

# Cardiac Remodelling in Thermally Acclimated Fish

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“You were made to soar, to crash to earth, then to rise and soar again.”

Alfred Wainwright (1907 –1991)

*Photo taken by Mike Fenna Jan, 2012*

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## ABSTRACT

Fish are subject to a variety of long and short term environmental and physical insults during their life; however they manage to adapt, ensuring physiological processes remain effective, enabling the animal to thrive in a wide range of conditions.

One major environmental fluctuation that can occur rapidly or over a long period of time is temperature. Teleost fish, such as the rainbow trout (*Oncorhynchus mykiss*) are ectothermic, meaning their body temperature is regulated by environmental temperature which can affect activity levels, oxygen availability, biochemical reactions, biophysical processes, and importantly, circulatory demand and cardiac output.

Rainbow trout heart is a relatively simple structure consisting of a single atrium and a single, double layered ventricle in an enclosed circulatory system. Cardiac output in the trout is largely intrinsically regulated in the short term by stretch, which modulates stroke volume.

Long term acclimation or adaptation to cold temperature in trout has been previously shown to cause a type of cardiac remodelling, cardiac hypertrophy: an increase in muscle mass of the heart. The increase in muscle mass is thought to mitigate against the increase in blood viscosity in cold conditions.

The aim of the present study was to further characterise this cardiac remodelling by using histological, immuno and genetic techniques to assess temperature dependent changes in the ventricle and atrium at the structural, proteomic and genetic levels, including defining the potential triggering events and mechanisms behind the changes.

Trout were acclimated to three different temperatures. There was extensive temperature dependent, chamber specific remodelling of the muscle, connective tissue, and gap junction conduction channels. Atrial changes largely opposed ventricular responses. Cold acclimation was associated with spongy layer hypertrophy, increased collagen throughout, and atrophy of the atria. The reverse was true in warm acclimation. Genetic and proteomic analysis revealed there to be significant muscle (e.g. VMHC and MLP) and collagen (e.g. MMP13 and TIMP2) gene regulation coupled with temperature dependent changes in gap junction levels (e.g. Cx43 and Cx30.9) and distribution. Moreover, markers for stretch (e.g. ANP and BNP), stress (e.g. GR1 and DNAJ2) and neuro-hormonal input (e.g. Beta-AR2 and Beta-AR3b), coupled with significant cell signalling protein activation (e.g. p38MAPK and PKB) revealed extensive cardiac remodelling from trigger to output.

Results from this study led to the conclusion that temperature acclimation causes extensive structural remodelling of the heart in order to satisfy the circulatory demand requirements of the animal in extremes of temperature. Cold fish are relatively inactive as shown by decreases in VEGF, and the heart remodels to pump blood around the body at low pressure, as shown by decreased compact layer thickness and atrophy of the atrium, and at low ejection volume, as shown by increases in stiffening collagen and spongy layer hypertrophy. A key trigger for the remodelling response is likely to be the degree of stretch the heart muscle is subject to, probably due to increased blood viscosity as shown by increases in stretch markers ANP and BNP in cold ventricle. To help ensure electrical harmony, gap junction remodelling occurs, as shown by significant changes in connexins in acclimated hearts. Due to structural observations and observed activation of signalling pathways such as GSK/PKB/NFAT, it was concluded that cardiac remodelling in rainbow trout shares similarities with physiological eccentric cardiac hypertrophy in mammals.

## ABBREVIATIONS

<b>AC</b>	Adenylate cyclase
<b>AKT</b>	Protein Kinase B
<b>ANG</b>	Angiotensin
<b>ANOVA</b>	Analysis of variance
<b>ANP</b>	Atrial natriuretic peptide (ANP)
<b>AP</b>	Action potential
<b>ATP</b>	Adenosine triphosphate
<b>AU</b>	Arbitrary unit
<b>AV</b>	Atrioventricular
<b>BLAST</b>	Basic logical alignment search tool
<b>BNP</b>	Basic natriuretic peptide
<b>BSA</b>	Bovine serum albumin
<b>CamK</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>CBE</b>	Creb response element
<b>cDNA</b>	Complementary DNA
<b>cGMP</b>	Cyclic guanosine monophosphate
<b>CIAKP</b>	Calf intestinal alkaline phosphatase
<b>CICR</b>	Calcium induced calcium release
<b>Col</b>	Collagen
<b>CTMax</b>	Maximum critical temperature
<b>Cx</b>	Connexin
<b>DAG</b>	Diacylglycerol
<b>DNA</b>	Deoxyribonucleic acid
<b>DNAJ2</b>	Heat shock protein
<b>dNTP</b>	Deoxyribonucleotide
<b>EBP</b>	Elastin Binding Protein
<b>EBS</b>	Extrabundular space
<b>E-C</b>	Excitation - contraction
<b>ECM</b>	Extracellular Matrix
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>ERG</b>	Delayed rectifier potassium channel
<b>ERK</b>	Extracellular-signal-regulated kinase
<b>ET</b>	Endothelin
<b>FA</b>	Focal adhesion
<b>FAK</b>	Focal adhesion kinase
<b>FGF</b>	Fibroblast growth factor
<b>GAP</b>	GTPase-Activating Protein
<b>GDP</b>	Guanosine diphosphate
<b>GEF</b>	Guanine nucleotide exchange factor
<b>GPCR</b>	G-protein coupled receptor
<b>GR</b>	Glucocorticoid receptor
<b>GSK</b>	Glycogen synthase kinase
<b>GTP</b>	Guanosine-5'-triphosphate

<b>HDAC</b>	Histone deacetylases
<b>HRP</b>	Horse radish peroxidase
<b>ID</b>	Intercalated disc
<b>Ig</b>	Immunoglobulin
<b>IHC</b>	Immunohistochemistry
<b>IL</b>	Interleukin
<b>ILK</b>	Integrated linked kinase
<b>IP3</b>	Inositol trisphosphate
<b>IRES</b>	Internal ribosome entry site
<b>JNK</b>	c-Jun N-terminal kinase
<b>Kda</b>	Kilodalton
<b>LTCC</b>	L-Type calcium channel
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MCIP</b>	Enriched calcineurin interacting proteins
<b>MKK/MEK</b>	Mitogen-activated protein kinase kinase
<b>MKP</b>	MAP kinase phosphatase
<b>MLCK</b>	Myosin light-chain kinase
<b>MLP</b>	Muscle LIM Protein
<b>MMP</b>	Matrix metalloproteinase
<b>MR</b>	Mineralcorticoid receptor
<b>mRNA</b>	Messenger ribonucleic acid
<b>NCX</b>	Sodium calcium exchanger
<b>NFAT</b>	Nuclear factor of activated T-cells
<b>NO</b>	Nitric oxide
<b>PBS</b>	Physiological buffered saline
<b>PCNA</b>	Proliferating cell nuclear antigen
<b>PCR</b>	Polymerase chain reaction
<b>PDE2</b>	Phosphodiesterase 2
<b>PDGF</b>	Platelet derived growth factor
<b>PI3K</b>	Phosphatidylinositide 3-kinases
<b>PIP2</b>	Phosphatidylinositol 4,5-bisphosphate
<b>PKA</b>	Protein Kinase A
<b>PKB</b>	Protein Kinase B
<b>PKC</b>	Protein Kinase C
<b>PKG</b>	Protein kinase G
<b>PLB</b>	Phospholamban
<b>PLC</b>	Phospholipase C
<b>POmax</b>	Maximum power output
<b>Qmax</b>	Maximum cardiac output
<b>qPCR</b>	Quantitative PCR
<b>RCAN</b>	Regulator of calcineurin (MCIP)
<b>RNA</b>	Ribonucleic acid
<b>RT</b>	Reverse transcriptase
<b>RVM</b>	Relative ventricular mass
<b>RyR</b>	Ryanodine receptor
<b>SA</b>	Sinoatrial

<b>SBP</b>	Systolic blood pressure
<b>SEM</b>	Standard error of the mean
	Sarcoplasmic endoplasmic reticulum calcium
<b>SERCA</b>	ATPase
<b>SL</b>	Sarcolemma
<b>SMLC2</b>	Small myosin light chain
<b>SR</b>	Sarcoplasmic reticulum
<b>SRF</b>	Serum response factor
<b>TGF</b>	Transforming growth factor
<b>TIMP</b>	Tissue inhibitor of metalloproteinase
<b>TM</b>	Transmembrane
<b>TRPC</b>	Transient receptor potential channel C
<b>UCRIT</b>	Critical swimming speed
<b>VEGF</b>	Vascular endothelial growth factor
<b>VMHC</b>	Ventricular myosin heavy chain
<b>WB</b>	Western blot
<b><math>\alpha</math>-SA</b>	Alpha skeletal actin
<b><math>\beta</math>AR</b>	Beta adreno receptor
<b><math>\beta</math>-MHC</b>	Myosin heavy chain

# **WORK NOT CARRIED OUT DIRECTLY BY THE AUTHOR**

Some of the work carried out in this thesis fell under the umbrella of collaborative lab output. All fish used in the study were fish that the author personally sourced, delivered, cared for and acclimated. In some cases these fish were used by other members of the lab to generate data used in this study. The author is grateful to Hamid Rizvi for carrying out the collagen staining and to Devmalya Sarkar for undertaking elastin staining. The decision to include this work was for several reasons: (1) The author was directly involved in the generation of data via experiment design, direction, mentoring and guidance (2) Although relatively minor contributions, the data generated is directly relevant to this thesis and aids in the interpretation of overall data (3) Due to the lack of acclimated fish at times when the lab was full, the author sacrificed some of his experiments by donating his own acclimated fish to other people on the basis the data generated could be used in this thesis; this was agreed from the outset by the supervisor. All other work was carried out solely by the author.

## **DECLARATION**

I declare that no portion of the work referred to in the thesis, other than the work mentioned above, has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Andrew James Fenna

Faculty of Life Sciences

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## **ABOUT THE AUTHOR**

Prior to studying for his PhD, Andrew started his career in the Royal Air Force, joining at the age of 18 as an Aircraft Engineer. Andrew left the RAF after 4 years to study for a BSc in Biomedical Science at the University of Sheffield (2003-2006), achieving First Class Honours. Whilst at University in Sheffield, Andrew was awarded a Summer Studentship in the Department for Developmental and Biomedical Genetics with Prof Philip Ingham FRS and Dr Mary Ann Price where he developed a keen interest in cell signalling mechanisms and genetics. Immediately after his BSc, Andrew worked as a Research Technician for a short while at Kings College London before moving into the world of scientific consultancy in the nuclear industry. After 2 years in this role, the academic and research drive within him led to Andrew undertaking a PhD at Manchester University, firstly in a lab looking into MAPK signalling, and then into a lab investigating cardiac remodelling where he remained for 4 years to complete his PhD.

Andrew lives with fiancé Alison in Newton-le-Willows, Merseyside and is very keen on the outdoors, especially the Lake District.

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## ORGANISATION OF THESIS

Below is a succinct description of how the key chapters: *Introduction, Materials and Methods, Results Chapters x 3, General Discussion, References* and *Appendix* are written and organised.

### **Introduction**

Chapter one of the thesis is presented with a long introduction covering, in detail, a literature review and background to the main topics discussed throughout the study and should be considered an over-arching, yet detailed introduction for each of the three results chapters as there is considerable overlap of the topics. Each results chapter contains its own “*Brief Introduction*”, drawing on the main concepts from the introduction and the introduction should be used in reference to these if required.

### **Materials and methods**

Chapter two of the thesis contains a detailed account of the materials and methods used throughout the study and is designed to be a reference for use with the “*General Methodology*” section of the results chapters if more detail is required. It is recommended to start at the “*General Methodology*” of the results chapters before selecting which sections of chapter two are read. There are common experimental procedures carried out in each of the results chapters, which is the main reason this approach was used.

### **Results chapters**

Results chapters are designed to be succinct and concise. A “*Brief Introduction*” contains key information as to the background of the study, what was known previously, a general insight into the logic behind the experimental procedure and what is being investigated and why. Any extra information can be gained from chapter one where a more detailed introduction is discussed. “*Key aims*” are bullet-pointed main goals of the current study

designed to focus the reader's mind on key reasons behind the experiments, concepts and topics in the particular results chapter. "*General methodology*" describes succinctly how the results in the results chapters were generated. They are designed to give a general, yet focussed overview of key procedures used in the study. For more technical information on each of the protocols mentioned see chapter two. "*Results*" are grouped together in subheadings of what is being examined, not concluded. Under each subheading is a succinct description of key, but not entire results related to that particular subheading. Directly underneath the subheading descriptions are the related figures which contain a detailed description of results including full results and statistics generated from that particular experiment. Immediately after the results section is the "*Discussion*" where key findings from the results are first presented in bullet point form, and then discussed either individually or in an over-arching related group context in terms of current knowledge, with the aim of understanding as best as possible what the available data is telling us, ultimately leading to a theory of what is happening. "*Study Limitations*" focus on any factor that could have led to a better interpretation of data or any experiment that would have helped prove or disprove a particular theory and which ideally would have been carried out if time or equipment permitted . A "*Summary*" is provided as brief revisit of the points covered in the discussion. In the "*Conclusions*" section, a concise overview of key results and key theories is presented with the aim of rounding off the study with key ideas that seeks to address and answer the title and key aims of each chapter succinctly. qPCR data in the results sections are presented as cold v warm, normalised to control; please see the "*Appendix*" for a full data set of mRNA data.

## **General Discussion**

The general discussion attempts to bring together all key findings from the three results chapters and consider the data and conclusions in each study collectively, with the aim of building an overall model that addresses the title of thesis based on current knowledge in

the related fields to date, and importantly, the data gathered throughout this study. This section also provides a table of key findings and a “*Summary*” of the entire thesis.

## **References**

References contain a full list of all references used in the entire thesis in order of usage.

## **Appendix**

The appendix contains full qPCR data used throughout the study as well as supplementary data generated throughout the study which is used in the overall interpretation of results.

## PREFACE

In the ancient Egyptian medical textbook, the *Edwin Smith Papyrus* which refers to medical practices from 3000BC, the importance of the physiology of blood circulation and the heart was noted. With reference to the pulse, the literature states: “It is there that the heart speaks”, and, “It is there that every physician and every priest of Sekhmet places his fingers, he feels something from the heart”. In appreciation of the significance of the heart on the functioning of the human body, the manuscript records: “There are vessels in him for every part of the body. It speaks forth in the vessels of every body part”.

Throughout the history of medicine, there has been great wonder, mystique and controversy surrounding the heart. Some believed the heart to be the source of the soul (cardiocentrists) whilst others believed the same was true of the brain (cerebrocentrists). Aristotle (384 BC – 322 BC) was a cardiocentrism who believed the heart must be the soul's origin as it was: “central, mobile, and hot, with connections to all parts of the body”. Plato (428/427 BC – 348/347 BC), a cerebrocentrist, who was not overly concerned about the heart, described it simply as: "the knot of veins and the fountain of the blood." Hippocrates (460 BC – 370 BC), a cerebrocentrist who had access to human hearts, carried out exquisite examinations of the organ. Despite flaws in his functional understanding of the heart, he accurately described ventricles, membranes, arteries, veins and valves.

It has long been the view that the heart does not respond well to insult. Gabriele Falloppio (Fallopius), the 16th century anatomist stated: “a cardiac wound could only be survived by a miracle. The heart could never heal as it was too hard, always in motion, and of an inflammatory heat.” This view was shared by Hippocrates who believed that all wounds to the heart were fatal. Although Fallopius and Hippocrates were probably referring to large

puncture wounds, and were basing their views on events at the whole organ level, there was certainly doubt over the heart's ability to adapt when faced with any kind of insult; whether it being trauma, or a more subtle physiological challenge.

Armed with our current understanding of cellular biology and cardiac physiology, as well as modern research techniques, we can now examine and appreciate the remarkable degree of long term and short term cardiac adaptation, both mechanically and structurally, and from cell surface to DNA level.

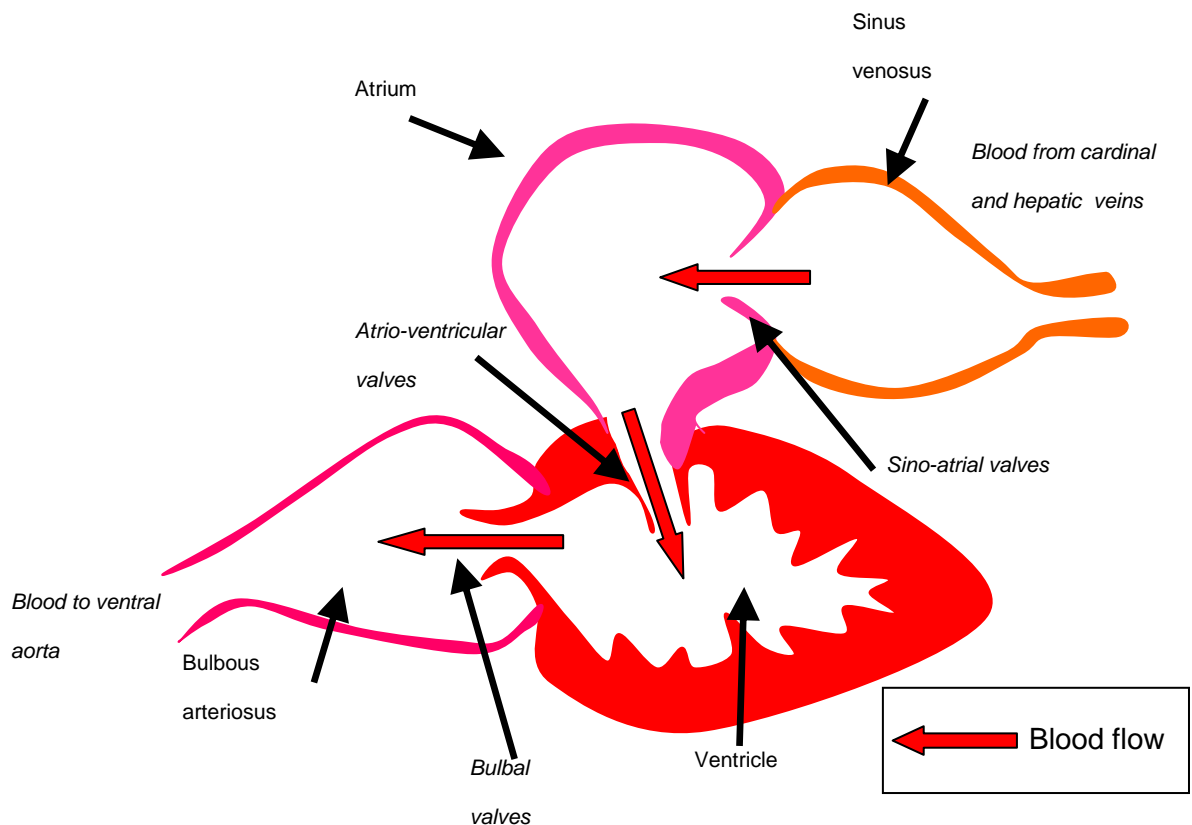
It is the purpose of this thesis to explore remodelling events and adaptation in one of the simplest hearts; the fish heart, when faced with the environmental insult of temperature change, which it faces in the short and long term, whilst maintaining adequate cardiac output.



# 1 Introduction

## 1.1 The Fish Heart

The heart in most fish represents ~0.1% of their body mass which is a small value in comparison with other vertebrate hearts. However some fish display significantly greater heart to body mass ratio such as skipjack tuna where it can be as large as ~0.4% [1].



**Figure 1.1 – Gross anatomy of fish heart.** Blood is ejected from the single muscular ventricle into the body via the ventral aorta. The highly elastic bulbous arteriosus acts as a pressure buffer. Blood returns from the body into the sinus venosus, then into the large sac-like structure, the atrium and finally returns to the ventricle.

Fish employ a closed circulatory system with a two chambered heart consisting of four main parts: the sinus venosus (a thin walled structure that collects deoxygenated blood from the body), the atrium (a thin walled, high volume chamber that facilitates blood

filling of the ventricle), the ventricle (a thick walled relatively low volume muscular chamber that is the main contractile unit of the heart responsible for pumping blood around the body) and the bulbus arteriosus (a tube like structure, more recently being referred to as an *out flow tract*, mainly comprised of connective tissue that acts as a pressure buffer). In elasmobranchs a conus arteriosus is present in place of the bulbus arteriosus. From the ventricle, blood flows via the bulbus arteriosus into the ventral aorta and then into the gills, from the gills to the rest of the body via a system of brachial arteries, and then returns to the atrium via the sinus venosus, liver and ducts of Cuvier [2].

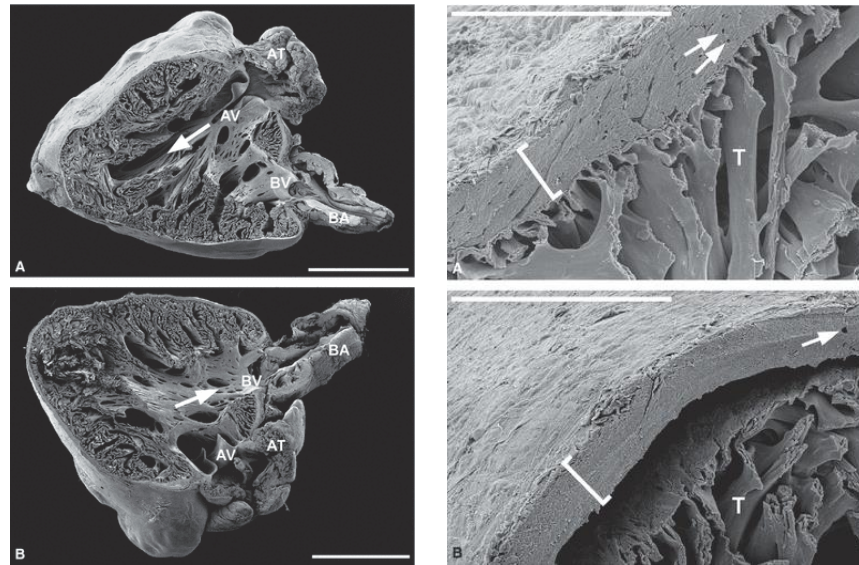
There are two main types of cardiac ventricle in fish: a spongy type and a mixed type. In the spongy ventricle, the muscle trabeculae form a sponge-like network; the spongiosa. Ventricle type is largely dictated by the activity level of the species. For example, fish that only employ a spongy layer are typically less active and include species such as cod and flounder [3]. More active fish such as salmon make use of a mixed-type ventricle. In the mixed ventricle, one or more superficial layers of compact tissue (compacta) enclose an inner spongiosa [4]. In salmonid fish, such as rainbow trout who employ the mixed type, and will be the focus of this study, the ventricle consists of an outer compact layer of circumferentially arranged cardiomyocytes encasing a spongy myocardium that spans the lumen (presence of a lumen is a distinct characteristic of active fish) of the ventricle with a fine arrangement of muscular trabeculae [5]. As well as the presence of a compact layer, more active fish have different overall ventricular morphology where it is pyramidal in appearance (as opposed to more spherical) with a distinct apex or tip at the base of the heart [6]. The apex consists of more tightly bound cardiomyocytes in a distinct orientation, and combined with the pyramidal shape, helps with force production in these active species [7].

The spongy layer consists of trabecular bundles of ~ 20 cardiomyocytes in varying orientations interspersed with extrabundular space filled with blood. The spongiosa and compacta are respectively associated with a lacunary and a vascularised blood supply [4].

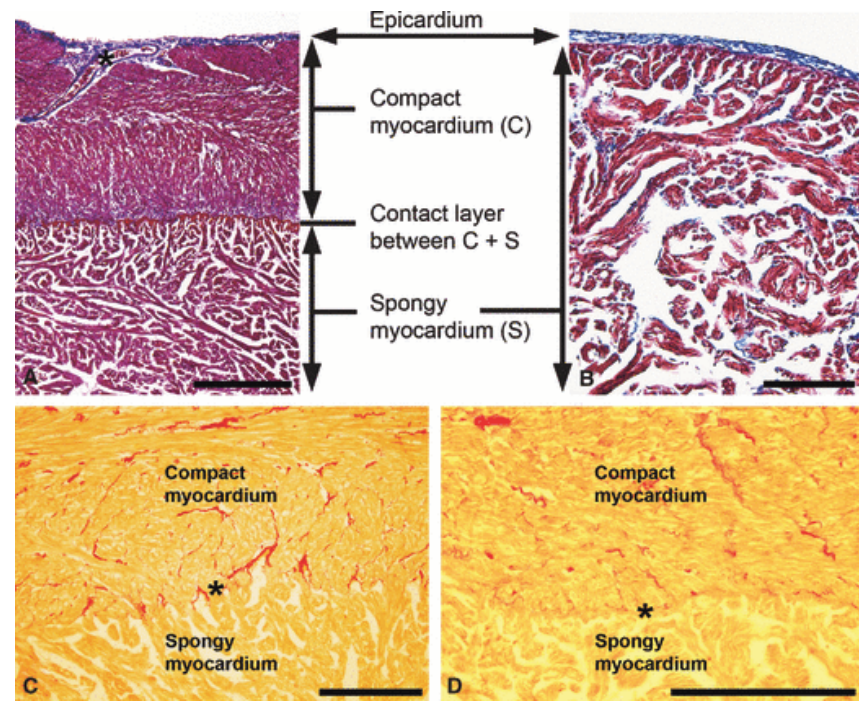
In comparison to the ventricle, the atrium commonly consists of a single thin sheet of cardiomyocytes and the atrium is of a significantly larger diameter than the ventricle. Indeed the blood filling volume of the atrium is larger than that of the ventricle and the atrium is able to respond easily to increased blood filling volume requirements of the ventricle [8].

The ventricle's main job is to pump blood around the body, whilst the atrium's predominant role is to facilitate one observed type of ventricle filling – *vis-a-fronte*; the second type, *vis-a-tergo* being dictated by the vacuum like state created when the ventricle relaxes after contraction [9] [10].

The bulbus arteriosus is not studied in this research; however it is key to the function of the heart and cardiac output in terms of modulating the levels of ventral aortic blood pressure required for effective cardiac output [11].



**Figure 1.2 – Salmonid heart internal structure.** Scanning electron micrographs show (top left and bottom left) two halves of the same adult sockeye salmon heart. Highly trabeculated inner spongy layer and the outer compact layer myocardium are clear. Top right and bottom right show the same heart at higher magnification, making clear the two separate layers and fibre orientation within each chamber. AT, atrium; BA, bulbus arteriosus; AV, atrioventricular valve; BV, bulboventricular valve. Scale bars (in white): left images; 2mm, right images 200µm. Arrows show blood flow (left) and blood entry (right) [5].



**Figure 1.3 – Compact and spongy layers.** Light microscopic images of Masson's trichrome stained (A, B) and picosirius red stained (C,D) paraffin sections through the adult sockeye salmon heart (A,C), through the tilapia heart (B) and through the rainbow trout heart (D). Images clearly show the presence of two distinct layers in the salmonid heart whereas the compact layer is completely missing in the tilapia. Purple and orange are muscle, blue is connective tissue, and red is collagen. Scale bar in (A) 4 mm, bar in (B) 1 mm, bars in (C,D) 100 µm [5]

### 1.1.1 Morphology of the fish myocyte

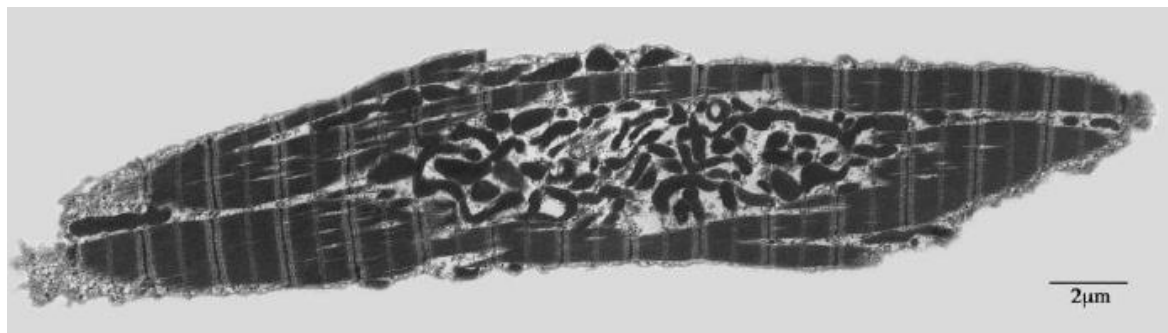
Cardiomyocytes are individual heart cells that contain the contractile units (sarcomeres) of the organ. Each myocyte on its own has the ability to respond to a variety of physiological insults, including stretch, through a variety of intracellular and extracellular proteins.

Together, through highly regulated communication and conduction pathways, and with interactions with the extra cellular matrix, myocytes enable the heart as a whole unit to collectively pump in a synchronised manner, and also adapt to fluctuating biological demands.

Fish cardiomyocytes are long and narrow in shape and predominantly mono-nuclear. It is thought this “spindle like” morphology is advantageous in heart physiology in terms of shorter ion diffusion distances in fish cardiomyocytes that lack T tubules, therefore facilitating adequate contraction. In rainbow trout of around 300g in mass, ventricular myocytes are approximately 180 $\mu\text{m}$  in length and 8 $\mu\text{m}$  in width. The surface area is 3000 – 4000 $\mu\text{m}^2$  with a volume of 2.5 – 3.5pl. Atrial myocytes are similar in length with a width of ~6 $\mu\text{m}$  and surface area of 2500 – 3000 $\mu\text{m}^2$  and cell volume of 2.0 – 2.5 pl [12].

In trout, the nucleus is central in location and is surrounded by a variable number of mitochondria, glycogen and a few fat droplets. Myofibrils, which account for ~ 40% of the cellular volume, are located in close proximity to the sarcolemma (SL). Sarcoplasmic reticulum (SR) of varying amounts surrounds the myofibrils. Caveoli have been observed in fish myocytes but have not been further characterised [13]. Peripheral SR forms couplings with the SL where processes 25nm wide and 10nm can be observed which are likely to be ryanodine receptors (RyR) [14]. Intercalated discs (IDs) are highly organised distinct structures within heart tissue and represent areas where cardiomyocytes interact

end to end. The IDs contain junction complexes including gap junctions, adherens junctions and desmosomes, as well as adhesion molecules such as N-Cadherin [5].



**Figure 1.4 – Fish cardiomyocyte structure.** A blue fin tuna cardiomyocyte in the longitudinal plane showing distinct sarcomeric organisation separated at either end by intercalated discs, with central nucleus and reticulum network [15].

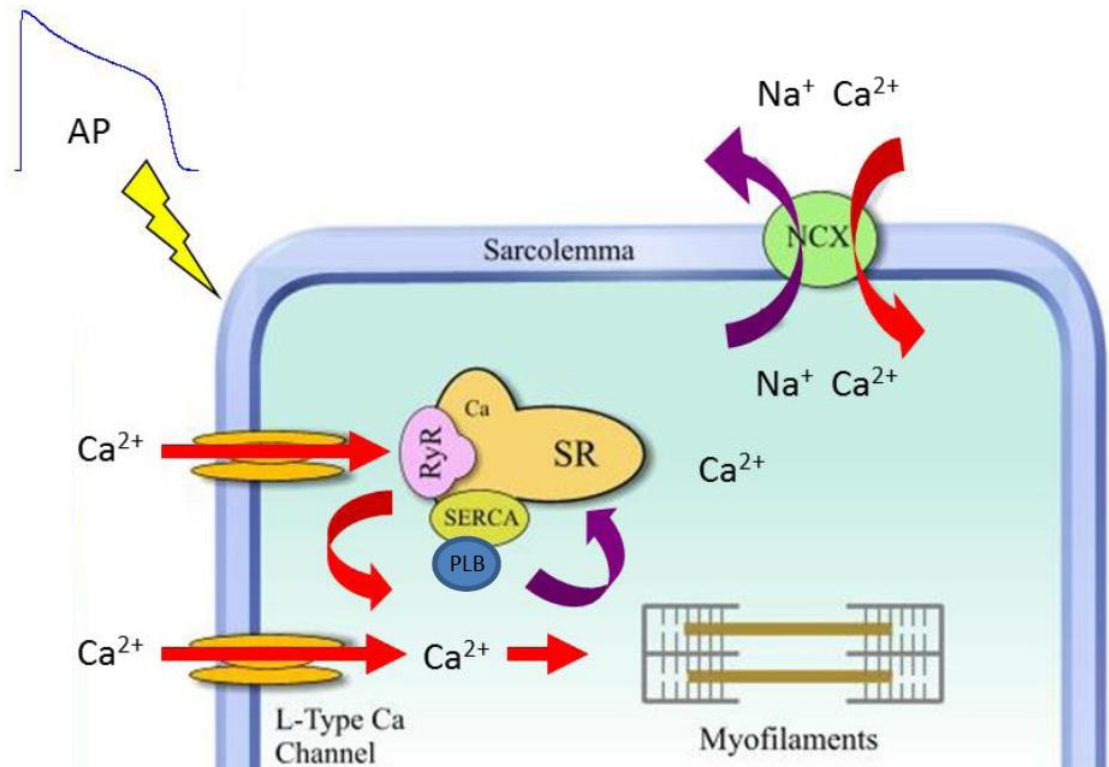
### 1.1.2 Cardiac contractile regulation in fish

Fish, in common with mammals, regulate their hearts via *stretch* which directly modulates *calcium dynamics*. Indeed, rainbow trout heart regulation is dependent on central venous pressure which modulates cardiac output via the *Frank Starling mechanism* where stretch of the cardiac muscle wall results in an increase in  $\text{Ca}^{2+}$  dependent contractile force [16].

#### 1.1.2.1 Calcium

$\text{Ca}^{2+}$  has two major functions in the fish heart: 1) Excitation – contraction (E-C) coupling and 2) Signalling. E-c coupling will be discussed here, and signalling will be discussed further on in the chapter. Both phenomena are vital to short term and long term adaptation of the heart to external (and internal) stimuli. In e-c coupling, an action potential, propagated by  $\text{Na}^+$  channels, and influenced by  $\text{K}^+$  channel kinetics, depolarises the SL membrane. This causes a trans-SL influx of  $\text{Ca}^{2+}$  through L-type  $\text{Ca}^{2+}$  channels which can directly activate contraction of the myofilaments. The rise in intracellular  $\text{Ca}^{2+}$  can also result in  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release (CICR) from the SR leading to a greater and rapid rise in intracellular  $\text{Ca}^{2+}$  which facilitates the contraction of myocytes via interaction with troponin. Trans-SL influx is the major source of  $\text{Ca}^{2+}$  (approximately 90%) in rainbow

trout heart for cardiac power production [17].  $\text{Ca}^{2+}$  is removed via sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) pump and  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger (NCX) pump activity.



**Figure 1.5 – E-C coupling in fish.** An incoming action potential results in depolarisation of the SL which allows  $\text{Ca}^{2+}$  to enter the cell via L-Type calcium channels, causing a release of calcium from the SR.  $\text{Ca}^{2+}$  is removed from the cell via SERCA and NCX activity following myofilament activation resulting in contraction (*graphic courtesy of Gina Galli, University of Manchester, 2012*).

Dilation of the ventricle, as a result of venous return, causes the contractile units of the cardiomyocytes, the sarcomeres, to increase in length, opening them up to greater activation via the effect of  $\text{Ca}^{2+}$  on troponin C, which requires Gln(29) and Asp(30) [18] causing a change in shape that allows myosin and actin to bind and generate increased force; termed the *Frank Starling mechanism* [19].

At the cellular level therefore, the method of fish cardiac output regulation can be partly attributed to increased myofilament  $\text{Ca}^{2+}$  sensitivity [20] where increased blood volume and increased contractility combine to increase cardiac output.

### **1.1.2.2 Stretch**

Mammals, birds and amphibians increase cardiac output by large increases in heart rate and relatively small increases in stroke volume (the difference between the end-diastolic and end-systolic volumes). However fish (except tuna), who have a much lower cardiac output than mammals, utilise large increases in stroke volume (up to 300%) and lesser changes in heart rate; ~50% [21] to modify their cardiac output. End-diastolic volume is a particularly important factor in fish stroke volume because fish are able to eject up to 95% of the blood content of their ventricle which is considerably higher than most mammals [19]. In vivo, cardiac output is partially regulated by sinus venosus pressure with venous pressure being more important than ventricular suction on atrial filling [10]. With an increase in central venous pressure comes greater blood filling which forces the atrium and ventricle to dilate. Indeed, blood volume responsive ANP release from stretched atrium is as much as 4 fold than atrium during normal swimming conditions [22]. Furthermore, there is evidence that stretch induced mechano – electrical feedback is present in the ventricle of the rainbow trout heart, potentially acting via TRPC channels [23].

#### **1.1.2.2.1 Chamber compliance**

With significant changes in ventricle and atrial filling causing large scale stretching of the chambers, there is clearly a requirement for resistive support governing the amount of dilation biophysically permissible, whilst at the same time maintaining structural integrity to generate sufficient force and containment. The degree or ability of stretch or dilation a chamber can undergo is termed *compliance* [24], and in many living systems and organs, including the heart, compliance is governed by two key proteins: elastin and collagen [25], both of which will be discussed in more details in terms of extracellular matrix remodelling further on in the chapter. Although to some degree, elastin provides support at lower pressures [26], elastin's main role is to *allow* stretch and facilitate recoil, whilst collagen provides resistive support, or dictates *stiffness*, especially at higher pressures [27]. Indeed,



the greater the elastin:collagen ratio, the more compliant the chamber is, a good example being in the highly compliant rainbow trout bulbus arteriosus where the ratio is ~14:1 [28]. On the contrary, the greater the collagen:elastin ratio, the stiffer the heart is [29]. For a heart to be able to increase in filling volume, it must become more compliant, however increased blood volume itself has been shown to increase ventricle stiffness [30] suggesting a degree of auto-regulation.

## **1.2 Cardiac remodelling in mammals**

Cardiac remodelling is essentially any significant change to the heart as a result of internal or external stimuli that results in a functional or morphological difference from the norm which can range from ionic changes through to obvious structural changes. Indeed, cardiac remodelling generally refers to any large change in protein quantity, function or location. Cardiac remodelling during temperature acclimation has been previously observed in fish and will be discussed in detail later on, however, most of our knowledge about cardiac remodelling has come from mammals.

The three main components of cardiac remodelling discussed in mammalian cardiac science, and which are to be explored in the next few sections, specifically relating to mammalian knowledge are: *cardiac hypertrophy*, *extracellular matrix remodelling* and *connexin remodelling*, with the last topic falling under the category of *electrical conduction remodelling*. The next few sections of this thesis will discuss mammalian cardiac remodelling and the remainder of the thesis will be focussed on exploring each of these remodelling characteristics in terms of fish.

## 1.2.1 Cardiac hypertrophy

Hypertrophy of any organ in literal terms means “an increase in size” [31]. Although tissues and organs can increase in size due to increases in cell numbers (hyperplasia), hypertrophy specifically relates to increases in size of cells via increased protein synthesis and is distinct from hyperplasia, increases in fluid volume or increases in extracellular matrix. In the heart, in general, it is believed that cardiac hypertrophy is due to cellular hypertrophy where cells become larger in all dimensions as a response to elevated periods of biomechanical stress. For example, arterial hypertension increases the wall tension on the heart, consistently stretching the cardiomyocytes. To compensate, cardiomyocytes and therefore the heart become larger via mechanisms which include increased mRNA production and greater protein synthesis as opposed to increased cellular volume (oedema) [32]. There is currently debate as to the extent of cardiomyocyte hyperplasia in hypertrophy, especially in disease states [33]. The end goal and desired result of hypertrophy is to normalise the increase in wall tension, and essentially mitigate the external insult of stretch, whether it be of volume or pressure related stress; a feature dictated by La Place’s law:

$$\sigma = \frac{PR}{2h}$$

$\sigma$  is ventricular wall stress, P is ventricular pressure, R is ventricular radius and h is ventricular wall thickness. For a given ventricular pressure, ventricular wall stress is higher with increased ventricular diameter. On the contrary, increasing ventricular wall thickness will decrease wall stress, as seen in cardiac hypertrophy [34].

### 1.2.1.1.1 Stretch, biomechanical strain and biomechanical stress

As described above, increased blood volume or increased blood pressure can impart a *stress* on blood vessels and this is often referred to as *stretch* in cardiac hypertrophy when discussing the degree of *force* applied to heart muscle walls as a result of factors including elevated blood pressure which is a trigger for hypertrophy. *Stretch*, however, when talked

about in terms of cardiac hypertrophy encompasses two key parameters: *biomechanical stress* defined as the “intensity of force per unit measure of tissue”, and *biomechanical strain* defined as “the extent of deformation relative to the tissue’s starting position.” Therefore blood pressure in the intact cardiovascular system imparts *stress* on vessels and structures and the resulting tissue deformation constitutes the *strain* component; *strain* being the actual triggering event, termed *stretch*. Throughout this thesis, where mentioned, *stretch* is a descriptive combination/ function of both *stress* and *strain*.

#### **1.2.1.2 Features of cardiac hypertrophy**

At its core, cardiac hypertrophy is associated with a net increase in protein synthesis leading to increased muscle mass which is influenced at all levels from gene transcription, gene translation, post translation control, trafficking and protein degradation control. This increase in metabolic demand, including a requirement for extra nourishment for the muscle tissue, requires increased blood supply and therefore cardiac hypertrophy is usually associated with increased expression of pro-angiogenic factors; the main one being vascular endothelial growth factor (VEGF) [35]. An increase in metabolic demand can also impart oxidative stress on the heart that can lead to heart failure over the long term, and this can be observed as an increase in stress related factors including *heat shock proteins* and several members of the histone deacetylase (HDAC) family [36]. As well as a distinct and significant increases in growth of the muscular component of the heart, there are other several well defined features and stages of hypertrophy in different contexts and in response to different triggering stimuli. These features range from structural to molecular alterations and include changes in connective tissue (particularly collagen) levels and distribution; termed fibrosis [37], and activation of highly specific signalling cascades (with considerable cross talk) and transcription events, including pathological activation of the *fetal gene program* – a termed used to describe a set of transcription factors (of the

MEF, NFAT, GATA and Nkx families) and genes ( $\alpha$ -SA,  $\beta$ -MHC, ANP, BNP), expressed in a pattern more commonly associated with early heart development [38].

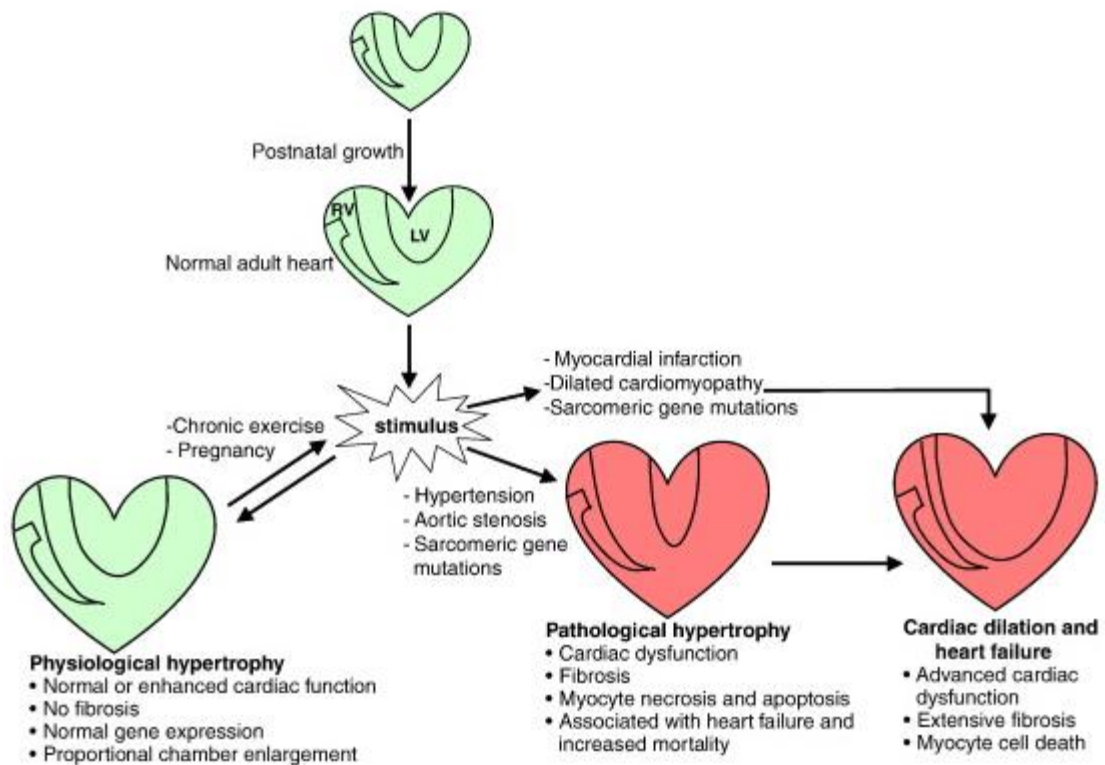
Importantly, cardiac hypertrophy can be either *physiological* or *pathological*, and each distinct type is characterised by differences in the triggering input, the mediation events (signal transduction) and the end structural and functional result. Indeed, hypertrophy can be both compensatory (in the case of e.g. athletes during chronic exercise training) or maladaptive (in the case of e.g. hypertension), and in each case, different but overlapping cell signalling systems are activated and each is governed by force and duration of the trigger. However the end result is the same: an increase in heart mass [32]. Furthermore compensatory hypertrophy in some cases can eventually lead to maladaptive hypertrophy and there is considerable overlap between physiological and pathological hypertrophy [39]. In the next few sections a generalised appreciation of the types of cardiac remodelling observed is discussed.

### ***1.2.1.3 Physiological cardiac hypertrophy***

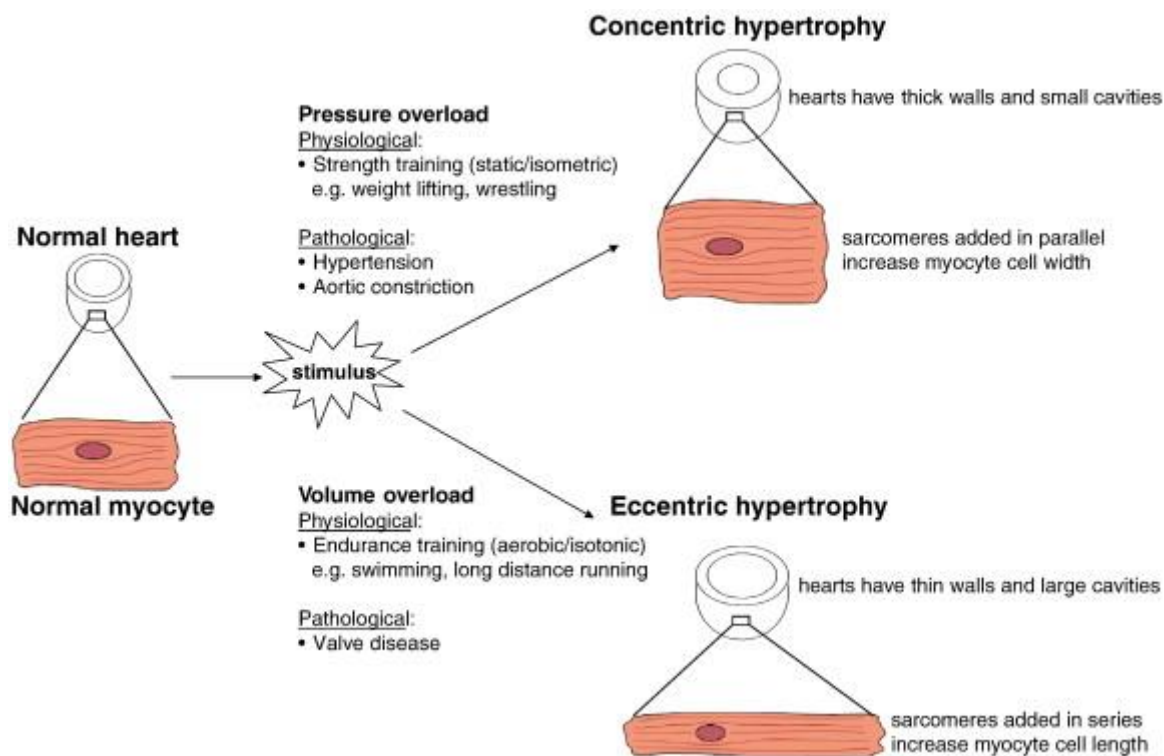
Physiological cardiac hypertrophy is an adaptive, reversible response in reaction to stimuli that dictates a requirement for additional cardiac output including physical exercise and pregnancy; the defining trigger status is predominantly *volume overload* and this leads commonly to *eccentric remodelling* where sarcomeres are added in-series, and although muscle thickness increases, chamber volume also increases in tandem, maintaining effective blood filling and ejection. Indeed, the heart predominantly displays *compensated* remodelling and cardiac output is generally enhanced. In physiological hypertrophy, as well as increases in muscle mass, there is also an increase in mitochondria density, and the heart enhances its ability to use fatty acids as an energy source [40].

#### ***1.2.1.4 Pathological cardiac hypertrophy***

Pathological cardiac hypertrophy is a maladaptive, largely irreversible response in reaction to stimuli that induces increased prolonged stress on the heart walls including chronic hypertension and restrictive valve disease. Other causes include myocardial infarction and certain familial genetic states, however in the majority of cases the defining trigger status is a *pressure overload* and this leads commonly to *concentric remodelling* where sarcomeres are added in-parallel resulting in increased thickness with no compensatory change in chamber size, which therefore limits blood filling and ejection capacity; also termed *de-compensated remodelling*. Muscle growth and remodelling observed in pathological cardiac hypertrophy is governed largely by activation of the *fetal gene program* and this reversion to a fetal-like genetic state is coupled with a reduced ability to use fatty acids as energy and instead, as in the developing heart, glucose is preferred [41]. Indeed, energy requirements are believed to influence the activation of fetal genes in an attempt to make the heart more energy efficient during a pathological state. For example, the greater ratio of  $\beta$  to  $\alpha$  form of MHC in hypertrophic hearts is to minimise energy loss through ATPase activity. A similar shift in dependence on skeletal as opposed cardiac contractile protein actin is observed to help improve contractility in the compromised heart. Although there is some overlap between physiological and pathological hypertrophy, the defining characteristics of the pathological type are a marked increase in fibrosis as well as cell death via necrosis and apoptosis [42, 43].



**Figure 1.6 – Physiological and pathological hypertrophy.** Cardiac hypertrophy can be classified as physiological, which is reversible and characterized by normal cardiac morphology and function. In contrast, pathological hypertrophy can lead to heart failure. Developmental hypertrophy is associated with the normal growth of the heart after birth until adulthood. RV: right ventricle, LV: left ventricle. Normal/ physiological heart growth is shown in green, pathological heart growth is shown in red [44].



**Figure 1.7 – Concentric and eccentric hypertrophy** – Concentric remodelling is associated with pressure overload, and eccentric remodelling is associated with volume overload. Both physiological and pathological states can influence the extent of concentric and eccentric remodelling [44].

### ***1.2.1.5 Signalling events in cardiac hypertrophy***

Growth of heart muscle as seen in hypertrophy requires triggering mechanisms, such as prolonged stretch, followed by transduction of the signal to the nucleus (cell signalling), and eventually, gene transcription of muscle genes. As in many cell signalling systems, it is generally accepted there is overlap in terms of physiological and pathological hypertrophy signalling as well as some key distinct cell signalling mechanisms [32, 40, 44] in the two different conditions, however below is a generalised acceptance of signalling mechanisms involved in cardiac hypertrophy as a whole.

#### **1.2.1.5.1 Calcium/ calcineurin NFAT signalling**

This signalling system is largely believed to be important in the pathological hypertrophic response, however is also involved physiological hypertrophy [45-47]. Calcineurin is a two subunit ( $\alpha$  catalytic and  $\beta$  regulatory) serine – threonine phosphatase expressed in a wide range of tissues and, of significant relevance to heart physiology, is sensitive to intracellular calcium concentrations acting via L Type Calcium Channel (LTCC) influx and calmodulin binding. Calcineurin's method of function is to de-phosphorylate NFAT type transcription factors allowing them entry into the nucleus to activate target genes [48] Constitutive activation of calcineurin in a transgenic mouse model induces heart enlargement and eventually heart failure. The same result is true if NFAT3 is made constitutively active [49]. CsA and FK506 (calcineurin blockers) have been shown to prevent cardiac hypertrophy in rat model systems [50]. A group of calcineurin inhibitory proteins called Myocyte Enriched Calcineurin Interacting Proteins (MCIPs) provide further evidence for the importance of calcineurin signalling pathways in the heart. MCIPs bind calcineurin at multiple domains and their over-expression has been shown to inhibit both physiological and pathological cardiac hypertrophy. Furthermore, there are several NFAT

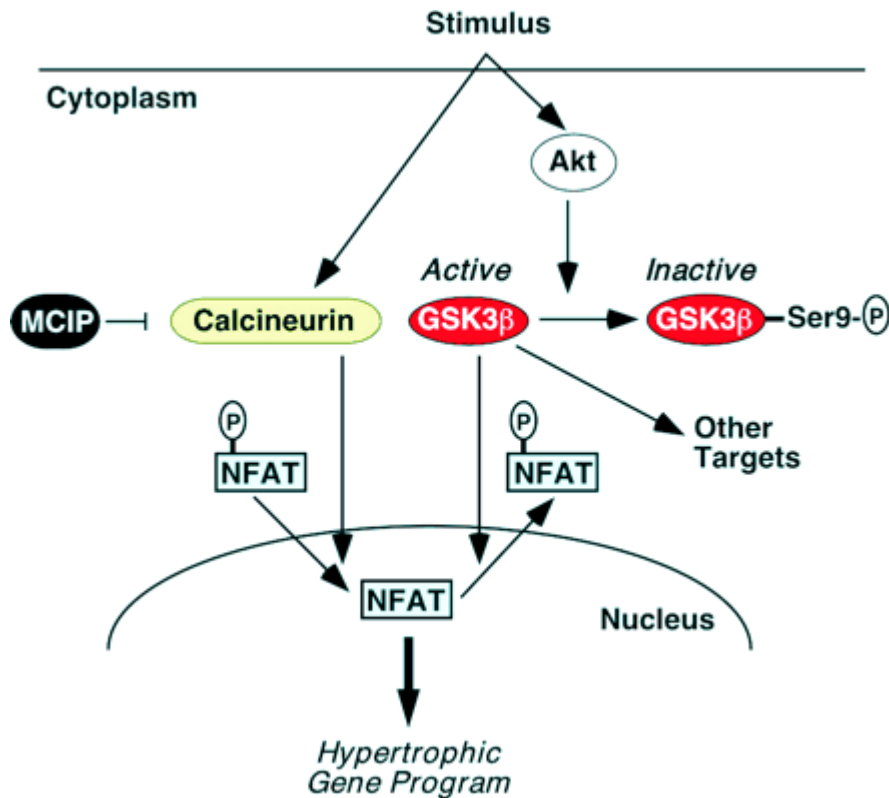
binding sites in the MCIP promoter region, suggesting a positive feedback loop. Indeed, MCIP is dependent on calcineurin for its own expression [51].

#### **1.2.1.5.2 PI3K/Akt/GSK – 3 $\beta$**

This signalling system is largely believed to be important in the physiological hypertrophic response [44, 52]. Phosphoinositide 3-Kinases (PI3Ks) are protein and lipid kinases mainly important in cell growth, survival and proliferation. PI3K's are activated by a variety of cell surface proteins including receptor tyrosine kinases and G protein coupled receptors (GPCRs) – including the cardio-physiologically important  $\beta$  - adrenergic receptor ( $\beta$ AR) family. When activated, PI3K's phosphorylate Protein Kinase B (PKB/Akt) which causes this protein to translocate to the cell membrane where it can phosphorylate and activate various target proteins. Over-expression of a constitutively active PI3K in transgenic mice leads to cardiac hypertrophy and over expression of PKB/AkT induces murine cardiac hypertrophy [53, 54].

An important target of Akt is GSK-3 $\beta$ . GSK 3- $\beta$  is a kinase expressed in numerous tissues that phosphorylates serine/threonine residues of NFAT transcription factors (in contrast to calcineurin which dephosphorylates these residues). Phosphorylation and subsequent inactivation of GSK – 3 $\beta$  promotes cardiac hypertrophy [55].





**Figure 1.8 – Different methods of hypertrophic gene control via Calcineurin/NFAT and AKT/GSK3β.** Many external stimuli, including  $\beta$ AR and calcium transients, can lead to activation of Akt and calcineurin respectively. Following Akt activation by phosphorylation, Akt phosphorylates GSK3 $\beta$  and in doing so inactivates it, repressing its ability to phosphorylate NFAT. Unphosphorylated NFAT is able to enter the nucleus and upregulate hypertrophic genes. Calcineurin directly dephosphorylates NFAT allowing a pro-hypertrophic response. Although both pathways cause hypertrophic growth, the calcineurin pathway is largely associated with pathological hypertrophic signalling whereas AKT/GSK3 $\beta$  is commonly associated with physiological signalling. [56]

### 1.2.1.5.3 PKC

The serine/threonine Protein Kinase C's (PKCs) are a family of 12 proteins expressed in various forms in all tissue. Gq/G11 coupled receptors are usually the source of the PKC activation stimulus. Both PKC –  $\alpha$ ,  $\beta$  and ETA when over-expressed individually have been implicated in causing cardiomyocyte hypertrophy [32].

### 1.2.1.5.4 MAPK

Mitogen Activated Protein Kinases (MAPKs) are amongst the most studied cell signalling molecules, being important mediators of diverse multi-level phosphorylation cascades, responding to numerous external stimuli such as stress, stretch and inflammation and causing activation of a vast array of transcription factors. They are commonly divided into three main groups/pathways (ERKs, JNKs, and p38 MAPKs) with many levels of cross

talk reported between the systems [57]. MAPK proteins are themselves activated by MAPKKs and MAPKKs are regulated by MAPKKKs via phosphorylation at each tier [58]. MAPKs are largely believed to be pro-hypertrophic. Indeed, over expression of MKP-1, a potent blocker of all three MAPK cascades was shown to block both agonist induced and pressure/volume overload induced cardiac hypertrophy [59], however in a knockout mouse, deletion of MKK7 was shown to promote heart failure, indicative of a protective function of MAPKs [60]. ERK1/2 has been shown to promote physiological hypertrophy [61], while JNK, p38MAPK and ERK5 have been implicated in pathological hypertrophy [62-64].

#### **1.2.1.5.5 G protein – coupled receptors and hypertrophy**

G protein coupled receptors (GPCRs) are key regulators of cardiac function and adaptation and are now thought to be involved in hypertrophy [65]. These proteins transduce their signal via direct interaction with the G proteins: Gs, Gq/G11 and Gi.

##### **1.2.1.5.5.1 Gq/G11**

After activation, Gq/G11 causes PIP2 cleavage by phospholipase 2, subsequently stimulating the IP3 and DAG signalling pathways. Pro-hypertrophic Ang2, ET1 and  $\alpha$  adrenergic receptors are all coupled to Gq/G11 and when these three proteins are over-expressed hypertrophy is induced. Furthermore, over-expression of Gq leads to cardiac hypertrophy [32].

##### **1.2.1.5.5.2 Gs**

When  $\beta$ AR1 cardiac receptor is activated, adenylate cyclase (AC) levels rise therefore increasing cAMP which subsequently activates protein kinase A (PKA), an enzyme which influences multiple cardiac functions including ion channel modulation and gene expression. Over-expression of  $\beta$ 1 receptor eventually leads to isoproterenol induced cardiomyocyte hypertrophy. Furthermore, over-expression of Gs leads to the same

cardiomyocyte morphology. Inhibition of  $\beta$ AR1 receptor by  $\beta$  blocker therapy has been shown to down-regulate hypertrophic genes [32].

