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The effect of hypothermia and rewarming on  
cardiac electrophysiology and mechanical  
function

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Submitted in fulfilment of the requirements for the degree of  
Doctor of Philosophy

May 2017

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## Abstract

Hypothermia is defined as a core body temperature of 35°C or less and can be induced (i.e. therapeutic) or accidental. It is well established that hypothermia leads to a positive inotropic response which causes an increase in the magnitude of cardiac contraction, however rewarming from hypothermia is associated with a negative inotropic response, and the underlying mechanisms of this remain unclear. Accidental hypothermia is further complicated by risk of ventricular arrhythmias and cardiac arrest. This contributes to high mortality rates among these patients. Although hypothermia is used extensively as a therapeutic intervention and survival is possible after extreme exposure, treatment of arrhythmias during rewarming is still challenging. In order to develop targeted anti-arrhythmic strategies in this very specific situation, we first need to understand the basis for pro-arrhythmia during cooling and rewarming. This study aimed to examine the effect of hypothermia and rewarming on aspects of cardiac inotropy and excitability.

An *in vitro* model of hypothermia and rewarming using isolated rat ventricular cardiomyocytes showed that following 3 hours of hypothermia there was a significant reduction in shortening upon rewarming. This was not accompanied by a change in intracellular  $Ca^{2+}$ , suggesting a rewarming induced decrease in myofilament sensitivity to  $Ca^{2+}$ . In separate experiments, animals underwent an *in vivo* hypothermia/rewarming procedure and displayed evidence of rewarming induced contractile dysfunction. Epicardial action potential (AP) measurements on these hearts showed a shortened AP duration (APD) when compared to normothermic control animals, which suggests that a sustained electrophysiological effect that could manifest as a shortened QT interval. In contrast to this, a period of transient hypothermia had alternative detrimental effects on the cardiac APD when compared to prolonged hypothermia, an effect that could predispose to the induction of long QT related arrhythmias and ventricular tachycardia.

Separate experiments assessed the effect of moderate (31°C) and severe (17°C) hypothermia on cardiac excitability in Langendorff perfused rabbit hearts. Moderate hypothermia prolonged PR and QT intervals whilst in severe hypothermia all ECG parameters were prolonged. Ventricular activation times were unaffected at 31°C whilst action potential duration (APD<sub>90</sub>) was significantly prolonged. At 17°C there were significant and proportionally similar delays in both activation and repolarisation. Ventricular fibrillation (VF) threshold was significantly reduced at 31°C (pro-arrhythmic), but at 17°C VF threshold was >2x baseline (37°C) (anti-arrhythmic). At 31°C, transverse conduction (CV<sub>t</sub>) was relatively insensitive to cooling versus longitudinal conduction (CV<sub>l</sub>) but at 17°C both CV<sub>t</sub> and CV<sub>l</sub> were proportionately reduced to a similar extent. The gap junction uncoupler heptanol had a larger relative effect on CV<sub>t</sub> than CV<sub>l</sub>, and at 31°C was able to restore the CV<sub>t</sub>/CV<sub>l</sub> ratio, returning VF threshold to baseline values. This suggests that moderate hypothermia creates repolarisation abnormalities and is pro-arrhythmic. These divergent effects appear to be linked to a lower temperature sensitivity of gap junctions, a conclusion supported by the anti-arrhythmic effect of heptanol at 31°C.

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## Acknowledgements

Firstly, my supervisor Professor Godfrey Smith, thank you for all the support, help and guidance you have given me. Thanks also to Dr Ole Kemi who encouraged me to apply for a PhD programme.

I would not have been able to carry out my experimental work without the help of Aileen Rankin and Mike Dunne, whose expertise has been invaluable throughout this project. I'd also like to thank all my colleagues in the Smith lab, especially Annabel for keeping me sane whilst writing up, and Allen for helping with proof reading.

My thanks to Professor Torkjel Tveita for allowing me the opportunity to spend time working at the University of Tromsø, also to Dr Timofei Kondratiev for help with the experimental work, and to Dr Erik Sveberg Dietrichs for making me feel so welcome on my visits.

Thanks to my friends and family for being there throughout my PhD, especially Jo, Leanne and Amanda. I feel like your scientific finding that a shot of alcohol cures hiccups deserves a thesis of its own.

A special thank you to my Dad. I couldn't have got here without all of your support and for that I am very grateful.

Finally, to my Mum. If I thought doing a PhD was challenging, it has been nothing in comparison to losing you. To say the last couple of years have been tough would be an understatement. Getting back to work and trying to find the focus to continue this work was not easy, and there were many days where I couldn't see how I was going to get to the end, but knowing the belief you had in me and how proud you would be to see me complete a PhD gave me the encouragement to continue, even on the toughest of days.

Thank you Mum, for everything, this one is for you.

## Author's Declaration

The *in vivo* surgery (Chapter 3) was carried out by Dr Timofei Kondratiev at the University of Tromsø. All other experimental work described in this thesis was carried out by myself and has not been presented as part of any other degree.

### Publications:

Erik Sveberg Dietrich E.S; McGlynn K.P; Allan A; Connolly A; Bishop M; Burton F; Myles R; Tveita T; Smith G.L. **The differential effects of hypothermia on cardiac conduction and excitability.**

# Abbreviations

AM	Acetoxymethyl ester
AP	Action potential
APD	Action potential duration
APD <sub>50</sub>	Action Potential Duration at 50% Repolarisation
APD <sub>75</sub>	Action Potential Duration at 75% Repolarisation
APD <sub>90</sub>	Action Potential Duration at 90% Repolarisation
AVN	Atrio-ventricular node
BDM	2,3-butanedione monoxime
cAMP	Cyclic adenosine monophosphate
CICR	Ca <sup>2+</sup> induced Ca <sup>2+</sup> release
CO	Cardiac output
cTnI	Cardiac troponin I
CV	Conduction velocity
CV <sub>l</sub>	Longitudinal conduction velocity
CV <sub>t</sub>	Transverse conduction velocity
DAD	Delayed afterdepolarisations
DHCA	Deep hypothermic circulatory arrest
DMSO	Dimethyl sulphoxide
Dn90	90% of sarcomere length relaxation
EAD	Early afterdepolarisations
E-C	Excitation-contraction
EDV	End diastolic volume
ESV	End systolic volume

FS	Fractional shortening
HR	Heart rate
$I_{CaL}$	L-type $Ca^{2+}$ current
$I_{KATP}$	ATP-Sensitive $K^+$ current
$I_{Kir}$	Inward rectifier $K^+$ current
$I_{Ks}$	Slow delayed rectifier $K^+$ current
$I_{Na}$	Sodium current
LA	Left atrium
LED	Light-emitting diode
LTCC	L-type $Ca^{2+}$ channel
LV	Left ventricle
MAP	Mean arterial pressure
NCX	$Na^+ / Ca^{2+}$ exchange
PCr	Creatine phosphate
Pes	End systolic pressure
Pi	Inorganic phosphate
PKA	Protein kinase A
PMCA	Plasma membrane $Ca^{2+}$ ATPase
RA	Right Atrium
ROS	Reactive oxygen species
RV	Right Ventricle
RyRs	Ryanodine receptors
SA	Sino-Atrial
SERCA	Sarco-Endoplasmic Reticulum $Ca^{2+}$ -ATPase
SV	Stroke volume



SW	Stroke work
VF	Ventricular fibrillation
$V_m$	Transmembrane potential

# Chapter 1: Introduction

Normal thermoregulation involves a dynamic balance between heat production and control of heat loss, with the aim of providing a constant core temperature. In humans, body temperature is controlled by the thermoregulatory centre in the hypothalamus. It receives input from two sets of thermoreceptors; receptors in the hypothalamus itself monitor the temperature of the blood as it passes through the brain (core temperature), whilst receptors in the skin monitor the external temperature. The thermoregulatory centre sends impulses to several different effectors to adjust body temperature. In humans, a normal adult core temperature ranges from 36.5°C to 37.5°C and temperatures out with this narrow range are poorly tolerated (Connolly and Worthley, 2000).

## Hypothermia

Hypothermia is defined as a core body temperature of 35°C or less and can be induced (i.e. therapeutic) or accidental.

## Stages of hypothermia

Guidelines distinguishing the depth or stages of hypothermia have differed between authors, and as a result there are various definitions within the published literature. It was initially suggested that hypothermia should be considered as either three stages; mild (35-32°C), moderate (32-22°C) and deep (22-8°C) (Popovic and Popovic, 1974) or four stages; mild (35-32°C), moderate (31-26°C), deep (25-20°C) and profound (19-14°C) (Wong, 1983). More recently it has been proposed that mild (35-32°C), moderate (32-28°C) and severe (<28°C) are more appropriate classifications, and these are currently used by the European Resuscitation Council (Moss, 1986; Soar et al., 2010). In slight contrast, the American Heart Association has arbitrarily adopted the definition of Polderman *et al* which states mild hypothermia as temperatures down to 34°C, moderate 34-30°C and severe <30°C (Polderman and Herold, 2009; Vanden Hoek et al., 2010). A single measurement of core temperature is often used to classify hypothermia as mild, moderate or severe. Frequently, the treatment of hypothermia patients depends on the severity level, therefore as our understanding of the pathophysiological mechanisms of hypothermia

improves it is important to have a general consensus on the stages so that treatment is appropriately targeted.

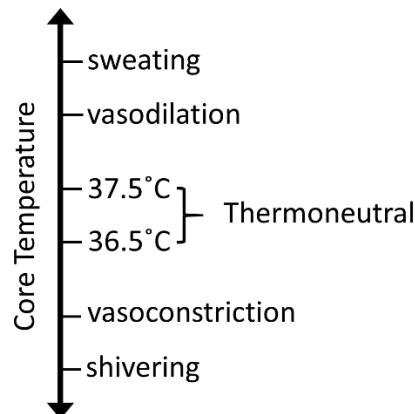
## **Accidental hypothermia**

Accidental hypothermia is traditionally referred to as the unintentional lowering of body temperature to below 35°C. The causes of accidental hypothermia are multifactorial and can be sub-divided based on the circumstances surrounding the cooling. Acute hypothermia occurs when there is a sudden and severe exposure to a cold stress which is so great that the body's intrinsic heat production is overwhelmed and the body cools before energy reserves are exhausted. In these circumstances the victim will rewarm once removed from the cold stress. This is most commonly seen following immersion in cold water, or being hypothermic whilst under the influence of alcohol in low ambient temperatures (Lloyd, 1979). Second is subacute (exhaustion) hypothermia. In this situation the cold stress is less severe, and cooling only occurs when energy reserves are exhausted. Spontaneous rewarming is less certain in these circumstances and therefore every route of heat loss must be prevented. This type of hypothermia is most commonly found in climbers exposed to the elements (moderate cold and wind/rain), hikers, in endurance sport or people who have been immersed in warmer water. Third is chronic hypothermia, where a person has been exposed to a moderate cold stress for a prolonged period of time (days). This is typically found in the elderly, where core temperature decreases over time. Finally, there is submersion hypothermia, where hypothermia occurs as a result of full body immersion in cold water. A number of reports have shown survival is possible in patients without oxygen for up to 60 minutes following submersion (Lloyd, 1996; Avellanas et al., 2012).

## **Physiological effects of hypothermia**

Hypothermia has profound systemic effects that involve many of the body's vital organs. The initial effect of hypothermia involves a sympathetic response that causes vasoconstriction followed by shivering, an involuntary muscular activity

that enhances heat production in an attempt to restore homeostasis (Figure 1.1).



**Figure 1.1** The process of thermoregulation.

In humans, core temperature is normally maintained within a tight range (36.5-37.5 °C) known as the thermo-neutral zone. When core temperature falls, thermoregulatory responses such as vasoconstriction and shivering are triggered. When temperatures rise above this range, vasodilation and sweating are typical homeostatic responses

There are numerous physiological effects of hypothermia, some of the non-cardiac ones are described in Table 1.1. Briefly, in mild hypothermia, patients may have vigorous shivering and cold white skin. As temperatures drop to moderate hypothermia, there may be evidence of mental status changes such as amnesia, confusion and apathy in addition to reduced shivering, slurred speech and loss of fine motor skills. Most severely hypothermic patients have no shivering and present with hallucinations, fixed dilated pupils, bradycardia, hypotension and pulmonary oedema (Mallet, 2002).

## Cardiovascular

Whilst the brain is protected from the effects of lack of oxygen during hypothermia, survival is dependent on other factors, namely having sufficient cardiac function to maintain adequate perfusion. In mild hypothermia there is an initial tachycardia and peripheral vasoconstriction alongside an increased myocardial oxygen consumption resulting in an increased cardiac output (CO). As temperature drops there is a progressive decrease in heart rate (HR) and CO. Experimental work in dogs has shown that at 25 °C, HR is reduced by almost 60% (Ohmura et al., 1979) alongside a 65% reduction in CO (Tveita et al., 1994).

**Table 1.1: Physiological effects of hypothermia**

	Core Temp (°C)	Respiratory	Central Nervous System	Neuromuscular
Mild	35-32	Initial tachypnea then progressive decrease in respiratory function; bronchospasm	Depression of cerebral metabolism; amnesia, impaired judgement; maladaptive behaviour	Ataxia; shivering
Moderate	32-28	Hypoventilation; loss of protective airway reflexes; 50% decrease in O <sub>2</sub> consumption	Hallucinations; EEG abnormalities; paradoxical undressing	Decreased reflexes; rigidity
Severe	<28	Pulmonary oedema; 75% decrease in O <sub>2</sub> consumption	Decline in cerebral blood flow; global loss of reflexes; further decrease in EEG activity	No motion; decreased nerve-conduction velocity; absent reflexes

Adapted from (Danzl and Pozos, 1994; Petrone et al., 2014)

The electrocardiographic (ECG) features of hypothermia can be nonspecific but can include prolongation of PR and QT intervals and can also include classic J (Osborn) waves in >50% of patients (Mustafa et al., 2005). The J wave, seen as an upward deflection at the junction of the QRS complex and the ST segment is not specific to hypothermia and can be found in a number of cardiac arrhythmia syndromes e.g. Brugada syndrome (Antzelevitch and Yan, 2015). Both atrial and ventricular arrhythmias can occur in hypothermia and asystole commonly occurs below 20 °C (Kempainen and Brunette, 2004).

## Epidemiology of accidental hypothermia

Media reports of accidental hypothermia will typically focus on outdoor enthusiasts who have been exposed to cold, harsh conditions in remote settings.

However, there are in fact two main sub-divisions people with accidental hypothermia fall into:

Firstly, those otherwise healthy individuals who suffer hypothermia due to exposure to extreme cold, wet or windy conditions often as a result of excessive exposure to snow, water or altitude. A 10 year review of accidental hypothermia in the French Alps found that of 48 patients admitted with severe hypothermia (16-28°C), 56% were as a result of exposure to cold environment, 27% as a result of avalanche and the remaining 17% from immersion in cold water (Debaty et al., 2015). These circumstances are relatively uncommon, however the temperatures involved can be very severe. The lowest recorded core temperature from which someone survived was as a result of exposure to extreme weather conditions. A skier survived with a good neurological outcome following 7 hours of circulatory arrest and a core temperature of 13.7°C following an accident in Northern Norway (Gilbert et al., 2000).

Secondly, and perhaps under-appreciated is the frequency with which hypothermia occurs in urban areas, particularly amongst the elderly, and those socially and economically challenged. The elderly are often immobile, either due to trauma or infirmity and often have a decreased muscle mass, which in combination with an age related decline in thermoregulatory capacity make them high risk candidates for hypothermia (Collins et al., 1977; Reuler, 1978). Two studies examining the incidence of accidental hypothermia in urban areas of Scotland found the average age of patients to be 69-76 years old (Ledingham and Mone, 1980; Rankin and Rae, 1984).

Those who are socially deprived are also at risk from hypothermia, this could be as a result of inadequate/no housing, or substance abuse. In Japan, hypothermia is the leading cause of death amongst the homeless irrespective of age (Suzuki et al., 2013). Substance abuse, in particular alcohol is commonly reported alongside hypothermia. A study examining hypothermia as a cause of death in British Columbia found during a 14 year period that over 40% of patients were under the influence of alcohol when hypothermia occurred (Stares and Kosatsky, 2015).

Contrary to what many believe, hypothermia does not only occur in cold countries. Whilst there is a much higher incidence in cold climates, hypothermia can also occur in more temperate regions. De Souza (2007) reported that in one

hospital in Sao Paulo, Brazil, 59 patients were admitted with hypothermia between 1988 and 2003, despite a tropical climate with mild annual average temperatures of 20-25°C. Similarly, between 2001 and 2005 there were 24 cases of fatal hypothermia in Sydney, Australia (Lim and Duflou, 2008), further highlighting the need for milder countries to be aware of the risk of hypothermia.

### **Rewarming strategies**

It is a priority to stabilise and rewarm victims of accidental hypothermia. The method of rewarming will depend on the stage and circumstances surrounding the hypothermic patient and there are various methods available (Table 1.2). For patients with mild hypothermia, passive external warming is usually sufficient, however as hypothermia becomes more severe then active methods of rewarming become necessary. For patients with a core temperature <28°C and cardiac arrest, the American Heart Association recommend extracorporeal warming (Vanden Hoek et al., 2010). Patients should therefore always be transported to the closest available centre based on their rewarming needs. Of 84 patients admitted to one hospital in the Netherlands, 14 different methods were used (van der Ploeg et al., 2010). This highlights the differing nature/circumstances surrounding accidental hypothermia and how each patient requires specialised treatment.



**Table 1.2: Rewarming techniques for hypothermia**

Passive	Removal from environmental exposure Dry patient Warm environment Blanket/clothing insulation
Active external	External convection heaters Immersion in heated water bath Heating pads, warm water bottles
Active internal	Heated IV solutions Peritoneal lavage Warmed inhalational agents
Extracorporeal	Cardiopulmonary bypass Haemodialysis

(Adapted from: Gregory et al., 1991; Daanen and Van de Linde, 1992; Danzl and Pozos, 1994)

## Mortality

Whilst not a common cause of death, accidental hypothermia still accounts for significant mortality. It has been reported that hypothermia is involved in more than 1500 deaths annually in the United States (Brown et al., 2012), whilst official figures from death certificates in Scotland state hypothermia is responsible for approximately 40 per year. There is a lot of variation in reported death rates following accidental hypothermia, with mortality rates between 17 and 69% (Ledingham and Mone, 1980; Farstad et al., 2001; Silfvast and Pettilä, 2003; van der Ploeg et al., 2010; Debaty et al., 2015). A possible reason for such variation in the mortality rates is that it is an inhomogeneous population affected by hypothermia, severity and rewarming methods can vary, and it can be recorded as the primary or secondary (to an underlying condition) cause of death.

## Therapeutic hypothermia

In contrast to accidental hypothermia, therapeutic hypothermia is a clinician driven modality aimed at *intentionally* decreasing a patient's core body temperature. The idea of using hypothermia therapeutically is not a new concept and has been around for centuries, however many abandoned the idea due to adverse effects (Alzaga et al., 2006). The current clinical application of therapeutic hypothermia stems in part from experimental observations of Bigelow and associates in the 1950s. Their work demonstrated a potential beneficial effect of therapeutic hypothermia for the brain and heart during cardiac surgery in a canine and monkey model (Bigelow et al., 1950; Bigelow and McBirnie, 1953). More recently, mild hypothermia (34°C) was shown to have beneficial neurological outcomes, without deleterious effects, if induced immediately post cardiac arrest in dogs. However, severe hypothermia (15°C) was shown to have the opposite effect, and could worsen cardiovascular and neurological outcome (Weinrauch et al., 1992).

Experimental research into therapeutic hypothermia continued from these studies, however it was the findings from two prospective, randomised controlled clinical trials in 2002 that led to a shift in treatment recommendations. Both looked at the effect of mild - moderate hypothermia in patients with anoxic brain injury that had suffered cardiac arrest. These trials, which were the largest to date, included several hundred patients and found a significant neurological protection in patients who were cooled to 32-34°C (Bernard et al., 2002; Holzer et al., 2003). Based on these trials the American Heart Association and European Resuscitation Council recommended therapeutic hypothermia in such patients due to the favourable outcome. Recent meta analyses strongly support the neuroprotective effect of mild-moderate hypothermia (Arrich et al., 2016; Schenone et al., 2016). The beneficial effect of hypothermia as a neuro-protectant is the result of a reduction in tissue oxygen consumption and metabolic demand. The basal metabolic rate falls by ~7-8%/°C and so reaches 75-85% of normal in mild hypothermia and 65-75% of normal at moderate hypothermia (Reuler, 1978).

Therapeutic hypothermia is also used to varying degrees in other conditions such as aortic arch surgery. Cerebral protection has been the cornerstone of successful aortic arch surgery for almost 40 years and deep hypothermic circulatory arrest (DHCA), with temperatures as low as 15-20°C has been a main strategy to achieve this (Di Luozzo and Griep, 2012). Cooling the brain down to hypothermic temperatures is sufficient to reduce brain metabolic requirements to such an extent that blood flow can be completely interrupted. Most notably, DHCA offers surgeons a bloodless operating field and extended surgical time limit while meeting the body's high metabolic demands. Whilst other perfusion techniques have been developed, many clinicians have favoured hypothermia as the preferred technique due to the relative ease at which it can be carried out. It provides cerebral protection whilst minimising problems with perfusion techniques. Its use has been supported by follow up studies involving more than 400 patients which yielded positive clinical results, with low rates of side effects (such as stroke and seizures) accompanied by a good cognitive function (Gega et al., 2007; Percy et al., 2009).

Despite positive findings, it has also emerged that timing of DHCA is important, with longer periods of hypothermia showing evidence of temporary neurologic dysfunction (Tang et al., 2013). Indeed, direct measurement of the Q10 temperature coefficient for the cerebral metabolic rate in human beings showed that the safe limit of DHCA at 15°C was only 30 minutes and that it was only 20 minutes at 20°C. The investigators in this study showed that maintaining a patient at 18°C for longer than 25 mins decreased memory and fine motor function and prolonged hospital stays (McCullough et al., 1999). Evidence would suggest that DHCA is therefore beneficial if it is performed within its safe limits however for more prolonged surgeries DHCA should be used in conjunction with other operative procedures.

In recent years there has been growing interest into the beneficial effects of therapeutic hypothermia following ischaemic stroke. The protective mechanisms of hypothermia affect the ischaemic cascade across several parallel pathways and it is therefore a condition where cooling may increase positive outcomes.

Animal studies have reported favourable results in both focal and global ischaemia (van der Worp et al., 2007; van der Worp et al., 2010). However, the impact on patients with ischaemic stroke has still to be fully explored. Its transition to human trials presents many challenges, including time of initiation relative to stroke onset, and also depth and duration of hypothermia (Zhang et al., 2013). Recently a large scale trial looked to replicate the beneficial findings reported in small pilot studies. Only 120 patients of the proposed 1600 were enrolled before the trial was stopped. Despite them reporting beneficial outcomes in the small sample size, mortality rates in the hypothermic group were double that in the control group (15.9% vs 8.8%) with pneumonia being a major cause of death (Lyden et al., 2016). Therefore, evidence suggests that the use of therapeutic hypothermia in stroke remains debatable and more work is required to bridge the knowledge gap between experimental and human trials (Tahir and Pabaney, 2016).

Traumatic brain injury represents a significant challenge to healthcare, and is a leading cause of death and permanent disability (Maas et al., 2008). Therapeutic hypothermia has been well established as a treatment in this scenario for over 40 years (Fox et al., 2010; Sadaka and Veremakis, 2012). It is based on the principle that cooling controls dangerously high intra-cranial pressure and mediates damage to neural tissue from hypoxic and metabolic mechanisms. However in recent years there has been growing evidence that therapeutic hypothermia is associated with unfavourable long term outcomes when applied clinically (Crossley et al., 2014; Honeybul, 2016). This has been shown in both meta-analysis of previous studies (Shaefi et al., 2016) and also in recent large scale clinical trials (Andrews et al., 2015), which was stopped early when it was shown that hypothermia did not improve clinical outcome.

Whilst *in vivo* studies have found favourable effects of therapeutic hypothermia, they have often not translated to clinical work. This highlights the important point that ultimately the full effectiveness of cooling cannot be completely understood until there are more robust, randomised clinical trials with large volumes of patients.

## Temperature and cell activity

Thermoregulation is the fine balance between heat production and heat elimination. This active equilibrium, which maintains the body's core temperature at  $\sim 37^{\circ}\text{C}$  allows the cellular enzyme systems to function within a narrow optimum metabolic window. Changes in temperature which affect physiological processes and can be quantified by  $Q_{10}$  (temperature coefficient), which describes the amount by which the processes change in response to a  $10^{\circ}\text{C}$  increase in temperature (the more temperature dependent a process is; the higher the  $Q_{10}$  value).

The cardiac excitation and contractile processes are markedly influenced by changes in temperature (Langer and Brady, 1968). Under normal circumstances the activity of channels, pumps and exchangers are tuned to  $37^{\circ}\text{C}$  but when temperature decreases, so too does the relative activity. However this change is not uniform,; gating kinetics (i.e. where the conformation of a channel changes through activation or inactivation) and exchangers (e.g.  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, (Puglisi et al., 1996)) can have a range of  $Q_{10}$  values, with some aspects more temperature dependent than others. There is also evidence of temperature-dependent slowing of the kinetics of a number of pumps and ionic currents ( $I_{\text{Ca}}$ ,  $I_{\text{Na}}$ ,  $I_{\text{K}}$ ) (McDonald and Trautwein, 1978; Eisner and Lederer, 1980; Colatsky, 1980; Cavalié et al., 1985; Kiyosue et al., 1993; Puglisi et al., 1996). Due to the complex processes involved, both  $\text{Ca}^{2+}$  handling mechanisms (Shattock and Bers, 1987) and myofilament  $\text{Ca}^{2+}$  sensitivity contractile properties (Harrison and Bers, 1989; Kusuoka et al., 1991; Stehle et al., 2002) are therefore highly temperature dependent.

## Excitation Contraction Coupling

Excitation contraction (E-C) coupling involves the cellular processes from the electrical signal through to the mechanical contractile response. Following the electrical excitation from the action potential (AP), the sarcolemma including the transverse tubules (t-tubules) are depolarised resulting in the activation of the voltage sensitive L-type  $\text{Ca}^{2+}$  channels (LTCC). These LTCC are located

primarily at the junction of the sarcolemma and sarcoplasmic reticulum (SR), the internal cardiomyocyte  $\text{Ca}^{2+}$  store. This inward  $\text{Ca}^{2+}$  current activates the nearby ryanodine receptors (RyRs) to release  $\text{Ca}^{2+}$  from the SR, in a process known as  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release (CICR). This graded increase in free intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]$ ) allows for more binding of  $\text{Ca}^{2+}$  to troponin C, which initiates contraction of the myofilaments through the conformational change that occurs in the actin-myosin complex leading to cross bridge formation. When this occurs in a co-ordinated fashion, the concurrent increase in  $\text{Ca}^{2+}$  leads to contraction of the whole cell, and when it occurs across the myocardium is the basis for systole (Calaghan and White, 1999; Bers, 2002).

During the relaxation phase, intracellular levels of  $\text{Ca}^{2+}$  must decline, allowing the dissociation of  $\text{Ca}^{2+}$  from troponin C, thus ensuring relaxation of the myocardium and consequent filling of the heart. The main process by which  $\text{Ca}^{2+}$  is re-sequestered within the cell is by the SR through the SR  $\text{Ca}^{2+}$  ATPase (SERCA), which in exchange for adenosine tri-phosphate (ATP) is able to uptake  $\text{Ca}^{2+}$  from the cytosol. Whilst SERCA is the predominant mechanism by which  $\text{Ca}^{2+}$  is removed from the cytosol (~70%), other mechanisms also contribute; the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange (NCX) (~28%), where high cytoplasmic  $\text{Ca}^{2+}$  initiates an outward  $\text{Ca}^{2+}$  and an inward  $\text{Na}^+$  flux, whilst the plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA) and the mitochondrial  $\text{Ca}^{2+}$  uniporter account for ~1% each (D. M. Bers, 2002).

Cooling has been shown to have multiple effects on EC-coupling mechanisms, including but not limited to; increasing AP duration (APD) (Shattock and Bers, 1987), slowing rate of NCX (Puglisi et al., 1996); reducing the magnitude of the L-type  $\text{Ca}^{2+}$  current (Cavalié et al., 1985), and increasing the open probability of SR  $\text{Ca}^{2+}$  release channels (Sitsapesan et al., 1991).

### **PKA signalling**

The heart provides an example of how two signalling pathways that are involved in elevating the levels of two intracellular second messengers, cyclic AMP (cAMP) and  $\text{Ca}^{2+}$ , can interact physiologically. As described, in response to depolarisation,  $\text{Ca}^{2+}$  enters the cytoplasm and triggers  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release. This influx of  $\text{Ca}^{2+}$  triggers the release of intracellular stores of  $\text{Ca}^{2+}$  from the SR,

and the ensuing intracellular  $\text{Ca}^{2+}$  transient results in activation of the myofilaments.

Beta-adrenergic stimulation has a positive inotropic effect on the  $\text{Ca}^{2+}$  handling in cardiac myocytes by activating adenylyl cyclase to produce cyclic-adenosine monophosphate (cAMP), which activates PKA. This kinase phosphorylates several proteins related to EC-coupling, including L-type  $\text{Ca}^{2+}$  channels, RyR and troponin I (Bers, 2002). On phosphorylation of the LTCC by PKA,  $\text{I}_{\text{Ca}}$  is increased (Bers, 2001; Reuter, 1987) increasing cytosolic  $\text{Ca}^{2+}$ , SR  $\text{Ca}^{2+}$  content and thereby increasing the amplitude of the  $\text{Ca}^{2+}$  transient (Bers, 2002).

