant 1.3-fold increase in *col1a2* expression in the spring compared to fall season (Tukey HSD, $p = 0.0161$). In summer, while having slightly lower mean expression than spring, no significant effect was observed (Tukey HSD, $p = 0.6665$). The slight increase in mean expression in summer compared to fall also showed no significant effect (Tukey HSD, $p = 0.1297$).

Interestingly, no significant effect was observed for either season (Fig 2.8C: Two-Way ANOVA, $F_{2,46} = 0.5122$, $p = 0.6025$) or sex (Two-Way ANOVA, $F_{1,46} = 0.7446$, $p = 0.3927$) in *col1a3* expression. Therefore, there was also no interactive effect between season and sex observed (Two-Way ANOVA, $F_{2,46} = 0.4804$, $p = 0.6216$).

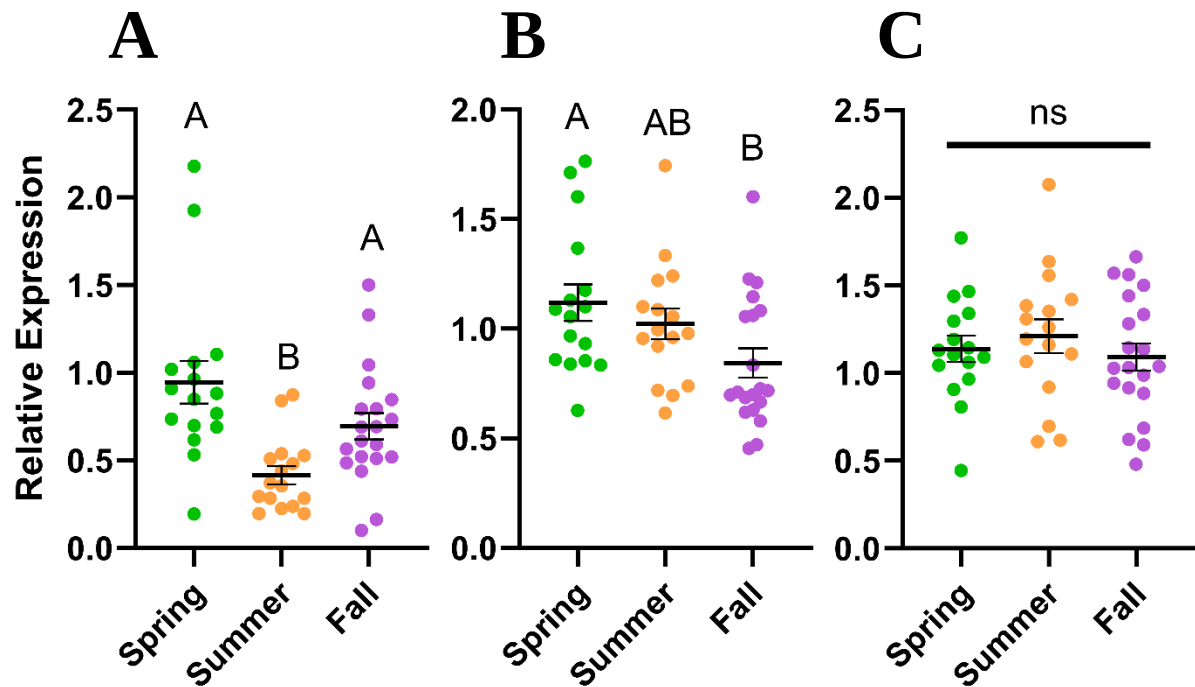


Figure 2.8 Gene expression of *col1a1*, *col1a2*, and *col1a3*.

Relative expression of the three collagen type I subunit mRNA transcripts (A) *col1a1*, B) *col1a2*, and C) *col1a3*) in the rainbow darter heart in spring, summer, and fall 2023. Black bars indicate the mean $\pm$ SEM of each group. Analysis through Two-way ANOVA, followed by Tukey post-hoc test. *col1a1* and *col1a2* were both sqrt transformed to meet test assumptions. No significant sex effect was found, so sexes for each season were combined in the graph. Different letters within graphs denote statistical significance. (n = 8 for males and females in both spring and summer; n = 10 for males and females in fall).

2.2.3 MicroRNA-29b binding sequence conserved in darter species

Figure 2.9 shows the alignment of the 3'UTR of the rainbow trout, Atlantic salmon, zebrafish, Arctic char, Arkansas darter, and rainbow darter. The nucleotides highlighted in yellow represent the binding sequence for *miR-29b*, either 7 or 8 nucleotides long. The Arkansas darter and rainbow darter have been added to this alignment based on the alignment performed by (Johnston et al., 2019). Both *col1a1* (Fig. 2.9A) and *col1a3* (Fig. 2.9C) align with the 7-nucleotide seed sequence for *miR-29b* (GGTGCTA), while *col1a2* aligns with the 8-nucleotide seed sequence (TGGTGCTA) binding sequence (Fig. 2.9B). This alignment shows that this binding sequence is conserved among these fish species and that it would be predicted that *miR-29b* would have the ability to bind to and act on these three transcripts.

2.2.4 MicroRNA-29b expression is significantly higher in females but no regression with collagen type I mRNA

A significant sex effect was observed in the expression of *miR-29b* in heart tissue between the males and females (Fig 2.10: Two-way ANOVA, $F_{1,46} = 8.358$, $p = 0.0058$). Females had a 1.4-fold increase in overall *miR-29b* expression compared to the males. Interestingly, no significant season effect was observed (Two-way ANOVA, $F_{2,46} = 2.329$, $p = 0.1088$). Additionally, no interaction effect was observed (Two-way ANOVA, $F_{2,46} = 2.051$, $p = 0.1402$). No significant regression slope was found between *miR-29b* and *col1a1* (Fig 2.11: Linear regression, slope = -0.1668, $F_{1,50} = 0.7174$, $p = 0.401$), *col1a2* (Fig 2.12: Linear regression, slope = -0.05759, $F_{1,50} = 0.1384$, $p = 0.7115$), or *col1a3* (Fig 2.13: Linear regression, slope = -0.1751, $F_{1,50} = 1.14$, $p = 0.2908$).

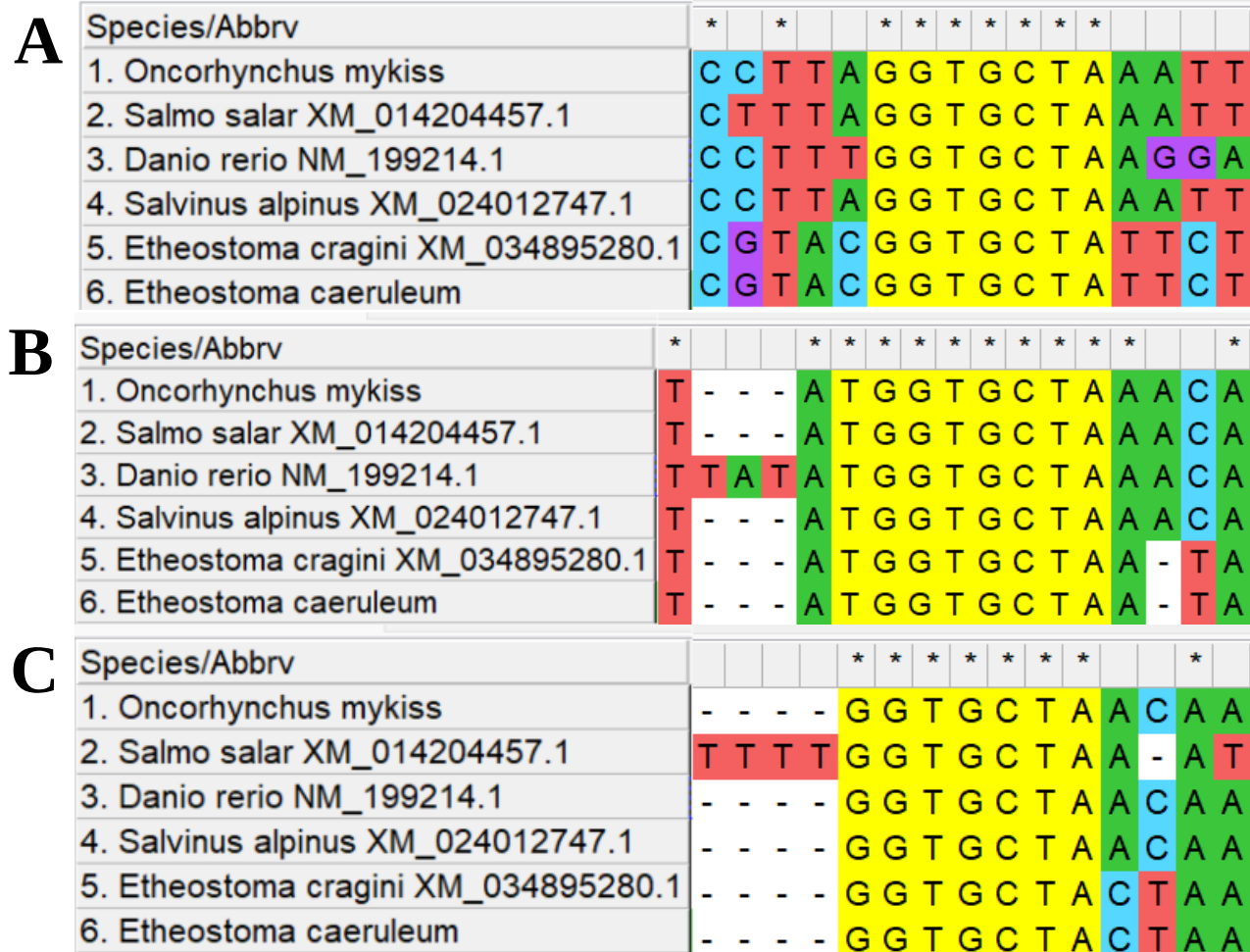


Figure 2.9 Sequence alignment of *miR-29b*.