#### **1.2.1.5.3 Gi**

Gi is the mediator for muscarinic and  $\beta$ 2 adrenergic receptors in the heart, and acts by inhibiting AC. By inhibiting AC, Gi signalling directly opposes Gs mediated signalling. Gi is upregulated in human heart failure as well as in hypertensive hypertrophy [32].

#### **1.2.1.5.6 Small GTP binding proteins and sarcomere induced hypertrophic signalling**

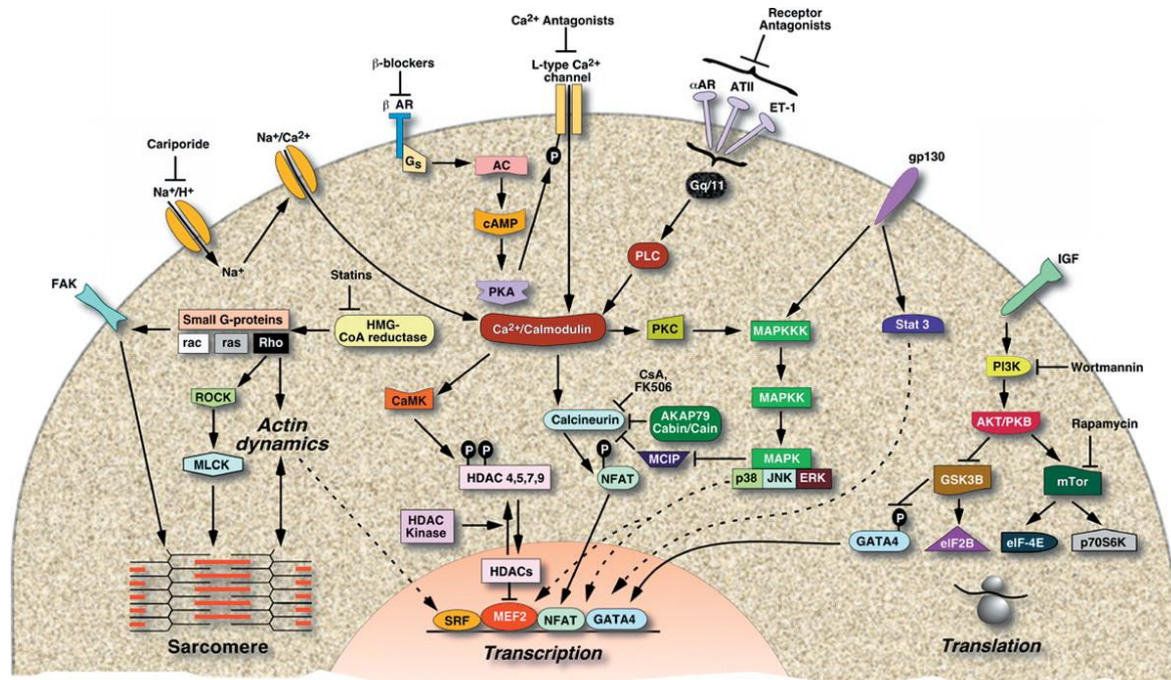
Small G proteins, namely the rho, ras, arfs, rab and ran families, are amongst the most abundant signalling mediators in cells, spatially and temporally modulating multiple signalling pathways and linking communication systems at numerous cascade levels. They are involved in a vast array of cellular processes including cell survival, proliferation and identity amongst many more. G proteins are activated by the binding of a molecule of GTP (modulated by guanine exchange factors, *GEFs*). When GTP is hydrolysed to GDP (a process coordinated by GTPase activating proteins, *GAPs*) the G protein is “switched off” and the protein is in an inactive state.

Over-expression of ras in transgenic mice has been shown to be sufficient for hypertrophy of the heart and of particular importance, it was shown that ras was significantly involved in the translocation of NFAT3 to the nucleus, implying it is of importance in the pro-hypertrophic calcineurin signalling pathway [66-68]. Moreover, rhoA is involved in sarcomeric organisation during hypertrophy [69] and rhoA is believed important in sarcomeric induced signalling in a calcineurin dependent manner

Furthermore, the novel muscle specific sarcomere protein STARS, via rhoA signalling, was shown to be upregulated in both pressure induced hypertrophy *and* calcineurin dependent cardiac growth [70].

### 1.2.1.5.7 Summary of cell signalling in hypertrophy

In summary, multiple, relatively well studied signalling pathways are involved in cardiac hypertrophy, however calcineurin/NFAT and Akt/ GSK 3 $\beta$  signalling appear to be the most prominent and influential cascades governing this condition in pathological and physiological states respectively, as well as MAPK cascades as shown in many cell systems which tend to promote the progression from adaptive hypertrophy to heart failure.



**Figure 1.9 – Signalling pathways in cardiac hypertrophy.** A diverse array of cell signalling mediators, activated by several receptors converge on only a few transcription factors. The pathways of interest in this study are the AKT/PKB/GSK3 $\beta$  pathway; far right, the MAPK pathway; middle right, and the calcineurin NFAT pathway; middle [32]

## **1.2.2 Extracellular matrix remodelling**

The extracellular matrix is the extracellular part of the structure of tissue within an organ, made up of cells, proteins and relatively large molecules that primarily provide scaffolding support and maintain the structural integrity of the organ via distinct connections with the cellular components (cardiomyocytes) of the heart. The ECM is exquisitely linked to the intracellular communication and signalling networks via a host of anchoring/adhesion and signalling proteins facilitating signalling of extracellular events, including stretch, right through to the nucleus of the cell. The ECM is therefore able to govern cellular behaviour in response to external events via protein expression. Indeed, this relationship is bidirectional, as the cell is also able to govern ECM behaviour, by influencing ECM regulatory proteins as discussed below [71]. Therefore, the ECM of the heart should always be considered in conjunction with the cardiomyocytes when discussing remodelling.

### ***1.2.2.1 Structure of the ECM in the heart***

Surrounding cardiomyocytes is a basal lamina made up of collagen type 4 and laminin, ECM cells; fibroblasts and macrophages, large carbohydrate polymers; glycosaminoglycans and proteoglycans and fibrous proteins; collagen and elastin. Glycosaminoglycans and proteoglycans are capable of large-scale hydration due to their ability to attract positively charged sodium ions and therefore water via osmosis, and in doing so, form a gel-like structure that facilitates hydration of the ECM and protection, resistive support, and lubrication of heart tissue during diastole and systole. The ECM also contains key signalling and anchoring proteins that interact with the cardiomyocytes and will be discussed in more detail further in the chapter [71].

Of particular importance to this study, are two ECM proteins; *collagen* and *elastin*.

### **1.2.2.1.1 Collagen**

In the human body, collagen is the most widely expressed protein. Collagen, which is synthesised in fibroblasts, is highly studied and in brief, consists of a tri-helical structure made up of  $\alpha$  chains which are Glycine – Proline – Hydroxyproline repeats, usually referred to as Gly – X – Y repeats. It is the variety of  $\alpha$  chains coming together that dictates the type of collagen formed. Indeed, there are believed to be 28 different types of collagen. The classic Gly – X – Y motif enables the formation of a highly stable structure through glycine and proline interaction. Collagen is synthesised as 3 soluble  $\alpha$  chains of pro-collagen that self-assemble inside the fibroblast and are then excreted into the extracellular space where proteolytic cleavage occurs, forming collagen proper. Multiple collagen tri-helices form covalent cross links with each other, largely facilitated by the enzyme lysyl oxidase, forming fibrils of collagen [72].

In the heart, collagen types 1 and 3 are structurally important; indeed fibrillar collagen in the heart is made up of varying amounts of type 1 and 3 collagen polymers and the ratio of the two has implications on structure and function. Collagen type 1, which makes up about 80% of total cardiac collagen, is more concerned with mechanical stiffness, whereas collagen type 3 dictates mechanical compliance of the polymer, suggesting overall collagen is there for robustness during normal cardiac function. Cardiac collagen forms three distinct networks: the epimysium (concerned with collagen in the epicardium and endocardium), perimysium (a middle layer broadly connecting the epimysium and myocytes within the endomysium) and endomysium (which generally surrounds, links and facilitates connections between all myocytes and capillaries). These three distinct layers vary in prominence throughout sub regions of the heart, and the distribution of each is believed to greatly influence cardiac performance in health and disease [73-75].

Collagen during cardiac remodelling is well studied, in particular during cardiac hypertrophy where, in line with an increased thickness of the ventricle wall, increases in collagen, particularly in the interstitial region, are observed (termed fibrosis) which is believed to aid in mitigating against the external insult of prolonged stretch by building stiffness and robustness [76]. Fibrosis in hypertrophied hearts is considered a pathological response due to the effects on diastole, where increased stiffness and reduced compliance reduces the blood filling capability of the ventricle therefore leading to decreased ejection [77-79]. In physiological hypertrophy, fibrosis does not occur, due to contextual cell signalling.

#### **1.2.2.1.1.1.1 Collagen regulation**

As mentioned above, collagen is manufactured in the fibroblasts, which make up about 65% of the entire myocardium. Collagen levels could therefore be dictated by the abundance of fibroblasts. Indeed, fibroblast numbers have been shown to change in health and diseases states: Ang-2 and TGF- $\beta$ , both released during hypertrophy, have been shown to directly increase their number [80]. Collagen is consistently produced in the heart, so mechanisms must exist to maintain desired levels of the protein. Ultimately, it is the role of two sets of enzymes that dictate the levels of collagen; the matrix metalloproteinases (MMPs) [81], which are negative regulators of collagen, and tissue inhibitor of metalloproteinases (TIMPs) [82], which are positive regulators of collagen.

#### **1.2.2.1.1.1.2 MMPs**

MMPs are a family of ~25 zinc dependent enzymatic proteins that are manufactured in both fibroblasts and cardiomyocytes. Their function is to degrade collagen and gelatine-like molecules and as such are collagenases and gelatinases. Some are membranous and some are secreted into the ECM. Like collagen, secreted MMPs are produced in the “pro” form, and N- terminal cleavage of the pro-peptide, with binding of a water molecule to the

inherent zinc ion, is required for catalytic activity of the enzyme [71]. Membrane MMPs are produced fully active and are tethered in the membrane via a transmembrane domain. Substrate specificity is determined by the C terminus and there is considerable overlap in their target protein, for example many gelatinase type MMPs will also act as collagenases. Multiple MMPs are implicated in cardiac remodelling, however of particular importance are MMPs 2, 9 and 13, which will be subject to study in this thesis. A key substrate of all three is the cardiac important collagen type 1, which will also be studied. TGF- $\beta$ , a factor released in cardiac hypertrophy is known to decrease MMP production, therefore promoting fibrosis [83-85].

#### **1.2.2.1.1.1.3 TIMPs**

Like most proteins, MMPs are subject to regulation at most levels from protein-protein interaction, cell signalling and transcription and translational control, however TIMPs are believed to be the key regulator of MMP activity. TIMPs are negative regulators of MMPs, and therefore positive regulators of collagen. There are 4 known TIMPs and cardiac muscle expresses each one. Moreover, all TIMPs have been shown to be important in cardiac remodelling in health and disease. TIMPs bind directly to MMPs, blocking the catalytic site of the MMP. TIMP1 is bioactive against most MMPs, TIMP2 is involved in suppression of endothelial cell proliferation, TIMP3 is important in inflammation induced responses of heart tissue, and TIMP 4 is believed to be highly expressed, purely inhibitory and bind with the highest affinity. Importantly for this study, TIMP2 has been shown to interact strongly with MMP2 and MMP9, as well as MMP13, and all three will be investigated during this study [84, 86-88].

#### **1.2.2.1.2 Elastin**

Elastin is a major fibrillar component of the ECM whose function is to promote compliance by allowing stretch and recoil in tissue, albeit with a small amount of resistance; it is an efficient energy store that is biophysically elastic. It is therefore ideally



suited for tissues and organs that undergo retraction and expansion such as the heart. The elastin gene contains 36 exons that are alternatively spliced to produce ~13 isoforms of elastin protein. Elastin protein, which itself interacts with proteins such as fibrillin, is characterised by hydrophobic regions rich in glycine and proline that cross link with each other in a manner facilitated by, in the same way as collagen, lysyl oxidase, and amino acids desmosine and isodesmosine. This cross linking, along with peptide regions between the hydrophobic areas and alternating alanine-lysine motifs, creates a rigid yet stretchy three-dimensional elastic  $\beta$ -sheet protein [89, 90].

Elastin communicates with cells partly by its interaction with elastin binding protein (EBP) which is a  $\beta$ -galactosidase coded by the GLB1 gene, S-Gal, neuramidase, and cathepsin A. This interaction, facilitated by several cell signalling cascades can alter MMP activity as well as cell division in certain tissues [91-93].

#### ***1.2.2.2 ECM and cytoskeletal signalling events in hypertrophy***

In hypertrophy, not only must the muscle components of the cardiomyocyte increase in size, but supporting structures of the cell both internal (e.g. cytoskeletal elements) and external (e.g. the ECM) must also diversify in order to accommodate increases in cell mass and reorientation of structural proteins and domains. With the contractile portions of the cardiomyocyte being intimately linked to the cytoskeleton, and the cytoskeleton being closely intertwined with the extracellular matrix, there are stringent communication events occurring between, and as a result of these relationships. Focal adhesions (FAs) are structures in cells where the cytoskeleton is linked to the ECM, usually mediated via the homodimer, integrin [94]. Extracellularly, integrin binds to the ECM via either the R-G-D sequence motif for proteins such as laminin, or DGEA/ GFOGER motifs for collagen isoforms. Intracellularly, integrin binds to the cytoskeleton via adaptor proteins including vinculin, talin, filamin and  $\alpha$  actinin. The point at which the cytoskeleton connects with

the ECM via accessory proteins is termed the FA. At the FA, other signalling proteins such as focal adhesion kinase (FAK) can attach and initiate signalling events as a result of biochemical force at the junction [95]. In a cardiomyocyte subject to constant workload, the FA (via biomechanical stress) is a potentially important regulator of hypertrophy [96]. One important downstream effector of FA signalling is the small G protein rac. Constitutive activation of rac has been shown to promote hypertrophy and when FA signalling is genetically stunted, repression of pro hypertrophic genes is apparent [32].

### **1.2.3 Gap junctions in hypertrophy**

In the intact hypertrophied heart, it is imperative that the integrity of the heart as a whole, and especially the electrical conduction system is maintained in order for the heart to continue its physiological role as a circulatory pump. This includes maintaining an adequate distribution and volume of electrical conduction channels, both spatially and temporally.

One key protein that has to take part in this overall remodelling is *connexin* which form *gap junctions*. Gap junctions facilitate effective cardiac conduction through the heart, enabling electrical synchrony and preventing potentially fatal arrhythmia brought about by impaired cell to cell communication and conduction [97]. Indeed, the genetic addition of one key connexin, Connexin 43 directly into the heart in mammals is directly correlated with an increase in conduction velocity in the ventricle [98, 99] and this action potential facilitation by Connexin 43 is partly due to direct interaction with sodium channels [100].

#### **1.2.3.1 Connexin structure and synthesis**

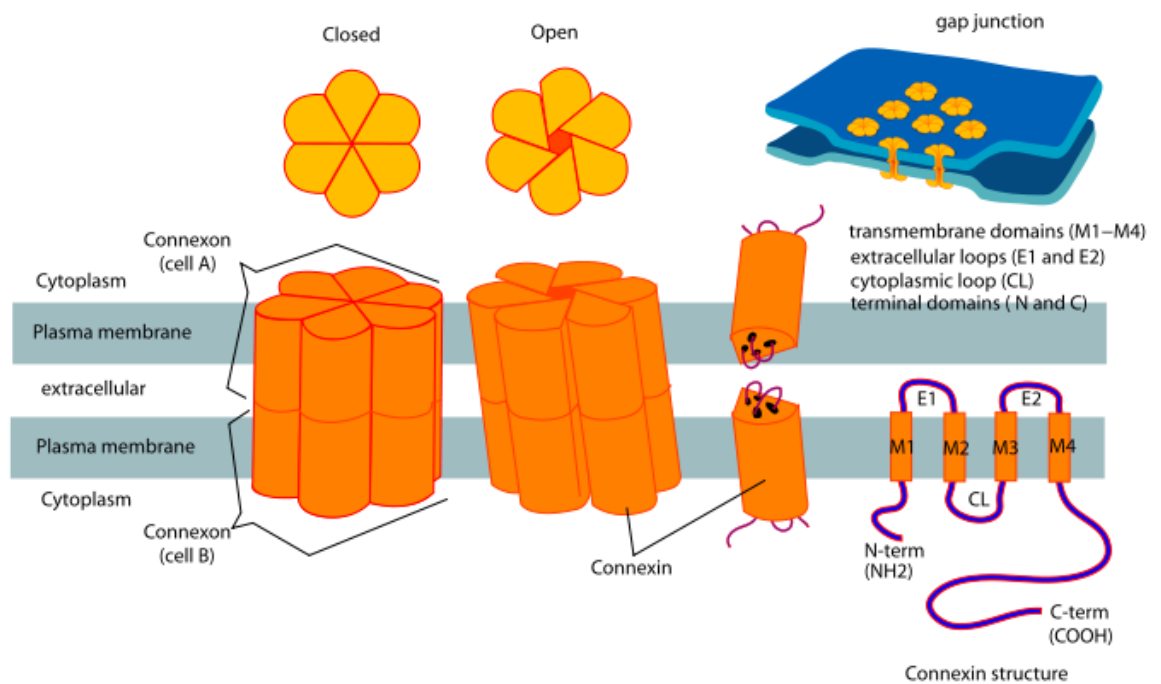
Connexins, sometimes referred to as *gap junction proteins*, are 26 – 60 Kda, ~380 amino acid transmembrane proteins that form heteromeric and homomeric junctional channels (gap junctions) between vertebrate cells, allowing the passage of ions, metabolites, and

signalling molecules [97] and are arguably one of the main electrical conduction channels or proteins in the heart. Gap junctions are formed by two hemi-channels of connexon from opposing cells that come together with the aid of other proteins and structures including cadherins and adherens junctions to form a large conduction pore [101]. Connexins are essential to the normal electrical conduction distribution within the heart by allowing the flow of necessary electrically important ions between cardiomyocytes.

Each connexon protein is composed of 6 connexin subunits, with each connexin being made from 4  $\alpha$  helix transmembrane domains (M1 – M4) with two highly conserved extracellular loops (E1, E2). E1 and E2 domains exhibit three cysteine residues believed to be essential for hemi channel to hemi channel cross linking. There is one intracellular loop, with the amino and carboxy termini being cytoplasmic. In the human genome there are twenty one genes coding for connexin proteins, whilst there are twenty in mouse [97].

Following expression of mRNA, connexins are inserted into endoplasmic reticulum membranes before processing in the golgi apparatus where connexons *per se* are formed. In common with all proteins, connexin expression is subject to regulation at transcriptional and post transcriptional levels. Highly coordinated rapid trafficking utilising the cytoskeleton and adaptor and motor proteins promote assembly of the gap junction in the cell membrane [102-104]. Regulation of connexin partly occurs at the level of assembly into functional gap junctions [105]. Recently, the importance of the C terminus of connexin has become apparent. A key piece of evidence stems from the complete removal of Cx43 C terminus in a mutant mouse model in which there are dramatic changes in spatial organisation of gap junction plaques [106]. Several regulatory proteins including c-src, tubulin, caveolins, and zonula occludens -1 (ZO -1) reportedly interact with the C

terminus region of the hemi channel subunit, raising the likelihood of a highly orchestrated network of connexin trafficking and distribution that is highly integrated with the cytoskeleton and ECM [107-109]. Other proteins shown to interact with the Cx43 C terminus are classic adhesion junction proteins and include N-cadherin,  $\beta$  catenin, desmoplakin,  $\gamma$ -catenin and PKP2 [110].



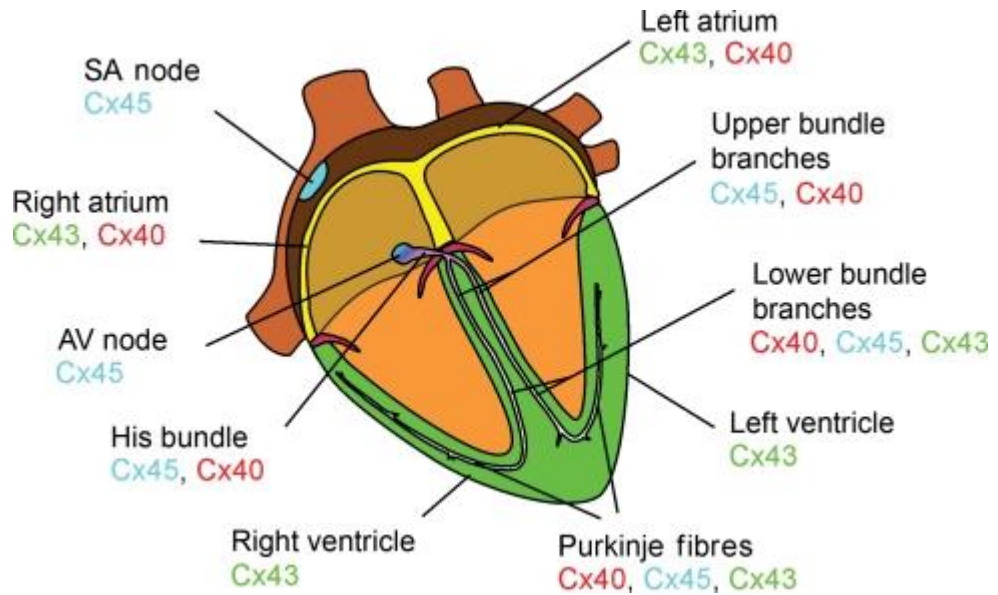
**Figure 1.10 – Gap junction structure.** Gap junctions are formed by two opposing connexons from two adjacent cells that come together to form a pore. Connexons are made of 6 connexins which are themselves a 4 transmembrane domain protein. ([www.http://cellbiology.med.unsw.edu.au](http://cellbiology.med.unsw.edu.au), no copyright.)

### 1.2.3.2 Gap junctions in the mammalian heart

In general physiology, gap junction mediated cell to cell communication is vital in tissue homeostasis and regulation of growth and development. Indeed, mutant forms of connexin

have been linked to several human disease states [111] such as Charcote-Marie-Tooth-Disease, a hereditary neuropathy [112].

In the heart, the role of the gap junction is to allow passage of ionic flow, key to the electrical conduction system, between cardiomyocytes and the facilitation of synchronous contraction of the heart. Connexin plaques in adult mammalian cardiomyocytes are found concentrated in the intercalated discs (IDs) at end to end cellular connections [113]. Several connexin subtypes are expressed in the heart including Cx43, Cx40, and Cx45, with Cx43 being by far the most prominent [114]. The working myocardium of the ventricle is heavily interconnected by Cx43 gap junctions, which are themselves co-localised with both fascia adherens junctions and desmosomes within the intercalated discs. Atrial myocyte gap junctions in most mammals tend to be constructed of both Cx43 and Cx40. Cx45 is expressed to a relatively much lesser extent however there are higher levels of this isoform in the atria compared with the ventricles. There are apparent species differences in terms of connexin expression. For example, Cx40 is not detectable in the rat conduction system [115, 116].



**Figure 1.11 – Connexin distribution in the human heart.** Cx43 is the main connexin found in the ventricle, whereas Cx40 is thought to be the main atrial connexin. Cx45 is largely concerned with specific conduction pathways [117].

### 1.2.3.3 Connexin control of conduction velocity

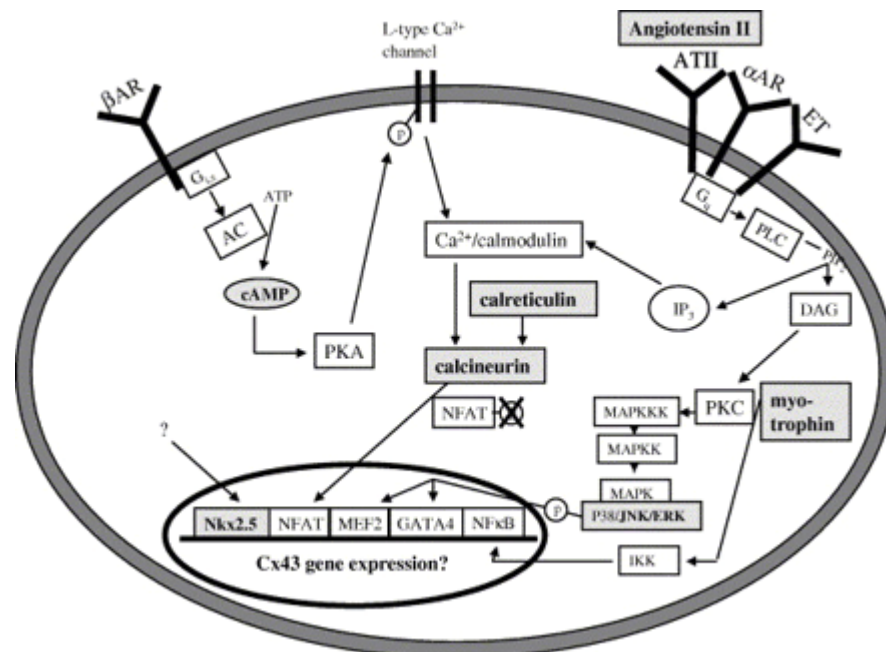
Several functional differences have been observed between different connexin family members, which give each connexin a distinct behaviour. These functional differences include pore conduction, size selectivity, charge selectivity, voltage gating, and chemical gating. It is the combination of these factors, and the specific distribution within cardiac tissue that allow connexins to control conduction velocity and action potential propagation through the heart [118, 119].

When analysing key domains in the heart for connexin subtype and abundance, it becomes apparent how connexins can influence cardiac conduction velocity. For example in the SA and AV nodes, gap junction complexes are less abundant, highly dispersed and composed predominantly of Cx45, which facilitates slowing of electrical conduction in these structures (a feature essential for correct electrical synchrony) [120]. *Bundle of His* cells, lower nodal cells, and posterior nodal extensions in mammals, where conduction velocity

is relatively high, co-express Cx40 and Cx43. The remainder of the cardiac conduction system myocytes express Cx40 predominantly [121]. The Purkinje/ ventricular interface is a site of particular importance both functionally and anatomically. Here, Purkinje cells co-express Cx40 and Cx45, and connect, via a sheet of transitional cells, with ventricular tissue that expresses Cx43 predominantly. In this area, imaging reveals that Cx45 may be involved in bridging Cx40 and Cx43 [117] thus providing exquisite control of conduction velocity via a buffering system dictated by connexins of differing pore size and conduction properties.

#### ***1.2.3.4 Connexin regulation***

Connexins exhibit a very short half life of only a few hours [122] implying that they form part of highly regulated and plastic physiological systems. Furthermore, multiple consensus sites of phosphorylation for a range of signalling cascades have been found in numerous species and phosphorylation of connexin has been implicated in trafficking, degradation, channel function, and channel assembly. Cx40 contains putative sites for PKC, PKA and PKG phosphorylation. Cx43 exhibits PKC, PKA, PKG and MAP Kinase target domains and Cx45 displays binding sites for PKC and PKA directed regulation [123]. Furthermore, initiators of these cascades including Ang2, FGF, PDGF, TGF $\beta$  and IL-1 $\beta$  have been shown to alter channel gating characteristics as well as connexin localisation and distribution. Importantly for this study, Cx43 signalling pathways, summarised in Figure 1.12, are similar to the hypertrophy signalling mechanisms. Indeed there is believed to be considerable cross talk between them, implying that hypertrophy and connexin remodelling are intimately linked, or at least share common physiological initiators and effectors [124].



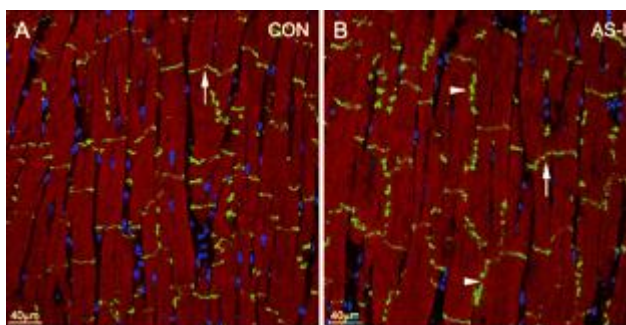
**Figure 1.12 - Cell signalling systems involved in connexin43 regulation** There are common pathways involved in both Cx43 regulation and hypertrophic signalling. A circled “P” represents phosphorylation. X represents dephosphorylation. Of particular interest to this study is the MAPK pathway, bottom right, and the calcineurin pathway, middle [124].

### 1.2.3.5 Connexin remodelling in cardiac hypertrophy

Lateralisation of connexin is a common feature of connexin remodelling in hypertrophy [125] and is believed to alter conduction properties of the cell [126]. Indeed, connexin remodelling promotes arrhythmia and sudden death [127]. This re-localisation appears to involve de-phosphorylation of connexin, and de-phosphorylation of connexin appears to be linked with an increased degradation of the protein as well as channel inhibition. In rabbit, a mechanical load model of hypertrophy resulted in rapid decline of connexin phosphorylation at ser368 and a subsequent reduction in Cx43 protein levels [128]. Many different groups report either up or down regulation of connexin protein levels in hypertrophic models. Ang2, a pro-hypertrophic stimulus, down-regulates Cx43 [129]. Conversely an alternative pro-hypertrophic stimulus,  $\beta$  adrenergic receptor activation, results in increased expression of Cx43 via cAMP signalling [130]. JNK, the MAPK



which has been shown to become activated upon biomechanical stretch, leads to a down-regulation of Cx43 [131].



**Figure 1.13 – Connexin lateralisation in cardiac hypertrophy** – Confocal images depict left ventricular tissue in humans; red being  $\alpha$ -actinin, green being Cx43, and blue being nuclei. On the left (A) is a control (CON) group taken from non-heart disease patients. Here, the arrow points to Cx43 (green) localisation at end-to-end cardiomyocyte borders in the IDs. On the right (B) is a hypertrophic patient with aortic stenosis (AS-1). Here, the two horizontal arrowheads indicate lateralisation of Cx43 and the arrow points to correct ID distribution [125].

There is evidence that physiological and pathological types of hypertrophy convey different outcomes on connexin remodelling. In a hamster model of physiological hypertrophy, Cx43 levels were seen to rise and no lateral movement of gap junction plaques was noticed [132]. This was also true in an exercise study in mice [133]. Numerous studies however, including those mentioned above, show that pathological hypertrophy models result in considerable relocation of connexin [125]. In mouse, within two weeks after aortic binding, Cx43 phosphorylation is markedly reduced, and gap junction plaques are diffuse. Furthermore, these changes result in progressive slowing of impulse propagation, providing a direct link between connexin remodelling and disruption to the electrical conduction system of the heart [134].

#### 1.2.4 Summary of cardiac remodelling in mammals

In mammals, cardiac remodelling is extensively studied and large amounts of evidence point to the fact that an increase in stretch within the heart, causes, through cell signalling cascades derived from both cardiomyocytes and ECM, an increase in muscle mass, an

increase in collagen via collagen regulatory proteins, and on balance, a redistribution of connexin gap junctions probably facilitated by connexin dephosphorylation. Hypertrophic remodelling can be both physiological and pathological in nature with pathological remodelling contributing to heart failure by disruption of cardiac output due to increased stiffening, reduced ejection efficiency and conduction problems.

### **1.3 Cardiac remodelling in fish**

Fish are subject to rapidly changing environments and have to maintain adequate cardiac output over a range of insults including drastic temperature ranges, pH, hypoxia, physical exertion and pollution that occur in the short and long term [135]. In this study, cardiac remodelling in fish as a response to long term temperature acclimation is to be studied. Below is a literature review of current knowledge on the effect of temperature on fish cardiac physiology.

#### **1.3.1 Temperature acclimation and the fish heart**

Acclimation to temperature is a biophysical response of the fish to being placed in warm or cold water for extended periods of time. In the wild, these conditions are brought about by seasonal changes with fish being subject to a wide variety of large but predictable temperature changes. In warm water, oxygen levels are low and fish become more active. On the contrary, in cold water, oxygen levels are high and fish are relatively less active [136]. In both instances however, the fish must be able to maintain an ability to efficiently pump oxygenated blood around the body. Therefore in temperature acclimation studies we are investigating changes in activity levels, oxygen handling capability/metabolic requirements and circulatory demand. Ultimately the combined effect leads to cardiac

changes at the whole heart structure, cardiomyocyte and ionic levels with the aim of maintaining adequate cardiac output.

#### **1.3.1.1 Cold acclimation**

Optimum temperatures in salmonids for maximum cardiac performance, aerobic scope and swimming are commonly around 15 – 18 °C with the preferred temperature range being 12 to 18 °C [137]. Despite this wide range of temperatures, the rainbow trout is able to maintain maximum cardiac output ( $Q_{max}$ ) and maximum power output ( $PO_{max}$ ). One method of achieving this is by an increase in ventricular mass as seen in species including carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), green sunfish (*Lepomis cyanellus*), goldfish (*Carassius auratus auratus*), small mouth bass (*Micropterus dolomieu*), and striped bass (*Morone saxatilis*) [138] and the rainbow trout [139-142].

When the rainbow trout is exposed to a temperature drop of 10 °C for 3 – 4 weeks, relative ventricular mass increases 20 – 40% and the proportion of compact myocardium decreases by 15 – 30% [143]. Cold acclimation increases the surface area of the cardiomyocyte by as much as 49.6% [144]. This increase in mass compensates for cold induced decreases in contractility, increases in blood viscosity, and decreases in end diastolic volume. Relative ventricular mass (RVM) is positively correlated with peak systolic pressure (SBP), mean blood pressure, and pulse pressure [145]. Furthermore, long term cold acclimation results in a higher basal heart rate with respect to warm acclimation (~44 bpm compared with ~38bpm). This is achieved by shortening of the duration of the ventricular action potential via modification of potassium channels resulting in a higher heart rate and a more excitable heart. In contrast, during acute cooling, the action potential duration is increased, allowing more  $Ca^{2+}$  into the cell for contraction. [146]. Cardiac enlargement is not solely dependent on temperature; an important point to consider when designing hypertrophy experiments and models in the laboratory. For example in one study, rainbow trout on a 12 hour: 12 hour light:dark cycle show little or no cardiac hypertrophy despite wide ranging

temperatures [147]. Furthermore, somewhat unexpectedly, protein synthesis was two-fold higher in trout heart tissue acclimated from 5°C to 15°C in comparison with a temperature change from 15°C to 5°C, and there was no overall cardiac enlargement in cold acclimated trout [148]. Taken together, these data show that potentially, temperature alone is not sufficient to induce cardiac hypertrophy, and seasonal effects need to be taken into account.

### ***1.3.1.2 Warm acclimation***

At maximum critical temperature (CT<sub>max</sub>) there is a significant reduction in work and power of the trout heart [149], indeed warm acclimated fish have a reduced ability to perform sustained aerobic exercise than cold fish, probably due to decreased blood oxygen content [150]. The pumping ability of the trout heart is affected by metabolic rate, critical swimming speed (U<sub>Crit</sub>) and ventricular morphology [151]. Warm acclimated trout have a higher U<sub>Crit</sub> value and swim harder and possibly more anaerobically [136] than their cold counterparts.

### ***1.3.1.3 Calcium dynamics***

Ca<sup>2+</sup> stores from the SR contribute to contraction in the fish heart, and the SR of fish heart is able to retain its Ca<sup>2+</sup> load at low body temperatures. Indeed, as discussed below, cold acclimation appears particularly important in SR involvement. Higher rates of contraction in cold temperatures are observed in the burbot (*Lota lota*) and inhibition of SR ryanodine receptors (RyRs) is able to reduce the force of contraction in cold fish only [14].

Furthermore another group reported the SR blocker ryanodine was only inhibitory in the cold trout [153]. SR in cold acclimated fish increases however the effects of this increase in SR are not seen until the fish is acutely warmed. Indeed, SR appears to supply a greater component of activating calcium after cold acclimation, in acutely warmed hearts, but not in acutely cooled hearts [154]. Acclimation to cold also increases the Ca<sup>2+</sup> uptake rate of trout cardiac SR [155] presumably as a measure to remove the extra Ca<sup>2+</sup> released by the

SR. Despite the increased involvement of the SR in cold acclimated fish, temperature acclimation has no effect on the expression of rainbow trout heart SR ryanodine receptor (RyR) [156], furthermore the ratio of SR RyRs and SL RyRs are not affected by temperature acclimation [14], leading to the possibility of other protein involvement in  $\text{Ca}^{2+}$  within the SR response of the fish cardiomyocyte. Indeed, calsequestrin, the SR binding protein was shown to be 1.5-2 times higher in cold acclimated than warm acclimated rainbow trout [157].

#### ***1.3.1.4 Oxygen availability***

In the wild, rainbow trout experience acute changes in both temperature and oxygen availability and exhibit behavioural selection of low temperatures during hypoxia that acts to reduce metabolism and alleviate the demands on the heart. At high oxygenation, maximum cardiac output and power output increase with temperature as a result of increased heart rate. In cold acclimation trout hearts are able to maintain routine cardiac output during a 20 minute period of anoxia. In warm acclimated trout, hearts fail sooner in the same anoxic conditions [137]. Hypoxia in trout is poorly tolerated with respect to the goldfish, possibly due to the low myoglobin affinity for oxygen [158]. However the salmonid heart does have the ability to adapt and  $\beta$ AR density in the spongy, but not compact layer was shown to increase by 14% in hypoxic conditions [159]. Severe hypoxia in trout is associated with bradycardia and unchanged cardiac output, whereas mild hypoxia is associated with a small but significant increase in cardiac output with no bradycardia [160]. For most teleost fish, the majority of the myocardial oxygen supply is provided by the oxygen reserve remaining in venous blood after other tissues have extracted oxygen. Despite clear evidence suggesting that oxygen availability affects trout cardiac performance, it has been suggested that because of a right-shift in the haemoglobin-oxygen dissociation curve at warm temperatures there is little difference in the oxygen supply in the cardiac circulation between warm and cold fish [161].

### ***1.3.1.5 Beta adrenergic system***

Stimulation of cardiac  $\beta$ -adrenergic receptors ( $\beta$ ARs) by physiological adrenaline-like substances increases cardiac output via inotropy, chronotropy and lusitropy.  $\beta$ 1AR exerts its effect via Gs's activation of adenylyl cyclase (AC) which increases cAMP and subsequently activates PKA which in turn activates L-Type calcium channels and SR protein phospholamban amongst other target proteins.  $\beta$ 2AR is coupled to both Gs and Gi proteins and therefore, via the Gi route is able to negatively influence cAMP production. However the Gs portion of  $\beta$ 2AR signalling is able to indirectly activate nearby LTTCs.  $\beta$ 3ARs operate via the nitric oxide (NO) stimulating guanylyl cyclase leading to production of cGMP which may activate protein kinase G (PKG) or phosphodiesterase 2 (PDE2) [71].

Adrenergic stimulation is believed to be crucial for maintaining cardiac output at all activity levels, regardless of temperature [162]. Indeed, SL  $\text{Ca}^{2+}$  was shown to be increased at all acclimation temperatures following adrenergic stimulation [149]. Furthermore, adrenergic stimulation enhances heart pump activity in the living trout without any oxygen waste [163]. It is believed that  $\beta$ AR density and affinity show massive species variation and these differences cannot be alone attributed to temperature [164]. In trout, cold acclimation up-regulates the beta adrenergic system and adrenaline strongly opposes the temperature-dependent deterioration of force production in cold-acclimated trout but not in warm-acclimated trout [140].

### ***1.3.1.6 Gender influences***

Sex is an important factor in cardiac remodelling in fish species. For example male rainbow trout exhibit ventricle enlargement three fold higher than their female counterparts, typically a 31% increase in cardiomyocyte length and an 83% increase in cross sectional area [165]. This gender effect is thought to be due mainly to testosterone

but has also shown to be dependent on hypertension and hypervolemia differences between the genders [11]. Furthermore mitochondria levels are higher in male heart and mitochondrial protein synthesis is markedly increased in males and this is strongly inhibited by decreased temperature [166]. Male ventricular strips have been shown to have a greater ionotropic response to glucose and testosterone compared with females subject to glucose and 17beta-estradiol. Furthermore, sexually maturing males show a greater ionotropic response than immature males or females and pretreatment with ryanodine, a blocker of SR Ca<sup>2+</sup> release abolished the sex steroid responses. Male trout hearts have a greater response to caffeine than females however when comparing Ca<sup>2+</sup> concentration and force production above baseline, females appear to be more Ca<sup>2+</sup> sensitive [167]. Despite the apparent differences at the cellular level however, using Doppler echocardiography and electrocardiography it has been shown that the systolic function is maintained over a wide range of heart sizes for both sexes and maturation state of males [168].

### **1.3.2 Connexins in fish**

Connexins in fish are much less studied than in mammals; this is particularly true of heart research, however with the emergence of zebrafish genetic tools of research, more information is coming to light on the importance of connexins in fish. Indeed, much of this research has revealed striking similarities between connexins in fish and mammals in terms of structure, function and regulation. As in mammals, dye passage experiments in fish embryos have revealed a key determinant of connexin function in fish is their electrochemical properties [169] Furthermore, in the atlantic croaker (*Micropogonias undulatus*) it has also been demonstrated that connexins can form heterologous as well as homologous gap junctions [170] as is widely accepted in mammals. Indeed, such is the similarity of fish and mammalian connexins, zebrafish Cx30.3 is now used as a viable model for human skin disorders and deafness [171] due to its high homology to Cx30.

### **1.3.2.1 Connexins in fish heart**

Connexins are highly conserved between species, and over the last decade it has become apparent the importance of connexins in fish heart, although the information to date is limited. Zebrafish mutant *fk* has severe heart development problems related to a loss of function of Cx36.7 [172]. Zebrafish Cx48.5 is expressed in the embryonic and adult heart and morpholino injection, disrupting Cx48.5 expression resulted in severe cardiovascular deficiencies [173]. Zebrafish mutant studies have shown that a Cx46 mutation in the heart, not only leads to disturbed electrical conduction in the ventricle, but this conduction impairment can directly cause changes in heart morphology, therefore directly influencing cardiac remodelling [174]. Furthermore Cx43.4 in zebrafish heart has been shown to be involved in symmetrical gene expression and is required for correct left-right axis formation in the heart [175]. In the adult zebrafish Cx45.6 is present in the atrium and ventricle [176].

### **1.3.2.2 Connexin 43 in fish**

Cx43 is the most important connexin in mammalian heart, however in the fish, the distribution and role of Cx43 is much more widespread. Cx43 has been cloned and sequenced in zebrafish and was found to contain multiple transcription sites and expression locations including notochord and brain, heart and vasculature, indicative of high regulation as well as similarity of function and expression with respect to mammalian Cx43 [177]. Furthermore, the *short fin* zebrafish mutant is caused by Cx43 mutations where it has been suggested that mutant Cx43 is unable to fulfil its role as a molecular “ruler” [178]. Cx43 has been shown to be expressed during zebrafish lens development [179] and Cx43 remodelling has been observed trout testes in response to seasonal change [180] where Cx43 expression and distribution in the trout testis is highly regulated [181] and dependent on cAMP signalling [180]. As in mammals, Cx43 has been found in fish



brain cells [182] but unlike mammals, Cx43 has been implicated in oocyte maturation in the rainbow trout [183].

### ***1.3.2.3 Connexin signalling and regulation in fish***

Zebrafish studies have defined high similarity between the motifs responsible for gene regulation in both fish and mammals [184]. Indeed the Cx32.2 gene itself contains three creb response elements (CBE) suggestive of PKA regulation [185] and PKC signalling is also known in fish connexin [186]. Perch Cx35 has been shown to be a target of cAMP/PKA signalling which ultimately acts to gate the channel [187]. Some connexins in zebrafish have been shown to be highly restricted in terms of their expression profile and one reason for this are highly specific promoter elements within the gene, as characterised when looking at the highly specific expression patterns of Cx55.5 and Cx52.6 in the retina [188]. PKA phosphorylation of Cx35 at Ser110 and Ser276 is key to this retinal modulation in zebrafish [189].

## **1.4 The fish as a cardiac research model**

There are ~30,000 species of fish, and due to evolutionary constraints, interspecies differences between fish heart anatomy are apparent. For example, highly athletic fish such as tuna have a much more powerful heart than relatively sedentary species such as carp. Furthermore, species such as eel, that is able to migrate across land, have a higher tolerance to myocardial hypoxia than species such as salmonids. These physiological differences provide the researcher with useful tools when dissecting cardiac function in different contexts. Within fish of the same species, cardiac plasticity is well documented and involves a variety of anatomical and physiological changes to the heart in response to prolonged environmental insults including temperature, photo-period, sexual maturation, food deprivation and hypoxia. With respect to cardiac remodelling, most of our current

knowledge has been gained from studies involving mammals including rats and mice. This is mainly due to the greater availability of transgenic strains of animals, a far more numerous supply of effective antibodies for immunohistochemistry work, and the relative ease of invasive experimental techniques and animal housekeeping in comparison to fish. The fish, however, represents an extremely useful tool when studying cardiac physiology as a whole, particularly cardiac plasticity and cardiac hypertrophy. Fish heart regulation is less dependent on neuro-hormonal input than mammals, allowing a greater focus on intrinsic properties and responses of individual cardiomyocytes in research models. Moreover, physiological cardiac hypertrophy is a common, natural phenotype in fish that can be easily induced in the laboratory without the need to administer agents commonly used to promote non-physiological types of hypertrophy in mammalian models.

## **1.5 Summary of introduction**

Cardiac remodelling is a response to physical stimuli that causes long and short term changes in ion handling and dynamics, molecular events and structural alterations in the heart. These changes are triggered by factors including pressure and volume overload, and mediated by several well defined cell signalling mechanisms including calcineurin/NFAT and AKT/GSK 3 $\beta$  which appear important in pathological and physiological remodelling respectively. In mammals, a common form of cardiac remodelling is cardiac hypertrophy that occurs as a result of physiological and pathological stimuli such as chronic exercise and chronic hypertension respectively. In tandem with hypertrophy, there are gap junction changes, and this highly regulated *connexin remodelling* is known to affect cardiac conduction. Fish, which have a relatively simple heart structure, also remodel their hearts in response to environmental stimuli; for example, temperature. Temperature induced remodelling in fish is known to involve Ca<sup>2+</sup> handling changes at the ionic and organelle levels, and hypertrophy of the heart has been observed during cold acclimation. It is the

purpose of this study to explore in more detail, cardiac remodelling in fish heart in terms of heart structure, connexin distribution and the potential triggers and mechanisms behind the changes.

## 2 Materials and methods

### 2.1 Fish origin and care

#### 2.1.1 Acclimation of fish

Female rainbow trout (*Oncorhynchus mykiss*), ~475 grams in weight, were obtained from Chirk Trout Farm, Wales and transported to the University of Manchester in 500 litre constantly aerated tanks. Fish were maintained in 1000 litre tanks with continuous circulation of aerated ground water ( $0.5\text{ l min}^{-1}$ ), kept under a 12h dark: 12h light photoperiod and fed three times a week with commercial fish pellets. A 50% water change was carried out 3 times a week and filters were changed once a week. Following transportation, fish were placed in 11°C tanks for 1 week in order to acclimatise to their new surroundings and minimise stress before acclimation. Fish were then separated at random into 3 cohorts:

- **Cold (4°C)** – all fish acclimated to 4°C, termed *Cold* throughout.
- **Control (11°C)** – all fish acclimated to 11°C, termed *Control* throughout.
- **Warm (19°C)** - all fish acclimated to 19°C, termed *Warm* throughout.

Following acclimatisation at 11°C for a week, cold fish were gradually subjected to a temperature change of 1°C/ day until 4°C was reached, control fish were left at 11°C, and warm fish were gradually subjected to a temperature change of 1°C/ day until 19°C was reached. To reflect as best as possible the most likely photoperiod experienced by fish in the wild, the cold acclimated group were started on an initial 12h dark: 12h light photoperiod which was changed 1hr every 3 days until a 18h dark: 6h light photoperiod

was achieved. This was to minimise as best as possible disruption to naturally occurring circadian rhythms. Fish were left for at least 3 months to acclimate before being sacrificed. For each acclimation cycle, a total of ~30 fish were acclimated at each temperature with the death rate being ~2% in the cold tanks, ~5% in the control tanks and up to ~20% in the warm tanks.

## **2.2 Measurement of heart chamber mass**

Body weight and heart weight were determined to establish gross remodelling following thermal acclimation. Following acclimation, fish from all three temperatures were stunned with a sharp blow to the head, the spine was severed just below the head, and the brain was destroyed by pithing, before the heart was removed, in line with local and national animal handling regulations. Excess blood was massaged out of the ventricle in physiological solution (NaCl 100, KCl 10, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 4, taurine 50, glucose 20, and Hepes 10, adjusted to pH 6.9 with KOH) before weighing of the bulbous, the atrium and the ventricle as well as whole fish body weight. Excess solution was blotted off using filter paper to ensure accuracy in measuring.

## **2.3 Staining**

### **2.3.1 Tissue preparation for Masson's trichrome staining**

The ventricles used for MT staining were also sectioned for immunostaining (see below). Therefore, these hearts were frozen and cryosectioned rather than using conventional paraffin embedded sections. The ventricle was placed on a silicone Petri dish kept on ice, pinned out to determine orientation and the position of the apex was recorded before the ventricle was surrounded by OCT embedding medium (BDH, Poole, UK) for cryoprotection. The embedded tissue was then frozen using iso-pentane cooled by liquid nitrogen, wrapped in foil and kept at -80°C until sectioning. Ventricles were sectioned using a Leica CM3050S cryostat from bulbous and atrial openings towards the apex. Serial

sections of 10µm were mounted onto Superfrost Plus glass slides (VWR International, Germany) kept at -80°C, with three sections per slide.

### **2.3.2 Tissue preparation for picosirius-red and elastin staining**

Following rapid heart removal as described above, ventricles of trout from all three acclimation temperatures were immediately placed in phosphate buffered saline (PBS) solution containing 4% formaldehyde (PFA) for 24h to fix the tissue. The tissue samples were dehydrated in the Shandon Citadel 2000 Tissue Processor, using graded alcohol solutions (70% ethanol, 90% ethanol, 3x 100% ethanol) followed by 3 xylene washes, for 1.5hrs per stage. Samples were then immersed in molten paraffin wax and embedded in paraffin blocks, which were allowed to solidify overnight. 5µm transmural tissue sections were taken using the Leica RM2125 rotary microtome. Sections were laid in a water bath pre-heated to 52°C to ensure any creases and air bubbles were removed and finally placed onto polylysine-coated slides and dried overnight at 37°C.

### **2.3.3 Masson's trichrome staining**

Masson's trichrome staining protocol was applied to frozen rainbow trout ventricle tissue sections. All stains were prepared following the protocol in Bancroft *et al.* (2002), and all chemicals were obtained from Sigma (Poole, UK). Frozen slides were immersed in Bouin's fluid overnight, and then dehydrated in 70% ethanol for 10 minutes, repeated 3 times. Slides were then stained in Celestine Blue for 5 minutes, rinsed in distilled water (dH<sub>2</sub>O) and immersed in Cole's Alum Haematoxylin for 5 minutes. After 15 minutes washing with tap water, tissue was stained in Acid Fuchsin for 10 minutes, and rinsed in dH<sub>2</sub>O until clear. Phosphomolybdic Acid was then applied for 5 minutes, with slides briefly drained, then submerged in Methyl Blue for 90 seconds then rinsed in dH<sub>2</sub>O. Slides were then dehydrated through a series of ethanol baths, 70% (20 seconds), 90% (20 seconds), and 100% (2 minutes x 2). Finally, tissue was prepared for mounting in clear solvent

Histoclear twice for 5 minutes each. Slides were then mounted with glass cover slips in DPX mountant (VWR International, Leicestershire, UK).

### **2.3.4 Picosirius red staining**

Paraffin wax was removed from the tissue sections by firstly immersing in xylene for 5 minutes followed by graded alcohol rehydration (100% ethanol, 90% ethanol and 70% ethanol) for three minutes per stage and finally two stages of distilled water for three minutes per stage. The sections were then stained in 0.1% Sirius red saturated in picric acid for a period of 1h and sections were blot-dried and mounted with cover slips.

### **2.3.5 Elastin staining**

Sections were first de-waxed using the IHC staining procedure for paraffin sections. This comprised running them through a series of solutions in the following order – two changes of xylene for 5 minutes each, two changes of ethanol (absolute) for 3 minutes each followed by 90% ethanol, 70% ethanol, 50% ethanol and finally distilled water for 3 minutes per stage. Slides were then stained for elastin using Aldehyde fuchsin as follows. The sections were rinsed with water, then oxidised in 1% potassium permanganate solution for 5 minutes and then rinsed in tap water. The permanganate stain was removed by treating the slides with 1% oxalic acid. Following this they were rinsed in tap water again. Slides were then treated in 70% ethanol and stained in Aldehyde fuchsin for a period of 20 minutes. The excess stain was then removed with 70% alcohol and the sections were washed in tap water. Sections were checked microscopically for staining of elastic fibres then rinsed with distilled water and counterstained with a light green working solution for 2 minutes. Sections were dehydrated by immersing in 95% ethanol and absolute alcohol. Following this, they were immersed in xylene; coverslips were mounted using a synthetic resin mountant and then left to dry.

### **2.3.6 Immunostaining**

All stages of the protocol were carried out at room temperature unless stated. A PAP pen (Sigma) was used to draw boxes on Superfrost Plus glass slides, around pre-mounted frozen tissue sections. Slides were next fixed in 10% buffered formalin (Sigma) for 30 minutes before three 10 minute washes in 0.01M PBS (phosphate buffered saline; Sigma), followed by 30 minute permeabilisation in 0.1% Triton-X100 (Sigma) in PBS. Slides were then washed 3 times in 0.01M PBS for 10 minutes before blocking of non-specific sites using 1% BSA (bovine serum albumin; Sigma) in PBS for 60 minutes. Primary antibodies in 1% BSA were then applied to individual squares and incubated at 4°C overnight. Primary negative controls involved applying 1% BSA without diluted antibody. The following day, slides were washed in PBS for 10 minutes, 3 times before application of the secondary antibody in 1% BSA incubated for 90 minutes. Following a final 3 PBS 10 minute washes, slides were mounted with cover slips in Vectashield (H-1000; Vector Labs, Peterborough, UK), and sealed with nail varnish.

### **2.3.7 Confocal microscopy and Masson's trichrome image analysis**

Both confocal microscopy and light microscopy images were taken with LSM 5 Pascal Laser Scanning Microscope (Zeiss, Germany) using Pascal software (Zeiss) for immunodetection and Axiovision software (Zeiss) for histology (Masson's trichrome stained sections). ImageJ software (Rasband, 2006) was used to analyse the resulting images for collagen and muscle content using a software plugin for Image J called *colour threshold* allowing specific stained tissue to be quantified by specific selectable colour. Inherent measurement tools within the core ImageJ program were used to determine bundle and cell areas, and compact layer thickness.

### **2.3.8 Bright field and polarised microscopy for elastin and collagen analysis**

Bright field and polarised images were required to calculate the total collagen content within the tissue sections. Bright field images were used to calculate elastin, muscle and



collagen whilst polarised light images were collagen specific. The polarised light allows visualisation of collagen due to picric acid binding to the collagen fibres and due to the fact collagen fibres are organised in such a way as to allow light to pass through in a single plane, therefore allowing collagen fibres to appear illuminated against a dark background. All images were taken using a Leica CTR5000 Microscope. Images were taken across the complete width of the tissue to form approximately 4-5 continuous micrograph images, resulting in ~50 images for each ventricle. The montages included images from the spongy myocardium and the compact layer. Camera settings are listed below:

**Table 2.1 – Microscope settings used to analyse slides and take images**

<b>Setting</b>	<b>Bright Field</b>	<b>Polarised Light</b>
Exposure	6.44ms	1.0s
Saturation	1.4	1.55
Gamma	0.74	1.3
Gain	1.4X	2.1X

## **2.4 Myocardial protein studies**

In order to investigate events at the protein level (connexins and cell signalling proteins) during acclimation, several procedures were carried out. Protein was taken exclusively from the spongy layer of the trout heart. This was primarily due to the difficulty in obtaining sufficient quantities of compact layer tissue, as opposed to the abundant spongy layer, which was required to satisfy the requirements of the procedures described below. Time was also a factor. Optimisation of antibodies and proteomic techniques took over a

year and this was all carried out on spongy layer tissue. It was decided that to repeat the same process on compact layer would not be time feasible.

#### **2.4.1 Homogenisation and centrifugation of heart tissue**

To isolate membranous and cytosolic connexion, in order to ascertain sub-cellular distribution, 1g frozen ventricular (spongy layer) tissue per 10ml homogenisation buffer (12mM HEPES, 300mM sucrose, 1 Complete mini EDTA free protease inhibitor cocktail (Roche)), was homogenised using a Dremel Tissue Tearor 395 over ice. Homogenates were spun at 2,500g for 10 minutes to remove unlysed cells, the nuclei, and unwanted cellular debris. Pellet was discarded and supernatant kept and termed the *total* fraction. Half the total fraction was further centrifuged at 100,000g for 30 minutes. The supernatant was kept and termed the *cytosolic* fraction, and the pellet was also stored for usage and called the *membrane* fraction. The pellet was resuspended in 300µl homogenisation buffer. All fractions were stored at -80°C until usage. Fractions were used directly in Western blotting.

#### **2.4.2 Dephosphorylation of protein**

One of the key proteins looked at in this study was Cx43. Cx43 is known to exist in phosphorylated and dephosphorylated states in different contexts. Dephosphorylation was looked at to determine the degree of resistance to dephosphorylation of the protein. 1µl calf intestinal alkaline phosphatase (CIAKP, Roche, Welwyn, UK) and 5ul phosphatase buffer (0.5M Tris – HCL, 1mM EDTA, pH 8.5) were added to 30µg of previously homogenised trout ventricular tissue and incubated at 37°C for 1 hr.

#### **2.4.3 Western blotting**

Previously homogenised and frozen rainbow trout and rat ventricle tissue was analysed for protein concentration using Bio Rad protein assay (Bio – Rad, Hertfordshire, UK) and absorbance read @ 595nm using a Gene Quant Pro reader (GE Healthcare,

Buckinghamshire, UK). 30µg protein per sample and 7.5µl NuPAGE LDS sample buffer (Invitrogen, Paisley, UK) per lane was prepared and heat shocked at 95°C for 5 minutes, before being loaded into NuPAGE Novex Bis-Tris 10% electrophoresis gel (Invitrogen) alongside either Full-range Rainbow Molecular Weight Marker (Amersham Biosciences, Buckinghamshire, UK) or MagicMark XP Western Protein Standard (Invitrogen).

NuPAGE MES SDS running buffer (5% in dH<sub>2</sub>O) filled the upper and lower chambers of electrophoresis unit (Invitrogen) and samples were run for 1hr 15 minutes at constant 200V. Protein on gels was then transferred to nitrocellulose membrane (0.2µm pore size; pre-soaked in methanol and NuPAGE Transfer Buffer [5% buffer with 10% methanol per membrane in dH<sub>2</sub>O]) using XCell II Blot module (Invitrogen) filled with transfer buffer in electrophoresis unit for 1 hour at constant 30V. After transfer, membranes were blocked in milk solution (2.5g skimmed milk powder in 47.5ml TBS-T [2.42g TrisBase, 8g NaCl, 1.5 ml Tween Z, in 1l dH<sub>2</sub>O]) 1 hour at room temperature. Membranes were then washed for 10 minutes in TBS-T before primary antibodies (please see full list of antibodies used in Table 2.2 and Table 2.3) were applied and left overnight at 4°C. Primary antibodies were then replaced once by TBS-T, followed by another three 10 minute TBS-T washes.

Secondary HRP conjugated swine anti-rabbit IgG antibody (Dako Labs, Cambridgeshire, UK) used at 1: 5000 in TBS-T was applied to membranes and left at room temperature for 1 hour then washed off with TBS-T four times for 10 minutes each. To visualise protein bands on photographic film 1ml of EZ-ECL chemiluminescent detection kit (Geneflow, Staffordshire, UK) was applied to each membrane for 1 minute then placed into a cassette (Kodak) and transferred onto Biomax MR imaging film (Kodak) and developed and fixed in a dark room.