The ryanodine receptor (RyR2) is also a target of PKA - this increases RyR2 sensitivity to  $\text{Ca}^{2+}$  (Bers, 2001) and further facilitates CICR and therefore increases cytosolic  $[\text{Ca}^{2+}]$  (Bers, 2002). This increase in systolic  $\text{Ca}^{2+}$  is largely responsible for the positive inotropic response to  $\beta$ -AR stimulation. SERCA2 is also a target for PKA phosphorylation following  $\beta$ -AR stimulation. Phospholamban (PLB) - an inhibitory protein which inhibits SERCA2 in the unphosphorylated state - relieves inhibition of SERCA2 when phosphorylated by PKA. This increases reuptake of  $\text{Ca}^{2+}$  from the cytoplasm and generates the positive lusitropy caused by  $\beta$ -AR stimulation. It also contributes to positive inotropy - a combination of increased systolic  $\text{Ca}^{2+}$  and increased uptake by SERCA2 increases the SR  $\text{Ca}^{2+}$  content (Bers, 2002).

cTnI is a key regulatory protein in cardiac muscle contraction and relaxation, and phosphorylation by PKA results in a decrease in the sensitivity of the contractile apparatus to  $\text{Ca}^{2+}$ . After phosphorylation the half-maximal ATPase activity and half-maximal tension are achieved at higher  $\text{Ca}^{2+}$  concentrations, an effect that may be due to decreased affinity of troponin I to troponin C (Filatov et al., 1999). Indeed the phosphorylation at specific sites (Ser23/24) on cTnI has been shown to be mechanistically involved in reduced myofilament sensitivity in various pathological situations (Gao et al., 1995; Tavernier et al., 2001; Chen and Ogut, 2006).

## Inotropic dysfunction

When normal EC coupling is disrupted it can cause a cascade of inotropic defects leading to reduced force production and a weakened contraction. An example situation where this occurs is in the disruption of normal cardiac metabolism e.g. following hypoxia or ischaemia (Allen and Orchard, 1987).

The metabolic demands of the heart are the highest of any organ in the body, and normal cardiac metabolism is required to fuel contractile function and viability. More than 95% of adenosine triphosphate (ATP), the immediate energy source, is generated by oxidative phosphorylation in the mitochondria. This ATP re-phosphorylates creatine to generate creatine phosphate (PCr), which then shuttles the metabolic energy to the sites of energy consumption (primarily the contractile proteins) where PCr is used to phosphorylate local adenosine diphosphate (ADP) via creatine phosphokinase. If aerobic metabolism is prevented, e.g. by hypoxia or malfunctioning mitochondria then ATP can be supplied anaerobically for a limited period of time. However, this switch from aerobic to anaerobic metabolism has subsequent consequences for the heart; a rapid fall in PCr levels (due to a mismatch in supply and consumption) and also a corresponding increase in inorganic phosphate (Pi) (Allen et al., 1985; Kusuoka et al., 1986) and production of lactic acid (Elliott et al., 1992), leading to an intracellular acidosis.

It has been known for some time that metabolic dysfunctions can lead to contractile dysfunction (Cingolani et al., 1970; Fabiato and Fabiato, 1978; Poole-Wilson and Langer, 1979; Eisner et al., 1987; Jeffrey et al., 1987). This effect has been linked to an impairment in  $\text{Ca}^{2+}$  release from the SR as well as blockade of  $\text{Ca}^{2+}$  channels in the sarcolemma (Fabiato and Fabiato, 1978; Poole-Wilson and Langer, 1979) although there is also evidence to support a myofilament  $\text{Ca}^{2+}$  sensitivity (Marban and Kusuoka, 1987; Orchard et al., 1991).



## Arrhythmias

Under normal circumstances, electrical conduction of the heart involves a propagating AP which will stimulate the atria and ventricle in a co-ordinated manner, continuing until refractory tissue is encountered (Weiss et al., 2010). However, abnormalities in electrical activity can lead to disrupted impulse propagation. Cardiac arrhythmias are clinically of high importance, as the compromised mechanical activation can lead to circulatory impairment and potentially to sudden cardiac death (Chugh et al., 2008).

Cardiac arrhythmias are generally produced by one of three mechanisms;

- i. Automaticity; enhanced automaticity can increase the rate at which an AP will be generated.
- ii. Triggered activity; resulting from premature activation of cardiac tissue either due to early afterdepolarisations (EADs), which correspond to phase 2 and 3 of the AP and are usually associated with a prolonged APD; or delayed afterdepolarisations (DADs) which occur in phase 4 when the membrane potential has returned to baseline.
- iii. Re-entry; where an AP doesn't terminate and re-excites regions that have recently repolarised (Antzelevitch et al., 2003) .

Arrhythmias are generally considered to have separate *trigger* and arrhythmogenic *substrate* entities (Coumel, 1993). Triggers can be EADs or DADs, which can lead to ectopic beats, whilst the substrate refers to some abnormality which predisposes to re-entry, e.g. increased dispersion of conduction or repolarisation or scar/fibrosed tissue (Antzelevitch and Burashnikov, 2011).

Clinically, long QT syndrome is a common finding in therapeutic hypothermia (Mattu et al., 2002; Khan et al., 2010; Kim et al., 2014; Salinas et al., 2015). The prolongation in APD associated with long QT syndrome may be a pro-arrhythmic substrate which can increase the propensity for afterdepolarisations (trigger).

Both polymorphic ventricular tachycardia or fibrillation and Torsades de pointes may develop in this setting, and are known complications of long QT syndrome (Weiss et al., 2010). Recent studies have shown that in temperatures associated with therapeutic hypothermia there are numerous electrophysiological changes to the heart including; enhanced dispersion of repolarisation (Piktel et al., 2011; Hsieh et al., 2009; Salama et al., 1998), slowing of conduction (Fedorov et al., 2008) and APD alternans (Hsieh et al., 2009). The enhanced heterogeneity of repolarisation that has been shown in hypothermia is associated with increased incidence of arrhythmias and re-entrant excitation. With the widespread use of therapeutic hypothermia, it is important to have a thorough understanding of the electrophysiological effects so that appropriate treatment can be given to patients.

## Aims

The main aim of this thesis was to study the effect of hypothermia and rewarming on cardiac electrophysiology and mechanical function. Hypothermia affects many aspects of cardiac function. Using temperatures that commonly occur in both therapeutic and accidental hypothermia, this thesis aimed to examine two main areas;

- 1.) Inotropy
- 2.) Excitability

The specific aims of this work were:

- i. To study the characteristics of electrophysiology and mechanical function in isolated Langendorff perfused hearts following *in vivo* hypothermia and rewarming.
- ii. To investigate whether transient hypothermia affects cardiac electrophysiology by the same mechanisms as long term cooling.
- iii. To study an *in vitro* model of hypothermia and assess contractile response to hypothermia and rewarming in isolated cardiomyocytes
- iv. To assess temperatures and mechanisms that may lead to hypothermia induced arrhythmias.
- v. To study whether pharmacological gap junction uncoupling can alter conduction and VF threshold during moderate hypothermia.

## Chapter 2: General Methods

## **Animals**

Animals used at the University of Glasgow (Chapters 4, 5 and 6) were killed in accordance with Home Office Guidelines of the Animals (Scientific Procedures) Act 1986.

Animals used at the University of Tromsø (Chapter 3) were killed in accordance with the Norwegian Animal Research Authority guidelines and experimental protocols were conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.III.1986).

## **Physiological extracellular solutions**

All experiments performed on isolated cardiomyocytes used a modified Krebs solution. For whole heart (rat and rabbit) experiments, a modified Tyrode's solution was used. All chemicals were obtained from Sigma-Aldrich (Saint Louis; MO, USA) unless otherwise stated.

### **Modified Krebs solution**

120 NaCl, 20 HEPES, 5.4 KCl, 0.52 NaH<sub>2</sub>PO<sub>4</sub>, 3.5 MgCl<sub>2</sub>·6H<sub>2</sub>O, 20 taurine, 10 creatine and 11.1 glucose (all concentrations in mM). Solution pH adjusted to 7.4 at 37 °C using NaOH.

### **Modified Tyrodes solution**

93 NaCl, 20 NaHCO<sub>3</sub>, 1 Na<sub>2</sub>HPO<sub>4</sub> (anhydrous), 1 MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 KCl, 25 Glucose, 20 C<sub>2</sub>H<sub>9</sub>NaO<sub>5</sub>, 1.8 CaCl<sub>2</sub> (all concentrations in mM). Solution continuously bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> to maintain pH 7.4. Before being used all Tyrode's solution was filtered through a 5µm filter membrane (Millipore; Billerica, MA, USA).

**Kraft-Bruhe (KB) solution**

70 KOH, 40 KCl, 50 L-glutamic acid, 20 taurine, 20 KH<sub>2</sub>PO<sub>4</sub>, 3 MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 glucose, 10 HEPES, 0.5 EGTA, pH 7.2 with KOH

**Langendorff perfused whole heart configuration****Rabbit**

Male New Zealand White rabbits (2.5-3kg) were euthanised with an intravenous (I.V) injection of 0.5ml/kg Na pentobarbital (200 mg/ml, Euthatal) mixed with 500IU of heparin via the left marginal ear vein. Hearts were quickly excised once absence of pain reflexes were confirmed, and placed in ice cold Tyrode's solution. Excess tissue was trimmed away and the heart was connected to a Langendorff perfusion system (Figure 2.1) and perfused with oxygenated Tyrode's (37°C) at a constant flow rate of 30ml/min (Skrzypiec-Spring et al., 2007; Bell et al., 2011). The retrograde flow closes the aortic valve and as a result the perfusion solution is displaced via the ostia into the coronary arteries. To ensure adequate perfusion of the coronary vessels, pressure was measured throughout all experiments using a calibrated pressure monitor (Pressure Monitor BP1, World Precision Instruments; Sarasota, FL, USA). The system was primed with Tyrode's solution prior to starting to ensure no bubbles were present which could compromise the coronary vessels.

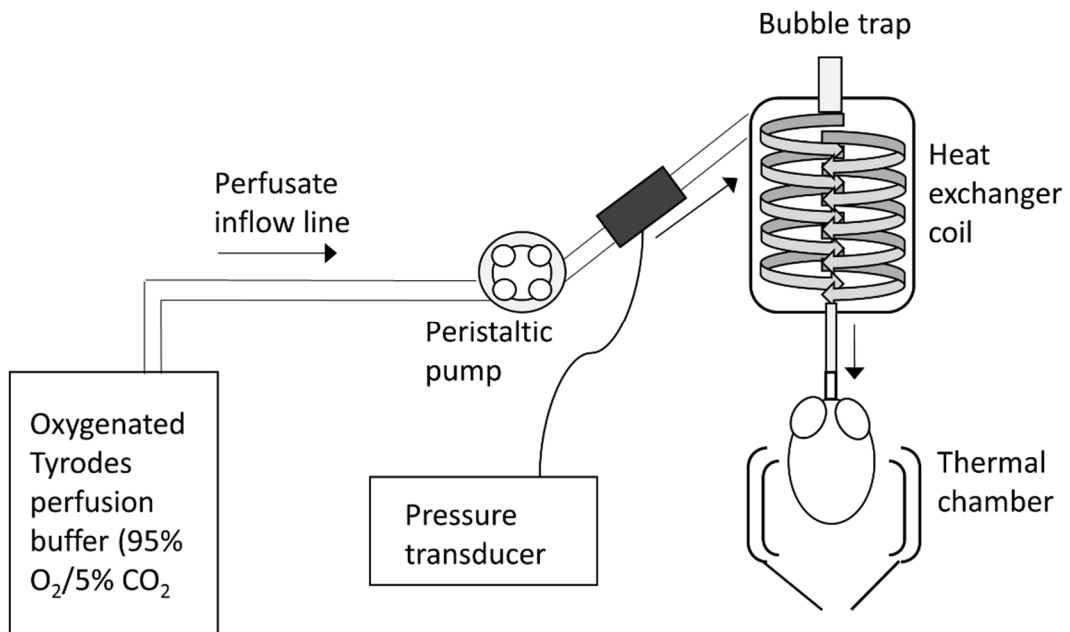


Figure 2.1 Schematic of Langendorff perfusion set up.

## Rat

The preparation for the rat Langendorff was similar to the rabbit. Male Wistar rats (250g-300g) were stunned with a blow to the head followed by a cervical dislocation, causing both ascending and descending injury to the spinal cord and brain stem. The xiphoid process was identified and the thoracic cavity was opened to allow the rapid removal of the heart which was subsequently placed in ice cold perfusion buffer (Krebs/Tyrode's) containing 25IU/ml heparin for anti-coagulation. Excess tissue was trimmed and hearts mounted and tied via the aorta on to the cannula of a Langendorff perfusion system. Hearts were perfused at 12ml/min until blood cleared from the coronary vessels and the perfusate ran clear.

## Cooling/rewarming protocol

Hypothermia and rewarming was carried out on isolated cardiomyocytes, Langendorff perfused rat and rabbit hearts and also *in vivo*. Due to the differences in optimal conditions between each of the experimental procedures, several cooling/rewarming protocols were carried out. Each chapter describes

the methods particular for that study. The temperatures used covered all aspects of hypothermia, from mild to severe. This wide spectrum allowed the study of temperatures routinely employed clinically and also those observed in victims of accidental hypothermia where core temperatures below 17°C are reported in patients with a positive neurological outcome after rewarming (Gilbert et al., 2000). All cooling was achieved gradually in order to mimic gradual whole body cooling and avoid rapid cooling contracture (Rebeyka et al., 1990, Lahorra et al., 1997). Every effort was made to keep cooling/rewarming time consistent within studies.

## **Dyes**

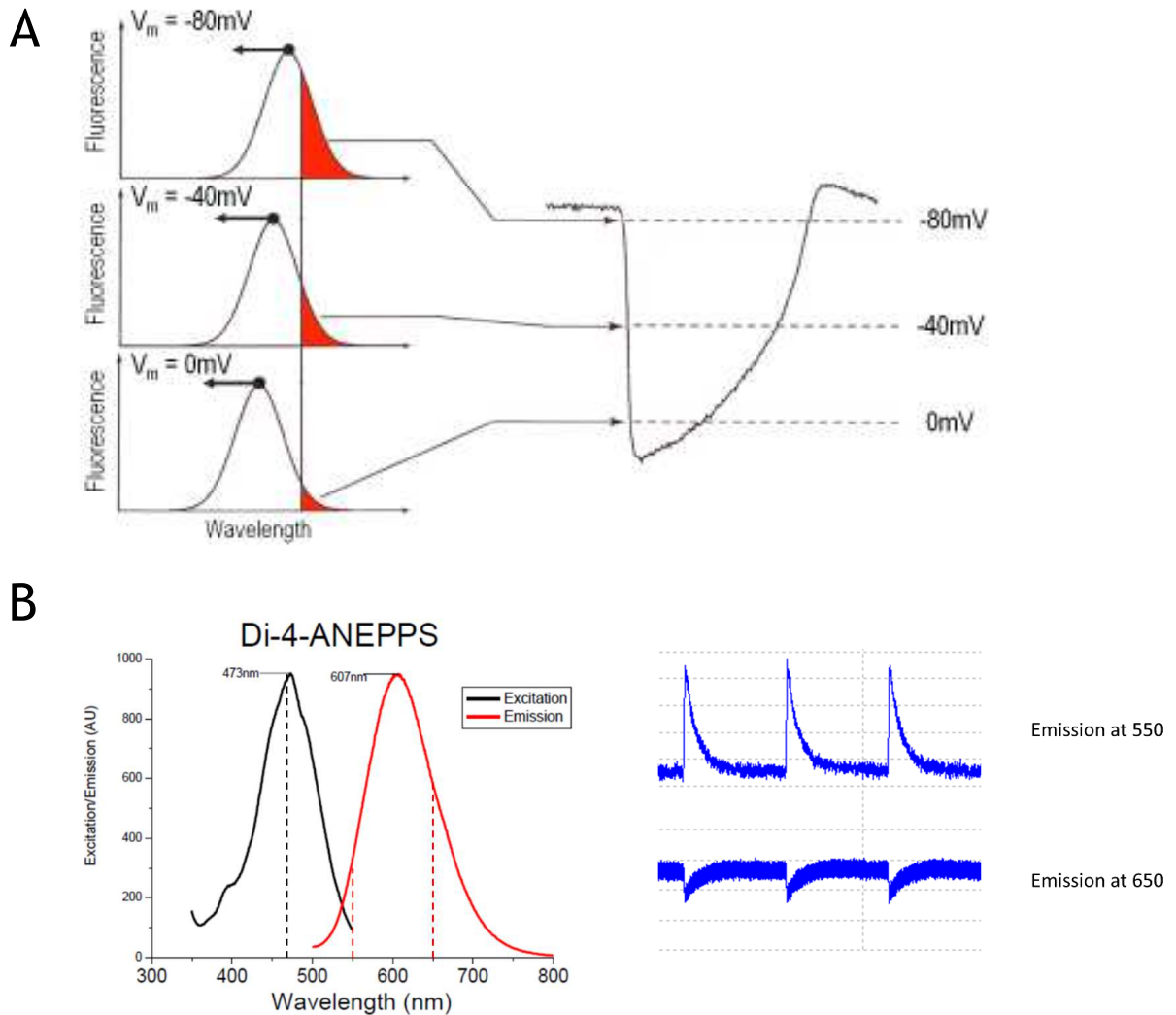
### **Voltage sensitive dye**

Voltage sensitive dyes are fluorescent compounds which have a high affinity for the plasma membrane of cells and change their spectral properties in response to changes in transmembrane potential (Loew, 2011). They are classified as fast and slow response dyes and the aminonaphthylethylpyridinium (ANEP) family of dyes are among the most commonly used due to their recording of voltage changes in the order of microseconds. They have been well established in measuring electrical activity in whole heart preparations (Choi and Salama, 2000; Efimov et al., 2004; Novakova et al., 2008). In particular, di-4-ANEPPs and di-8-ANEPPS are often utilised to accurately represent changes in membrane potential via changes in dye fluorescence (Morad and Salama, 1979). Both dyes yield a uniform 10% change in fluorescence per 100mV change in membrane potential but di-4-ANEPPS rapidly internalizes in cells, thus making it useful only for short-term experiments (Herron et al., 2012).

Di-4 ANEPPs delivery to whole heart preparations is generally through bolus injection into the coronary arteries via the perfusion system. The dye molecules intercalate into the extracellular side of the cell membrane due to affinity between the pair of hydrocarbons on the dye molecule and the amphiphilic lipid molecules in the membrane surface (Loew, 2011). The part of the molecule that gives it colour, also known as the chromophore, orientates perpendicular to the membrane surface. During excitation, the dye molecules undergo a charge redistribution which causes a shift in the emission spectrum when a membrane



potential change occurs. As shown in Figure 2.2, upon membrane depolarisation, there is a spectral shift to shorter wavelengths.



**Figure 2.2: Excitation and emission spectra of di-4 ANEPPS**

**A:** During rapid upstroke of the action potential, a depolarisation of the cell from its resting transmembrane potential ( $V_m$ ) of  $-80\text{mV}$  toward less negative voltage, e.g.  $V_m = -40\text{mV}$  and  $0\text{mV}$ , causes the fluorescence emission spectrum of the dye to shift to the left (shorter wavelengths), producing an inverted action potential signal. **B:** shows example traces of emission at both wavelengths.

The specific loading of di-4-ANEPPS is described in more detail in Chapters 3 and 5, however preparation of the dye was the same in all experiments. The dye was made up of  $1\text{mg/ml}$  stock solution in dimethyl sulphoxide (DMSO). Stock solution was stored in darkness at room temperature.

## Ca<sup>2+</sup> sensitive dye

The central concept underlying the use of Ca<sup>2+</sup> indicators is that an acute increase or decrease in their fluorescence intensity reflects a change in intracellular Ca<sup>2+</sup> concentration. Ca<sup>2+</sup> sensitive fluorescent indicators can be broadly divided into ratiometric (dual wavelength) or single wavelength indicators based on their response to Ca<sup>2+</sup> elevation.

Single wavelength indicators display a change in their fluorescence emission when Ca<sup>2+</sup> signals occur. In contrast to this, ratiometric indicators when bound to Ca<sup>2+</sup> change the optimum excitation or emission wavelength of the indicator (Grynkiewicz et al., 1985). For example, Fura-2, a ratiometric indicator, in the unbound form has a peak excitation wavelength of ~380nm, whereas the peak excitation wavelength when Ca<sup>2+</sup> bound is ~340nm (Figure 2.3). Ratiometric indicators are among the most popular due to their higher robustness compared with single wavelength counterparts. A major advantage being that Ca<sup>2+</sup> estimated with ratiometric compounds is not affected by uneven dye loading, photo-bleaching, optical path length or illumination intensity (Wokosin et al., 2004). Therefore, using ratiometry avoids many of the problems related to absolute fluorescence values.

Fura-2 has a limited sensitivity to Ca<sup>2+</sup> concentrations above 1µM, however other Fura derivatives with a lower Ca<sup>2+</sup> binding affinity are available, including Fura 4F-AM which has a response range shifted to detect higher Ca<sup>2+</sup> concentrations. This acetoxymethyl (AM) ester derivative is useful for non-invasive intracellular loading. Modification of carboxylic acids with AM ester groups results in an unchanged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound (Grynkiewicz et al., 1985).

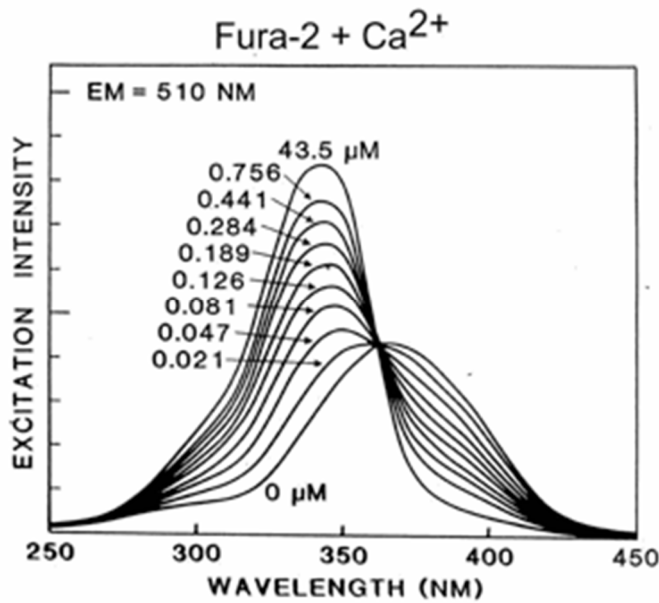


Figure 2.3: Excitation and emission spectra of Fura.

Most experiments using calcium sensitive dyes such as Fura are carried out at a constant temperature (e.g. 37°C). However, performing experiments at unphysiological temperatures results in cellular responses that would not be observed under physiological conditions. Therefore, if temperature is being varied, the change on the fluorescent probe must also be considered during data interpretation. One important consideration is a variation in the binding characteristics of the chelator. Various studies have documented that for dyes such as Indo, Fura and fluo-3 that the binding equilibria are shifted at lower temperatures, therefore leading to an increased dissociation constant ( $K_d$ ) and thus a lower affinity (Shuttleworth and Thompson, 1991; Howarth et al., 1995; Larsson et al., 1999). Fortunately this type of error is easily avoided using the appropriate temperature corrected  $K_d$  for a given dye (Shuttleworth and Thompson, 1991). Another important consideration when temperature is changed during the course of an experiment is that the fluorescence itself is affected by temperature, regardless of binding characteristics. As temperature increases, fluorescence intensity decreases. Likewise when temperature is cooled the fluorescence intensity increases, due to increases in quantum yield (Haynes et al., 1993).

These two effects of temperature on calcium sensitive dyes (i.e on binding characteristics and on fluorescence) are important when measuring free calcium concentrations. It is possible to avoid the problem of the  $K_d$  effects by using the ratio of the wavelengths fluorescence intensities for the calcium bound to calcium free fluorophores. However this method does not control for the effects of temperature on the fluorescence intensity at those two wavelengths (Larsson et al., 1999).

Preparation involved adding DMSO to 25mg Fura 4F-AM (Life technologies, Thermo Fisher Scientific; Waltham, MA USA). Throughout different experiments, Fura 4F-AM was used at various concentrations and loading times differed. As such, the experiment-specific use of Fura 4F-AM is described in detail in the relevant chapters (3 and 4).

### **Excitation-contraction (E-C) uncoupling with blebbistatin**

E-C uncouplers are commonly used in cardiac optical mapping/conduction experiments for suppression of motion. The basis of these compounds is to suppress muscle contraction whilst having little to no adverse effects on the electrical activity of the heart. Several compounds are available including cytochalasin D (Cyto D), 2, 3- butanedione monoxime (BDM) and more recently blebbistatin.

Blebbistatin is an inhibitor of the adenosine triphosphatases (ATPases) associated with class II myosin isoforms in an actin-detached state (Kovacs et al., 2004, Fedorov et al., 2007, Allingham et al., 2005). Its behaviour in this non-bound state is advantageous because it prevents any stiffness which may result from a constant actin-myosin crosslink. Blebbistatin has been shown to have minimal effects on ECG parameters and atrial and ventricular electrophysiology compared with other E-C uncouplers (e.g.BDM) (Lou et al., 2012), and it has also been characterised in various experimental preparations in different species, including rat and rabbit (Farman et al., 2008; Fedorov et al., 2007).

A main disadvantage of blebbistatin is photosensitivity and phototoxicity. Studies have shown that both UV and blue light photo-inactivate blebbistatin, resulting in toxicity from the production of free radicals causing damage to the surrounding cells (Sakamoto et al., 2005; Kolega, 2004). Additionally, a recent study suggests that blebbistatin may have adverse effects in whole heart rabbit experiments, including APD prolongation and reduced VF threshold (Brack et al., 2013). Therefore, it is important to consider these effects when evaluating experimental results.

Preparation involved adding 1ml of DMSO to 5mg blebbistatin (Enzo Life Sciences). Blebbistatin was used at both 3 $\mu$ M (Chapter 4) and 10 $\mu$ M (Chapters 5 and 6). Prior to adding the blebbistatin, the solution was heated, allowing it to fully dissolve in the solution and minimise the chances of any precipitate forming in the coronary vessels (Swift et al., 2012).

## **Chapter 3: Cardiac electrophysiology following *in vivo* hypothermia and rewarming**

## Introduction

Rewarming from hypothermia is associated with significant mortality and despite advances in medical therapy, has been associated with physiological effects including hypotension, reduced CO and diminished ventricular work (Prec et al., 1949; Bigelow et al., 1950; Berne, 1954; Blair et al., 1956). Data generated from a rat model of hypothermia/rewarming has shown that upon rewarming from sustained severe hypothermia there is ~50% reduction in CO when compared to starting values (Tveita et al., 1996; Kondratiev et al., 2006a; Kondratiev et al., 2006b). HR and mean arterial pressure (MAP) were maintained as was oxygen supply and the relative oxygenation of cardiac tissue but this was alongside a substantially reduced CO. Reducing temperature has a marked influence on many components of EC-coupling, and whether the dysfunction we see on rewarming is a consequence of the cooling, or from the increasing temperatures remains unclear.

Hypothermia is known to have effects on ventricular repolarisation, and has been associated with a prolonged APD, resulting in a prolongation of the QT interval (Yan and Antzelevitch, 1996; Mattu et al., 2002). Multiple ionic currents which control repolarisation are particularly susceptible to hypothermia (Liu et al., 1991; Sprung et al., 1995; Marshall et al., 2002). Despite numerous studies examining cooling and rewarming (Egorov et al., 2012; Fedorov et al., 2008; Hsieh et al., 2009; Piktel et al., 2011) to date, there has been no assessment of electrical function using the rat model of rewarming shock.

## Aims

The main aims of this chapter were:

Part 1:

to assess cardiac electrophysiology and mechanical function in isolated perfused rat hearts following *in vivo* hypothermia and rewarming

Part 2:

to assess cardiac electrophysiology in isolated perfused rat hearts during transient hypothermia and rewarming

## **Part 1: The electrophysiological effects of hypothermia and rewarming *in vivo***

### **Methods**

#### **Animals**

Male Wistar rats (250-300g) were used for all experimental work in this chapter. All of the surgery was carried out within the Anaesthesia and Critical Care Research Group, Institute of Clinical Medicine, University of Tromsø, Norway.

#### ***In vivo* hypothermia and rewarming procedure**

Animals were assigned to either a normothermic (n=9) or hypothermic (n=9) *in vivo* procedure. The surgery took 60-90 minutes on average, and all animals, irrespective of group received the same surgical procedure.

#### **Anaesthesia and respiratory support**

Animals were given an initial intraperitoneal injection containing sodium (Na) pentobarbital (50mg/kg) and fentanyl (50µg/kg) which was then followed by a continuous infusion (7.5mg/kg/hr) via an intravenous (I.V.) line inserted in the right jugular vein. Animals in the normothermic control group received anaesthesia throughout the duration of the experiment whilst animals in the hypothermic group had the infusion terminated at 30°C due to reduced drug metabolism in hypothermia (Zhou and Poloyac, 2011; Wood and Thoresen, 2015). Infusion of anaesthesia was resumed at 30°C during rewarming. Throughout all procedures animals were observed for any sign of discomfort by monitoring haemodynamics and also by toe-pinch, a recognised testing method for rodents under anaesthesia (Collier et al., 1961).

At temperatures >20°C animals have spontaneous and sufficient ventilation, however at core temperatures <20°C ventilation had to be supported. The trachea was opened and a tracheal tube inserted at the start of the procedure. When the animal was no longer able to support its own ventilation this tracheal



tube was then attached to a volume controlled small animal respirator (New England rodent ventilator, model 414, New England Instruments, Medway, MA) using room air.

Ventilation was adjusted in accordance to blood gas analysis. Samples (0.15ml) were drawn from the left femoral artery and analysed using a commercially available blood gas analyser (ABL 800 blood gas analyser, Radiometer, DK). These samples were analysed at 37°C using the alpha-stat strategy which does not require correction for pH and blood gas values in hypothermic conditions (Ashwood et al., 1983).

### **Core cooling and rewarming**

Animals in the hypothermic group were cooled by circulating cold water through a U-shaped polyethylene tube which was placed in the lower bowels. To further assist in cooling, the operating table was constructed of hollow double layered aluminium to allow the circulation of temperature adjusted water. For normothermic animals, warm water (37°C) was circulated around the polythene tube and the aluminium table. A thermocouple wire connected to a thermocouple controller (Thermoalert TH-5, Columbus Instruments, Columbus, OH, USA) was placed in the oesophagus to continuously monitor core temperature. Temperature was cooled from 37°C to 15°C over 60-90 mins (Figure 3.1). Animals then remained at 15°C for 3 hours before being gradually rewarmed to 37°C, again over 60-90 mins. Effort was taken to ensure that cooling and rewarming times were consistent between animals. Normothermic animals remained at 37°C for 5 hours. Previous work using this experimental model has used 4 hours of stable hypothermia (Tveita et al., 1996; Kondratiev et al., 2006). However, the decision to use 3 hours within this study was made to maximise the number of animals that would survive the procedure.