Sequence alignment of 3'UTR of rainbow trout, Atlantic salmon, zebrafish, Arctic char, Arkansas darter, and rainbow darter. Aligned *miR-29b* binding site highlighted in yellow, either the 7-nucleotide (GGTGCTA) or 8-nucleotide (TGGTGCTA) seed sequence. A) *col1a1* transcript. B) *col1a2* transcript. C) *col1a3* transcript. Non-darter species sequences from alignment performed by (Johnston et al., 2019).

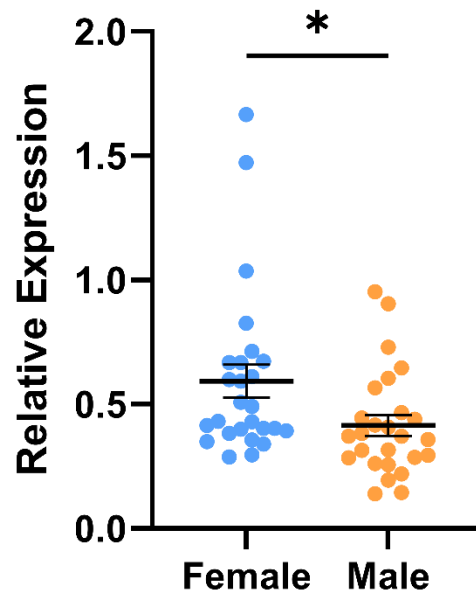


Figure 2.10 Gene expression of *miR-29b*.

Relative expression of *miR-29b* in the rainbow darter heart in spring (May 29), summer (August 22), and fall (October 31) 2023. Black bars indicate the mean of each group. Analysis through Two-way ANOVA. Data was log transformed to meet test assumptions. No significant season effect was found, so seasons for each sex were combined in the graph. Different letters within graphs denote statistical significance. (n = 8 for males and females in both spring and summer; n = 10 for males and females in fall).

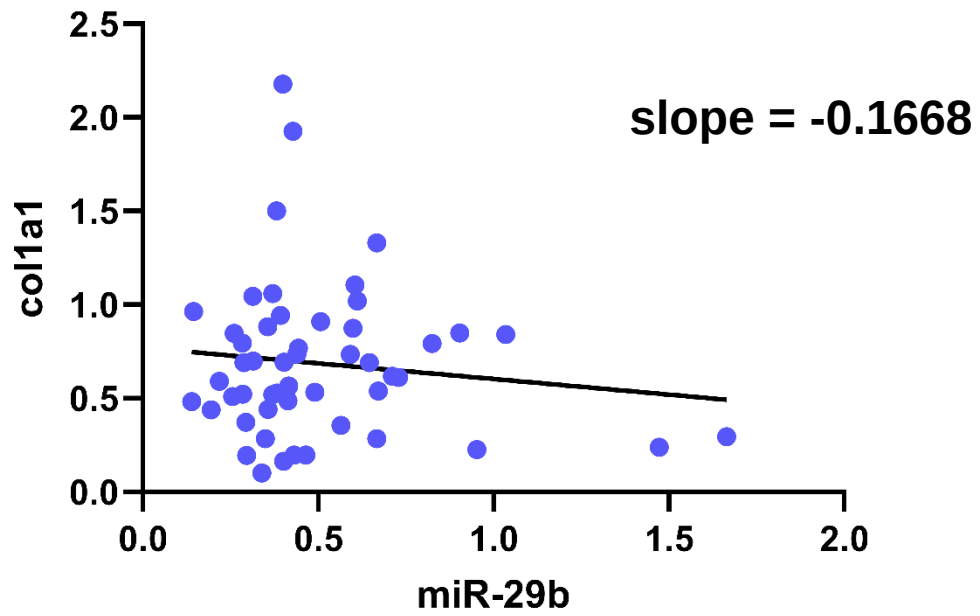


Figure 2.11 Regression of *miR-29b* & *col1a1*.

Regression of *miR-29b* expression and *col1a1* expression. Expression values include all three seasons. Slope of regression line = -0.1668 ($p > 0.05$).

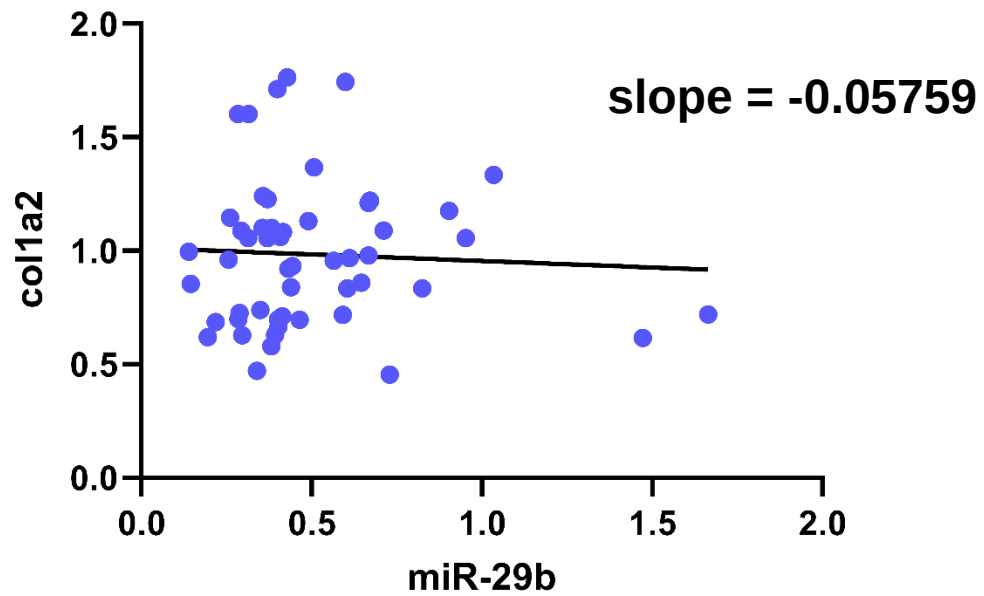


Figure 2.12 Regression of *miR-29b* & *col1a2*.

Regression of *miR-29b* expression and *col1a2* expression. Expression values include all three seasons. Slope of regression line = -0.05759 ($p > 0.05$).

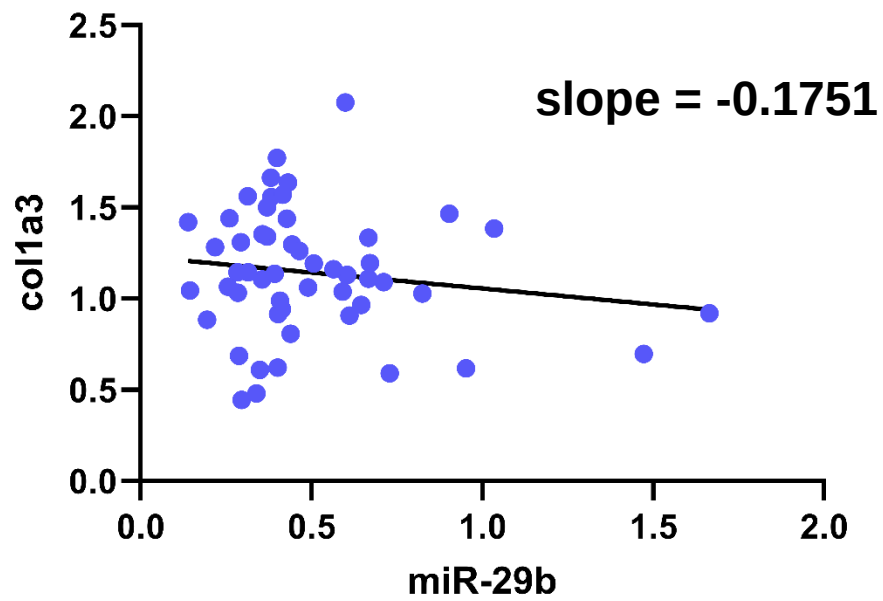


Figure 2.13 Regression of *miR-29b* & *col1a3*.