**Table 2.2 – Primary antibodies used in this study**

<b>Primary antibody (IgG)</b>	<b>Method (WB/ IHC)</b>	<b>Dilution</b>	<b>Host</b>	<b>Source</b>
Cx43 (H – 150)	IHC	1:50	Rabbit	Santa Cruz, California, US.
Cx43 (H – 150)	WB	1:1000	Rabbit	Santa Cruz, California, US.
ILK1 (4G9) Rabbit mAb #3856	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP™ 4370	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK
Phospho-Akt (Ser473) (D9E) XP™ #4060	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP™ #4511	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK
p44/42 MAPK (Erk1/2) (137F5) #4695	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK

Phospho-Smad3 (Ser423/425) (C25A9) #9520	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK
Smad3 (C67H9) #9523	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK
Phospho-GSK-3 $\alpha/\beta$ (Ser21/9) #9331	WB	1:1000	Rabbit	New England Biolabs, Hertfordshire, UK

Primary antibodies used in this study were selected first based on manufacturer's instructions that reactivity in zebrafish had either been observed or predicted to a high degree of confidence. Following this, at least 9 western blots were carried out in control trout alongside rat tissue to confirm target protein selectivity. (WB = used in western blot, IHC = used in immunohistochemistry).

**Table 2.3 – Secondary antibodies used in this study**

<b>Secondary antibody</b>	<b>Dilution</b>	<b>Source</b>	<b>Combined with primary antibodies</b>
Donkey anti-mouse Cy3	1: 400	Chemicon International, Harrow, UK	Cx43 (H – 150) for IHC
Anti rabbit HRP	1:5000	Dako labs, Cambridgeshire, UK	All primary antibodies used for WB

Both secondary antibodies were subject to intensive optimisation studies. This involved applying secondary antibody without the presence of the primary to ensure no inherent activity. (WB = used in western blot, IHC = used in immunohistochemistry)

## **2.5 Genetic analysis**

In order to analyse events at the genetic level during temperature acclimation, a PCR and qPCR approach was used to target and quantify various markers of growth and regulation as well as to specifically find certain genes including connexins. Tissue was taken from the spongy layer only. Although it was easier to obtain genetic material as opposed to protein material from the compact layer, it was decided to remain with spongy layer only due to 1) consistency with protein work and 2) time constraints which would have doubled the amount of experiments and would not have been feasible at the time of experiments.

### **2.5.1 RNA extraction**

RNeasy Microkit, Qiagen was used to extract RNA from the ventricle and atrium. 5mg of freshly isolated atrium or spongy myocardium of the ventricle was excised. Sample tissue was homogenised with 30 second bursts of a Tissue-Tearor in a solution of 150µl BufferRLT containing 0.01% β-Mercaptoethanol (β-ME). 295 µl RNase-free water and 5

$\mu\text{l}$  QIAGEN Proteinase K was added to the homogenate and incubated at  $55^{\circ}\text{C}$  for 10 minutes to digest protein within the homogenate. Homogenate was centrifuged for 3 minutes at  $10,000 \times g$  at room temperature. Pellet was discarded and the supernatant was transferred to a new tube; with  $225 \mu\text{l}$  of 96-100% ethanol added to precipitate out nuclear material. The sample was then transferred to an RNeasy MinElute spin column, placed within a 2ml collection tube. Supernatant was centrifuged for 15 seconds at  $8000 \times g$ .  $350 \mu\text{l}$  Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 seconds at  $8000 \times g$  to clean the spin column membrane. Following this,  $10 \mu\text{l}$  DNase I stock solution was added to  $70 \mu\text{l}$  Buffer RDD. The  $80 \mu\text{l}$  DNAase I Incubation mix was placed directly into the RNeasy MiniElute spin column membrane to degrade and left to incubate for a period of 15 minutes at room temperature to remove unwanted DNA.  $350 \mu\text{l}$  of Buffer RW1 was then added to the RNeasy MiniElute column and centrifuged at  $8000 \times g$  for a period of 15 seconds to clean the spin column membrane. The RNeasy MinElute spin column was then placed in a new 2 ml collection tube and centrifuged at  $8000 \times g$  for 15.  $500 \mu\text{l}$  of 80% ethanol was added to the RNeasy MinElute spin column and centrifuged for 2 minutes at  $8000 \times g$  to clean the column membrane. The RNeasy MiniElute spin column was then placed in a new 2 ml collection tube and centrifuged at full speed for 5 minutes with the eppendorf lid fully open to fully evaporate any unwanted ethanol. Finally, the RNeasy MiniElute column was placed into a 1.5 ml collection tube.  $14 \mu\text{l}$  of RNase-free water was added to the centre of the spin column membrane which was centrifuged at full speed for a minute to elute the RNA. A nanodrop machine (NanoDrop ND-1000) was used to determine the amount and quality of RNA present within the sample. The RNA concentration was  $200\text{ng}/\mu\text{l} \pm 50\text{ng}$ . RNA was stored at  $-80^{\circ}\text{C}$  until required for use.

### **2.5.2 cDNA production**

SuperScript III First-Strand Synthesis System, Invitrogen was used to make cDNA.  $1.6 \mu\text{l}$  of sample RNA,  $1 \mu\text{l}$  of random hexamers and  $1 \mu\text{l}$  of 10mM dNTP mix were mixed and

placed into 0.2ml PCR tubes. Mixtures were incubated at 65°C for 5 minutes and then placed on ice for 1 minute. 2µl of RT Buffer, 4µl of 25mM MgCl<sub>2</sub>, 2µl of 0.1M DTT, 1µl of RNaseOUT and 1µl of SuperScript III RT was added to each of the tubes containing the incubated RNA/ primer mix. The full reaction mix was then incubated at 25°C for 10 minutes, 50°C for 50 minutes and 85°C for 5 minutes then cooled on ice for 5 minutes. 1µl of RNase H was added to each tube to remove any remaining RNA and incubated at 37°C for 20 minutes. cDNA was stored at -20°C until required for use.

### 2.5.3 Primer design, testing and usage

All primers used in this study were taken from published work, where the primer had been used successfully before in rainbow trout, or designed using Primer 3 (<http://frodo.wi.mit.edu/>) online software from full and complete rainbow trout mRNA sequences available on PUBMED. Primers were fully tested using conventional PCR until a single PCR product band of the expected size was observed. If more than one band was seen, or any bands were different from the expected product length, then primers were redesigned until they fulfilled the criteria. Successful primers were then used for qPCR studies. The table below shows all primers used to generate qPCR data in this study:

**Table 2.4 - Primers used in this study**

Gene	Forward sequence	Reverse sequence	Notes
(Ventricular myosin heavy chain) VMHC	TGC TGA TGC AAT CAA AGG AA	GGA ACT TGC CCA GAT GGT T	Marker of hypertrophy
Muscle LIM Protein (MLP)	AGT TCG GGG ACT CGG ATA AG	CGC CAT CTT TCT CTG TCT GG	Marker of hypertrophy
Small myosin light	TCT CAG GCG	CGT AGC ACA	Marker of



chain 2 (SMLC2)	GAC AAG TTC A	GGT TCT TGT AGT CC	hypertrophy
Vascular endothelial growth factor (VEGF)	AGT GTG TCC CCA CGG AAA	TGC TTT AAC TTC TGG CTT TGG	Marker of angiogenesis
Proliferating cell nuclear antigen (PCNA)	AGC AAT GTG GAC AAG GAG GA	GGG CTA TCT TGT ACT CCA CCA	Marker of hyperplasia
Collagen 1 alpha 1 (Col1A1)	GCT TTT GGC AAG AGG ACA AG	GCA GAT AAC TTC GTC GCA CA	Marker of fibrosis
Collagen 1 alpha 2 (Col1A2)	GGC TGA TCG GCT CTG TAC TC	TGG CTC TGC TGG TAT CAC TG	Marker of fibrosis
Collagen 1 alpha 3 (Col1A3)	CCC TGC TTT TTA TGG TTG GA	GCA GGG TTC TGG TTT CCA TA	Marker of fibrosis
Matrix metalloproteinase 2 (MMP2)	TGT ATT GGG CAA CAT CAG GA	CCC AGG AGA CGA TAG TCC AA	Inhibitor of fibrosis
Matrix metalloproteinase 9 (MMP9)	GGT CCA GTT TTC GTC ATC GT	AGA CAT GGG AGC CTC TCT GA	Inhibitor of fibrosis
Matrix metalloproteinase 13 (MMP13)	TCT GAT GTG GTT TGC TGC TC	CAG ATA AGC CCG ACC CTA CA	Inhibitor of fibrosis

Tissue inhibitor of metalloproteinases 2 (TIMP2)	CAG GCC ATC CAC CTA CTG TT	TGT TGC TCT CTT GCA TAC GG	Inhibitor of MMP
Connexin degenerate primer forward reverse 1 (F1R1)	ATG GGT GAC TGG AGY KYC YTR G	ACC ACC ARC ATR AAG AYR ATG AAG	Connexin degenerate primers
Connexin degenerate primer forward reverse 2 (F2R2)	GGC TGY RAS AAY GTC TGC TAY G	GCC KGG ARA YRA ARC AGT CCA C	Connexin degenerate primers
Connexin 43 (Cx43)	CCT ACA GGG CTC TCC ACT C	ACG GTT GAG TTT CTC CTC C	Connexin 43
Atrial natriuretic peptide (ANP)	CCA CAG AGG CTC TCA GAC G	ATG CGG TCC ATC CTA GCT C	Marker of heart stretch
Brain natriuretic peptide (BNP)	TGG CCT TGT TCT CCT GTT CT	GGA GAC TCG CTC AAC CTC AC	Marker of heart stretch
Mineralcorticoid receptor (MR)	CAG CGT TTG AGG AGA TGA GA	CCA CCT TCA GAG CCT GAG AC	Marker of stress
Glucocorticoid receptor 1 (GR1)	AGG TTG TCT CAG CCG TCA AA	GCA GCT TCA TCC TCT CAT CAT	Marker of stress

Glucocorticoid receptor 2 (GR2)	ACT CCA TGC ACG AGA TGG TT	CGG TAG CAC CAC ACA GTC AT	Marker of stress
Chaperone protein 2 (DNAJA2)	TTG TAA TGG AGA AGG TGA GG	TGG GCC GCT CTC TTG TAT GT	Heat shock protein
Beta actin (BACTIN)	AGA GCT ACG AGC TGC CTG AC	GTG TTG GCG TAC AGG TCC TT	Control/ housekeeping gene
Beta adrenoreceptor 2 (BETA2AR)	GTG ATG TGC ATC GTC TTT GG	CCA GGT GTT GAG GAG GAT GT	Beta 2 adrenoceptor
Beta adrenoreceptor 3a (BETA3aAR)	CCT CAA GAC CTT GGG CAT TA	ATG TTA AAT CGT GGG GTG GA	Beta 3a adrenoceptor
Beta adrenoreceptor 3b (BETA3bAR)	CAT AGT GCT CTA CGC GGT CA	GCG TGC TAT AGC GAT GAT GA	Beta 3b adrenoceptor
Regulator of calcineurin (RCAN)	AGT TTC CGG CGT GTG AGA	GGG GAC TGC CTA TGA GGA C	Marker of hypertrophic signalling

Primers selected in this study have either all been used in previous published rainbow work, or designed from full trout mRNA sequences. All primers were tested on trout cardiac tissue in a PCR in order to detect a single distinct band before being used in qPCR.

#### **2.5.4 PCR**

*Taq* PCR Core Kit (Qiagen), containing 10x PCR buffer, 25mM MgCl<sub>2</sub>, 10mM dNTP and *Taq* polymerase was used as follows: 10µl of 10x PCR buffer, 4µl of MgCl<sub>2</sub>, 2µl of dNTP, 0.5µl of *Taq* polymerase and 80.5µl of distilled water was used as standard for each reaction. 1µl of both forward and reverse primers, together with 1µl sample cDNA was used (see section 2.5.2 for information on cDNA production). PCR was performed as follows: three minute initial denaturation at 94°C, followed by 3-step cycling, which consisted of denaturation at 94°C for 45 seconds, annealing for 45 seconds at a temperature ~5°C lower than the melting pointing of the primer and extension at 72°C for 1 minute. The 3-step cycling segment was repeated 34 times. This was followed by a final extension at 72°C for 10 minutes.

#### **2.5.5 Gel Electrophoresis of PCR Product**

A 1% Agar (Lonza) gel was used to separate PCR bands. Agar was dissolved in 1xTAE buffer (pH8) made from a 50x TAE buffer stock solution made from 242g of Tris base dissolved in ~750ml deionised water. 57.1ml of glacial acid and 100ml of 0.5M EDTA (pH 8.0). 2µl of ethidium bromide per 100ml gel was added to the agar solution and the solution was poured into an electrophoresis cast and allowed to set. Once the gel had set in the cast, 1x TAE was poured on top of it. 20µl PCR product was mixed with 5µl loading buffer, obtained from Bionline. 5µl of Hyperladder IV (Bionline) was used as a marker. The gel was run for 90 minutes at 50V and imaged under UV light.

#### **2.5.6 Extraction and purification of PCR band from gel**

PCR bands of interest were extracted and purified using QIAquick Gel Extraction Kit, Qiagen. PCR band of interest was excised out of the gel using a clean, sharp scalpel blade. 3 volumes of Buffer QG were added to 1 volume of gel and incubated at 50°C for 10 min

(or until the gel slice has completely dissolved). 1 gel volume of isopropanolol was added to the sample and mixed. Sample was then added to the QIAquick column, and centrifuged for 1 min at 13000rpm and flow-through discarded. To ensure all agarose is removed, a further 0.5 ml of Buffer QG was added to the QIAquick column and centrifuged for 1 min at 13000rpm and flow-through discarded. To wash, 0.75 ml of Buffer PE was added to the QIAquick column, left to stand for 5 minutes and centrifuged for 1 min at 13000rpm. Flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 13,000rpm. To elute the DNA, 30  $\mu$ l of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane, left to stand for 1 minutes and the column was centrifuged for 1 min at maximum speed.

### **2.5.7 Sequencing**

DNA sequencing was carried out by the University of Manchester's DNA Sequencing Facility, who were provided with ~200ng DNA (extracted PCR product) and relevant primers. The output of the sequencing was a series of text files containing nucleotide sequences of the DNA samples. 10 – 20 nucleotide base pairs were trimmed off either end of the text file, and the remaining sequence was inputted directly into PUBMED's nucleotide BLAST algorithm.

### **2.5.8 qPCR**

SYBR Green I pre-mixed chemo-technology was used for qPCR. 1 $\mu$ l of both forward and reverse primers, 1 $\mu$ l of 1:10 cDNA, 5 $\mu$ l of SYBR Green mix and 3.5 $\mu$ l deionised H<sub>2</sub>O was used per reaction within a 96 well qPCR plate, centrifuged @ 1200g for 2 minutes immediately prior to qPCR. qPCR was carried out in a 7900HT sequence detection system (Applied Biosystems) using the following step-wise procedure: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a dissociation stage at 95°C for 15 seconds, 60°C for 15 seconds, and a slow ramp to 95°C.

Gene *cycle threshold* (Ct) values, generated by the qPCR machine, multiplied by primer efficiency (Pe) were used to determine gene expression levels: Expression (e) =  $1 / (Ct * Pe)$ . All expression levels were normalised to housekeeping gene  $\beta$ -actin to determine absolute expression levels for comparison at each acclimation temperature.  $\beta$ -actin was used in preference to GAPDH as GAPDH is involved in metabolic cascades which could be influenced by temperature.

## **2.6 Statistical analysis**

Throughout the thesis, all acclimation groups in each individual experiment (e.g. one specific qPCR marker or one mass parameter) were statistically analysed against each other using a One Way ANOVA test using SigmaPlot 11.0 software followed by the Holm-Sidak post-hoc test with a significance threshold of  $P < 0.05$ . N numbers and SEM values are quoted in the legend under each respective experiment figure.

### **3 Structural adaptations in rainbow trout heart during temperature acclimation**

#### **3.1 Brief introduction**

Fish, which are active over a wide range of temperatures in the short and long term, must be able to maintain adequate cardiac output despite varying physical and physiological insults. Although changes in  $\text{Ca}^{2+}$  flux and the proteins involved in  $\text{Ca}^{2+}$  dynamics during temperature change have been well studied [12, 20], and help facilitate cardiac adaptation, fish also undergo large-scale structural changes during thermal adaptation including muscle and connective tissue remodelling [5, 143].

Chronic temperature change affects several key parameters that influence cardiac demand including oxygen availability, activity levels and blood viscosity [190]. Cold acclimated salmonids are less active, have to contend with relatively more viscous blood, and live in water with relatively high oxygen saturation. Conversely, chronically warm salmonids are more active, have relatively less viscous blood to pump around their bodies and ambient water is less oxygenated [102, 141, 161]. In turn, this therefore dictates the volume of blood required per cardiac stroke, and the force with which this blood is pumped out of the ventricle and through the cardiac system. Therefore stroke volume and force/pressure are key parameters in maintaining effective cardiac output during thermal acclimation in fish.

The fish heart must have measures in place to alter force of contraction and blood volume to cope with thermal challenges. In fish such as rainbow trout, cardiac output regulation is governed by mechanical (stretch) responses (Frank Starling mechanism), and neuro-hormonal input (e.g. adrenaline) which work in concert to preserve cardiac function during

environmental change [16, 146].  $\text{Ca}^{2+}$  dynamics alter force at the cardiomyocyte and sarcomeric level [20], complemented by adaptation of structural/ECM proteins [143, 147]. Muscle is the force provider and connective tissue is the stiffness/ compliance governor. Combined, they provide pumping power yet allow chamber filling and emptying to meet cardiac demand.

Cold acclimation of fish has been shown in a number of species to induce hypertrophy in the heart [138], presumably to counteract an increase in blood viscosity which increases vascular resistance thus requiring greater pumping force to maintain cardiac output [190]. Hypertrophy however has not been analysed in any great detail prior to the start of this thesis work. Indeed, it was unknown if the observed gain in heart mass was limited to ventricular hypertrophy, to what degree ventricular spongy and compact layers were affected, how this change in mass affected stroke volume, whether the gain in ventricular mass was due to changes in cardiomyocyte *size* or *number* and whether there was any change to the extracellular matrix components including collagen and elastin. Importantly, there was no clear molecular evidence that cold acclimation was actually inducing cardiac muscle growth as seen in mammalian hypertrophy; the gain in mass could be attributed to non-muscle phenomena such as energy storage or increases in cytosolic volume.

Furthermore, there was no data available on the effects of *warm* acclimation on the complete morphology of the heart, and whether this could induce opposite remodelling to cold acclimation (i.e. atrophy). Moreover, there was a lack of any evidence to the effects of temperature on the equally important atrium.

It was therefore important to address all these issues in order to gain an overall understanding of the nature and extent of rainbow trout cardiac thermal remodelling. It was



also important to elicit the benefit, based on structural evidence, that remodelling conferred on the animal during acclimation when adapting to fluctuating operating environments.

### **3.2 Key aims**

- Deduce whether temperature acclimation causes cardiac remodelling of the heart at the structural level
- Compare the remodelling of the atrium and ventricle
- Characterise the extent of remodelling *within* the ventricle, including the spongy layer, compact layer, cardiomyocyte trabecular bundles, extrabundular space and connective tissue
- Gain evidence of muscle and connective tissue remodelling at the molecular level
- Use available evidence to understand the nature of the cardiac pump in different environmental contexts

### **3.3 General Methodology**

Full details of the methodologies are in Chapter 2, however below is a concise summary of the methods used to generate the results in this chapter.

In order to address all points of interest above, a single sex, female cohort, acclimated at the University of Manchester was studied in order to examine the ventricle *and* atrium, without the pro-growth male specific gender effect (testosterone/androgens) present. Using staining techniques, muscle and connective tissue were visualised in the ventricle to understand overall structural remodelling. Using genetic techniques, gene expression and regulation was assessed to help ascertain the *type* of cardiac remodelling and to reinforce any structural observations.

Following 3 months of temperature acclimation to cold (4°C), control (11°C) and warm (19°C) temperatures (as described in more detail in Chapter 2) the following was carried out:

1. Measurement of mass of fish, heart and heart chambers by wet weighing
2. Masson's trichrome, picosirius red and aldehyde fuchsin staining of ventricular sections to observe muscle, connective tissue, collagen and elastin components
3. Microscopic imaging (bright field and polarised light) of stained sections
4. ImageJ analysis of microscope derived digital images for muscle content, connective tissue content, collagen and elastin content, spongy and compact layer analysis and extrabundular space

Following the initial assessment (above) a more detailed approach was used to reveal genetic evidence of remodelling:

1. RNA extraction from ventricle spongy layer and atrial tissue
2. cDNA production
3. qPCR of various markers of growth and collagen regulation (detailed below)

Cardiac muscle remodelling was looked at by investigating levels of three muscle-growth genes; namely Ventricular Myosin Heavy Chain (VMHC), Muscle LIM protein (MLP) and Small Myosin Light Chain (SMLC2), with the aim of determining the degree of cardiac muscle gene activation over the range of temperatures. For analysis of whether or not cell number, as opposed to cell size was important in cardiac remodelling, Proliferating Cell Nuclear Antigen (PCNA), a marker of cell division, mRNA levels were looked at. To investigate whether cardiac muscle remodelling correlated to the abundance of blood supply vessels, as well as using blood supply as a marker of cardiac metabolic demand, Vascular Endothelial Growth Factor (VEGF) levels were examined to determine the

degree of angiogenesis. Furthermore, collagen content and regulation was analysed using qPCR to investigate levels of collagen genes (Colla1, Colla2 and Colla3) as well as negative collagen regulators (MMP2, MMP9, MMP13) and positive regulator TIMP2.

### 3.4 Summary of aims, methods and expected outcomes

**Table 3.1 – Summary of aims, methods and expected outcomes (chapter three)**

<b>Aim</b>	<b>Method</b>	<b>Expected outcome</b>	
<b>Determine remodelling of whole heart during temperature acclimation</b>	Weighing of whole heart, atrium and ventricle with respect to fish weight	Whole heart mass determined	
		Relative ventricle mass determined	
		Relative atrium mass determined	
<b>Determine remodelling of cardiac muscle during temperature acclimation</b>	Masson's trichrome staining of ventricle sections and analysis of images using imageJ software	Ventricle spongy layer cardiomyocyte bundle cross sectional area determined	
		Ventricle compact layer thickness determined	
		Ventricle extrabundular space determined	
		qPCR of VMHC, MLP and SMLC2	Determine expression level of muscle-growth important genes in ventricle
			Determine expression level of muscle-growth important genes in atrium
		qPCR of PCNA	Determine whether cardiomyocyte hyperplasia is occurring in the ventricle
			Determine whether cardiomyocyte hyperplasia is occurring in the atrium
		qPCR of VEGF	Determine the extent of vascularisation of cardiac muscle in the ventricle
			Determine the extent of vascularisation of cardiac muscle in the atrium
<b>Determine remodelling of connective tissue during temperature acclimation</b>	Masson's trichrome staining of ventricle sections and analysis of images using imageJ software	Determine overall connective tissue content in ventricle spongy layer	
		Determine overall connective tissue content in ventricle compact layer	
		Picosirius red staining of ventricle sections	Determine collagen levels in ventricle spongy layer
			Determine collagen levels in

		ventricle compact layer
	Elastin staining of ventricle sections using aldehyde fuchsin	Determine elastin content in ventricle
	qPCR of col1a1, col1a2 and col1a3	Determine levels of collagen mRNA in the ventricle
		Determine levels of collagen mRNA in the atrium
	qPCR of MMP2, MMP9, MMP13	Determine levels of negative collagen regulators in the ventricle
		Determine levels of negative collagen regulators in the atrium
	qPCR of TIMP2	Determine levels of positive collagen regulator in the ventricle
		Determine levels of positive collagen regulator in the atrium

### 3.5 Results

Data are presented first as a paragraph of key relative values, followed immediately by directly related figures where full absolute values are provided in the figure legend for each respective experiment. In the appendix are qPCR raw values for cold, control and warm.

#### 3.5.1 Whole heart and chamber masses during acclimation

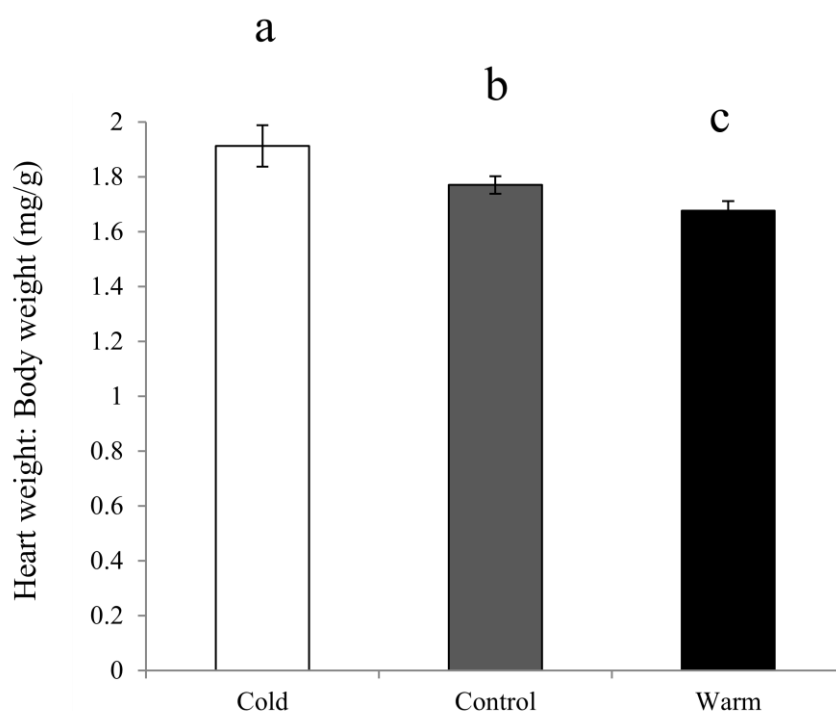
Table 3.2 shows raw acclimation data. After a 3 month acclimation, there was a significant difference in the mass of the hearts between all temperature groups; namely the cold acclimated were 8% and 14% respectively larger in mass compared to control and warm (Figure 3.1) with respect to body mass. When the separate chambers were examined, there were also significant differences, with the ventricle increasing in mass during cold acclimation and the reverse being true of the atrium. Relative ventricular mass (the mass of ventricle with respect to the mass of the fish) in cold acclimated fish was 23% greater than that of the warm group (Figure 3.2). Moreover, the atrium of warm acclimated fish was 17% greater in relative mass in comparison to cold acclimated atrium (Figure 3.3). With respect to the mass of the heart, ventricles from cold acclimated fish were 9.8% greater in

weight compared to warm (Figure 3.4), and atria were of 42% greater mass in warm fish with respect to cold (Figure 3.5).

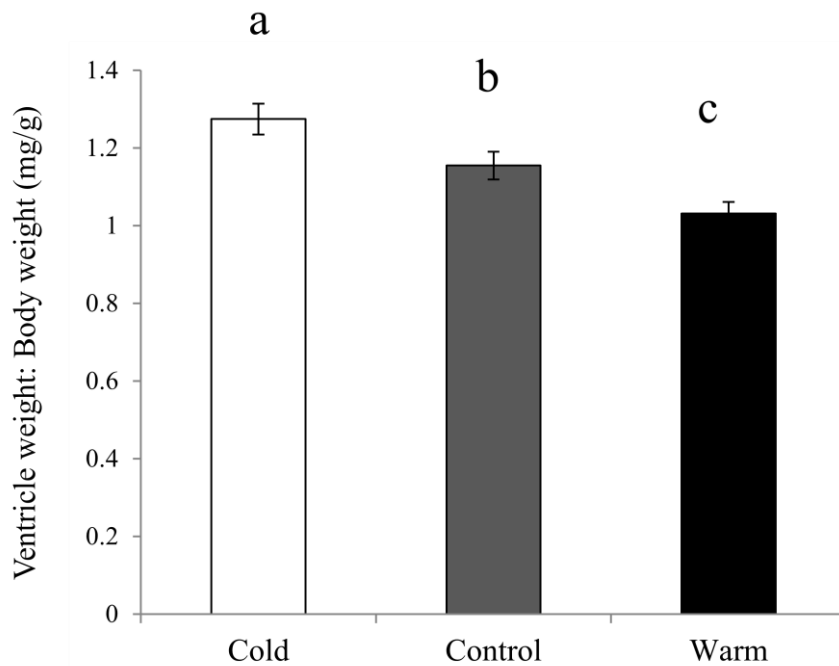
**Table 3.2 - Fish, heart and chamber wet weights after 3 months acclimation**

Acclimation temp (°C)	Fish weight (g)	Atrium weight (g)	Ventricle weight (g)	Bulbous weight (g)	Total heart weight (g)
4	475 ± 4 <sup>a</sup>	0.11 ± 0.01 <sup>a</sup>	0.6 ± 0.02 <sup>a</sup>	0.19 ± 0.02 <sup>a</sup>	0.9 ± 0.04 <sup>a</sup>
11	505 ± 4 <sup>b</sup>	0.12 ± 0.01 <sup>a</sup>	0.58 ± 0.02 <sup>a</sup>	0.19 ± 0.02 <sup>a</sup>	0.89 ± 0.01 <sup>a</sup>
19	515 ± 4 <sup>c</sup>	0.14 ± 0.01 <sup>b</sup>	0.53 ± 0.02 <sup>b</sup>	0.19 ± 0.02 <sup>a</sup>	0.86 ± 0.02 <sup>b</sup>

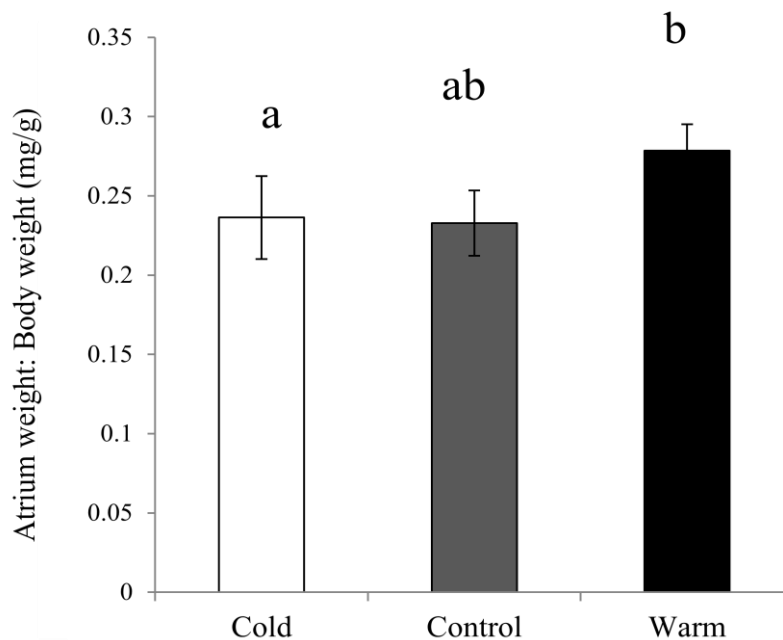
Data is presented as the mean of n = 24 fish per acclimation group with standard error of the mean. Superscripted dissimilar letters within *each column exclusively* represents where significance as dictated by a one way ANOVA statistical test results in p < 0.05.



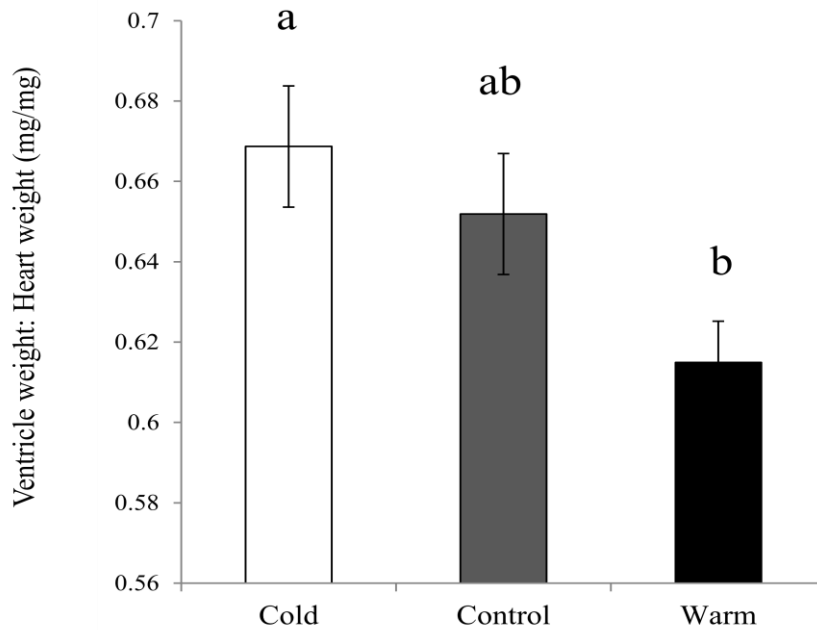
**Figure 3.1- Heart mass as a ratio of body mass.** With respect to whole body mass, cold acclimated hearts were significantly greater in mass (1.91mg/g ± 0.08) than both control (1.77mg/g ± 0.03) and warm hearts (1.68mg/g ± 0.04) representing a difference of 8% and 14% respectively. Data is presented on the histogram as the mean of n = 24 fish from each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represents where significance as dictated by a one way ANOVA statistical test results in p < 0.05.



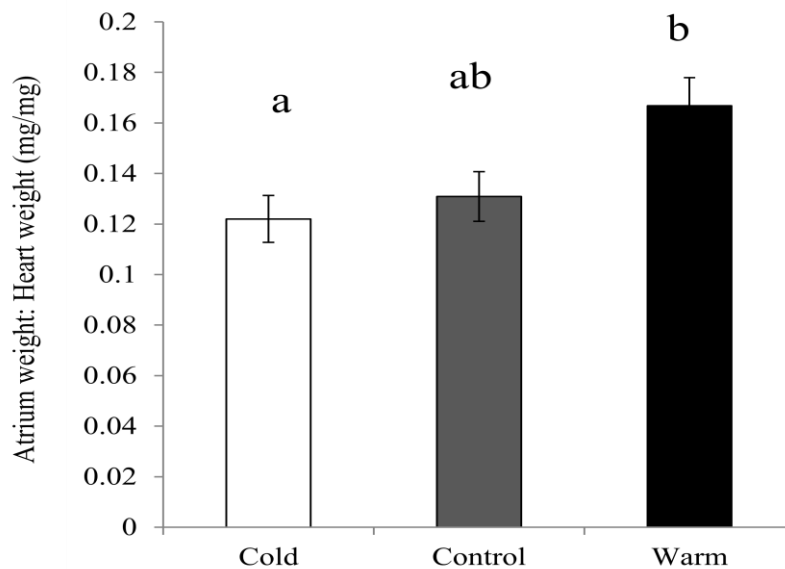
**Figure 3.2 - Ventricle mass as a ratio of fish mass.** With respect to whole body mass, cold acclimated ventricles were significantly greater in mass ( $1.3\text{mg/g} \pm 0.04$ ) than both control ( $1.2\text{mg/g} \pm 0.04$ ) and warm hearts ( $1\text{mg/g} \pm 0.03$ ) representing a difference of 10% and 23% respectively. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represents where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



**Figure 3.3- Atrium mass as a ratio of fish mass.** With respect to whole body mass, warm acclimated atria were significantly greater in mass ( $0.28\text{mg/g} \pm 0.02$ ) than both control ( $0.23\text{mg/g} \pm 0.02$ ) and cold hearts ( $0.23\text{mg/g} \pm 0.02$ ) representing a difference of 17% and 17% respectively. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represents where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



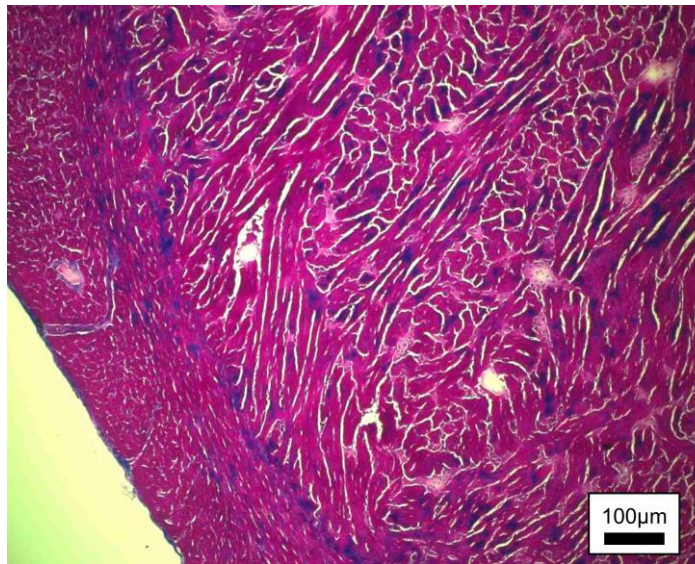
**Figure 3.4- Ventricle mass as a ratio of heart mass.** Cold acclimated ventricles were significantly greater in relative mass ( $0.67\text{mg/mg} \pm 0.02$ ) with respect to warm ventricles ( $0.61\text{mg/mg} \pm 0.01$ ) representing a difference of 9.8%. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



**Figure 3.5- Atrium mass as a percentage of heart mass.** Warm acclimated atrium were significantly greater in relative mass ( $0.17\text{mg/mg} \pm 0.01$ ) with respect to cold atrium ( $0.12\text{mg/mg} \pm 0.01$ ) representing a difference of 42%. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .

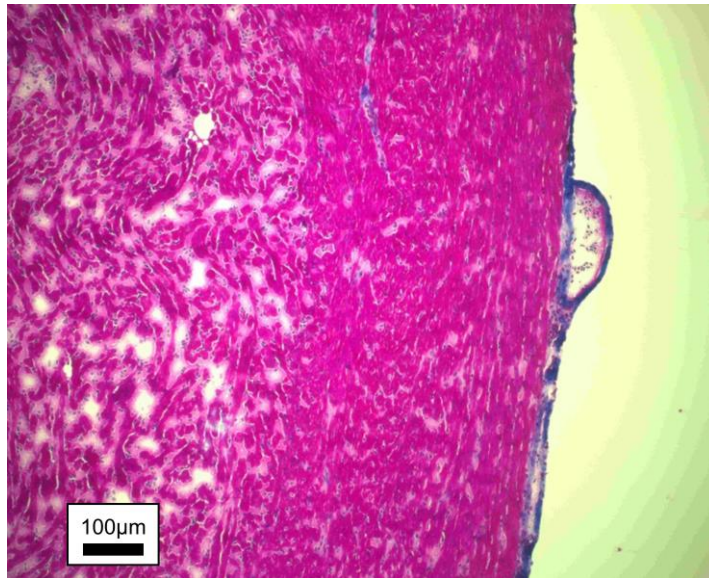
### 3.5.2 Spongy and compact layer muscle remodelling during acclimation

After 3 months acclimation, Masson's trichrome staining of cold and warm cryo-preserved ventricle sections (Figures 3.6 and 3.7 respectively) revealed clear structural differences. Bundles were confirmed for measurement by using anti-Cx43 antibody to reveal cardiomyocyte membranes (Figure 3.8) showing each bundle comprised of ~15 cardiomyocytes. Cold spongy layer bundles increased in size by 77% with respect to warm and warm bundles were 17% smaller than bundles from the control group (Figure 3.9). Increases in bundle size correlated with decreases in extra-bundular space (EBS). Warm spongy layer had 23% more EBS than control and cold had 7.7% EBS less than control (Figure 3.10). As well as spongy layer remodelling there was significant compact layer remodelling. Warm acclimated fish had a 13.3% thicker compact layer than the cold cohort and the cold group's compact layer was 4% thinner than control (Figure 3.11).

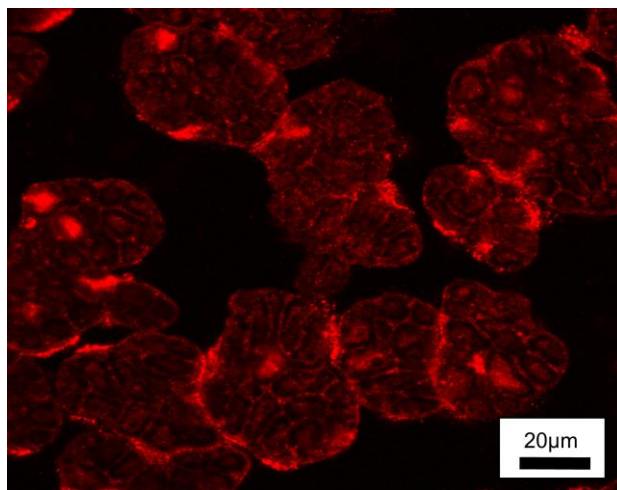


**Figure 3.6 - Masson's trichrome stained section of a typical ventricle from a cold acclimated female rainbow trout** with the distinct compact layer on the left and the spongy layer on the right. Pink/ purple is muscle, blue is connective tissue, white is "extra-bundular" space. Scale bar shown is 100  $\mu\text{m}$ .

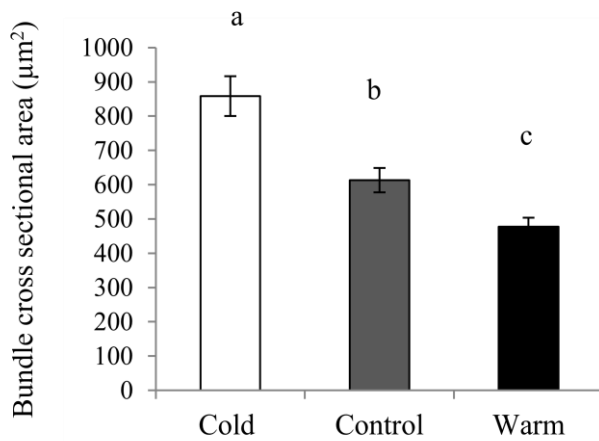




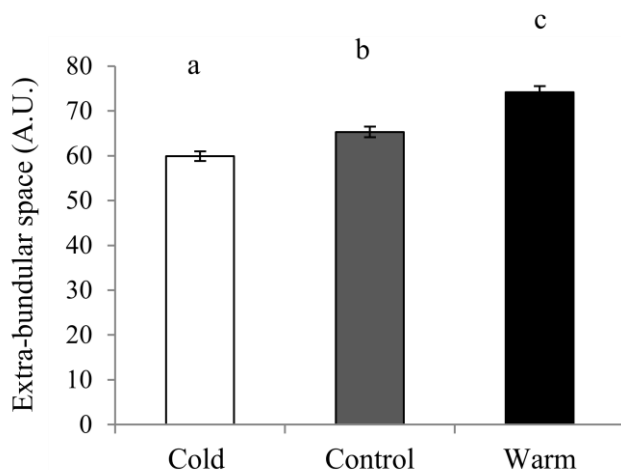
**Figure 3.7 - Masson's trichrome stained section of a ventricle from a warm acclimated female rainbow trout** with the distinct compact layer on the right and the spongy layer on the left. The bubble-like object is a blood vessel with blood cells within. Pink/ purple is muscle, blue is connective tissue, white is "extra-bundular" space. Scale bar shown is 100  $\mu\text{m}$ .



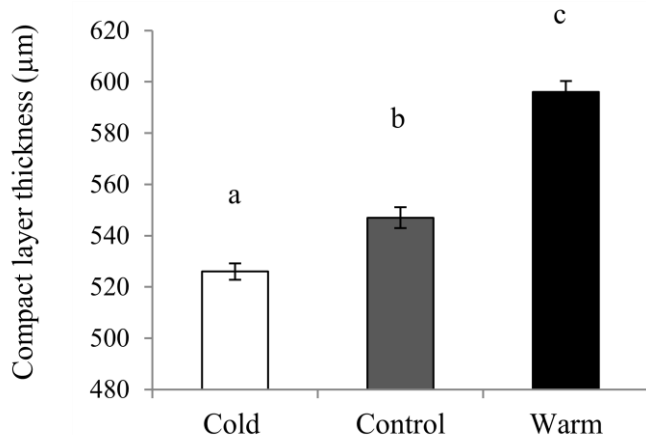
**Figure 3.8 – Anti-Cx43 antibody membrane protein stained section of a typical ventricular spongy layer** from a rainbow trout revealing that "bundles" are made up of ~15 -20 cardiomyocytes arranged cylindrically. Scale bar shown is 20  $\mu\text{m}$ .



**Figure 3.9 – Spongy layer trabecular bundle cross sectional area.** Cold acclimated spongy layer bundles were significantly greater in cross sectional area ( $858 \mu\text{m}^2 \pm 58$ ) with respect to warm spongy layer bundles ( $477 \mu\text{m}^2 \pm 27$ ) representing a difference of 77%. Warm bundle cross sectional areas were 17% less than control conditions. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group with 100 measurements taken for each fish (sample size of 2400 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



**Figure 3.10 – Spongy layer extra-bundular space.** Warm acclimated ventricles had significantly greater extra-bundular space (74 A.U.  $\pm$  1.3) with respect to cold spongy extra-bundular space (60 A.U.  $\pm$  1.1) representing a difference of 23%. Cold extra-bundular space was 7.7% less than control conditions. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



**Figure 3.11 – Compact layer thickness.** Warm acclimated compact layer thickness was significantly greater ( $596\mu\text{m} \pm 4.3$ ) with respect to the cold compact layer thickness ( $526\mu\text{m} \pm 3.2$ ) representing a difference of 13.3%. Cold compact layer thickness was 4% less than control conditions. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group with 100 measurements taken for each fish (sample size of 2400 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .

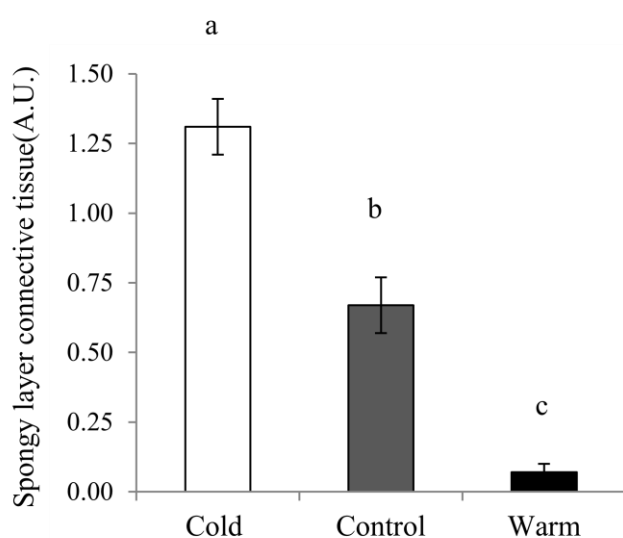
### 3.5.3 Spongy and compact layer connective tissue remodelling during acclimation

*Picosirius staining was carried out by Hamid Rizvi under the author's direct supervision.*

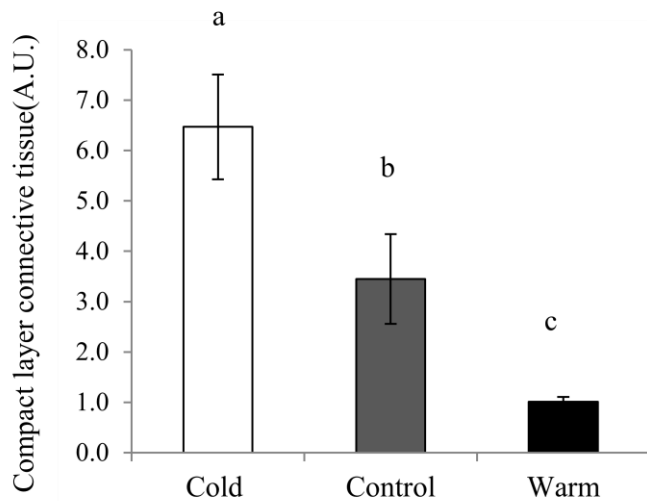
*Aldehyde fuchsin staining was carried out by Devmalya Sarkar in a collaborative study using fish from the author's acclimation groups. Masson's trichrome staining was carried out solely by the author.*

Significant connective tissue remodelling was observed at the structural level. After analysis of Masson's trichrome stained sections (Figures 3.6 and 3.7), in the spongy layer, cold acclimated fish had 1200% more connective tissue than warm and warm fish had 87% less connective tissue than control (Figure 3.12). In the compact layer, cold acclimated fish had 540% more connective tissue than warm and warm had 71% less connective tissue than control (Figure 3.13). Picosirius red sections (Figures 3.14 and 3.15) revealed collagen differences. Overall collagen content in cold acclimated fish ventricle was 100% more than warm, however, there was significantly less collagen in the control fish compared to warm (Figure 3.16). In both the spongy and compact layers there was 100% more collagen in the cold group compared with the warm (Figures 3.17 and 3.18) and

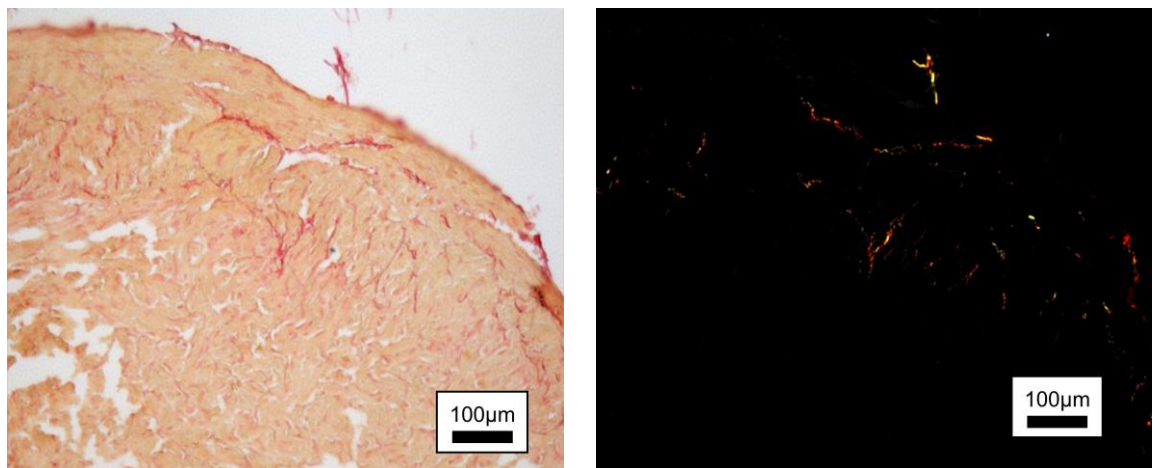
again in both layers the amount of collagen was significantly less in the control than the warm cohort. Aldehyde fuchsin stained sections were used to look for elastin content (Figure 3.19). When elastin content was assessed it revealed warm acclimated fish hearts contain 117% more elastin than their cold counterparts, however there was no difference in elastin content between control and cold acclimated fish (Figure 3.20).



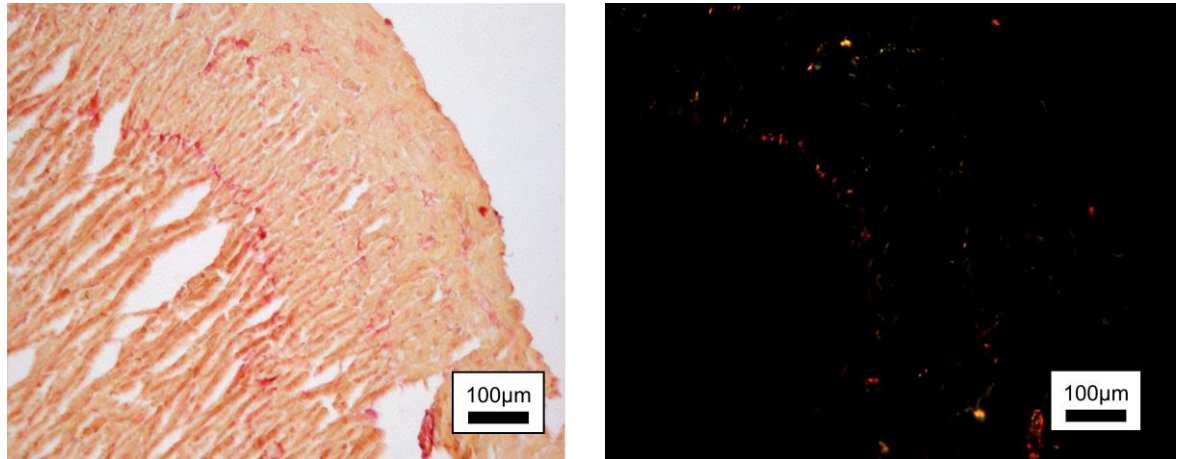
**Figure 3.12 – Spongy layer connective tissue.** Cold acclimated spongy layer connective tissue content ( $1.3 \text{ A.U.} \pm 0.1$ ) was significantly greater than warm spongy layer connective tissue content ( $0.1 \pm 0.02$ ) representing a difference of 1200%. Warm spongy layer connective tissue content was 83% less than control conditions. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group with 100 measurements taken for each fish (sample size of 2400 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



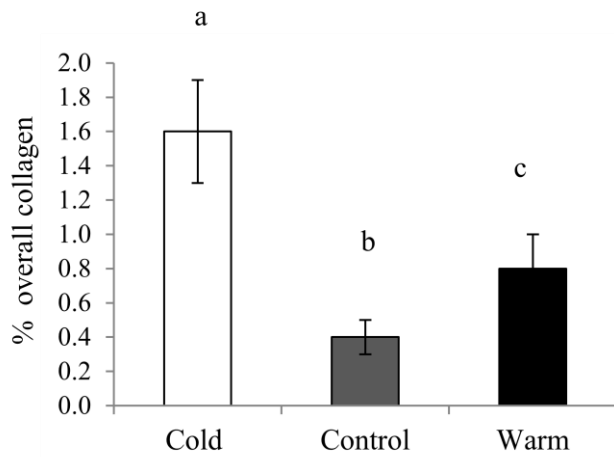
**Figure 3.13 – Compact layer connective tissue.** Cold acclimated compact layer connective tissue content ( $6.4 \text{ A.U.} \pm 1.1$ ) was significantly greater than warm spongy layer connective tissue content ( $1.0 \pm 0.1$ ) representing a difference of 540%. Warm compact layer connective tissue content was 71% less than control conditions. Data is presented on the histogram as the mean of  $n = 24$  fish from each acclimation group with 100 measurements taken for each fish (sample size of 2400 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



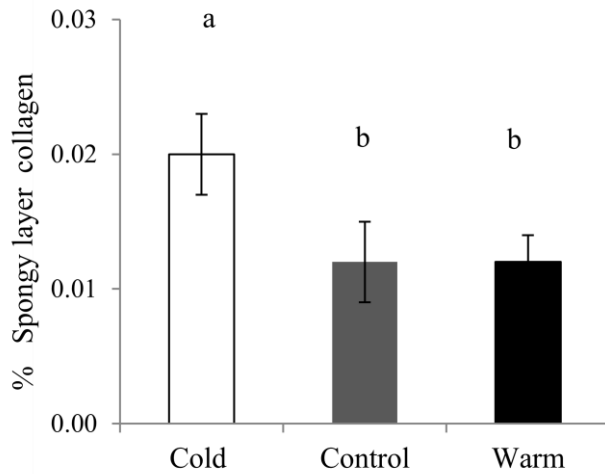
**Figure 3.14 – Picosirius red staining for collagen in cold acclimated ventricle.** On the left is a typical Picosirius red stained ventricle section from a cold acclimated rainbow trout showing muscle in orange and collagen in red. On the right is the same image viewed using polarised light to emphasize the collagen fibres. Scale bar shown is  $100\mu\text{m}$ .