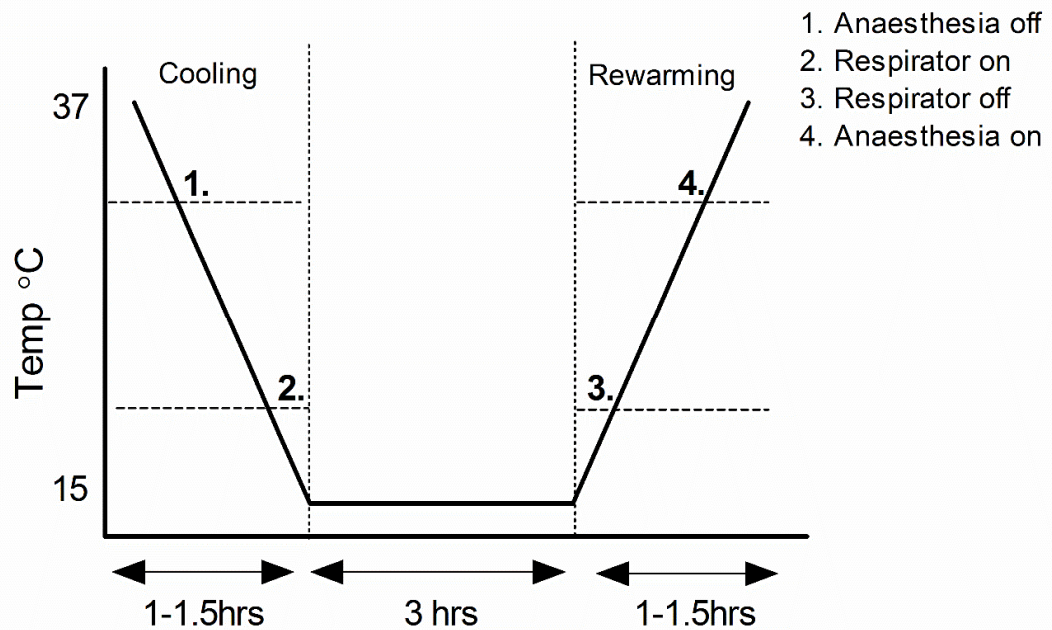


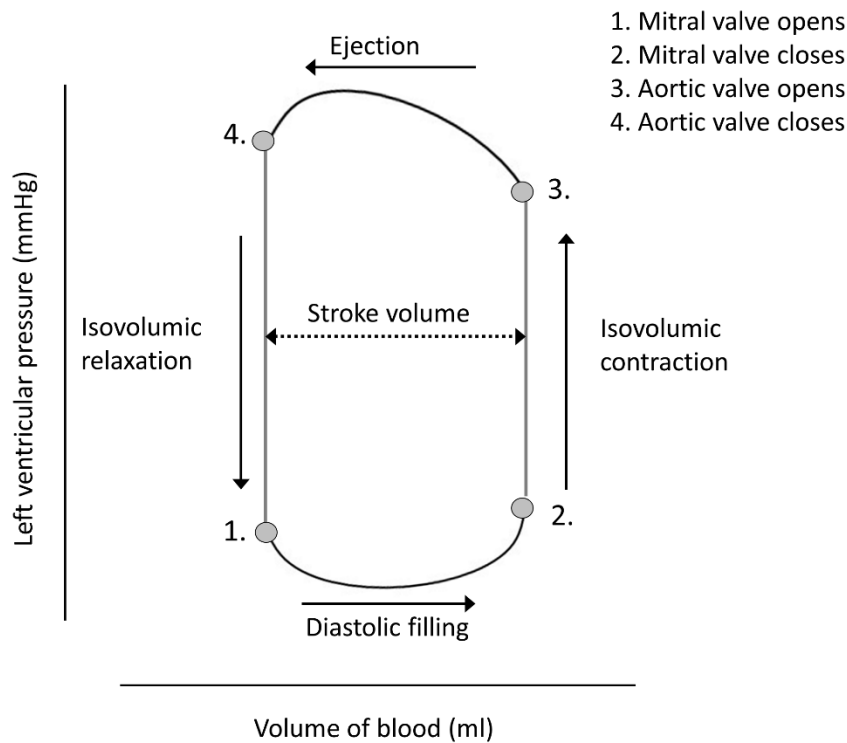
Figure 3.1: Temperature timeline for hypothermia rewarming *in vivo* surgery.

Shown are the temperatures points where anaesthesia was switched off/on and also where respiration was supported. Normothermic animals remained at 37°C throughout.

### Haemodynamic measurements

To assess ventricular haemodynamics during the *in vivo* procedure, a Millar P-V conductance catheter system was used (SPR - 838, Millar Instruments, Houston, TX). This miniaturised (2.0 French) was inserted into the right carotid artery and advanced into the LV.

Conductance catheter technology allows simultaneous measurement of pressure and volume within the LV and as such, the 4 phases of the cardiac cycle, isovolumic contraction, ejection, isovolumic relaxation and ventricular filling, can be conveniently visualised in a diagram (Figure 3.2). Under steady-state conditions and with a constant time interval between beats, this loop is repeated with each contraction. The use of P-V loop measurements are well established in rodents (Burkhoff et al., 2005; Pacher et al., 2008) and it serves as a particularly useful tool in the assessment of *in vivo* LV mechanical function during normothermia and hypothermia.



**Figure 3.2: Pressure-volume loop.**

Idealised pressure-volume diagram highlighting the 4 phases of the cardiac cycle.

The catheter measures relative changes in the conductance of blood during the cardiac cycle, reflective of ventricular filling and ejection during the cardiac cycle, which is then calibrated to achieve a measure of true volume. Special considerations related to the use of conductance measurements and volume determination are required at low core temperatures. The measured conductance should be corrected for parallel conductance induced by the alternating current passing through the blood into the surrounding LV wall or septum. At the end of experiments, parallel conductance is usually measured by a bolus of saline (Kass et al., 1986). However due to the multiple temperatures used in this study, this method was not applied. Blood viscosity is affected by temperature and the use of a saline bolus to calibrate at each temperature would be fatal. As a result of the lack of calibration, the measurements included parallel conductance (Lankford et al., 1990).

To more accurately assess volume, the cuvette calibration method was used. Insulator-type cuvettes of known diameters (2-7mm) were filled with heparin treated blood. These were placed in a thermo-controlled water circulator allowing the temperature of the blood to be adjusted during calibration.

Therefore, the specific temperatures used in this experimental procedure could be corrected. Slopes and y-intercepts were applied to the analysis software to adjust conductance units to true volume units ( $\mu\text{L}$ ) (Tveita and Sieck, 2012)

LV pressure and volume signals were digitized at 1Hz, and recorded by ADInstruments Chart software. The recorded data was analysed off-line using a cardiac PV loop analysis program (LabChart7). Recorded data included;

Heart Rate (HR)

Stroke Volume (SV): End diastolic volume (EDV) - End systolic volume (ESV)

Cardiac Output (CO): Volume of blood pumped per minute

$dP/dt_{\text{max}}$ : maximum rate of pressure change in the ventricle

$dP/dt_{\text{min}}$ : minimum rate of pressure change in the ventricle

Stroke Work (SW): the work done by the ventricle to eject a volume of blood (i.e. SV) into the aorta.

LV end systolic pressure (Pes): The maximal pressure developed by the ventricle during systole.

Additionally, to assess peripheral vascular responses, a fluid filled catheter (22G) attached to a pressure transducer was inserted into the left femoral artery to measure mean arterial pressure (MAP: average arterial pressure during a single cardiac cycle).

### ***In vivo* assessment of ECG**

Electrocardiogram (ECG) recordings to assess the electrical activity of the heart during hypothermia and rewarming were carried out using lead II ECG limb leads. This method was advantageous as it did not require any further invasive procedure to provide an assessment of the electrical activity of the heart. ECG signals were amplified and recorded on LabChart7. The main ECG parameters extracted from the software were PR, QRS and QT intervals (Figure 3.4).

Corrected QT (QTc) was also measured using Fridericia's formula to correct for HR variability. The software used was semi-automated with cursors identifying the different complexes of the ECG, these could be manually over-ridden if required. Reliable measurements require traces of a good quality, this can be problematic during cooling and rewarming as the animal will display a shivering response whereby the leads will experience a lot of movement. Recording for ECG was therefore continuous throughout the whole experiment and only traces with clear P, QRS and T waves were included in the results.

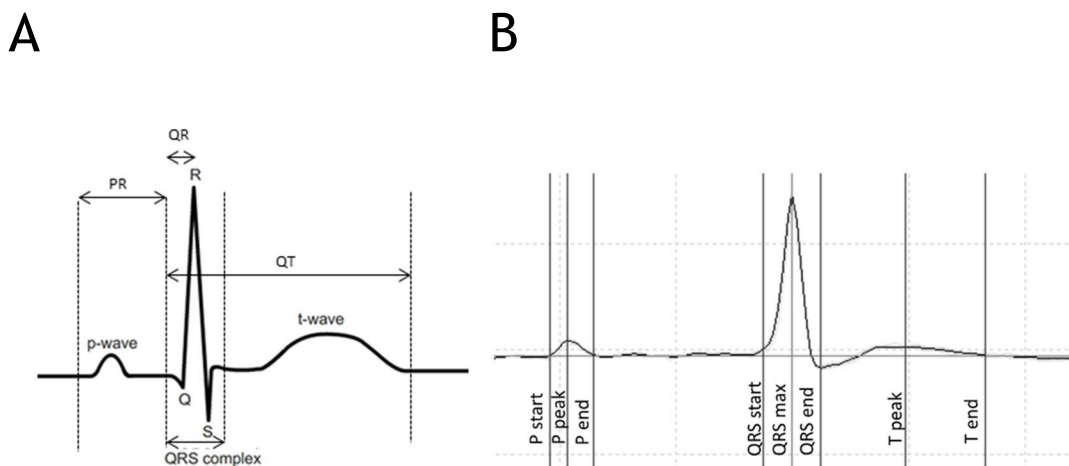


Figure 3.4: In vivo ECG measurements.

A: Example of the parameters measured in a standard ECG complex. B: Example from LabChart7 analysis of ECGs *in vivo*. Shown are the automated peaks that had been detected by the programme, all of which could be adjusted manually.

### Langendorff perfusion of hearts following in vivo cooling and rewarming

Following rewarming, animals received an overdose of Na pentobarbital and hearts were rapidly removed and placed in cold Tyrode's solution. Hearts were then Langendorff perfused (see Chapter 2 for more details) with Tyrode's solution at 37°C until perfusate ran clear. Hearts were paced at 200ms (5Hz) via hook electrodes in the RA.

### Intraventricular pressure

Hearts were given 15 minutes to physiologically stabilise before assessing LV function *ex vivo*. As a measure of contractility, intraventricular function can be

measured using special, compliant balloons. These have been widely used and are effective in small rodents as a method of assessing cardiac function on isolated perfused hearts (Curtis et al., 1986; Sutherland et al., 2003). The left atrium was trimmed and a balloon tipped (latex) catheter was inserted into the LV through the mitral valve. The balloon provided a closed system from which pressure measurements could be made, and intraventricular (i.e. left ventricular developed pressure [LVDP]) determined. The balloon was attached to a fluid filled catheter and connected to a pressure transducer and bridge amplifier.

### **Optrode recordings**

Following contractility measurements, hearts were perfused with mechanical uncoupler Blebbistatin ( $3\mu\text{M}$ ) to reduce contraction-based movement. To assess the electrophysiology of these hearts, an optrode system was used (Cairn Research, UK). This fibre-optic light guide allowed near simultaneous recordings of optical APs and optical  $\text{Ca}^{2+}$  transients from the same site on the LV. Figure 3.5 shows the fibre optic light-guide (2mm diameter) placed against the heart.

### **Voltage**

$50\mu\text{L}$  di-4-ANEPPS was added to the heart as a bolus just prior to recordings. A light emitting diode (LED) emitted light at 470nm to excite the dye, with emission spilt at 580nm and measured at wavebands 550nm and 650nm. To minimise the effect of bleaching this LED was only switched on for 5 sec intervals to record a train of APs.

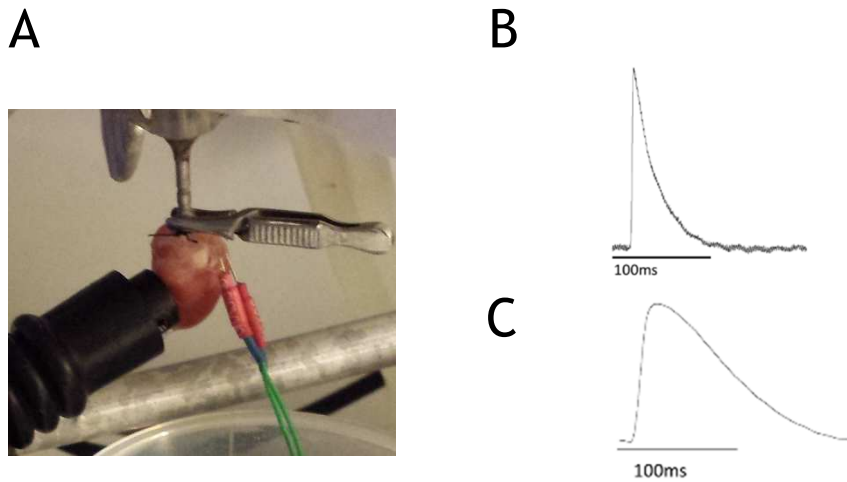


Figure 3.5: Optrode placement on heart.

A: Optrode placed against LV. Example recording of B: AP and C: Ca<sup>2+</sup> transient.

### Ca<sup>2+</sup> measurements

To record Ca<sup>2+</sup> transients Fura-4F was used. As described in Chapter 2, Fura-4F is excited at 340nm and 380nm, with emission collected at 510nm. The benefits of this is allowing for a ratiometric measurement of emitted fluorescence to be taken due to two excitation wavelengths. However, excitation at 340nm is difficult using the current LED based system as the emitted wavelengths are of a very low power. For this reason, Fura-4F was excited with a 360nm light from a longer wavelength LED. This wavelength is referred to as the isobestic point as it represents a Ca<sup>2+</sup> insensitive sector of the excitation spectrum (Figure 2.3). It is still possible to yield a ratiometric measurement of Ca<sup>2+</sup> using this approach.

Prior to Fura-4F loading, background readings were taken. 30µM Fura-4 was then perfused onto the heart over 5 minutes. This was carried out at half the normal perfusion rate to allow time for the Fura-4F to enter the heart. Additionally, 2.5mM probenecid (Sigma Aldrich, Saint Louis; MO, USA) was added to the Tyrode's solution and perfused onto the heart to inhibit anion transport and prevent Fura-4 secretion. The heart was left for a further 10 minutes to allow the acetomethyl groups to be removed by intracellular esterases. Recordings were then made by exciting with a 360nm optoLED (5secs) and then immediately switching to 380nm optoLED for a further 5 secs. Fluorescence was collected at 510nm.

Near simultaneous AP and Ca<sup>2+</sup> recordings could be carried out on each of the hearts due to the different excitation wavelengths. By switching between 360, 380 and 470nm at 5 sec intervals it was possible to make sequential ratiometric recordings of both APs and Ca<sup>2+</sup> transients.

### **Analysis**

Signals were recorded using WinEDR software (SIPBS; University of Strathclyde, UK) and analysed using custom software developed in house (F. Burton; University of Glasgow, UK): RatioAverager for APs and CelloPTIQ for Ca<sup>2+</sup> transients. These programmes averaged recorded signals to a mean AP/Ca<sup>2+</sup> transient. Parameters such as time between 10 and 90% of the upstroke (Trise), time to peak (Tpeak), amplitude and duration of transients could be measured.

### **Statistical analysis**

Data are expressed as mean ± SEM. Haemodynamic and ECG measurements were assessed by one-way ANOVA for repeated measures. Optrode data (normothermic vs hypothermic) was compared using an unpaired t-test. When significant differences were found, data were post-hoc analysed using Tukey's test. All statistical tests were performed using GraphPad Prism software.



## Results

### Haemodynamic measurements

Animals either underwent a normothermic (n=9) or hypothermic (n=9) surgical procedure. All animals except one in the hypothermic group survived. This animal developed VF at 30°C upon rewarming. In this instance only haemodynamic measurements recorded during cooling were included, with all rewarming data excluded. Figure 3.5 shows typical PV loop traces from hearts at 37°C and 15°C, with considerable effects on both pressure and volume evident.

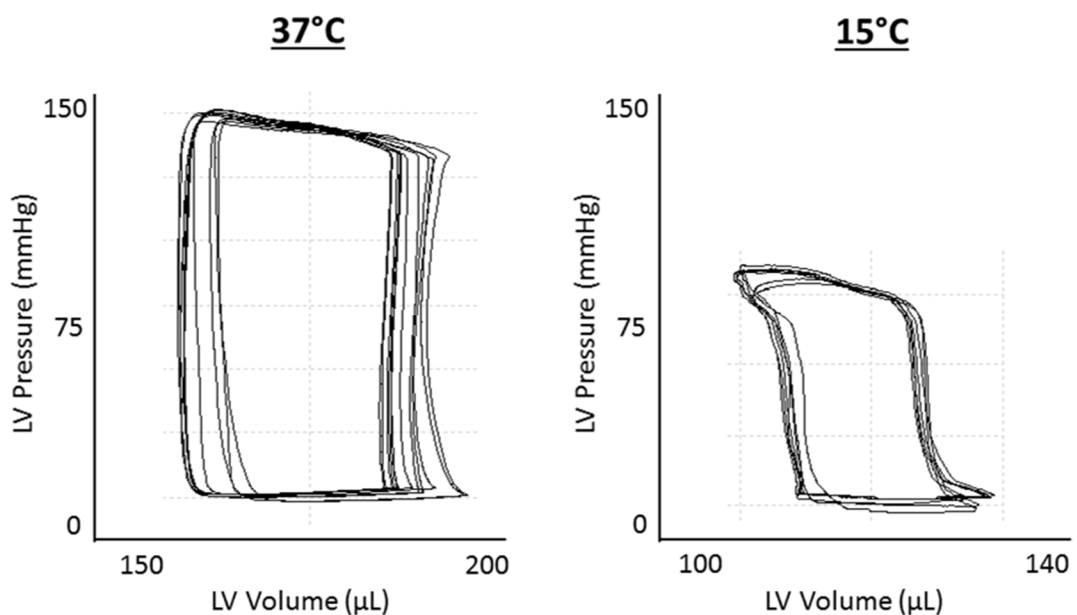


Figure 3.6: Pressure-volume loops at 37°C and 15°C.

Representative trace from one animal at baseline (37°C) and during stable hypothermia (3 hours at 15°C).

### Hypothermia and rewarming

Cooling to 15°C showed significant changes in all haemodynamic measurements (Figure 3.7, left panel). Following 3 hours of stable hypothermia, MAP decreased by  $71.9 \pm 2.4\%$  ( $117.8 \pm 3.9$  vs  $33.8$  mmHg,  $p < 0.05$ ) when compared to 37°C baseline. Gradual rewarming returned MAP to within baseline. HR gradually decreased and after stable hypothermia was reduced by  $84.2\%$  ( $427.6 \pm 11.1$  vs  $64.2$  bpm,  $p < 0.05$ ). Rewarming to 37°C increased HR, to  $115.5 \pm 15.1\%$  ( $494.1 \pm 8.9$  bpm) which was not different from baseline values. CO showed an

initial increase to  $147.6 \pm 17.6\%$  (vs  $37^\circ\text{C}$ ,  $p < 0.05$ ) when cooled to  $34^\circ\text{C}$ , which then gradually decreased as cooling continued (Figure 3.7C). Following 3 hours of stable hypothermia at  $15^\circ\text{C}$  CO had decreased by  $82.3 \pm 1.1\%$  ( $p < 0.05$ ). Upon rewarming to  $37^\circ\text{C}$ , CO did not return to baseline values, showing a *reduction* of  $35.7 \pm 4.1\%$  ( $p < 0.05$ ). Due to errors with the cuvette calibration equation, the absolute values of CO and SV, when compared to previous data from the lab, were out by a factor of 10, and as a result are only presented as  $\Delta\%$ . Despite this, the reduction in CO upon rewarming was evident in all hearts. SV increased throughout cooling to a maximum of  $238.4 \pm 32.8\%$  of baseline at  $15^\circ\text{C}$  ( $p < 0.05$ ) however normalised following 3 hours of stable hypothermia ( $141.2 \pm 25.1\%$ ). Rewarming did not significantly change SV when compared to pre-hypothermic values however there was a trend towards a reduction ( $100 \pm 0.0$  vs  $65.5 \pm 8.6\%$ ).

Ventricular performance during hypothermia and rewarming was also assessed by examining dP/dt max and min. dP/dt max (Figure 3.7E) showed a similar trend to CO, with an initial increase during cooling. At  $32^\circ\text{C}$  it was increased to  $150.6 \pm 7.3\%$  of baseline ( $15,277.5 \pm 1011.5$  vs  $10,256 \pm 756.1$  mmHg/s,  $p < 0.05$ ), however further lowering of temperatures showed a decrease in contractility. During stable hypothermia dP/dt max had decreased by  $89.2 \pm 1.4\%$  when compared to  $37^\circ\text{C}$  ( $p < 0.05$ ), however it normalised upon rewarming. dP/dt min, SW and end systolic pressure all showed significant reductions during cooling to  $15^\circ\text{C}$  ( $p < 0.05$ ) but returned to baseline values upon rewarming to  $37^\circ\text{C}$ . These haemodynamic results confirm that animals in the hypothermia group had evidence of cardiac dysfunction or 'rewarming-shock'.

### **Normothermia**

Data from normothermic control animals can be seen in Figure 3.7 (right panels). No differences were found in any haemodynamic measurements throughout the 5-hour *in vivo* procedure.

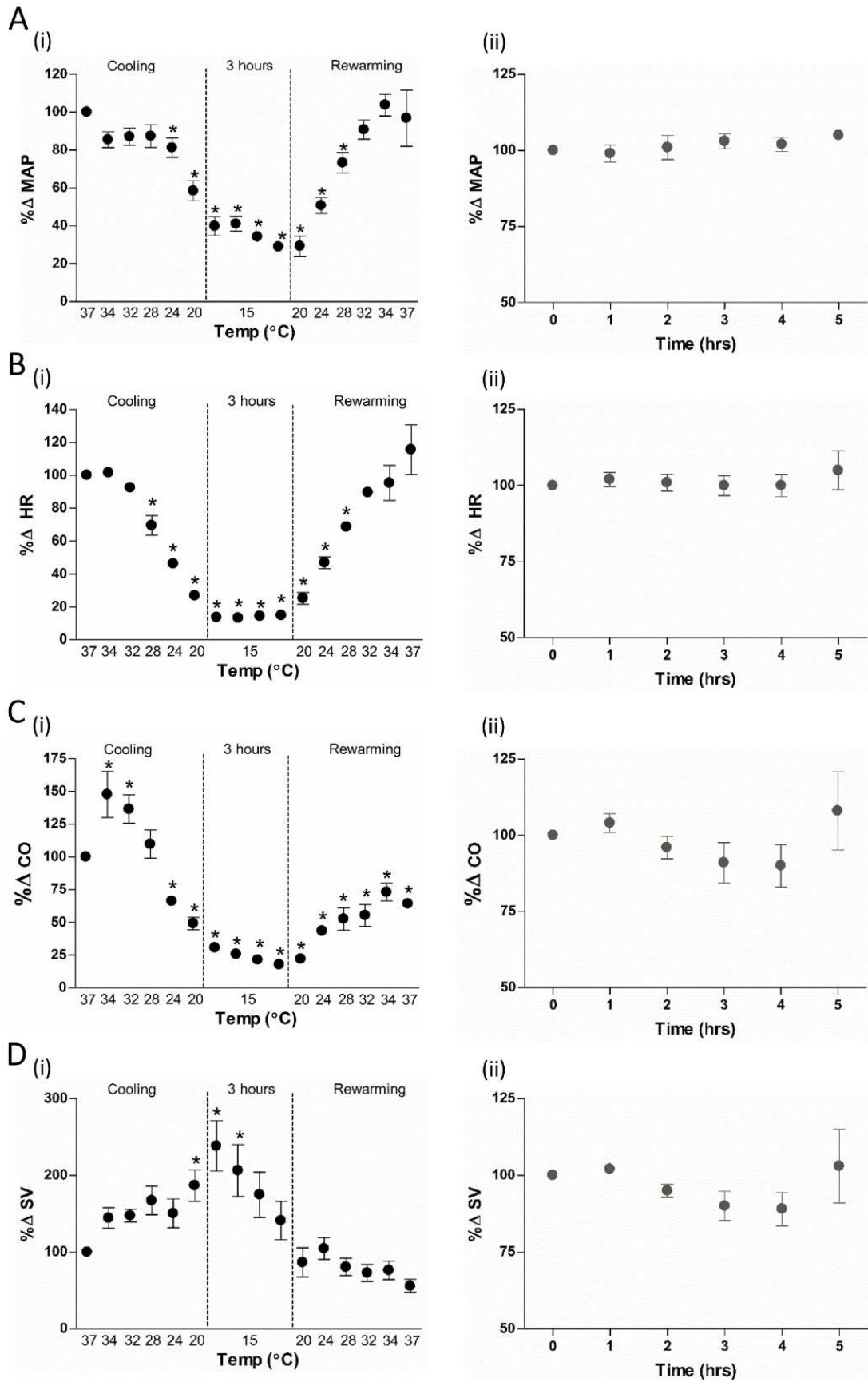


Figure 3.7- Haemodynamic measurements from *in vivo* surgery.

Left panel shows measurements during cooling and rewarming whilst right panel shows normothermic control data. All data presented as percentage change from baseline (37°C) A: MAP B: HR C: CO D: SV \*p<0.05 compared to 37°C. Continued overleaf.

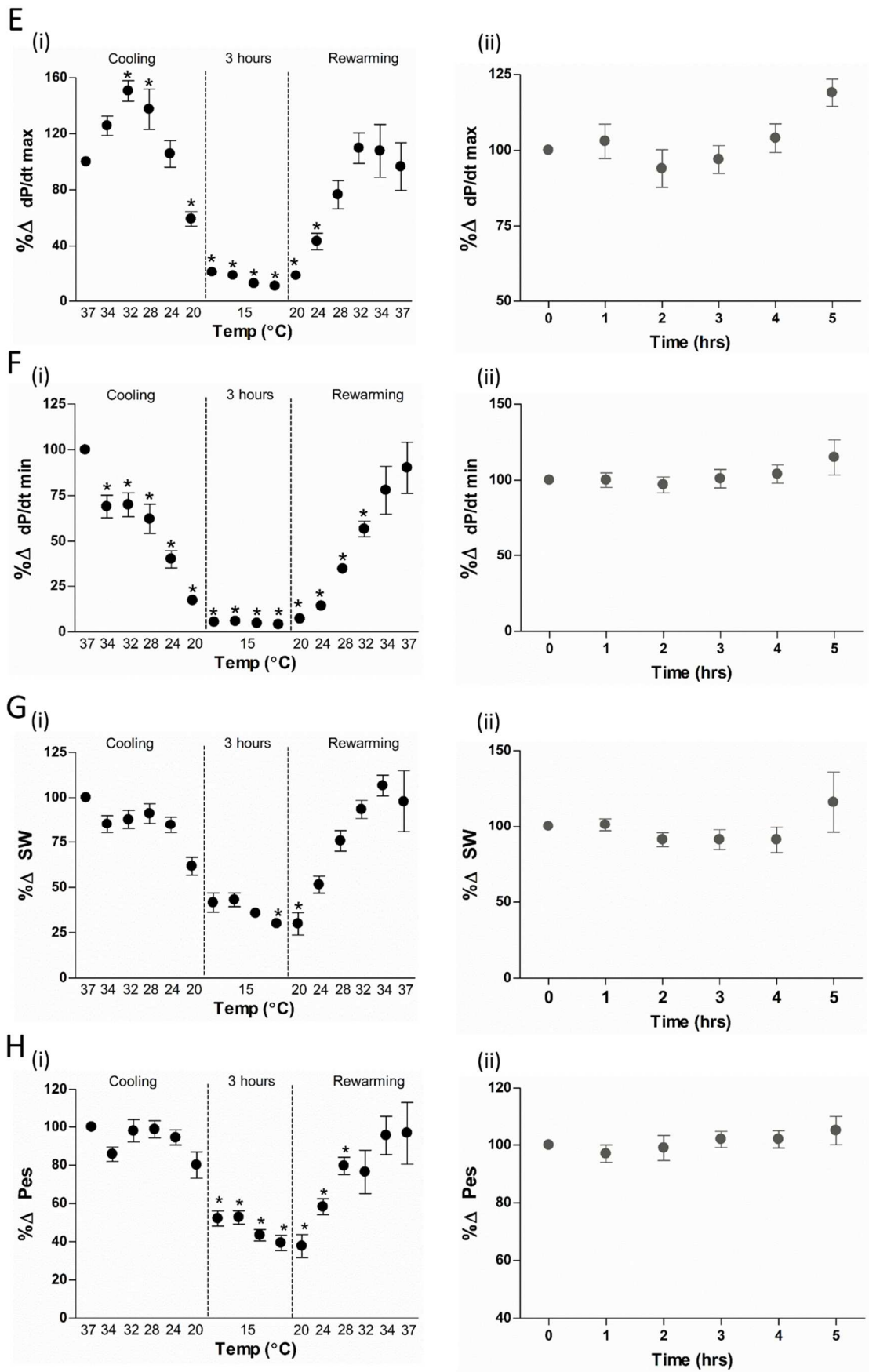


Figure 3.7 Haemodynamic measurements from *in vivo* surgery *cont.*.

Left panel shows measurements during cooling and rewarming whilst right panel shows normothermic control data. All data presented as percentage change from baseline (37°C) E: dP/dt max. F: dP/dt min. G: SW. H: Pes. \*p<0.05 compared to 37°C baseline.

### ***In vivo* assessment of ECG**

ECG was recorded in all animals throughout cooling and rewarming via 3 limb leads. Figure 3.8A shows example traces from different temperatures. Traces from several animals were excluded due to the noise in the signal. Due to missing data, statistical analysis was carried out using a One-Way ANOVA (Tukeys post hoc). This differed from the Repeated Measures test carried out for the PV loop data, which may explain some differential findings.