Regression of *miR-29b* expression and *col1a3* expression. Expression values include all three seasons. Slope of regression line = -0.1751 ($p > 0.05$).

2.2.5 Histological analysis shows evidence of cardiac hypertrophy

HrSI measurements show an increase in relative heart mass compared to body mass (with gonad mass removed) in the spring and fall, compared to summer (Fig 2.14: Two-way ANOVA, $F_{2,46} = 20.99$, $p < 0.0001$). No significant sex (Two-way ANOVA, $F_{1,46} = 0.5391$, $p = 0.4665$) or interaction effect was found (Two-way ANOVA, $F_{2,46} = 2.398$, $p = 0.1022$). Spring saw a 1.4-fold increase in relative heart mass compared to summer (Tukey HSD, $p = 0.0198$). Fall saw a 1.6-fold increase compared to spring (Tukey HSD, $p = 0.0031$) and a 2.3-fold increase compared to summer (Tukey HSD, $p < 0.0001$). GSI measurements also see seasonal differences in both males (Fig 2.15A: One-way ANOVA, $F_{2,30} = 324.9$, $p < 0.0001$) and females (Fig 2.15B: One-way ANOVA, $F_{2,31} = 158.1$, $p < 0.0001$). In males, spring had a 16-fold increase in relative gonad mass compared to summer (Tukey HSD, $p < 0.0001$). In fall, males had a 1.5-fold increase compared to spring (Tukey HSD, $p = 0.0004$) and a 23.7-fold increase compared to summer (Tukey HSD, $p < 0.0001$). Female rainbow darters had their highest GSI during the spring, with a 4.4-fold increase compared to summer (Tukey HSD, $p < 0.0001$) and a 1.8-fold increase compared to fall (Tukey HSD, $p = 0.0006$). The fall season in females had a 2.5-fold increase in relative gonad mass compared to summer (Tukey HSD, $p < 0.0001$). Measurements of epicardium thickness from the histological sections did not show a significant difference between seasons (Fig 2.16: One-way ANOVA, $F_{2,12} = 1.502$, $p = 0.2617$). Picro-sirius red staining showed a trend of lower mean collagen type I content in the summer compared to both the spring and fall seasons (Fig. 2.17). Spring and fall mean collagen type I content were relatively similar, with a slight increase in the fall, likely due to the higher variation between fall samples.

2.2.6 Western blot analysis

The western blots for collagen type I show a significant seasonal effect (Fig. 2.18: One-way ANOVA, $F_{2,14} = 3.970$, $p = 0.0431$). Follow-up Tukey test was not able to discern individual group differences between seasons (Tukey HSD, $p > 0.05$), but there is a trend that the mean differences between seasons may be driven by an increase in the collagen type I content in the spring.

Comparison of mean collagen type I and mean *miR-29b* expression in each season through linear regression found no significant regression slope (Fig. 2.19: Linear regression, slope = -0.7137, $F_{1,1} = 0.066199$, $p = 0.8398$).

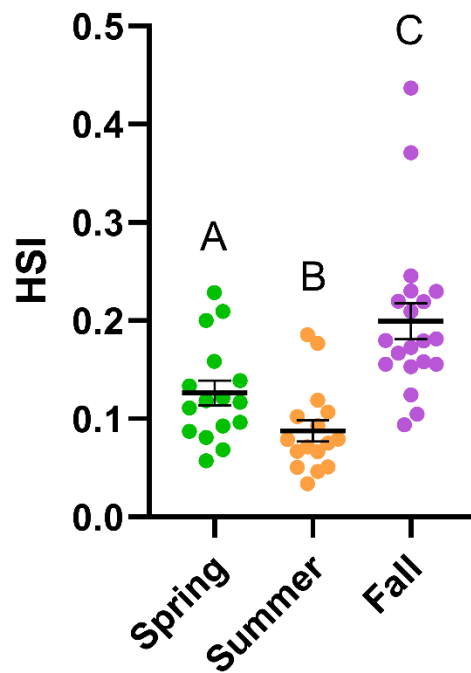


Figure 2.14 Heart somatic index of collected rainbow darters.

Heart somatic index (HrSI) of collected rainbow darters. Gonad mass removed from total body mass in HrSI calculation to remove reproductive state effects on total mass ($\text{HrSI} = [\text{heart mass (g)} / (\text{total mass (g)} - \text{gonad mass (g)})] * 100$). Black bars represent mean with $\pm$ SEM for each group. Analysis through two-way ANOVA to test for season and sex effects. Data was log transformed to meet test assumptions. No significant sex effect was found. Tukey post hoc test performed to find season differences. ($n = 16$ for spring and summer; $n = 20$ for fall).

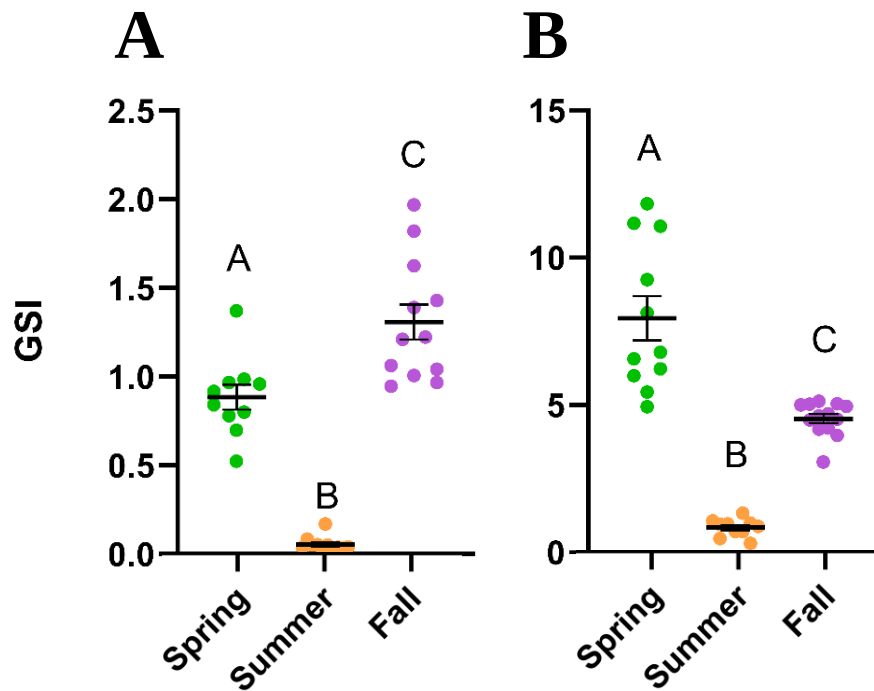


Figure 2.15 Gonad somatic index of collected male and female rainbow darters.

Gonad somatic index (GSI) for male (A) and female (B) rainbow darters. Black bars represent mean with $\pm$ SEM for each group. Analysis through one-way ANOVA followed by Tukey post hoc test. One outlier was removed from GSI in the female during summer (Grubbs test, $G = 2.993$, $\alpha = 0.05$). Data was sine transformed and log transformed for male GSI and female GSI, respectively, to meet test assumptions. ($n = 10$ for spring and summer for each sex; $n = 12$ for fall for each sex).

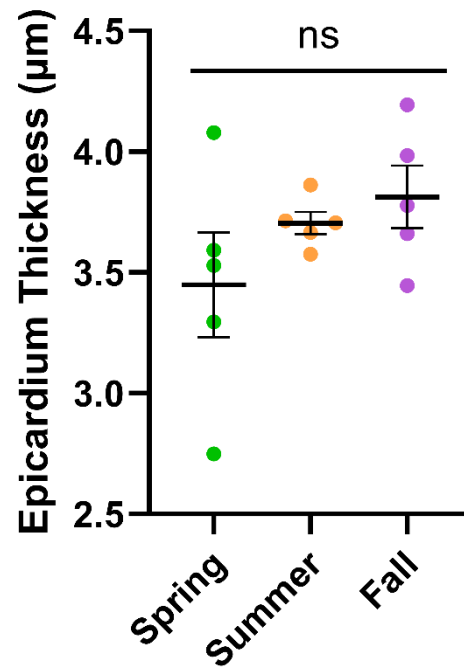


Figure 2.16 Epicardium thickness of rainbow darter hearts.

Epicardium thickness (μm) of the rainbow darter heart tissue. Black bars represent mean $\pm$ SEM for each group. Analysis through one-way ANOVA. Sexes combined due to low available tissue. One sample in summer was removed, as it was determined to be an outlier (Grubbs test, $G = 2.032$, $\alpha = 0.05$). Data was Y^2 transformed to meet test assumptions. ($n = 5$ for all seasons).