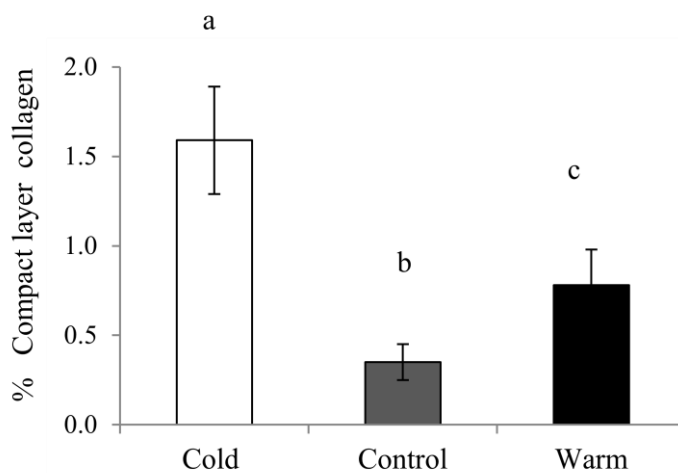
**Figure 3.15 – Picosirius red staining for collagen in warm acclimated ventricle.** On the left is a typical Picosirius red stained ventricle section from a warm acclimated rainbow trout showing muscle in orange and collagen in red. On the right is the same image viewed using polarised light to emphasize the collagen fibres. Scale bar shown is 100µm. (staining carried out by Hamid Rizvi, 2011 under the author's supervision)



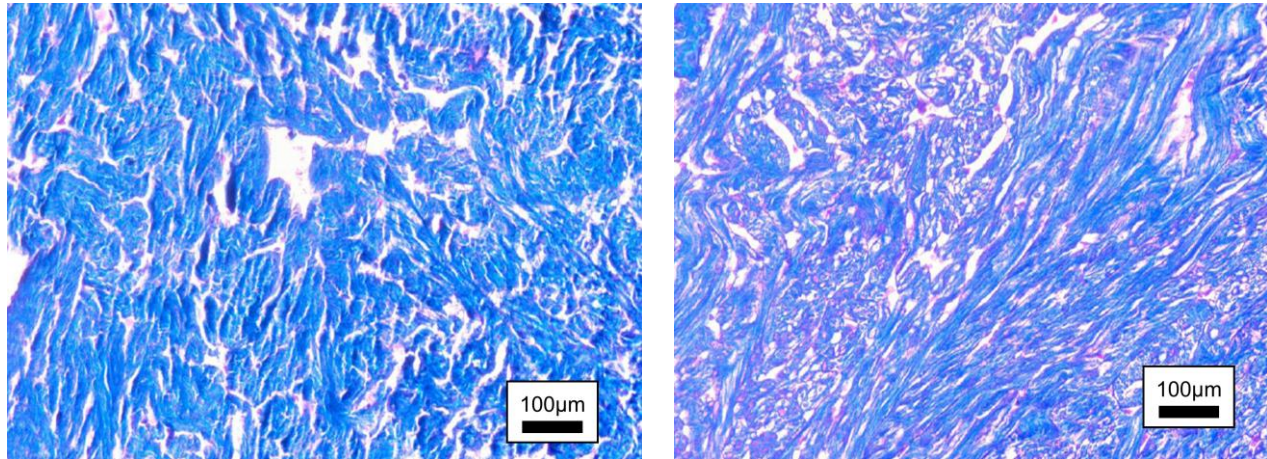
**Figure 3.16 – Overall collagen in the ventricle.** Cold acclimated ventricle collagen content ( $1.6\% \pm 0.3$ ) was significantly greater than warm collagen content ( $0.8\% \pm 0.2$ ) representing a difference of 100%. In control conditions there was less collagen than both cold (75%) and warm (50%). Data is presented on the histogram as the mean of  $n = 3$  fish from each acclimation group with 3 measurements taken for each fish (sample size of 9 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



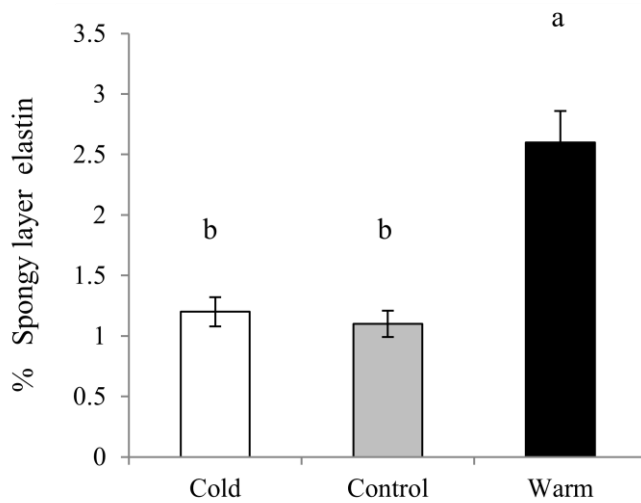
**Figure 3.17 – Collagen in the spongy layer.** Cold acclimated spongy layer collagen content ( $0.02\% \pm 0.003$ ) was significantly greater than warm spongy layer collagen content ( $0.012\% \pm 0.002$ ) representing a difference of 100%. There was no difference between warm and control. Data is presented on the histogram as the mean of  $n = 3$  fish from each acclimation group with 6 measurements taken for each fish (sample size of 18 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$



**Figure 3.18 – Collagen in the compact layer.** Cold acclimated compact layer collagen content ( $1.6\% \pm 0.3$ ) was significantly greater than warm collagen content ( $0.8\% \pm 0.2$ ) representing a difference of 100%. In control conditions there was less collagen than both cold (75%) and warm (50%). Data is presented on the histogram as the mean of  $n = 3$  fish from each acclimation group with 6 measurements taken for each fish (sample size of 18 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



**Figure 3.19 – Elastin staining in the spongy layer.** On the left is a typical aldehyde fuchsin stained ventricle section from a cold acclimated rainbow trout showing spongy layer muscle in blue and elastin in purple. On the right is a warm acclimated fish section using the same stain. Scale bar shown is 100µm. (staining carried out by Devmalya Sarkar, 2012)



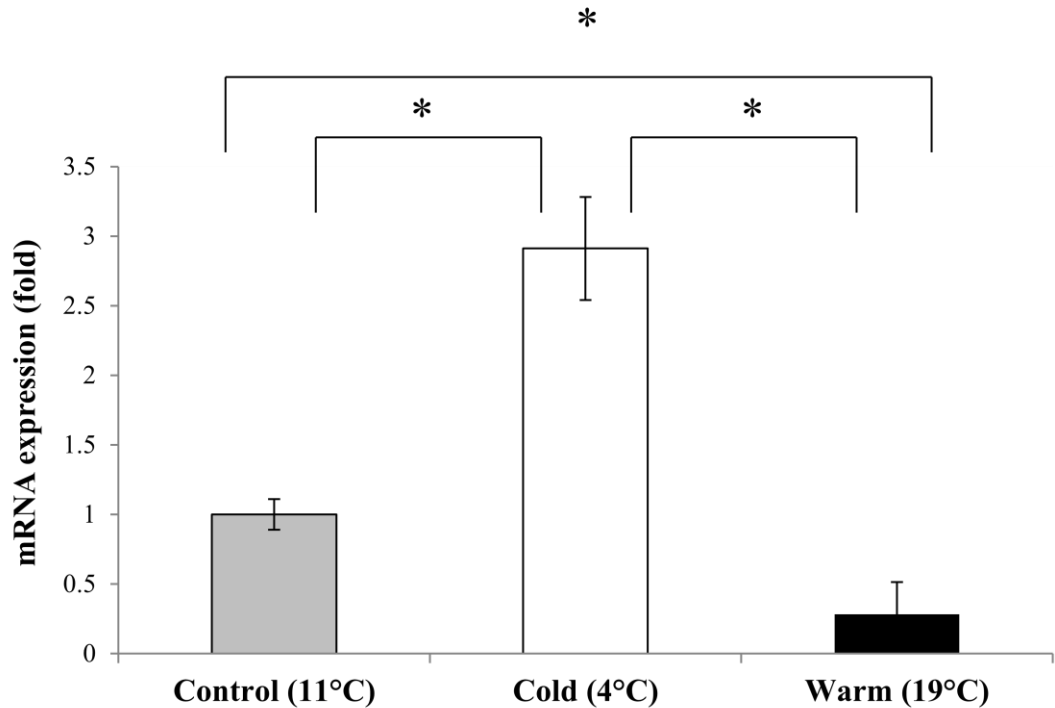
**Figure 3.20 – Elastin in the spongy layer.** Warm acclimated spongy layer elastin content was significantly greater ( $2.6\% \pm 0.24$ ) with respect to the cold spongy elastin content ( $1.2\% \pm 0.11$ ) representing a difference of 117%. Data is presented on the histogram as the mean of  $n = 3$  fish from each acclimation group with 6 measurements taken for each fish (sample size of 18 per acclimation group). Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



### **3.5.4 Cardiac muscle gene activity during temperature acclimation**

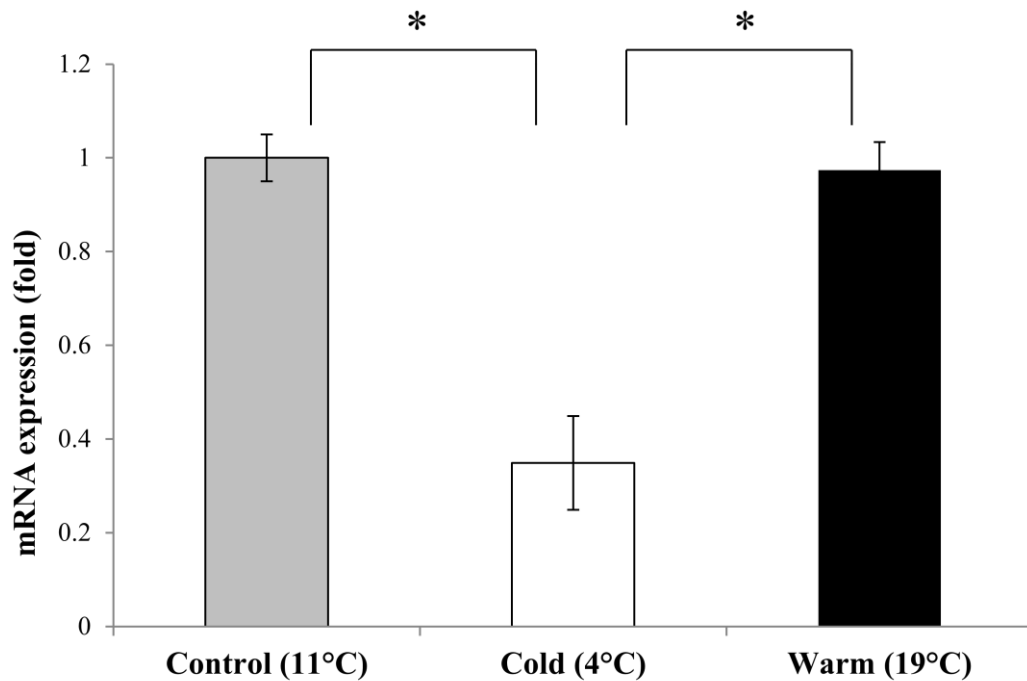
qPCR analysis of mRNA levels during acclimation reveals significant up-regulation of cardiac muscle specific genes in cold acclimated ventricles, whereas the reverse is true in atrium. VMHC levels in cold acclimated ventricles are 2.6 fold higher than warm ventricles (Figure 3.21) whereas VMHC levels in warm acclimated atrium are 0.2 fold higher than warm (Figure 3.22). MLP mRNA levels in cold acclimated ventricles are 1.6 fold higher in cold ventricle compared to warm (Figure 3.23) whereas there is no difference between MLP levels in cold and warm atrium (Figure 3.24). SMLC2 levels are 2.4 fold higher in cold ventricle compared with warm (Figure 3.25) however SMLC2 levels are 0.9 higher warm atrium than they are in warm atrium (Figure 3.26).

## VMHC VENTRICLE



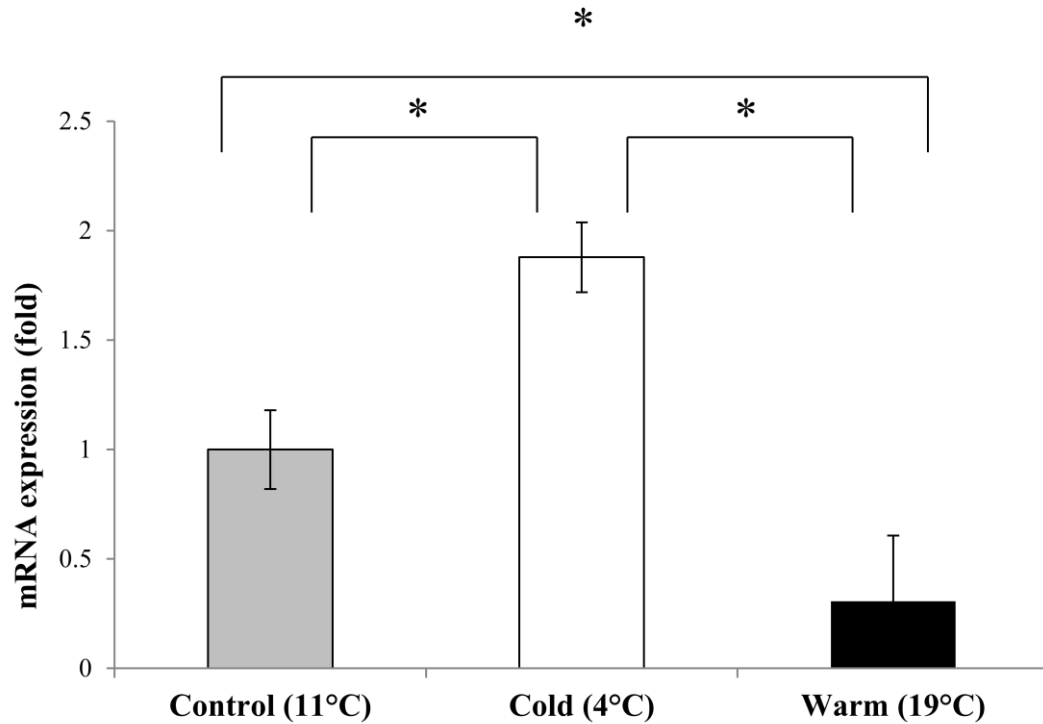
**Figure 3.21 - Expression of ventricular VMHC mRNA during cold and warm acclimation of rainbow trout heart.** VMHC in the cold ventricle was expressed  $2.91 \text{ fold} \pm 0.37$  with respect to control. VMHC in the warm ventricle was expressed  $0.28 \text{ fold} \pm 0.23$  with respect to control. Expression levels of VMHC in the cold ventricle were 2.63 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## VMHC ATRIA



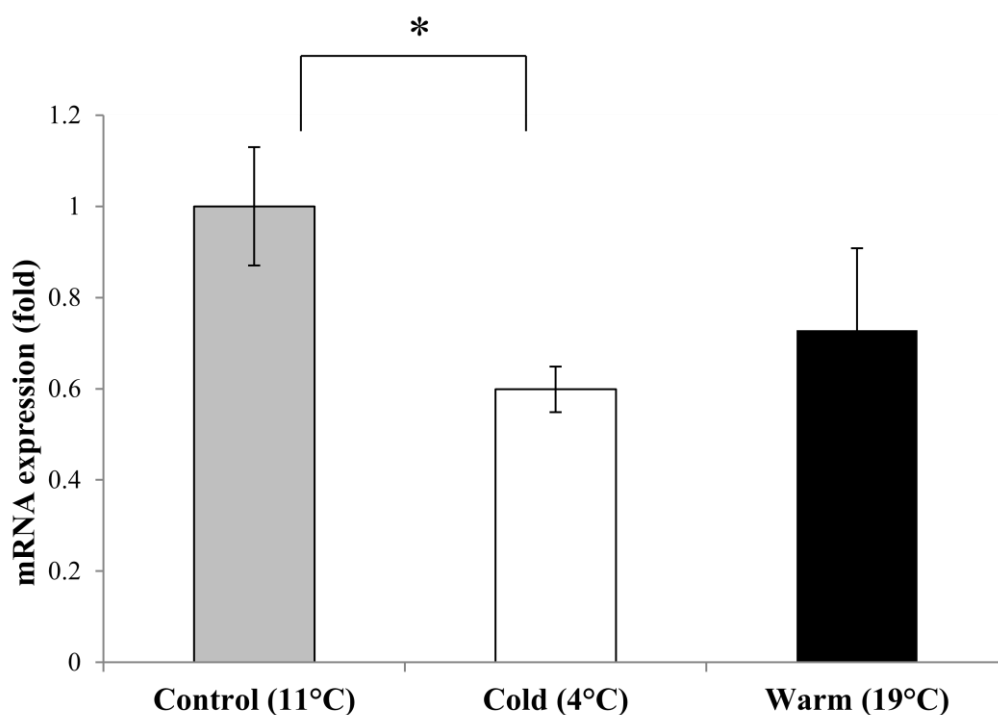
**Figure 3.22 - Expression of atrial VMHC mRNA during cold and warm acclimation of rainbow trout heart.** VMHC in the cold atria was expressed 0.35 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of VMHC in the atria. Expression levels of VMHC in the warm group were 0.62 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MLP VENTRICLE



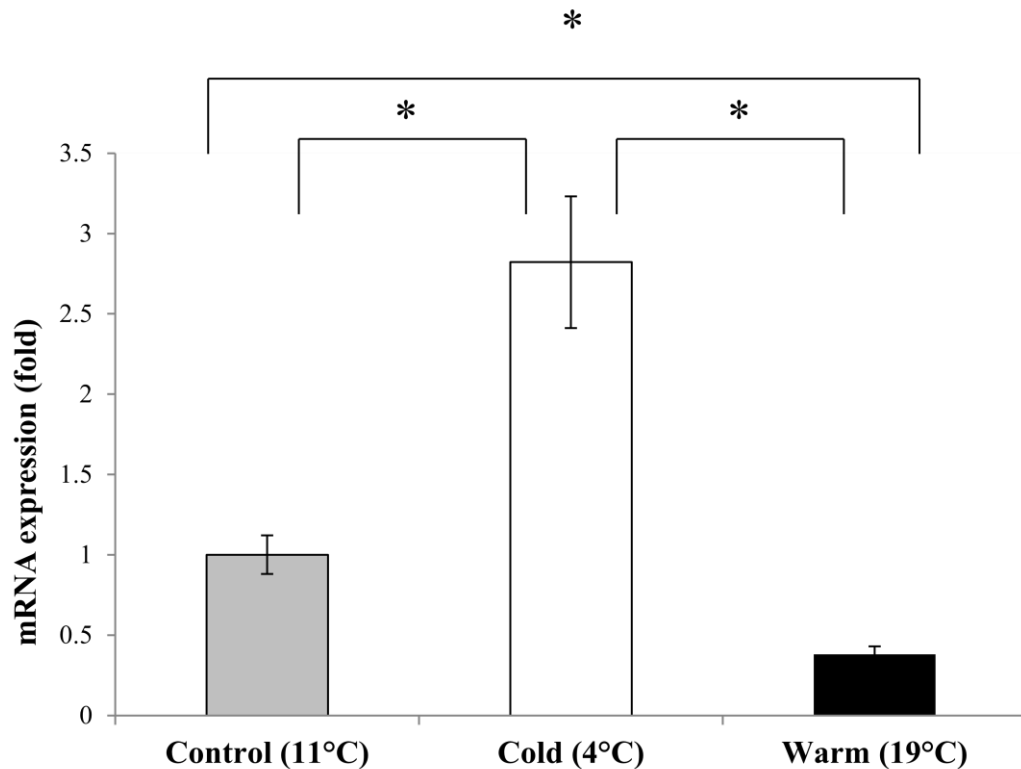
**Figure 3.23 - Expression of ventricular MLP mRNA during cold and warm acclimation of rainbow trout heart.** MLP in the cold ventricle was expressed 1.88 fold  $\pm$  0.16 with respect to control. MLP in the warm ventricle was expressed 0.31 fold  $\pm$  0.3 with respect to control. Expression levels of MLP in the cold ventricle were 1.57 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MLP ATRIA



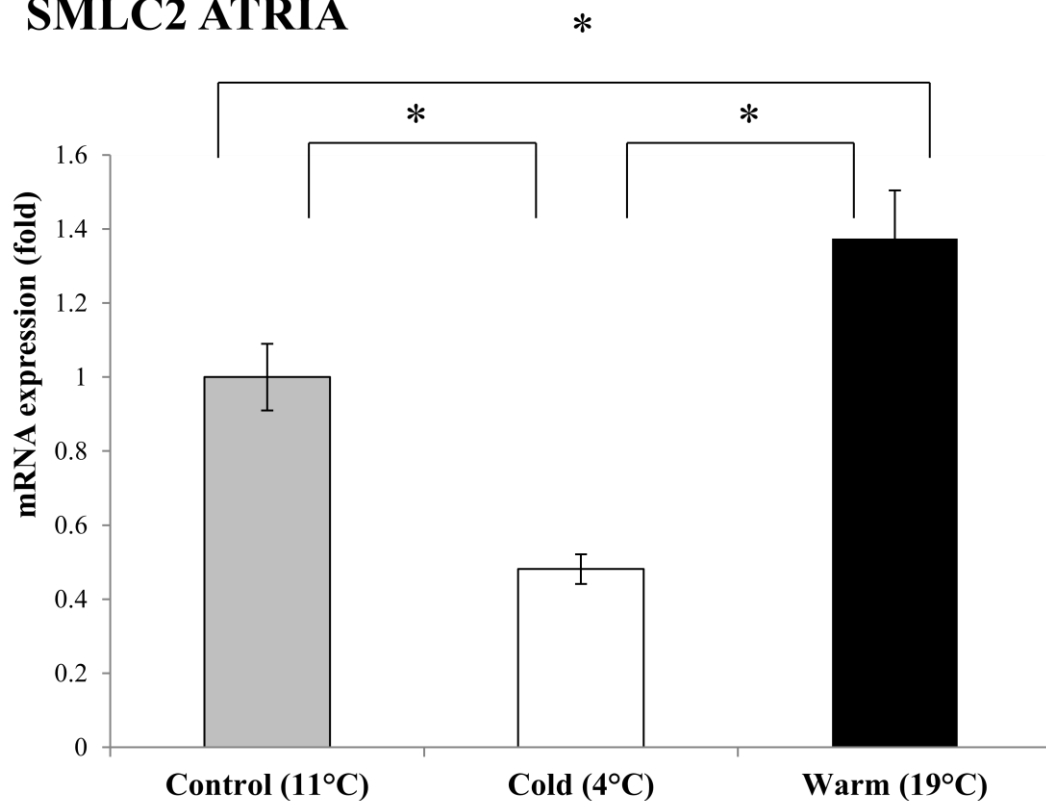
**Figure 3.24 - Expression of atrial MLP mRNA during cold and warm acclimation of rainbow trout heart.** MLP in the cold atria was expressed 0.6 fold  $\pm$  0.05 with respect to control. There was no difference between warm and control expression levels of MLP in the atria. There was no difference between warm and control expression levels of MLP in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## SMLC2 VENTRICLE



**Figure 3.25 - Expression of ventricular SMLC2 mRNA during cold and warm acclimation of rainbow trout heart.** SMLC2 in the cold ventricle was expressed  $2.82 \text{ fold} \pm 0.41$  with respect to control. SMLC2 in the warm ventricle was expressed  $0.38 \text{ fold} \pm 0.05$  with respect to control. Expression levels of SMLC2 in the cold ventricle were 2.44 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## SMLC2 ATRIA

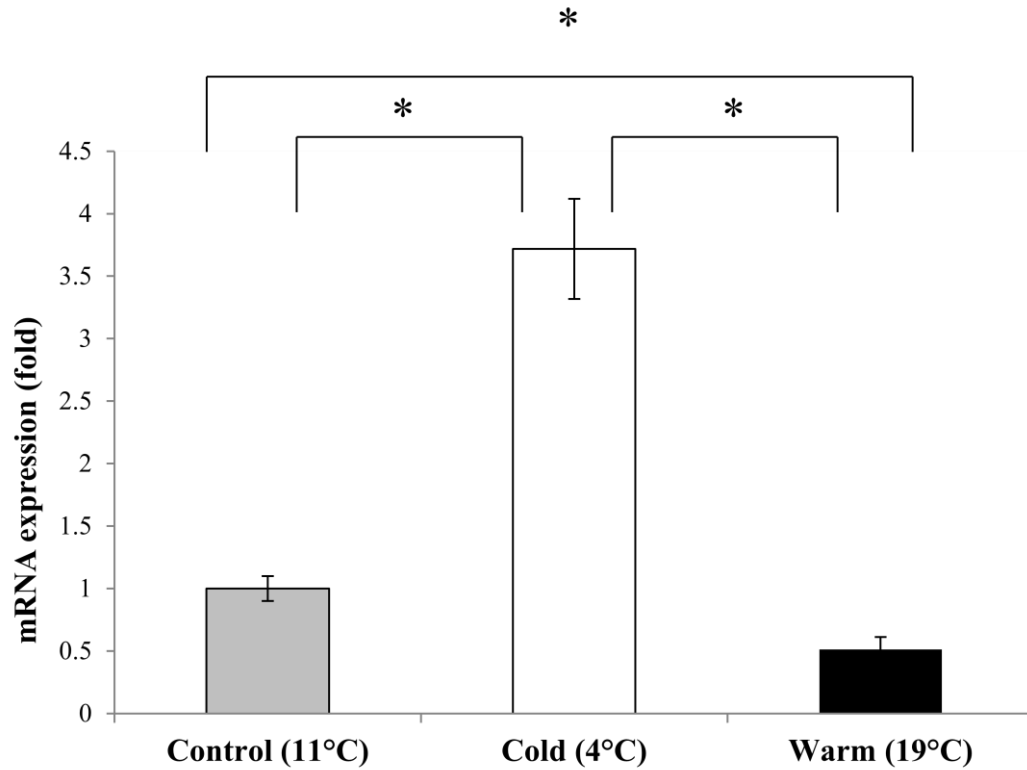


**Figure 3.26 - Expression of atrial SMLC2 mRNA during cold and warm acclimation of rainbow trout heart.** SMLC2 in the cold atria was expressed 0.48 fold  $\pm$  0.04 with respect to control. SMLC2 in the warm atria was expressed 1.37 fold  $\pm$  0.13 with respect to control. Expression levels of SMLC2 in the warm group were 0.89 fold higher than cold with respect to control. qPCR readings were taken from a sample size of n = 7 fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ . Angiogenesis during temperature acclimation

### 3.5.5 Angiogenesis during temperature acclimation

qPCR revealed the marker for angiogenesis, VEGF, to be up-regulated in both cold ventricle and warm atrium. VEGF mRNA levels were 3.2 fold higher in cold ventricle compared to warm (Figure 3.27) whereas atrial levels of VEGF were 2.9 fold higher in warm compared with the cold cohort (Figure 3.28).

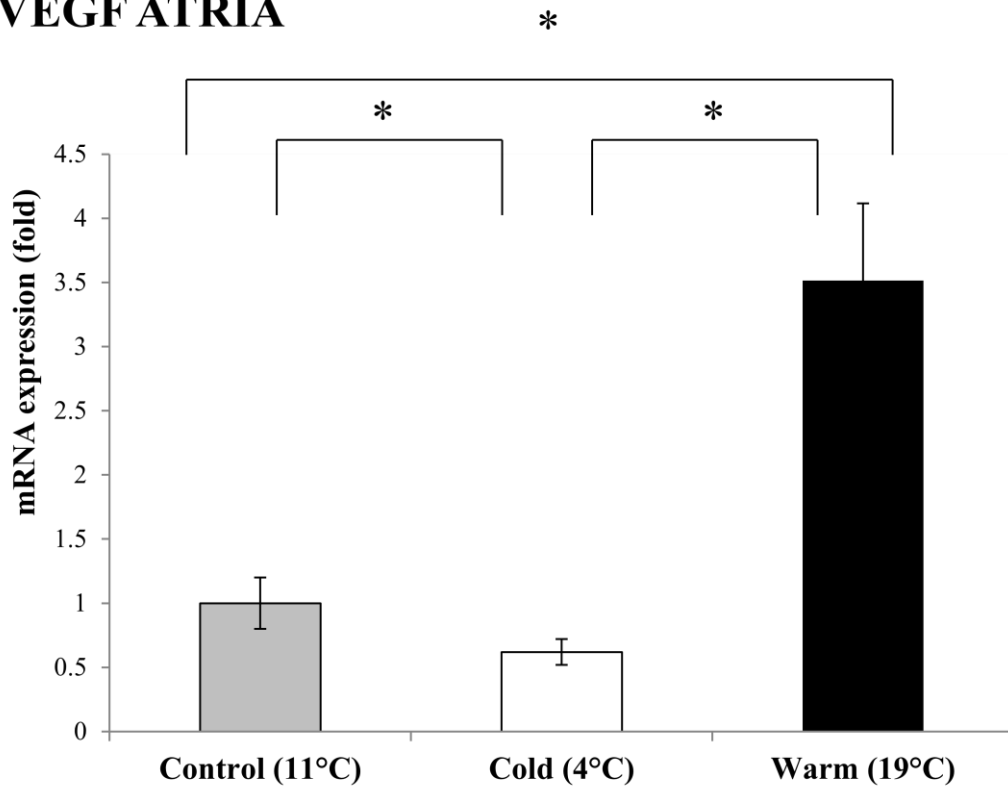
## VEGF VENTRICLE



**Figure 3.27 - Expression of ventricular VEGF mRNA during cold and warm acclimation of rainbow trout heart.** VEGF in the cold ventricle was expressed 3.72 fold  $\pm$  0.4 with respect to control. VEGF in the warm ventricle was expressed 0.51 fold  $\pm$  0.1 with respect to control. Expression levels of VEGF in the cold ventricle were 3.21 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .



## VEGF ATRIA

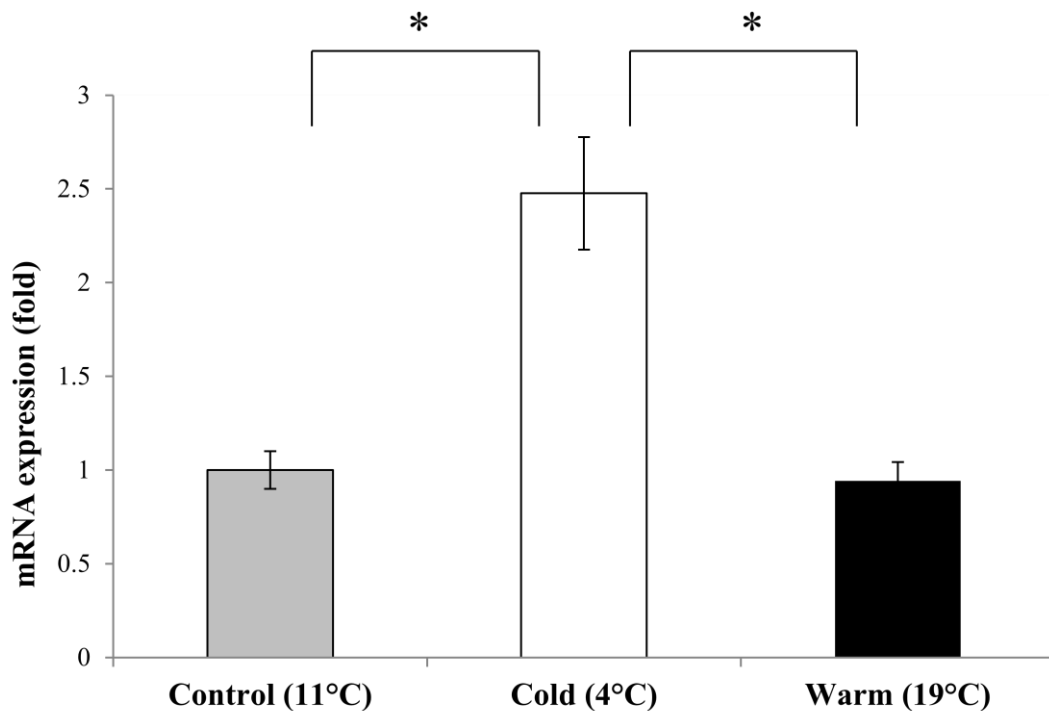


**Figure 3.28 - Expression of atrial VEGF mRNA during cold and warm acclimation of rainbow trout heart.** VEGF in the cold atria was expressed 0.62 fold  $\pm$  0.1 with respect to control. VEGF in the warm atria was expressed 3.52 fold  $\pm$  0.6 with respect to control. Expression levels of VEGF in the warm group were 2.9 fold higher than cold with respect to control. qPCR readings were taken from a sample size of n = 7 fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

### 3.5.6 Hyperplasia during temperature acclimation

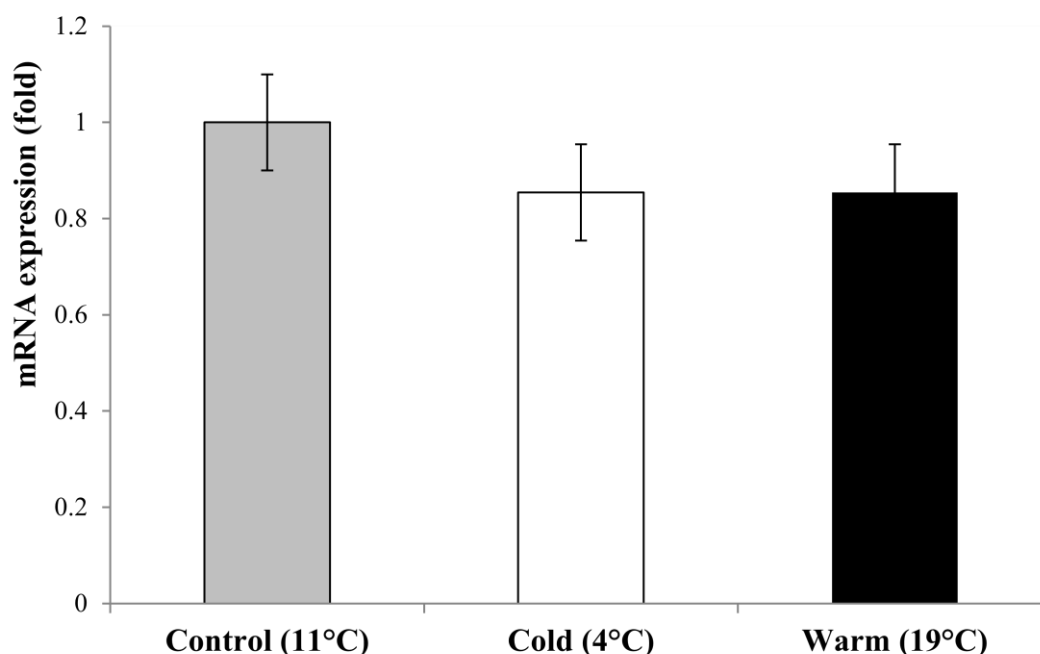
PCNA is a marker for hyperplasia. PCNA mRNA levels, as shown by qPCR, were 1.5 fold higher in cold ventricle compared with warm (Figure 3.29). There was no difference in PCNA levels in the atrium of either temperature (Figure 3.30).

## PCNA VENTRICLE



**Figure 3.29 - Expression of ventricular PCNA mRNA during cold and warm acclimation of rainbow trout heart.** PCNA in the cold ventricle was expressed 2.48 fold  $\pm$  0.3 with respect to control. There was no difference between warm and control expression levels of PCNA in the ventricle. Expression levels of PCNA in the cold ventricle were 1.54 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## PCNA ATRIA

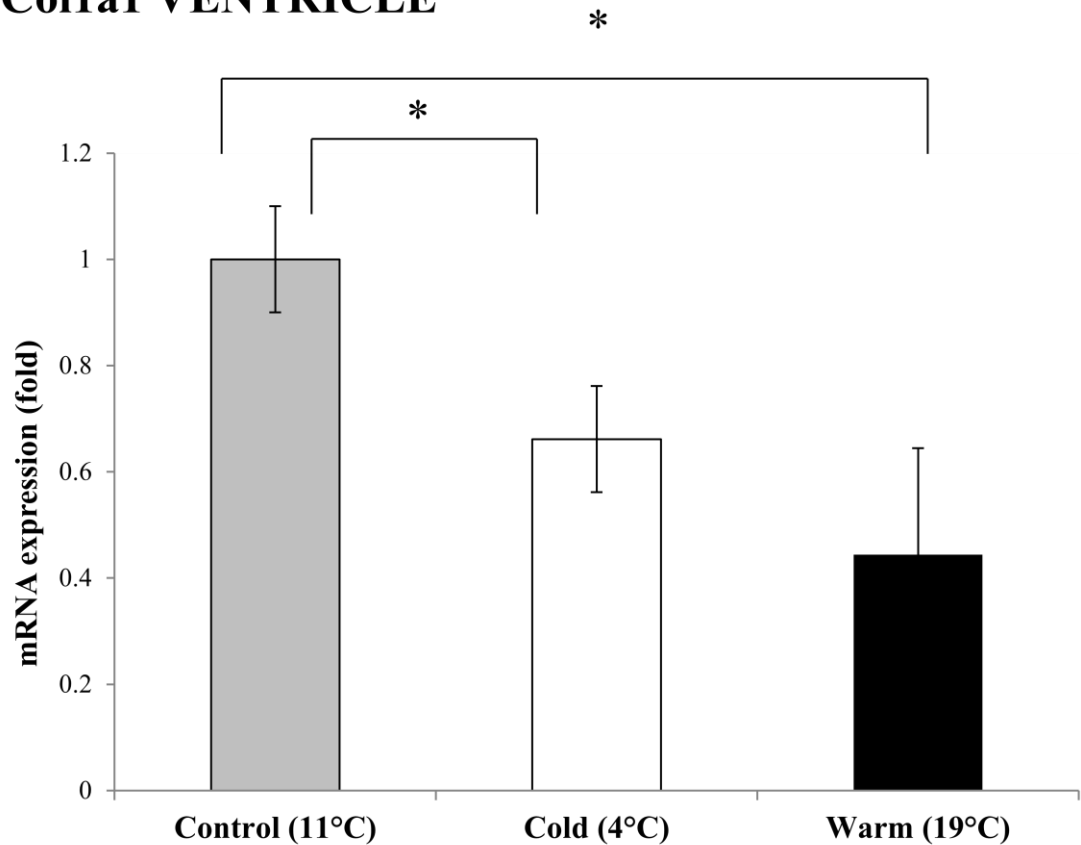


**Figure 3.30 - Expression of atrial PCNA mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of PCNA in the atria. There was no difference between warm and control expression levels of PCNA in the atria. There was no difference between cold and warm expression levels of PCNA in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ . Collagen regulation during temperature acclimation.

Using qPCR on collagen and collagen-regulatory genes, significant collagen regulation at the genetic level was observed. Col1a1 and Col1a2 mRNA were both expressed at significantly lower levels in cold and warm atrium and ventricle compared to control, however there was no difference between cold and warm levels of each in any chamber (Figures 3.31 – 3.34). Col1a3 however was expressed at significantly higher levels in both cold ventricle and cold atrium. Col1a3 mRNA was expressed at 1.4 fold higher levels in cold ventricle compared with warm ventricle (Figure 3.35) and the same gene was expressed 1.8 fold more in cold atrium compared with warm atrium (Figure 3.36). MMPs are negative regulators of collagen. In the ventricle, MMP2 was expressed 1.2 fold higher

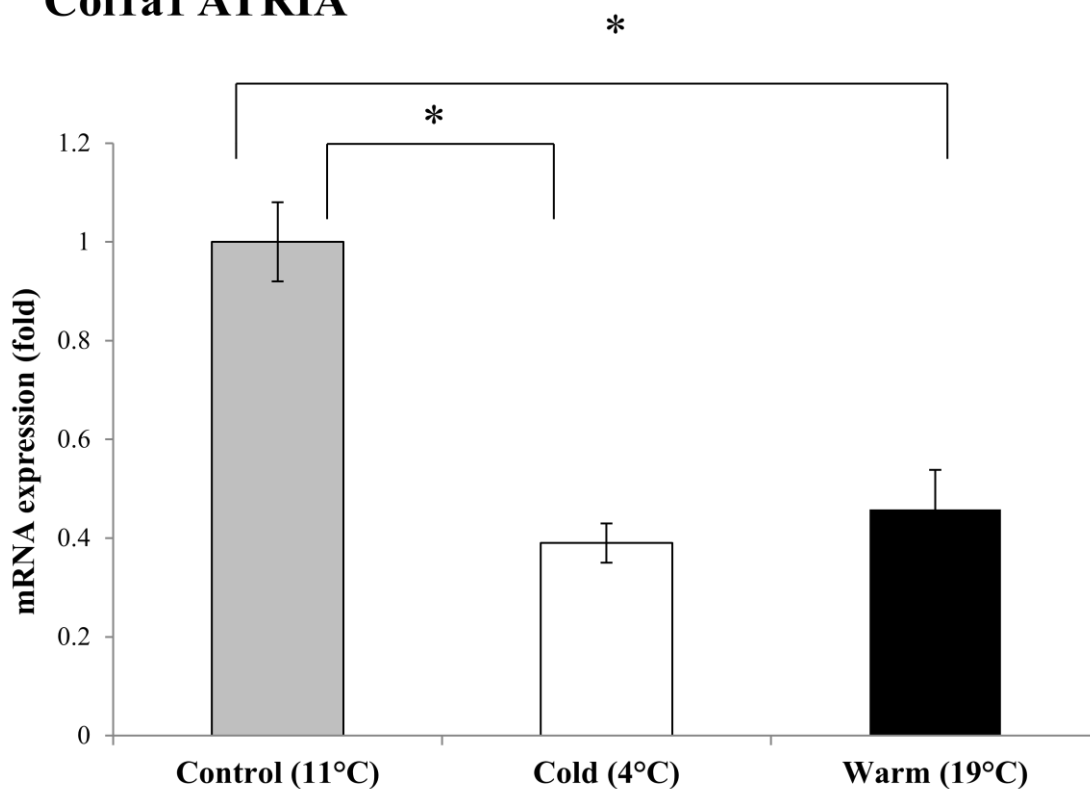
in the warm fish compared with cold, and in the atrium, MMP2 was expressed 1.2 fold more in warm than cold (Figures 3.37 and 3.38). MMP9 levels were indifferent in cold and warm ventricle (Figure 3.39) however the atrium expressed 1.4 fold more MMP9 mRNA in warm fish compared to cold (Figure 3.40). MMP13 was expressed 0.6 fold higher in warm compared with cold ventricle (Figure 3.41) and in the atrium; the same gene was expressed 0.4 fold higher in warm fish in comparison with cold. TIMPs are negative regulators of MMPs and therefore positive regulators of collagens. In the cold ventricle, TIMP2 is expressed 2.3 fold higher than in the warm fish (Figure 3.43), whereas TIMP2 in the atrium is unaffected by temperature (Figure 3.44).

## Col1a1 VENTRICLE



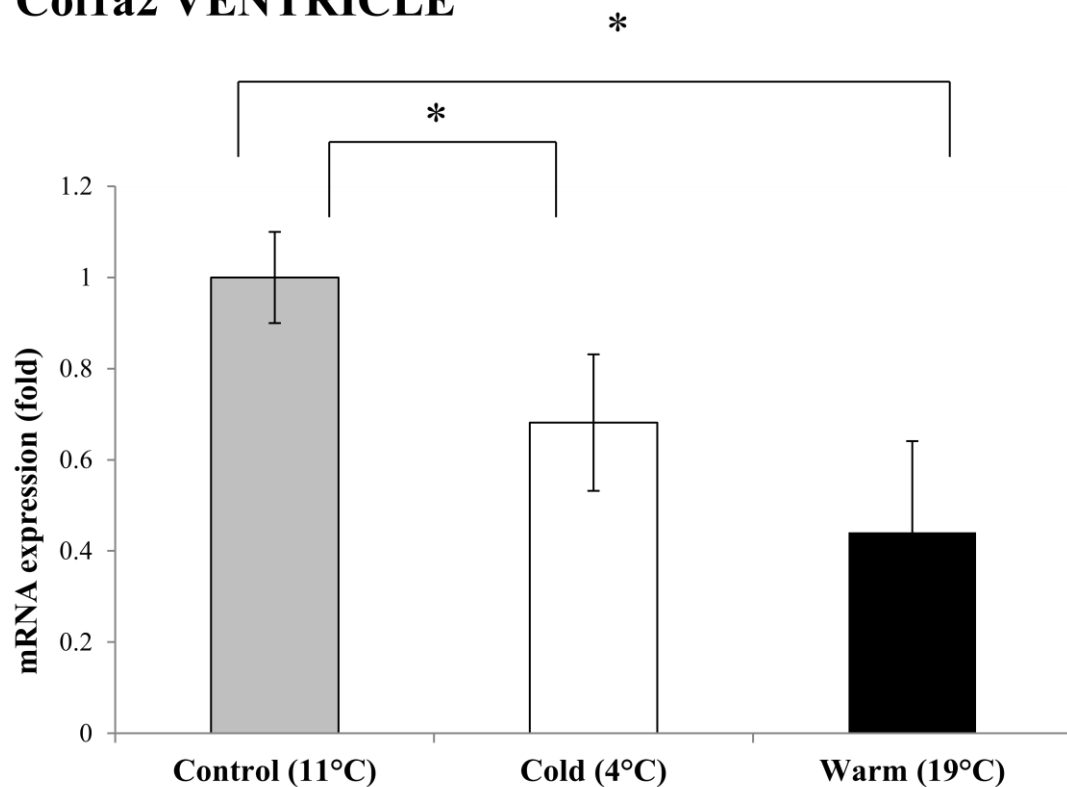
**Figure 3.31 - Expression of ventricular Col1a1 mRNA during cold and warm acclimation of rainbow trout heart.** Col1a1 in the cold ventricle was expressed 0.66 fold  $\pm$  0.1 with respect to control. Col1a1 in the warm ventricle was expressed 0.44 fold  $\pm$  0.2 with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## Col1a1 ATRIA



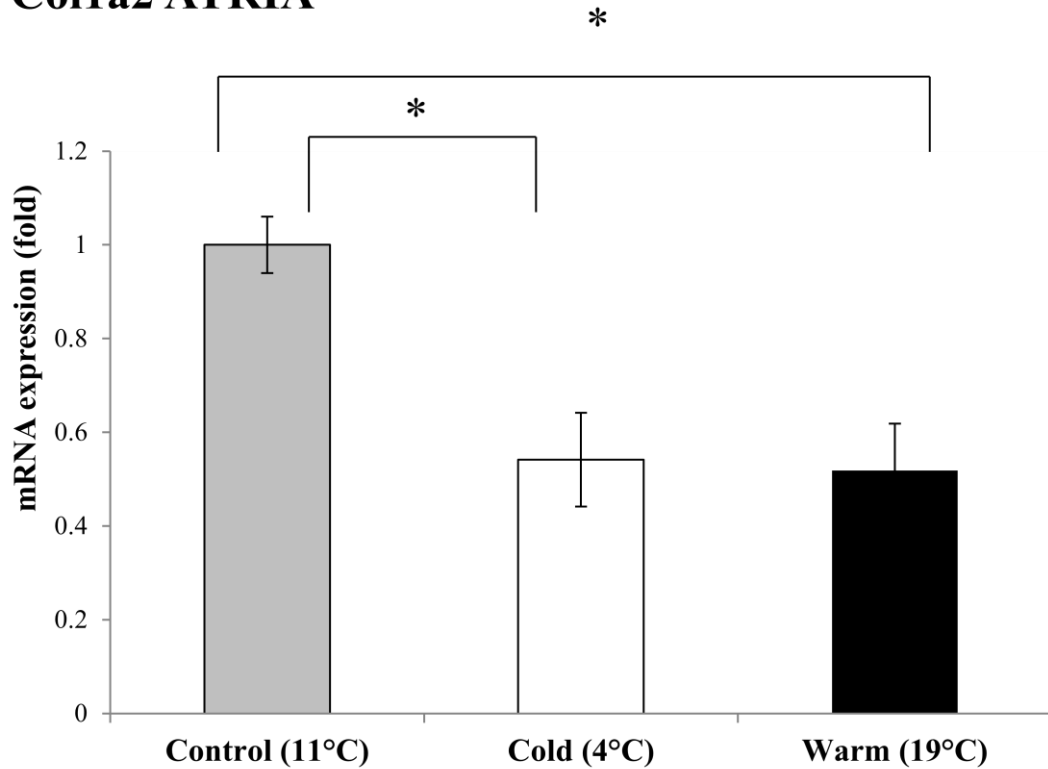
**Figure 3.32 - Expression of atrial Col1a1 mRNA during cold and warm acclimation of rainbow trout heart.** Col1a1 in the cold atria was expressed 0.39 fold  $\pm$  0.04 with respect to control. Col1a1 in the warm atria was expressed 0.46 fold  $\pm$  0.08 with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## Col1a2 VENTRICLE



**Figure 3.33 - Expression of ventricular Col1a2 mRNA during cold and warm acclimation of rainbow trout heart.** Col1a2 in the cold ventricle was expressed 0.68 fold  $\pm$  0.15 with respect to control. Col1a2 in the warm ventricle was expressed 0.44 fold  $\pm$  0.2 with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

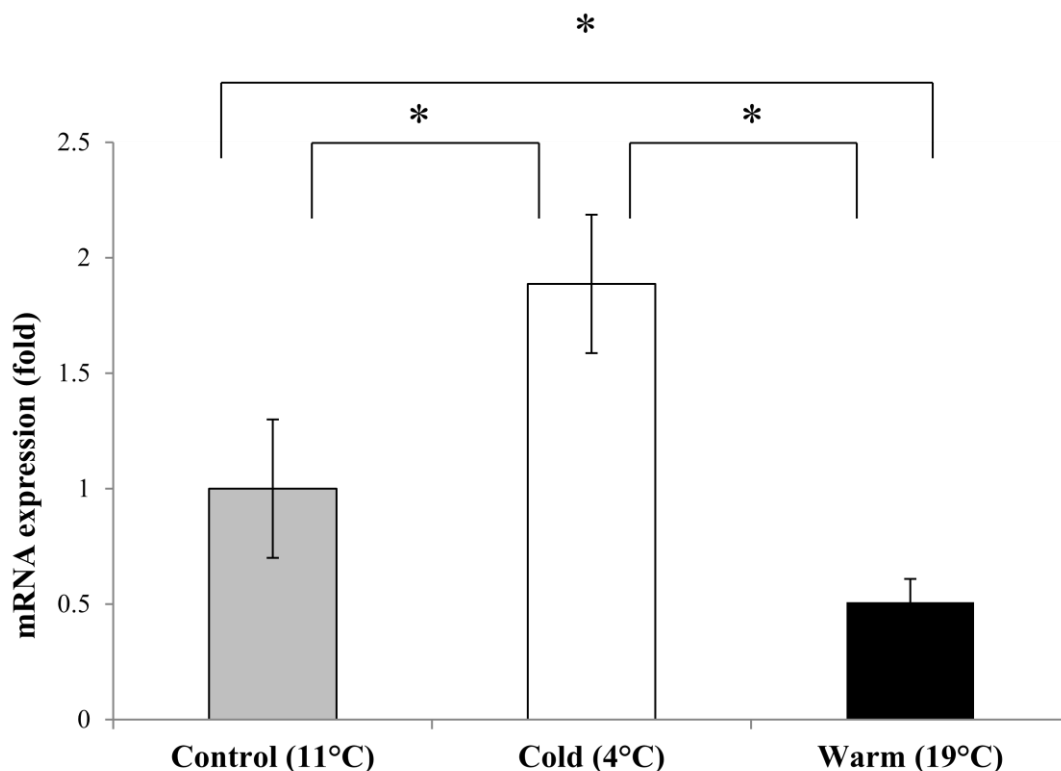
## Col1a2 ATRIA



**Figure 3.34 - Expression of atrial Col1a2 mRNA during cold and warm acclimation of rainbow trout heart.** Col1a2 in the cold atria was expressed 0.54 fold  $\pm$  0.1 with respect to control. Col1a2 in the warm atria was expressed 0.52 fold  $\pm$  0.1 with respect to control. qPCR readings were taken from a sample size of n = 7 fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

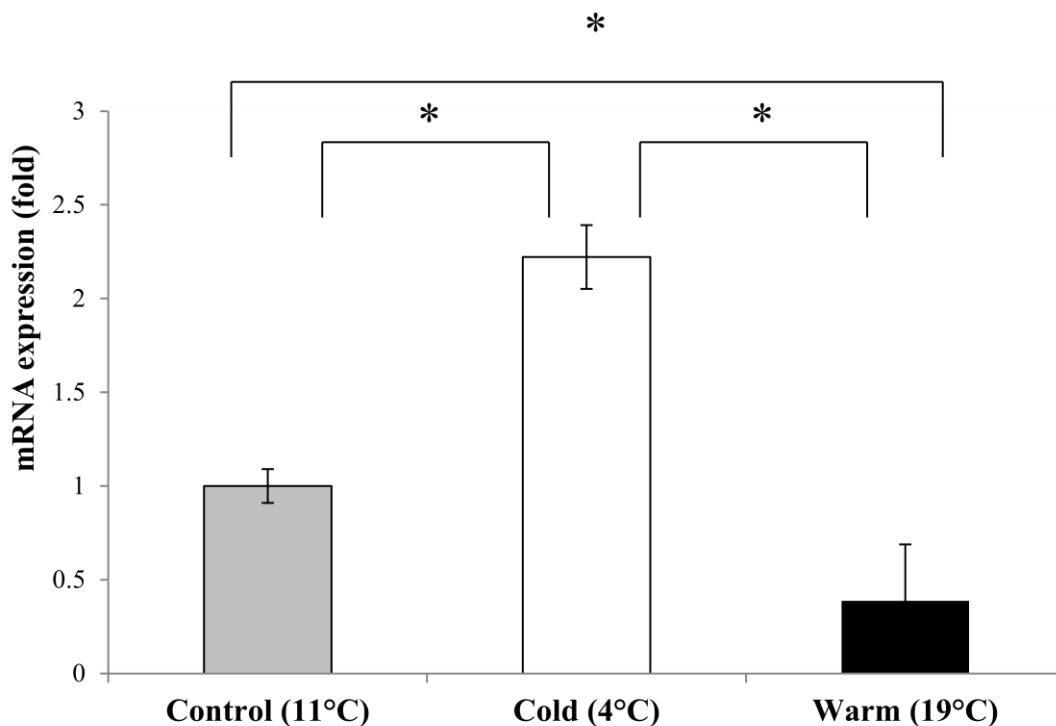


## Col1a3 VENTRICLE



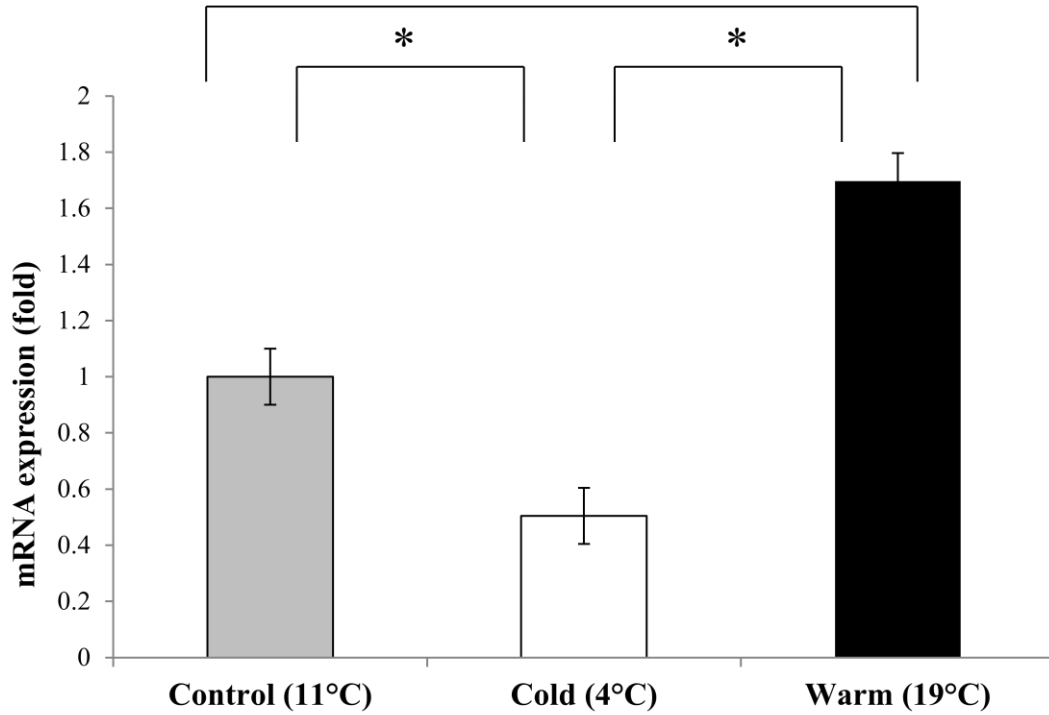
**Figure 3.35 - Expression of ventricular Col1a3 mRNA during cold and warm acclimation of rainbow trout heart.** Col1a3 in the cold ventricle was expressed 1.89 fold  $\pm$  0.3 with respect to control. Col1a3 in the warm ventricle was expressed 0.51 fold  $\pm$  0.1 with respect to control. Expression levels of Col1a3 in the cold ventricle were 1.38 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## Col1a3 ATRIA



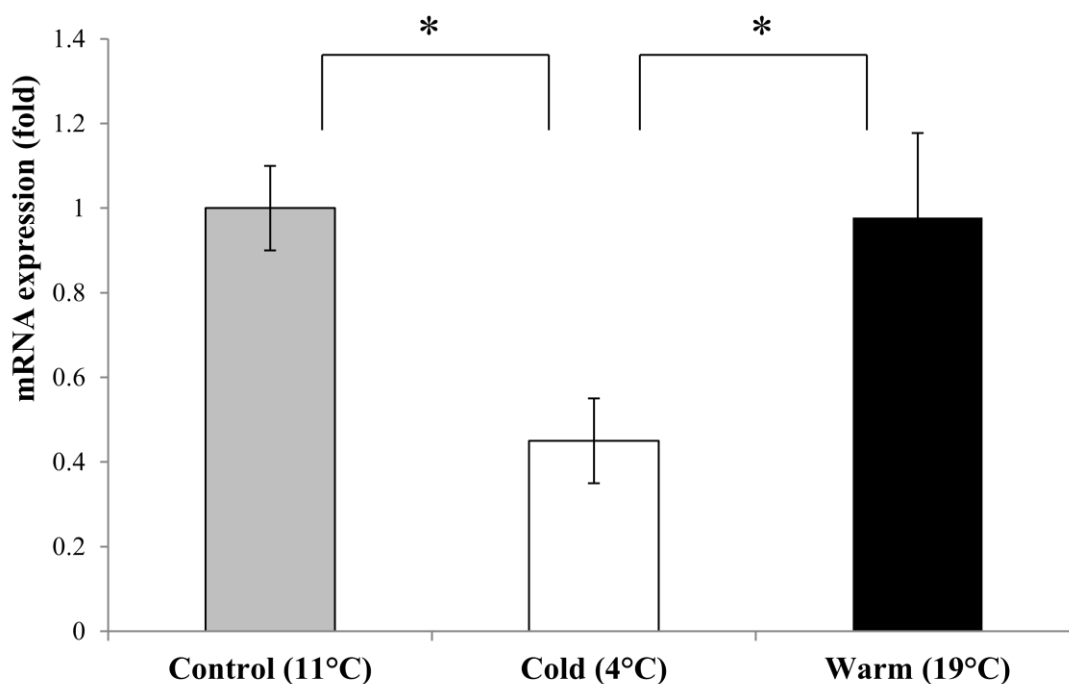
**Figure 3.36 - Expression of atrial Col1a3 mRNA during cold and warm acclimation of rainbow trout heart.** Col1a3 in the cold atria was expressed 2.22 fold  $\pm$  0.17 with respect to control. Col1a3 in the warm atria was expressed 0.39 fold  $\pm$  0.3 with respect to control. Expression levels of Col1a3 in the cold atria were 1.83 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MMP2 VENTRICLE \*



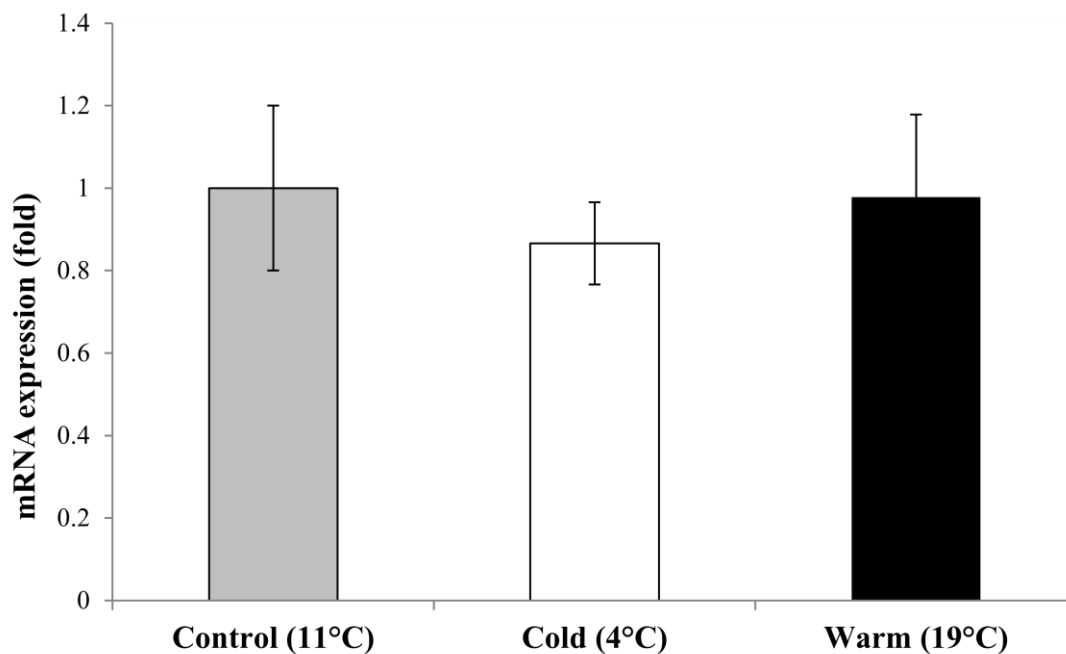
**Figure 3.37 - Expression of ventricular MMP2 mRNA during cold and warm acclimation of rainbow trout heart.** MMP2 in the cold ventricle was expressed 0.5 fold  $\pm$  0.1 with respect to control. MMP2 in the warm ventricle was expressed 1.7 fold  $\pm$  0.1 with respect to control. Expression levels of MMP2 in the warm group were 1.2 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MMP2 ATRIA