### **Hypothermia and rewarming**

Cooling to 15°C slowed both HR and prolonged ECG parameters (Figure 3.8B/3.9, left panels). As seen with the P-V loop recordings in the previous section, there was a significant slowing of HR ( $404.1 \pm 6.9$  vs  $55.3 \pm 6.3$  bpm,  $p < 0.05$ ) during cooling. However, upon rewarming HR increased above baseline values ( $477.9 \pm 9.1$  vs  $404.1 \pm 6.9$ ,  $p < 0.01$ ), a finding not replicated in the P-V loop analysis. Following 3 hours of stable hypothermia there was significant slowing of both the PR ( $43.7 \pm 2.1$  vs  $291.5 \pm 21.1$  ms,  $p < 0.05$ ) and QRS ( $31.9 \pm 9.2$  vs  $86.7 \pm 6.5$  ms,  $p < 0.05$ ) intervals when compared to 37°C baseline. Clear T waves were not always evident in ECG traces. Therefore, the QT and heart rate corrected QT (QTc) are from  $n=4$  in the hypothermic group and  $n=3$  in the normothermic group. The differences in the values at 37°C are quite evident, with a QT interval of  $59.9 \pm 16.8$  ms compared to a QTc value of  $112.7 \pm 31.2$  ms. Both parameters showed the greatest slowing following one hour of stable hypothermia, but returned to within baseline values upon rewarming.

### **Normothermia control**

Data from normothermic control animals can be seen in Figure 3.8/9 (right panels). No differences were found in any ECG parameters throughout the 5-hour *in vivo* procedure.

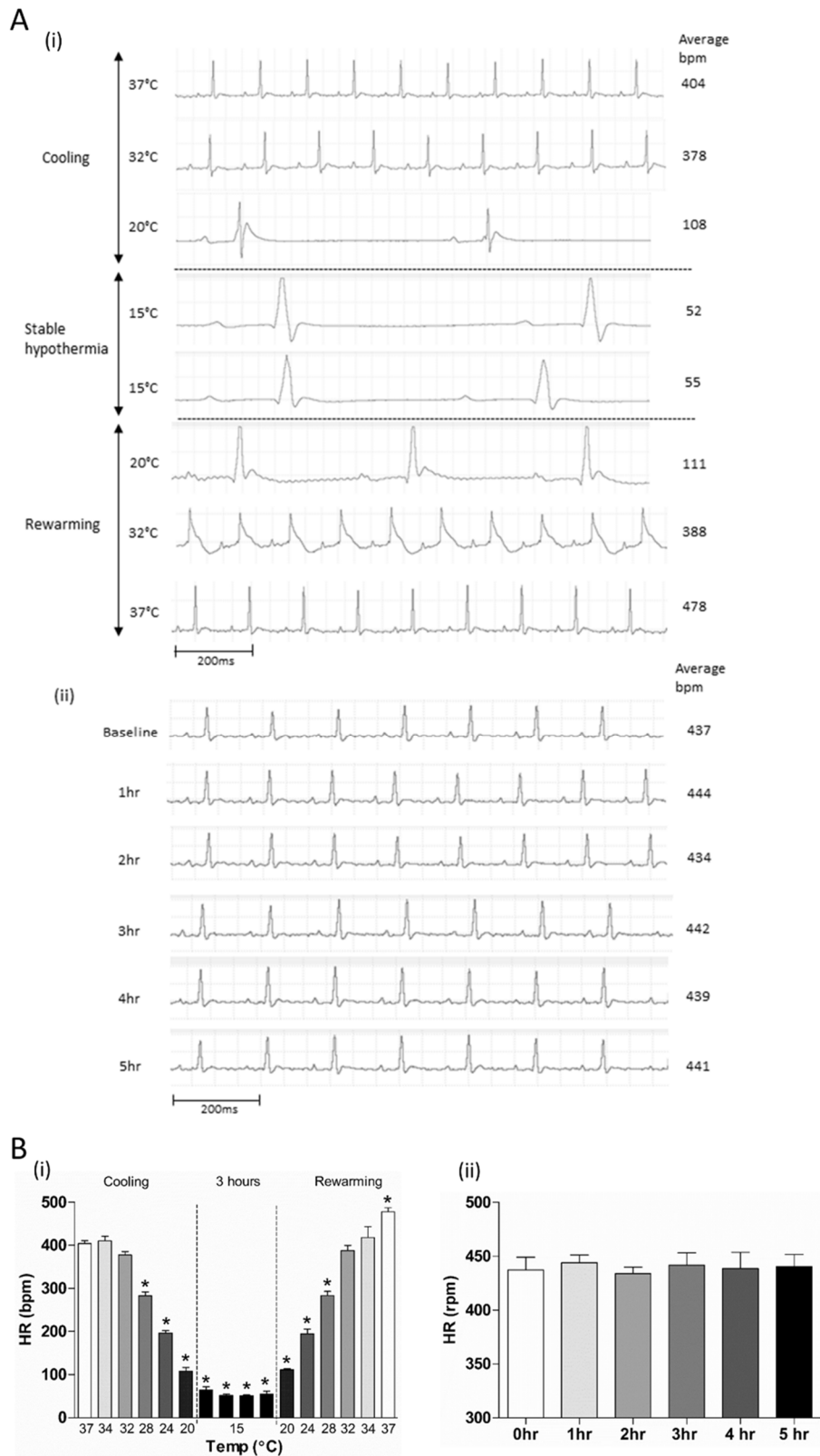


Figure 3.8: ECG traces and heart rate data from *in vivo* surgery.

**A:** Example traces of ECG recorded using 3 limb leads. Shown are the temperatures recorded during hypothermia and rewarming (i) and also normothermic control animals (ii). **B:** Heart rate data from ECG leads. Left panel (i) shows measurements during cooling and rewarming whilst right panel (ii) shows normothermic control data. \* $p < 0.01$  compared to compared to 37°C baseline.

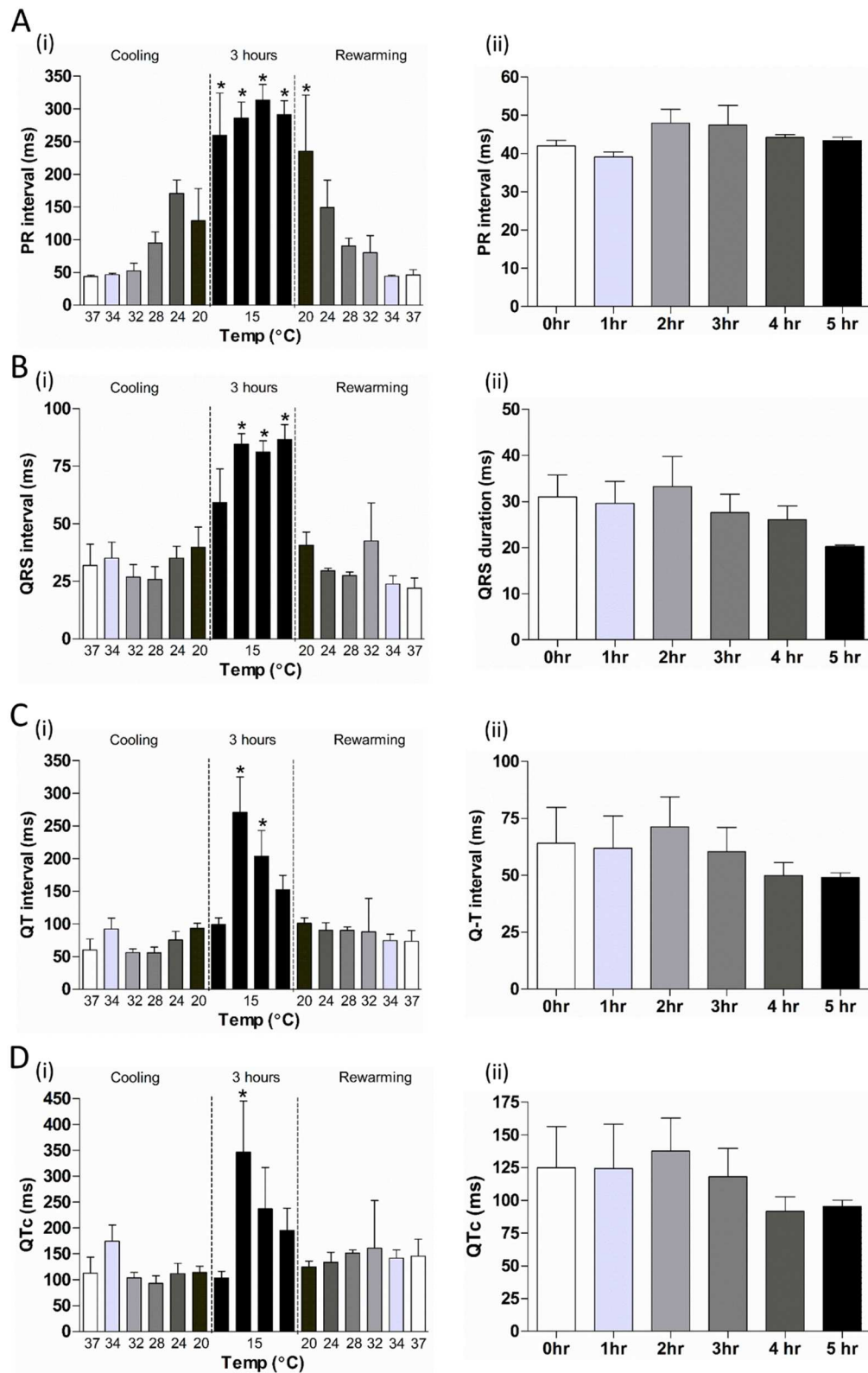


Figure 3.94 ECG measurements during *in vivo* surgery.

Left panel shows measurements during cooling and rewarming whilst right panel shows normothermic control data. A: PR interval. B: QRS interval. C: QT interval. D: Corrected QT interval (QTc) using Fridericia's correction formula. \* $p < 0.01$  compared to 37°C baseline.

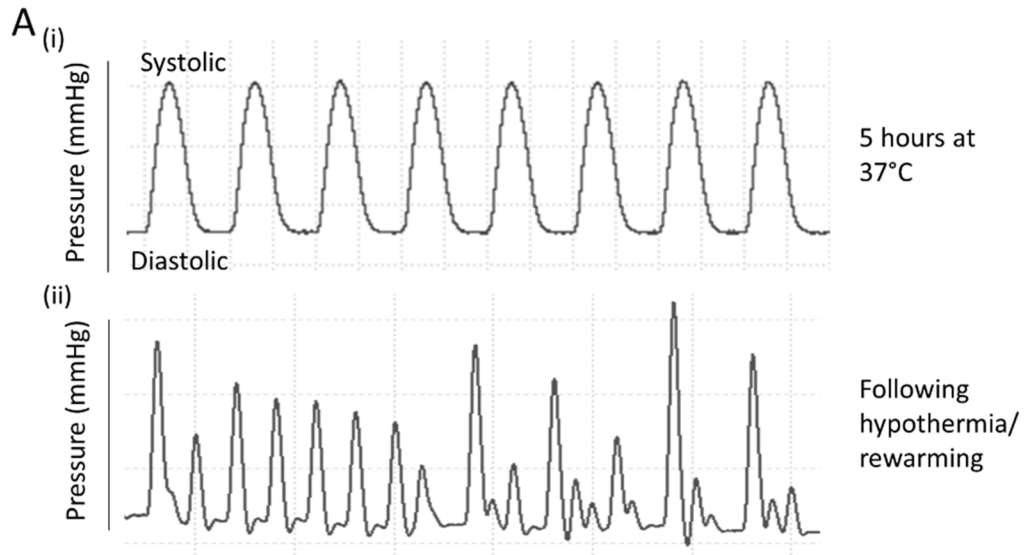
### **Langendorff perfused heart preparation following *in vivo* surgery**

Following rewarming to 37°C hearts were removed and Langendorff perfused with Tyrode's solution. Initial experiments on separate non-surgical animals attempted to minimise multiple temperature changes to hearts. These animals (n=2) were euthanised and hearts removed and placed in Tyrode's solution warmed to 37°C. However, because these hearts continued to beat it was technically very difficult to mount them onto the Langendorff cannula without air entering the coronary vessels. For this reason, all subsequent experiments used cold Tyrode's solution.

### **Intraventricular pressure recordings on isolated rewarmed hearts**

Pressure measurements were made on a subset of hearts (n=3 in each of normothermic and hypothermic groups) from a balloon inserted in the LV (Figure 3.10). Hearts that underwent the *in vivo* surgery but remained at 37°C throughout displayed normal pressure traces. In contrast to this hearts that had been rewarmed following 3 hours of hypothermia began to fibrillate immediately upon placement on the balloon. These hearts were given time to recover (bolus of KCl used where necessary) however any adjustment to the balloon (inflation/deflation) caused the hearts to go into an abnormal rhythm. Comparisons between hypothermic and normothermic hearts were not made due to the irritability of these hearts.





**Figure 3.10: Inter ventricular pressure on hearts isolated post-surgery.**

Hearts removed following surgical procedure and Langendorff perfused. Typical traces recorded by balloon placement in the LV.

## Voltage measurements on isolated rewarmed hearts

Due to the difficulties in obtaining intraventricular pressure measurements, separate hearts were used for the optrode recordings (hypothermia n=4, normothermia n=4). AP differences can be seen in Figure 3.11A. Hearts that underwent hypothermia and rewarming had a shorter AP than those that were maintained at a stable 37°C. Analysis of APs showed no difference in TRise, Tpeak or Trepol. APD<sub>90</sub> was significantly shorter in hypothermic hearts (Figure 3.12) compared to normothermic controls ( $40.7 \pm 1.1$  vs  $52.6 \pm 3.8$  ms,  $p < 0.05$ )

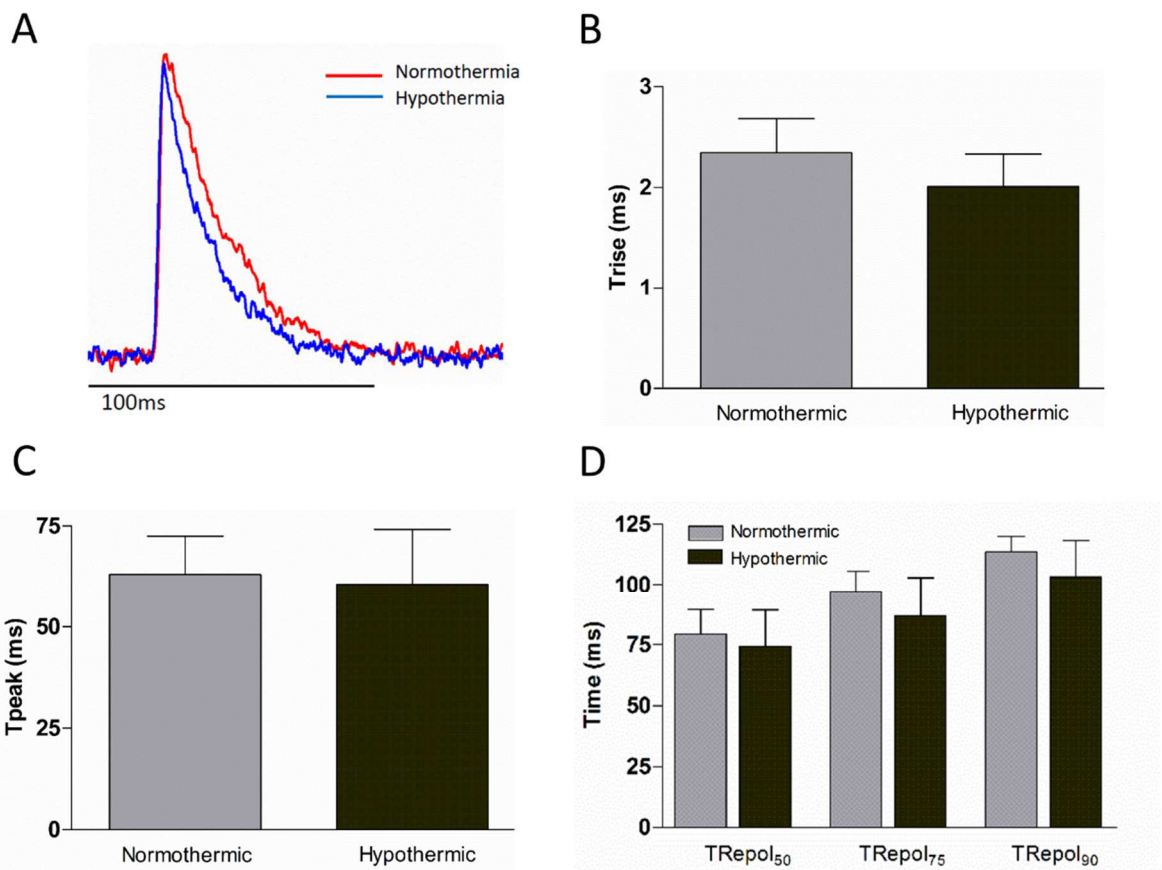


Figure 3.11: Voltage measurements on isolated hearts following surgery.

Hearts removed following *in vivo* surgical procedure and Langendorff perfused. Following loading with di-4 ANEPPS Optrode was placed against the LV. A: Example traces of normothermic (red) and hypothermic (blue) APs. B: Rise time (TRise) of AP. C: Time to peak of AP. D: Time to Repolarisation 50%, 75% and 90% (TRepol<sub>50/75/90</sub>). Light grey bars represent hearts that underwent the normothermic surgery whilst black bars represent hearts that were cooled and rewarmed.

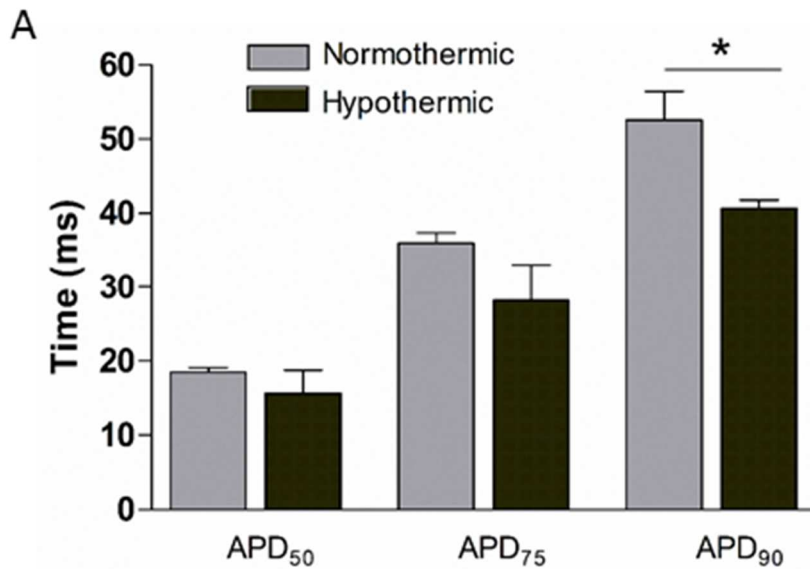


Figure 3.12: Action Potential Duration (APD) values from isolated hearts following surgery. Hearts removed following *in vivo* surgical procedure and Langendorff perfused. Following loading with di-4 ANEPPS Optrode was placed against the LV. A: APD 50%, 75% and 90% (APD<sub>50/75/90</sub>). Light grey bars represent hearts that underwent the normothermic surgery whilst black bars represent hearts that were cooled and rewarmed. \* $p < 0.05$  difference in APD<sub>90</sub> between hypothermic and normothermic hearts.

### Ca<sup>2+</sup> measurements on isolated rewarmed hearts

Due to problems with equipment, Ca<sup>2+</sup> transients were only available from 1 normothermic and 2 hypothermic hearts, therefore it is not possible to draw any conclusions from the data. The main difficulties were that the traces were noisy, due to interference in the system and not physiological issues. Figure 3.13 shows parameters measured. Although no statistical tests were able to be used, there does not appear to be any difference in amplitude, Trise or Tpeak of the Ca<sup>2+</sup> transients. Time to 90% Ca<sup>2+</sup> decay shows a trend towards a slowing (142.2 vs 115.5 ± 20.8 ms) in the hypothermic hearts however further experimental work would be required to comment further.

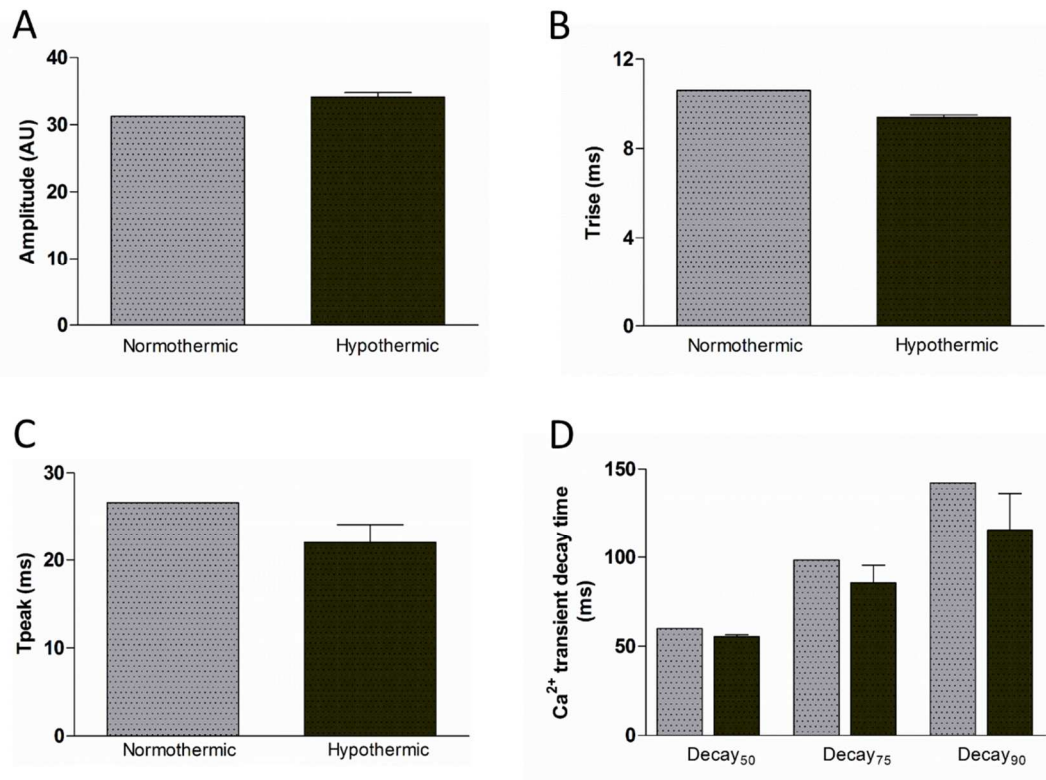


Figure 3.13: Ca<sup>2+</sup> transient analysis from isolated hearts following surgery.

Hearts removed following *in vivo* surgical procedure and Langendorff perfused. Following loading with Fura-4F optrode was placed against the LV. A: Ca<sup>2+</sup> transient amplitude (arbitrary units, AU). B: Rise time (TRise) of Ca<sup>2+</sup> transient. C: Time to peak of Ca<sup>2+</sup> transient. D: Time to 50/75/90% Ca<sup>2+</sup> transient decay (Decay<sub>50/75/90</sub>). Light grey bars represent hearts that underwent normothermic surgery whilst black bars represent hearts that were cooled and rewarmed.

## Part 2: Transient hypothermia and rewarming on isolated perfused rat hearts

The aim of this work was to examine the effect of short term cooling and rewarming on cardiac electrophysiology. The animals used in this work had not undergone any prior *in vivo* surgery.

### Methods

Male Wistar rats (n=10) were given an intraperitoneal overdose of Na pentobarbital and hearts were removed and Langendorff perfused as previously described. The optrode protocol was the same as described in the previous section.

### Transient cooling and rewarming

Hypothermia was induced by running Tyrode's solution through a water-coupled heat exchanger before allowing the solution to perfuse the heart. Cooling from baseline (37°C) was achieved through reduction in the temperature of the water bath to 34, 32, 28, 25, 22, 20, 17 and 15°C. Cooling to 15°C took approximately 80 minutes, with hearts maintained at each target temperatures ( $\pm 0.2^\circ\text{C}$ ) for ~5 mins prior to recordings. Rewarming (to 22, 32 and 37°C) took approximately 25 mins. RA pacing by hook electrodes was adjusted at each temperature (Figure 3.14B).

## Results

Table 3.1 shows the number of hearts that signals were successfully acquired from during cooling and rewarming. Voltage measurements were recorded from 37°C down to 15°C and through rewarming. Due to the length of the experiments, a second bolus of di-4-ANEPPS was required in most hearts. No extra Fura-4F was required however Ca<sup>2+</sup> transients recorded below 17°C had such a small amplitude that they were not able to be reliably analysed.

**Table 3.1: Voltage and Ca<sup>2+</sup> heart data**

<i>Temp (°C)</i>	37	34	32	28	25	22	20	17	15	22	32	37
<i>Voltage (n=)</i>	10	7	7	7	7	6	7	6	5	4	5	5
<i>Ca<sup>2+</sup> (n=)</i>	5	7	7	7	7	4	5	1	-	2	3	4

Shown are the temperatures used and number of hearts that voltage and Ca<sup>2+</sup> data was acquired from. Shaded boxes refer to rewarming temperatures.

### Effect of transient hypothermia on action potential characteristics

Transient cooling and rewarming showed distinct changes in AP morphology. Figure 3.14A shows typical AP traces from a single heart at selected temperatures though cooling and rewarming. The AP from rewarmed hearts displays a markedly prolonged shape when compared to baseline. Ventricular activation (TRise) progressively slowed through cooling, with temperatures below 22°C significantly different when compared to baseline ( $1.8 \pm 0.08$  ms at 37°C vs  $22.17 \pm 1.22$  at 15°C,  $p < 0.05$ ). Rewarming returned Trise towards baseline values however it did not recover completely ( $5.83 \pm 0.7$  vs  $1.8 \pm 0.1$  ms). This difference was not found to be different when applying a repeated measure ANOVA and post hoc analysis, however comparing these two points using a student t-test indicated a significant difference ( $p < 0.01$ ). Time to repolarisation (TRepol90) gradually slowed through cooling with temperatures below 25°C significantly different from baseline ( $p < 0.05$ ).

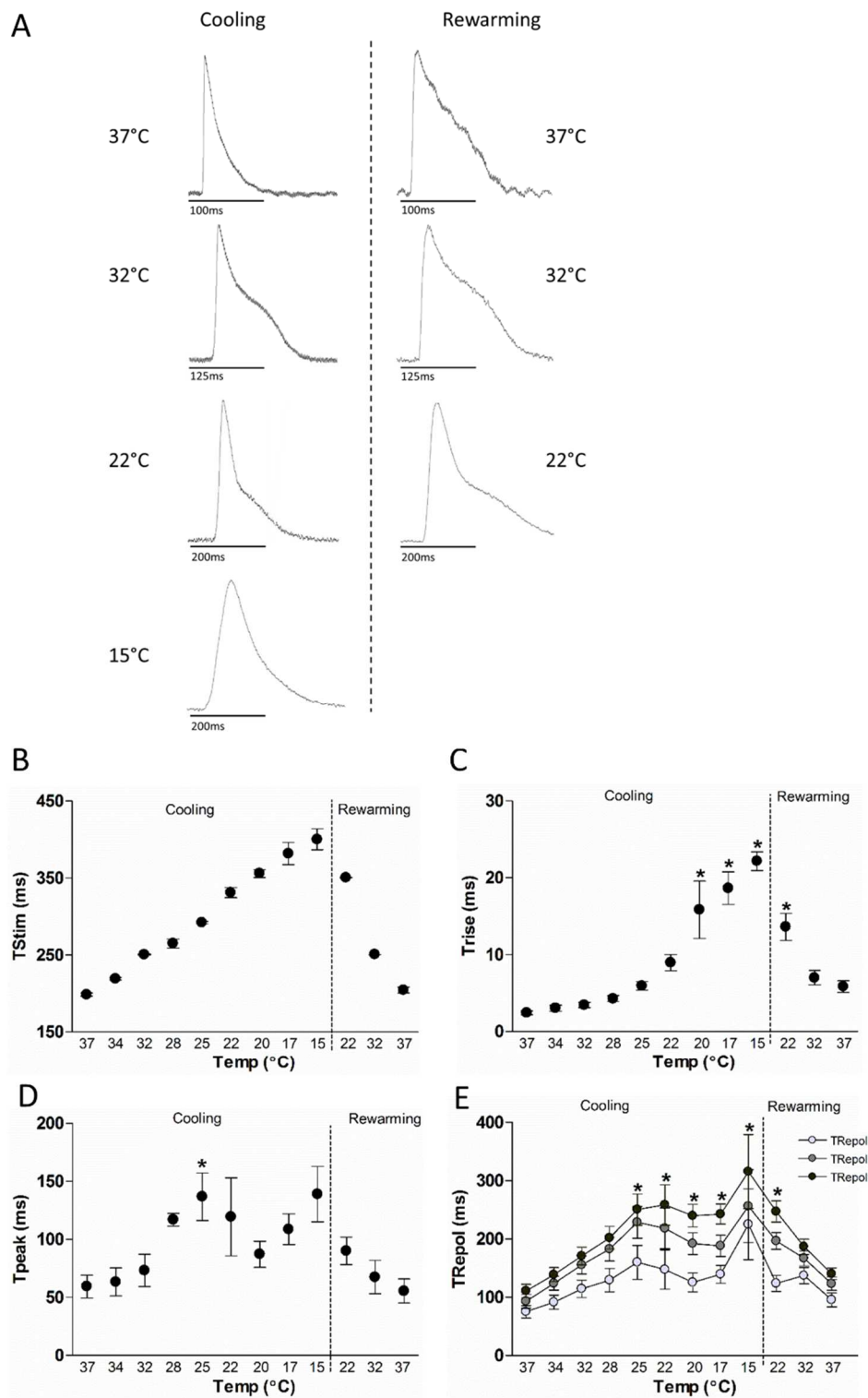


Figure 3.14 Voltage measurements on isolated hearts undergoing transient hypothermia/rewarming.