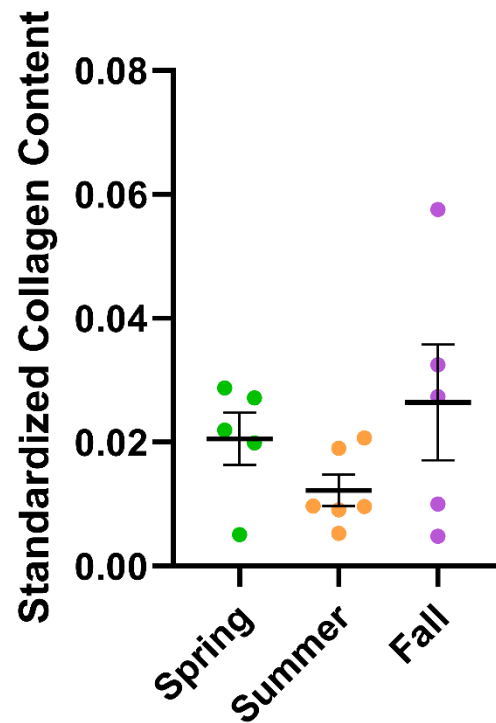


Figure 2.17 Histological collagen protein content in rainbow darter heart tissue.

Collagen type I area (μm^2) in rainbow darter cardiac muscle stained with picro-sirius red stain, standardized to total section area. Picro-sirius red stain binds to collagen fibres, colouring them a deep red. Quantity of red in the section is used to determine total collagen content compared to the rest of the section. Separated Hue image components revealed deep red colours as hue values of 221-256.

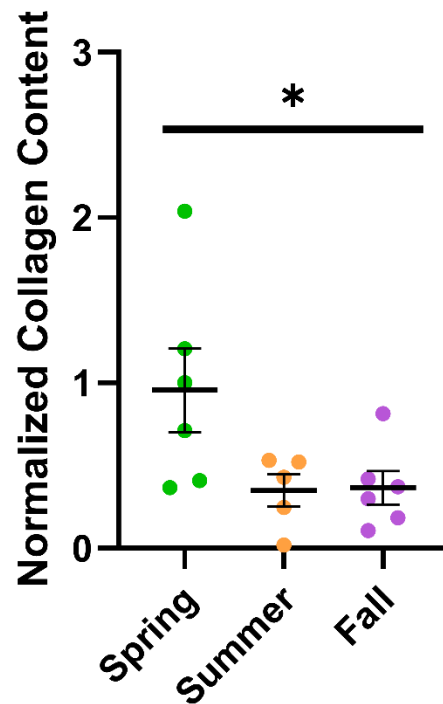


Figure 2.18 Western blot seasonal collagen type I content in rainbow darter heart tissue.

Collagen type I content determined by western blot in the rainbow darter heart in spring, summer, and fall, normalized to protein on the gel images. Black bars represent the mean with $\pm$ SEM for each group. Analysis through one-way ANOVA with follow-up Tukey post hoc test. Male and female sexes were combined in each group due to low sample number. One sample was removed from summer as it was determined to be an outlier (Grubbs test, $G = 1.983$, $\alpha = 0.05$). Statistically significant season effect denoted with asterisk (*). ($n = 6$ for spring and fall; $n = 5$ for summer).

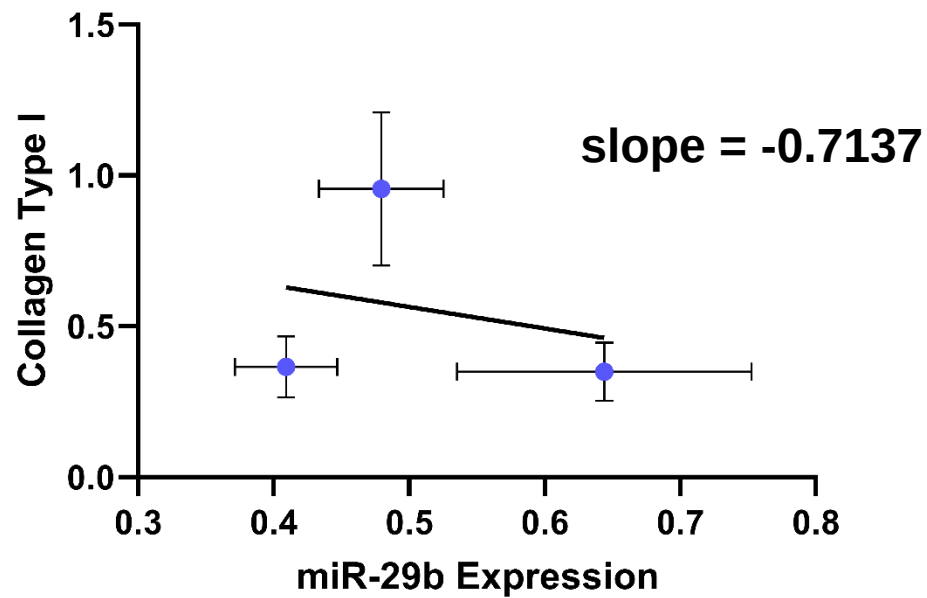


Figure 2.19 Regression of miR-29b & collagen type I.

Regression of *miR-29b* expression and collagen type I protein determined from western blot. Season means and $\pm$ SEM (black bars) for expression and protein used for each of the three points. Slope of regression line = -0.7137 ($p > 0.05$).

Chapter 3

Discussion

3.1.1 River Temperature Analysis

River water temperature across the three seasons saw very different temperature profiles (Fig 2.4, Fig. 2.5, & Fig. 2.6). Unsurprisingly, water temperature fluctuated daily, with fluctuations matching closely with the changes in air temperature. However, it was interesting to observe that the average water temperature of the recorded summer temperatures in August was 0.5°C lower than the average water temperature in early June. Evidently, this is only a single year of measurements, and it would be expected that average summer temperatures could be quite a bit higher than average spring temperatures, especially during El Niño years, which could increase in frequency with climate change (McPhaden et al., 2020; Sandweiss & Maasch, 2022). It is also interesting to note the much lower variation in fall temperatures, having only average water temperature fluctuations of 1.7°C. This demonstrates that not only are there different average water temperatures across seasons, but also overall different season water temperature fluctuation profiles. This adds another layer of interest in understanding how seasons relate to temperature profiles.

It is worth noting that the observed water temperature fluctuations in this study represent a different seasonal temperature profile than the use of static temperature used in previous studies examining the heart remodeling response (Johnson et al., 2014; Jørgensen et al., 2014; Keen et al., 2016, 2018). The absolute difference in these static temperatures between treatment groups within these lab trials approaches the bounds of the fluctuations observed in the spring and summer seasons of this study (Johnson et al., 2014; Jørgensen et al., 2014; Keen et al., 2016, 2018). For example, Keen et al., (2016) utilized temperatures of 5°C ± 1 for cold treatment, 10°C ± 1 for control treatment, and 18°C ± 1 for warm treatment for their rainbow trout cold and warm acclimation study. Similarly,

Johnson et al., (2014) used 27°C for control and 20°C for cold treatment in their zebrafish cold acclimation study and Jørgensen et al., (2014) use 14°C for control and 19°C for warm treatment in their Atlantic salmon study. Observed Grand River daily temperature fluctuations reached as high as 8.5°C and 5.5°C in spring and summer, respectively. With two-week average fluctuations at 6.8°C and 3.9°C, respectively, it is evident that water temperatures in shallow river systems, such as the Grand River, will consistently oscillate between the treatment temperatures of those past studies. It remains to be studied whether such large swings in temperature would impact a longer-term acclimation/acclimatization heart remodeling response since the fluctuations occur on a relatively short time scale. However, it is clear that these are not insignificant changes in temperature and that current lab methods for representing season in the study of a proposed seasonal response may be insufficient for capturing the true thermal nature of those seasons. Therefore, fluctuating temperatures should be examined further in more controlled settings (Turko et al., 2023).

3.1.2 Gene Expression

The alignment of the 3'UTR of *col1a1*, *col1a2*, and *col1a3* all demonstrate that *miR-29b* has a high likelihood of being able to target those mRNA transcripts (Fig 2.9), however no seasonal changes in *miR-29b* expression were observed. The sex effect observed (Fig 2.10) was largely driven by higher variation in the females during the summer (see Appendix Fig A.4 for an alternate graphical view), so it is difficult to determine what the significance of the sex difference may be. Testosterone has been demonstrated to be pro-hypertrophy in trout (Davie & Thorarensen, 1997) and estrogen to be anti-hypertrophy in mammalian models (Babiker et al., 2002; Behrendt, 1977; Schaible et al., 1984), in the context of cardiac hypertrophy, however given that *miR-29b* is linked with collagen remodeling and not cardiac hypertrophy (even though both often occur together), the hormone effects exhibited in those studies likely don't translate to microRNA. Whether either hormone influences

miR-29b would have to be investigated further. There has been evidence in mammal models of effects of testosterone on microRNA expression (Chodari et al., 2017) but it is also possible that life history differences between male and female rainbow darters, perhaps linked to reproduction, could be a stronger driver of these sex differences.