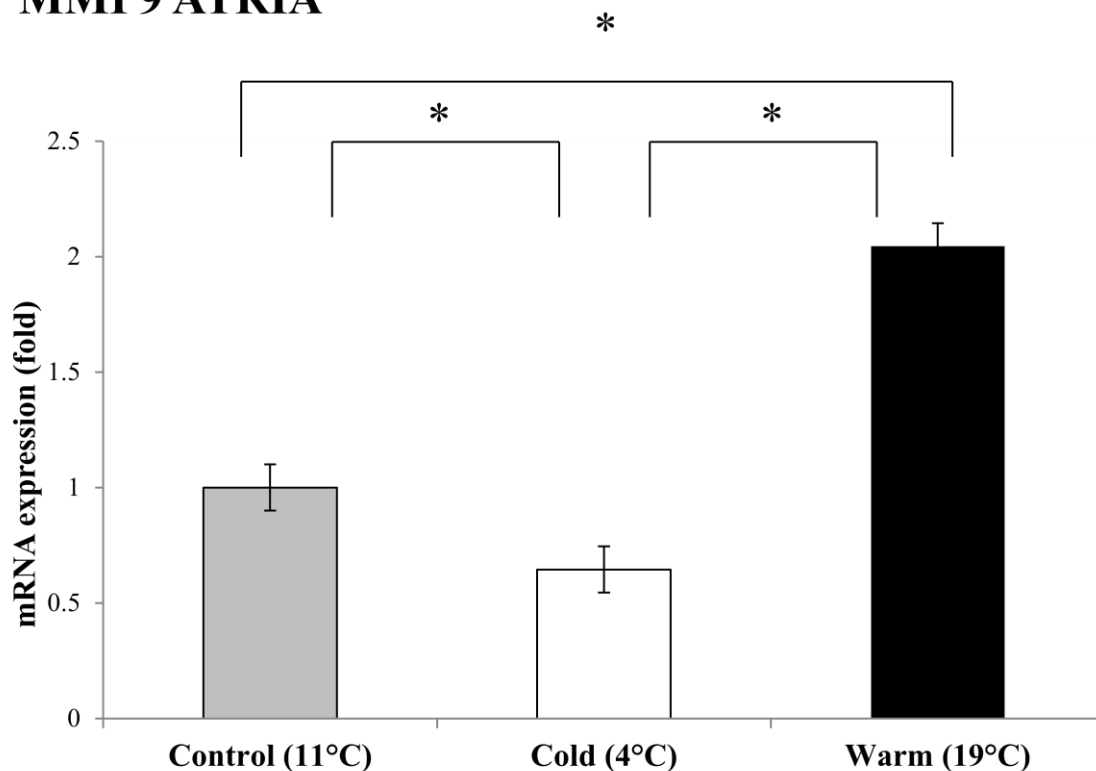
**Figure 3.38 - Expression of atrial MMP2 mRNA during cold and warm acclimation of rainbow trout heart.** MMP2 in the cold atria was expressed 0.45 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of MMP2 in the atria. Expression levels of MMP2 in the warm group were 0.53 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MMP9 VENTRICLE



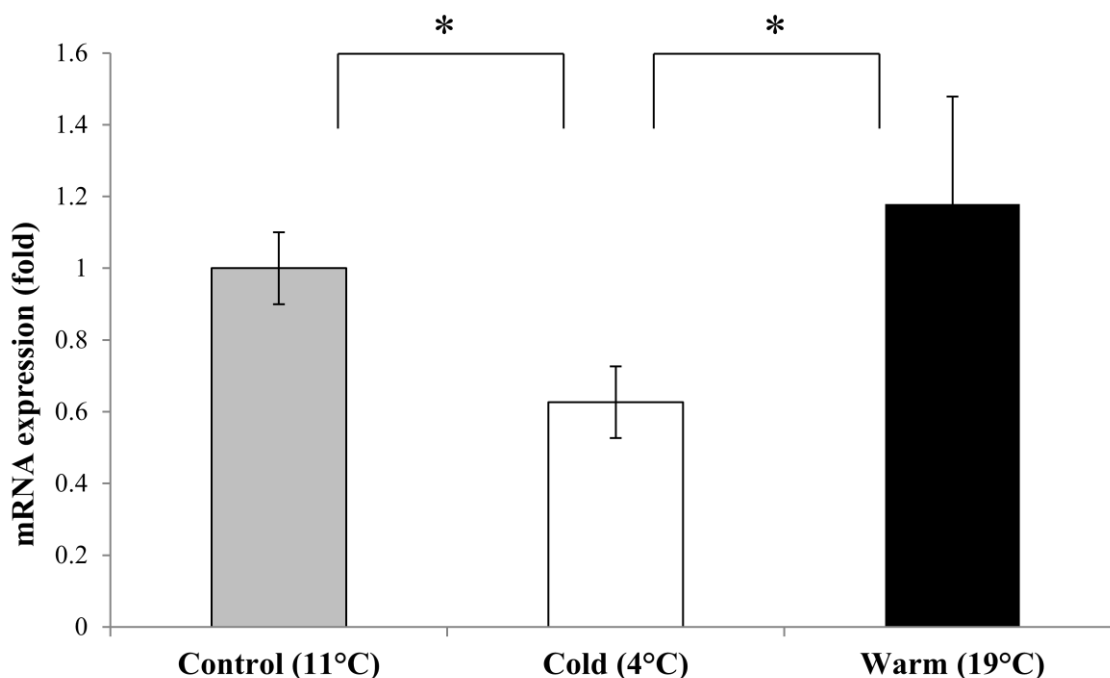
**Figure 3.39 - Expression of ventricular MMP9 mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of MMP9 in the ventricle. There was no difference between warm and control expression levels of MMP9 in the ventricle. There was no difference between warm and control expression levels of MMP9 in the ventricle. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MMP9 ATRIA



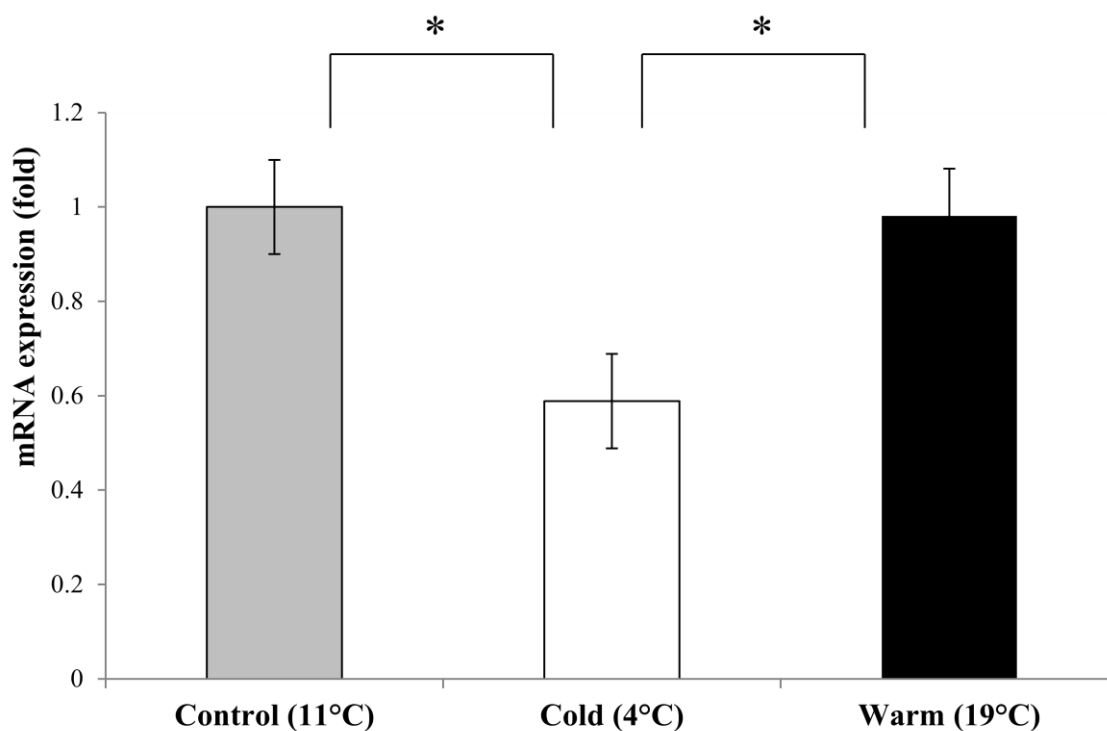
**Figure 3.40 - Expression of atrial MMP9 mRNA during cold and warm acclimation of rainbow trout heart.** MMP9 in the cold atria was expressed 0.65 fold  $\pm$  0.1 with respect to control. MMP9 in the warm atria was expressed 2.05 fold  $\pm$  0.1 with respect to control. Expression levels of MMP9 in the warm group were 1.4 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## MMP13 VENTRICLE



**Figure 3.41 - Expression of ventricular MMP13 mRNA during cold and warm acclimation of rainbow trout heart.** MMP13 in the cold ventricle was expressed 0.63 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of MMP13 in the ventricle. Expression levels of MMP13 in the warm group were 0.55 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

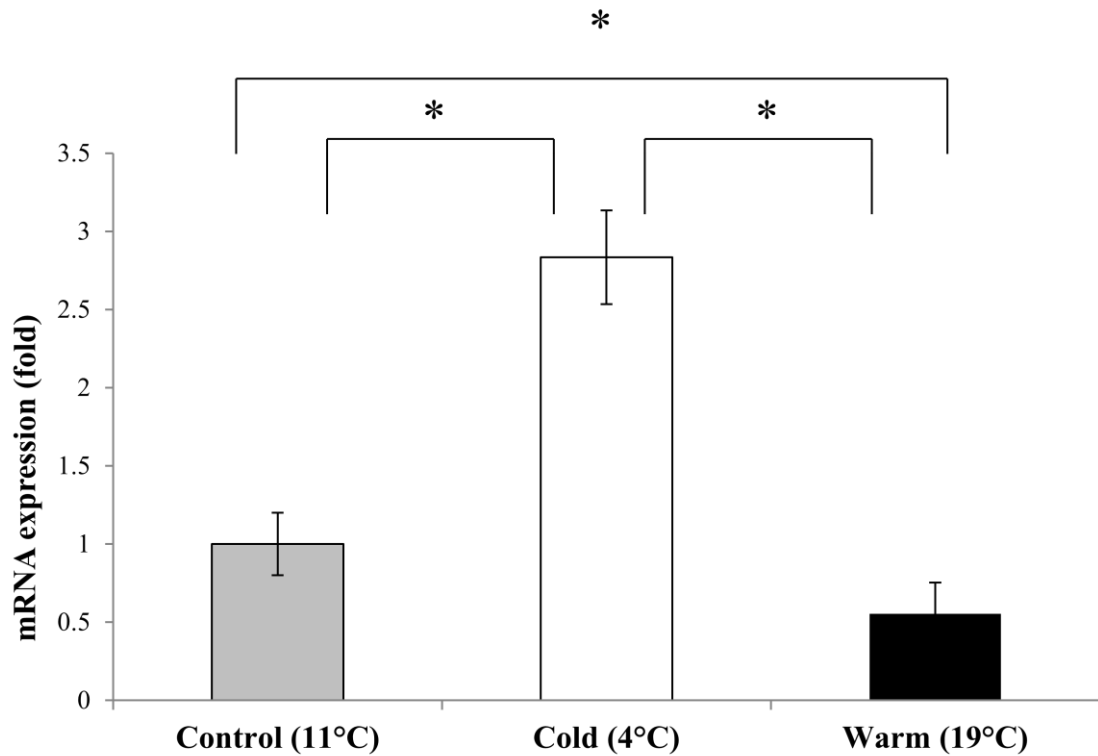
## MMP13 ATRIA



**Figure 3.42 - Expression of atrial MMP13 mRNA during cold and warm acclimation of rainbow trout heart.** MMP13 in the cold atria was expressed 0.59 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of MMP13 in the atria. Expression levels of MMP13 in the warm group were 0.39 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

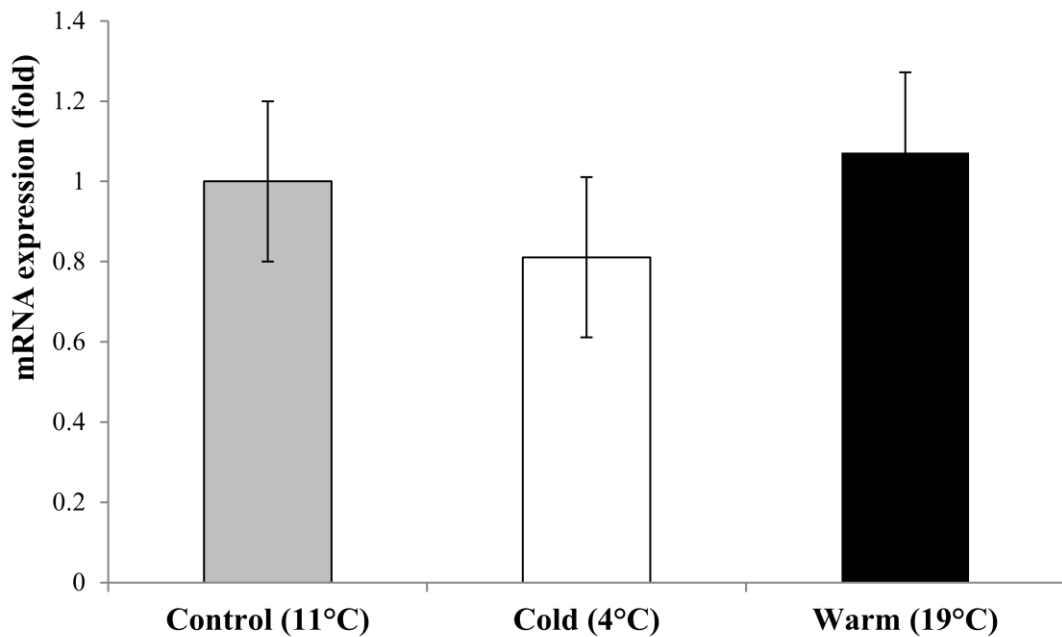


## TIMP2 VENTRICLE



**Figure 3.43 - Expression of ventricular TIMP2 mRNA during cold and warm acclimation of rainbow trout heart.** TIMP2 in the cold ventricle was expressed 2.83 fold  $\pm$  0.3 with respect to control. TIMP2 in the warm ventricle was expressed 0.55 fold  $\pm$  0.1 with respect to control. Expression levels of TIMP2 in the cold ventricle were 2.28 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## TIMP2 ATRIA



**Figure 3.44 - Expression of atrial TIMP2 mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of TIMP2 in the atria. There was no difference between warm and control expression levels of TIMP2 in the atria. There was no difference between warm and control expression levels of TIMP2 in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## 3.6 Discussion

### 3.6.1 Key findings:

1. Cold acclimation causes hypertrophy of the whole heart
2. Temperature acclimation causes significant cardiac remodelling at the structural level in both muscle and connective tissue components
3. Temperature acclimation causes significant chamber-specific remodelling whereby ventricular changes in mass oppose atrial changes in mass
4. Cold acclimation promotes overall ventricular hypertrophy via spongy layer hypertrophy and muscle gene regulation
5. Warm acclimation promotes overall ventricular atrophy via spongy layer atrophy and muscle gene regulation
6. Warm acclimation promotes thickening of the compact layer
7. Cold acclimation causes atrial atrophy with the reverse being true in warm acclimation
8. Evidence consistent with hyperplasia is apparent in the cold acclimated ventricle
9. There is evidence consistent with increased angiogenesis where overall chamber muscle mass is increased
10. Cold acclimation causes a generalised increase in collagen throughout the heart via collagen regulatory gene expression with the reverse being true in warm acclimation
11. Warm acclimation results in increased elastin in the ventricle with the reverse being true in cold fish

### **3.6.2 Cold acclimation causes ventricular hypertrophy and atrial atrophy**

In the present study, a female only cohort subject to temperature acclimation was assessed for cardiac remodelling at the structural and genetic levels to ascertain the degree of muscle and connective tissue changes, with the aim of determining the likely function of the heart as a physical unit during acclimation based on structural evidence.

#### ***Whole heart observations***

In agreement with several past studies where it has been shown that a temperature drop from ~ 10°C to ~ 4°C is sufficient for significant cardiac hypertrophy [144, 191-194], cardiac hypertrophy was observed at the whole heart level during cold acclimation. Furthermore, this trend continued into the ventricle where warm acclimated fish (19°C) ventricles were significantly smaller than that of the cold acclimated fish (4°C) and importantly, both whole hearts and ventricles from warm fish were significantly smaller than that of the control group. This is the first evidence in trout that warm acclimation can promote atrophy of the heart. Despite this clear increase in cardiac growth in cold fish, it was actually relatively small; 8% with respect to the control group which is much smaller than many groups report (~45%) [197, 198]. However, in the majority of cases, it was unclear whether a mixed cohort of fish was used; indeed, no study (prior to the start of this research) has focussed purely on a female-only group of fish in the context of temperature acclimation induced cardiac growth. Males were specifically excluded from the study due to significant prior evidence suggesting males exhibit cardiac hypertrophy during sexual maturation, independent of temperature and in direct response to testosterone [139, 195]. With this in mind, it is highly likely that significant cold acclimation induced cardiac hypertrophy reported in several studies was at the very least, *enhanced* by the presence of pro-growth androgens which exaggerated the effects of any cardiac hypertrophy observed.

One surprising, yet fascinating result to come out of this study, was the observation that atrial responses in cardiac muscle mass largely oppose the ventricle response; namely, cold acclimation causes overall ventricular hypertrophy and atrial atrophy. These changes, observed at both the structural and genetic levels provide one explanation as to why overall cardiac hypertrophy, in terms of mass, was not as high as other groups have reported due to the offset of muscle mass between the two chambers. Furthermore, these differences in muscle mass between the chambers provide an initial indication into the role and/or structural requirements of the chambers at extremes of temperature acclimation in the heart.

### *Atrium*

The atrium, in effect, is the last structure within the cardiac and circulatory system receiving blood from the body and facilitating filling of the ventricle via *vis-a-fronte* or *vis-a-tergo* where force is provided by from the front or from behind (via ventricular suction) respectively. The extent to which each filling type is relied on is under much debate. For example, while opening of the pericardium and thus elimination of *vis-a-fronte* filling reduces maximum *in situ* cardiac output by 44% in the rainbow trout [147] *in vivo* measurements suggest that *vis-a-tergo* is the primary filling mechanism in resting fish of this species [196]. It is however clear, that the atrium is both subject to pressure, and can generate its own force via inherent contraction of 0.25 – 0.5 kPa [12]. Muscle mass, clearly, is associated with providing force against a resistance, with the results of this study suggesting in cold acclimation, the atrium is either subject to less mechanical load or pressure than the warm atrium or the cold atrium is required to produce less force than its warm counterpart to maintain adequate cardiac output. When combined with evidence from the ventricle, we can begin to understand what the function of the atrium is as a structural unit in the context of the biophysical ramifications of temperature acclimation.

### *Ventricle as a whole*

There were significant differences at the chamber level between cold and warm acclimated fish in the ventricle. Indeed, relative ventricle mass (RVM) was 23% greater in cold fish with respect to warm, giving clear evidence that temperature acclimation alone is sufficient to induce ventricular remodelling. A 23% increase in RVM however, is relatively small in comparison to sexual maturation induced RVM in males where relative ventricular mass increases by as much as 90% compared with females at the same temperature [197, 198]. One interesting observation in this study was the inter-chamber remodelling within the ventricle itself: spongy layer hypertrophy is associated with compact layer atrophy in the cold, with the reverse being true in warm. As in the differences between ventricle and atrium, the spongy and compact layer differences allude to potential differences in cardiac function at extremes of temperature.

### *Spongy layer*

It was apparent that overall ventricular hypertrophy during cold acclimation was caused by spongy layer hypertrophy of the trabecular bundles where they were of 77% greater cross sectional area than warm and importantly, 30% greater than control. There was therefore a direct correlation between a decrease in acclimation temperature and an increase in spongy layer hypertrophy at all temperatures. At the genetic level (mRNA), there were also clear and significant differences between growth specific gene levels at all three temperatures and these genetic changes provide strong evidence that increases or decrease in mass within the trout heart are largely down to muscle changes, indicative of genuine cardiac hypertrophy as seen in mammals and not through other causes such as increases in cytosolic volume. Indeed, ventricular myosin heavy chain (VMHC), muscle LIM protein (MLP) and small myosin light chain (SMLC2) levels were 2.6, 1.6 and 2.4 fold greater in cold acclimated spongy layer with respect to warm which are very similar to levels seen in another, non-temperature induced model of hypertrophy in the same species [199]. As well

as muscle gene evidence of cardiac hypertrophy in cold acclimated fish, vascular endothelial growth factor (VEGF) mRNA levels provide insight into the extent of angiogenesis during cardiac remodelling in the spongy layer [199]. Angiogenesis is often observed in mammalian cardiac hypertrophy [200, 201] and this increased vascularisation is needed to nourish the ever increasing mass of muscle. It was however slightly surprising to observe angiogenesis markers in the spongy layer, where blood supply is predominantly lacunary in nature as opposed to the compact layer which is classically vascularised [4]. However, vascularisation of the spongy layer does occur in some fish species [202] and thus it is possible that cold acclimation has led to vascularisation of the ventricular spongiosa in this study. It is also possible however that VEGF derived from the spongy layer dictates generalised increases in blood vascularisation to the heart, including the compact layer. In line with the present study, wild rainbow trout in winter conditions have been shown to increase angiogenesis [194]. Interestingly, hyperplasia, as shown by expression levels of proliferating cell nuclear antigen (PCNA) being 1.5 fold higher in cold acclimated spongy layer compared to warm, is an important factor in ventricular increases in mass. Hyperplasia has been observed in cold acclimated fish before [203] and in a model of hypertrophy in zebrafish, hyperplasia was shown to be a key factor in cardiac remodelling [204] with hyperplasia documented to be firstly, wnt signalling dependent, and secondly, variable between cardiac layers [205]. This phenomenon of cardiac hyperplasia in the physiological response of fish is in contrast to many mammalian studies where cardiac hyperplasia is typically only observed in pathological cardiac states, however there is currently much debate about the presence of non-developmental cardiac hyperplasia in mammals [206, 207]. It was therefore extremely surprising to have evidence of both extensive hypertrophy and hyperplasia in the spongy layer that is not reflected in the overall RVM at the same temperature. This suggests that mass was being lost elsewhere in the heart and other structures were compensating for increases in the spongy layer; later found to be the compact layer as discussed in the next section.

Overall, the spongy layer data provides evidence that the cardiac response in cold acclimated fish is largely facilitated by increases in muscle mass within this chamber layer.

### *Compact layer*

It was surprising to discover significant thinning of the compact layer in cold acclimated fish, as one would expect this portion of the chamber to increase in thickness in response to an increase in blood viscosity. Indeed, cold ventricle exhibits a 4% and 13% narrower compact layer than control and warm respectively giving the first evidence that the ratio of spongy layer to compact layer is a key parameter in temperature induced cardiac remodelling in trout. It was not however surprising to see compact layer hypertrophy in warm acclimated fish which are considerably more active than cold fish and have to contend with lower oxygen availability [161], so thickening of the compact layer would be ideally suited to sustain cardiac output in these conditions. Thickening of the compact layer in fish in both temperatures would have been akin to physiological and pathological types of hypertrophy as seen in mammals where in both cases the ventricular wall thickness increases, but via different mechanisms and diverse stimuli (chronic exercise and chronic hypertension being two important contextual triggers). It was therefore clear that two different types of hypertrophy (both spongy and compact) were being observed in the fish heart in different contexts that are distinct from classic mammalian hypertrophy, and these distinct types of hypertrophy must convey benefits to cardiac output in different operating temperatures.

By looking at the key features of hypertrophy in mammals and male salmonids, we can begin to explore the potential functional significance of the thickness of the compact layer.



### *Concentric and eccentric hypertrophy: lessons from mammals*

As discussed in chapter one, ventricular hypertrophy caused by chronic exercise (physiological) and chronic hypertension (pathological) both display thickening of the left ventricle wall, however closer inspection reveals distinct types of ventricular hypertrophy; *eccentric* and *concentric*.

*Eccentric* remodelling is concerned largely with *volume overload*. In this instance the ventricle is able to mitigate against the insult by adding sarcomeres *in-series* with sarcomeres already present. The net result is an increase in length of the contractile unit of the heart, as opposed to an increased thickness; ultimately allowing the heart to dilate and accept a greater volume of blood. Ventricular wall thickness increases in line with an increase in chamber radius so blood filling volume is not compromised. Both stroke volume and stroke pressure are maintained in eccentric remodelling [44].

Concentric remodelling however is concerned largely with *pressure overload* where chronic hypertension causes sarcomeres to be added in-parallel to inherent sarcomeres. The key difference here is that despite a much thicker ventricle wall capable of high pressure output, the chamber size does not change in tandem with this increased thickness. The net result in mammals over time is reduced ventricle filling leading to diastolic and eventually systolic dysfunction. Concentric remodelling therefore maintains stroke pressure but at the expense of maintained stroke volume [44].

In warm acclimated rainbow trout, which are more active than their cold counterparts, we see an increase in compact layer thickness of 13% and an increase in extra-bundular space

of 23%, therefore outer wall thickness increases and potential blood filling volume (also dictated by compliance as discussed later) is maintained. This is remarkably similar to *eccentric remodelling* seen in mammalian cardiac hypertrophy and is in-line with a mammalian chronic exercise response. We were unable to measure true luminal volume changes via histology, however preliminary pressure-volume curves in isolated perfused hearts suggest greater maximum filling volume and greater ventricular compliance of the warm ventricle (Shiels lab, unpublished).

In cold acclimated rainbow trout however, we do not see the opposite, concentric remodelling, as the outer or compact layer in fact undergoes atrophy. However, it could be argued that an effort is made to maintain stroke volume by decreasing compact layer thickness in line with spongy layer hypertrophy; indicative of a physiological as opposed to pathological response. However, due to the increased collagen deposition and therefore likely stiffness in cold acclimated hearts (discussed in the next section), blood filling volumes at best, remain the same, or reduce. It may simply be the case in cold acclimated trout that hypertrophied spongy layer contraction is sufficient to meet the reduced oxygen and circulatory demands of a fish with reduced metabolic demand. How the bi-directional relationship (in terms of differential growth) between the spongy layer and compact layer is managed, and which layer dominates the other, remains elusive, however this study attempts to realise the triggers behind spongy layer hypertrophy in chapter five, further shedding light on potential mechanisms.

#### ***Activity levels promote compact layer hypertrophy: lessons from male salmonids***

Male fish were excluded from this study due to evident temperature independent effects on hypertrophy, however we can learn from male studies to gain an understanding as to the “goal” of ventricular hypertrophy in certain contexts. Indeed, we know that males become

extremely active during spawning periods and these increases in activity levels dictate the need for greater cardiac output. Furthermore, increases in male hypertrophy are associated with compact as opposed to spongy layer hypertrophy [139, 208] and ventricle hypertrophy in male fish has been previously shown to be associated with increased vascularisation of cardiac tissue [209] . Combined, these cardiac remodelling events in male fish increase maximum cardiac stroke volume and cardiac output, which may be beneficial to increasing the oxygen transport capacity in male fish, strongly suggesting that activity levels are a driving factor in ventricular hypertrophy [198] and in this study warm acclimated fish are presumed to be operating at greater activity levels [161] (which was observed but not quantified in this study).

Taken together, the compact layer appears largely concerned with warm acclimation, and the requirement for a thick compact layer appears dictated by activity level, which is known to increase in warm acclimated trout [161]. Both warm and cold acclimated trout hearts display signs of compensation, whereby methods are employed to maintain blood volume despite structural changes to the spongy and compact layers. Thus, temperature induced cardiac remodelling in fish is more in-tune with eccentric remodelling as seen in mammals, which would point to, as expected, a physiological type of cardiac remodelling in fish.

In summary of this section, cold acclimation promotes spongy layer hypertrophy whilst warm acclimation drives compact layer growth. One key, if not sole, factor is the activity level of the fish, which is secondary to temperature. Downstream, the atrium appears to respond to events at the ventricle level by directly responding to compact layer size and therefore compact layer force output.

### **3.6.3 Cold acclimation increases cardiac stiffness and warm acclimation promotes cardiac compliance**

In the spongy layer there is a significant increase of overall connective tissue (shown by Masson's trichrome staining) during cold acclimation. Indeed, warm acclimation reduced the amount of connective tissue in this sub-chamber, revealing an inverse relationship between temperature and connective tissue. Moreover, this pattern was also true of the compact layer. Masson's trichrome stains all connective tissue, however using picosirius red, collagen was isolated. Again, there was a significant increase in collagen in both cold acclimated spongy and compact layers. It was however surprising to find that collagen, as labelled by picosirius red, was unchanged between control and warm in the spongy layer, and actually appeared to increase in warm compact layer, although only half as much as cold. There is therefore a slight contradiction in the Masson's trichrome stain and picosirius staining especially in the warm response, indicative of multiple types of connective tissue including different collagen isoforms present in the fish heart that perhaps show differential staining. What is clear however, is that cold acclimation significantly increases the amount of connective tissue/collagen in heart, akin with fibrosis as seen in pathological hypertrophy in mammals which is perhaps surprising in a physiological response such as natural temperature acclimation in trout. This would therefore dictate that collagen deposition in cold acclimated fish hearts is part of a physiological response, and functions to facilitate effective cardiac output.

Elastin, the biophysically elastic protein is unchanged in cold acclimated trout hearts, however levels significantly rise in the spongy layer during warm acclimation in line with a generalised reduction in collagen, implying that warm acclimation in the ventricle promotes compliance.

Collagen fibrils and networks are made of numerous types of collagen proteins, each made up of a vast array of alternatively spliced collagen mRNAs and post-translational modified proteins [210]. In this study, collagen 1 was investigated at the mRNA level as collagen 1 in mammals is a key cardiac collagen [73]. This yielded surprising results whereby Col1a1 mRNA and Col1a2 mRNA in both atrium and ventricle were significantly reduced with respect to control in both cold and warm, suggesting strongly a non-temperature dependent response. Moreover these mRNA levels do not tally with events observed at the structural level. Col1a3 mRNA levels however showed temperature dependency in both chambers and in a pattern in line with the ventricular structural events. The reasons behind these contradicting pieces of evidence are open to speculation, however there are certain possibilities: the Col1a1 and Col1a2 results tally with a heat shock or stress type response, whereby gene activity is promoted by extremes of temperature giving rise to cellular stress. It is also a possibility, although unlikely, that mRNA levels do not tally directly with protein levels due to effects such as alternative splicing and posttranslational modifications. Another possibility is that Col1a1 and Col1a2 are not required for temperature dependent structural responses in trout heart and have an alternative role. Col1a3 however *does* share an expression profile in line with the protein observations leading to a possibility that this collagen isoform is structurally important in the trout heart. The most compelling evidence of temperature dependent connective tissue remodelling in the trout heart comes from analysis of collagen regulatory genes; MMP (anti-collagen) and TIMP (pro-collagen). As MMPs and TIMPs show large scale cross-reactivity (e.g. each MMP can interact with numerous overlapping types of collagen) [85] they are ideally suited to study general increases or decreases in overall collagen/ connective tissue at the molecular level at least. Indeed cold acclimation induces a pro-collagen state via MMP regulation; MMP2 and MMP13 mRNA levels decrease significantly in cold acclimation in

the atrium and ventricle and MMP9 levels decrease in the atrium. Furthermore in warm acclimation, MMP2 and MMP9 levels rise significantly in ventricle and atrium respectively. Moreover, the pro-collagen regulatory enzyme TIMP increases significantly in ventricular cold acclimation.

Taken together, these data allude to cold acclimation promoting increases in connective tissue and collagen which likely occur via temperature dependent expression of collagen regulatory proteins; MMPs and TIMPs. Moreover, MMPs and TIMPs show chamber specific expression, adding a further level of cardiac remodelling control.

Cold acclimation therefore promotes cardiac stiffness and a more robust heart, with warm acclimation promoting compliance via the combined influence of collagen and elastin content in different temperatures.

#### **3.6.4 Temperature acclimation in trout dictates the *nature of the pump*; low pressure or high pressure**

In the previous two sections, cold acclimation has been described as causing spongy layer hypertrophy, compact layer atrophy, atrial atrophy and a generalised large-scale increase in collagen. Warm acclimation on the other hand causes spongy layer atrophy, compact layer hypertrophy and atrial hypertrophy coupled with a generalised decrease in collagen and increase in elastin. The key question remains though; how do these structural observations relate to the function of the heart in extremes of temperature? In order to understand the significance of these changes, it was important to look at the hearts of fish that live in extreme environments.

### ***Volume driven pump: lessons from the ice fish***

The Channichthyidae or "icefish" is a teleost that lives in the Antarctic where temperatures are low and oxygen concentration is high. Very low oxygen-binding hemoglobin and myoglobin proteins present in these fish result in relatively low oxygen consumption in which the circulatory system responds by increasing blood volume, increasing heart size (hypertrophy) and increasing cardiac output [4]. The icefish heart has the ability to displace large systolic volumes at a low rate and relatively low pressure, with large ventricular fillings necessary for this to occur [211]. The cardiac ventricle of the icefish is characterized by hypertrophy of the spongy layer. It functions as a specialized volume pump which moves large stroke volumes at a low heart rate, but is not able to produce high pressures [4].

### ***Pressure driven pump: lessons from the tuna***

Tuna are highly active, live in warmer climates compared to the ice fish, have high myoglobin levels and the highest relative mass and proportion of the compact layer (40-70%) among fishes [212]. The presence of this large compact layer gives these types of hearts the potential to act as pressure pumps: they move small volumes at a relatively high rate and high pressure [4].

When comparing cold acclimated rainbow trout with ice fish, there are similarities: spongy layer hypertrophy and a relatively thin compact layer. Likewise, there are similarities between warm acclimated trout and tuna: namely a thick compact layer, and relatively atrophied spongy layer.

### **3.6.5 Conclusions**

#### ***Cold acclimation***

Cold acclimation results in a trout heart well adapted to pumping blood around the body at low pressure. The fish is relatively immobile in an environment of high oxygen availability and although it does not require a large stroke volume, it *does* require a heart suitable of overcoming biomechanical stress associated with increases in blood viscosity. This non-contractile structural robustness is provided by increases in collagen via downregulation of negative collagen regulators and increases in collagen expression, while contractile structural integrity is provided by spongy layer hypertrophy brought about by upregulation of muscle specific genes and hyperplasia. Because only a low pressure is required, the compact layer atrophies and the relatively weak circumferential compact layer contraction combined with contraction of hypertrophied spongy layer are sufficient to meet the circulatory demands of the fish. Blood filling volume is dictated by extrabundular space and ventricle compliance. Both extrabundular space and compliance are reduced in the cold acclimated heart; however the relatively small blood volume is sufficient in this high oxygen, low metabolic demand environment. In the cold, the circulatory system is operating at low pressure, therefore the atrium, the last part of the system, is subject to relatively low return pressure and responds by atrophying, however it maintains structural integrity via increases in collagen.

#### ***Warm acclimation***

In warm acclimation, the emphasis is on creating an efficient pressure pump, presumably due to an increased need by metabolising tissue for oxygen in a reduced oxygen environment, which would also dictate the need for a greater stroke volume. In order to meet these requirements, the compact layer hypertrophies, with the aim of providing force, and both extrabundular space and compliance increase to facilitate increased blood filling. Increased compact layer thickness can help provide structural integrity to the heart,



reducing the need for connective tissue support. In the warm, the circulatory system is operating at high pressure therefore the atrium, the last part of the system, is subject to relatively high pressure and it responds by hypertrophying. Due to the increased muscle mass in the atrium, the need for collagen support is decreased in the atrium.

### **3.6.6 Summary**

Temperature acclimation of rainbow trout involves structural and functional changes in the heart allowing the heart to maintain sufficient cardiac output over a wide range of oxygen and activity levels. Although calcium handling is important in the acclimation response, this study shows profound differences in heart morphology between cold and warm acclimated fish hearts and it is likely that these changes convey more dominance over events at the cellular level in maintaining adequate cardiac output in temperature acclimation – by *modifying the nature of the pump* from a low pressure, low volume pump in cold to a high pressure, high volume pump in warm. The acclimation response in fish heart has strong similarities with varying degrees of eccentric physiological hypertrophy as seen in mammals.

### **3.6.7 Study limitations**

#### **1. Blood volume and pressure not quantified**

In vivo measurements of blood volume and pressure in ventricle and atrium would help show (or not) a direct link between structure and function, as well as activity levels in different temperatures.

#### **2. No staining of atrium**

Structural evidence gained from histological staining of the atrium would have allowed a more accurate analysis of the structure and function of the atrium during temperature acclimation.

#### **3. No cell growth/ apoptosis markers/ chromatin staining/ cell count**

Hyperplasia was only measured using one mRNA marker, with no other evidence taken into account which is vital to prove genuine hyperplasia. Indeed, cell count (as opposed to cell size) was not investigated. Moreover, no attempt was made to look at methods of atrophy (other than gene regulation) so apoptosis and necrosis, which are key to pathological cardiac remodelling in mammals cannot be ruled out or in.

#### **4. Genetic analysis was of spongy layer tissue only**

All molecular evidence was taken by analysis of genes in the spongy layer only. By carrying out the same experiments on the compact layer, it would be more clear as to the differential remodelling observed structurally.

## **4 Connexin remodelling in the rainbow trout heart following temperature acclimation**

### **4.1 Brief introduction**

Significant structural cardiac remodelling of acclimated rainbow trout was described in chapter three and these muscle and connective tissue changes allow the heart to function despite physical insults and metabolic demands brought about by temperature acclimation. Despite these large scale structural alterations that are in place to mitigate factors such as blood viscosity and stretch, the heart must continue to pump in synchrony and in correct time and space. In order for this to occur, highly organised conduction pathways must still be able to function and facilitate effective electrical communication throughout the heart. With structural changes taking place, these electrical conduction pathways have the potential to become disjointed due to factors including increased/ decreased travelling distances, altered cell-to-cell and bundle-to-bundle ionic flow due to three dimensional positional changes, and physical obstructions such as connective tissue build up/ fibrosis and muscle alignment [213]. Moreover, during temperature acclimation, these conduction pathways are operating at different temperatures resulting in variable conduction kinetics [214]. Therefore in the acclimated fish heart, the conduction system has to deal with physical as well as kinetic constraints.

With this in mind, it is clear that during temperature acclimation, the electrical conduction system has to adapt or remodel in-line with the structural changes in order to allow the heart to function effectively and maintain efficient cardiac output regardless of operating environment.

As described in the overall introduction, gap junctions are the most important conduction channels within the mammalian heart, facilitating electrical conduction and cell to cell communication through connexin proteins [215]. Connexins themselves have been shown to remodel during cardiac remodelling of the mammalian heart; named *connexin remodelling* [101], and furthermore, cardiac remodelling has also been shown to alter conduction within the heart [98, 99].

It was therefore the aim of this section to investigate connexin remodelling within the acclimated rainbow trout heart by quantifying mRNA and protein levels and distribution.

## **4.2 Key aims**

- Determine types of connexins in rainbow trout ventricle and atrium
- Investigate levels of connexins during temperature acclimation at the mRNA level
- Investigate distribution of connexin protein generally and during temperature acclimation

## **4.3 General Methodology**

Full details of the methodologies are in Chapter 2, however below is a concise annotation of the methods used to generate the results in this chapter.

In order to determine the types of connexin present in the rainbow trout heart, it was important to start at the genetic level, therefore gene expression was examined using two pairs of degenerate primers (F1R1 and F2R2), previously shown in other studies to successfully detect the full range of connexin mRNA present in target tissue of various species [181, 220]. Following PCR on cDNA derived from RNA extraction from atrium

and ventricle, PCR bands of interest were excised directly from the gel, purified and sequenced. Nucleotide sequences were entered directly into a Pubmed Nucleotide BLAST search and connexin subtype was determined directly from the % alignment with a minimum of  $80 \pm 1\%$  threshold required for satisfactory recognition. If the % alignment was less than 90%, other factors were considered, such as species similarities to target sequence, and tissue location of connexin in the target sequence. For example, if the target sequence was zebrafish from heart tissue, and the trout sequence was 80% then this was accepted.

PCR using F1R1 and F2R2 on acclimated atrium and ventricle was carried out to qualitatively investigate connexin mRNA levels at different temperatures, followed by qPCR (using a published primer for one of the connexins identified and F2R2 for the remaining connexins), to determine to a greater degree of accuracy via quantitation; ventricular and atrial connexin levels during acclimation.

To investigate connexin protein levels, Western blotting and immunostaining were used. Homogenates from ventricle tissue taken from the three acclimation temperatures were centrifuged at two distinct speeds to generate two fractions: cytosolic and membrane. The two fractions were run on a gel and Western blotting probing using anti-Cx43 antibody, followed by HRP-conjugated secondary antibody, in order to assess connexin distribution within the two fractions. The same 1° antibody was used on ventricular cryosections and tagged with a secondary fluorescent antibody to visualise connexin in intact sections.

## 4.4 Summary of aims, methods and expected outcomes

Table 4.1 – Summary of aims, methods and expected outcomes (chapter four)

Aim	Method	Expected outcome
<b>Identify connexins present in rainbow trout heart</b>	PCR using degenerate primers followed by sequencing and sequencing alignment	Connexins present in ventricle determined to high level of confidence
		Connexins present in atrium determined to high level of confidence
	Western blotting of ventricular tissue homogenate using anti Cx43 antibody	Determine connexin protein is present in ventricle
<b>Determine connexin distribution in rainbow trout heart</b>	Western blotting of ventricular homogenate cytosolic and membrane fractions using anti Cx43 antibody	Determine cellular distribution of connexin protein in ventricle
	Immuno staining of fluorescence-tagged anti Cx43 antibody on ventricle sections	Visualise connexin distribution/ location in ventricle
<b>Determine extent of connexin remodelling in rainbow trout heart</b>	PCR and qPCR using specific Cx43 primer and degenerate primers shown only to pick up connexins	Determine levels of Cx mRNA in ventricle during temperature acclimation
		Determine levels of Cx mRNA in atrium during temperature acclimation
	Western blotting of ventricular homogenate cytosolic and membrane fractions using anti Cx43 antibody over three acclimation temperatures	Determine cellular distribution of connexin protein in ventricle over three acclimation temperatures

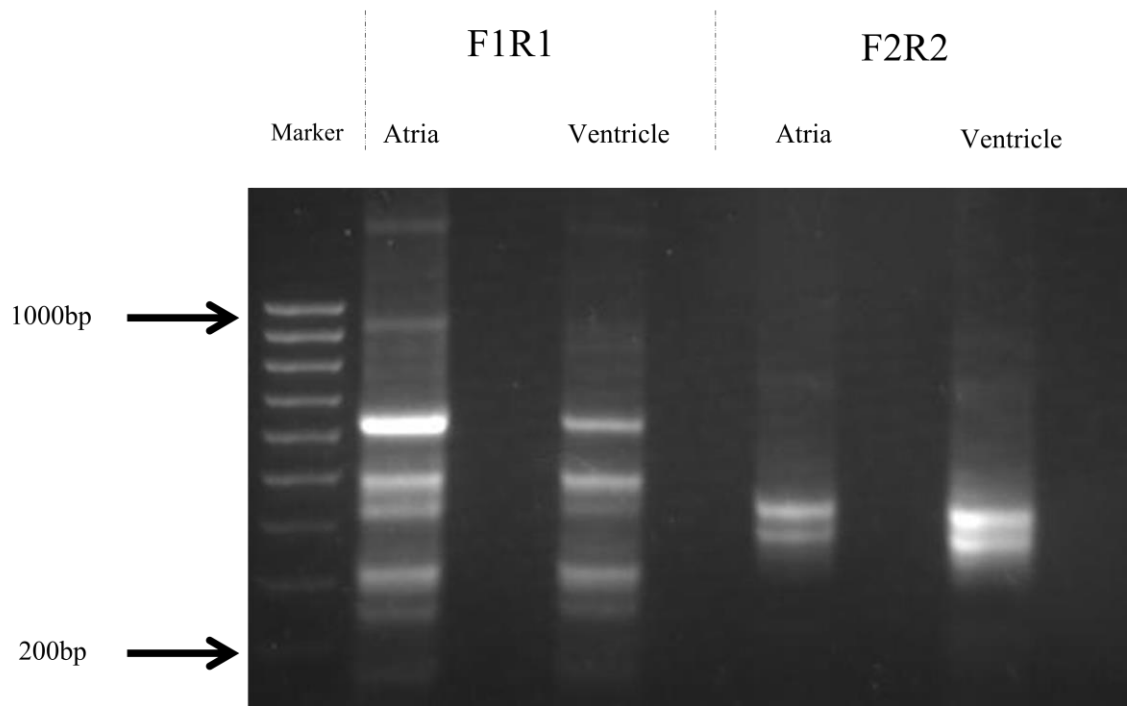
## 4.5 Results

*Please note that certain parts of these results refer to supporting material generated in this study and documented in the Appendix, Chapter 8.*

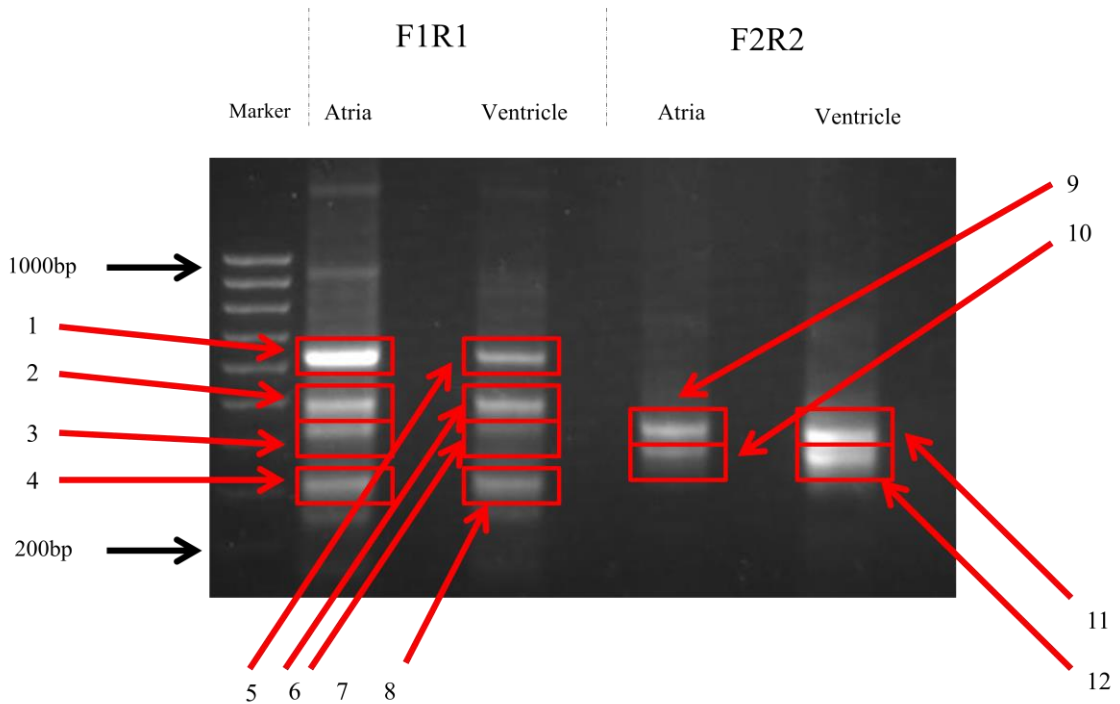
### 4.5.1 Identification of connexins in rainbow trout heart

Using PCR of degenerate primer sets F1R1 and F2R2 (Figure 4.1), followed by sequencing of determined bands (Figure 4.2), BLAST searches revealed the connexins present in rainbow trout ventricle and atrium to be: Cx43, Cx30.9 and Cx48.5 (Figures 4.3 – 4.5).

Also found using the degenerate primers were a kinase and a kinesin (Table 4.1).



**Figure 4.1 – Connexin expression profile using degenerate primers.** Using connexin degenerate primer sets “F1R1” and “F2R2” in a PCR on warm acclimated rainbow trout atrium and ventricle cDNA side by side, the above electrophoresis gel (inverted colour) was obtained. Marker on the left handside ranges from 1000 nucleotide base pairs (bps) to 100 bps with the range 1000 – 200bp shown.



**Figure 4.2 - Identification of PCR bands for sequencing.** The 12 most prominent bands from the F1R1/ F2R2 primer sets were excised, purified and sequenced.

> [ref|NM\\_001124563.1|](#) **UG** *Oncorhynchus mykiss* connexin 43 (cx43), mRNA  
[gb|DQ204869.1|](#) **UG** *Oncorhynchus mykiss* connexin 43 (Cx43) mRNA, complete cds  
 Length=1285

GENE ID: [100136315](#) cx43 | connexin 43 [*Oncorhynchus mykiss*]

Score = 1020 bits (552), Expect = 0.0  
 Identities = 584/600 (97%), Gaps = 0/600 (0%)  
 Strand=Plus/Plus

```

Query 1   TTACTCCACGGCTGGAGGGAAGGTGTGGCTCTCTGTCCCTTTCAICTTCAGGATCCTGGT 60
          |||
Sbjct 126 TTACTCCACGGCTGGAGGGAAGGTGTGGCTCTCTGTCCCTTTCAICTTCAGGATCCTGGT 185

Query 61   GTTGGGAACGGCTGTGGAGTCTGCCTGGGGGACGAGCAGTCCGCCTTCAAGTGCAACAC 120
          | |||
Sbjct 186   GCTGGGAACCGCTGTGGAGTCTGCCTGGGGGACGAGCAGTCCGCCTTCAAGTGCAACAC 245

Query 121  CCAGCAACCTGGTTGTGAGAAATGTGTGTTATGACAAATCTTTTCCTATATCGCATGTACG 180
          |||
Sbjct 246   CCAGCAACCTGGTTGTGAGAAATGTGTGTTACGACAAATCCTTTTCCTATATCACATGTACG 305

Query 181  GTTCTGGGTGCTACAGATTACTTTGTCTCGACGCCGACTCTTCTCTACCTTGCCCATGT 240
          |||
Sbjct 306   GTTCTGGGTGCTACAGATTACTTTGTCTCGACGCCGACTCTTCTCTACCTTGCCCATGT 365

Query 241  GTTCTACCTGTTGCGAATAGAGCAGAAGATTAACCGCAAAGAGGAAGGGCTGAAAACCAT 300
          |||
Sbjct 366   GTTCTACCTGTTGCGAATAGAGCAGAAGATTAACCGCAAAGAGGAAGGGCTAAAACCAT 425

Query 301  CCAGAACGACGGAGGCGACGTGGACGTACCTCTAAAGAAGATTGAGTTAAAAAAGCTCAA 360
          |||
Sbjct 426   CCAGAACGACGGAGGCGATGTGGACGTACCTCTGAAGAAGATTGAGTTAAAAAAGCTCAA 485

Query 361  GCATGGGCTGGAGGAGCATGGGAAGGTTAAGATGAAGGGAGCCCTCTTGAGAACCTACAT 420
          |||
Sbjct 486   GCATGGGCTGGAGGAGCATGGGAAGGTTAAGATGAAGGGAGCCCTCTTGAGAACCTACAT 545

Query 421  ATTACAGCAttttttCAAGTCCATTTTTGAGGTGGGCTTCCTGGTCATACAGTGGTACAT 480
          |||
Sbjct 546   CGTCAGCATTTCCTTTAAGTCCATCTTTGAGGTGGGCTTCCTGGTCATACAGTGGTACAT 605

Query 481  TTACGGCTTCAGCTTGGCTGCTGTCTACACCTGTGAGAGGTCCCCCTGCCCCACAGAGT 540
          |||
Sbjct 606   TTACGGCTTCAGCTTGGCCGCTGTCTACACCTGTGAGAGGTCCCCCTGCCCCACAGAGT 665

Query 541  AAACGTTTTCTCTCCAGACCTACTGAGAAAACCTGCTTCATCAICTTCATGCTGGTGGT 600
          |||
Sbjct 666   AAACGTTTTCTCTCCAGACCCACTGAGAAAACCGTCTTCATCAICTTCATGCTGGTGGT 725
  
```

**Figure 4.3 - Identification of Connexin 43.** Following sequencing, a nucleotide BLAST search was carried out. Bands 1 and 6 were confirmed as having a 97% identity ( $\pm 2\%$ ) to *Oncorhynchus mykiss* connexin 43. Sequencing was carried out 3 times on 3 separate bands/fish.



>|gb|GU363512.1| UGM Danio rerio connexin 48.5 mRNA, complete cds, alternatively spliced  
Length=3865

GENE ID: 387533 qja3 | gap junction protein, alpha 3 [Danio rerio]  
(Over 10 PubMed links)

Score = 343 bits (380), Expect = 5e-91  
Identities = 330/419 (79%), Gaps = 11/419 (3%)  
Strand=Plus/Plus

```
Query 6 CATCCGCTTCTGGN-GTTCAGATCATCTTTGTGTCCACGCCACCCATCTACCTGGG 64
      |||
Sbjct 664 CATCCGCTTCTGGGTGCTCCAGATCATCTTCGTGTCCACGCCGACGCTCATCTACCTGGG 723

Query 65 TCACGTACTGCACATCGTCCGCATGGAGGAGAAGCGGAAAGAGAAGGAGGAGGAGCTGCG 124
      |||
Sbjct 724 CCACGTCTGCACATCGTTCGTATGGAGGAGAAGCGGAAAGAGCGTGAGGAGGAGTTCG 783

Query 125 AGAGGCCATCAGACTCCAGGAGGAAAAGGAACCTCTTA---CAATGAA---GGAGGGGA 178
      |||
Sbjct 784 AAAGGCCAGCCGCTCCAGGAGGAGAAAAGAACTCCTGTATAGAAATGGAGGGGGAGGGGA 843

Query 179 TGCAGGGGGAGGGGGAGGTGGT---AAAAAGACAACCCCTCTGCGGGATGATCCGGG 235
      |||
Sbjct 844 GCCTGGTGGACGGGGTGGGGCGGCAAAAAGGAAAAGCCCAATCAGAGACGAGCATGG 903

Query 236 CGAGATCTGTGGCTGGGGTGCCTGCTCCACCTAIGTGTCCAC-GGATATCAAAC 294
      |||
Sbjct 904 CAAAATCCGATTAGAGGTGCTTGTTCGCGACCTACGTGTTCAACATCATTTCAGAC 963

Query 295 CCTTTTGAAGTGGGGTTCATTTAGGTCATGATTTACTCTATGGCTTCCAGCTGAGGCC 354
      |||
Sbjct 964 CCTGTTGAAGTGGGGTTCATTTAGGTCAGTATTTCTCTATGGTTTCCAGTTGCGGCC 1023

Query 355 GCTATACAAGTGTGCACGGTGGCCCTGCCCAAACTGTGGACTGCTTCATCTCCCGGC 413
      |||
Sbjct 1024 CCTGTATAAGTGTGCGCGGTGSCCTTGCCCAACACGCTGGACTGCTTCATTCCCGGC 1082
```

**Figure 4.4 - Identification of Connexin 48.5.** Following sequencing, a nucleotide BLAST search was carried out. Bands 9 and 11 were confirmed as having a 79% identity ( $\pm 1\%$ ) to *Danio rerio* connexin 48.5. Sequencing was carried out 3 times on 3 separate bands/fish.

>|gb|HQ315553.1| Onchorhynchus kisutch connexin 30.9 mRNA, complete cds  
Length=1088

Score = 625 bits (338), Expect = 1e-175  
Identities = 342/344 (99%), Gaps = 0/344 (0%)  
Strand=Plus/Plus

```
Query 22 CGTCTCTGGTGCCTGCAGCTGATCTTCGTGTCCACACCGGCCCTGCTGGTGGCCATGCAC 81
      |||
Sbjct 264 CGTCTCTGGTGCCTGCAGCTGATCTTCGTGTCTACACCGGCCCTGCTGGTGGCCATGCAC 323

Query 82 GTGACTTACAGGAAGCGTGGGGACAAGAAGCACATCATCGCGACGGCCTTGCACAGTGGT 141
      |||
Sbjct 324 GTGACTTACAGGAAGCGTGGGGACAAGAAGCACATCATCGCGACGGCCTTGCACAGTGGT 383

Query 142 GGTGATGACAAGACCAGGCAGGTGGACCTAGAGGCCCTGAAGAGTCGTCTGCTGCCCATC 201
      |||
Sbjct 384 GGTGATGACAAGACCAGGCAGGTGGACCTAGAGGCCCTGAAGAGTCGTCTGCTGCCCATC 443

Query 202 ACGGGGCCCTCTGTGGTGGACGTACACCTGCAGCCTGTTCTTCCGCCTGATTTTGGAGGCT 261
      |||
Sbjct 444 ACGGGGCCCTCTGTGGTGGACGTACACCTGCAGCCTGTTCTTCCGCCTGATTTTGGAGGCT 503

Query 262 GGCTTTAIGTACGCCCTCTACTTTTGTGTTACGATGGCTTTGCGATGCCTCGCTGGTGAAG 321
      |||
Sbjct 504 GGCTTTAIGTACGCCCTCTATTTTGTGTTACGATGGCTTTGCGATGCCTCGCTGGTGAAG 563

Query 322 TGTGAGCAGTGGCCCTGCCCAAAACAAGGTGGACTGCTTCATCTC 365
      |||
Sbjct 564 TGTGAGCAGTGGCCCTGCCCAAAACAAGGTGGACTGCTTCATCTC 607
```

**Figure 4.5 - Identification of Connexin 30.9.** Following sequencing, a nucleotide BLAST search was carried out. Bands 10 and 12 were confirmed as having a 99% identity ( $\pm 1\%$ ) to *Onchorhynchus kisutch* connexin 30.9. Sequencing was carried out 3 times on 3 separate bands/fish.

**Table 4.2 – Genes detected from sequencing of PCR bands**

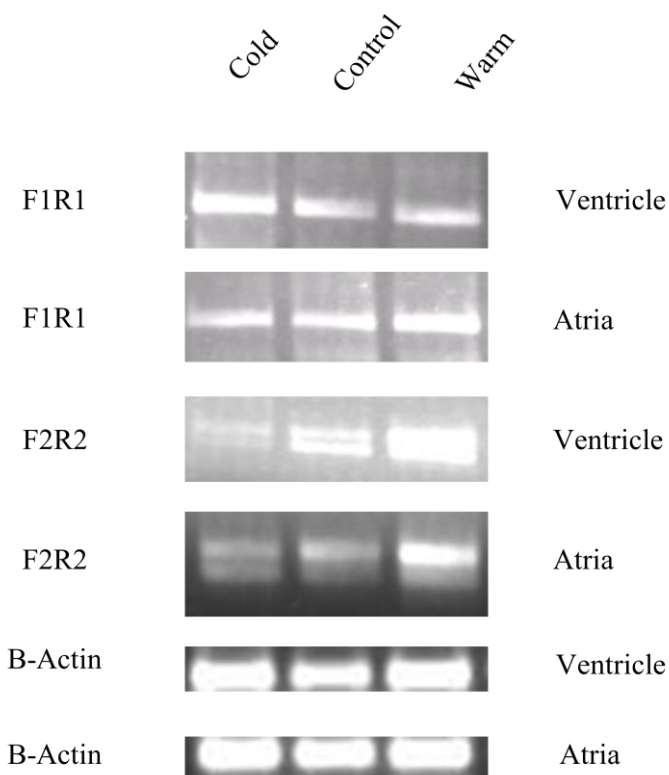
<b>Band</b>	<b>Gene identified via BLAST</b>
1	<b>Connexin 43 (Cx43)</b>
2	A kinase
3	Unknown
4	Kinesin
5	<b>Cx43</b>
6	A kinase
7	Unknown
8	Kinesin
9	<b>Cx48.5</b>
10	<b>Cx30.9</b>
11	<b>Cx48.5</b>
12	<b>Cx30.9</b>

Bands obtained from PCR using primers sets F1R1 and F2R2 (shown in figure 4.2) were sequenced (shown in figures 4.3,4.4 and 4.5) to determine with a high degree confidence, the connexins and other genes being picked up by the degenerate primers. F2R2 only picked up connexin genes (48.5 and 30.9).