Langendorff perfused hearts loaded with di-4 ANEPPS and optrode was placed against the LV. Cooled and rewarmed over 90 mins. A: Example traces of APs during cooling (37°C, 31°C, 22°C and 15°C) and rewarming (22°C, 31°C and 37°C). B: Stimulation Time (TStim), rate hearts were stimulated at throughout. C: Rise Time (TRise). C: Time to peak (TPeak). D: Time to Repolarisation 50%, 75% and 90% (TRepol50/75/90). White circles refer to TRepol50, light grey bars to TRepl75 and black to TRepol90. \*p<0.05 compared to 37°C baseline. (Not shown on graph TRepol75, 28°C, 25°C, 22°C, 20°C and 17°C different to 37°C baseline, p<0.05). No differences observed between temperatures in TRepol50.

Mean APD differences can be seen in Figure 3.15. As shown in the example records there is a prolongation of APD throughout cooling and rewarming. APD<sub>90</sub> is significantly slower than baseline at temperatures below 32°C ( $p < 0.001$ ). At 15°C APD<sub>90</sub> is almost 3x longer than at 37°C ( $53.5 \pm 2.6$  vs  $143.2 \pm 10.1$  ms,  $p < 0.05$ ). Unlike activation parameters, rewarming from 15°C to 22°C showed an increase in APD<sub>90</sub> ( $143.2 \pm 10.1$  vs  $172.9 \pm 11.1$  ms). Post hoc analysis using Tukeys test did not find this to be different, however comparison by paired t-test showed that at 22°C APD<sub>90</sub> was significantly longer than at 15°C ( $p < 0.05$ ). Rewarming to 37°C showed that APD<sub>90</sub> remained prolonged with a duration almost double starting values ( $53.5 \pm 2.6$  vs  $92.2 \pm 4.9$  ms,  $p < 0.05$ ).

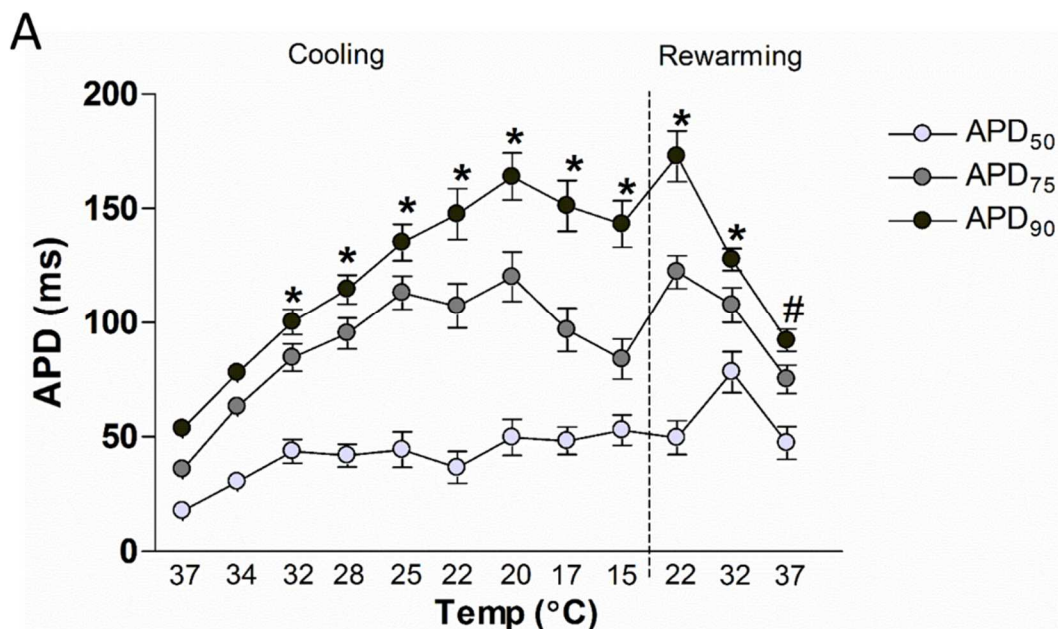


Figure 3.15: APD in isolated hearts during transient cooling and rewarming.

Langendorff perfused hearts loaded with Di-4 ANEPPS and optrode was placed against the LV. Cooled and rewarmed over 90 mins. A: APD<sub>50/75/90</sub>. White circles refer to APD<sub>50</sub>, light grey to APD<sub>75</sub> and black to APD<sub>90</sub>. \* $p < 0.001$  compared to 37°C baseline. #  $p < 0.05$  compared to 37°C baseline. (Not shown on graph, all points that are different at APD<sub>90</sub>, also significant at APD<sub>75</sub>,  $p < 0.05$ . For APD<sub>50</sub>, 31°C, 28°C, 25°C, 20°C, 17°C, 15°C, 22°C and 31°C different to 37 baseline,  $p < 0.05$ ).



**Effect of transient hypothermia on intracellular  $\text{Ca}^{2+}$** 

Example traces of  $\text{Ca}^{2+}$  recorded from isolated perfused rat hearts during transient cooling and rewarming can be seen in Figure 3.16. Cooling showed non-significant variation in rise time and time to peak.  $\text{Ca}^{2+}$  transient decay (diastole) was prolonged at temperatures below  $32^\circ\text{C}$  and up to  $32^\circ\text{C}$  upon rewarming ( $p < 0.05$ ), however at  $37^\circ\text{C}$  no differences compared to baseline were detected. Despite missing data at  $15^\circ\text{C}$  there does not seem to be a similar rewarming change in  $\text{Ca}^{2+}$  that was seen in APD90.

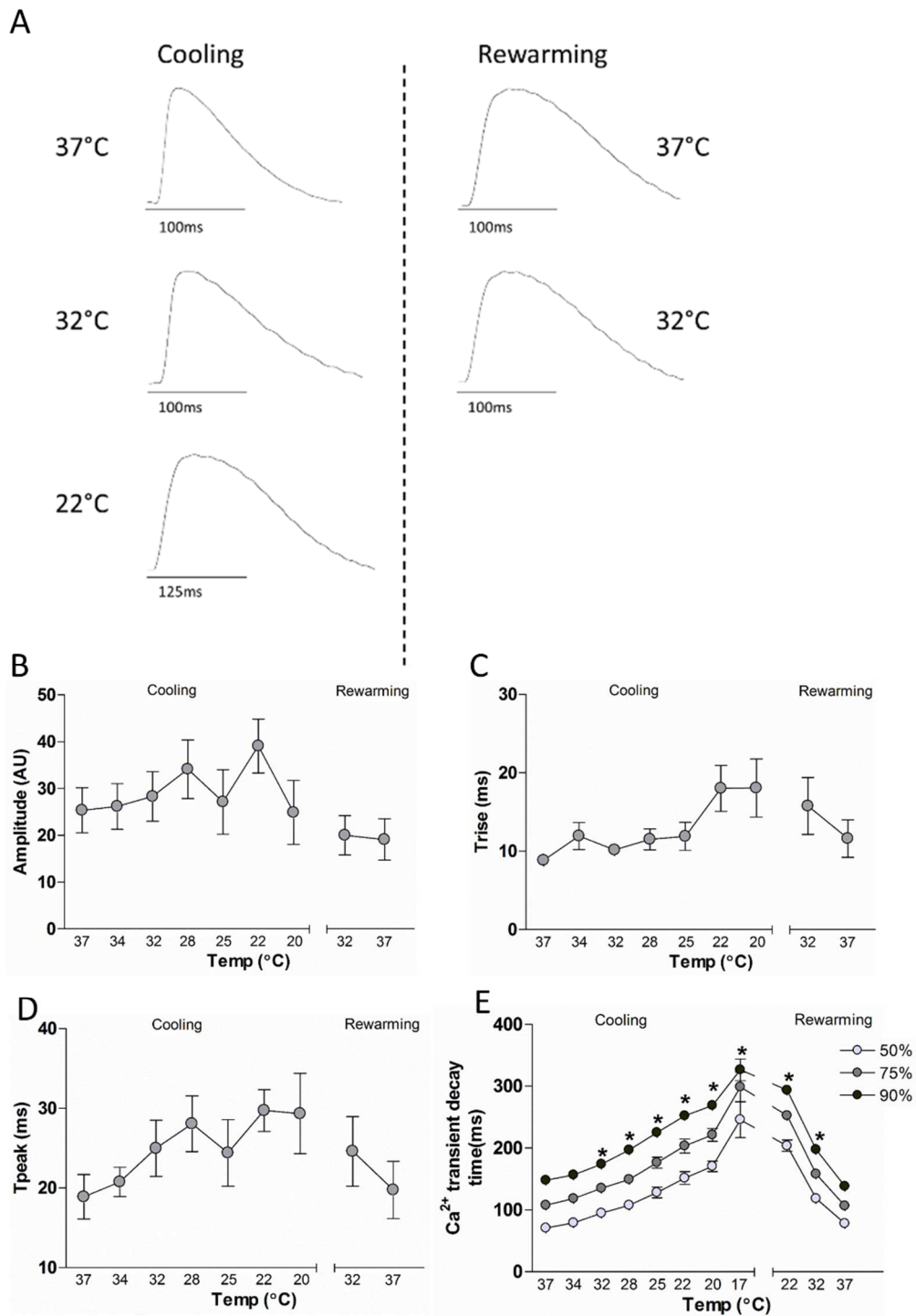


Figure 3.5: Optrode  $\text{Ca}^{2+}$  measurements during transient hypothermia and rewarming.

Langendorff perfused hearts loaded with Fura-4F and optrode placed against the LV. Cooled and rewarmed over ~100 mins. **A**: Example traces of  $\text{Ca}^{2+}$  transients during cooling (37°C, 32°C, 22°C) and rewarming (32°C and 37°C). **B**:  $\text{Ca}^{2+}$  transient amplitude (arbitrary units, AU). **C**: Rise time (Trise) of  $\text{Ca}^{2+}$  transient. **D**: Time to peak (Tpeak). **E**: Time to 50/75/90%  $\text{Ca}^{2+}$  transient decay (Decay50/75/90). Figures B, C and D: no data available below 20°C, E: no data available at 15°C. \* $p < 0.05$  compared to 37°C baseline. No differences were found in Decay50/75.

## Discussion

This chapter looked to examine the electrophysiology of isolated hearts following short and long term hypothermia. There are several key findings from this work, which are detailed below.

### Summary of findings

Part 1 examined electrophysiology and mechanical function following an *in vivo* hypothermia/rewarming procedure:

- 1 Animals that underwent an *in vivo* hypothermia rewarming procedure suffered from rewarming shock, with evidence of a reduction in CO at 37°C, and a compromised SV whilst other haemodynamic measurements stabilised. These findings are in line with previous studies on both dog (Prec et al., 1949; Blair et al., 1956; Tveita et al., 1994) and rat models (Tveita et al., 1996; Kondratiev et al., 2008).
- 2 Whilst previous work using this model has used 4-5 hours of stable hypothermia (Tveita et al., 1996; Kondratiev et al., 2006a; Kondratiev et al., 2008; Wold et al., 2013), this study confirmed that after 3 hours there was a significant reduction in contractile function, indicative of the rewarming effect (Tveita et al., 2012).
- 3 Following *in vivo* hypothermia and rewarming there is a *shortening* of APD<sub>90</sub>.

Part 2 examined the effect of transient hypothermia on cardiac electrophysiology:

- 1 Transient cooling and rewarming appears to affect cardiac behaviour in a different manner than long term *in vivo* cooling.
- 2 Following transient hypothermia and rewarming there is a *prolongation* of APD<sub>90</sub>.

- 3 No change in intracellular  $\text{Ca}^{2+}$  upon rewarming was detected. This is in line with isolated cell findings from Chapter 3 which found no change in  $\text{Ca}^{2+}$  following a hypothermia/rewarming procedure.

### **APD shortening following *in vivo* hypothermia and rewarming**

It has been extensively shown in experimental studies that hypothermia prolongs APD (Fedorov et al., 2008; Piktel et al., 2011; Egorov et al., 2012), however in this model of rewarming induced shock we have been able to show that there is a markedly shorter APD.

Contraction is initiated by an action potential, and the action potential duration (APD) is determined by a critical balance between inward and outward membrane currents. The AP consists of 4 phases;

Phase 0: rapid depolarisation, where the voltage dependent  $\text{Na}^+$  channel conducts a rapid inward current of  $\text{Na}^+$  ( $I_{\text{Na}}$ ), which depolarises the myocyte leading to upstroke of the AP

Phase 1: early repolarisation,  $\text{Na}^+$  channels rapidly inactivate and a transient outward  $\text{K}^+$  current ( $I_{\text{to}}$ ) causes early partial repolarisation

Phase 2: the plateau contraction begins during this phase where a second inward current, mainly of extracellular  $\text{Ca}^{2+}$  ions ( $I_{\text{Ca}}$ ) maintains a prolonged depolarisation. An inward  $\text{Na}^+$  current through NCX maintains the late stage of phase 2.

Phase 3: final repolarisation, outward  $\text{K}^+$  currents ( $I_{\text{Kv}}$  and  $I_{\text{Kir}}$ ) terminate the plateau

The shortening of APD that we see in this study could be as a direct result of the anaerobic environment that develops in hypothermia/rewarming. In other situations, where metabolic inhibition has occurred (e.g. ischaemia and hypoxia), the ATP-sensitive  $\text{K}^+$  ( $I_{\text{K(ATP)}}$ ) channel has been implicated as a cause of APD shortening (Weiss and Lamp, 1989; Fujita and Kurachi, 2000; Tang et al., 2012) and provides a link between cellular metabolism and excitability. Gated by intracellular nucleotides, ATP and ADP, this type of  $\text{K}^+$  channel can be found

on two subcellular membranes; sarcoplasmic (sarcoK<sub>ATP</sub>) and mitochondrial (mitoK<sub>ATP</sub>). When ATP decreases, adenosine removes the ATP inhibition and opens the channels, of which there are a high density in cardiomyocytes (Donald M. Bers, 2002) and increases the K<sup>+</sup> efflux, and shortening the APD. Evidence to support K<sub>ATP</sub> channels as a contributor to shortening of APD has been determined from studying the effect of Gilbenclamide, a selective K<sub>ATP</sub> channel blocker. Studies have shown that it is able to reduce or prevent APD shortening in hypoxia and ischaemia (Kantor et al., 1990; Venkatesh et al., 1991). It has been shown that low intracellular ATP (<0.1mM) is enough to activate less than 1% of channels, which can result in an APD shortening of ~50% (Weiss et al., 1992). Within this study, hearts that had been cooled and rewarmed showed a reduction in APD of ~20% when compared to normothermic hearts. Therefore, a small decrease in ATP could have activated the opening of these channels, terminating the AP early and this could be a component in reduced contractile force.

It has previously been shown that ATP was significantly reduced both during hypothermia and rewarming (Ytrehus and Aspang, 1994), alongside an increase in Pi (Tveita et al., 1996). In isolated myocytes it has been shown that ATP depletion inhibits the Na<sup>+</sup>/K<sup>+</sup> ATPase leading to an increase in intracellular Na<sup>+</sup>. Alongside this there is a significant increase in K<sup>+</sup> conductance due to the opening of K<sub>ATP</sub> channels as a consequence of decreased ATP (Lederer et al., 1989). These findings, in combination with the results from this study suggest that metabolic consequences of hypothermia and rewarming are significant enough to cause activation of the K<sub>ATP</sub> channels.

### **APD prolongation following transient hypothermia**

Transient hypothermia showed a significant prolongation in APD in isolated rat hearts that didn't recover upon rewarming. APD prolongation is a common finding in hypothermia (Shattock and Bers, 1987; Yan and Antzelevitch, 1996; Mattu et al., 2002). During hypothermia it is known that ionic currents (e.g. I<sub>Ca</sub>, I<sub>Na</sub>, I<sub>K</sub>, I<sub>Ca</sub>) are affected by decreasing temperature (McDonald and Trautwein,

1978; ; Colatsky, 1980; Cavalié et al., 1985; Sprung et al., 1991; Kiyosue et al., 1993; Puglisi et al., 1996).

The findings from this study showed no change in AP rise time throughout hypothermia or rewarming which suggests that there is a change in repolarising currents that does not occur during sustained hypothermia.

These results would suggest that transient cooling and rewarming is more detrimental to the heart than prolonged hypothermia. Whilst there is significant contractile dysfunction following *in vivo* cooling and rewarming, the AP has returned to a shorter duration. In contrast, rewarming hearts from transient hypothermia does not return AP to normal, suggesting that these hearts are more affected by the speed of which the low temperatures were induced.

## Conclusions

Following *in vivo* hypothermia/rewarming where hearts showed a reduced contractile function there was a chronically shorter APD which suggests a sustained electrophysiological effect that would manifest as a shortened QT interval, a change that has been linked to arrhythmic behaviour in human hearts (Iribarren et al., 2014). This APD effect could potentially reduce the amplitude of the intracellular  $\text{Ca}^{2+}$  transient and subsequent contraction, but measurements of  $\text{Ca}^{2+}$  with Fura4 failed to detect a significant decrease in transient amplitude. Thus the reduced SV would appear to be the result of reduced myofilament sensitivity to  $\text{Ca}^{2+}$ . In contrast to this, a period of transient hypothermia has alternative detrimental effects on the cardiac APD when compared to prolonged hypothermia, an effect that would predispose to the induction of long QT related arrhythmias and ventricular tachycardia.

## **Chapter 4: Examination of the inotropic response in an *in vitro* model of hypothermia/rewarming**

## Introduction

The mortality rate from accidental hypothermia remains high and has not improved in the last 40 years despite medical advances (Walpoth et al., 1997). The incidence of arrhythmias and cardiac output insufficiency that occur during rewarming are documented, however there remains an incomplete understanding of the underlying physiological mechanisms.

### Rewarming induced shock

Whilst many studies focus on the physiological effects of cooling, there are substantial detrimental side effects that occur during rewarming from accidental hypothermia. The inability to maintain CO and blood pressure, known as “rewarming shock” is a common threat.

Early canine studies examining hypothermia/re-warming found evidence of this ‘rewarming shock’. When cooled to 30-33°C, dogs displayed prolongation of systole and isovolumetric relaxation. However, on rewarming to normothermia there was an immediate and rapid increase in heart and respiration rate that rose with temperature with evidence of circulatory collapse (characterised as low CO that didn’t return to normal, hypotension and diminished ventricular work) (Prec et al., 1949; Bigelow et al., 1950; Berne, 1954; Blair et al., 1956). More recently a reproducible experimental rat model of rewarming induced shock was developed. Anaesthetised animals were cooled to 13-16°C and maintained at this temperature for 4-5 hours before being rewarmed to 37°C. In line with the early canine work, data generated from this model has shown that upon rewarming from sustained severe hypothermia there is ~50% reduction in CO compared to baseline values (Tveita et al., 1996; Kondratiev et al., 2006a; Kondratiev et al., 2006b). HR and blood pressure were maintained as was oxygen supply and the relative oxygenation of cardiac tissue but this was alongside a substantially reduced CO. This lowered CO would not normally be compatible with adequate perfusion of the tissue and the cellular basis for this remains unclear.

In terms of therapeutic hypothermia, a similar cardiac deterioration has been reported following surgery, however in such cases this is attributed to



myocardial stunning, where mechanical dysfunction persists after the restoration of blood flow following ischaemia (Chalkias and Xanthos, 2012).

The findings from the previous chapter showed that there was a significant reduction in CO. This would suggest that it is a cardiovascular response, however although the SV was not significantly different upon rewarming, there was a trend towards a reduction upon rewarming to 37°C. CO depends on both SV and HR, however in order to determine whether the rewarming dysfunction is cardiovascular or peripheral response we aimed to assess whether rewarming shock could be recapitulated *in vitro* using isolated ventricular myocytes.

### **Cellular contractile dysfunction and temperature**

In order to understand the mechanisms involved in rewarming shock it is necessary to consider the fact that cardiac contraction is a result of the process of EC-coupling. There has been a vast amount of research into the mechanisms involved in this process, in both whole heart preparations but also in isolated cardiomyocytes (Fabiato, 1985; Frampton et al., 1991; Hattori et al., 1991; Satoh et al., 1997).

It is well established that hypothermia leads to a positive inotropic response which causes an increase in the magnitude of cardiac contraction (Langer and Brady, 1968; Bjornstad et al., 1993). One study showed that when cooling rabbit ventricular muscle from 37°C to 25°C there was a 400% increase in force production (Shattock and Bers, 1987). Changes in contractile function have been observed across species, and hypothermia has been shown to increase the amplitudes of contraction recorded from rabbit, ferret and cat cardiomyocytes at 25°C (Puglisi et al., 1996).

Conversely, rewarming has been associated with a negative inotropic response. Previous work has shown that following a period of hypothermia and rewarming, hearts displayed a Ca<sup>2+</sup> content that was six to seven fold greater than control hearts (Kondratiev et al., 2008). A recent study therefore investigated whether an increase in global Ca<sup>2+</sup> may be a possible reason for cardiac insufficiency upon rewarming. Using the same animal model as the previous study, Wold *et al* (2013) examined whether the reported Ca<sup>2+</sup> increase occurred during the

hypothermia or rewarming stage. They found that the rise in global myocardial  $\text{Ca}^{2+}$  was present following 4 hours of severe hypothermia ( $15^{\circ}\text{C}$ ) and that it remained elevated following rewarming. Based on these results, they suggested that the  $\text{Ca}^{2+}$  overload was as a result of the hypothermia, and that the dysfunction seen following rewarming was not induced by the rewarming process per se.

It is clear that reducing temperature has a marked influence on many components of EC-coupling, and whether the dysfunction we see on rewarming is a consequence of the cooling, or from the increasing temperatures remains unclear. Animal models have provided a vast amount of data; however, a similar model has not been reproduced *in vitro* to assist in providing a better understanding of this process.

### **Aims**

The main aim of this chapter was to assess whether ‘rewarming shock’, a phenomenon evident *in vivo*, could be recapitulated in an *in vitro* model using isolated rat ventricular cardiomyocytes.

## Methods

### Cardiomyocyte Isolation

Male Wistar rats (250-300g) underwent a Schedule 1 procedure in accordance with Home Office Guidelines. Animals were stunned followed by a cervical dislocation causing both ascending and descending injury to the spinal cord and brain stem. The xiphoid process was identified and the thoracic cavity was opened to allow the rapid removal of the heart which was subsequently placed in an ice-cold perfusion buffer containing Krebs solution and heparin (25IU/ml) for anti-coagulation. Excess tissue was trimmed before the heart was mounted and tied via the aorta onto the cannula of a Langendorff perfusion system (Figure 2.1). Hearts were perfused (12ml/min) with Krebs solution (37°C) until blood cleared from the coronary vessels and perfusate ran clear. Perfusion was then switched to a Krebs solution containing 0.05% (w/v) bovine serum albumin (BSA) and type 1 collagenase (0.68mg/ml) to dissociate single ventricular myocytes. Once the initial solution had run through, the enzyme was collected and re-circulated. Enzymatic digestion was continued until tissue felt softened to the touch, in most cases this was between 12-14 minutes. Hearts were removed from the perfusion system and ventricles diced into small segments in KB solution containing 0.5% (w/v) BSA. Tissue was gently triturated with a Pasteur pipette in order to dissociate myocytes and the supernatant filtered through polyamide mesh (pore size 250µm) before being re-suspended in KB solution. Cell solution was gradually brought up to 1.8mM Ca<sup>2+</sup> concentration using CaCl<sub>2</sub>. This was carried out in a stepwise manner using increasing Ca<sup>2+</sup> concentration over a period of 60 min.

### *In vitro* cooling and rewarming

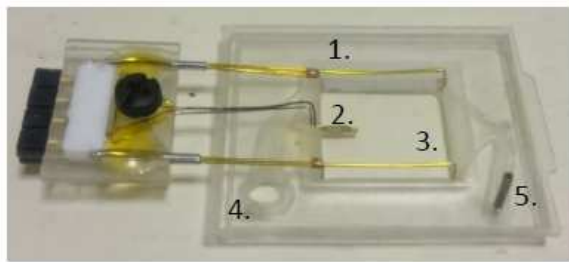
Ventricular myocytes from each animal were split into hypothermic and normothermic groups. For the hypothermia group, the cell suspension was gradually cooled by lowering the temperature of the waterbath that the Eppendorf was semi-immersed in. Cooling to 15°C took approx. 20 mins and was maintained for 4 hours. Myocytes were either stimulated (MyoPacer Field Stimulator, IonOptix) at 1Hz (40V; 2ms duration) at 15°C for 4 hours to ensure contractile activity or remained as a quiescent control. Samples of cells were

taken at each time point and gradually rewarmed to 37°C over 10 min. A metal heat plate was used to ensure temperature remained at 37°C throughout measurements. A further control group of ventricular cardiomyocytes underwent the same dissociation protocol but were maintained at 37°C for 4 hours (stimulated and quiescent).

### Cell Shortening

Cardiomyocytes were placed in a cell chamber (CellMicroControls, Norfolk, VA, US) (Figure 4.1) which was then secured onto a heat plate. Cells were allowed to settle for 10 minutes then were perfused throughout experiments with 1.8mM Ca<sup>2+</sup> Krebs (37°C) to ensure availability of substrates for contraction and to maintain rewarming temperature. Temperature was monitored via the thermistor in the cell bath. Individual myocytes were identified and twitch-stimulated by electrical field stimulation at a set frequency of 1Hz at 2ms duration (DS2A and DG2A, Digitimer Ltd) via the stimulation electrodes on either side of the chamber.

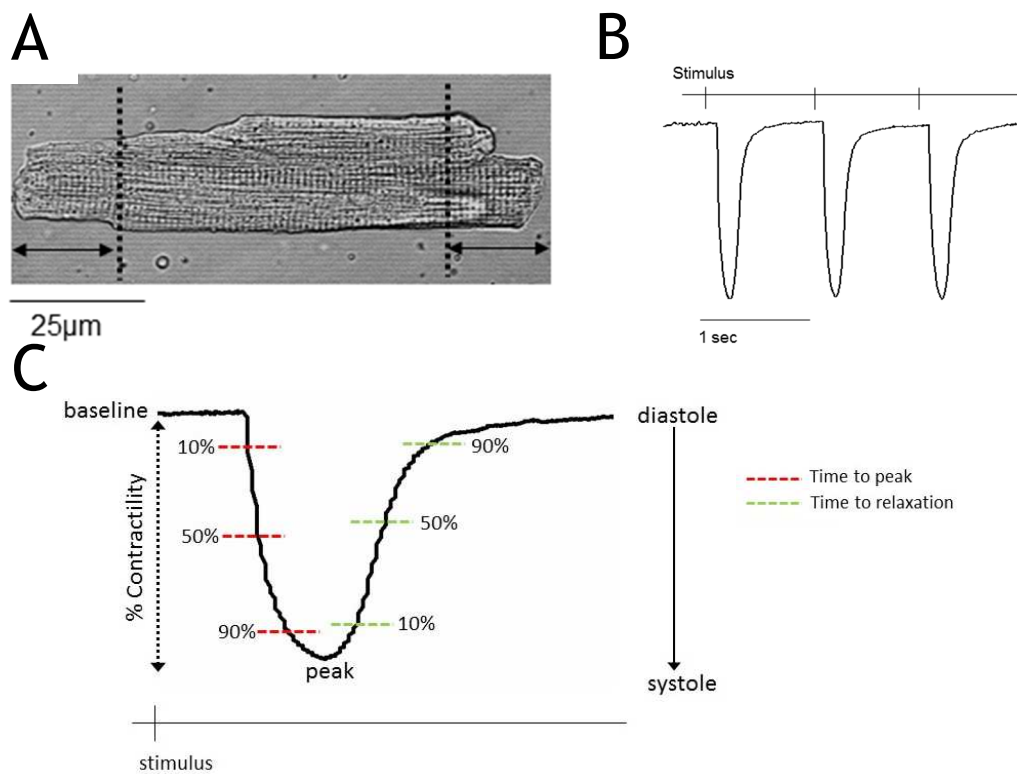
Video-based edge detection was used to record cell shortening with each contraction detected by light microscopy (Light source: Optoled lite - Cairn Research Ltd, Faversham, UK; Microscope: Nikon Diaphot 200, 40x objective). The velocity of contraction and relaxation and the amplitude of cell shortening was measured using light/dark contrast of the cell using signals which followed the left and right edges of the myocyte (Figure 4.2). These cell tracers were used to determine the relative cell length and calibrate it to absolute cell length. Both the left and right edges were scanned from the outside in to maximise edge detection. Images were digitised and viewed in IonWizard6.1 (IonOptix Ltd). Analysis of the traces (Figure 4.2B) provided information on the time course of contraction and relaxation and the relative fractional shortening (FS).



- 1.) Platinum Iridium stimulation wires
- 2.) Central chamber – 13x12mm (0.3ml volume)
- 3.) Inflow tube
- 4.) Thermistor
- 5.) Area for aspirating solution

**Figure 4.1: Cell bath**

Cell bath chamber has an inflow tube and an area for aspirating solution. The stimulator/thermistor assembly presses into the chamber holder, this design allowed the monitoring of temperature of solution in the bath.



**Figure 4.2: Cell shortening recording**

**A:** Brightfield image of Isolated ventricular myocyte. Arrows refer to the tracking of the left and right edges for edge detection. **B:** Example trace of fractional shortening (FS). **C:** Parameters measured by IonWizard (semi-automated). FS is expressed at percent (%) difference between baseline length (diastole) and peak height (systole).

**Intracellular Ca<sup>2+</sup> recordings following hypothermia and rewarming**

Intracellular Ca<sup>2+</sup> was measured in cardiomyocytes following the hypothermic and rewarming protocol. Myocytes were loaded with 5µM of Ca<sup>2+</sup> sensitive dye Fura-4F (Chapter 2). Cells were suspended in dye for 10 min at 15°C. Cells were then centrifuged, supernatant removed and re-suspended in Krebs solution (1.8mM Ca<sup>2+</sup>) and rewarmed to 37°C. Myocytes were then stimulated in the same manner as for cell shortening. Cells were loaded with Ca<sup>2+</sup> sensitive Fura-4F prior to rewarming in the hypothermic group and at 3 hours in the normothermic control group. Recording of Ca<sup>2+</sup> transients was by epifluorescence microscopy (Nikon eclipse T/C. 40x/1.3 NA oil-immersion lens). In order to eliminate background fluorescence an aperture was placed around the cell so that there were no other cells in the optical field. Myocytes were excited using a xenon arc lamp as a light source with a monochromator transmitting at 340 and 380nm wavelengths alternatively at 6ms intervals, and fluorescence emission was recorded through one photomultiplier tube (PMT) at 510 nm, with transients viewed in IonWizard 6.1. The optical settings for the epifluorescence are described in Figure 4.3. Analysis of the traces provided information on the time course of Ca<sup>2+</sup> transient decay, amplitude and time to peak.