Observed differences in collagen type I gene expression (Fig 2.8) suggest possible changes in collagen type I content in the cardiac tissue of the rainbow darter across seasons. The primary differences are lower overall expression of *col1a1* in the summer and lower expression of *col1a2* in the fall (Fig 2.8A, B). Given that all three collagen type I transcripts are required for fish to produce the collagen type I protein, a reduction in any of the transcripts would theoretically result in less protein. Therefore, the expression data would predict that there would be lower collagen type I protein in the summer due to a decrease in *col1a1* expression and lower protein in fall due to a decrease in *col1a2*. However, gene expression does not necessarily reflect protein abundance, due to the numerous post-transcriptional and translational processes prior to producing a functional protein. Interestingly, *col1a3* saw no significant difference in expression across seasons. This transcript is of particular interest due to it being unique to fish and, therefore, has been proposed as a mechanism to regulate removal of collagen from the heart tissue (Johnston et al., 2019; Keen et al., 2016). As discussed previously, mammals lack the ability to remove heart collagen, which leads to fibrosis pathologies, and they also lack the *col1a3* transcript (Keen et al., 2018; Pöschl et al., 1988; Saito et al., 2001). This a primary reason *col1a3* was proposed as a mechanism for fish cardiac collagen removal (Keen et al., 2018), since *col1a3* was most responsive to temperature and *miR-29b* effects compared to the other two collagen type I mRNA transcripts (Johnston et al., 2019; Keen et al., 2016). However, there are likely a combination of mechanisms, including the TGF- β 1 pathway described previously, that enable collagen removal in the heart. The exact reason why no *col1a3*

effects were observed in the rainbow darter is unknown, but it could suggest a different mechanism of regulation in these fish or the possibility that the sampling time at each season timepoint missed the ‘window’ of gene expression change responsible for the changes in heart collagen. It is also possible that *miR-29b* is acting on other collagen transcripts, as it has been predicted to target *col4a4* (Johnston et al., 2019) and has been demonstrated to alter expression of COL3A1 and COL4A2 proteins in mice (Abonnenc et al., 2013). Additionally, it has been demonstrated to alter expression of MMP2 and TIMP2 proteins in mice (Abonnenc et al., 2013), suggesting that *miR-29b* could act on collagen removal more indirectly than targeting its specific mRNA transcripts.

It has not yet been fully explored when gene expression changes occur for this acclimatization response. Acclimation studies in the lab range from 4 weeks to several months, meaning the window of expression changes could be easy to miss (Johnson et al., 2014; Jørgensen et al., 2014; Keen et al., 2016, 2018). Additionally, rainbow darter heart samples in this study were collected in the later part of each season, meaning there was a higher likelihood of missing the ‘window’ of gene expression. This could explain why there was unexpectedly no seasonal difference in *miR-29b* expression. If *miR-29b* is involved in the removal of collagen mRNA transcripts during this cardiac remodeling, it would be expected to be most active during the gene expression window. Regression analysis of *miR-29b* and collagen type I mRNA demonstrated that there is no observed relationship between them at the time of sampling, as there would have been an expected inverse relationship present given that microRNAs reduce the abundance of their target transcripts (Bartel, 2004).

There is no current literature that examines the *in vivo* function of *miR-29b* with respect to this cardiac response, so it is also plausible that it does not act on collagen type I transcripts in this particular context. Given the alignment of *miR-29b* with the collagen type I transcripts, even if *miR-*

29b is not involved in this response, it likely is involved with collagen type I in other situations (Johnston et al., 2019). Either way, more focused studies are needed to discern the true role of *miR-29b* on collagen type I in the fish heart.

3.1.3 Heart Morphology

Histological analysis was able to show that the rainbow darter does not appear to have a compact myocardium layer, as only the epicardium and a uniform myocardium (resembling spongy myocardium) were present in any of the sections. This information has yet to be documented in the literature due to very little research on rainbow darter heart physiology or morphology. The lack of a compact myocardium was expected since the rainbow darter is a benthic fish and the compact myocardium layer it is typically reserved for more athletic fish (Farrell, 1996).

There was evidence of cardiac hypertrophy in both the spring and fall based on HrSI measurements (Fig 2.14). Cardiac hypertrophy at colder temperatures has been widely documented in fishes (Farrell, 1987; Farrell et al., 1988; Goolish, 1987; Graham & Farrell, 1989), so these findings are not surprising. Cold temperatures cause the blood to become more viscous, so the heart must compensate by increasing muscle mass to handle the increased stress on the heart tissue (Graham & Farrell, 1989). Given the removal of gonad weight from total body weight for the HrSI calculations, these results demonstrate that changes in reproductive state on body mass was not the main driver of HrSI differences. GSI for both males and females showed significant seasonal differences (Fig 2.15) which aligned with their reproductive state and visual observations made during sampling. Females had the largest gonads in the spring, followed by the fall, which is consistent with rainbow darter gonad development for the spring spawning season (Hicks et al., 2023). Gonad size is known to increase again in fall before the winter (Hicks et al., 2023). Male GSI in the fall was quite high compared to the other seasons, but the testes are very small, so the odds of inaccurate measurements

in the males is much higher than the females, which may explain this discrepancy. Overall, these GSI measurements show the collected darters were in their expected reproductive cycles.

The trends of the cardiac hypertrophy observed also follow a logical pattern based on the expected average temperatures leading up to the sampling dates, with the highest heart mass being in fall and the lowest being in summer. While the average spring temperature recorded (Fig 2.4) was very similar to that of summer (Fig 2.5), it would be expected that temperatures in the weeks and months before the end of May would have been noticeably colder than those in the summer. This would mean that cardiac hypertrophy during the spring may be the lingering result of environmental temperature effects from the previous colder season, with the extra size then being lost in the warm summer months. The fall experienced the coldest temperatures of any of the collected fish, so it is logical that those fish would have undergone the most cardiac hypertrophy.

Epicardium measurements showed no significant change between seasons despite evidence of cardiac hypertrophy. This is consistent with the findings of Procopio et. al. (2024) in the pumpkinseed sunfish, which experienced collagen remodeling and cold temperature hypertrophy but no changes in epicardium thickness. This further supports that in fish that lack a compact myocardium, the role of the epicardium does not function like that of the compact myocardium during hypertrophy, as was initially proposed by Procopio et al. (2024) but was then dismissed after finding no evidence of epicardium change themselves either.

3.1.4 Collagen Type I Protein Analysis

Histological analysis showed differing trends in collagen content in the heart tissue, although there was a considerable amount of variation in the data, which may have been caused by sectioning issues that arose due to the tissue being noticeably brittle from tissue processing. This led to more focus being placed on the western blots to determine collagen content, especially as the blots related

to collagen type I specifically. Through western blot analysis, collagen type I content in the rainbow darter heart was found to be significantly different across seasons. While exact season differences could not be determined statistically, it is evident that much of the seasonal differences are driven by an increasing trend in mean collagen in the spring. Between the spring and summer collection timepoints, collagen content was reduced in the heart tissue, which could signify that full remodeling from winter to summer had not occurred at the time of spring sampling. Additionally, the similar collagen levels between summer and fall could indicate that the remodeling for winter had not yet started, especially since the onset of colder temperatures had only just begun by October 31. Therefore, it appears that the rainbow darter may share a similar collagen remodeling profile to the rainbow trout, where colder temperatures cause increased collagen, and warmer temperatures cause decreased collagen (Fig 1.2) (Keen et al., 2016; Klaiman et al., 2011), however more investigation into the specific seasonal collagen changes is required. Additionally, comparison with the pumpkinseed sunfish reveals yet another instance of a differing collagen response in relation to season. The pumpkinseed sunfish experienced lower collagen during cold seasons and higher collagen during warm seasons (Procopio et al., 2024), which is the opposite of the possible response observed in the rainbow darter. This demonstrates that there may be differing environmental pressures on these two species that trigger these opposing responses. The two populations investigated by Procopio et al. (2024) displayed collagen remodeling despite differing pressures of seasonality, which appeared to impact cardiac hypertrophy responses. It is clear that further investigation is needed regarding the conditions these organisms experience that could potentially drive this response. See Figure 3.1 for the current summary of heart collagen remodeling.

The regression analysis performed between mean collagen type I content and mean *miR-29b* expression at the season timepoints did not reveal any trend (Fig. 2.19). This is unsurprising given

that no season effect was found independently with *miR-29b* expression, and no significant linear regression was observed between *miR-29b* and the collagen mRNAs. Despite this, it was still interesting to see if there was any link between overall protein content and the microRNA expression, particularly if *miR-29b* was acting on collagen removal more indirectly through regulation of MMPs/TIMPs, but it is evident that further study is needed with respect to that question.