#### **4.5.2 Connexin mRNA levels during temperature acclimation**

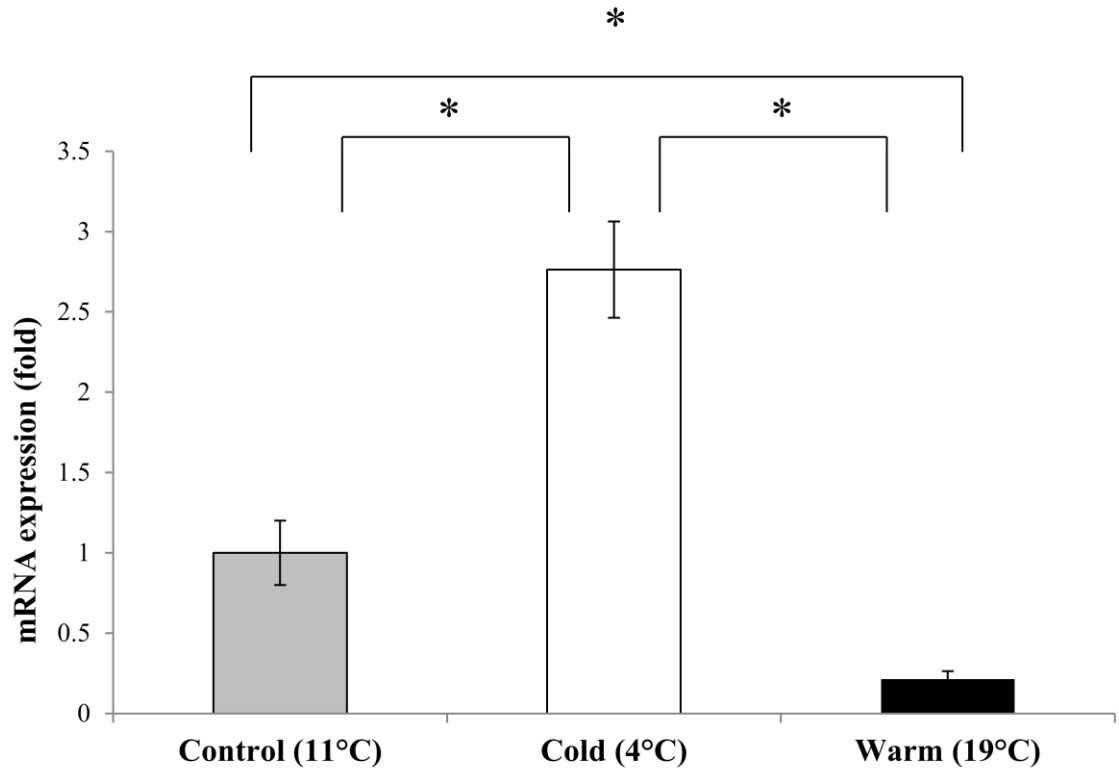
Using degenerate primers F1R1 and F2R2 for PCR of atrial and ventricle tissue taken from three acclimation temperatures, significant qualitative patterns were observed. Focusing on the Cx43 band of F1R1, Cx43 mRNA increases with decreased acclimation temperature in the ventricle, whilst the reverse is true in the atrium. F2R2 picks up two bands (Cx30.9 and Cx48.5) and mRNA levels of both increase with increased acclimation temperature in the ventricle and atrium (Figure 4.6). For a more quantitative approach, qPCR using a Cx43

specific primer set was used. In the ventricle, Cx43 mRNA levels were 2.5 fold higher in the cold ventricle compared to the warm ventricle (Figure 4.7). In the atrium, Cx43 was expressed 2.9 fold higher in the warm fish compared with cold (Figure 4.8). qPCR using degenerate primer set F2R2 revealed that Cx30.9 and Cx48.5 mRNA combined was 2.8 fold higher in warm as opposed to cold ventricle (Figure 4.9). In the atrium, Cx30.9 and Cx48.5 combined was also higher where the difference between cold and warm was 2.5 fold (Figure 4.10).



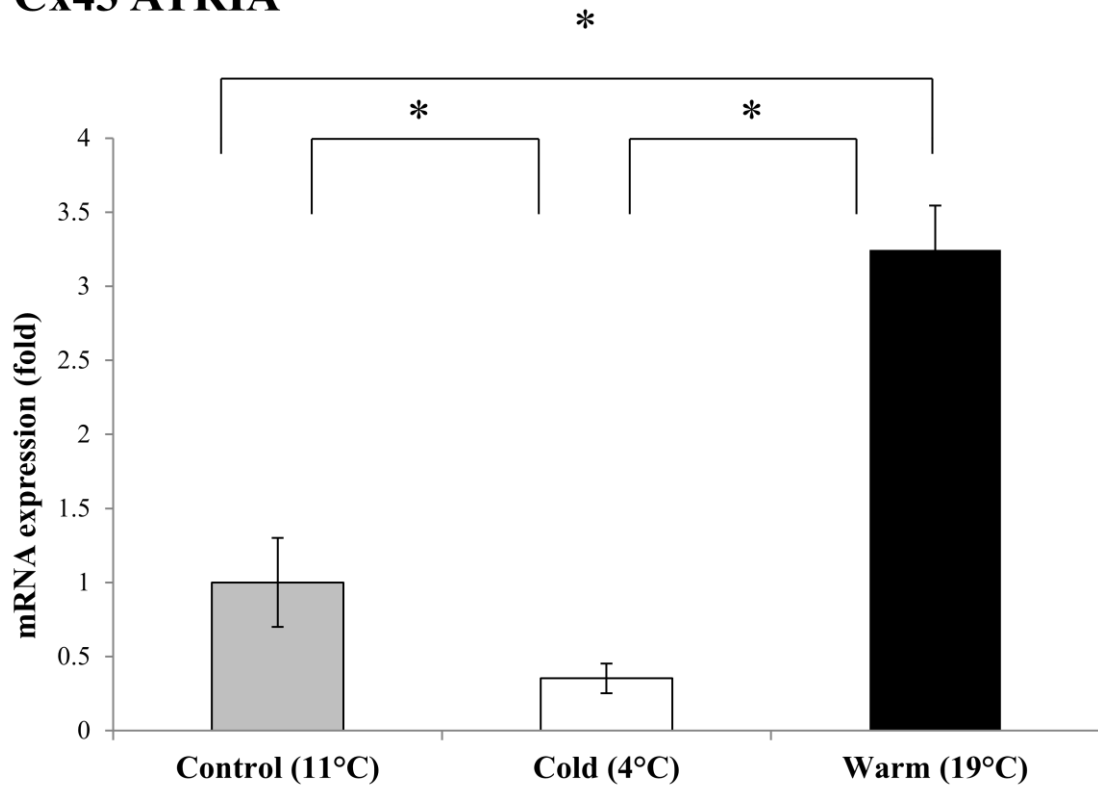
**Figure 4.6 – Connexin mRNA levels in ventricle and atrium during acclimation (qualitative).** Comparison of F1R1 and F2R2 amplification of cDNA taken from fish of three different temperatures gives initial indications that Cx43 (F1R1) rises in cold ventricle and increases in warm atrium. F2R2 products (Cx48.5 and Cx30.9) appear to increase in both warm ventricle and warm atrium. Beta actin was used as a load control for both atria and ventricle. PCR experiments shown are typical results taken from a combined pool of  $n = 7$  fish, repeated 3 times per fish (total sample size, 21).

## Cx43 VENTRICLE



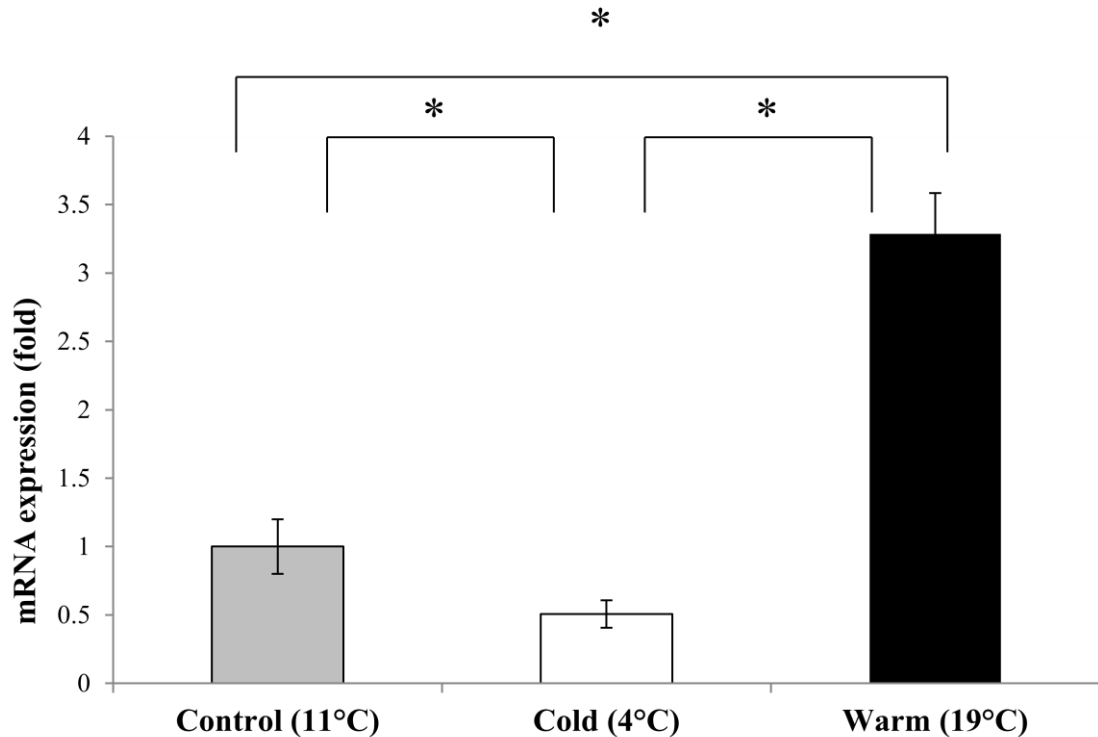
**Figure 4.7 - Expression of ventricular Cx43 mRNA during cold and warm acclimation of rainbow trout heart.** Cx43 in the cold ventricle was expressed 2.76 fold  $\pm$  0.3 with respect to control. Cx43 in the warm ventricle was expressed 0.21 fold  $\pm$  0.05 with respect to control. Expression levels of Cx43 in the cold ventricle were 2.55 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## Cx43 ATRIA



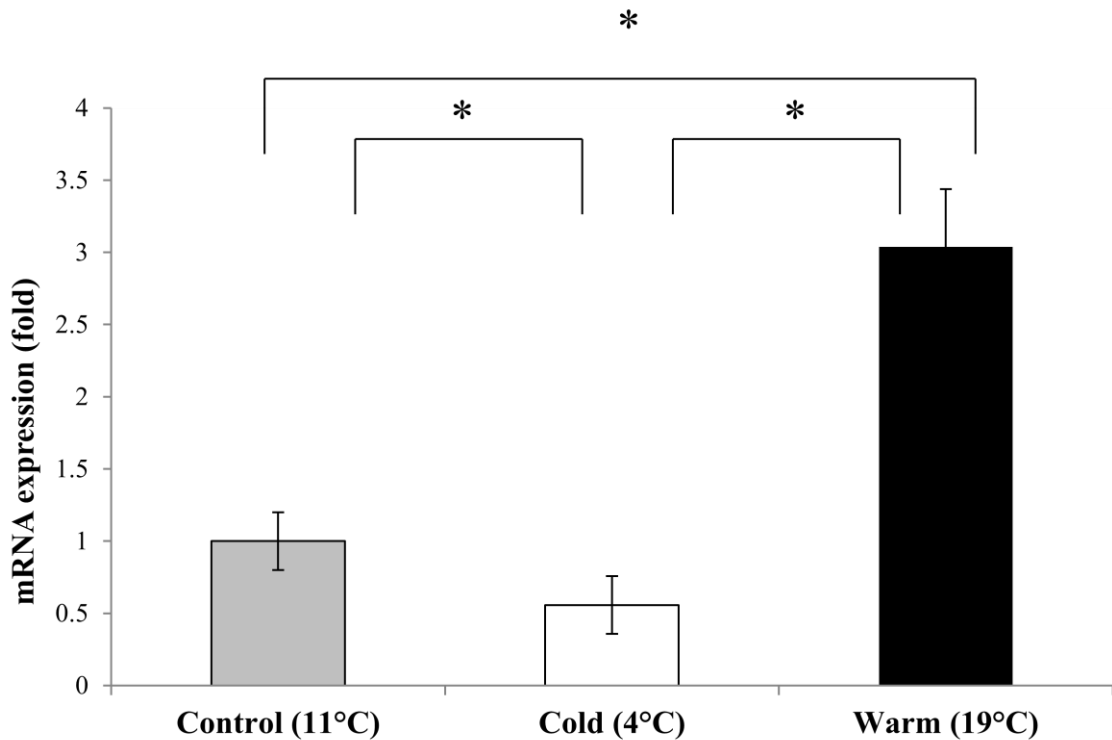
**Figure 4.8 - Expression of atrial Cx43 mRNA during cold and warm acclimation of rainbow trout heart.** Cx43 in the cold atria was expressed 0.35 fold  $\pm$  0.1 with respect to control. Cx43 in the warm atria was expressed 3.25 fold  $\pm$  0.3 with respect to control. Expression levels of Cx43 in the warm group were 2.9 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## Cx30.9 + Cx48.5 VENTRICLE



**Figure 4.9 - Expression of ventricular Cx30.9 and Cx48.5 mRNA during cold and warm acclimation of rainbow trout heart.** Cx30.9 + Cx48.5 in the cold ventricle were expressed 0.51 fold  $\pm$  0.1 with respect to control. Cx30.9 + Cx48.5 in the warm ventricle were expressed 3.29 fold  $\pm$  0.3 with respect to control. Expression levels of Cx30.9 + Cx48.5 in the warm group were 2.78 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## Cx30.9 + Cx48.5 ATRIA

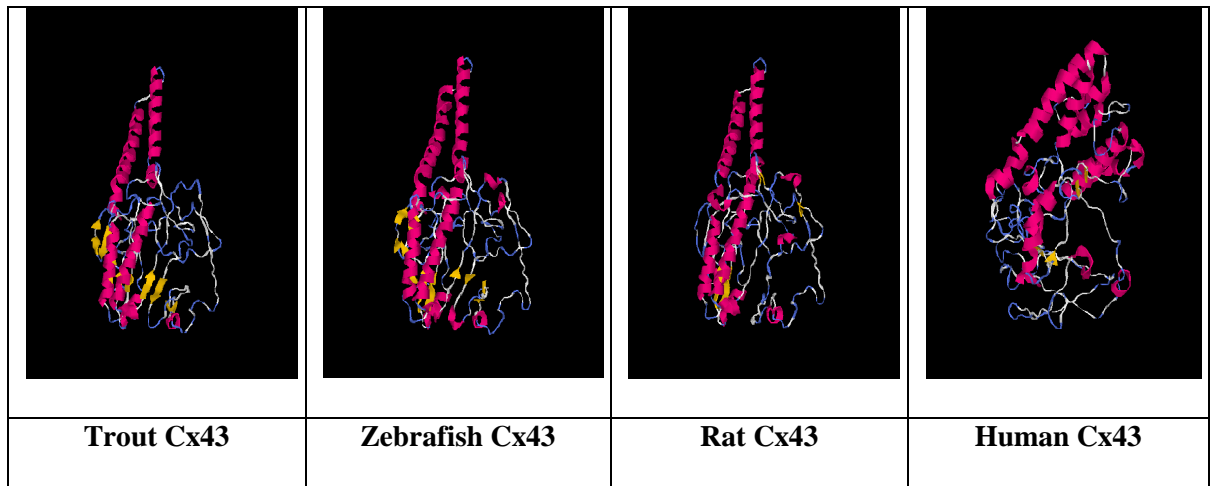


**Figure 4.10 - Expression of atrial Cx30.9 and Cx48.5 mRNA during cold and warm acclimation of rainbow trout heart.** Cx30.9 + Cx48.5 in the cold atria were expressed 0.56 fold  $\pm$  0.2 with respect to control. Cx30.9 + Cx48.5 in the warm atria were expressed 3.04 fold  $\pm$  0.4 with respect to control. Expression levels of Cx30.9 + Cx48.5 in the warm group were 2.48 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ . Connexin protein distribution during acclimation.

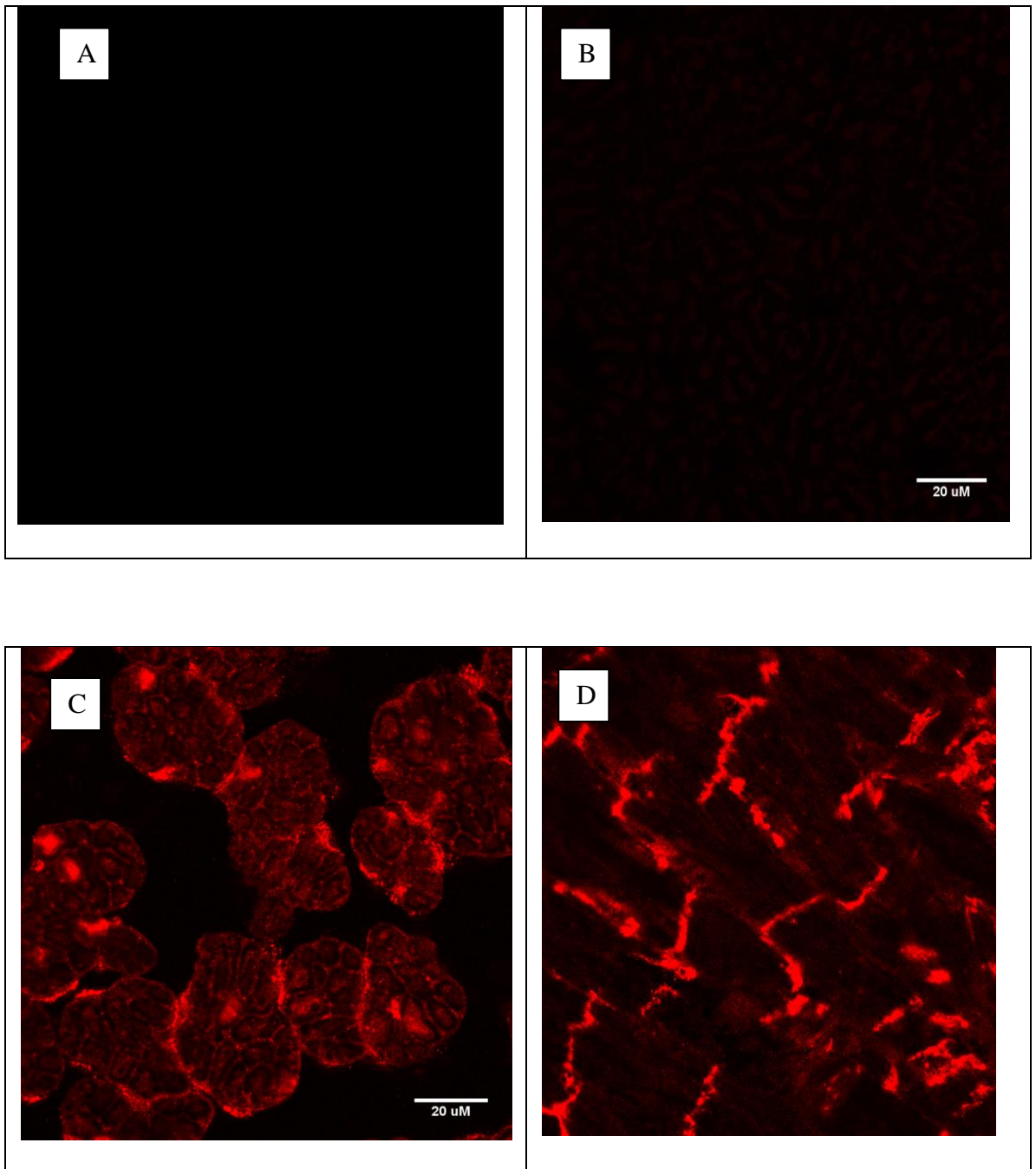
Predicted Cx43 protein structures were examined using iTASSER taken from published full Cx43 mRNA sequences, and trout Cx43 was shown to have a very similar structure to zebrafish and rat Cx43, with four transmembrane domains clearly visible. Human Cx43 was shown to be relatively different in structure to trout, zebrafish and rat, although four TM domains are apparent (Figure 4.11). Anti-Cx43 antibody was used to stain for connexin protein in trout spongy layer sections which revealed strong connexin distribution around the entire cardiomyocyte membrane, as well as distinct cytosolic staining. This antibody was also used on rat ventricle tissue as a positive control to show connexin selectivity (Figure 4.12). Using the same anti-Cx43 antibody against trout ventricular tissue alongside rat in a western blot, it appears to show a protein band slightly larger than rat Cx43, with strong reactivity, estimated to be around 48Kda. Furthermore, in

trout, again this preparation showed there to be a *cytosolic* as well as membrane component to this protein (Figure 4.13), which is not present when using an identical centrifugation preparation of rat ventricular tissue (Figure 4.14). Moreover, this band can be dephosphorylated using alkaline phosphatase, with warm acclimated ventricle tissue appearing to be more resistant to dephosphorylation compared to cold acclimated fish at the same experimental temperature (*see Appendix Figure 8.1*). Anti- Cx43 antibody directed to the highly conserved region of connexin picks up several protein bands of interest in the expected range of connexins; in particular, three clear bands appear at 30Kda, 43Kda and 48.5 Kda (*see Appendix Figure 8.2*) which are in-line with connexin 48.5, 43 and 30.9 mRNA previously found via sequencing (Figures 4.3 – 4.5). Densitometry revealed that the 48Kda band was present at 900% higher levels in the cytosol compared with the membrane (*see Appendix Figure 8.3*). Cytosolic 43Kda protein was present at 33% higher levels in cold spongy layer compared to control, and this cytosolic band was completely absent in warm acclimated fish. Moreover there is 100% more 43Kda protein in the membrane fraction of cold spongy layer compared to warm (*see Appendix Figure 8.4*). Analysis of the 30Kda protein band showed there to be 50% more of this protein present in the cytosol of cold acclimated fish hearts compared to the warm cohort, and 175% higher levels of this protein in the membranes of warm acclimated fish compared with cold (*see Appendix Figure 8.5*).

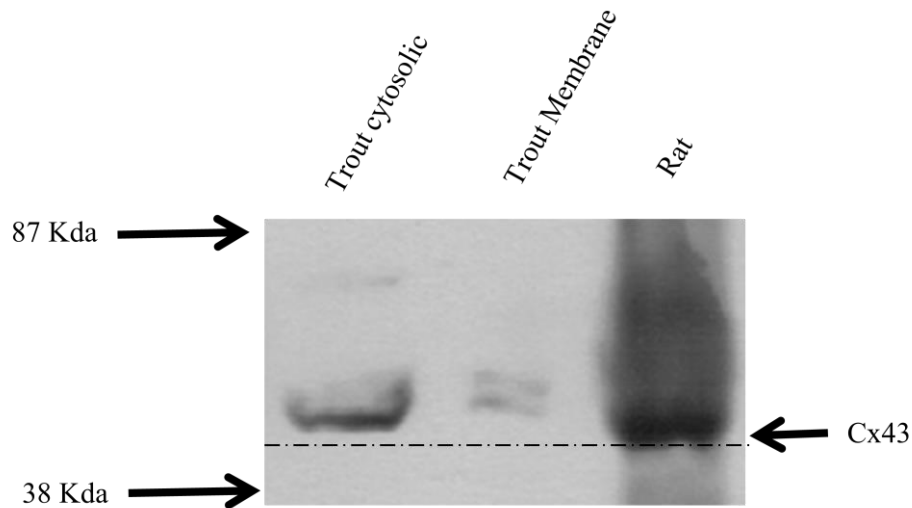




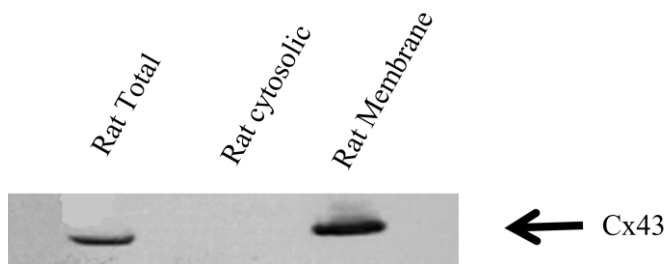
**Figure 4.11 - Predicted Cx43 protein structures from 4 different species.** Using iTASSER protein modeller (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>), and the fully sequenced mRNA of trout, zebrafish, rat and human Cx43, the structure of Cx43 including the distinctive 4 TM domains (in pink) can be clearly seen. Human Cx43 is the most dissimilar out of the four, with rat being remarkably similar to trout and zebrafish.



**Figure 4.12(A-D) - Immunofluorescence staining using anti-Cx43 antibody on trout and rat ventricle.** (A) is a spongy layer section with only primary anti-Cx43 antibody applied. (B) is a spongy layer section with only secondary antibody applied. (C) is a typical trout ventricle spongy layer stained with anti-Cx43 antibody and secondary cyr tagged secondary antibody. Bundles can be seen which contain clearly definable cardiomyocyte membranes with connexin present around the entire cardiomyocyte membrane. There also appears to be strong staining at bundle – bundle borders. Furthermore, cytosolic staining can be seen. (D) is a rat ventricle using the same antibodies. Connexin is present in the intercalated discs where cardiomyocytes adjoin at distal borders. Scale bar applies to both images.



**Figure 4.13 - Anti Cx43 antibody against trout cytosolic ventricular fraction and membrane fraction.** As discussed in more detail in Chapter Two, centrifugation was used to isolate cellular cytosolic and membranous connexin in order to begin to understand sub-cellular distribution. Tissue was taken from the spongy layer of control group fish. Lysed tissue homogenates were span for 10 minutes @ 2,500g to remove cellular debris and nuclei. The resulting supernatant was span for 30 minutes @ 100,000g. The resulting pellet was the “membrane” fraction, and the supernatant was the “cytosolic” fraction. Using anti Cx43 antibody, with a rat control, it is evident this antibody is picking up a protein band that is larger than rat Cx43 and is estimated to be between 45 and 50 Kda. Furthermore, use of fractionating centrifugation techniques has revealed a cytosolic as well as membrane presence of this protein. Western blot shown is representative of 9 experiments conducted. Dotted line is to highlight the difference in height of the rat Cx43 band and the trout bands.



**Figure 4.14- Anti Cx43 antibody against rat cytosolic and membrane Cx43.** Using the same fractionating (as above) centrifugation techniques on rat ventricular tissue, revealed that Cx43 in rat is entirely membranous.

## 4.6 Discussion

Please note this discussion also relates to sections of the study documented as supporting material drawn from the Appendix, Chapter Eight.

### Key findings from this chapter are:

1. Trout heart expresses Cx43, Cx48.5 and Cx30.9 mRNA
2. Connexin in trout is both cytosolic and membranous in nature
3. Membranous connexin is distributed around the entire cardiomyocyte perimeter
4. Connexin remodelling in trout involves changes in connexin levels, intracellular distribution and resistance to dephosphorylation
5. Cx43 levels increase in cold acclimated ventricle but decrease in cold acclimated atrium

### 4.6.1 Trout heart expresses Cx43, Cx30.9 and Cx48.5 in ventricle and atrium

Connexin subtype, location and expression levels are important in determining the overall electrical function of the heart due to connexins ability to regulate ionic flow in a subtype dependent manner, thus modulating electrical activity. Electrical events within the heart can broadly be compartmentalised into *pacemaker activity* and *general electrical conduction propagation*. This study revealed there to be Cx43, Cx30.9 and Cx48.5 in trout heart, with each one displaying distinct characteristics as discussed below.

#### **Cx43**

It was of no surprise to find Cx43 mRNA in trout heart as this connexin is widely regarded as the most important cardiac connexin in mammals expressed in both the ventricle and atria and is found in many species [221] [222] [223]. Furthermore, Cx43

has been previously found in zebrafish heart [224] [225] and fish Cx43 regulation and expression share high similarity with mammalian Cx43 [177]. Moreover, Cx43 is necessary for proper heart development and function in fish [226]. It is therefore likely that Cx43 is the main gap junction in trout heart and the role of Cx43 in fish heart is therefore likely to be, as in mammals, impulse propagation between cardiomyocytes where it acts as a fast conductor [227] and does not display pacemaker activity [121].

### ***Cx30.9***

Cx30.9 was found using a nucleotide BLAST search that revealed a group working on trout had already found this sequence not in heart, but in ovary tissue [228]. Because this group has already named this mRNA sequence in trout and there was such a high alignment it was decided by the author to keep with their nomenclature. However, as seen below (Figure 4.15), Cx30.9 was found, one year prior [229] to be *gap junction beta 6* (GJB6), which is a homolog of mammalian Cx30, an important cardiac connexin [230]. There are several studies pointing to the importance of Cx30 in the mammalian heart. Cx30's role appears to be in ensuring correct atrioventricular nodal firing in time and space via co-localisation with Cx45 and Cx40 [231] [232] in a notch signalling dependent manner [233] and forming heteromeric channels with other connexins [234]. Moreover, Cx30 is believed to modulate heart rate [230] by decreasing impulse propagation through the pore [235-237] which it does by employing two different gating mechanisms sensitive to both positive and negative potential differences [238]. Cx30 has also been found in zebrafish heart [239]. Cx30.9 in trout therefore is likely to be largely concerned with heart rate regulation.

Description	Max score	Total score	Query cover	E value	Max ident	Accession
<a href="#">Oncorhynchus kisutch connexin 30.9 mRNA, complete cds</a>	623	623	88%	3e-175	99%	<a href="#">HQ315553.1</a>
<a href="#">Salmo salar Gap junction beta-6 protein (cx6), mRNA &gt;qb BT045577.1 Salmo salar clone ssal-rqf-525-198 Gap junction beta-6 protein</a>	551	551	88%	2e-153	96%	<a href="#">NM_001140280.1</a>

**Figure 4.15 – Cx30.9 is the equivalent of mammalian Cx30.** Yamamoto named the mRNA sequence found in this study Cx30.9 however BLAST searches reveal Cx30.9 to be Atlantic salmon (*Salmo salar*) GJB6, otherwise known as Cx30.

### Cx48.5

Cx48.5 is the fish homolog of mammalian Cx46 where it is required for normal heart development [173]. In mammalian heart studies this connexin is relatively unexplored however gene knockout studies have revealed Cx46 to be key for conduction, contraction and heart morphology [174] [240] where it functions by being highly selective for ion size [241]. Interestingly, like Cx30, Cx46 has been shown to be important in pacemaker control by directly controlling the speed of impulse propagation [242]. Furthermore it shares the same gating characteristics as Cx30 [238].

Several connexins found in zebrafish heart were not found in this study: namely, Cx45.6 [243], Cx30.3 [171] and Cx36.7 [172] indicating species differences in connexin expression between fish.

In summary, Cx43 is likely to be the key conduction channel in trout involved in fast electrical propagation, and both Cx30.9 and Cx48.5 are probably mainly concerned with heart rate modulation by slowing conduction via ion selectivity and passage control.

## 4.6.2 Temperature acclimation causes connexin remodelling

Changes in temperature affect ion kinetics and could therefore affect cardiac conduction, so fish must employ methods to regulate this change in order to restore balance.

Furthermore, as described in chapter three, there are significant structural changes, not least to cardiomyocyte size, which could also affect conduction efficiency [244].

Moreover, in the previous chapter it was shown that the *nature of the pump* changes in acclimation with structural evidence suggesting each chamber is subject to varying amounts of pressure with which it has to provide a contractile force against. This dictates the need for variable chamber specific conduction, provided by gap junctions. It was therefore not surprising to observe considerable connexin remodelling in temperature acclimated fish heart.

In the spongy layer, Cx43mRNA levels rose two fold during cold acclimation and decreased 3 fold in the warm. This is in contrast to mammalian hypertrophy where connexin levels commonly decrease [245]. These observed increases in Cx43 are supported by other studies in conduction velocity in trout heart where cold acclimation is associated with a 60% increase in conduction velocity across the epicardial surface of the ventricle (M, Reza Kariman, Masters Thesis, University of Manchester, 2011) and is also in-line with genetic addition of Cx43 and the resulting increases in conduction velocity in mammals [246]. It was surprising to see an opposing response in the atrium where Cx43 mRNA levels decreased in cold acclimation and increased in warm acclimation one fold and two fold respectively. Together, this would suggest that temperature alone is not the driving factor behind Cx43 remodelling due to the opposing chamber specific response. It would therefore point to a more functional response whereby Cx43 levels appear more correlated with chamber size; increases in muscle mass could dictate the need for more Cx43 to facilitate effective conduction throughout the hypertrophied tissue. It is however unlikely that temperature does not influence Cx43 levels at all, but the extent to which this plays a part is unknown.

Cx30.9 and Cx48.5, unlike Cx43 are more involved with pacemaker activity and they do this by closely controlling the speed (namely slowing down) of the passage of ions through their pore. Again this could be affected by temperature and again, it would be expected that some compensation would occur so that extremes of temperature could not adversely affect heart rate and impulse propagation. For example, in the warm it can be envisaged that ions have greater kinetic ability and therefore to mitigate against this, and restore balance, an increased ratio of restrictive Cx30.9 and Cx48.5 would slow the passage of ions, and normalise heart rate. Indeed, this is what was observed in both chambers: in the warm Cx30.9 and Cx48.5 levels increase two fold and decrease one fold in the cold.

Furthermore, increased conduction velocity in the cold ventricle, where Cx30.9 and Cx48.5 levels are relatively low, would support the idea of conduction inhibition by these proteins. Together, this would suggest that the Cx30.9 and Cx48.5 response is largely temperature dependent and features to modulate heart rate during extremes of temperature.

#### **4.6.3 Connexin distribution suggests cytosol to membrane trafficking as a method for connexin remodelling**

Using anti-Cx43 antibody, it was confirmed that connexin staining, as expected, is located in the cardiomyocyte membrane (Figure 4.12) however, unlike in adult mammals where Cx43 is located in the IDs, Cx43 in trout cardiomyocytes appears to be around the entire perimeter of each cell. This is reminiscent of Cx43 staining in rat neonatal heart cells [247]. Indeed fish cardiomyocytes are remarkably similar in morphology to neonatal mammal cardiomyocytes; for example, both do not contain t-tubules that are present in mammalian adult heart cells [248]. Furthermore, Cx43 staining at bundle-to-bundle boundaries is relatively heavy, in similarity to mammalian IDs. Together this suggests that cells in trout heart are heavily interconnected electrically via gap junctions and this is



presumably to promote efficient communication in a spongy layer comprising cardiomyocytes orientated in numerous different planes.

### *Cytosolic connexin pool*

A surprising observation, shown by both western blot and immuno staining, was the heavy presence of cytosolic connexin. Exactly where in the cytosol this connexin is located is unknown however Cx43 has been found localised to mitochondria [249, 250] as well as being trafficked to and from the nucleus for signalling purposes [251]. This concept of a “cytosolic pool” of cardiac protein in an inactive state has been observed (using centrifugation techniques similar to those in this study) for the protein troponin [252] as well as contractile proteins including myosin heavy chain [253]. In a fish which can experience rapidly changing temperatures, but needs to quickly adapt and promote efficient electrical conduction through the heart, this *connexin pool* could represent a quick method of remodelling whereby this resource could be quickly tapped into as opposed to time-consuming fresh connexin synthesis, therefore ensuring connexin remodelling is rapid. In mammals, who operate at a constant temperature and therefore far less likely to experience rapidly changing temperature and kinetic demands, connexin is confined solely to the IDs. Indeed, using centrifugation in this study, rat connexin was entirely membranous.

Further evidence of this “pool” of connexin comes from western blot studies where there are clear temperature dependent patterns of connexin cytosol and membrane distribution. Indeed, there is a positive correlation of the 43Kda band with an increase in both membrane and cytosol connexin with a decrease of temperature. Moreover, in warm acclimation, connexin is completely absent from the cytosol component. Not only does this tally with events at the Cx43 mRNA level, it gives strong evidence that cold acclimation significantly increases the amount of Cx43 protein available to the cardiomyocyte; indeed

it is produced in surplus probably to ensure the essential Cx43 system is not compromised in conditions of reduced ionic kinetics when temperature change is rapid.

When looking at the 30Kda band, there are similar patterns which also correlate with Cx30 mRNA levels. Here, an increase in temperature strongly promotes the amount of Cx30 in the membrane with a reduction in the amount in the cytosol.

Unlike the pattern observed at the Cx48.5 mRNA level, the 48Kda band appears to show no temperature dependency, however this protein has the largest cytosolic to membrane ratio of all three proteins, suggesting it is required for heart function and its role is non-temperature critical. It may also allude to the fact that Cx30.9 is the more important pacemaker related connexin in trout heart.

Quite how this cytosol to membrane trafficking is managed remains elusive however connexin is known to have a short half life implying it is highly regulated [254]. What *is* known is that Cx43 is transported via microtubules in a kinesin dependent manner [255], and association with actin can sequester connexin in the cytosol [256], thus preventing full forward travel to the membrane and providing a possible mechanism of pool formation.

As shown in mammalian models of hypertrophy, connexin is largely modulated by phosphorylation status where dephosphorylation is associated with gap junction degradation and connexin relocation [257] [258]. In this study, connexin was found to be phosphorylated in the natural state; dephosphorylation was possible using alkaline phosphatase. Strikingly, this dephosphorylation was resisted in warm acclimated spongy

layer with respect to cold, at the same experimental temperature, suggesting a temperature and phosphorylation dependent method of connexin remodelling control in trout heart.

#### **4.6.4 Conclusions**

Trout heart expresses the fast conducting connexin Cx43 as well as two pacemaker connexins Cx30.9 and Cx48.5, all of which have homologs in mammalian hearts. Cold acclimation causes an increase in Cx43 in-line with chamber mass increases, and warm acclimation causes an increase in Cx30.9, potentially to modulate and compensate for temperature dependent changes in heart rate. Connexin in trout is cytosolic as well as membranous and this cytosolic pool may aid quick conduction remodelling during rapid temperature change. Temperature dependent resistance to dephosphorylation is one potential method of regulating the membrane:cytosol ratio of connexin.

#### **4.6.5 Summary**

During temperature acclimation of fish heart where biophysical insults as well as ionic kinetics occur, there is structural as well as electrical conduction remodelling in order to achieve effective cardiac output. Electrical remodelling involves changes in gap junction levels, location and activation status. In the trout heart, electrical harmony is achieved over a wide range of temperatures by varying ratios and sub-cellular locations of the fast conducting Cx43 gap junction and slow conducting, pacemaker gap junctions Cx30.9 and Cx48.5.

#### **4.6.6 Study limitations**

##### **1. Antibody not trout specific and selective for any particular connexin**

To be much more accurate on connexin protein levels and distribution, specific trout antibodies for Cx43, Cx30.9 and Cx48.5 should be designed. Moreover specific phosphorylated and dephosphorylated antibodies should be designed.

##### **2. Western blot not done on atrium**

Connexin levels in the atrium were analysed purely by mRNA data which were not backed up with protein data. This would help in determining the likely function of connexins in the atrium and shed more light on the opposing levels of Cx43 in the atrium and ventricle

##### **3. Accurate connexin cytosolic location not determined**

To investigate to a greater degree the exact location of cytosolic connexin and the phenomenon of pooling, other markers should be used and overlaid; e.g. DAPI blue for nuclear localisation or  $\alpha$ -actinin for trafficking.

##### **4. No separate primer for Cx30.9 and Cx48.5**

Although qualitative studies revealed mRNA levels of both these connexins displayed the same temperature dependent levels of expression, to be much more accurate, separate primers should be used, especially in light of the misnomer between Cx48.5 mRNA and the corresponding 48Kda protein band levels.

##### **5. Cannot rule out other connexins present due to degenerates used**

Although the degenerate primers used in this study have been successfully used by other groups and are believed to pick up all connexins present, there is a possibility that several connexins went undetected, which could potentially lead to misinterpretation of results.

##### **6. Connexin distribution throughout the whole heart unknown**

Connexin levels and general localisation of the protein were studied to a fair level of detail in this study, however to really appreciate the impact of connexin

remodelling on the function of the heart, higher level studies must be carried out specifically looking for connexin density in different areas of the heart, which would add much value to conduction studies, especially for Cx30.9 and Cx48.5, which if like their respective mammalian homologs would reveal distinct patterns of distribution.

## 5 Molecular mechanisms of cardiac remodelling during rainbow trout temperature acclimation

### 5.1 Brief introduction

In the two previous chapters extensive remodelling to the muscle and connective tissue components of the rainbow trout heart during temperature acclimation, in tandem with significant remodelling of connexin gap junction proteins was observed. Combined, these remodelling events allow the heart to function effectively over a wide range of temperatures, structurally and electrically. One key question remains though; how does this happen? Indeed, what are the *triggers* and *mechanisms* that underpin cardiac remodelling in rainbow trout? What *type* of response is it? What we *do* know is that these responses have at their core; a change in temperature that is prolonged. Furthermore, these changes are large-scale structural and muscular alterations, which suggest a significant physical force is a driving factor behind the remodelling or mitigation; i.e. muscle by its nature is there to create a force, and that force is required for *some* reason. In mammalian studies, we know of two main methods (excluding pregnancy) that cause or trigger cardiac hypertrophy. One is chronic hypertension, and the other is chronic exercise [259]. Chronic hypertension causes prolonged stretch (pressure overload) of the left ventricular wall above the norm and the heart responds by increasing muscle mass in that chamber – to mitigate against stretch under the governing rules of Laplace’s law. Chronic exercise causes hypertrophy due to an increase in venous return to the heart (volume overload) which is partially facilitated by increased neuro-hormonal stimulation to the heart [67]; an increase in exercise rate demands greater blood/oxygen supply and the body increases cardiac output via adreno-stimulation of the heart. In the fish, we know that cardiac function is predominantly regulated in the short term at least via stretch, with neuro-hormal input being relatively small, yet required at all temperatures [140]. With this in mind, it is a

strong possibility that the degree of stretch of the ventricular wall in fish is also the triggering event that initiates the chain reaction to varying degrees of long term cardiac remodelling. In fish, stretch of the heart wall could vary due to blood viscosity, downstream vascular resistance levels and venous return [211, 260]; with activity levels influencing the latter two [161]. Stretch is therefore a good place to start investigating the mechanisms behind cardiac remodelling in fish. We cannot, however, ignore the neuro-hormonal influence. It is known that beta-adreno sensitivity increases with cold acclimation [146], implying that beta-adreno signalling is relied on to some degree to maintain heart function in temperature change. Furthermore, we cannot disregard the possibility that the triggering event may purely be a response to temperature, *only*. If this is the case, this type of response would come under a *stress* or *heat shock* type of response.

Whether the trigger is stretch, neuro-hormonal input or stress, there has to be a mediator. Mediators link the initial trigger with the eventual outcome, and in biological terms, mediators tend to be cell signalling cascades of which there are hundreds, if not thousands of these pathways in most living cells. Indeed, many of the signalling events in mammalian cardiac hypertrophy have been well characterised from cell surface level to gene regulation. In rainbow trout, cell signalling (other than  $\text{Ca}^{2+}$ ) in the heart is largely untouched, and therefore a good place to start would be to base investigations on known, key, cell signalling players in mammalian studies in a comparative sense.

## **5.2 General Methodology**

Full details of the methodologies are in Chapter 2, however below is a concise summary of the methods used to generate the results in this chapter.

To address all the issues above, a generalised approach was initially required to begin to understand if the *trigger* for cardiac remodelling in acclimated rainbow trout is *stretch*, *stress/heat shock* or *neuro-hormonal*, or, a mixture of all three. These were examined using various mRNA markers and quantified using qPCR. Following investigations into potential *triggers*, key mammalian *mediators* of cardiac remodelling were looked into using western blotting for several cell signalling proteins which included qPCR of one gene known to be a direct regulator of mammalian NFAT signalling.

## **5.2.1 Finding the trigger**

### **5.2.1.1 Stretch**

Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP) are well characterised markers of cardiac hypertrophy in mammals, as mRNA levels of both increase in response to stretch of cardiac muscle, prior to release of the peptide itself [261, 262]. Therefore, qPCR of both genes was carried out on rainbow trout hearts from three acclimation temperatures.

### **5.2.1.2 Stress**

To investigate stress as a potential trigger, markers of stress: Mineral Corticoid Receptor (MR), Glucocorticoid Receptors 1 and 2 (GR1 and GR2) as well as heat shock gene (DNAJ2) mRNA levels were examined using qPCR. MR, GR1 and GR2 levels increase in response to increased stress hormones, such as cortisol, in the blood [199]. DNAJ2, a heat shock protein family member, is up-regulated in response to cellular stress [263].

### **5.2.1.3 Neuro-hormonal input**

To investigate neuro-hormonal influences during temperature acclimation, beta-adrenoceptor ( $\beta$ AR types 2, 3a and 3b) levels in the hearts of acclimated rainbow trout were assessed using qPCR.



## 5.2.2 Investigating the mediators

Mediators in many cells come in the form of cell signalling proteins. Cell signalling proteins transduce their signal, in many cases by phosphorylation. It was therefore important to look at activated cell signalling *protein* levels (and not mRNA) to observe a potential activated signalling cascade. Western blotting was therefore chosen to detect protein levels of activated cell signalling proteins. Choice of cell signalling molecules to examine was based on several factors; the primary being relevance to cardiac remodelling as a whole (as studied in mammals), and the secondary being confirmation of antibody reactivity in fish:

**Integrin linked kinase (ILK)** – ILK is indirectly linked to ECM protein integrin as well as stretch activated focal adhesion kinase (FAK). Upregulation of ILK has been shown to promote hypertrophy in mammalian models. Furthermore, an indirect downstream target of ILK is PKB/ AKT as discussed below [264, 265]. This gene was used as a potential mediator of ECM induced signalling in trout heart via stretch sensing.

**P – Akt** – also known as PKB is a well-studied cell signalling protein involved heavily in promoting cell growth and proliferation and shown to be important for pro physiological hypertrophic signalling and connexin remodelling [266-268].

**P - GSK - 3 $\beta$**  – Downstream target of Akt. GSK3- $\beta$  is largely believed to facilitate the physiological hypertrophic response. NFAT transcription factor is an important downstream target of this protein which is a key hypertrophic regulator in mammalian models [269-272].

**Smad 3 and p-smad 3** – Smad 3 and its activated form p-Smad 3 are important downstream regulators of TGF- $\beta$  signalling traditionally involved in pro apoptotic or growth inhibitory pathways in the heart so this was chosen as a potential negative regulator of cardiac hypertrophy [273].

**MAP kinases – p-P38 MAP kinase, ERK ½ and p – ERK ½.** Probably the most well studied cell signalling molecules involved in stress response and cellular growth. All these proteins have been shown to be involved in cardiac hypertrophy and due to their ability to phosphorylate target proteins, are also good candidates for connexin phosphorylation/ regulation [274] [275-277].

**RCAN** – Also known as MCIP, a direct regulator of the calcineurin, NFAT cascade important in pathological hypertrophic remodelling [278-281]. A successful antibody was not found for this protein, however mRNA levels were assessed using qPCR.

### 5.2.3 Observing a direct link between a trigger and cardiac remodelling

Application of synthetic *adrenaline analogues* such as isoprenaline to mammalian hearts results in increased expression of connexin 43 [282-285]. Isoprenaline and salbutamol, both beta receptor agonists, were applied (at the same experimental temperature) directly to strips of rainbow trout ventricle tissue from three acclimation temperatures for 15 minutes, and Cx43 mRNA levels were assessed by PCR and qPCR.

## 5.3 Key aims

- Investigate key potential triggers for cardiac remodelling in rainbow trout
- Investigate potential mediators in cardiac remodelling in rainbow trout
- Observe a direct link between a trigger and cardiac remodelling in acclimated fish

## 5.4 Summary of aims, methods and expected outcomes

**Table 5.1 – Summary of aims, methods and expected outcomes (chapter five)**

<b>Aim</b>	<b>Method</b>	<b>Expected outcome</b>
<b>Investigate stretch in acclimated hearts</b>		Degree of stretch determined in ventricle
		Degree of stretch determined in atrium
<b>Investigate stress in acclimated hearts</b>	qPCR to find mRNA levels of MR, GR1, GR2 and DNAJ2	Degree of stress determined in ventricle

		Degree of stress determined in atrium
<b>Investigate neuro-hormonal input to acclimated hearts</b>	qPCR to find mRNA levels beta-AR 2, 3a and 3b.	Degree of neuro-hormonal response determined in ventricle
		Degree of neuro-hormonal response determined in atrium
<b>Investigate potential mediators in acclimated hearts</b>	Western blotting against cell signalling proteins p-p38 MAPK, ERK ½ and p-ERK ½, p-PKB/Akt, ILK, Smad-3 and p-Smad-3, p-GSK3β. qPCR of RCAN.	Degree of cell signalling activation levels determined in ventricle
<b>Observe a direct link between potential trigger and cardiac remodelling</b>	Application of adrenaline-like drugs to ventricular tissue and assess Cx43 mRNA levels using qPCR	Determined a link between physiological response and cardiac remodelling

## 5.5 Results

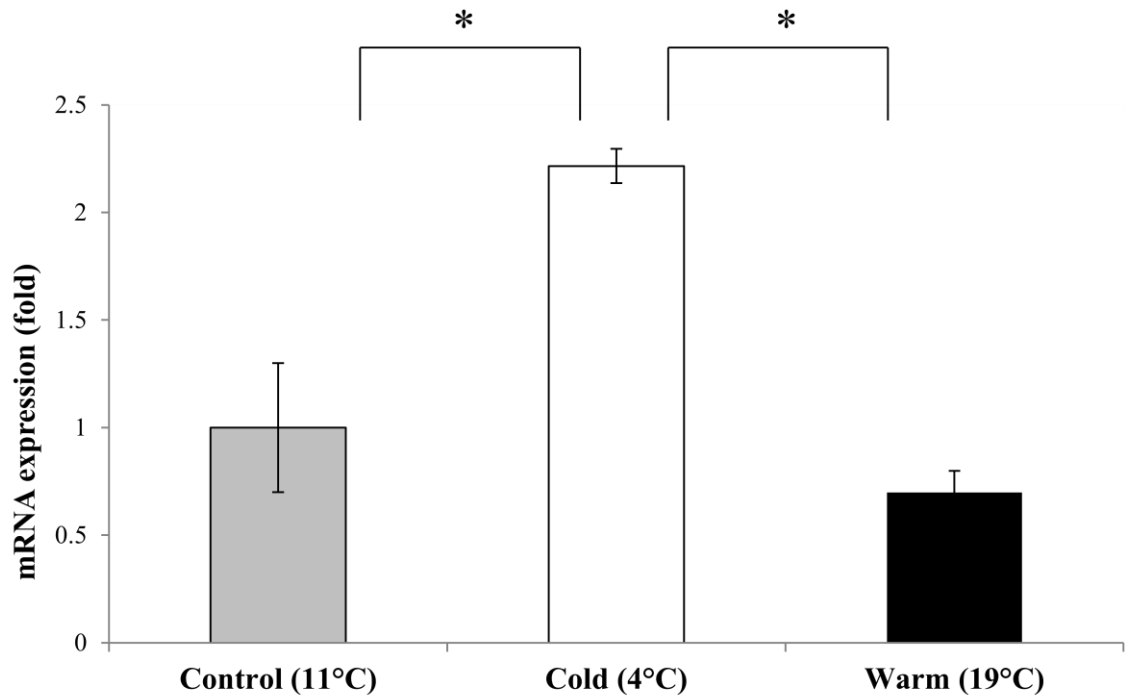
*Please note some of the results referred to below are taken from supporting material, also generated in this study, which are documented in the Appendix, Chapter Eight.*

### 5.5.1 Stretch during acclimation

Markers of stretch; ANP and BNP were assessed using qPCR in the atrium and ventricle.

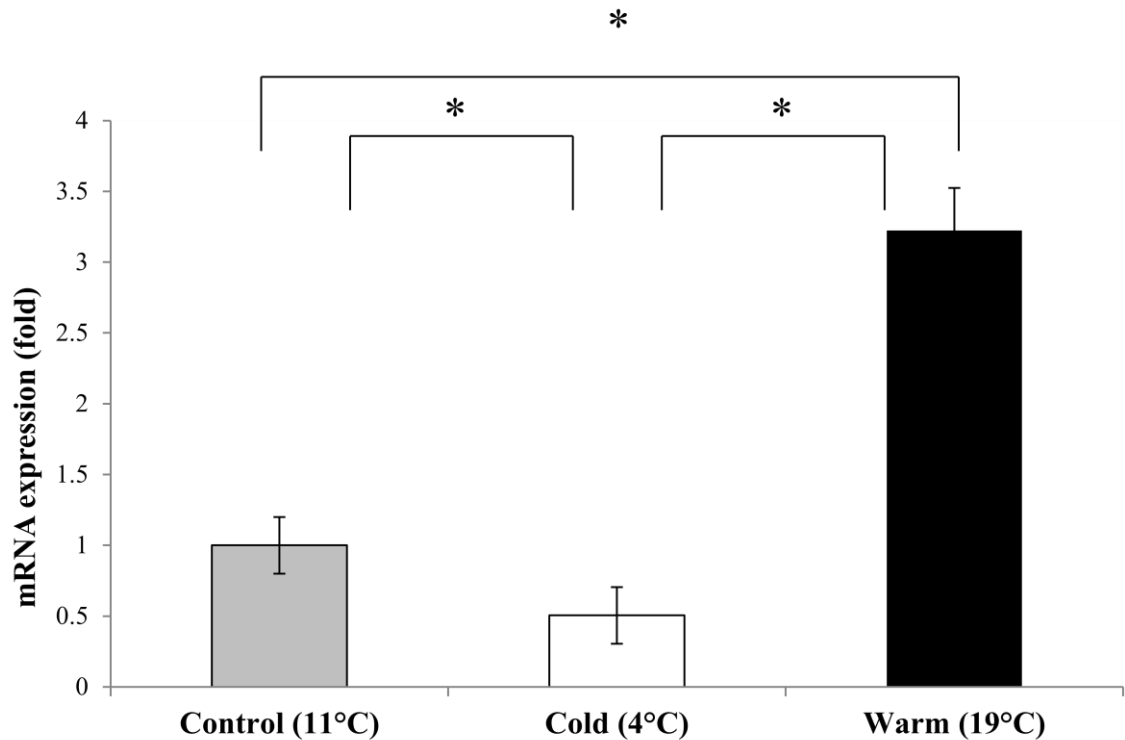
ANP expression was 1.5 fold higher in the ventricle of cold acclimated fish compared to warm (Figure 5.1) whereas ANP expression was 2.7 fold higher in warm atrium as opposed to cold atrium (Figure 5.2). BNP expression was 1.4 fold higher in the ventricle of cold acclimated fish compared to the warm group (Figure 5.3) however there was no difference in the levels of BNP between the atrium of either cold or warm fish (Figure 5.4).

## ANP VENTRICLE



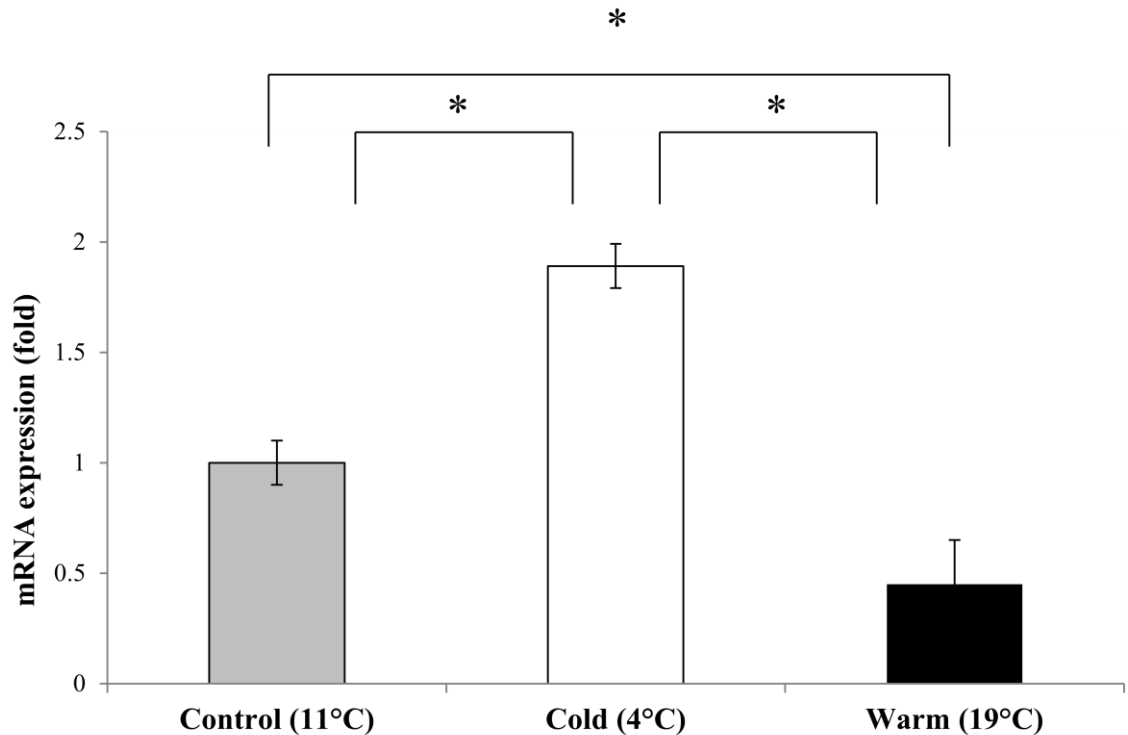
**Figure 5.1 - Expression of ventricular ANP mRNA during cold and warm acclimation of rainbow trout heart.** ANP in the cold ventricle was expressed 2.22 fold  $\pm$  0.08 with respect to control. There was no difference between warm and control expression levels of ANP in the ventricle. Expression levels of ANP in the cold ventricle were 1.52 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## ANP ATRIA



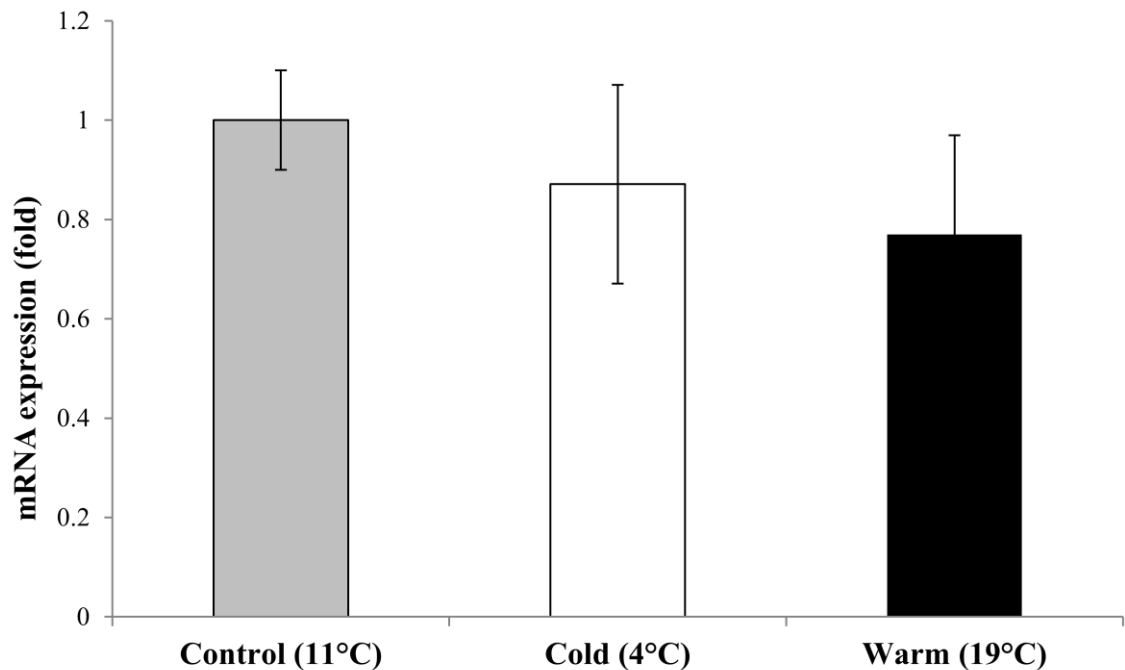
**Figure 5.2 - Expression of atrial ANP mRNA during cold and warm acclimation of rainbow trout heart.** ANP in the cold atria was expressed 0.5 fold  $\pm$  0.2 with respect to control. ANP in the warm atria was expressed 3.22 fold  $\pm$  0.3 with respect to control. Expression levels of ANP in the warm group were 2.72 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## BNP VENTRICLE



**Figure 5.3 - Expression of ventricular BNP mRNA during cold and warm acclimation of rainbow trout heart.** BNP in the cold ventricle was expressed  $1.89 \text{ fold} \pm 0.1$  with respect to control. BNP in the warm ventricle was expressed  $0.45 \text{ fold} \pm 0.2$  with respect to control. Expression levels of BNP in the cold ventricle were 1.44 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## BNP ATRIA

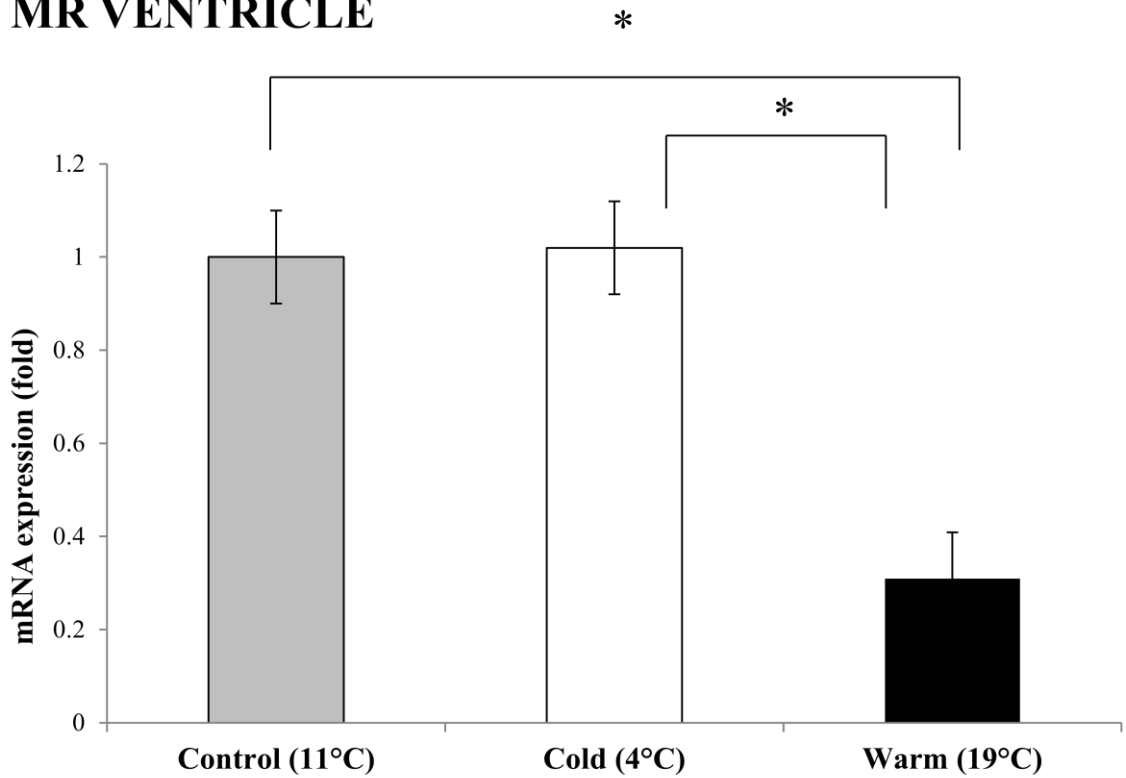


**Figure 5.4 - Expression of atrial BNP mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of BNP in the atria. There was no difference between warm and control expression levels of BNP in the atria. There was no difference between warm and control expression levels of BNP in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

### 5.5.2 Stress during acclimation

Stress receptor (MR, GR1 and GR2) levels and heat shock protein DNAJ2 were assessed using qPCR. MR was expressed 0.71 fold higher in cold compared to warm acclimated ventricles (Figure 5.5) however there was no difference in MR levels within atrium of fish from either acclimation temperature (Figure 5.6). GR1 levels were 2.6 fold higher in warm ventricles compared with cold (Figure 5.7) and the same receptor was 1.7 fold more highly expressed in the atrium of warm acclimated trout compared with cold (Figure 5.8). GR2 levels were 1 fold higher in warm acclimated ventricles compared with cold (Figure 5.9) and 0.5 fold higher in warm atria with respect to cold (Figure 5.10). DNAJ2 expression increased in both warm and cold atrium and ventricle 2.4, 2.4, 2.8 and 3.3 fold respectively (Figures 5.11 and 5.12).