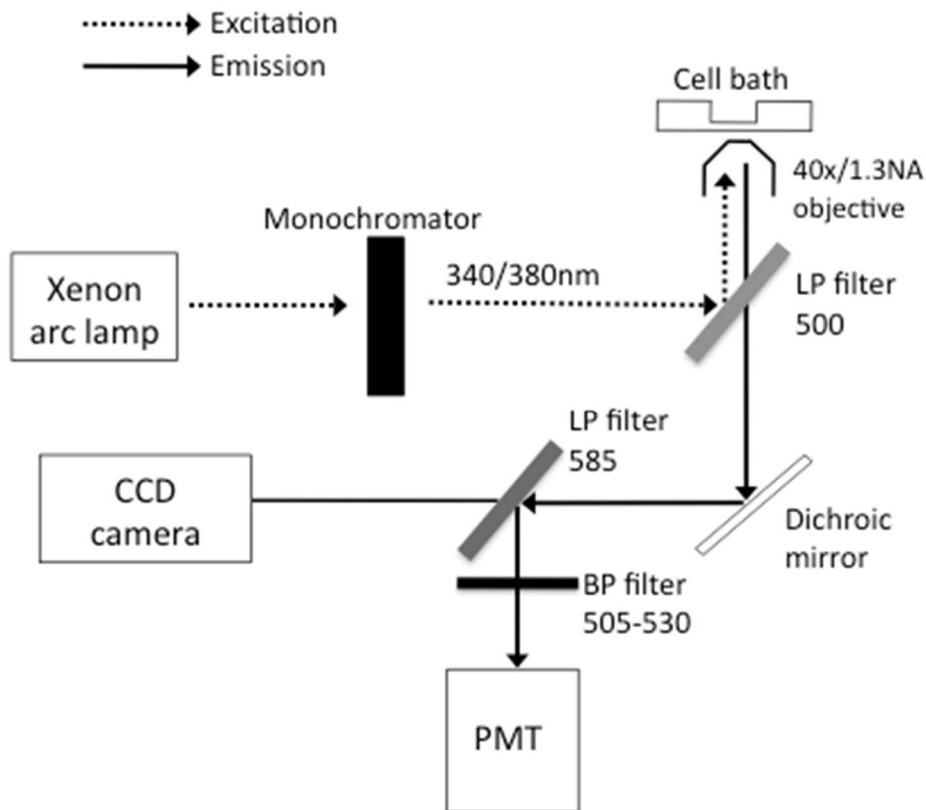
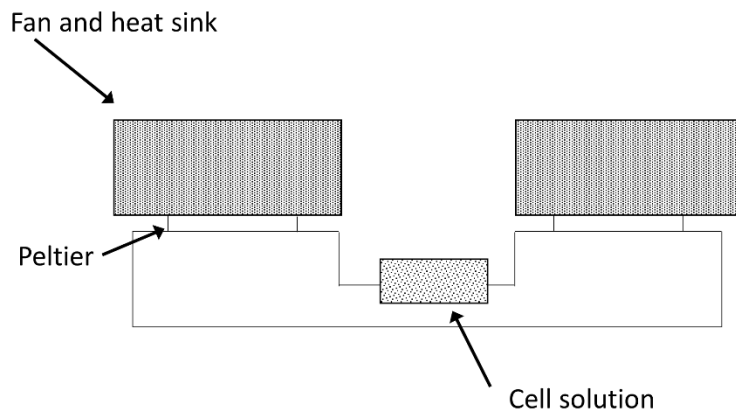


Figure 4.3: Schematic of optical settings for epifluorescence microscopy.

Light from the xenon arc lamp is directed onto the monochromator which displays light at 340nm and 380nm. The monochromator alternates between each excitation wavelength at 6ms intervals. Emitted fluorescence passes through a long pass (LP) filter at 500nm to split it from the excitation wavelength. A further LP filter at 585nm directs emitted light to a photo multiplier tube (PMT) through a band pass (BP) filter at 505-530nm, which corresponds to Fura 4F emission wavelengths. Light of wavelengths longer than 600nm (from microscope lamp) was directed to the CCD camera to enable cell edge detection measurements.

## Sarcomere length assessment during cooling and rewarming

Separate experiments used a custom made cooling plate to ensure rate of temperature change was the same in all experiments (Figure 4.4).



**Figure 4.4: Cooling plate design**

Metal plate that cooled cells to 17°C and rewarmed to 37°C. Two 35mm dishes were fitted into the plate, one of which was stimulated by graphite electrodes throughout the 3 hours, whilst the other remained as a quiescent control.

Two wells of myocytes were either cooled or maintained at 37°C, one stimulated at 1Hz (MyoPacer, 2ms, 40V) whilst the other remained quiescent. Cells could then be identified and the co-ordinates stored, therefore the same cells could be recorded at multiple time-points, before, during and after hypothermia. The throughput of this system was considerably higher than the previously described method and so an average of 10-15 recordings were made at each time point.

Cells were identified and position was recorded on the CellOptiq electrophysiology platform (Clyde Bioscience Ltd., Glasgow, UK). Sarcomere length shortening was calculated using a system which utilised a high resolution digital camera (Hamamatsu C11440) in conjunction with a multiple objective Olympus microscope. Images of the contracting cell were recorded for 10 s (1000, frames/s) on HCLImage Live (V4.2.003). Analysis was carried out using an in house developed macro for ImageJ. Examination of the recorded average pixel intensity in each frame allowed the production of a graphical representation of contraction using a fast fourier transform (FFT) algorithm (Figure 4.5).



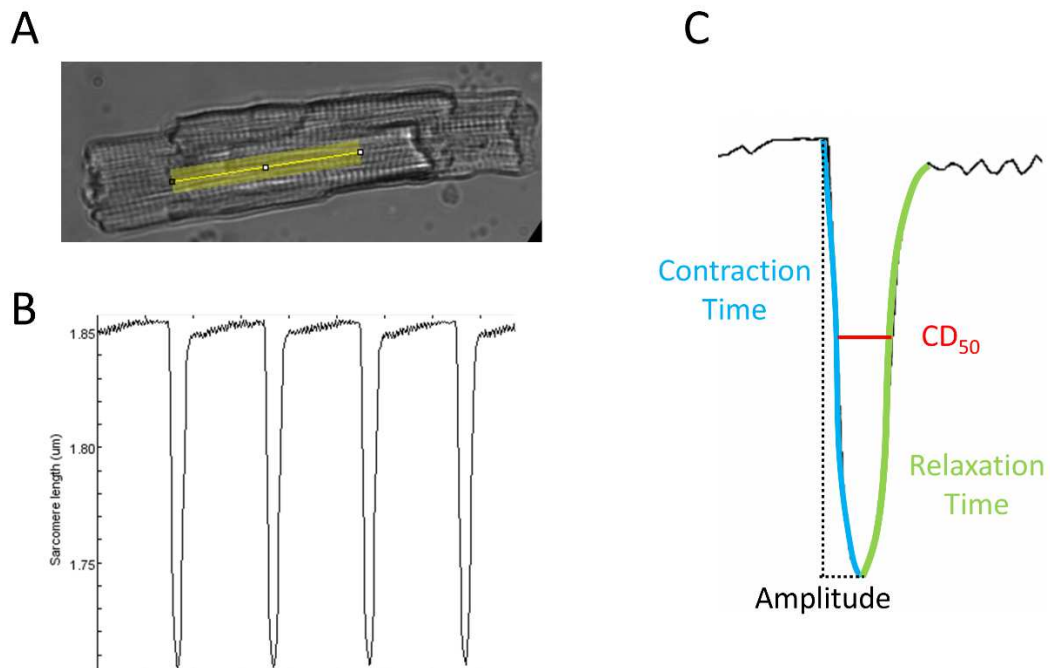


Figure 4.5: Assessment of sarcomere length.

**A:** Brightfield image of cell, the yellow box was fitted over a section of visible sarcomeres. **B:** Example trace of sarcomere length shortening. **C:** Measurements taken from each transient. Contraction difference 50 (CD<sub>50</sub>).

### CellOptiq assay to study intracellular Ca<sup>2+</sup>

Cells were loaded with 8 $\mu$ M Fura-4F at the start of the experiment. Repeated measurements were then taken from the cells at the same time points as sarcomere length assessment. As with the previous Ca<sup>2+</sup> measurements, an aperture was placed around the cell to eliminate background fluorescence. Myocytes were excited by an OptoLED (Cairn Research; Faversham, UK), alternating between 360 and 380nm with emission collected at 510nm. Recordings were made at 100Hz and could be viewed and analysed in custom software designed for this system (Dr Francis Burton, University of Glasgow). Ca<sup>2+</sup> transients were averaged and amplitude and time-course measurements taken.

### Statistical analysis

Data are presented as mean  $\pm$  SEM, and assessed by unpaired t-test or one-way ANOVA for repeated measures where appropriate. Where significant differences

were found, data were post-hoc analysed using Tukey's test. All statistical tests were performed using GraphPad Prism software.

## Results

### Contractility in isolated ventricular cardiomyocytes following hypothermia/rewarming

This study looked at the effect of cooling and rewarming on contractility in isolated ventricular myocytes. Figure 4.6A shows example traces of FS from cells at 3 hours following hypothermia/rewarming or normothermia. Immediately following dissociation, FS was  $8.9 \pm 0.4\%$  (Figure 4.6). Following hypothermic incubation ( $15^\circ\text{C}$ ) and subsequent re-warming to  $37^\circ\text{C}$ , cells which had been paced at 1Hz throughout showed a reduction in FS of 38% ( $5.5 \pm 0.7\%$ ,  $p < 0.01$ ), 57% ( $3.8 \pm 0.6\%$ ,  $p < 0.001$ ) and 54% ( $4.1 \pm 0.5\%$ ,  $p < 0.01$ ) at 2, 3 and 4 hours respectively when compared to freshly isolated cells. Cardiomyocytes stimulated at  $37^\circ\text{C}$  for 4 hours showed a reduction when compared to fresh cells ( $p < 0.05$ ). In quiescent cells (Figure 4.6B(i)) a significant reduction in FS was also only evident at 4 hours following hypothermia/rewarming. When comparing quiescent and stimulated cells following hypothermia and rewarming, myocytes at 2 and 3 hours were significantly different from one another, with stimulated cells showing a significantly lower FS ( $8.3 \pm 1.0$  vs  $5.5 \pm 0.7$  and  $6.4 \pm 0.9$  vs  $3.8 \pm 0.6$ ,  $p < 0.05$ ).

The reduced contractility evident following 3 hours of hypothermic incubation was not accompanied by a change in time to peak or time to relaxation (Figure 4.6C/D). All measurements of contractility can be seen in Table 3.1.

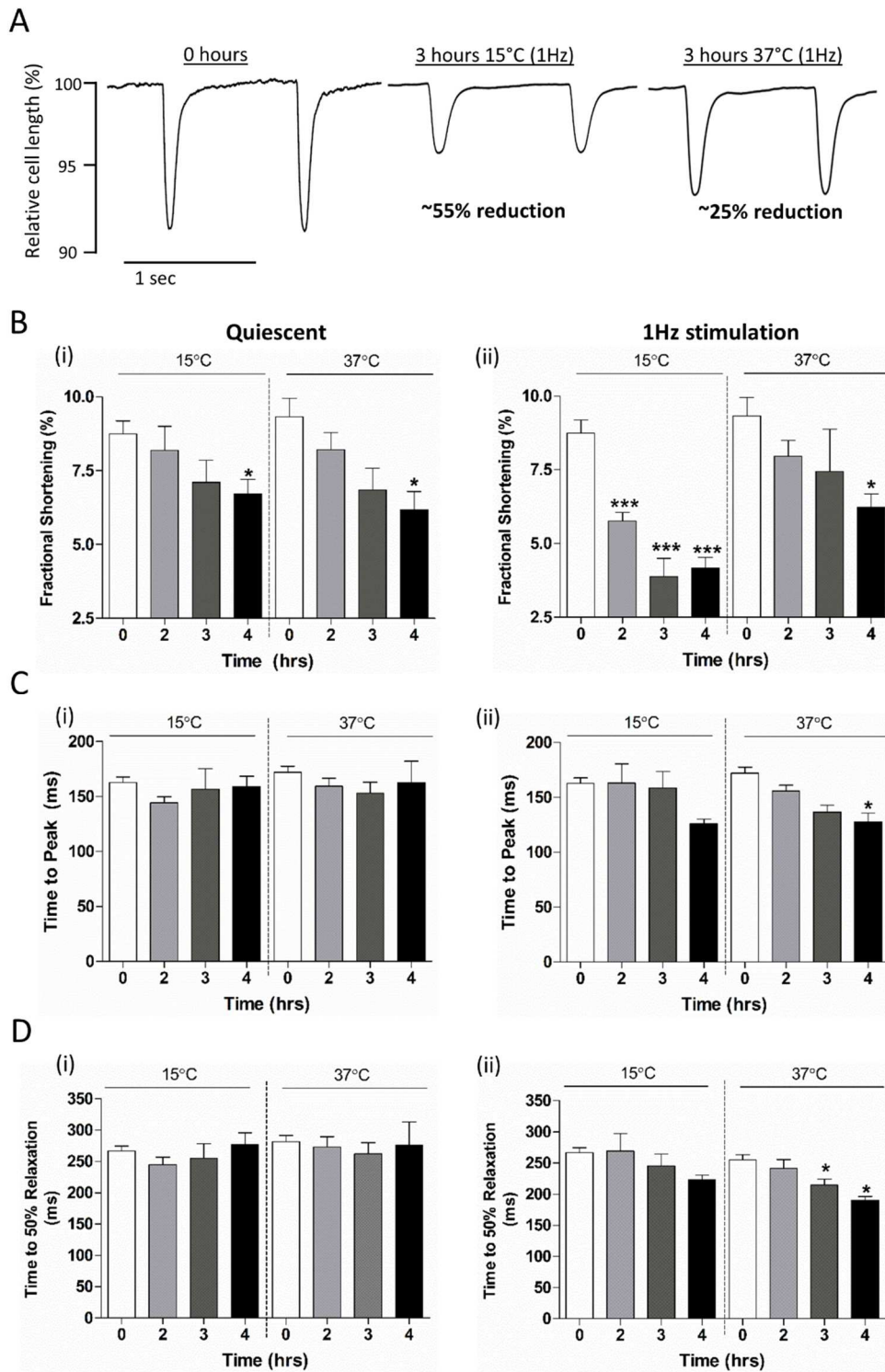


Figure 4.6: Fractional shortening (FS) measurements in isolated ventricular myocytes.

**A:** Example traces from fresh cells and following 3 hours at 15 °C and 37 °C (1Hz). Cells at 15 °C were rewarmed to 37 °C prior to shortening measurements. Traces are representative of FS shortening but not other parameters. **B:** Mean fractional shortening in quiescent (i) and stimulated (ii) myocytes. **C:** Time to peak in quiescent (i) and stimulated (ii) cells, and **D:** Time to 50% relaxation in quiescent (i) and stimulated (ii) cells. n=12 at 0hrs, n=11 at 2hrs, n=9 at 3hrs, and n=4 at 4 hrs. Numbers refer to the number of animals that myocytes were isolated from \*p<0.05, \*\*\*p<0.001

Table 4.1: Contractility measurements for all time points during cooling and rewarming in quiescent and stimulated ventricular cardiomyocytes.

	Hypothermia (15°C)				Normothermia (37°C)			
	0hrs	2hrs	3hrs	4hrs	0hrs	2hrs	3hrs	4hrs
FS(%)	8.8±0.4	8.7±0.9	6.5±0.7	6.7±0.5*	9.5±0.6	8.2±0.6	6.1±0.5	5.8±0.8*
Time to peak (ms)	162.4±5.2	150.3±5.8	138.9±6.8	152.6±8.3	171.9±5.8	159.2±7.3	153.1±9.8	149.4±16.3
10%	41.5±2.8	44.9±4.9	45.5±6.1	39.7±4.1	39.2±3.2	44.5±5.2	38.0±5.2	49.9±9.4
50%	70.0±2.3	72.5±4.6	69.3±5.8	69.0±4.1	70.7±2.7	71.2±4.3	64.5±4.9	70.4±8.2
90%	114.4±3.1	110.2±4.2	103.6±5.2	110.1±5.0	118.7±4.0	112.9±4.2	110.3±7.1	107.9±9.2
Time to relaxation (ms)								
10%	208.7±7.0	192.4±8.9	178.5±9.5	200.1±12.7	223.1±7.4	209.3±10.8	198.5±12.4	191.2±22.6
50%	266.9±8.5	244.7±12.1	235.2±13.4	267.8±18.1	283.5±10.2	272.8±16.7	262.2±17.7	248.9±29.5
90%	413.7±14.6	367.5±18.5	380.1±28.9	419.4±26.7	431.2±18.2	435.1±25.6	410.4±35.5	377.2±37.4

## Quiescent

	Hypothermia (15°C)				Normothermia (37°C)			
	0hrs	2hrs	3hrs	4hrs	0hrs	2hrs	3hrs	4hrs
FS(%)	8.8±0.4	5.8±0.8***	3.8±0.6***	4.2±0.3***	9.5±0.6	6.5±0.5	7.4±1.4	6.2±0.4*
Time to peak (ms)	162.4±5.2	148.2±24.8	158.6±15.1	129.1±2.5	171.9±5.8	155.8±5.4	133.3±4.7	125.6±6.3*
10%	41.5±2.8	38.8±8.7	45.1±7.9	44.6±7.4	39.2±3.2	62.7±7.8	60.7±5.0*	58.2±11.9
50%	70.0±2.3	67.0±8.8	69.5±6.1	65.4±5.9	70.7±2.7	88.3±6.1	79.5±4.1	76.6±11.1
90%	114.4±3.1	108.0±15.1	113.9±8.2	97.8±3.3	118.7±4.0	122.9±5.4	107.6±4.4	101.3±7.6
Time to relaxation (ms)								
10%	208.7±7.0	189.9±33.4	194.8±18.5	164.9±3.4	223.1±7.4	189.8±7.6*	161.5±5.8*	150.9±4.9*
50%	266.9±8.5	246.9±4.5	245.2±19.6	223.3±7.2	283.5±10.2	265.9±12.4	204.9±10.6*	186.7±5.9*
90%	413.7±14.6	368.2±66.5	397.1±50.4	375.3±25.2	431.2±18.2	356.9±26.2	314.6±21.0*	298.9±18.6*

## 1HZ

### **Intracellular Ca<sup>2+</sup> measurements in isolated ventricular cardiomyocytes following hypothermia/rewarming**

In separate experiments the role of Ca<sup>2+</sup> was assessed in a bid to understand the reduced contractile function of isolated myocytes following hypothermia and rewarming. Whilst the previous results showed that at 4 hours there was reduced FS, the number of viable cells was vastly reduced compared to earlier time points. This, combined with the finding of a significant effect of hypothermia/rewarming at 3 hours led to the decision to assess Ca<sup>2+</sup> at a 3-hour time-point in all subsequent experimental work. Cells were loaded with Ca<sup>2+</sup> sensitive Fura-4F prior to rewarming in the hypothermic group and at 3 hours in the normothermic control group.

Figure 4.7 shows typical Ca<sup>2+</sup> transients at 0hrs (fresh cells) and following 3 hours in hypothermic and normothermic conditions (stimulated at 1Hz). No differences were found in Ca<sup>2+</sup> transient amplitude between fresh myocytes and following cooling/rewarming, in either the quiescent or 1Hz stimulated group. Similarly, there was no significant differences in time to peak or the decay time of the Ca<sup>2+</sup> transient when comparing hypothermic and normothermic cells after 3 hours. Therefore, the first part of this study was able to find that hypothermia/rewarming leads to significant contractile dysfunction in cells which is not accompanied by a change in intracellular Ca<sup>2+</sup>.

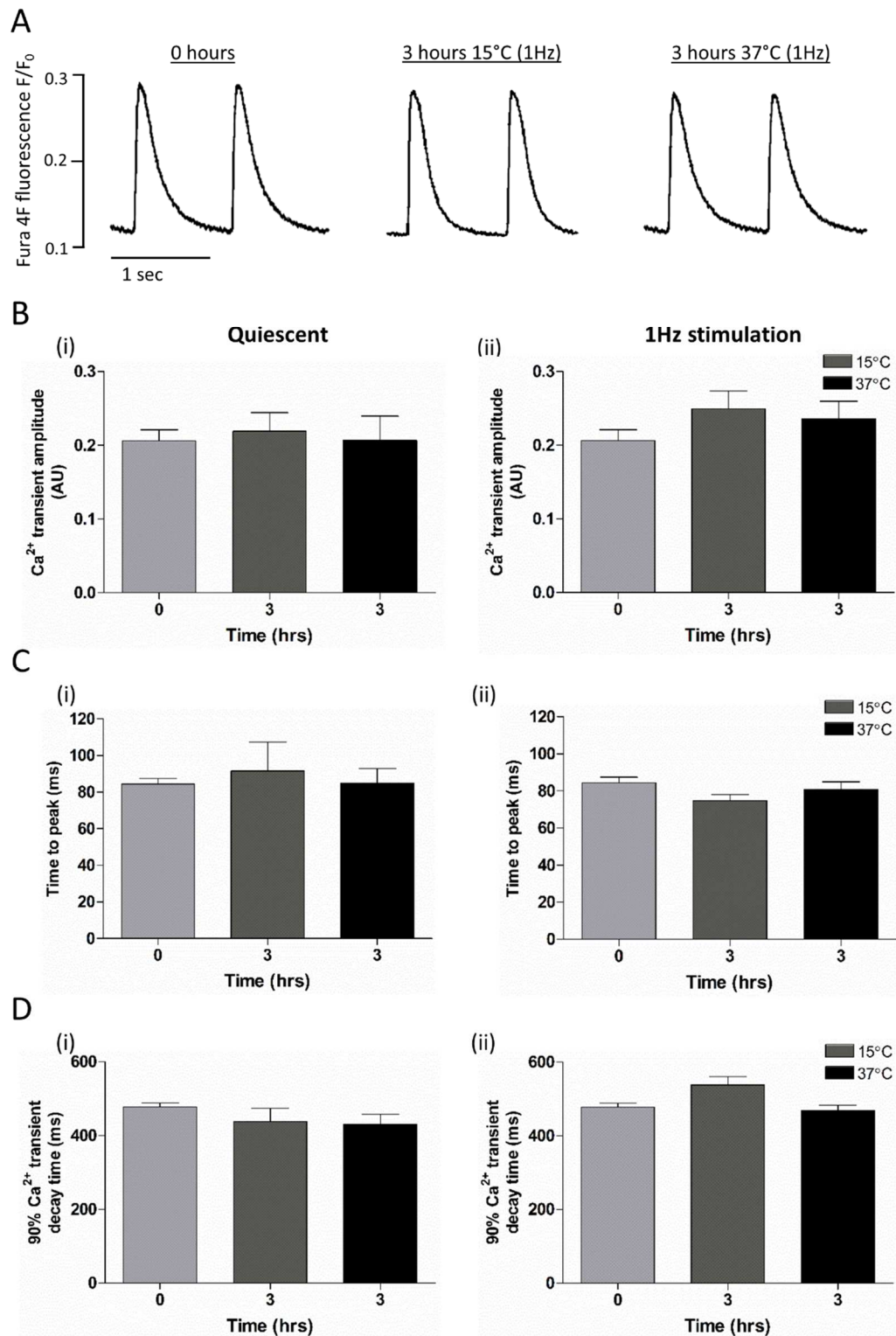


Figure 4.7:  $Ca^{2+}$  measurements in isolated ventricular myocytes.

A: Example traces (not averaged) from fresh cells and following 3 hours at 15 °C and 37 °C (1Hz). Cells at 15 °C were rewarmed to 37 °C prior to shortening measurements. B:  $Ca^{2+}$  transient amplitude in quiescent (i) and stimulated (ii) myocytes. C: Time to peak in quiescent (i) and stimulated (ii) cells, and D: 90%  $Ca^{2+}$  transient decay time. n=10

### Assessment of sarcomere length during cooling and rewarming

The previous work has shown evidence of contractile dysfunction in isolated myocytes when cooled and rewarmed. Due to the system used for these measurements, cells were cooled separately and samples taken at each time-point, therefore measurements were made on different cells each hour. The work presented in both this and the following section was carried out using a system whereby the same individual myocytes could be tracked throughout the cooling and rewarming procedure. This also ensured that the rate of temperature change was identical throughout all experiments.

Figure 4.8 shows example traces of sarcomere length shortening alongside  $\text{Ca}^{2+}$  transients measured at the same time points. As cells could be 'tracked' through cooling and rewarming, measurements were taken every hour up to 3 hours. Quiescent cells were only measured at the end of the 3 hours.

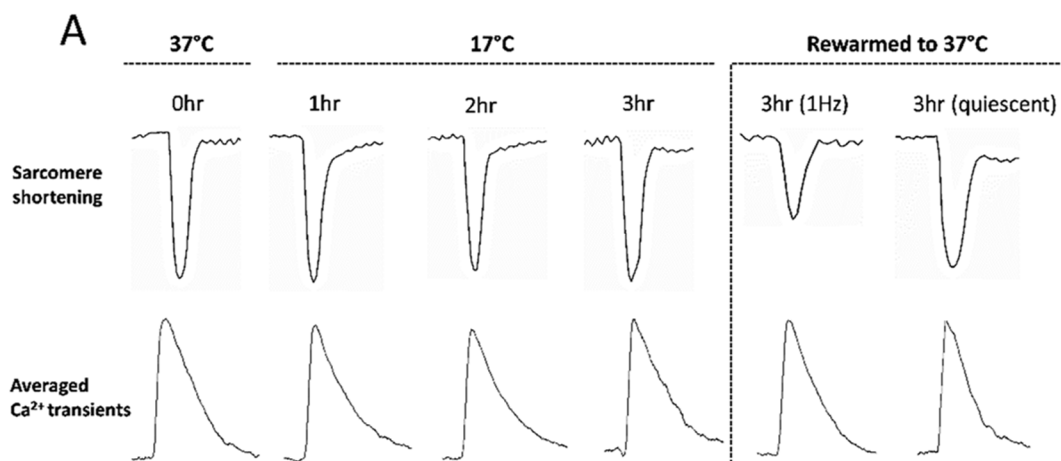


Figure 4.8: Typical traces of sarcomere shortening and averaged  $\text{Ca}^{2+}$  transients.

Traces from one cell which was tracked through hypothermia and rewarming. At 1, 2 and 3 hrs the cell was continually paced at 1Hz. 3hr (quiescent) is a separate cell.

Sarcomere length results can be seen in Figure 4.9. Due to variations in baseline values between animals, all data is normalised. Cooling to  $17^\circ\text{C}$  more than doubled contraction time ( $\text{Up}90$ ) at 1, 2 and 3 hours ( $p < 0.05$ ) when compared to fresh cells. Rewarming to  $37^\circ\text{C}$  returned contraction time to within baseline



values (at 1Hz). Quiescent rewarmed cells were also not different from starting values.

Throughout the 3 hours of hypothermia there was no evidence of significant variation in amplitude. However, upon rewarming, the amplitude of shortening was 53% shorter than baseline ( $p < 0.05$ ). This difference was not seen in cells that were quiescent through the hypothermia. The reduction in amplitude in stimulated cells mirrors the FS data previously described, and reinforces the finding of a rewarming-induced contractile dysfunction in ventricular cardiomyocytes. The relaxation time course (Dn90) showed a high degree of variance but overall no differences during hypothermia or rewarming. 50% contraction duration (a marker of the time course of contraction increased during hypothermia ( $p < 0.05$ ) but normalised upon rewarming.

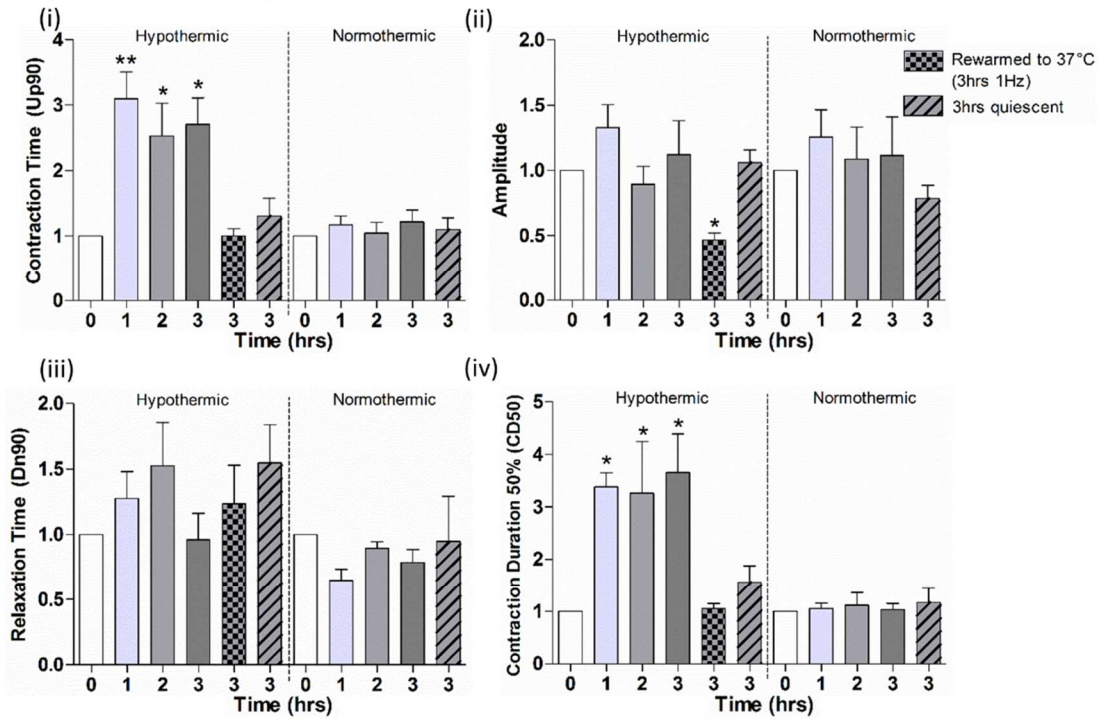
Normothermic control cells which were maintained at 37°C for 3 hours showed no difference in any of the recorded parameters.