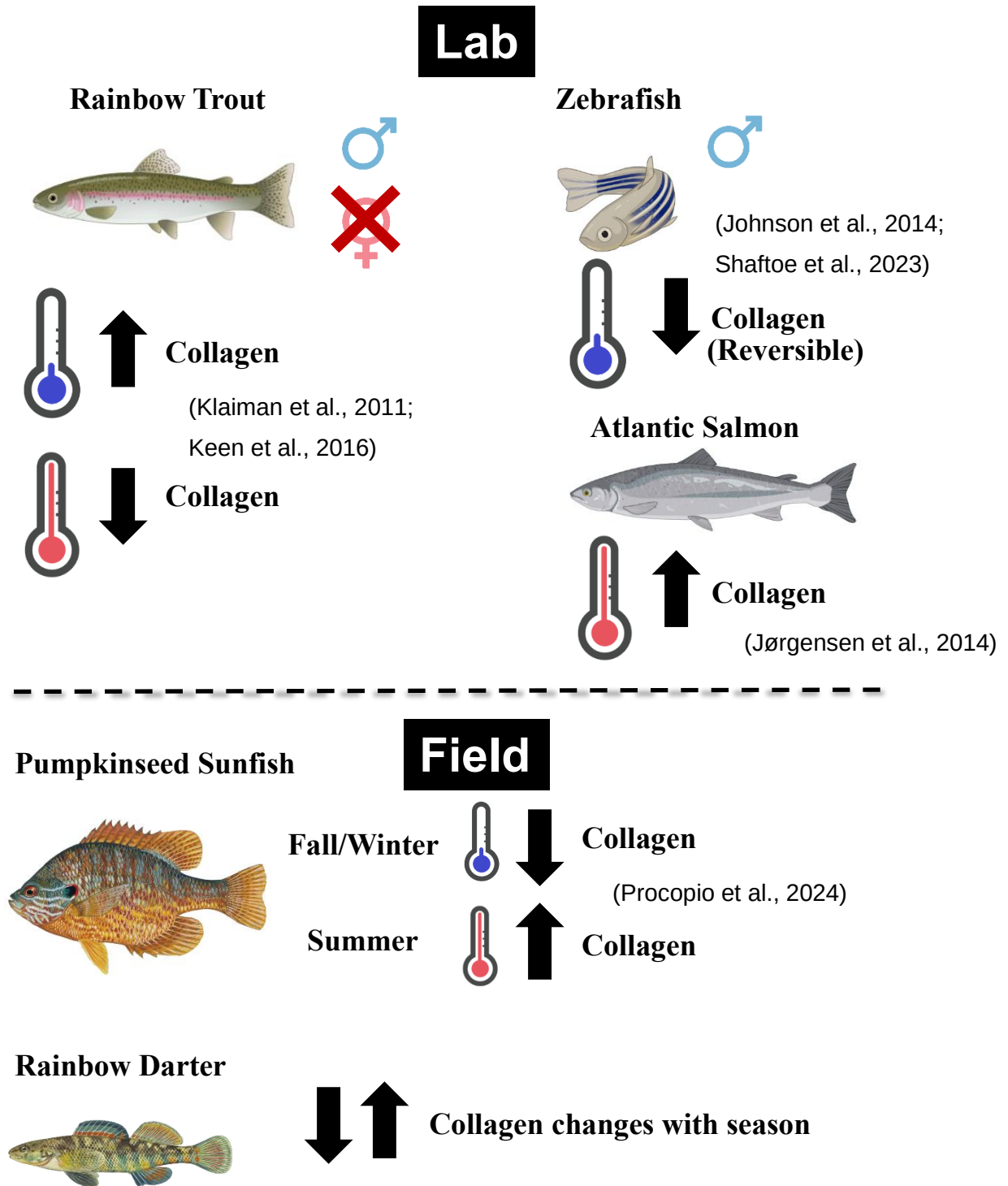


Figure 3.1 Summary of heart collagen remodeling response.

Overview of heart-collagen remodeling response in lab and field studies, including the response of the rainbow darter observed in this study.

3.1.5 Environmental Perspectives

Much like the study on the pumpkinseed sunfish, studying organisms in the natural environment comes with drawbacks. In the context of this study, it is difficult to separate the many biotic and abiotic factors that could have contributed to the results observed. For example, while temperature is a significant part of seasonality, photoperiod (Evans, 1984), dissolved oxygen (Marques et al., 2008), and maturation (Franklin & Davie, 1992) could all be responsible for triggering and influencing this remodeling response. However, based on previous controlled lab studies, it is evident that temperature still does play an important role in this response and temperature effects are highly likely to be the main reason for why these remodeling changes are needed even if they are not ultimately the trigger. Therefore, there is a good likelihood that temperature changes observed in this study did influence the results observed.

These results were also obtained from a single population of rainbow darters. While this study is still representative of the species, wild habitats offer very different environments and pressures. The pumpkinseed sunfish investigated by Procopio et al. (2024) were from two separate populations that had differing historical experiences with seasonality, and this was found to result in different observations in cardiac hypertrophy responses. While rainbow darter populations across the Grand River, ON would not have significantly differing exposure to seasonality, rainbow darter population comparisons have been common practice with respect to anthropogenic effects on physiology, particularly those caused by wastewater effluent and climate change (Gauvreau et al., 2022; Robichaud & Craig, 2022; Tetreault et al., 2014). If the effects of said anthropogenic effects on cardiac remodeling were to be investigated, the rainbow darter would be a good model.

While these drawbacks are caused by the complicated systems of a natural environment, the strengths of this study also are derived from those same systems. This remodeling mechanism has

been proposed as a seasonal response, but with limited studies in the natural environment, it has been difficult to fully understand its triggers and drivers. Adding more studies, such as the one present, helps give insight into whether our conclusions from the lab are supported in the context for which they are being applied to.

3.1.6 Conclusions and Future Directions

This study demonstrates that the rainbow darter has seasonal changes in the collagen type I content of its cardiac tissue. Spring appears to have a trend of more collagen than summer and mid fall, which could signify that colder temperatures in the winter require more collagen and warmer temperatures in the summer require less collagen. *MiR-29b* needs to be further investigated since no seasonal effect was observed, indicating that the window of its involvement may have been missed or its interactions with *col1a1*, *col1a2*, and *col1a3* may occur in contexts beyond heart remodeling since alignment predictions indicate interactions between those transcripts. Evidence of cold temperature cardiac hypertrophy is consistent with the literature and in conjunction with the lack of a compact myocardium layer, provides documentation of heart morphology not previously investigated in the rainbow darter.

This study is now one of very few that examines heart-collagen remodeling in the natural environment. Based on recorded temperature data, there are very clear differences between the season temperature profiles of a natural habitat and the lab. In particular, fluctuating temperatures are prevalent in most of the seasons, which could have significant impacts on the trigger and progress of the remodeling response. In future, it would be prudent to continue to investigate this response in parallel between the lab and natural environment, so lab-based hypotheses can be tested to see if their predicted conclusions match the actual responses of a complex ecosystem. Furthermore, results from natural studies can inform the controlled experiments designed in lab. For example, examining the

effects of a fluctuating temperature system in the lab would be an interesting study because it could help tease apart the mechanisms and triggers that underlie this response. This is not to say that past lab studies are inaccurately demonstrating the heart remodeling response, but that combining both lab and field studies will allow for the combined strength of both approaches while also being able to cover many of their inherent weaknesses.

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Appendix A

Table A.1 Two-way ANOVA F-statistics for gene targets.

Targets/Measurement	Two-way ANOVA [F (DFn, DFd) = F-ratio, p-value]		
	Season	Sex	Season x Sex
<i>col1a1</i>	F (2,46) =8.972; p=0.0005	F (1,46) =0.2600; p=0.6126	F (2,46) =0.01072; p=0.9893
<i>col1a2</i>	F (2,46) =4.460; p=0.0170	F (1,46) =0.07156; p=0.7903	F (2,46) =2.353; p=0.1064
<i>col1a3</i>	F (2,46) =0.5122; p=0.6025	F (1,46) =0.7446; p = 0.3927	F (2,46) =0.4804; p=0.6216
<i>miR-29b</i>	F (2,46) =2.329; p=0.1088	F (1,46) =8.358; p=0.0058	F (2,46) =2.051; p=0.1402
<i>HrSI</i>	F (2,46) =20.99; p<0.0001	F (1,46) =0.5391; p=0.4665	F (2,46) =2.398; p=0.122

*Note: DFn= degrees of freedom numerator; DFd= degrees of freedom denominator ($\alpha=0.05$), significant results are shown in bold.

Table A.2 One-way ANOVA F-statistics for histological analysis, collagen, and HrSI.