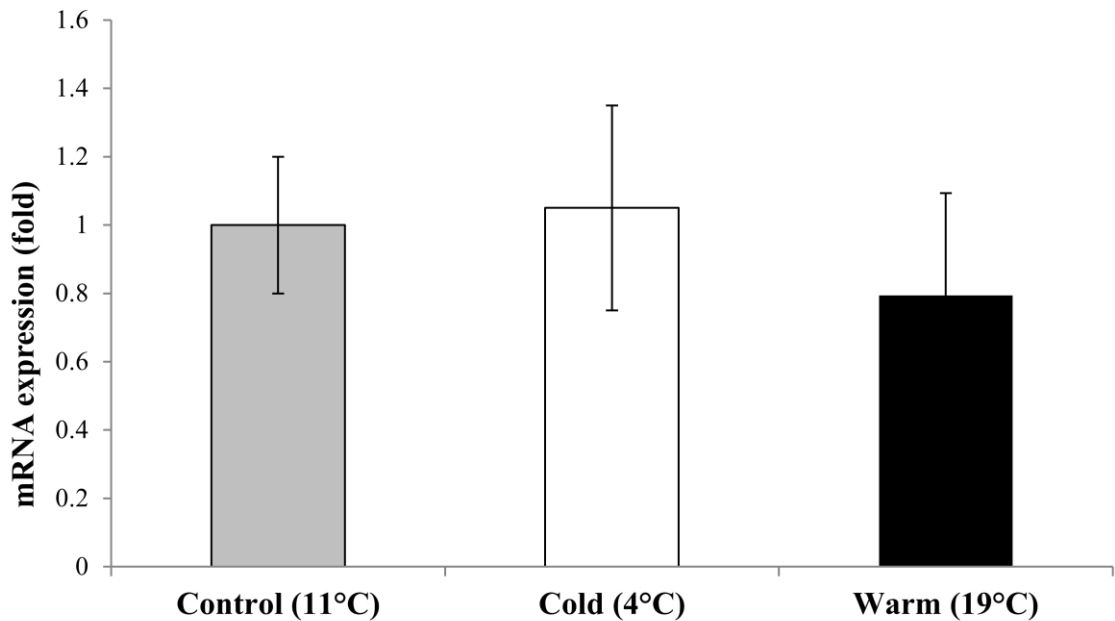
## MR VENTRICLE



**Figure 5.5 - Expression of ventricular MR mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of MR in the ventricle. MR in the warm ventricle was expressed 0.31 fold  $\pm$  0.1 with respect to control. Expression levels of MR in the cold ventricle were 0.71 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

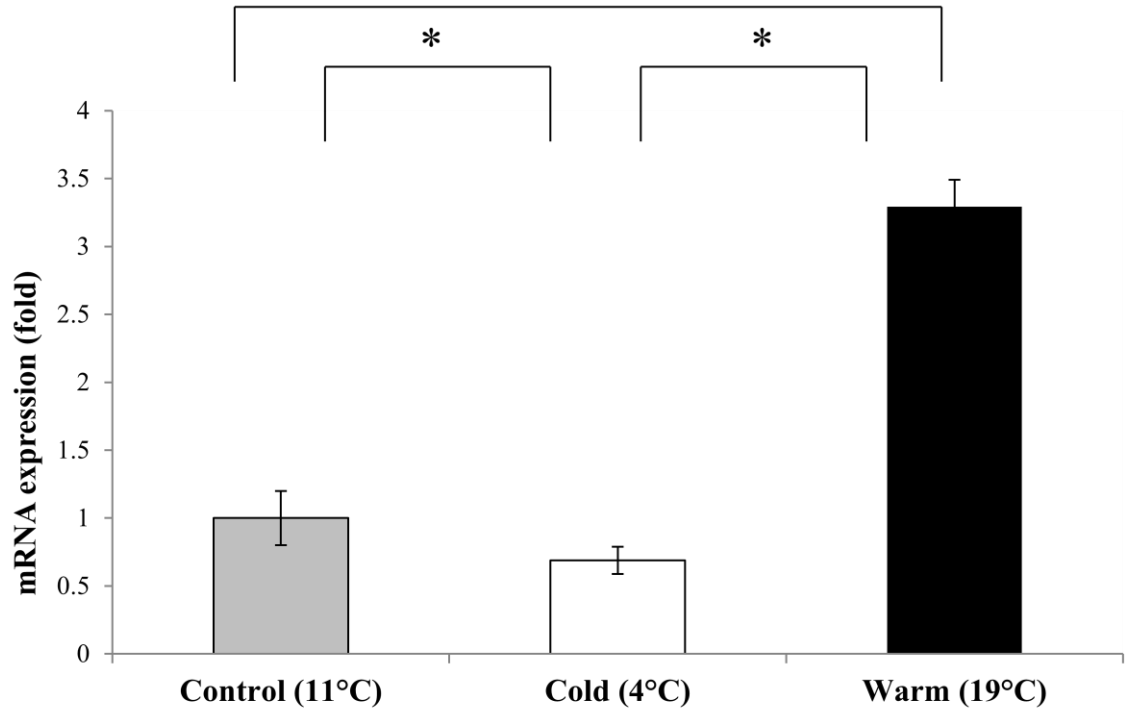


## MR ATRIA



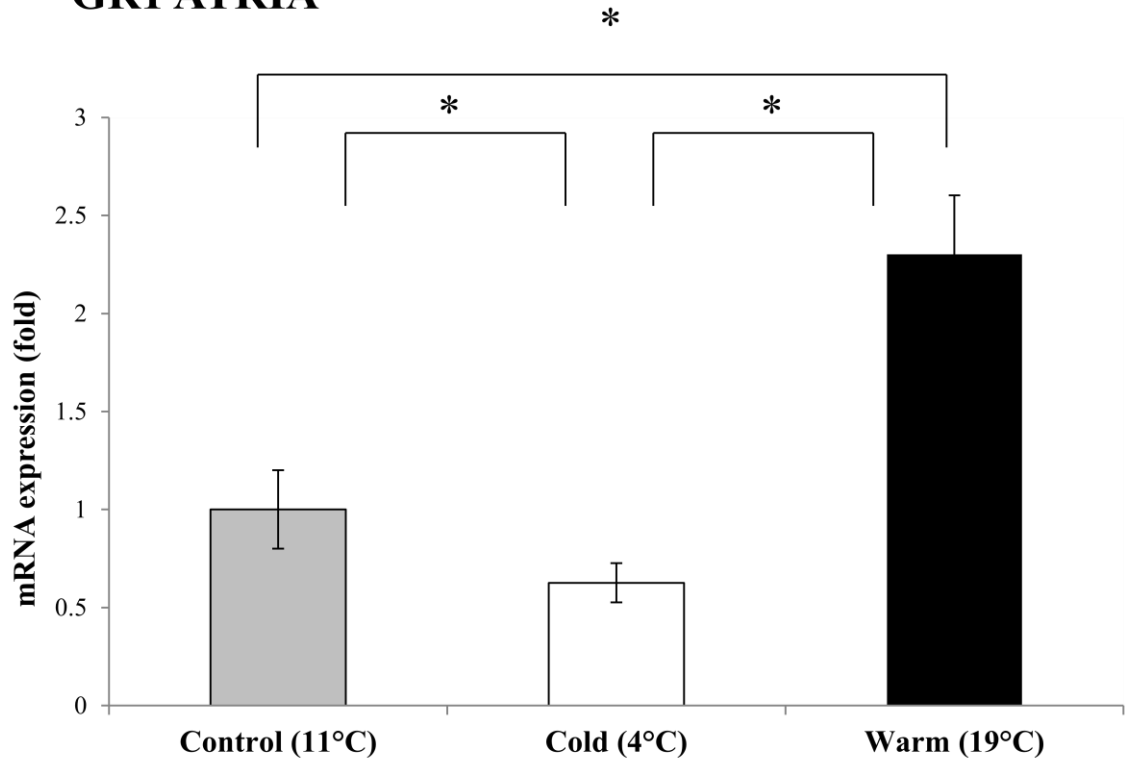
**Figure 5.6 - Expression of atrial MR mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of MR in the atria. There was no difference between warm and control expression levels of MR in the atria. There was no difference between warm and control expression levels of MR in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## GR1 VENTRICLE



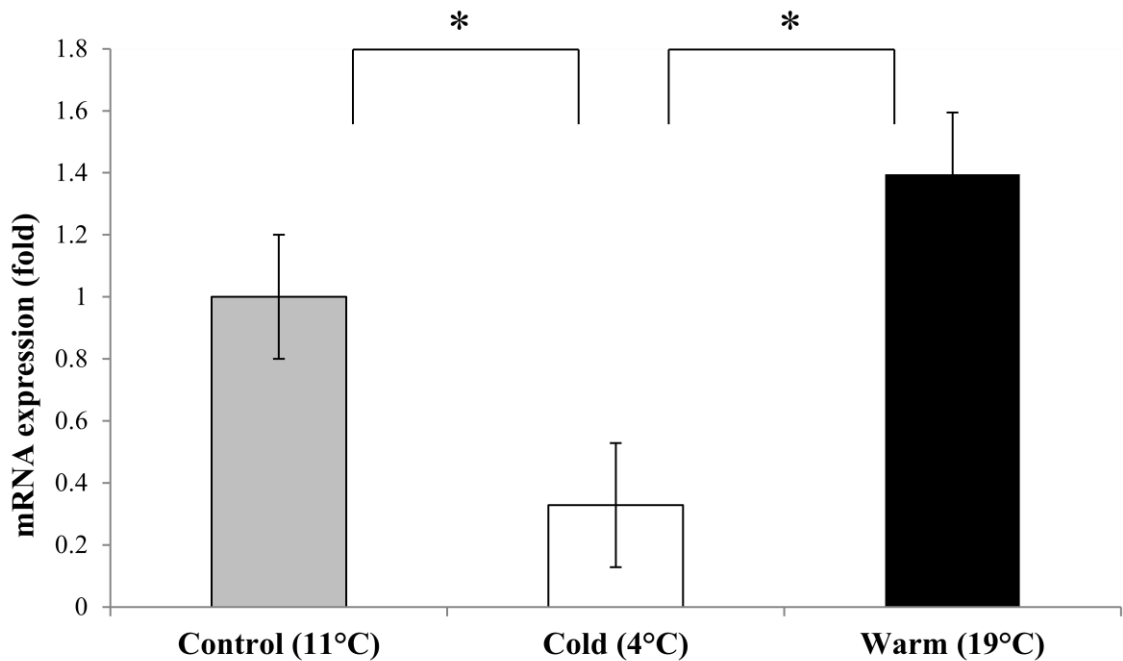
**Figure 5.7 - Expression of ventricular GR1 mRNA during cold and warm acclimation of rainbow trout heart.** GR1 in the cold ventricle was expressed  $0.69 \text{ fold} \pm 0.1$  with respect to control. GR1 in the warm ventricle was expressed  $3.29 \text{ fold} \pm 0.2$  with respect to control. Expression levels of GR1 in the warm group were 2.6 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## GR1 ATRIA



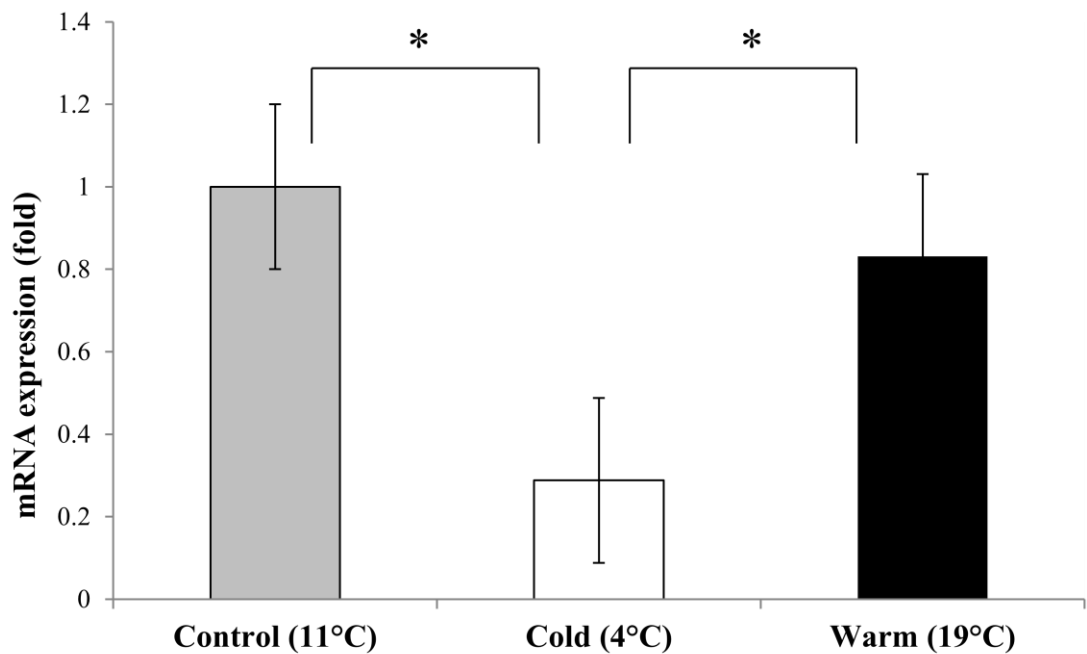
**Figure 5.8 - Expression of atrial GR1 mRNA during cold and warm acclimation of rainbow trout heart.** GR1 in the cold atria was expressed 0.63 fold  $\pm$  0.1 with respect to control. GR1 in the warm atria was expressed 2.3 fold  $\pm$  0.3 with respect to control. Expression levels of GR1 in the warm group were 1.67 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## GR2 VENTRICLE



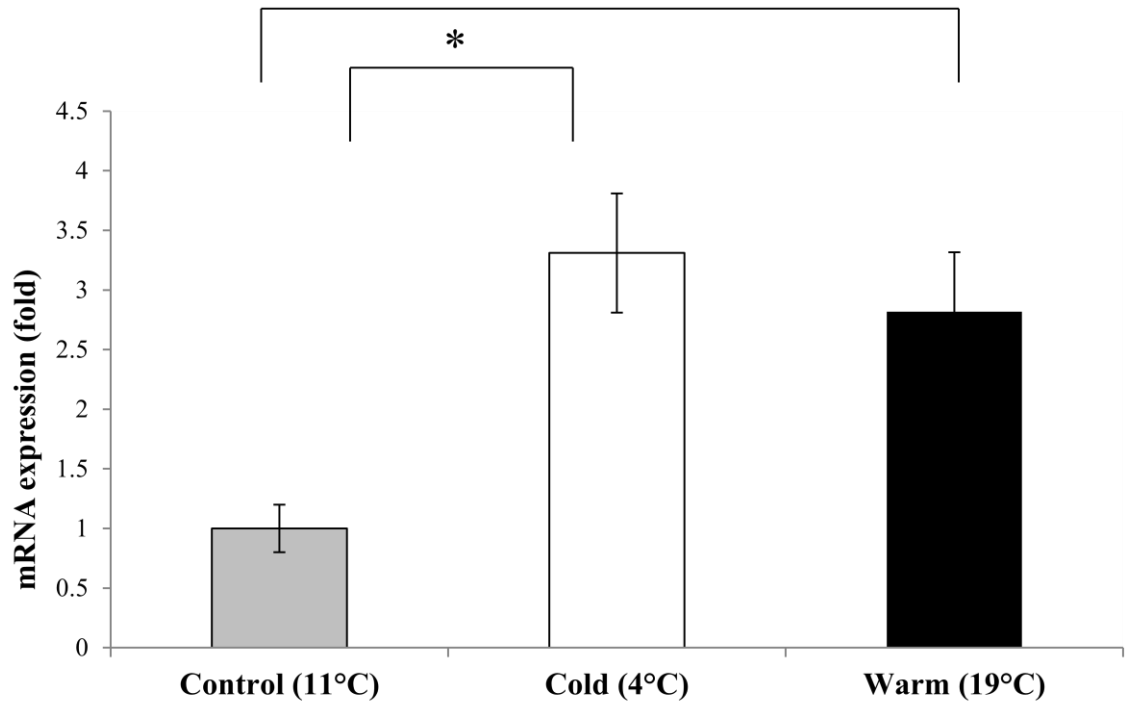
**Figure 5.9 - Expression of ventricular GR2 mRNA during cold and warm acclimation of rainbow trout heart.** GR2 in the cold ventricle was expressed 0.33 fold  $\pm$  0.2 with respect to control. There was no difference between warm and control expression levels of GR2 in the ventricle. Expression levels of GR2 in the warm group were 1.06 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## GR2 ATRIA



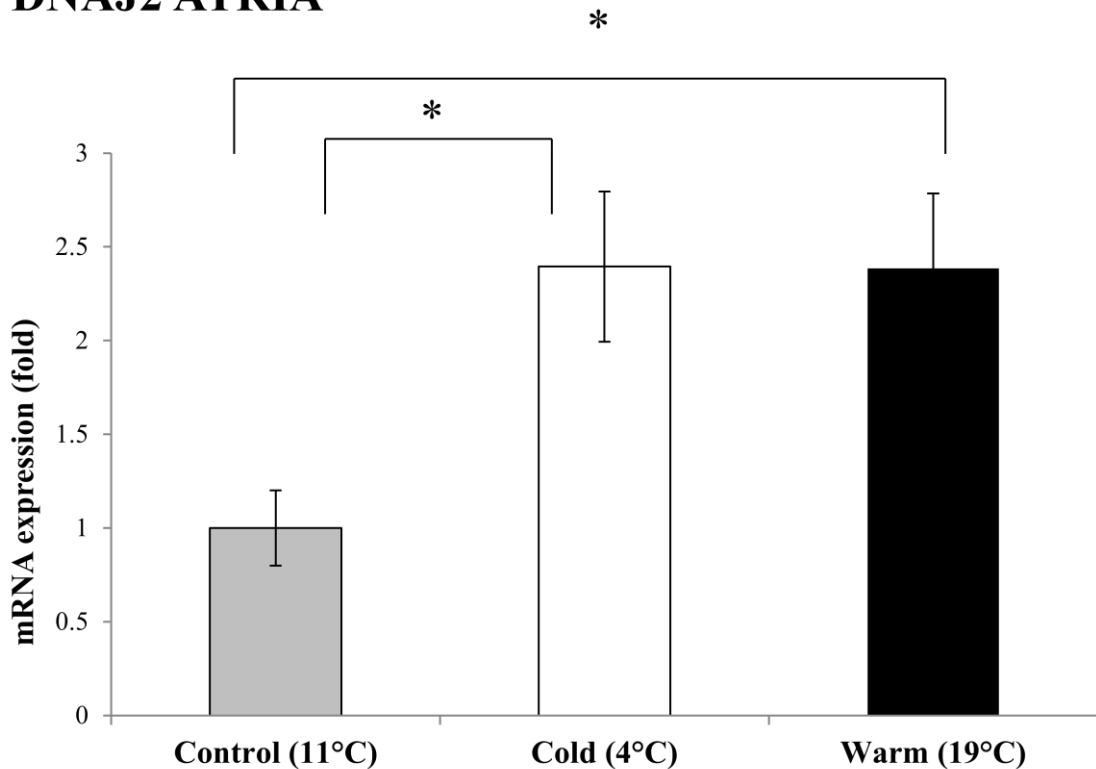
**Figure 5.10 - Expression of atrial GR2 mRNA during cold and warm acclimation of rainbow trout heart.** GR2 in the cold atria was expressed 0.29 fold  $\pm$  0.09 with respect to control. GR2 in the warm atria was expressed 0.83 fold  $\pm$  0.1 with respect to control. Expression levels of GR2 in the warm group were 0.54 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## DNAJ2 VENTRICLE



**Figure 5.11 - Expression of ventricular DNAJ2 mRNA during cold and warm acclimation of rainbow trout heart.** DNAJ2 in the cold ventricle was expressed 3.31 fold  $\pm$  0.5 with respect to control. DNAJ2 in the warm ventricle was expressed 2.82 fold  $\pm$  0.5 with respect to control. There was no difference between warm and control expression levels of DNAJ2 in the ventricle. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## DNAJ2 ATRIA

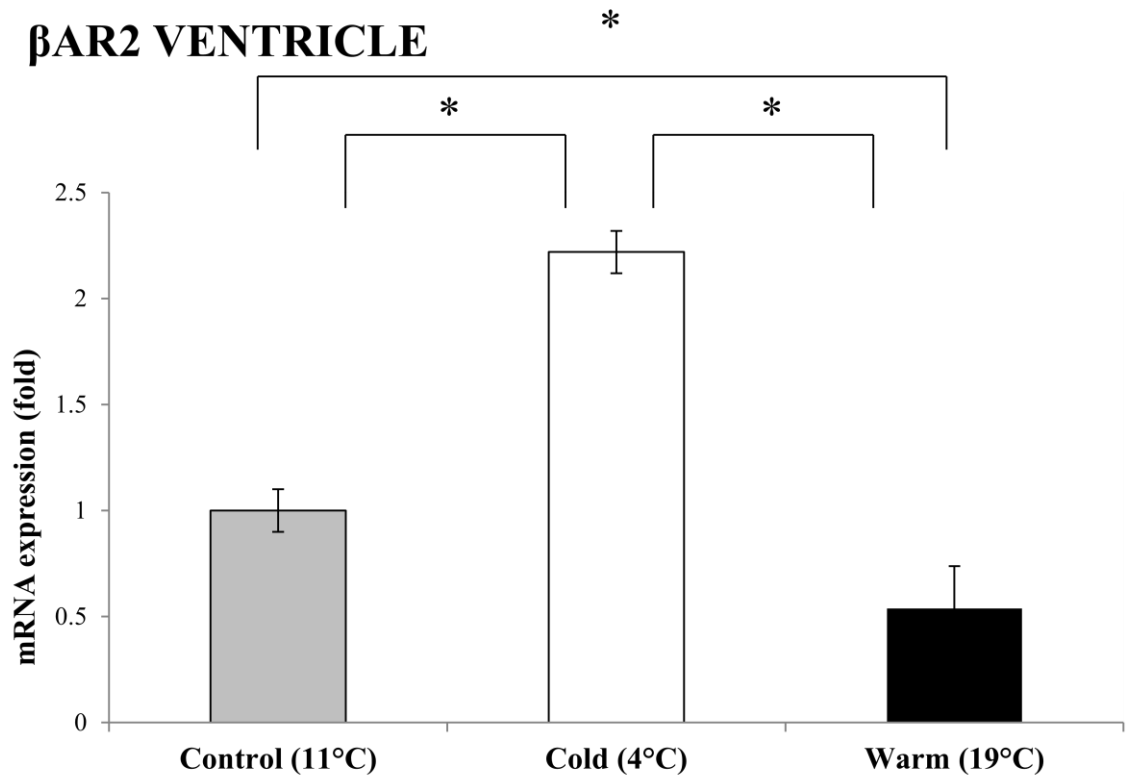


**Figure 5.12 - Expression of atrial DNAJ2 mRNA during cold and warm acclimation of rainbow trout heart.** DNAJ2 in the cold atria was expressed 2.39 fold  $\pm$  0.4 with respect to control. DNAJ2 in the warm atria was expressed 2.39 fold  $\pm$  0.4 with respect to control. There was no difference between warm and control expression levels of DNAJ2 in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

### 5.5.3 Beta-adrenoceptor levels during acclimation

Beta adrenoceptor levels were investigated as a potential marker of the degree of neuro-hormonal input into the hearts of acclimated fish. Receptors Beta2, Beta 3a and Beta 3b were examined. Beta2 receptor was expressed 1.7 fold more in cold acclimated ventricles compared with warm (Figure 5.13) however in the atrium, Beta2 was expressed 0.5 fold higher in the warm acclimated cohort with respect to cold (Figure 5.14). Beta3a was expressed at 0.7 fold higher levels in cold acclimated ventricles compared with warm (Figure 5.15) and the same receptor was 0.4 fold more expressed in warm atrium in comparison to cold (Figure 5.16). There were no differences in the expression levels of Beta3b in the ventricle and atrium at any acclimation temperature (Figures 5.17 and 5.18).

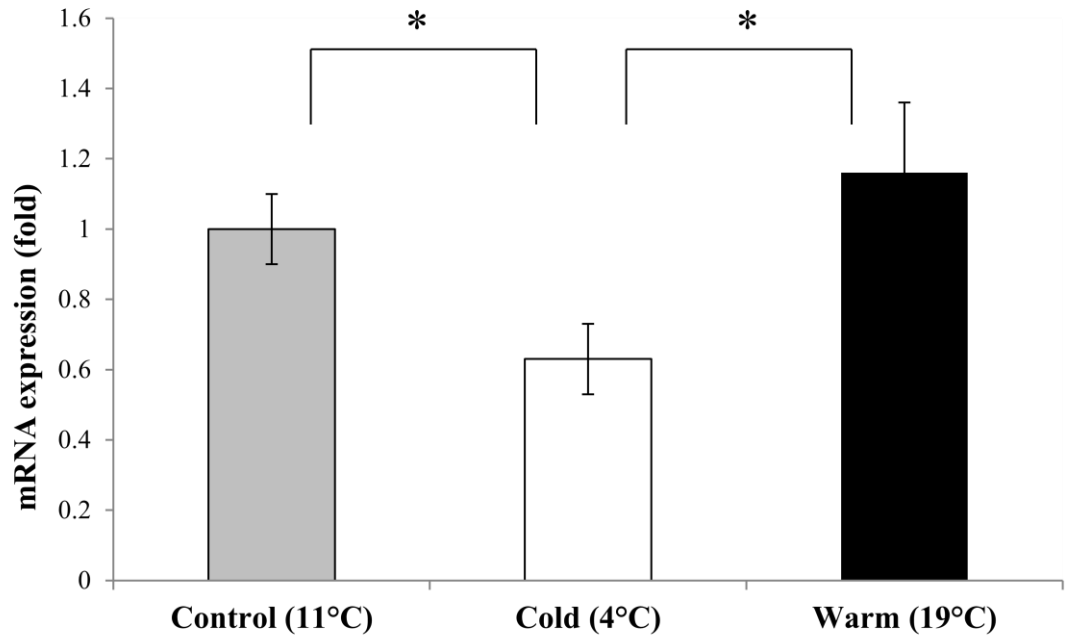
## βAR2 VENTRICLE



**Figure 5.13 - Expression of ventricular Beta 2 Adrenoceptor mRNA during cold and warm acclimation of rainbow trout heart.** BAR2 in the cold ventricle was expressed 2.22 fold  $\pm$  0.1 with respect to control. BAR2 in the warm ventricle was expressed 0.54 fold  $\pm$  0.2 with respect to control. Expression levels of BAR2 in the cold ventricle were 1.68 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

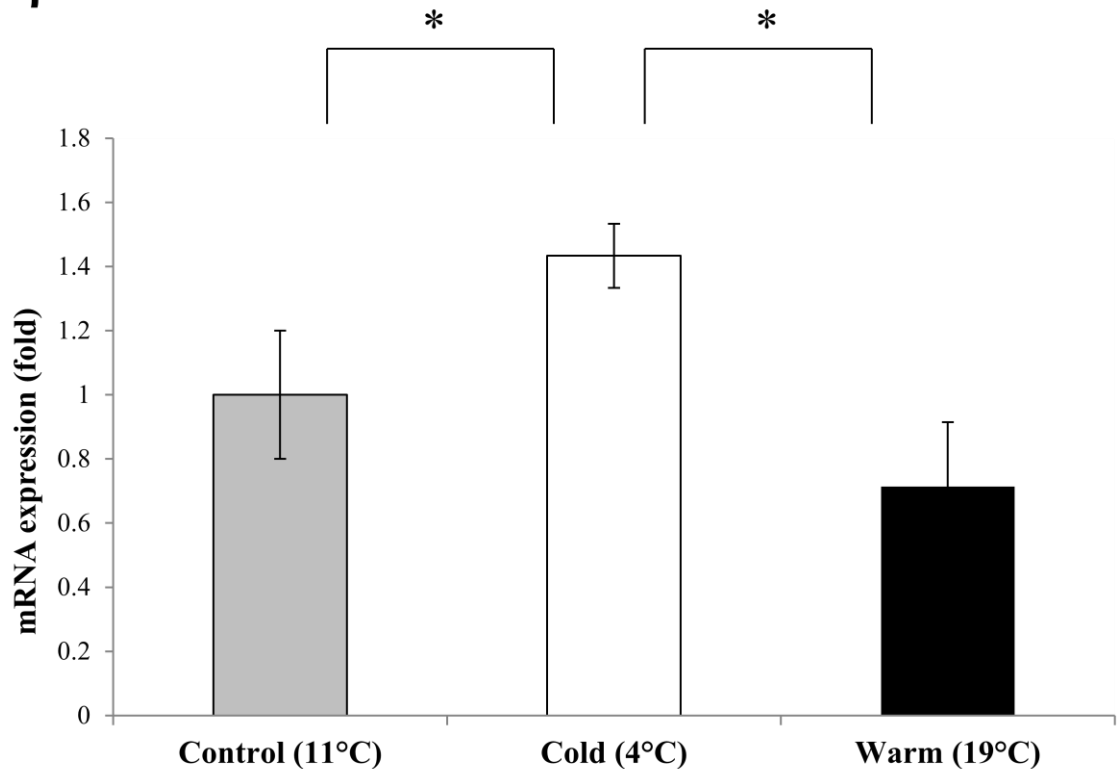


## βAR2 ATRIA



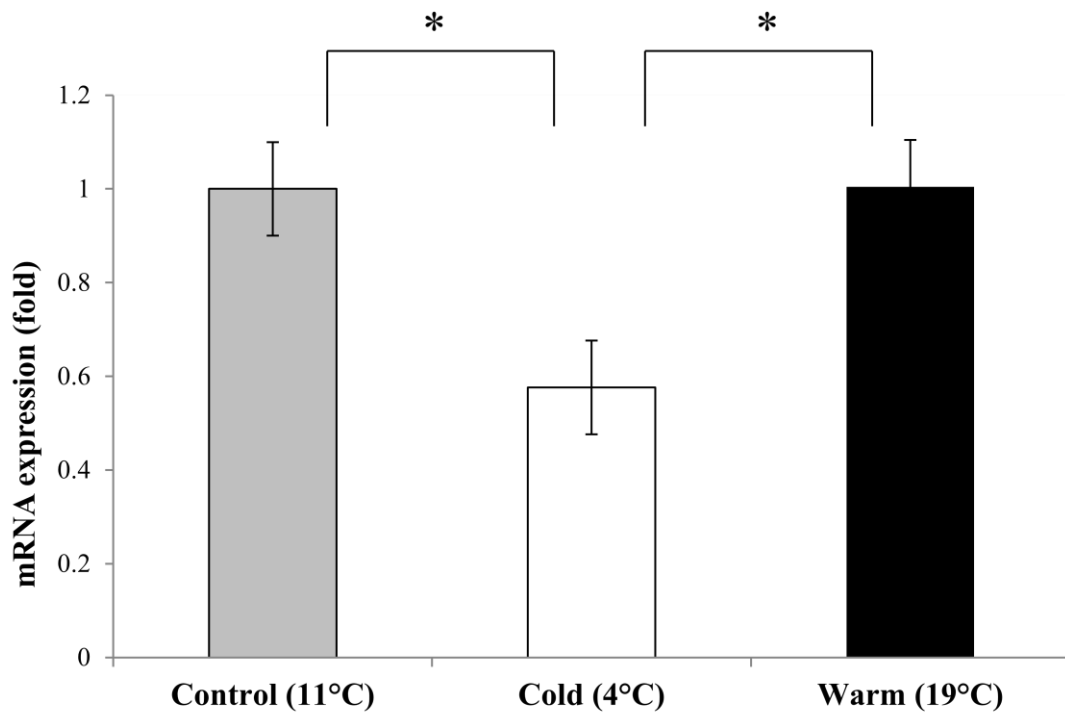
**Figure 5.14 - Expression of atrial Beta 2 Adrenoceptor mRNA during cold and warm acclimation of rainbow trout heart.** BAR2 in the cold atria was expressed 0.63 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of BAR2 in the atria. Expression levels of BAR2 in the warm group were 0.53 fold higher than cold with respect to control. qPCR readings were taken from a sample size of n = 7 fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## $\beta$ AR3a VENTRICLE



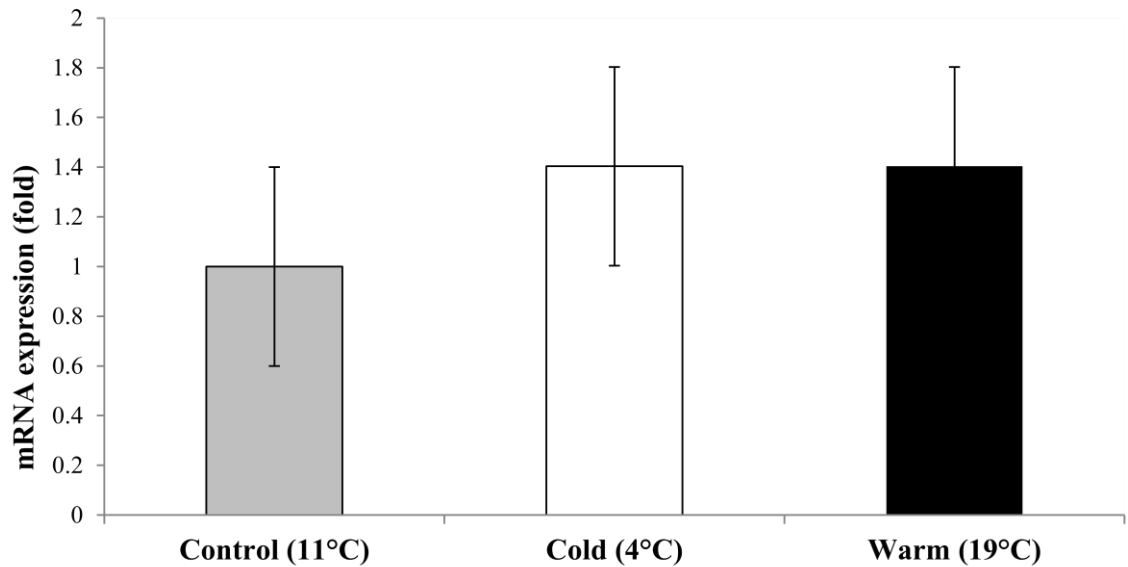
**Figure 5.15 - Expression of ventricular Beta 3a Adrenoceptor mRNA during cold and warm acclimation of rainbow trout heart.** BAR3a in the cold ventricle was expressed 1.43 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of BAR3a in the ventricle. Expression levels of BAR3a in the cold ventricle were 0.72 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## $\beta$ AR3a ATRIA



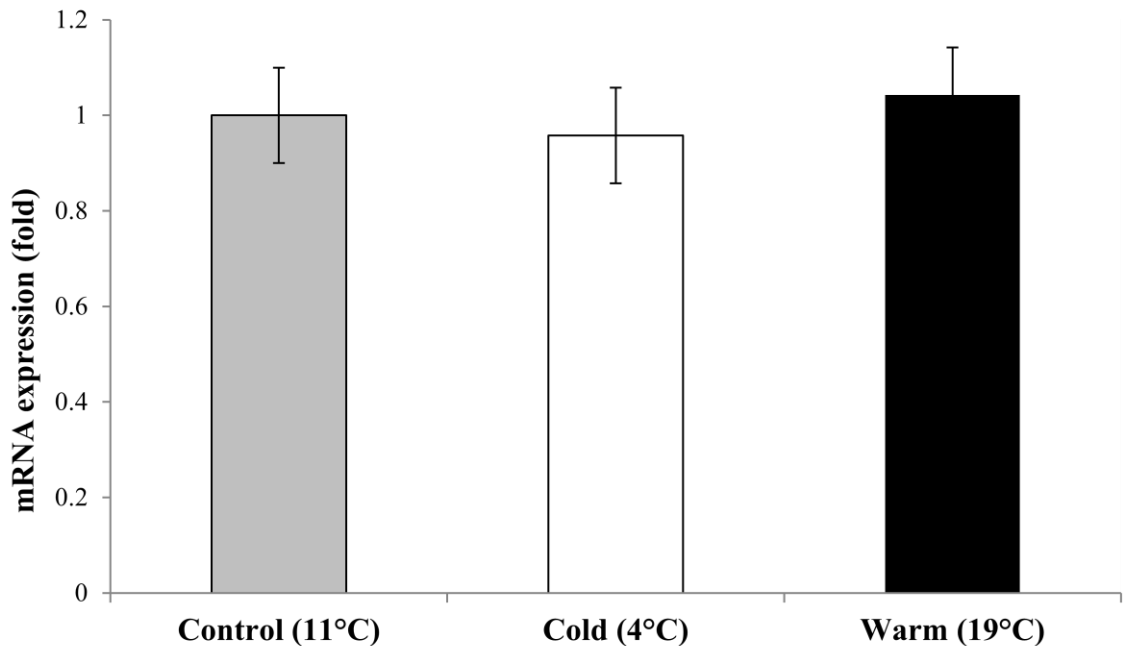
**Figure 5.16 - Expression of atrial Beta 3a Adrenoceptor mRNA during cold and warm acclimation of rainbow trout heart.** BAR3a in the cold atria was expressed 0.58 fold  $\pm$  0.1 with respect to control. There was no difference between warm and control expression levels of BAR3a in the atria. Expression levels of BAR3a in the warm group were 0.42 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## $\beta$ AR3b VENTRICLE



**Figure 5.17 - Expression of ventricular Beta 3b Adrenoceptor mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of BAR3b in the ventricle. There was no difference between warm and control expression levels of BAR3b in the ventricle. There was no difference between warm and control expression levels of BAR3b in the ventricle. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

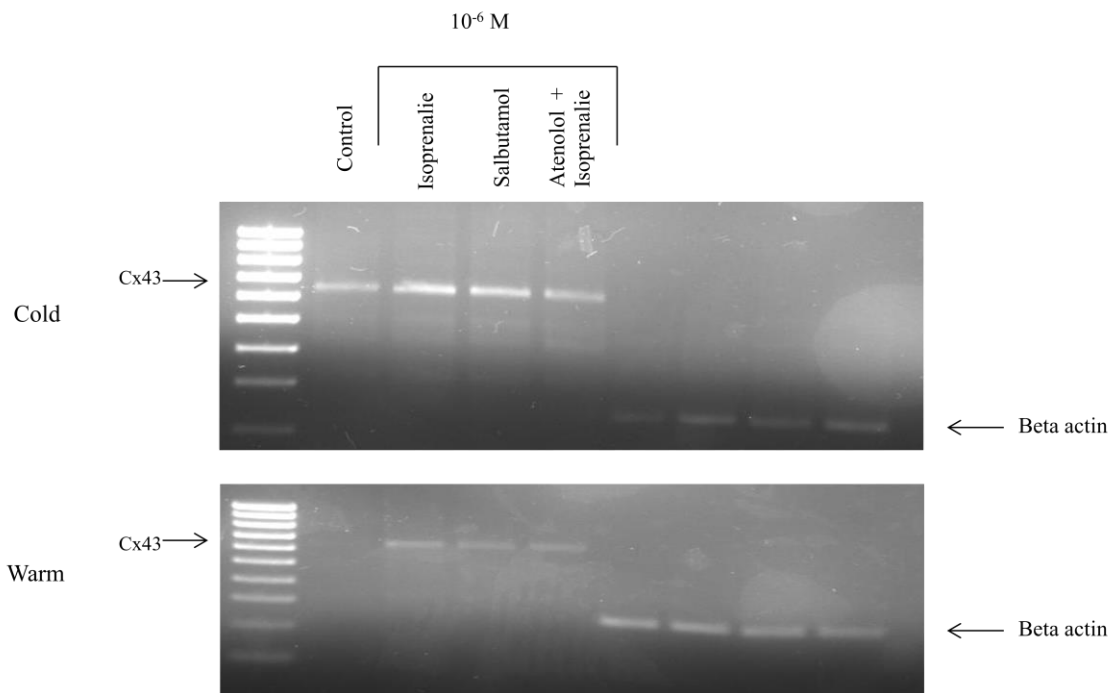
## $\beta$ AR3b ATRIA



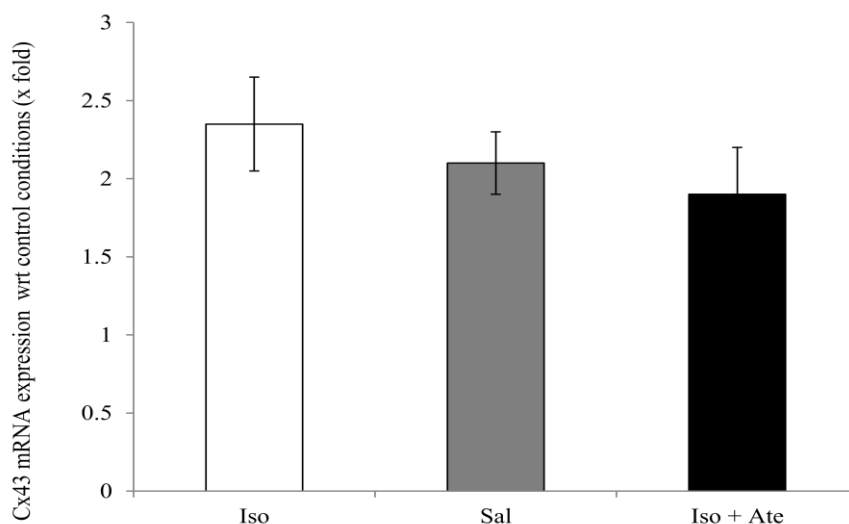
**Figure 5.18 - Expression of atrial Beta 3b Adrenoceptor mRNA during cold and warm acclimation of rainbow trout heart.** There was no difference between cold and control expression levels of BAR3b in the atria. There was no difference between warm and control expression levels of BAR3b in the atria. There was no difference between warm and control expression levels of BAR3b in the atria. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ . Effects of adreno-stimulation of trout heart on Cx43 expression

### 5.5.4 Effects of adreno-stimulation of trout heart on Cx43 expression

Adreno stimulation of ventricular tissue was assessed to observe a direct link with a potential triggering receptor (beta receptor) and cardiac remodelling (Cx43 expression) independent of any other trigger. Application of isoprenaline alone, salbutamol alone and a mixture of isoprenaline and atenolol to ventricular strips resulted in a qualified increase of Cx43 mRNA in both cold and warm acclimated fish, however the cold increase was noticeably greater (Figure 5.19). Using qPCR to quantify the response in control conditions, this increase of Cx43 expression in response to beta adreno stimulation was found to be 2 fold (Figure 5.20).



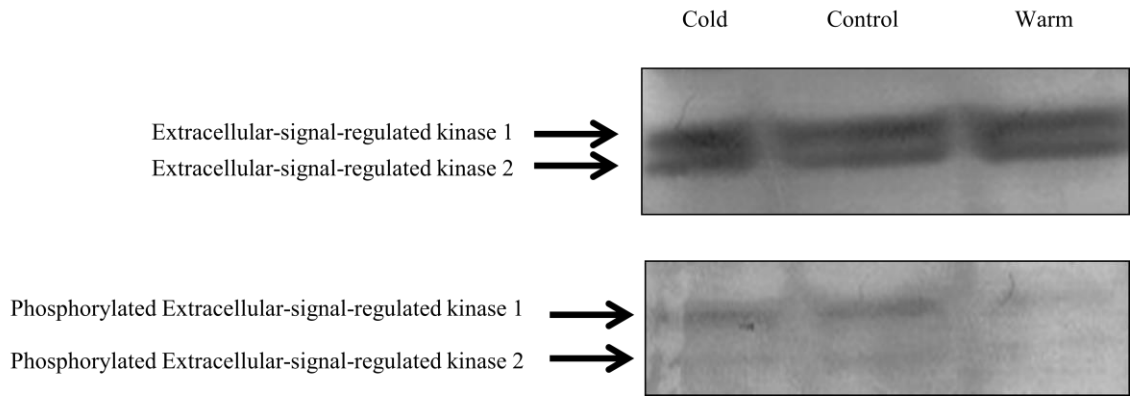
**Figure 5.19 – Qualitative effects of maximal response adreno stimulation on Cx43 expression.** Isoprenaline, the non-selective beta receptor agonist, increased the expression of Cx43 mRNA in both cold and warm ventricular strips incubated for 15 minutes at 12°C. Salbutamol, the beta 2 receptor agonist also increased expression of Cx43 in both groups however the cold group responded to a greater degree. The introduction of Atenolol, the beta 1 receptor antagonist to Isoprenaline treated heart strips did not abolish the expression of Cx43 mRNA. Beta actin loading controls are shown for each respective control and treatment group on the same gel. A 1µM concentration of each drug was added to induce a maximal response.



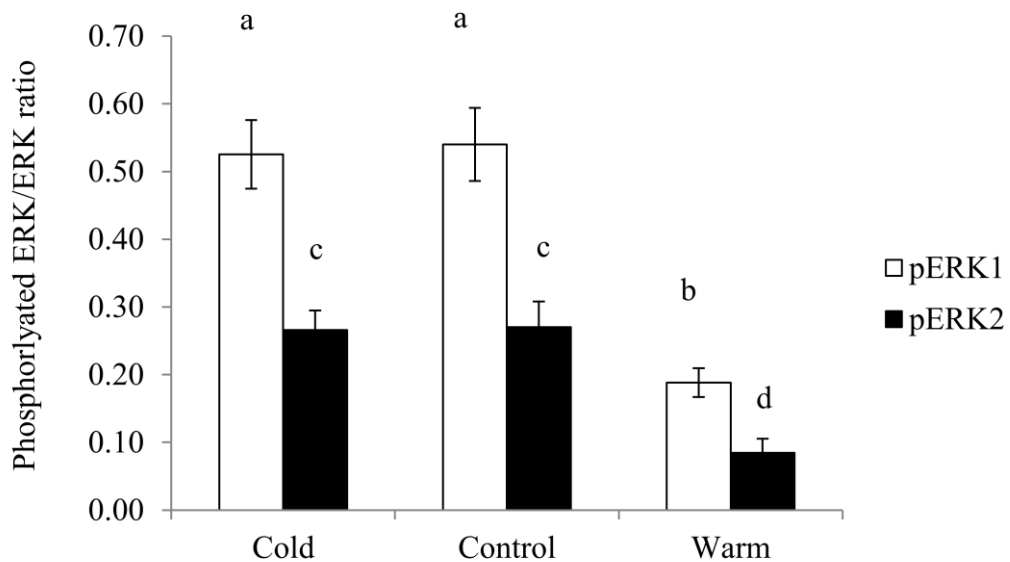
**Figure 5.20 - Quantitative effects of maximal adreno stimulation on Cx43 expression.** Isoprenaline and salbutamol, as well as a mixture of atenolol and isoprenaline increased the expression of Cx43 mRNA in control ventricular strips incubated for 15 minutes at 12°C by an average of 2 fold ±0.3. Data is presented on the graph as the mean expression levels (x fold) normalised to control (y = 0) conditions of n = 3 fish, with the qPCR repeated 3 times for every fish (9 samples). Error bars represent the standard error of the mean. A 1µM concentration of each drug was added to induce a maximal response.

### 5.5.5 Cell signalling mediator activity during acclimation

A range of cell signalling molecule activity was assessed in acclimated hearts as potential mediators of cardiac remodelling. Phosphorylated p38 MAP Kinase levels were 229% higher in cold acclimated ventricles compared with warm (*see Appendix Figures 8.6a and 8.6b*). There was no difference in ERK ½ levels however activated ERK ½ levels were 185% higher in cold ventricles compared to warm (Figures 5.21a and b). Phosphorylated PKB/ Akt levels were 138% higher in cold acclimated fish compared to warm (*see Appendix Figures 8.7a and 8.7b*). ILK levels were 17% higher in the cold cohort with respect to warm (*see Appendix Figures 8.8a and 8.8b*). Smad-3 levels were unchanged between the acclimation groups, however activated Smad-3 was completely missing in warm, unlike in cold (Figures 5.22a and b). p-GSK3B levels were 200% higher in cold acclimated ventricles compared with warm (*see Appendix Figures 8.9a and 8.9b*). RCAN mRNA levels were 3 fold higher in cold acclimated ventricles compared with warm (Figure 5.23) and RCAN levels are 0.9 fold higher in warm atrium compared with cold atrium (Figure 5.24).

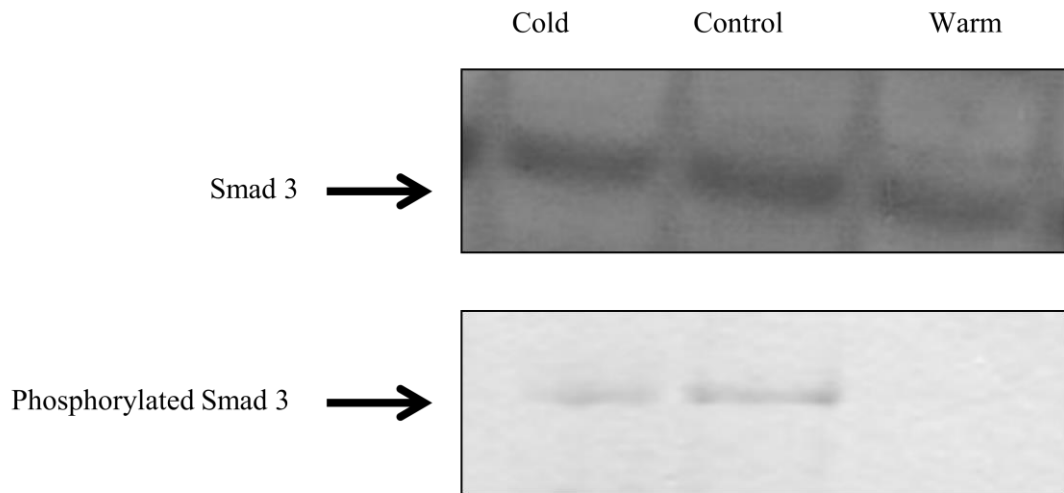


**Figure 5.21a – Western blot of rainbow trout spongy layer tissue from three acclimation temperatures using anti – ERK 1/2 antibody (top) and anti – phosphorylated ERK 1/2 (bottom).**

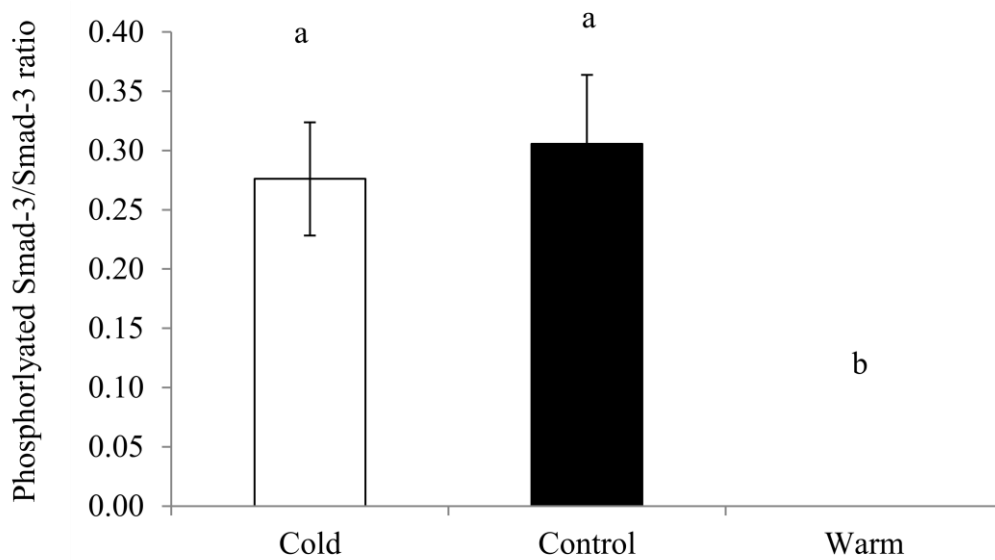


**Figure 5.21b – pERK 1/2 levels with respect to total ERK 1/2 levels.** Cold acclimated fish had a significantly greater phosphorylated(p) ERK 1 and ERK 2 ratio ( $0.53. \pm 0.05$  and  $0.27. \pm 0.03$  respectively) compared to warm ( $0.19. \pm 0.02$  and  $0.08 \pm 0.02$ ) acclimated fish representing a difference of 179% and 237% respectively. Data is presented on the graph as the mean of  $n = 9$  for each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters (a and b for ERK 1 and b and c for ERK 2 exclusively) represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



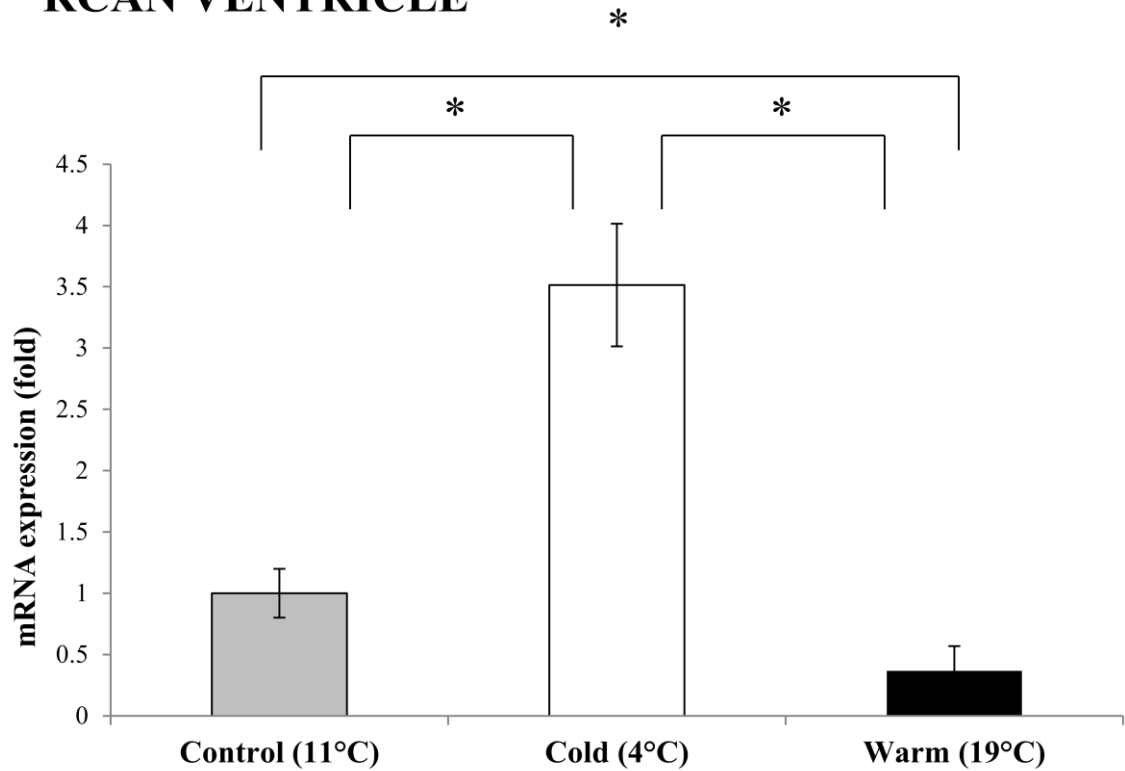


**Figure 5.22a – Western blot of rainbow trout spongy layer tissue from three acclimation temperatures using anti – Smad 3 (top) and anti- phosphorylated Smad 3 (bottom) antibodies.**



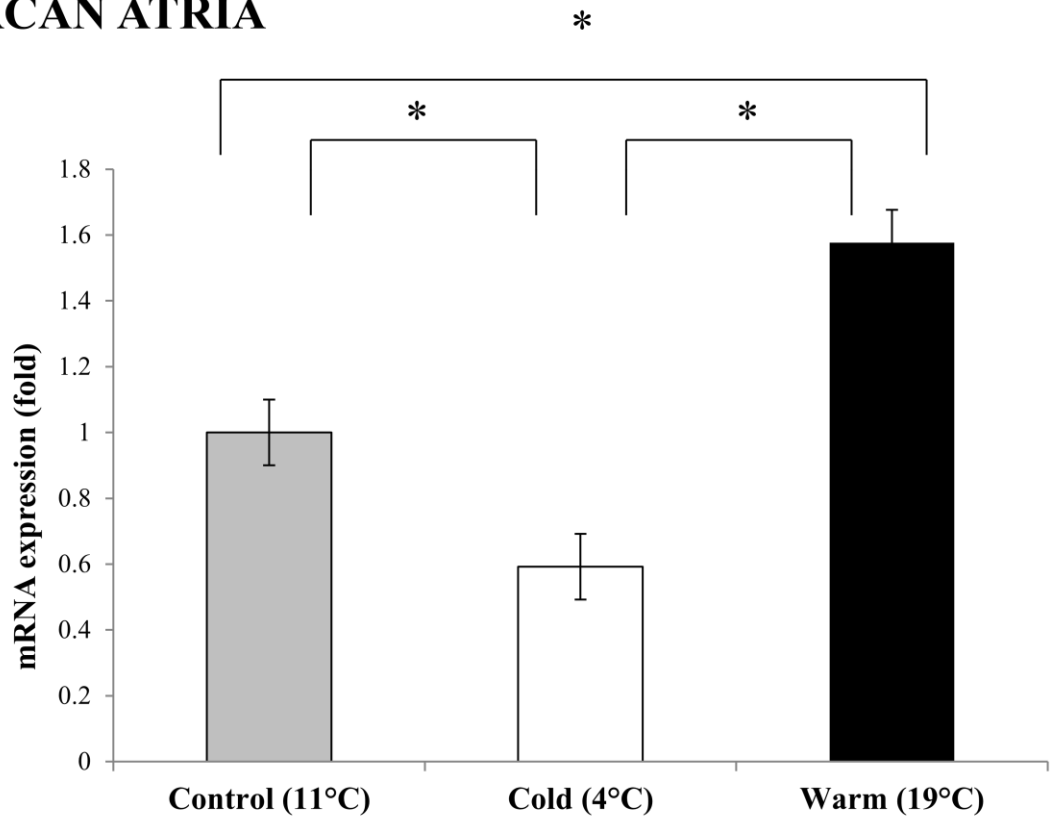
**Figure 5.22b – Phosphorylated Smad 3 levels with respect to total Smad 3 levels.** Phosphorylated Smad 3 was completely absent from warm acclimated fish. Data is presented on the graph as the mean of n = 9 for each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .

## RCAN VENTRICLE



**Figure 5.23 - Expression of ventricular RCAN mRNA during cold and warm acclimation of rainbow trout heart.** RCAN in the cold ventricle was expressed 3.51 fold  $\pm$  0.5 with respect to control. RCAN in the warm ventricle was expressed 0.37 fold  $\pm$  0.2 with respect to control. Expression levels of RCAN in the cold ventricle were 3.14 fold higher than warm with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## RCAN ATRIA



**Figure 5.24 - Expression of atrial RCAN mRNA during cold and warm acclimation of rainbow trout heart.** RCAN in the cold atria was expressed 0.59 fold  $\pm$  0.1 with respect to control. RCAN in the warm atria was expressed 1.58 fold  $\pm$  0.1 with respect to control. Expression levels of RCAN in the warm group were 0.99 fold higher than cold with respect to control. qPCR readings were taken from a sample size of  $n = 7$  fish from each acclimation group, repeated 3 times. Data is presented as the mean expression (fold, where control is normalised to 1 fold) and error bars show the standard error of the mean (SEM). \* represents where significance between acclimation group pairs as shown by One Way ANOVA resulted in  $p < 0.05$ .

## 5.6 Discussion

*Please note this discussion also relates to sections of the study documented as supporting material drawn from the Appendix, Chapter Eight.*

### **Key findings from this chapter are:**

1. Stretch markers increase in cold acclimated ventricle and warm acclimated atrium
2. Both cold and warm acclimation induce stress responses in the trout heart
3. Cold acclimation increases beta2 and beta3a adrenoceptors in the ventricle but not in the atrium
4. Adreno-stimulation of ventricle causes a direct increase in Cx43
5. Pro growth cell signalling molecules are active in cold acclimated ventricle with respect to warm

### **5.6.1 Stretch is a likely trigger for cardiac remodelling in trout**

ANP and BNP were chosen as markers for cardiac muscle stretch as it is well known that ANP and BNP serum levels increase in mammalian hypertrophy. Furthermore, mechanical stretch of cardiomyocytes leads directly to natriuretic peptide release [286-290]. ANP and BNP release in mammals are common features of pathological (pressure overload) hypertrophy or heart failure in mammals [278, 291, 292], however it is known that ANP and BNP regulation differ between fish and mammals [293] implying they may have different functions in different contexts and different species.

The results of this study show increases of ANP mRNA in cold acclimated ventricle spongy layer and warm acclimated atria. Furthermore there is an increase of BNP mRNA in cold acclimated ventricle. Together this suggests an increase in stretch in these chambers during these conditions. Moreover, this correlates with hypertrophy seen in this study in

the same chambers indicating a direct link between natriuretic peptide levels and the extent of hypertrophy, as seen in mammals.

ANP has been previously shown in wild migrating salmon to increase with a decrease of temperature [294], suggesting that temperature effects, and not activity levels are an important factor in determining the level of stretch in the ventricle. Furthermore, another study showed direct correlation with cold temperature, cardiac hypertrophy and ANP release in salmon [295]. Morphological data from chapter one suggests that cold acclimated hearts have less capacity for blood filling than the warm, therefore the ANP and BNP results of this study are in contradiction to one group who suggest volume overload is the driving force behind NP release in the trout heart [296]. However another study has shown increases of ANP in trout is known to decrease venous return to the ventricle via decreases in venous compliance [22], which would correlate well with the findings of this study where increases in ANP in the ventricle are associated with less filling capacity in cold acclimation as well as reduced compliance of the heart. This contradiction is a *cause or effect* debate. Do ANP and BNP levels modulate venous return prior to cardiac remodelling? Or, does the heart remodel first and then alter venous return via ANP and BNP release? Clearly, this would require much greater scrutinisation via experimental design, but in reality it is probably a mixture of the two.

On balance, it is likely that stretch is involved in the hypertrophic response in thermally acclimated rainbow trout heart as well characterised markers for mammalian cardiac stretch; ANP and BNP mRNA levels are increased in areas where hypertrophy is observed. Furthermore the physiological functions of ANP and BNP (reduction in venous return)

correlate with the morphological adaptations shown in the heart (namely blood filling space and compliance), suggesting a positive feedback loop.

Stretch is therefore not only involved in remodelling but potentially facilitates natriuretic peptide release which modulates venous return to a remodelled heart suitably adapted to work with the incoming blood volume and pressure in different temperatures.

### **5.6.2 Cardiac remodelling in captive thermally acclimated trout involves a stress response**

Stress responses during cardiac remodelling were analysed in this study after one group found that trout more sensitive to stress displayed greater cardiac hypertrophy. Indeed, trout with greater RVM all had increased levels of MR, GR1 and GR2, which are correlated with increases of the stress hormone cortisol [297, 298]. Their results are in contradiction to this thesis however where there were diverse levels between all three corticoid receptors. MR, GR1 and GR2, which are well studied in rainbow trout, are known to show highly different patterns of expression within the same and indeed different tissues [299-301] which is one explanation to the contradicting evidence.

Captivity is known to cause stress in fish [302, 303] and acute exposure to stress is repeatable in wild fish after a 1 year period [304] together showing that fish are highly sensitive to stress and this response cannot be ruled out of any experimental design, especially in captivity.

In this study, there appeared to be a temperature, but not chamber size correlation with stress receptor levels. Indeed, GR1 levels increased in the warm heart generally, whereas GR2 levels decreased in the cold heart in both chambers despite different degrees of hypertrophy occurring between chambers. With respect to control conditions therefore, each receptor may govern the stress response in difference temperatures, which is not surprising as they have been shown to be highly distinct in their functionality from each other [305]. This result may also allude to the fact that different stress hormones are being released depending on the extreme of temperature. The heat shock protein DNAJ mRNA levels were significantly higher in both cold and warm ventricle and atria indicative of a classic stress response in these tissues during extremes of temperature, which wasn't apparent in control. Heat shock in fish is known to alter glycolytic, gluconeogenic, protein metabolic and antioxidative enzymes in an attempt to preserve metabolic integrity [306] during extremes of temperature. Interestingly, in the heart, DNAJ is believed to be anti-hypertrophic [307] again, providing evidence that the observed stress response in this study is more correlated with temperature as opposed to chamber size.

In mammals, direct introduction of artificially high levels of cortisol have been shown to promote cardiac hypertrophy [308-310] and furthermore, in patients on corticosteroid therapy, this heart growth is abolished when medication is stopped, by directly affecting levels of myosin heavy chain [311].

Taken together, it is apparent that fish in this study, especially the ones subjected to cold and warm temperatures were undergoing a stress response. Although this appeared to be more temperature dependent and not necessarily a direct cause of hypertrophy, it cannot be

ruled out that stress was a component of the cardiac remodelling stimulus as it is widely accepted that increases in stress hormones influence cardiac remodelling in mammals.

### **5.6.3 Beta adrenergic receptor activation is a potential trigger of cardiac hypertrophy in trout**

$\beta$  adrenergic receptors ( $\beta$ ARs) were investigated in this study for two main reasons. Firstly, chronic activation of  $\beta$ ARs in mammals has been shown in numerous studies to induce cardiac hypertrophy [312-315] and secondly, trout heart is known to become more sensitive to adreno-stimulation in cold acclimation [146] [157, 316].

Prior to the start of this study, there was no confirmation as to which  $\beta$ ARs were present in the trout heart, as adreno-studies were assessed purely by the response to pharmacological agents [149, 317, 318]. Using PCR it was determined that both atrium and ventricle express  $\beta$ AR2, 3a and 3b.

In ventricle, there is an increase in  $\beta$ AR2 mRNA in cold acclimation and a decrease in warm, indicating a clear temperature dependent and chamber mass correlated response. This increase may explain why cold acclimated myocytes are more sensitive to adreno-stimulation. The increase in  $\beta$ AR2 is presumably to facilitate greater e-c coupling capacity when ion kinetics are reduced in cold temperatures; indeed, the SR  $\text{Ca}^{2+}$  stores become much more important during cold acclimation [157, 316, 319]. Increases in cytoplasmic  $\text{Ca}^{2+}$  and increases in  $\text{Ca}^{2+}$  entry via LTCC could directly lead to greater activation of the calcineurin/NFAT system [320, 321] thus promoting hypertrophy. However changes in LTCC induced contractility are not always directly linked with differences in hypertrophy



[322], indeed a reduction of LTCC activity in one study actually promoted hypertrophy [323]. As well as  $\beta$ AR2 positively influencing calcium dynamics in the cardiomyocyte, it can also activate other signalling molecules, importantly Akt [324-328], which, as discussed later on, is shown to be activated in cold acclimated hypertrophied ventricle and is a key physiological driver of hypertrophy in mammals.

It was surprising to see a reduction of  $\beta$ AR2 in the cold atrium, opposing the ventricular response which would suggest a mass dependence as opposed to temperature dependence response. This could also dictate a reduction in the contractile requirement of the atrium, which is in-line with qPCR and morphological data suggesting the atrium is subject to low mechanical load in cold conditions. This reduction in  $\beta$ AR2 number however, could be slightly offset by the fact that atrial rates of contraction are higher compared to ventricle cardiomyocytes which is facilitated by a low phospholamban/SERCA ratio and not calsequestrin levels [157, 316]. The relatively low density of  $\beta$ AR2 expression in the atrium correlates with relatively low chamber mass in the cold, and reduced levels could lead to reduced activation of calcineurin/NFAT and Akt, thus reducing the potential for hypertrophic signalling.

$\beta$ AR3a expression, although lower than  $\beta$ AR2, showed the same pattern of expression; namely increases in the cold ventricle and decreases in cold atria suggesting this receptor could be important in the temperature and hypertrophic response in trout heart.  $\beta$ AR3 receptors have been previously described in fish heart [329-331].  $\beta$ 3ARs operate via nitric oxide (NO) stimulating guanylyl cyclase leading to production of cGMP which may activate protein kinase G (PKG) or phosphodiesterase 2 (PDE2) and this cGMP/PKG pathway has been implicated in cardiac hypertrophy [332-336].

In summary, increases in  $\beta$ AR2 are associated with cold acclimation in the ventricle where hypertrophy is observed. This increase in receptor density could lead to a greater influx of  $\text{Ca}^{2+}$  which combined with an increase in cytosolic  $\text{Ca}^{2+}$  known to occur in cold acclimation could directly promote cardiac hypertrophy via calcineurin interaction. Furthermore,  $\beta$ AR2 could induce activation of Akt, promoting hypertrophy via a separate route. The presence of  $\beta$ AR3a in a similar expression to  $\beta$ AR2 opens up scope for a cGMP/PKG control of cardiac remodelling in trout heart.

#### **5.6.4 Cold acclimation in trout heart induces cell signalling cascades associated with mammalian physiological hypertrophy**

As seen in chapter one, cold acclimation is associated with hypertrophy of the spongy layer during cold acclimation. In the current chapter, investigation of key cell signalling systems involved in mammalian hypertrophy were assessed to help to understand the type of hypertrophy; pathological or physiological.

In mammals, the Akt/GSK-3 $\beta$  pathway is thought largely to be concerned with the physiological hypertrophic response [337, 338] whereas the RCAN(MCIP)/calcineurin/NFAT pathway appears more concerned with pathological signalling [320, 339-341]. Both pathways converge on their control over NFAT which is required to be in a dephosphorylated state to enter the nucleus and regulate hypertrophic genes. Calcineurin, which is inhibited by RCAN can directly dephosphorylate NFAT, therefore RCAN is anti-hypertrophic. GSK-3 $\beta$  is able to phosphorylate NFAT but only when in its active state. Deactivation of GSK-3 $\beta$  by Akt phosphorylation therefore promotes NFAT entry into the nucleus via the physiological cascade [56]. GSK-3 $\beta$  also imparts control over another pro-hypertrophic gene regulator, GATA4. GATA4, like

NFAT, is able to upregulate genes in the non-phosphorylated state. With Akt activated, GATA4 is therefore able to upregulate hypertrophic genes due to GSK-3 $\beta$  being unable to phosphorylate GATA4, allowing it entry into the nucleus [342]. GATA4 has been implicated with both types of hypertrophy however is required for physiological functioning [343] [344].

In the cold acclimated spongy layer we see an increase in activated Akt, which in turn, as shown by Western blot, could lead to phosphorylation of GSK-3 $\beta$ . This in theory would prevent phosphorylation of NFAT, promoting its entry into the cell via the physiological cascade. Furthermore this would promote GATA4 regulated expression of pro hypertrophic genes. Intriguingly, we also see an increase in RCAN mRNA in cold acclimated spongy layer. If protein levels represented the mRNA levels, this would inhibit calcineurin, in turn preventing entry of NFAT into the nucleus via the pathological signalling system.

In fish heart the calcineurin/NFAT system is largely unexplored, however it is known that this system is required for proper heart development in zebrafish, indicating a physiological role in non-hypertrophic conditions [345, 346]. Moreover, very little is known about the role of GSK-3 $\beta$  in fish heart other than its requirement in correct cardiomyocyte development [347, 348].

Akt is activated by numerous proteins including  $\beta$ AR2 [326, 327, 349-351], which as discussed in the previous section is upregulated in cold acclimated ventricle, potentially providing a direct link between adreno stimulation and cardiac hypertrophy in the trout heart. Another activator of Akt is integrin linked kinase (ILK), which again was shown to

be expressed at higher levels in cold with respect to warm. ILK in mammals is both upregulated in hypertrophy and can induce cardiac remodelling [352]. ILK is traditionally known as a “survival kinase” linking stretch events within the heart to signalling events via integrins (linked to the ECM) and Akt signalling [353] and is therefore likely to be a key mediator in the hypertrophic response which it does to promote repair mechanisms in a compromised heart in conditions such as pressure and volume overload [354]. In zebrafish, it has been shown that ILK is critical for correct heart function, where it acts via Akt signalling to mediate effective mechano-stretch sensing [355]. In the trout therefore, it is likely that the increase in ILK in cold hearts is a both a result of increased levels of stretch and a source of increased Akt signalling. This could imply increased activation of pro-hypertrophic GSK-3 $\beta$  signalling seen in cold acclimated trout heart. Furthermore, the observation of increased ECM shown in chapter one would correlate with an increase in ILK due to links via integrin, known to be influenced by ECM induced events [356] providing an exquisite stretch sensing apparatus spanning from connective tissue to the nucleus of each cardiomyocyte.

Mitogen-activated protein kinase (MAPK) signalling pathways are important regulators of cell growth, proliferation, and stress responsiveness. Phosphorylated p38 MAP kinase was shown to increase in cold acclimated fish hearts whilst activated ERK 1/2 was higher in cold with respect to warm. MAPKs are generally believed to be pro-hypertrophic and can act in both pathological [59, 357, 358] and physiological [359-361] mammalian hypertrophy. Furthermore, p38 MAPK is involved in promoting cardiomyocyte differentiation [362], which would correlate with hyperplasia observed (via PCNA marker) in this study. It is therefore likely that p38 MAPK and ERK1/2 are involved in cold acclimation induced cardiac hypertrophy in rainbow trout heart in a temperature dependent pro-growth manner.

Activated smad3 in this study was shown to be completely absent in warm acclimated spongy ventricle only. This is in contradiction to a study in mouse that showed depletion of Smad3 in a hypertrophic model [363] and another study showing that repression of smad3 promotes increases in muscle mass [364]. However, phosphorylation of smad3 is known to be involved in cardiac remodelling events largely associated with ECM remodelling via the TGF- $\beta$  signalling pathway [365, 366]; indeed hypertrophic growth is believed to be smad3 independent [367]. More conflict arises from one report in a smad3 knock out mouse where both collagen deposition and cardiac hypertrophy were accelerated [368] which is in direct contradiction to many studies showing that smad3 mediates the formation of fibrosis and promotes cardiac stiffness [366, 369-371]. Furthermore, smad3 in zebrafish has been shown to be important in effective cardiac repair by mediating TGF- $\beta$  directed signalling modulating the balance between scar and healthy heart muscle tissue [372]. Together, the evidence suggests that smad3 is largely involved in ECM remodelling and collagen deposition as opposed to muscle mass changes. This would explain the absence of activated smad3 in warm acclimated trout ventricle in-line with a large reduction of collagen.

In summary, in cold acclimated trout, physiological hypertrophic signalling systems are activated in the ventricle: namely Akt/GSK-3 $\beta$ . Pathological systems appear to be repressed which is evident by high levels of RCAN mRNA, the negative regulator of calcineurin. Akt can be activated by  $\beta$ AR2 signalling as well as by ILK, where we see increases in this “survival kinase” in cold acclimated hearts and it potentially acts as a key stretch sensor triggering the hypertrophic response. MAPKs appear to be associated with increased hypertrophy in trout heart and smad3 is likely involved in ECM remodelling.

### **5.6.5 Beta adrenergic stimulation directly causes connexin remodelling in trout heart**

Addition of beta-adrenergic agonists directly to strips of control ventricular tissue for 15 minutes resulted in a 2 fold increase in Cx43 mRNA. This is in agreement with numerous studies that application of adrenaline-like agents causes a direct increase in Cx43 mRNA and protein in cardiomyocytes [373-377]. This can directly lead to increased conduction velocity in rat neonatal cardiomyocytes [377] which is in agreement with increases in conduction velocity described in chapter four. Furthermore, in this study, there was a qualitative increase in this beta-adrenergic evoked Cx43 increase in cold acclimated ventricular strips, which is in-line with an increase in  $\beta$ AR2 mRNA receptor levels. Furthermore, this supports numerous reports from mammalian studies that beta-adrenergic stimulation promotes both Cx43 increases and cardiac hypertrophy [373, 374, 376-379]. This provides strong evidence, that similar to mammals, trout heart is able to remodel connexin in direct response to beta-adrenergic stimulation. Furthermore it suggests that  $\beta$ AR mediated signalling is a high level governor of both structural and electrical remodelling, which would make sense as an increase in heart mass would require an increase in compensatory conduction channels to maintain adequate function. Cx43 remodelled in response to adrenergic stimulation is known to be dependent on various signalling cascade including increases in activated p38 MAPK and ERK1/2 [284] which we see in cold acclimated hearts.

### **5.6.6 Conclusions**

Cold acclimation causes an increase in stretch in the spongy layer of the trout heart resulting in an increase of ANP and BNP mRNA; when the peptides themselves are released they alter vascular properties and venous return, further influencing cardiac remodelling. In cold acclimation,  $\beta$ ARs increase in the ventricle which stimulate structural and electrical remodelling through a combination  $\text{Ca}^{2+}$  dependent signalling, Akt activation

and MAPK phosphorylation. ILK, the mechano-stress sensor detects changes in heart stretch via interactions with both cardiomyocytes and extracellular matrix, and is able to influence cardiac remodelling via modulation of Akt activation. Akt is able to deactivate GSK-3 $\beta$  in the cold ventricle, which allows upregulation of hypertrophic genes likely via the action of NFAT and GATA transcription factors akin with physiological hypertrophy in mammalian hearts. Furthermore, ECM is modelled by the influence of Smad3, likely working in the TGF- $\beta$  pathway. Temperature acclimation involves a stress response and it is likely that this stress has a direct influence on cardiac remodelling, likely by the direct action of stress hormones such as cortisol.

### **5.6.7 Summary**

In order for large-scale remodelling of rainbow trout heart in response to temperature acclimation, there have to be triggers in place, and molecular methods to transduce the signals which ultimately regulate genes to action the remodelling of a vast array of proteins. In the trout heart, the main trigger is likely to be the degree of stretch the heart is subject to in any given temperature. Stretch causes the release of vasoactive peptides which help with venous control and return, possibly facilitating heart remodelling. Stretch is also able to directly trigger signalling mechanisms leading to the regulation of cardiac remodelling genes. As well as stretch, increased adreno-stimulation in the hearts of cold acclimated fish can directly cause structural and electrical remodelling by activation of several different cell signalling pathways.

### 5.6.8 Study limitations

1. **No biophysical measurements taken for stretch or stress** – although ANP and BNP are well characterised markers of stretch, accurate *in vivo* measurements would shed more light on the degree of stretch the heart is subject to. Blood samples of cortisol and/or other stress hormones would give a more accurate indication of the involvement of stress in the remodelling response in acclimated fish.
2. **Adreno-stimulation not carried out in whole hearts** – using adreno-stimulation on ventricular strips to investigate Cx43mRNA is not as accurate as using whole hearts due to the potential effects of altered signalling brought about by tissue damage and unnatural associations with communication and extracellular matrix pathways.
3. **Cell signalling only in the spongy layer** – all cell signalling investigations were carried out on the spongy layer and not the atrium or compact layer which would give much more overall understanding as to the potential roles of activated signalling cascades and the chamber/ sub-chamber specific levels of activation.
4. **Single cell work not carried out** – Single cell work on cardiomyocytes opens up the opportunity to explore cell signalling mechanisms by the addition of blockers and activators that is not possible in the whole organ.



## 6 General Discussion

The aim of this thesis was to investigate cardiac remodelling in fish acclimated to cold and warm extremes of temperature, in the physiological range. Prior to the start of this study, there was already evidence that temperature induced cardiac remodelling occurred in fish, however this was largely at the ion channel level [12, 149, 212, 219, 250]. There was also consistent evidence that cold acclimation caused cardiac enlargement in several species of fish [138] [139-142], which was the basis of this work. This study therefore sought to gain greater understanding of the extent of cardiac remodelling at the structural, proteomic, genetic and electrical levels, as well as to investigate potential mechanisms by which these adaptations occur. A secondary aim was to compare cardiac remodelling in fish to cardiac remodelling in mammals, thus exploring the possibility of fish being a viable model for cardiac remodelling in a therapeutic sense.

There was significant cardiac remodelling observed at all levels in a temperature dependent manner. In agreement with previous studies, cold acclimation caused a significant increase in the overall mass of the heart as well as within the ventricle; however overall cardiac enlargement was significantly less in comparison to other studies of cold acclimation in rainbow trout, likely due to the fact that females only were used in this study which do not experience the significant effect of pro-hypertrophic testosterone. Notwithstanding, there was significant ventricular hypertrophy observed in the cold group and this was due to spongy layer hypertrophy which is characteristic of fish that live in extreme cold environments such as the ice fish. Moreover, in the warm group, despite there being overall ventricular atrophy, there was significant hypertrophy of the compact layer, which is also observed in highly active fish in warm temperatures such as the tuna. This spongy versus compact layer offsetting was quite a surprising result but it gave an initial clue as to the aim of cardiac remodelling in different contexts.

Another surprising result was the fact that atrial mass changes opposed the ventricular changes; namely cold acclimation induced atrial atrophy, implying this chamber is subject to decreasing mechanical load in the cold. Moreover, in the warm we see atrial hypertrophy suggestive of greater mechanical load. Combined with the spongy and compact layer observations, with anatomical information from the ice fish and tuna, as well as studies on activity levels in acclimated trout and compact layer growth in migrating fish, it was concluded that cold acclimation produces a low pressure pump and warm acclimation promotes the formation of a high pressure pump; however this would require *in vivo* blood pressure measurements to be more conclusive.

Activity levels in different temperatures were deemed to be a key driving factor in cardiac remodelling; a key feature of eccentric physiological hypertrophy in mammals. This is further backed up by the fact that in warm acclimated trout, there was an increased blood filling potential brought about by likely increases in compliance via an increased elastin:collagen ratio, as well as increases in extrabundular space. Spongy layer growth was deemed to be genuine hypertrophy as opposed to e.g. oedema or energy storage due to the significant upregulation of muscle specific genes and a pro-angiogenic factor indicative of muscle growth.

A key finding of this study was the identification of three connexins, all of which have mammalian homologs. Cx43, a key mammalian connexin and a fast conduction channel was found in the ventricle and atrium. Moreover, Cx30.9 and Cx48.5, the equivalents of Cx30 and Cx46 respectively, which are known as slow conductors that regulate pacemaker activity and speed of electrical propagation, were also present in both chambers. Single cell expression and electrophysiological experiments are required to fully characterise these

connexins. In agreement with mammalian cardiac hypertrophy, there was significant connexin remodelling observed during temperature induced cardiac remodelling in trout, however unlike in mammals, Cx43 levels increased with hypertrophy. Indeed Cx43 levels in trout appear dependent on muscle mass which is probably due to the fact that membranous connexin is situated around the entire periphery therefore an increase in cell size would dictate an increased requirement for membranous protein. Cx30.9 and Cx48.5 appear to be the temperature dependent regulators of conduction in the heart as in both chambers their expression is negatively correlated with an increase in temperature, presumably as a measure to balance conduction kinetics. Indeed, conduction velocity recordings taken from another study revealed an increased conduction in cold hearts, where we see a reduction in both chambers of conduction-inhibitory Cx30.9 and Cx48.5.

A surprising find in this study was the detection of a large cytosolic pool of connexin, which is not present in mammals. Indeed, the cytosolic:membrane ratio of connexin is affected by temperature and is potentially regulated by phosphorylation, where in warm ventricle there appears to be increased resistance to dephosphorylation. It was concluded that this pooling may aid in fast remodelling in fish which experience rapid temperature change. Connexin remodelling in temperature acclimated fish is a fundamental part of cardiac remodelling as whole.

This study generated useful information about potential triggers and mechanisms that are behind the cardiac remodelling observed. Stretch in fish heart is known as a significant regulator of short term cardiac output acting via the *Frank Starling mechanism* and this study revealed that stretch may also be a regulator in the long term during temperature acclimation. Indeed, ANP and BNP mRNA levels were higher in hypertrophied chambers.

There is also evidence that ECM stretch signalling may play a part in the hypertrophic response due to an increase in ILK in cold acclimated spongy layer with respect to warm, where we also see an increase in connective tissue. ILK is able to activate the physiological pro-hypertrophic Akt/GSK3- $\beta$  pathway via stretch interactions with the ECM and in cold acclimated ventricle we see increased activation of this pathway relative to warm. As well as stretch, there is also the likelihood, as seen in mammals, that  $\beta$ AR stimulation is a trigger for hypertrophic growth acting via both  $\text{Ca}^{2+}$  and Akt. Indeed, in hypertrophied chambers, we see an increase in  $\beta$ AR2 receptor mRNA. In trout hypertrophied ventricle, there is activation of pro-hypertrophic signalling molecules p38MAPK and ERK1/2. P38MAPK is largely associated with pathological signalling in mammals whilst ERK1/2 is mainly concerned with physiological, however both are pro-growth. Furthermore in hypertrophied ventricle and atria, there is a significant increase of RCAN mRNA suggesting repression of the calcineurin/NFAT pathway, traditionally associated with pathological mammalian hypertrophy.

It was apparent that the fish in this study, especially the cohorts experiencing temperature extremes were affected by stress; however this stress response did not correlate with cardiac remodelling in any specific chamber. It is however likely that the stress response of the animals contributed to the output of the experiments as is described in previous fish and mammalian studies. A stress response could be a normal phase of the acclimation response, or it could be due to animal captivity. It will be imperative to address this issue in any future experiments of temperature acclimation in captive fish.

All findings from this study are presented in the table below:

**Table 6.1 – Overall findings**

	Cold acclimation			Warm acclimation	
	Atrium	Ventricle		Atrium	Ventricle
<b>General Morphology</b>					
Mass	↓	↑		↑	↓
Extrabundular space	*	↓		*	↑
Compact layer thickness	*	↓		*	↑
Connective tissue	*	↑		*	↓
Collagen protein	*	↑		*	↓
Elastin protein	*	↓		*	↑
<b>Muscle genes</b>					
VHMC	↓	↑		↑	↓
MLP	↓	↑		—	↓
SMLC2	↓	↑		↑	↓
<b>Angiogenesis</b>					
VEGF	↓	↑		↑	↓
<b>Hyperplasia</b>					
PCNA	—	↑		—	—
<b>Collagen regulation</b>					
Colla1	—	—		—	—
Colla2	—	—		—	—

Col1a3	↑	↑		↓	↓
MMP2	↓	↓		—	↑
MMP9	↓	—		↑	—
MMP13	—	↓		↓	—
TIMP2	—	↑		—	↓
<b>Connexins</b>					
Cx43 mRNA	↓	↑		↑	↓
Cx30.9 + Cx48.5 mRNA	↓	↓		↑	↑
Cytosolic 48Kda band	*	—		*	—
Membranous 48Kda band	*	—		*	—
Cytosolic 43Kda band	*	↑		*	↓
Membranous 43Kda band	*	↑		*	↓
Cytosolic 30Kda band	*	↓		*	↑
Membranous 30Kda band	*	↑		*	↓
<b>Stretch</b>					
ANP	↓	↑		↑	—
BNP	—	↑		—	↓
<b>Stress</b>					

MR	–	–		–	↓
GR1	↑	↓		↓	↑
GR2	–	↓		↓	–
DNAJ2	–	–		–	–
<b>Neuro-hormonal</b>					
βAR2	↓	↑		–	↓
βAR3a	↓	↑		–	–
βAR3b	–	–		–	–
<b>Cell signalling</b>					
p-P38 MAPK	*	↑		*	↓
p-ERK	*	↑		*	↓
p-Akt	*	↑		*	↓
ILK	*	↑		*	↓
p-Smad3	*	–		*	↓
p-GSK3-β	*	↑		*	↓
RCAN mRNA	↓	↑		↑	↓

Data is presented as a comparison between cold and warm acclimated fish. ↑ = a significant increase with respect to the opposing temperature and ↓ = a decrease. \* indicates where there is no data available/ non applicable. If there was no significance between subject and control or no significance between cold and warm “–“is displayed.

## 6.1 Summary

Fish experience wide ranging environmental conditions; one of which is temperature fluctuation. Despite temperature change in the short and long term, fish hearts are able to continue effective cardiac output by remodelling events that occur at the whole heart, chamber, sub chamber, cardiomyocyte, protein and genetic levels. During temperature

acclimation, activity levels, oxygen availability and metabolic demand alter which results in changes to overall cardiac requirements and function. In the cold, fish are relatively inactive, oxygen availability is high, and ion kinetics are reduced. Blood viscosity increases in cold conditions which imparts mechanical stress on the heart. In these conditions the body requires less cardiac output than in warm conditions however the heart must be able to contend with an increased vascular resistance. The heart responds to this stress by releasing vasoactive peptides to alter downstream vascular characteristics. Furthermore the hearts remodels, via molecular signalling pathways, activated by stretch and neuro-hormonal stimulation to regulate muscle and connective tissue genes, thereby changing the *nature of the pump*. In the cold, the result is a heart laden with connective tissue to provide structural support, a hypertrophied spongy layer sufficient to pump blood around the body at low pressure, and an atrium experiencing low returning blood pressure. In the warm, where there is an increased requirement for blood, the heart adapts to become a high pressure pump, with increased blood filling capacity due to a reduction of collagen brought about by regulation of connective tissue enzymatic proteins. In both cold and warm conditions, electrical activity and conduction in the heart has to be harmonised and this is achieved via gap junction or connexin remodelling. Indeed, the fish heart has the ability to tap into a cytosolic pool of connexin ensuring rapid remodelling and specific connexins regulate both general conduction and pacemaker activity. Fish heart remodelling is remarkably similar to eccentric remodelling as seen in mammals; indeed they display similar morphology and appear to share commonality in the activation of cell signalling pathways.



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## 8 Appendix

### 8.1 Full qPCR data

In the main thesis, mRNA data is presented as cold v warm, both normalised to control.

Below (Table 8.1) are absolute values for qPCR on each gene.

**Table 8.1 – Full qPCR data for every gene investigated in this thesis**

Gene	Atrium			Ventricle		
	Cold (4°C)	Control (11°C)	Warm (19°C)	Cold (4°C)	Control (11°C)	Warm (19°C)
PCNA	0.088 ±0.01	0.103 ±0.01	0.088 ±0.01	0.260 ±0.03	0.105 ±0.01	0.099 ±0.01
SMLC2	0.443 ±0.04	0.920 ±0.09	1.264 ±0.13	3.691 ±0.41	1.308 ±0.12	0.498 ±0.05
VHMC	0.251 ±0.01	0.719 ±0.05	0.700 ±0.06	4.117 ± 0.37	1.414 ± 0.11	0.401 ± 0.023
MLP	0.666 ±0.05	1.112 ±0.13	0.810 ±0.78	2.096 ±0.16	1.116 ±0.18	0.342 ±0.03
RCAN	0.186 ±0.02	0.314 ±0.03	0.495 ±0.04	1.100 ±0.09	0.313 ±0.03	0.115 ±0.02
ANP	0.207 ±0.02	0.410 ±0.03	1.322 ±0.11	0.698 ±0.08	0.315 ±0.03	0.220 ±0.01
BNP	0.446 ±0.05	0.512 ±0.05	0.394 ±0.05	0.764 ±0.06	0.404 ±0.04	0.182 ±0.02
VEGF	0.137 ±0.04	0.221 ±0.02	0.777 ±0.06	0.792 ±0.06	0.213 ±0.01	0.109 ±0.01
MR	0.336	0.320	0.254	0.310	0.304	0.094

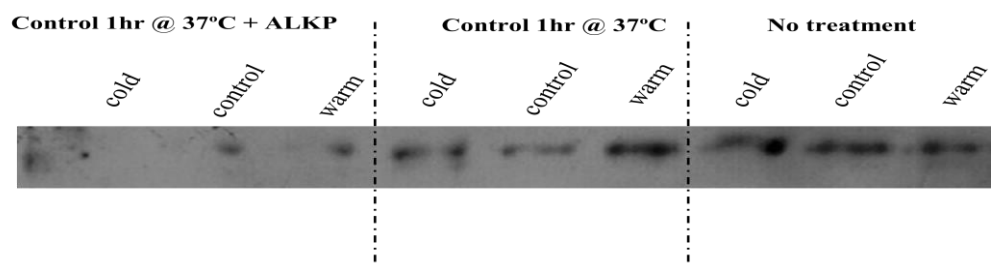
	±0.03	±0.02	±0.03	±0.03	±0.03	±0.07
GR1	0.201 ±0.02	0.321 ±0.03	0.739 ±0.07	0.217 ±0.01	0.315 ±0.02	1.037 ±0.14
GR2	0.087 ±0.09	0.302 ±0.03	0.251 ±0.01	0.103 ±0.02	0.314 ±0.02	0.438 ±0.04
DNAJ2	0.522 ±0.05	0.218 ±0.02	0.520 ±0.04	0.705 ±0.06	0.213 ±0.02	0.600 ±0.07
Colla1	0.379 ± 0.028	0.716 ±0.81	0.328 ±0.37	0.475 ±0.39	0.718 ±0.06	0.319 ±0.02
Colla2	0.332 ±0.02	0.613 ±0.06	0.318 ±0.03	0.413 ±0.04	0.606 ±0.04	0.267 ±0.02
Colla3	2.051 ±0.17	0.923 ±0.09	0.358 ±0.03	1.728 ±0.16	0.916 ±0.08	0.466 ±0.05
MMP2	0.180 ±0.02	0.400 ±0.03	0.391 ±0.04	0.210 ±0.03	0.416 ±0.03	0.706 ±0.05
MMP9	0.258 ±0.03	0.400 ±0.03	0.818 ±0.09	0.362 ±0.04	0.418 ±0.05	0.409 ±0.04
MMP13	0.126 ±0.01	0.214 ±0.01	0.210 ±0.01	0.193 ±0.02	0.308 ±0.03	0.363 ±0.03
TIMP2	0.090 ±0.01	0.111 ±0.01	0.119 ±0.01	0.876 ±0.07	0.309 ±0.03	0.171 ±0.01
Cx43	0.148 ±0.01	0.419 ±0.03	1.360 ±0.10	1.680 ±0.10	0.608 ±0.07	0.130 ±0.01
Cx30.9 + Cx48.5	0.176 ±0.02	0.316 ±0.04	0.960 ±0.08	0.105 ±0.01	0.207 ±0.02	0.680 ±0.05
BAR2	0.827	0.517	0.600	1.123	0.506	0.272

	±0.07	±0.05	±0.06	±0.01	±0.04	±0.02
BAR3a	0.125 ±0.01	0.217 ±0.02	0.218 ±0.02	0.291 ±0.03	0.203 ±0.03	0.145 ±0.01
BAR3b	0.113 ±0.01	0.118 ±0.01	0.123 ±0.01	0.160 ±0.01	0.114 ±0.01	0.160 ±0.01

Data is presented as the mean relative mRNA expression in comparison to  $\beta$ -actin housekeeping gene with standard error of the mean. For example, a value of 0.5 would mean half as much subject gene mRNA was present with respect to  $\beta$ -actin and a value of 2 would mean the subject gene was present at double the amount.  $\beta$ -actin had a raw qPCR threshold value (CT) of  $20 \pm 1.8$ .

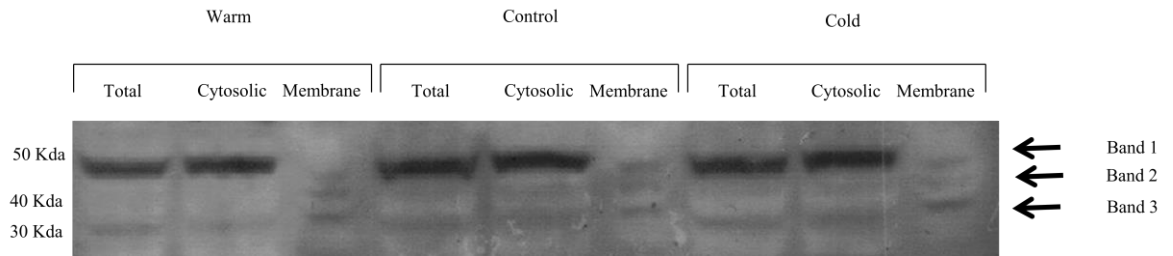
## 8.2 Connexin Western blot data

For loading controls during connexin Western blotting in this study, BioRad Protein Assay and Ponceau Red staining was used, however due to difficulties relating to the species studied, an antibody based loading control (e.g.  $\beta$  actin was not used.) It was therefore decided to place this part of the data in the appendix due to absolute protein loading uncertainty.

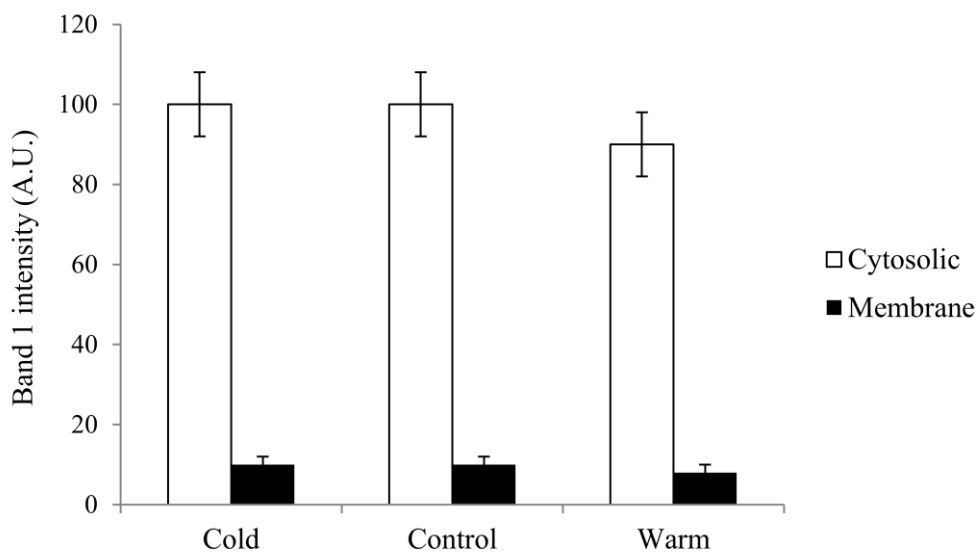


**Figure 8.1 - Dephosphorylation of anti Cx43 band.** Following treatment with alkaline dephosphatase (ALKP), the anti Cx43 band from ventricular cytosol is degraded. Interestingly, warm acclimated ventricular tissue appears to be more resistant to degradation at 37°C as shown in the centre panel of the figure.

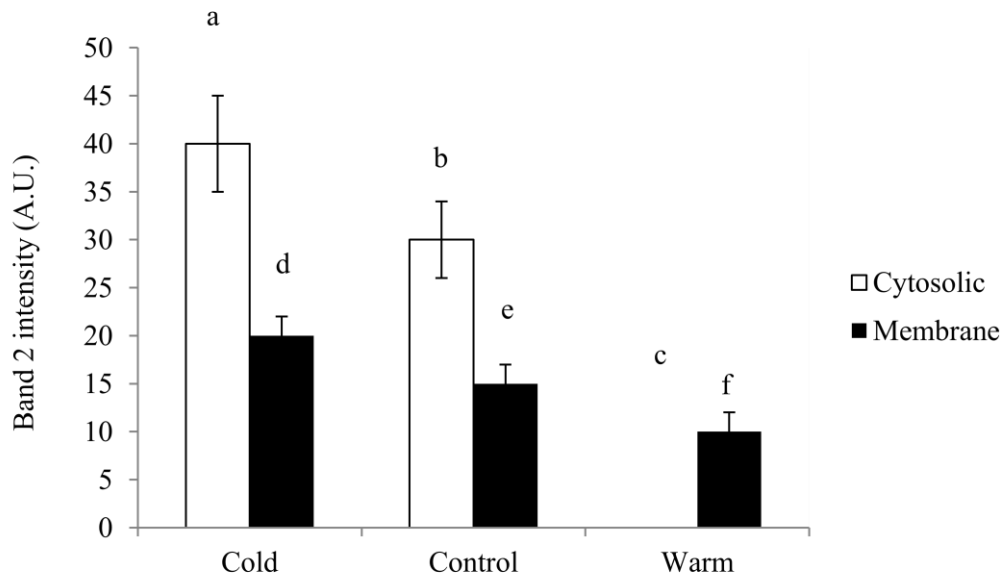




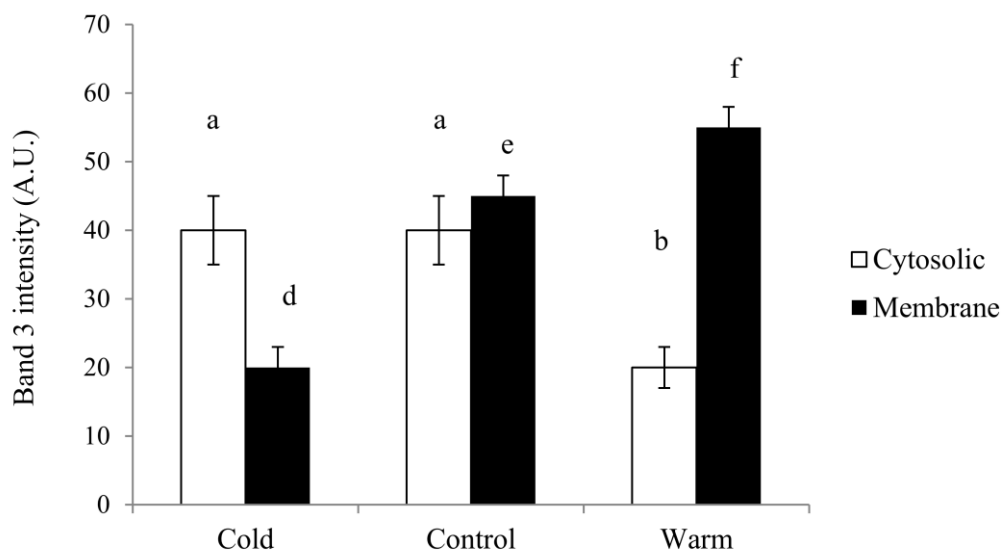
**Figure 8.2 - Full connexin protein western blot using anti-Cx43 antibody.** Following several optimisations of antibody concentration and protein quantity, the above western blot of total, cytosolic and membrane ventricular spongy layer fractions, side by side, for all three acclimation temperatures was obtained. Three bands appeared between 30Kda and 50Kda; appearing at Band 1: 48Kda, Band 2: 43Kda and Band 3: 30Kda. Although it is possible that the strongest band is Connexin 43, due to the fact that this band appears at about 48Kda, the PCR and sequencing evidence revealed Cx43, Cx48.5 and Cx30.9, along with the protein bands appearing at the respective Kda positions, it is more likely that Band 1 is Cx48.5, Band 2 is Cx43 and Band 3 is Cx30.9. Due to the uncertainty however, these bands are named Band 1, Band 2 and Band 3.



**Figure 8.3 - Band 1 (48 Kda) protein levels.** Band 1 appears at 48Kda. There was no significant difference between levels of the cytosolic protein of all three acclimation groups, nor was there any difference between the membrane fractions. However, the cytosolic component was 900% greater on average than the membrane content. Errors bars represent standard error of the mean taken from n = 9 fish of each temperature group.



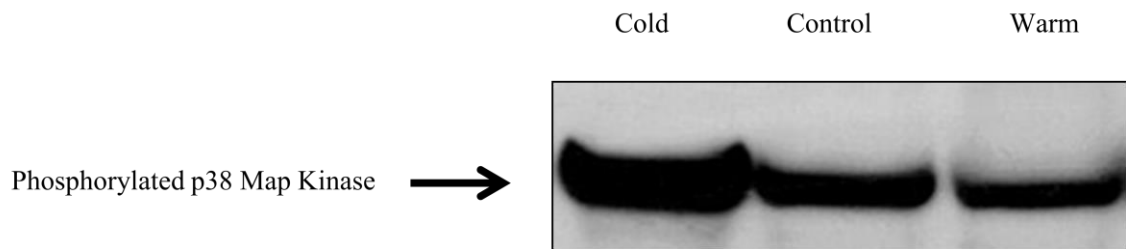
**Figure 8.4 - Band 2 (43 Kda) protein levels.** Band 2 appears at 43Kda. This 43Kda band was expressed at  $33\% \pm 4.5$  higher levels in the cold cytosolic fraction compared with the control. The warm cytosolic band was completely absent. In the membrane fraction, cold fish expressed  $100\% \pm 2$  more than their warm counterparts. Errors bars represent standard error of the mean taken from  $n = 9$  fish of each temperature group. Dissimilar letters (a-c for cytosolic, and d-f for membrane exclusively) show where a 1 way ANOVA statistical test results in  $p < 0.05$  significance between different temperatures.



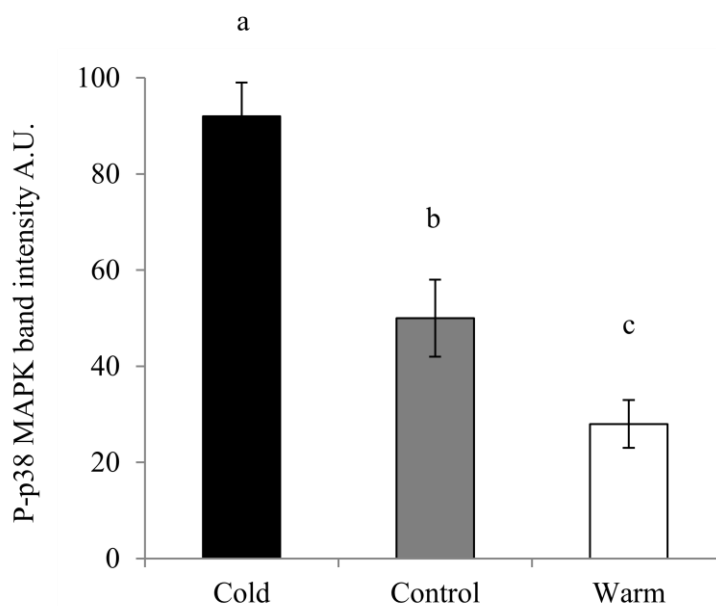
**Figure 8.5 - Band 3 (30 Kda) protein levels.** Band 3 appears at 30Kda. This 30Kda band was expressed at  $50\% \pm 4$  lower levels in the warm cytosolic fraction compared with the cold. In the membrane fraction, warm fish expressed  $22\% \pm 3$  and  $175\% \pm 3$  more than their control and cold counterparts respectively. Errors bars represent standard error of the mean taken from  $n = 9$  fish of each temperature group. Dissimilar letters (a-b for cytosolic, and d-f for membrane exclusively) show where a 1 way ANOVA statistical test results in  $p < 0.05$  significance between different temperatures.

### 8.3 Cell signalling Western blot data

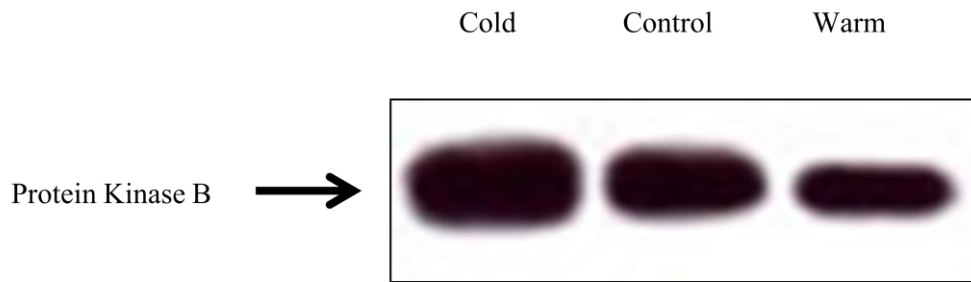
For loading controls during cell signalling Western blotting in this study, BioRad Protein Assay and Ponceau Red staining was used, however due to difficulties relating to the species studied, an antibody based loading control (e.g.  $\beta$  actin was not used.). It was therefore decided to place this part of the data in the appendix.



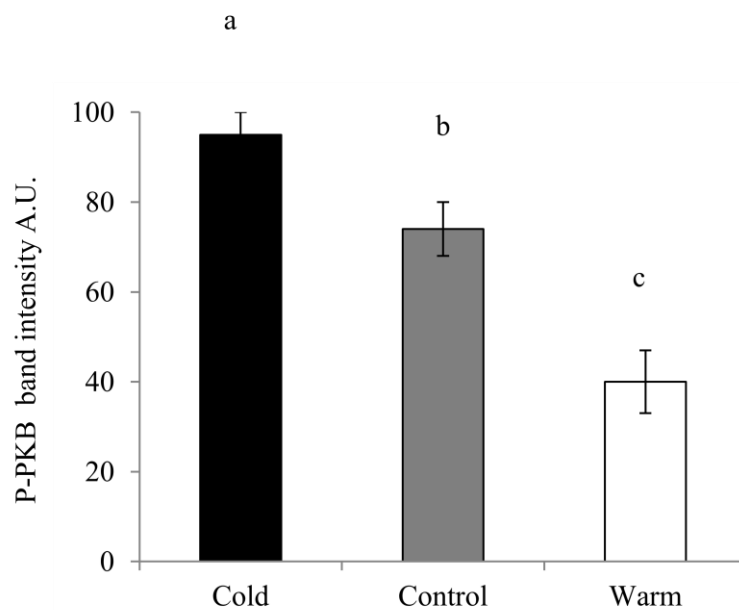
**Figure 8.6a** – Western blot of rainbow trout spongy layer tissue from three acclimation temperatures using anti - phosphorylated p38MAPK antibody.



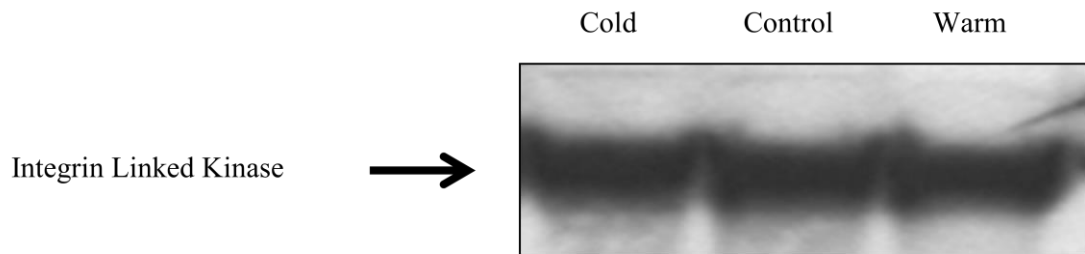
**Figure 8.6b** – p-p38 protein levels Cold acclimated fish had significantly greater phosphorylated p38 MAPK levels (92 A.U.  $\pm$ 7) compared to the control (50 A.U.  $\pm$ 8) and warm (28 A.U.  $\pm$ 5) acclimated groups representing a difference of 84% and 229% respectively. Data is presented on the graph as the mean of n = 9 for each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



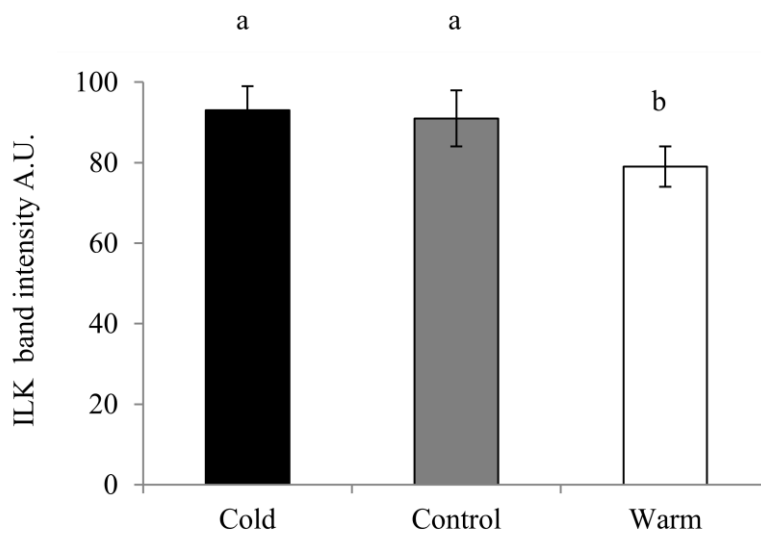
**Figure 8.7a** – Western blot of rainbow trout spongy layer tissue from three acclimation temperatures using anti - phosphorylated PKB/ AKT antibody.



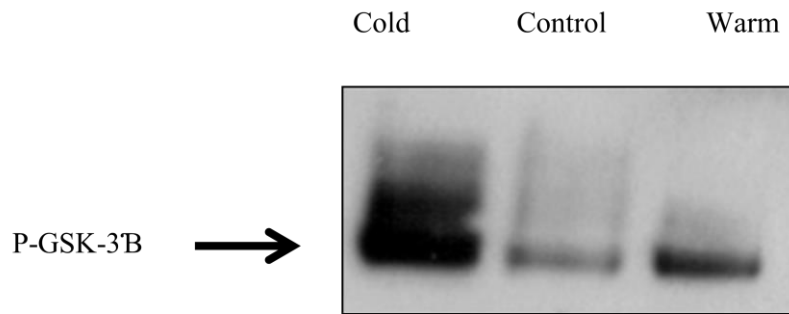
**Figure 8.7b** – p-PKB/ AKT levels. Cold acclimated fish had significantly greater phosphorylated Protein Kinase B levels (95 A.U. ±5) compared to the control (74 A.U. ±6) and warm (40 A.U. ±7) acclimated groups representing a difference of 28% and 138% respectively. Data is presented on the graph as the mean of n = 9 for each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



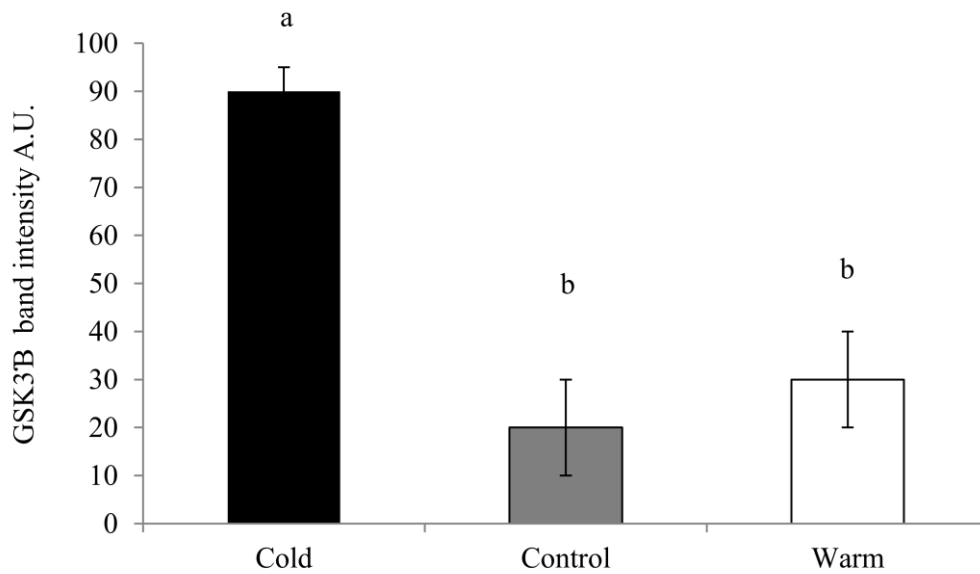
**Figure 8.8a** – Western blot of rainbow trout spongy layer tissue from three acclimation temperatures using anti - ILK antibody.



**Figure 8.8b** – ILK levels. Cold acclimated fish had significantly greater Integrin Linked Kinase levels (93 A.U. ±6) compared to warm (79 A.U. ±5) acclimated fish representing a difference of 17%. Data is presented on the graph as the mean of n = 9 for each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .



**Figure 8.9a – Western blot of rainbow trout spongy layer tissue from three acclimation temperatures using anti- phosphorylated GSK3B antibody.**



**Figure 8.9b – GSK3B levels.** Cold acclimated fish had significantly greater GSK3B levels (90 A.U. ±5) compared to warm (30 A.U. ±10) acclimated fish representing a difference of 200%. Data is presented on the graph as the mean of n = 9 for each acclimation group. Error bars represent the standard error of the mean. Dissimilar letters represent where significance as dictated by a one way ANOVA statistical test results in  $p < 0.05$ .

**END**