### **Ca<sup>2+</sup> measurements during cooling and rewarming using CelloPTIQ**

Intracellular Ca<sup>2+</sup> was also measured in these cells immediately following sarcomere length assessment. Cells were loaded with Fura-4F at room temperature prior to the experiment starting and measurements were made in the same cells throughout the 3 hours. Figure 4.9B shows averaged Ca<sup>2+</sup> transient analysis. No differences were found in Ca<sup>2+</sup> transient amplitude, time to peak or 90% Ca<sup>2+</sup> transient decay time during hypothermia or rewarming. Rise time was significantly slowed during the 3 hours of hypothermia ( $p < 0.05$ ) but normalised upon rewarming. These results reinforce the findings from the previous section that intracellular Ca<sup>2+</sup> is not responsible for the contractile dysfunction that is seen upon rewarming from hypothermia.

Normothermic control cells which were maintained at 37°C for 3 hours showed no difference in any of the recorded parameters.

**A: Sarcomere Length**



**B: Calcium Transients**

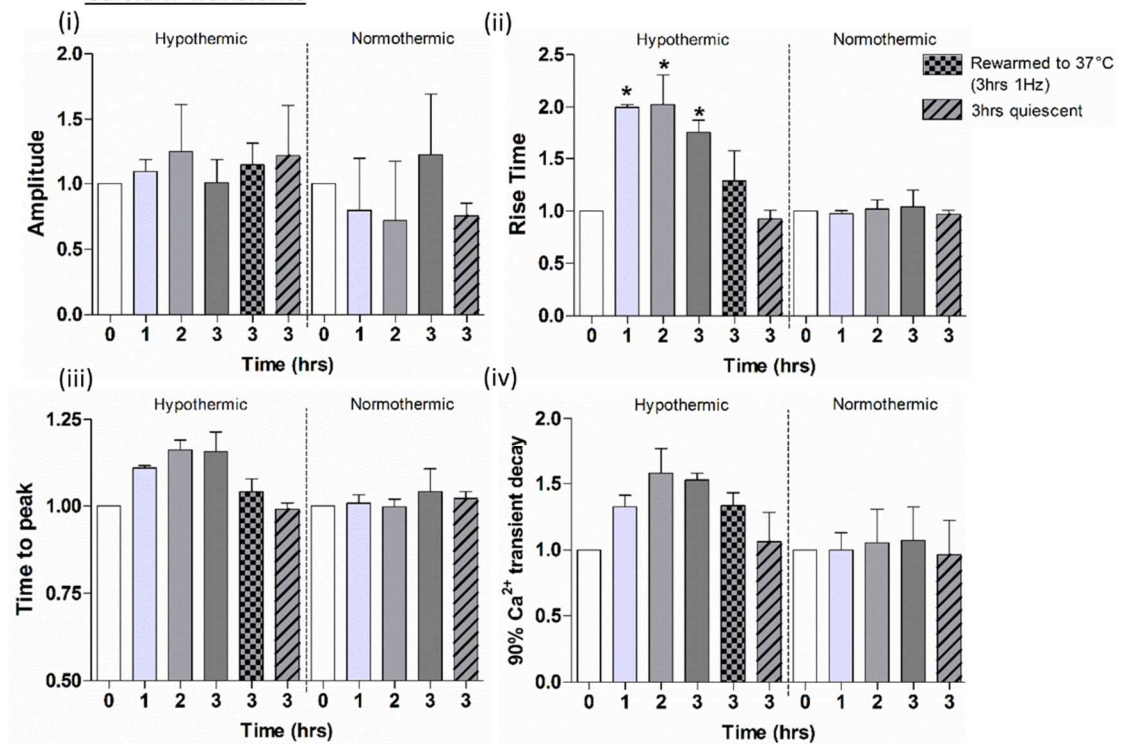


Figure 4.9: Sarcomere length and intracellular Ca<sup>2+</sup> measurements during hypothermia/rewarming.

**A:** Mean sarcomere length recorded as an indicator of contractility function: (i) Time to peak contraction (Up90). (ii) Amplitude of contraction, (iii) Relaxation time (Dn90) (iv) 50% Contraction duration (CD50). **B:** Averaged Ca<sup>2+</sup> transient results (i) Amplitude of Ca<sup>2+</sup> transient, (ii) Rise time, (iii) Time to peak, (iv) Time to 90% Ca<sup>2+</sup> transient decay. \*p<0.05, \*\*p<0.01.

## Discussion

The main aim of this work was to assess hypothermia and rewarming in isolated ventricular cardiomyocytes. Following hypothermia and rewarming, stimulated myocytes showed a reduction in contractile function that was not seen in quiescent or normothermic controls. These findings were confirmed by additional experimental work on a different system that allowed assessment of sarcomere length in hypothermic and rewarmed myocytes. The reduction in contractility was not accompanied by a change in intracellular  $\text{Ca}^{2+}$  transients. These findings are in line with a recently published study by Schaible *et al* (2016). Using a similar model to the one described in this chapter, they found that upon rewarming from hypothermia (3hrs) there was a shortening of ~3.5% compared to ~8% in freshly isolated cells. They also assessed  $\text{Ca}^{2+}$  throughout and whilst they witnessed an increase during the hypothermic phase, it normalised upon rewarming.

The mechanisms underlying excitation contraction coupling and force generation appear to be disrupted following hypothermia and rewarming. This is in line with other animal models that have shown that systolic function or force generation is significantly reduced following hypothermia and rewarming (Zhang *et al.*, 1995; Tveita *et al.*, 1998; Groban *et al.*, 2002; Han *et al.*, 2010). This could normally be explained by changes in cytoplasmic  $\text{Ca}^{2+}$  or sensitivity of the myofilaments to  $\text{Ca}^{2+}$ . However, the results of the current study, as in the study of Schaible *et al*, found no change in cytosolic  $\text{Ca}^{2+}$ . Taken together these results would suggest that the reduction in contraction is as a result of myofilament sensitivity, which has been shown to be temperature dependent in previous studies of cooling (de Tombe and Stienen, 2007) and rewarming (Harrison and Bers, 1989).

It has been suggested that the underlying mechanism of hypothermia/rewarming induced contractile dysfunction may be due to high total cellular  $\text{Ca}^{2+}$  (Wold *et al.*, 2013). Intracellular  $\text{Ca}^{2+}$  may increase in different cellular structures, e.g. SR, mitochondria or cytoplasm, and there are several different mechanisms by

which it could have an effect on myofilament sensitivity and contractile dysfunction;

### **i. Mitochondrial damage**

Firstly, there is a potential role of metabolism in contractile dysfunction. Under normal circumstances the heart uses oxidative phosphorylation to provide metabolic energy for contraction. ATP is produced aerobically by the mitochondria and generates creatine phosphate (PCr) which shuttles the metabolic energy to the sites of energy consumption e.g. the contractile proteins, where it is used to re-phosphorylate local adenosine diphosphate (ADP) via creatine phosphokinase. When cytoplasmic  $\text{Ca}^{2+}$  rises, the myofilaments are activated in a  $\text{Ca}^{2+}$ -dependent manner, thereby transducing the chemical signal and chemical energy (ATP) into mechanical force or shortening (Bers, 2002). If aerobic metabolism is prevented, e.g. by hypoxia or by damaged mitochondria then ATP can be generated anaerobically for a limited period of time. This switch to anaerobic metabolism comes with consequences for the heart, e.g a fall in PCr and subsequent rise in Pi.

$\text{Ca}^{2+}$  overload can lead to mitochondrial damage and a disruption in oxidative phosphorylation. When oxidative phosphorylation is inhibited, e.g. in hypoxia or due to mitochondrial damage, there is a decrease in contraction that isn't a direct result of a decrease in systolic  $\text{Ca}^{2+}$  (Allen and Orchard, 1983). There are two possible metabolic changes which could be responsible; an increase in intracellular Pi concentration (Allen et al., 1985; Kusuoka et al., 1986), or an intracellular acidosis due to lactic acid build up following anaerobic metabolism and reduced contractility (Allen et al., 1985; Eisner et al., 1987). Separate studies on cardiac myofilament sensitivity showed that acidic environment and increased Pi both reduced force production in isolated hearts and papillary muscles (Fabiato and Fabiato, 1978; Kentish, 1986).

However, experimental work on isolated rat cardiomyocytes with pharmacologically blocked mitochondrial function (cyanide) did not observe decreased cell shortening and contrasts with the work on whole hearts (Eisner et

al., 1989). The reason for the difference is unknown but may be related to the different measures of contractile activity, i.e. cell shortening (isotonic contraction) via force/pressure measurements (isometric). However, this latter study does suggest that hypothermia-induced reduced cell shortening is not due to blockade of mitochondrial metabolism.

## ii. PKA dependent pathway

If metabolism is not the modifying factor in the reduced contractility, then myofilament sensitivity must be altered via another pathway. It has been proposed that myofilament  $\text{Ca}^{2+}$  sensitivity following rewarming may be due to a Protein Kinase A (PKA)-dependent phosphorylation of cardiac troponin I (cTnI). Briefly, beta-adrenergic stimulation activates adenylyl cyclase to produce cyclic-adenosine monophosphate (cAMP), which activates PKA. This kinase phosphorylates several proteins related to EC-coupling, including L-type  $\text{Ca}^{2+}$  channels, RyR and troponin I (Bers, 2002). cTnI is a key regulatory protein in cardiac muscle contraction and relaxation, and phosphorylation by PKA results in a decrease in the sensitivity of the contractile apparatus to  $\text{Ca}^{2+}$ . After phosphorylation the half-maximal ATPase activity and half-maximal tension are achieved at higher  $\text{Ca}^{2+}$  concentrations, an effect that may be due to decreased affinity of troponin I to troponin C (Filatov et al., 1999). Indeed the phosphorylation at specific sites (Ser23/24) on cTnI has been shown to be mechanistically involved in reduced myofilament sensitivity in various pathological situations (Gao et al., 1995; Tavernier et al., 2001; Chen and Ogut, 2006).

Following hypothermia and rewarming it has been shown that there is an increased cTnI phosphorylation in rat papillary muscle (Han et al., 2010) and in isolated ventricular cardiomyocytes (Schaible et al., 2016). However, as yet there is no evidence to support  $\text{Ca}^{2+}$  overload in rewarming leading to increased cAMP activated PKA.

### iii. Oxygen derived free radicals

A final mechanism which could cause the myofilament sensitivity and reduced contractile function in this study are oxygen derived free radicals. Changes in mitochondrial function as a consequence of increased total  $\text{Ca}^{2+}$  may lead to an increase in reactive oxygen species (ROS) which could in turn lead to mitochondrial dysfunction (Brookes et al., 2004). It has been shown that ROS can reduce  $\text{Ca}^{2+}$  sensitivity in skinned rabbit muscle fibres (Perkins et al., 1997). Whilst it is believed that  $\text{Ca}^{2+}$  dysfunction plays a major role in the generation of ROS, the molecular mechanisms at this time remain elusive and further work is required, however it should be considered as a potential factor in hypothermia and rewarming.

## Conclusions

This study has shown that hypothermia and rewarming can induce a contractile dysfunction in isolated cardiomyocytes. No change in  $\text{Ca}^{2+}$  transients would suggest that the dysfunction is as a result of altered myofilament sensitivity but it would be speculative to say what the underlying mechanism to this reduced force is at this time.

# **Chapter 5: The differential effects of hypothermia on cardiac conduction and excitability**

## Introduction

Accidental hypothermia is complicated by risk of ventricular arrhythmias and cardiac arrest. This contributes to high mortality rates among these patients, reported to be between 29% (van der Ploeg et al., 2010) and 80% (Maclean and Emslie-Smith, 1977) depending on rewarming methods. However, several case reports in recent years have demonstrated that successful resuscitation is possible even from core temperatures below 20°C (Wanscher et al., 2012). The apparent beneficial effect of hypothermia, i.e. reducing the detrimental effects of prolonged cardiac arrest, is utilised in aortic arch surgery, where severe hypothermia down to 15°C can be used (Di Luozzo and Griep, 2012). Hypothermia has also been applied therapeutically in comatose survivors of cardiac arrest, where temperatures above 30°C are considered safe (Polderman and Herold, 2009). Although hypothermia is used extensively as a therapeutic intervention and survival is possible after extreme exposure, treatment of arrhythmias during rewarming is still challenging. Current guidelines provide only general suggestions for the use of defibrillator and preventative pharmacologic treatment (Vanden Hoek et al., 2010) when rewarming victims of accidental hypothermia. In order to develop targeted anti-arrhythmic strategies in this very specific situation, we first need to understand the basis for pro-arrhythmia during cooling and rewarming.

In humans, hypothermia-induced arrhythmias commonly appear at core temperatures below 28°C, including atrial fibrillation, nodal rhythms, ventricular extra systoles, atrioventricular blocks and ventricular fibrillation (VF) (Tveita, 2000). The pathophysiology behind development of VF in the hypothermic heart remains unknown. Recent studies have shown that hypothermia can induce conduction block and re-entrant VF in canine wedge preparations. In this study VF or tachycardia was most common during rewarming, and was associated with transmural dispersion of repolarisation (DOR) (Piktel et al., 2011) and in a separate study epicardial DOR (Salama et al., 1998). Combined with slowed conduction velocity (CV) at 30°C in rabbit hearts (Hsieh et al., 2009), these circumstances may favour unidirectional block and induction of VT/VF. Optical mapping of rabbit hearts cooled to 17°C have also



shown that severe hypothermia can induce spatial alterations in CV, a known predictor of VF (Egorov et al., 2012). However, it is unclear from previous studies whether electrophysiological changes and arrhythmic risk is directly proportional to the degree of hypothermia.

### **Aims**

The aim of this chapter was to assess whole heart electrophysiology in isolated Langendorff perfused rabbit hearts using a series of techniques. Measurements were carried out following gradual cooling and rewarming of hearts to temperatures occurring in therapeutic (31°C) and accidental (17°C) hypothermia.

## Methods

### Langendorff perfused heart

Male New Zealand White Rabbits (~3-3.5kg) were used for all experimental work in this chapter. Hearts were isolated and Langendorff perfused (as described in Chapter 2). Blebbistatin (10 $\mu$ M) was used for all of the following studies with the exception of whole heart conduction timings (section 5.2.3). Hearts were perfused with blebbistatin for 25 minutes prior to any measurements being made. Due to the light sensitive nature of blebbistatin, all work was carried out with minimal room lighting.

### Hypothermia and rewarming protocol

The temperatures used in this study were typical for moderate/therapeutic (31°C) and severe (17°C) hypothermia and are comparable to previous publications (Egorov et al., 2012). These temperatures are routinely employed therapeutically and observed in victims of accidental hypothermia, where core temperatures below 17°C have been reported in patients with a good neurological outcome after rewarming (Gilbert et al., 2000). Perfusion rate of hearts was constant (30 ml/min) during experiments. Whilst cooling to severe hypothermia *in vivo* decreases cardiac output, studies have also found coronary blood flow to be preserved or increased in hypothermia, partly because diastole is more extended than systole in cold animals (Tveita, 2000). In order to study electrophysiology under the same conditions at different temperatures, we therefore maintained perfusion at a constant rate. Hypothermia was induced by running Tyrode's solution through a water-coupled heat exchanger, before allowing the solution to perfuse the hearts. Cooling from baseline (37°C) was achieved through gradual reduction of the temperature in the water bath. Cooling and rewarming between 37°C and 31°C took approximately 20 minutes each, while both cooling and rewarming between 31°C and 17°C took 30 minutes (Figure 5.1). This gradual change of temperature was carried out in order to mimic gradual whole body cooling and avoid rapid cooling contracture (Bers et al., 1989). Both cooling and rewarming was temporarily stopped for ~5 minutes at 31°C and 17°C to allow hearts to stabilise for recordings. Hearts underwent both right atrial (RA) and right ventricular (RV) pacing at cycle lengths of 300ms

at 37°C, 450ms at 31°C and 1700ms at 17°C. These cycle lengths were based on previous work within the lab which assessed intrinsic heart rate using the same temperature protocol.

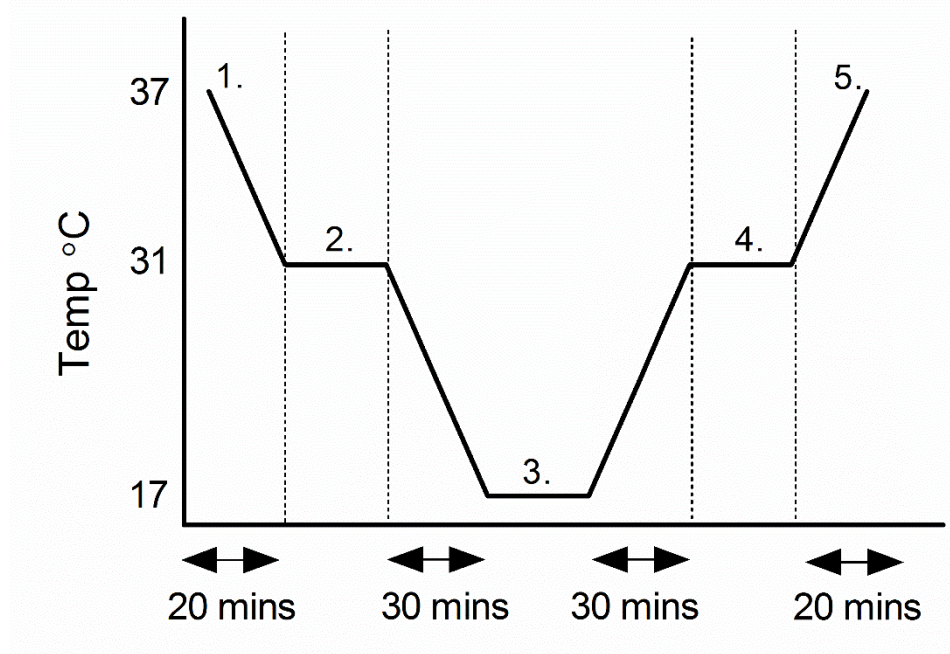


Figure 5.1: Temperature timeline of experimental procedures. Points 2-4 refer to the time where the heart was allowed to stabilise at each temperature (5 mins).

### Whole heart conduction timings

After establishing Langendorff perfusion, a small section of the right atrium was removed and the anatomical region of the AV node (AVN) identified (n=6). A quadripolar electrode catheter (Figure 5.2) was then placed across the tricuspid valve adjacent to the AVN such that the proximal poles recorded atrial and ventricular activation times at the level of the AVN and the distal poles recorded activation in the RV apical endocardium. Additional catheter electrodes were placed in the RV against the septum and in the left ventricle (LV) against the endocardium. Finally, platinum hook electrodes were placed on the epicardial surface of the LV and into the RA to allow for pacing (Figure 5.7A).



**Figure 5.2: Recording and pacing electrodes. A: Quadripolar electrode catheter. B: Platinum hook electrodes**

### Panoramic optical mapping

In a subset of experiments, hypothermic (n=8) and normothermic (n=6) optical action potential characteristics were compared. Hearts were placed in a custom built chamber and perfused via a computer-controlled rotatable centrepiece connected to the aortic cannula, which allowed for easy rotation of the heart (Figure 5.3). Once the hearts were stabilised and aligned in the chamber, the preparation was stained with voltage sensitive dye, di-4-ANEPPS (100  $\mu$ l of 1 mg/ml). A pair of platinum hook electrodes was passed through the wall of the right atrium (RA); a second pair contacted the right ventricle (RV) for pacing. The heart was illuminated with an annular array of LEDs (OptoLED, Cairn Research Ltd.; Faversham, UK) with peak excitation wavelength 470 nm. Emitted fluorescence was collected, filtered with a long pass filter (665 nm) and focussed on a CCD chip (Redshirt Imaging; Decateur, GA USA). An area of epicardium  $\sim$ 43x43 mm was imaged at a working distance of  $\sim$ 140 mm. Images were acquired every 1ms at a resolution of 80x80 pixels. Using a stepping motor, the heart inside the chamber was rotated through fixed angles ( $\pm$  120° from a central position) acquiring data sequentially from each viewpoint (LV, RV, posterior).

Data analysis for the panoramic optical mapping was performed using custom software (Optiq; developed locally by F.Burton, University of Glasgow). The automated algorithm was able to detect selected AP trace characteristics. Activation time (TAct90) was determined as time to 90% of the OAP upstroke whilst repolarisation time (TRepol90) was time to 90% repolarisation. Upstroke (Trise) was calculated as the time between 10% and 90% of activation time.

Action potential duration (APD<sub>90</sub>) was calculated by repolarisation time - activation time at the steepest upstroke.

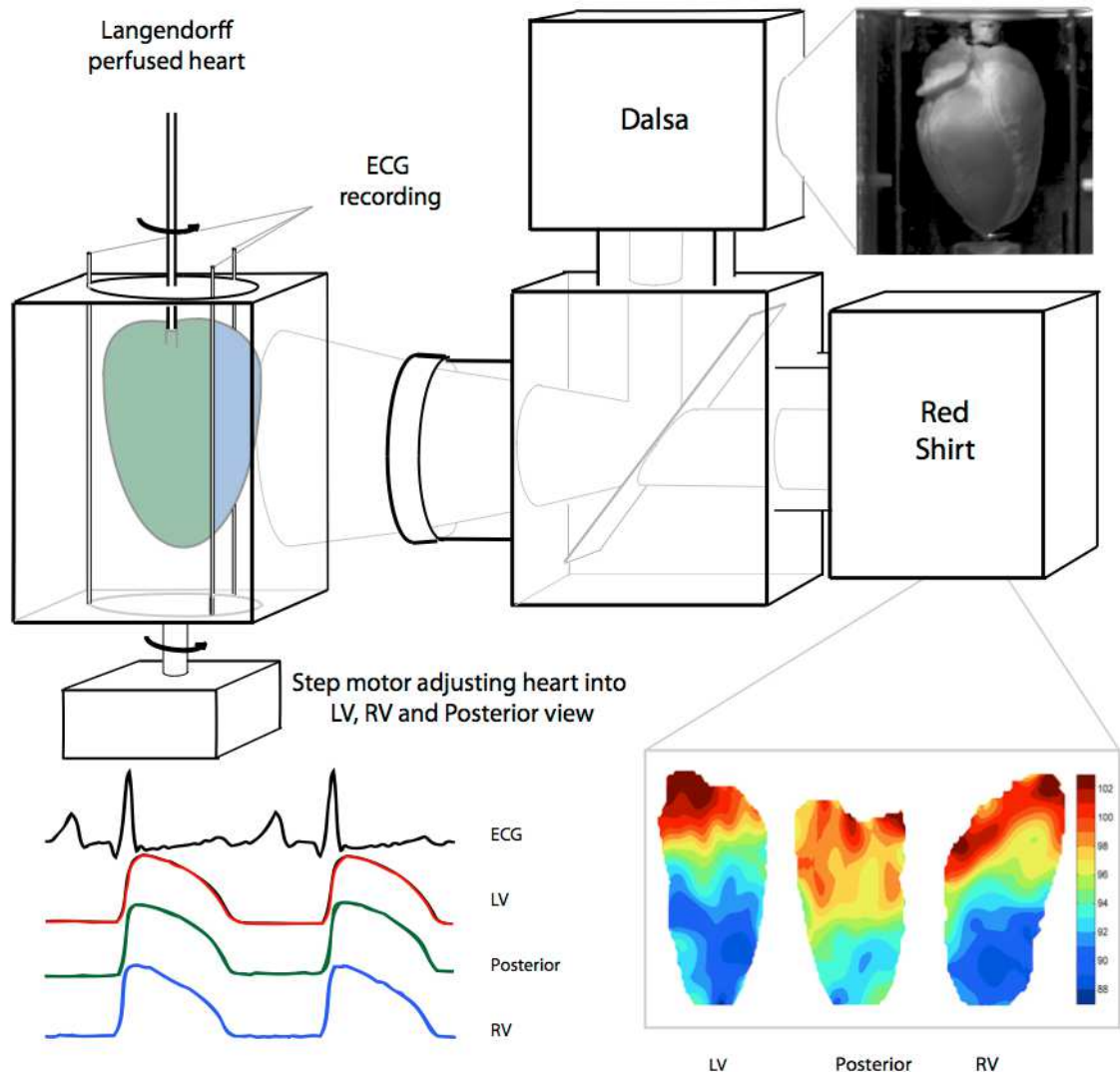


Figure 5.3: Optical mapping setup

Hearts were Langendorff perfused and placed in a plexi glass chamber. In order to record panoramic images, a step motor turned the heart to LV, posterior and RV views during the experiments. A lens captured light emitted from the dye-loaded heart and a dichromic mirror split the light, which was projected on to the Redshirt or Dalsa camera. ECG was recorded through two metal bars in the fluid filled plexi glass chamber and the cannula connecting the heart to the Langendorff system. (Schematic from Dr Erik Sveberg Dietrichs, University of Tromsø, Norway).

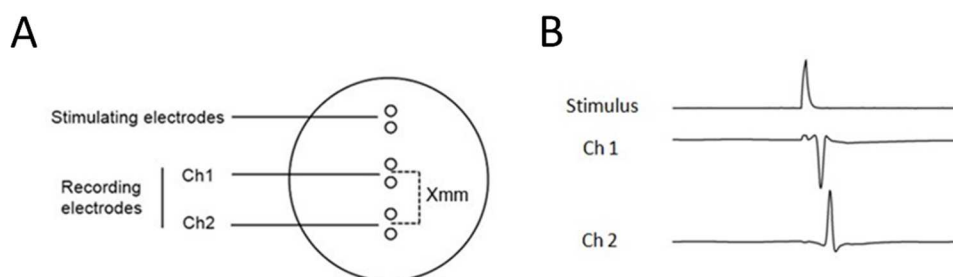
### ECG recording and analysis

Pseudo lead-II ECG recordings were made using the steel bars in the custom built chamber (Figure 5.3). These signals were amplified with a custom built

amplifier and displayed on an oscilloscope throughout experiments. Alongside optical action potentials, ECG signals were recorded using a Redshirt Imaging control box digitiser (DAP820, Redshirt, Decatur, GA USA). Continuous recordings of ECG signals were made using a laptop computer with a USB compatible digitiser (DI-158, Dataq Instruments Inc; Akron, OH USA). Signals were analysed using LabChart7 (ADInstruments; Sydney AUS) software (for full description of analysis see Chapter 4). The main parameters examined in these hearts were PR, QR, QRS and QT intervals.

### Conduction velocity

Conduction velocity (CV) was measured using custom-built electrodes which consisted of silver bipolar stimulating electrodes and two sets of silver bipolar recording electrodes (Channel 1 and Channel 2) which were fixed distances apart (Figure 5.4A). CV was recorded by pacing continuously at a fixed interval and recording the signals from the Channel 1 and Channel 2 recording electrodes (20 kHz). The difference in time between the peaks of both channels was taken as the difference in activation time. As the distance between the electrodes was known, CV could be calculated using:  $V=d/t$  (where  $V$  = Velocity (cm/s),  $d$  = distance (cm) and  $t$  = time (s)). Recordings were made using software developed in house: ACQ1 (F Burton, University of Glasgow).

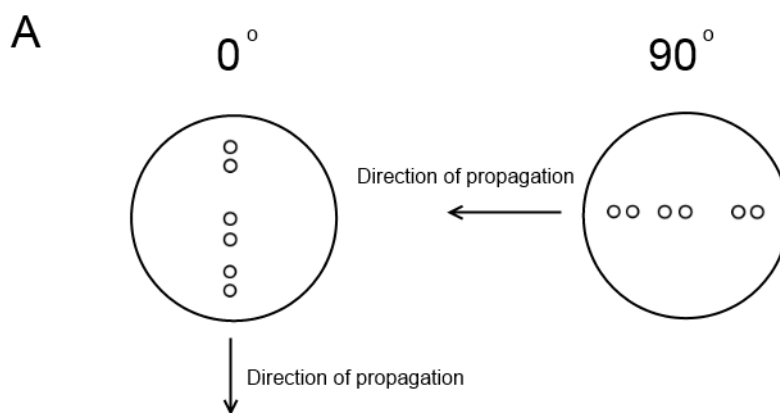


**Figure 5.4 CV Electrode Design.**

**A:** Diagram of electrode design, showing the stimulating electrodes and two sets of recording electrodes.  $X$  mm is the distance between electrode pairs and is between 1.6 and 1.8mm, as more than one set of electrodes was used during these experiments. **B:** Example traces taken from an experiment showing the stimulus artefact and the peaks on the Ch1 and Ch2 recording electrodes.

Initial CV experiments (n=4) looked at conduction in both the atria and ventricle. The electrodes were placed flat against the epicardium of the LV, and also against the surface of the RA. CV was recorded throughout hypothermia and rewarming.

Separate experiments (n=5) used two sets of electrodes to record CV in more than one axis. CV is dependent on fibre orientation with longitudinal conduction being ~2x faster than transverse conduction (Valderrabano, 2007). The epicardial surface has a fibre orientation such that the long axis of the cell runs ~90° from this angle to the vertical axis. Once the fastest conduction time in the long axis was identified, the electrodes were rotated 90° from this angle to record the transverse conduction (Figure 5.5). Such is the arrangement of fibres within the myocardium this is indicative of endocardial to epicardial (transmural) conduction. Both longitudinal (CV<sub>l</sub>) and transverse (CV<sub>t</sub>) conduction were recorded during hypothermia and rewarming.



**Figure 5.5: Electrode orientation**

Diagram showing the angle of the electrodes and the direction of propagation at 0° and 90°.

### Measurement of ventricular fibrillation threshold

In separate experiments (n=7), ventricular fibrillation (VF) threshold was measured with RV endocardial electrodes. At each temperature, a train of 100

constant current pulses of 4 ms duration, 10 ms apart was delivered. The train spanned the refractory period at all experimental temperatures. Current strength was increased in steps of 5 mA until VF was sustained for a period longer than 4 secs. Between measurements, hearts were allowed to recover for 1 minute after normal sinus rhythm was established. At each temperature, thresholds were averaged over 3 measurements according to the work of Cha *et al* (1993).

### **Statistical analysis**

Data are expressed as mean  $\pm$  SEM. Conduction time, VF threshold, optical imaging, ECG and CV measurements were assessed by one-way ANOVA for repeated measures. When significant differences were found, data were post-hoc analysed using Tukey's test.