One-way ANOVA [F (DFn, DFd) = F-ratio, p-value]	
Measurement	Season
<i>Epicardium thickness</i>	F (2,12) =1.502; p=0.2617
<i>Western blot collagen type I</i>	F (2,14) =3.970; p=0.0431
<i>GSI (male)</i>	F (2,30) =324.9; p<0.0001
<i>GSI (female)</i>	F (2,31) =158.1; p<0.0001

**Note: DFn= degrees of freedom numerator; DFd= degrees of freedom denominator ($\alpha=0.05$), significant results are shown in bold.*

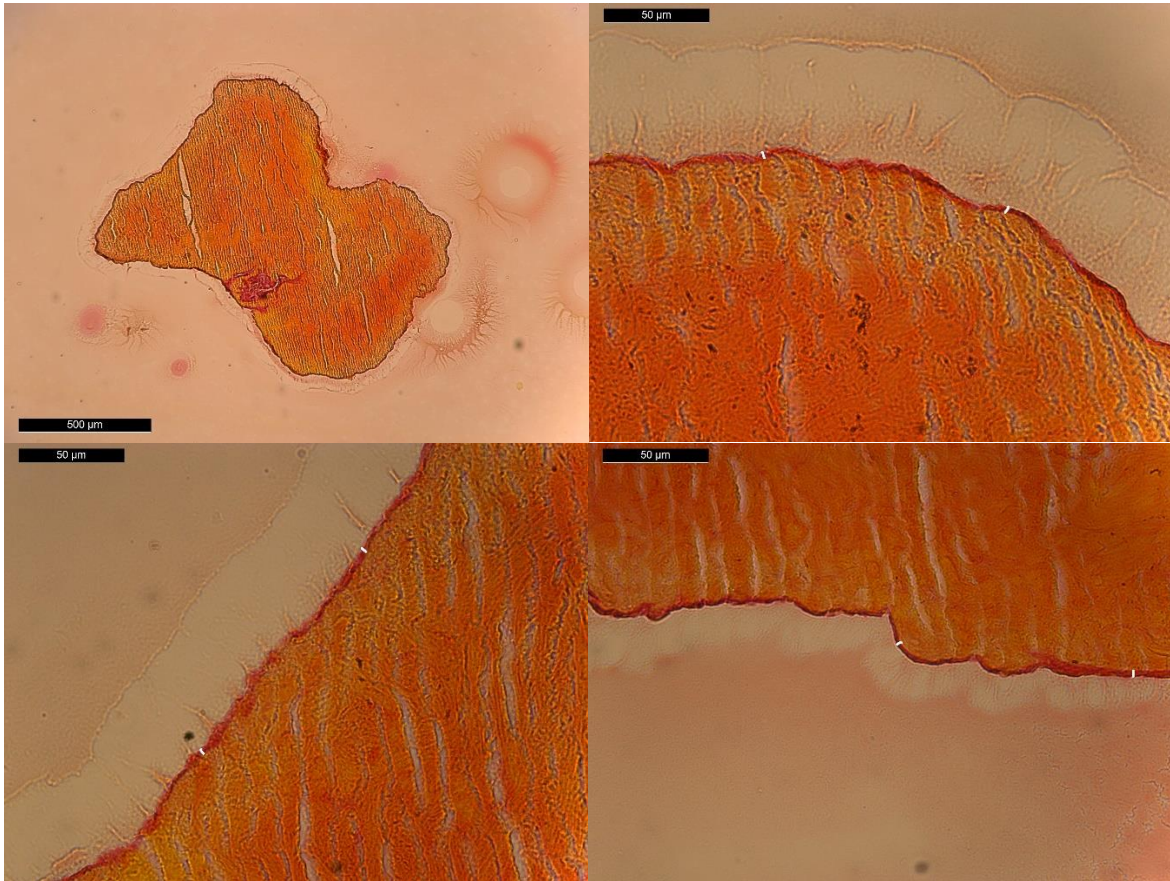


Figure A.1 Spring season histology collagen and epicardium measurements.

Measurements of rainbow darter heart collagen (through picro-sirius red staining) and epicardium thickness from tissue sections during the spring season (May 29, 2023 sampling date) ($n = 5$). Collagen content was determined through relative red hue (hue range 221-256 (Rich & Whittaker, 2005; Shaftoe et al., 2023)) compared to tissue area (50X magnification). Epicardium thickness determined by averaging six random epicardium measurements (Procopio et al., 2024) denoted by white lines (400X magnification).

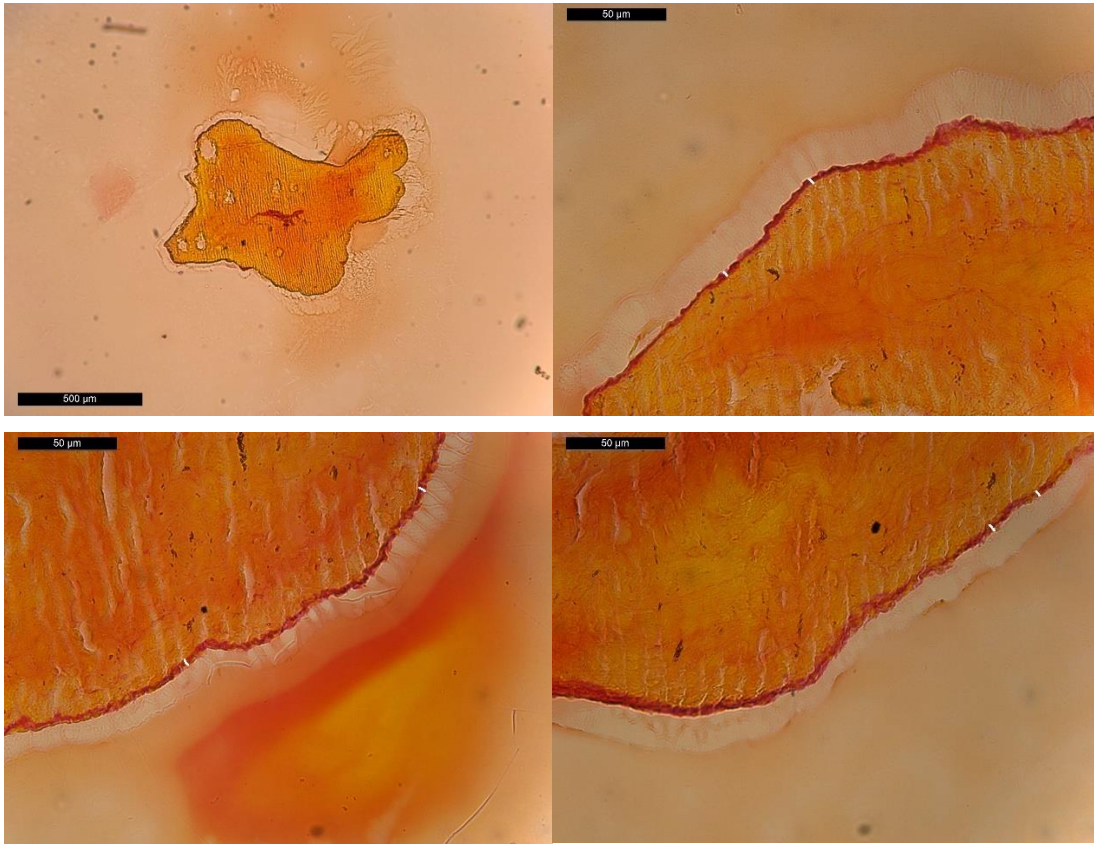


Figure A.2 Summer season histology collagen and epicardium measurements.

Measurements of rainbow darter heart collagen (through picro-sirius red staining) and epicardium thickness from tissue sections during the summer season (August 22, 2023 sampling date) ($n = 6$). Collagen content was determined through relative red hue (hue range 221-256 (Rich & Whittaker, 2005; Shaftoe et al., 2023)) compared to tissue area (50X magnification). Epicardium thickness determined by averaging six random epicardium measurements (Procopio et al., 2024) denoted by white lines (400X magnification).

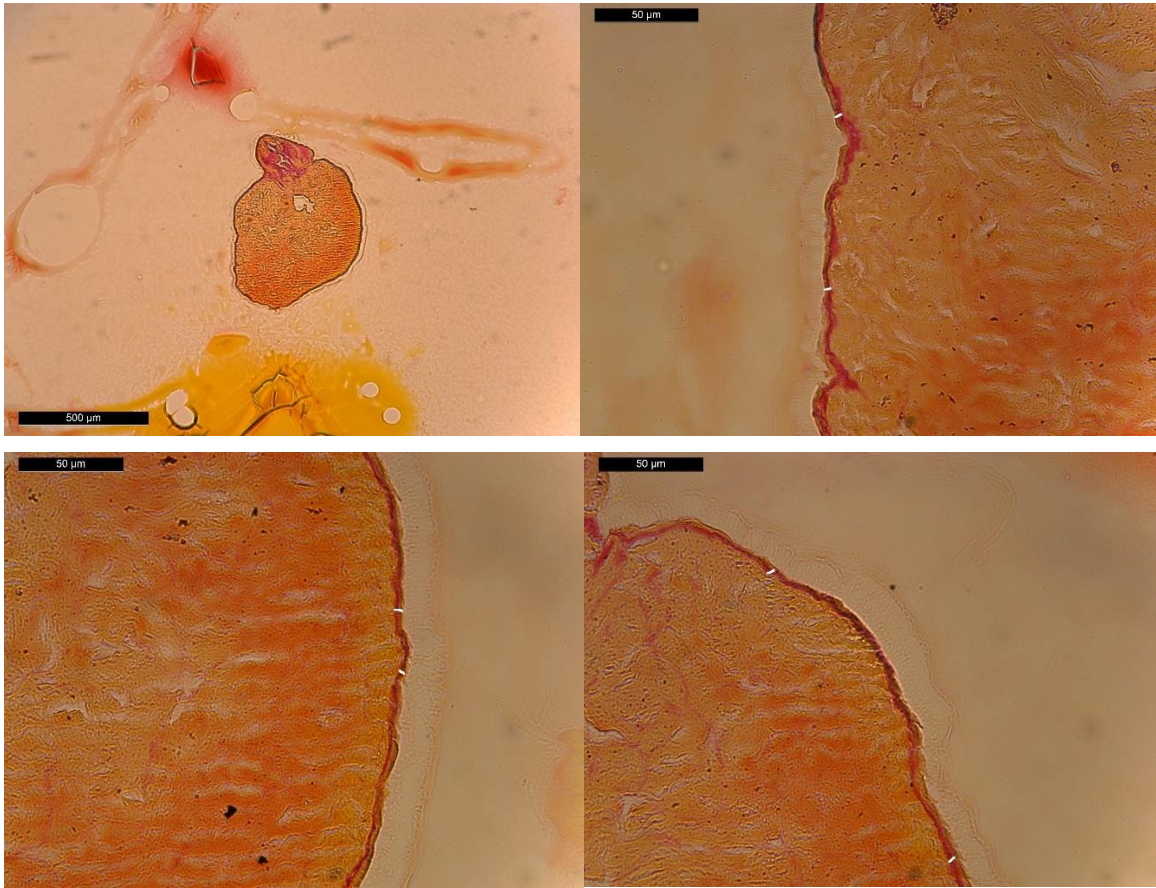


Figure A.3 Fall season histology collagen and epicardium measurements.

Measurements of rainbow darter heart collagen (through picro-sirius red staining) and epicardium thickness from tissue sections during the fall season (October 31, 2023 sampling date) ($n = 5$). Collagen content was determined through relative red hue (hue range 221-256 (Rich & Whittaker, 2005; Shaftoe et al., 2023)) compared to tissue area (50X magnification). Epicardium thickness determined by averaging six random epicardium measurements (Procopio et al., 2024) denoted by white lines (400X magnification).

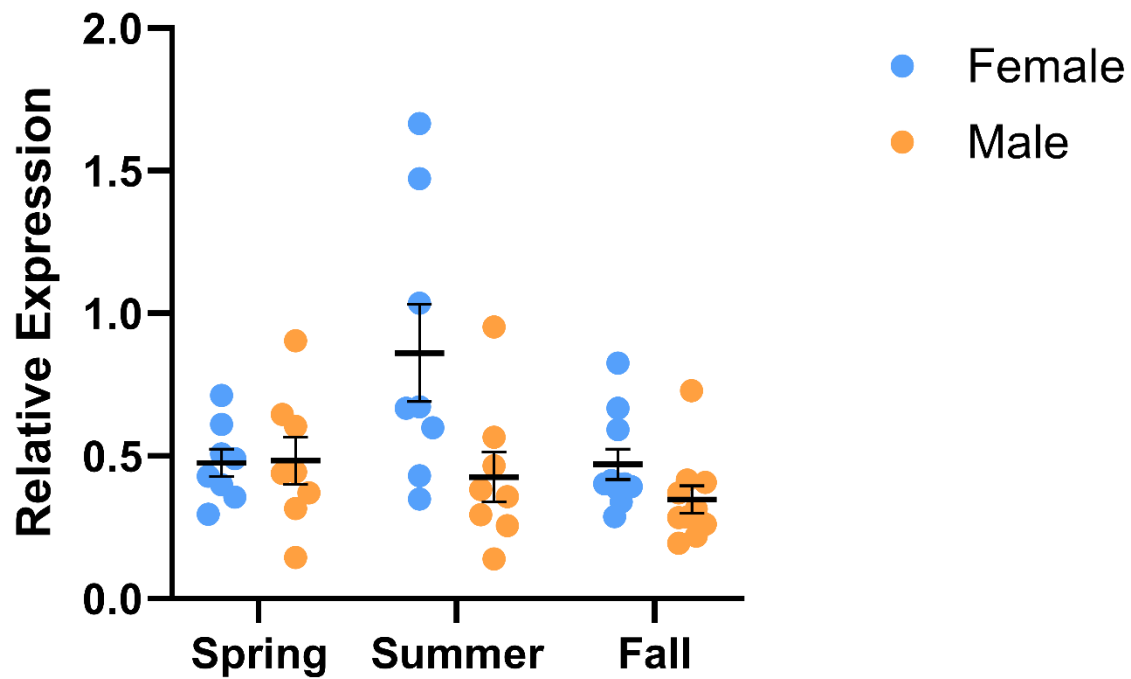


Figure A.4 Alternate miR-29b expression graph depicting sexes separated.

Gene expression of *miR-29b* shown with the sexes separated in each season to show the differences in variation in each group. Statistics are not shown but are the same as in Figure 2.10. Black bars represent mean $\pm$ SEM for each group.

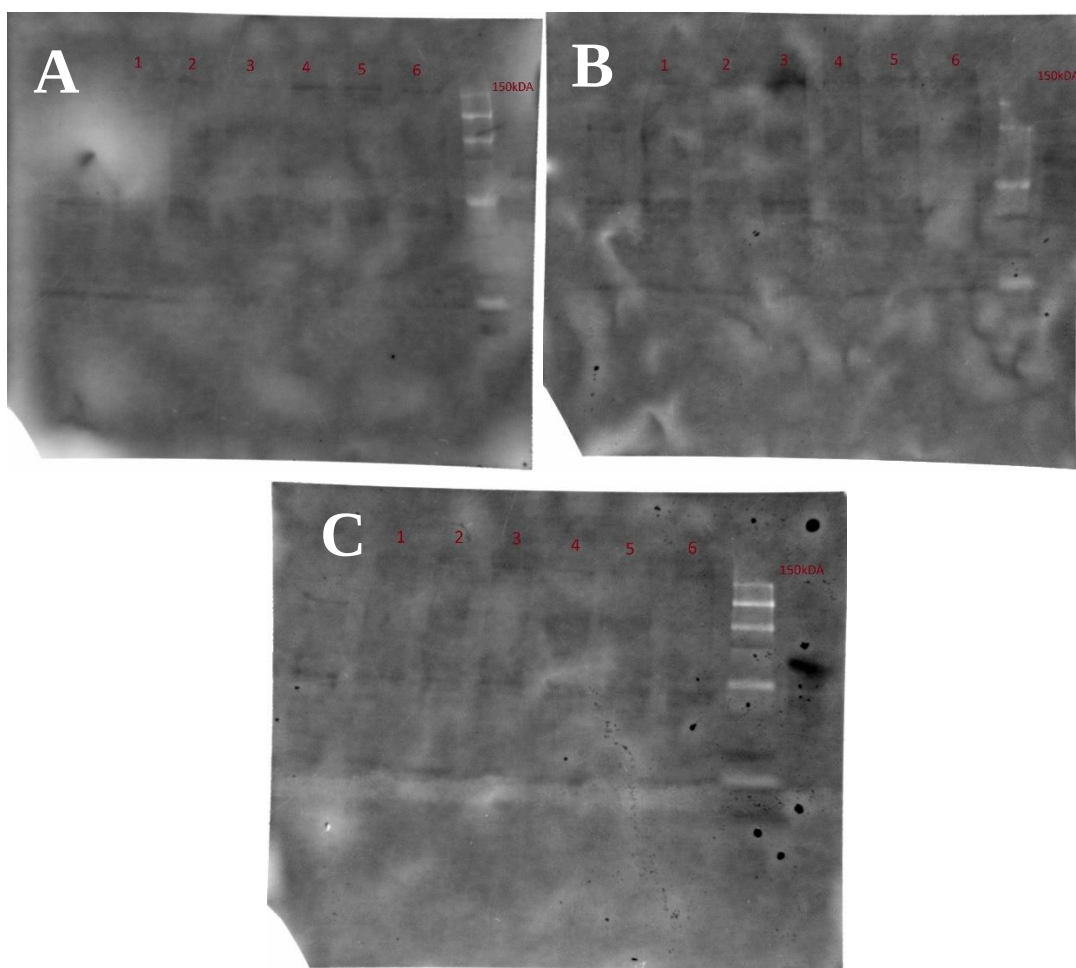


Figure A.5
collagen type I.

Western blot analysis of

Western blots measuring collagen type I (150kDa) in the rainbow darter heart in spring (A; n = 6), summer (B; n = 5), and fall (C; n = 6). Numbers denote the sample in each lane. Sample #3 in summer was removed as it was determined to be an outlier through statistical testing (Grubbs test, $G = 1.983$, $\alpha = 0.05$) and visual inspection of the blot shows a high degree of 'noise' around the band.