## Results

### ECG in isolated rabbit hearts during cooling and rewarming

The mean changes in ECG measured from isolated Langendorff perfused hearts are shown in Figure 5.6. On cooling to 31 °C, PR interval showed significant changes, increasing to 130% of normothermic baseline levels ( $73.3 \pm 10.8$  vs.  $97.8 \pm 13.7$  ms,  $p < 0.05$ ). QT interval also increased to 150% ( $178.8 \pm 21.4$  vs.  $267.9 \pm 32.6$  ms,  $p < 0.05$ ) but changes in QRS and the QR intervals were absent; in particular QR interval was on average 99% of normothermic baseline values.

During further cooling to 17 °C both QR and QT time were prolonged, respectively ( $25.5 \pm 4.1$  vs.  $53.8 \pm 9.3$  ms,  $p < 0.05$ ) ( $178.8 \pm 21.4$  vs.  $591.8 \pm 74.7$  ms,  $p < 0.05$ ). During rewarming to 31 °C, QR interval returned to baseline values, but QT time was still prolonged ( $178.8 \pm 21.4$  vs.  $294.1 \pm 35.5$  ms,  $p < 0.05$ ). After rewarming to 37 °C, both parameters returned to baseline values.

### Whole heart conduction timings

To examine electrical activity through the heart during cooling and rewarming, catheters were placed inside the right and left ventricles (Figure 5.7A).

Example traces showing the relative timing of whole heart conduction can be seen in Figure 5.7B. Measurements were taken at the peak following maximum deflection (Figure 5.7C) representing the sequence of activation through the atria and ventricles. Typical records (Figure 5.7D) show that following stimulation, atrial conduction was unchanged at 31 °C but considerably slowed at 17 °C. The first ventricular measurement, taken after the AV-node delay shows a steep increase in conduction time at 17 °C that is not present at 31 °C or 37 °C.

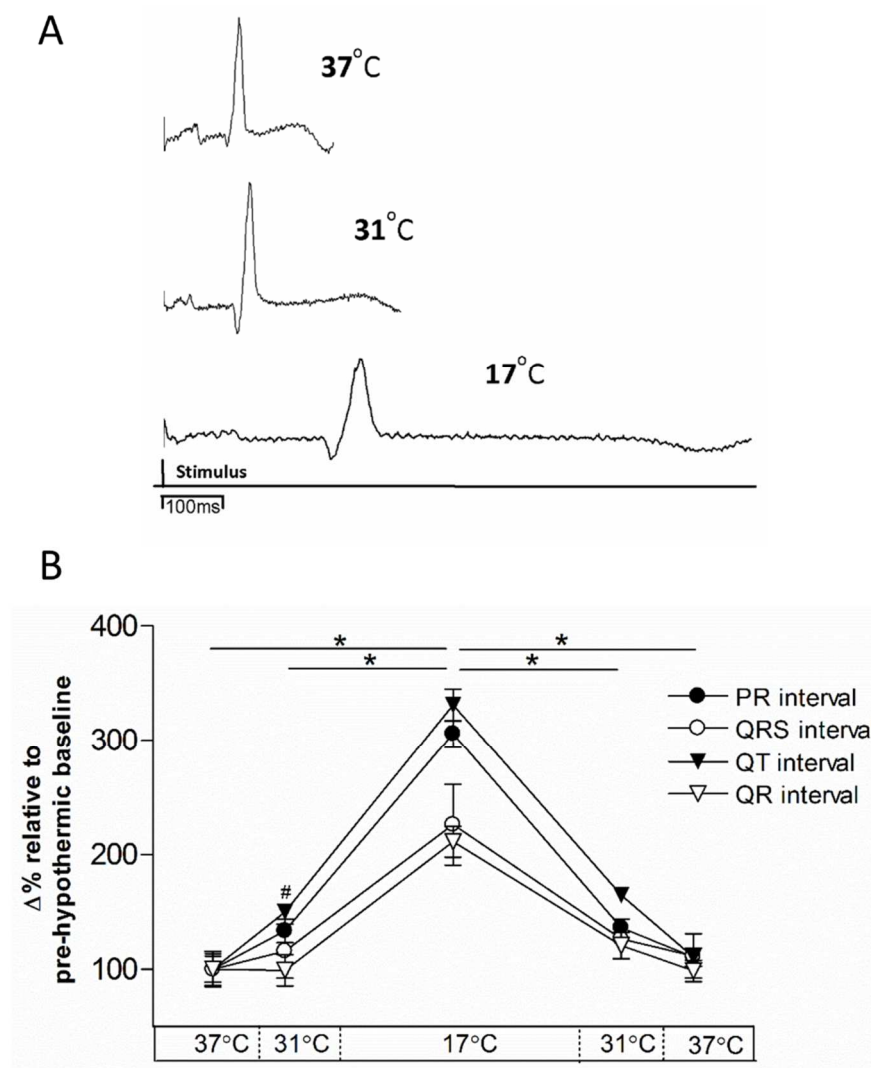
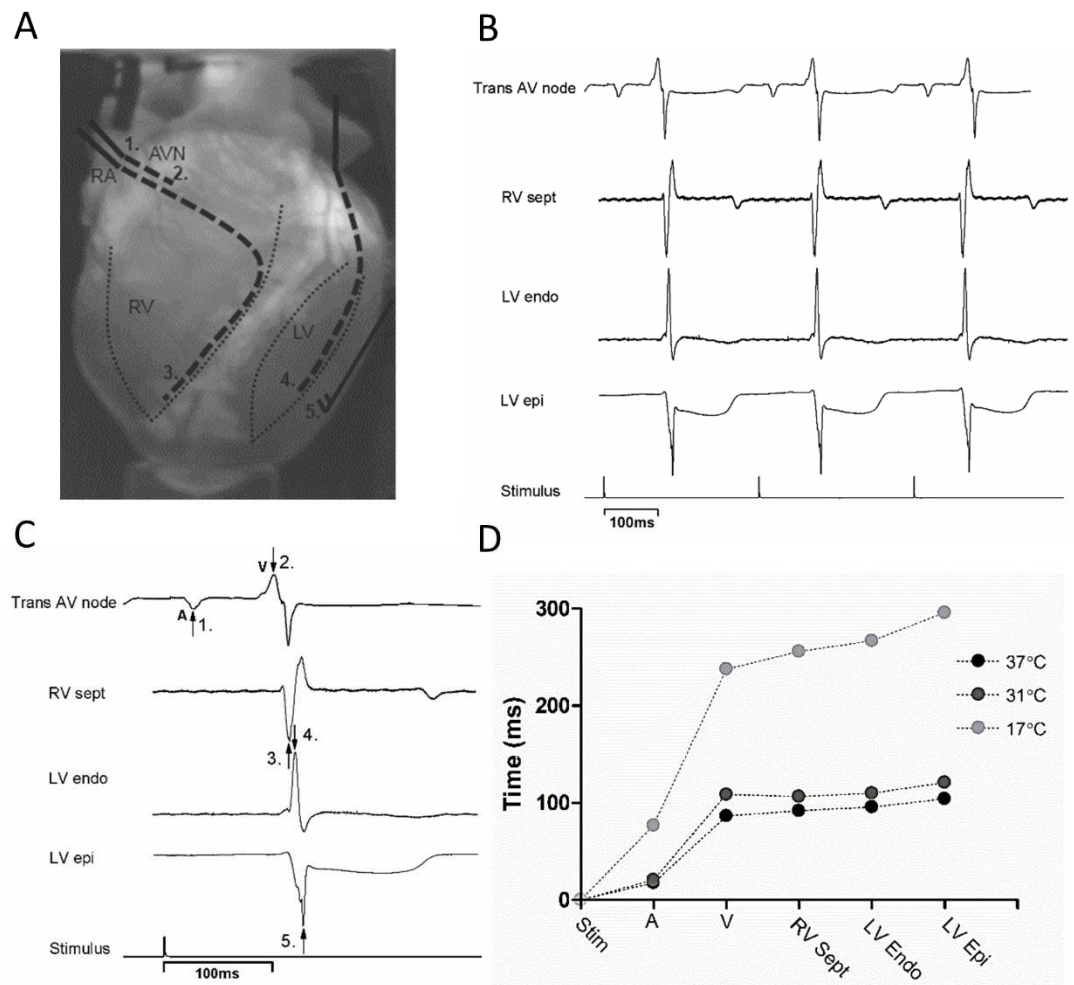


Figure 5.6 ECG parameters during cooling and rewarming

A: Example ECG traces at 37°C, 31°C and 17°C. B: Percentage change of ECG characteristics during cooling and rewarming compared to normothermic baseline. \*Significant difference (p<0.05). # Significant difference between PR/QT intervals at 31°C and baseline (p<0.05).



**Figure 5.7** Regional electrical activity measurements to assess whole heart conduction.

**A:** Placement of electrodes indicated by dashed lines (1-2) trans AV node, (3) RV septum, (4) LV endocardium, (5) LV epicardium. **B:** Example traces of recorded electrical activity. **C:** Single example traces indicating the peaks where recordings were measured. **D:** Representative trace from one heart showing the changes in activation times during cooling

The mean changes in electrical activity during cooling to 31°C (Figure 5.8A) showed no difference in activation times from stimulus to either atrial or ventricular points when compared to 37°C. During cooling to 17°C, all times were significantly slowed compared to baseline and 31°C. In particular, time from stimulus to atrial activation was more than 4 times longer at 462% of baseline ( $76.9 \pm 4.5$  vs.  $17.5 \pm 1.2$  ms,  $p < 0.01$ ), compared to activation of the ventricles which was ~280% of baseline ( $p < 0.001$ ). Upon rewarming to 31°C there was no difference between atrial or ventricular activation times, and at 37°C all values returned to baseline.

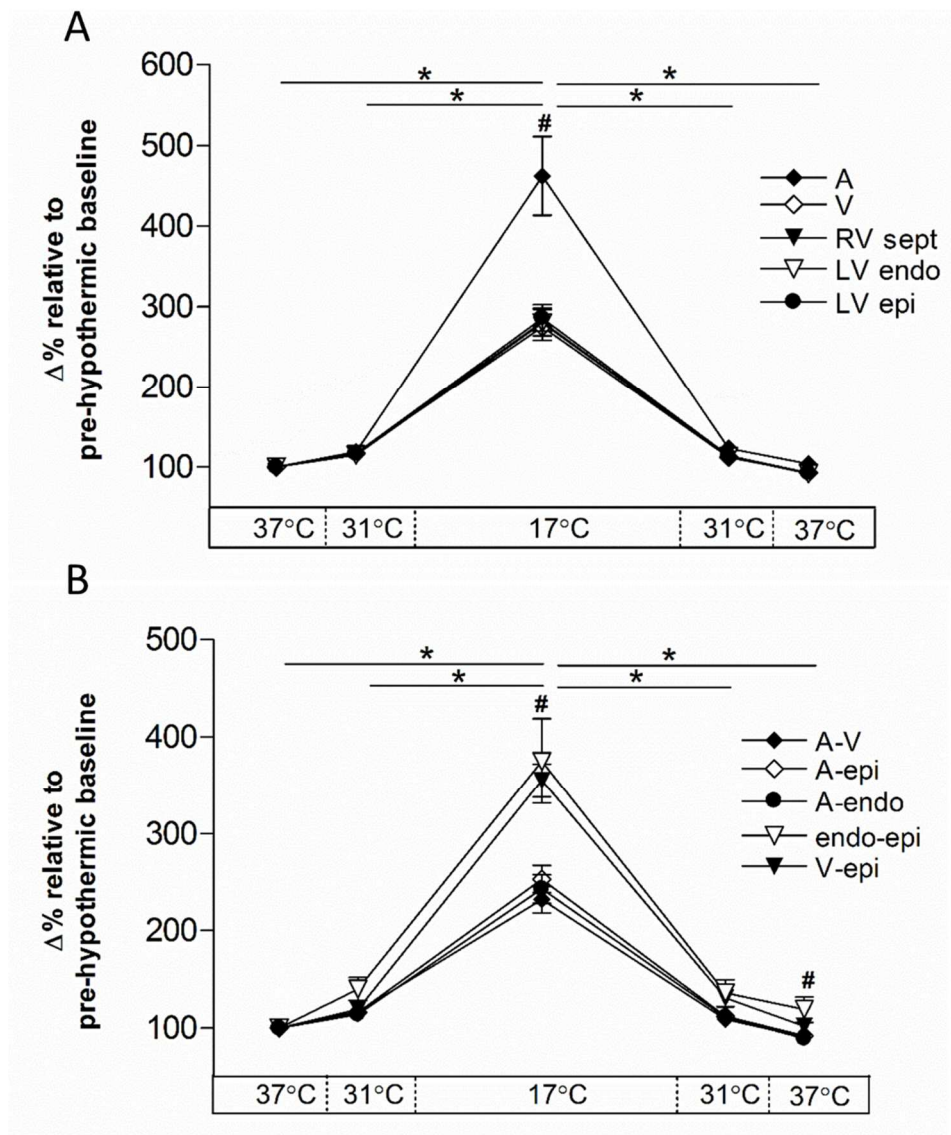


Figure 5.8: Changes in regional electrical activity compared to normothermic baseline.

A: Percentage change at individual areas of heart. B: Differences between regions.

\*Significant difference between temperatures. # Significant difference within temperature.

To investigate whether there were temperature dependent effects within specific pathways of the heart, differences between regions were examined (Figure 5.8B). At 37°C, all pathways showed similar timings, but when hearts were cooled to 17°C, all paths were significantly slower when compared to baseline ( $p < 0.001$ ). In particular, endo-epi delay was 375% greater than control whilst V-epi was 355% of baseline; both of these delays were prolonged more than A-V (232%), A-epi (253%) and A-endo (243%) delays ( $p < 0.01$ ). Upon

rewarming to 31°C, there were no differences between groups; at 37°C endo-epi times did not completely return to baseline values (119%) in contrast to all paths involving atrial conduction which were fully reversed.

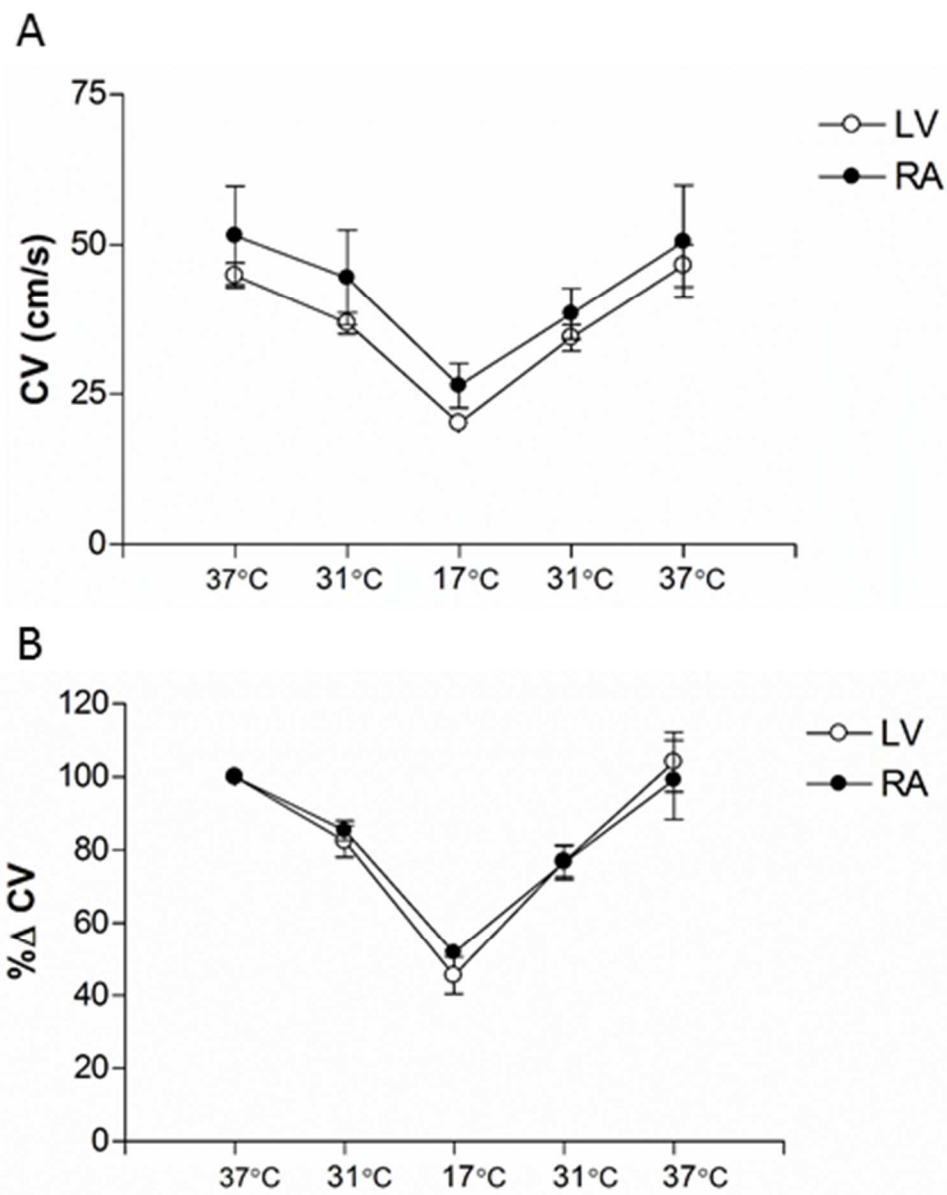


Figure 5.9 Conduction velocity measurements in atria and ventricle during cooling and rewarming.

A: Absolute CV values in right atria and left ventricle recorded by contact electrodes. B: Percentage change in CV in atria and ventricle. One-way ANOVA ( $p < 0.01$ ) shows differences between temperature but no difference between atria and ventricle.

### Atrial vs ventricular conduction during hypothermia and rewarming

These findings, combined with the ECG data showing differences in PR and QT intervals raised the question of whether there were temperature-dependent

effects between the atria and the ventricle. CV was measured using contact electrodes placed on the right atria (RA) and left ventricle (LV) in a subset group (n=4). Figure 5.9 shows that cooling to 31 °C slowed RA conduction to 85% ( $51.5 \pm 8.2$  vs  $44.5 \pm 7.9$  cm/sec) and LV to 82% ( $44.8 \pm 2.1$  vs  $36.8 \pm 1.8$  cm/sec) compared to baseline. At 17 °C RA conduction was further reduced to 52% and LV to 46% of baseline. Rewarming returned values to baseline. Statistical analysis showed that there were differences within the group ( $p < 0.001$ ) however these differences were only between temperature, with no difference in conduction between atria and ventricle.

### **VF threshold**

Taken together these results indicated that hypothermic temperatures had effects on whole heart conduction. However, the arrhythmogenic risk that accompanied these changes in ECG and conduction at 31 °C and 17 °C was unclear. Therefore, we examined the effect of temperature on VF threshold as an indicator of arrhythmia. At 37 °C, VF threshold was  $30.5 \pm 6.8$  mA (Figure 5.10). Cooling hearts to 31 °C decreased VF threshold compared to 37 °C ( $30.5 \pm 6.8$  mA vs  $14.0 \pm 2.2$  mA,  $p < 0.05$ ) indicating a more pro-arrhythmic state. In all but one heart VF threshold decreased at 31 °C (n=7). However, further cooling to 17 °C *increased* VF threshold ( $64.2 \pm 9.9$  mA,  $p < 0.05$ ) to a value higher than that seen at 37 °C. During rewarming to 31 °C and subsequently to 37 °C, a reversed sequence of VF threshold changes was observed.

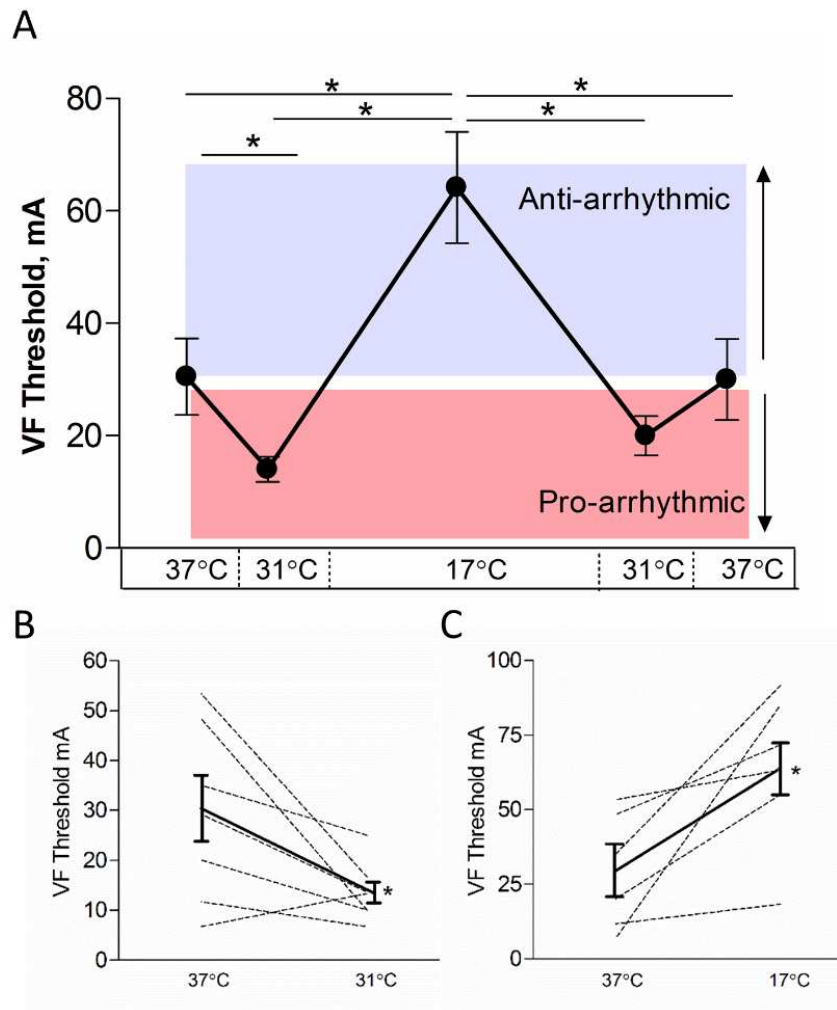


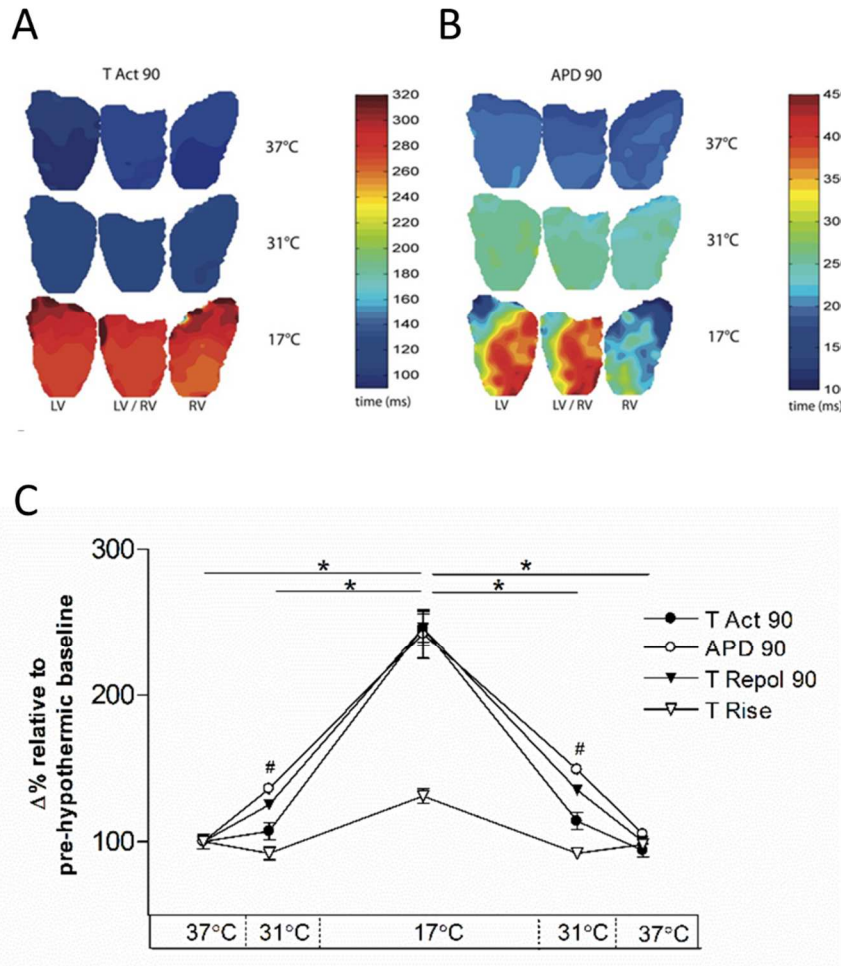
Figure 5.10 VF threshold during cooling and rewarming.

A: Mean VF threshold at all temperatures. B: Individual values from each heart (dashed lines) showing cooling to 31 °C (n=7) and to (C) 17 °C (n=6). Solid line shows mean values. \*Significant difference between temperatures (p<0.05).

### Whole heart action potential characteristics

Measurements of epicardial optical action potentials revealed changes in activation and repolarisation times (Figure 5.11). Data associated with ventricular activation (TAct90 and Trise) showed no significant changes at 31 °C while repolarisation characteristics (TRepol90 and APD90) were prolonged by 124% (305.2 ± 6.3 ms vs. 380.2 ± 8.4 ms, p<0.05) and 136% (176.92 ± 4.2 ms vs. 241.0 ± 2.9 ms, p<0.05) respectively. Compared to baseline both APD90 and TAct90 were prolonged to the same degree when hearts were cooled to 17 °C. APD90 showed a 242% prolongation (135.5 ± 6.6 vs 333.2 ± 7.8 ms, p<0.05) and TAct90 was prolonged by 246% (176.9 ± 4.2 vs 428.2 ± 29.2 ms, p<0.05). During rewarming to 31 °C, Tact90 returned to baseline levels while APD90 was

prolonged compared to control ( $176.9 \pm 4.2$  vs  $263.0 \pm 5.2$  ms,  $p < 0.05$ ). During rewarming to  $37^\circ\text{C}$  all parameters returned to baseline levels. Normothermic control hearts showed no significant differences in any of these parameters over the same period).



**Figure 5.11 : Activation and repolarisation characteristics during cooling and rewarming.** Compared to normothermic baseline. Optical mapping of hearts to show (A) Time to 90% Activation (Tact90) and (B) Action Potential Duration at 90% repolarisation (APD90) is displayed alongside percentage values relative to pre-hypothermic baseline (C). \*Significant difference between temperatures. # Significant difference within temperature.

### CV measured by panoramic optical mapping

Epicardial CV over a large area of the ventricle was measured in the same hearts with epicardial pacing on the RV/LV border and analysing the subsequent spread of activation in the longitudinal axis. CV was reduced to 66% of baseline during



cooling to 31°C ( $52.8 \pm 7.3$  vs  $35.2 \pm 3.7$  cm/sec,  $p < 0.05$ ). After cooling to 17°C a reduction to 37% of baseline was observed ( $19.7 \pm 3.7$  cm/sec,  $p < 0.05$ ). When rewarmed to 31°C, CV returned to values similar to that seen on initial cooling ( $36.9 \pm 5.7$  cm/sec). Upon rewarming to 37°C, CV values returned to within baseline values.

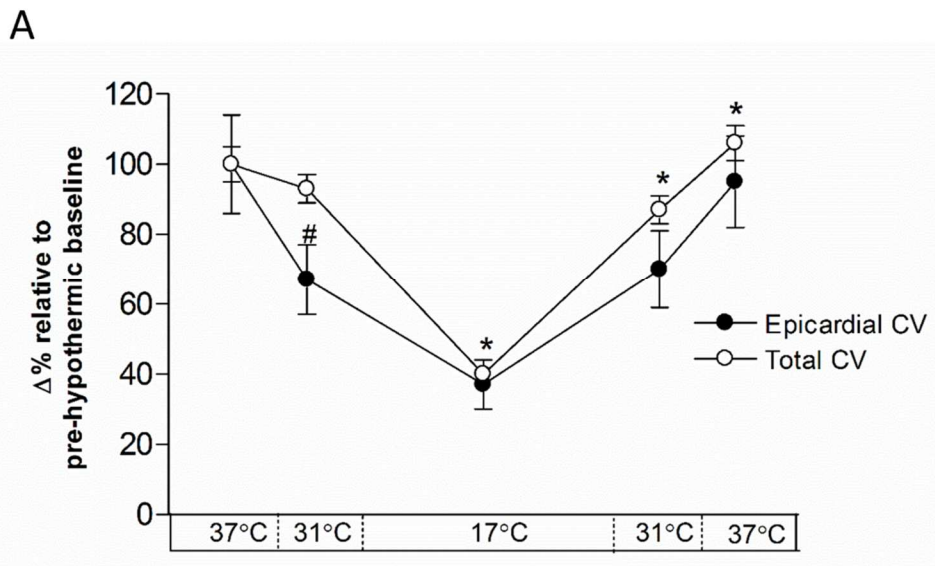


Figure 5.12 Total cardiac vs epicardial CV

Total cardiac (RA pacing) vs epicardial CV (RV pacing). \*Significant difference between temperatures. # Significant difference within temperature.

### Longitudinal vs transverse conduction

Further examination of CV explored the differences between  $CV_l$  and  $CV_t$  throughout cooling and rewarming (Figure 5.13). In experiments using contact electrodes, cooling to 31°C decreased CV in the longitudinal axis by 32% ( $76.3 \pm 11.9$  vs  $51.8 \pm 10.1$  cm/sec) and by only 13.5% in the transverse ( $22.6 \pm 1.4$  vs  $19.5 \pm 1.2$  cm/sec). At 17°C,  $CV_l$  decreased by 73.2% ( $76.3 \pm 11.9$  vs  $20.7 \pm 4.2$  cm/sec) and  $CV_t$  decreased by 52.3% ( $22.6 \pm 1.4$  vs  $10.5 \pm 0.9$  cm/sec) ( $p < 0.01$ ). Rewarming to 31°C increased both  $CV_l$  and  $CV_t$  and achieved values that were similar to that seen on initial cooling. After rewarming to 37°C, CV values were not different from pre-cooling values.

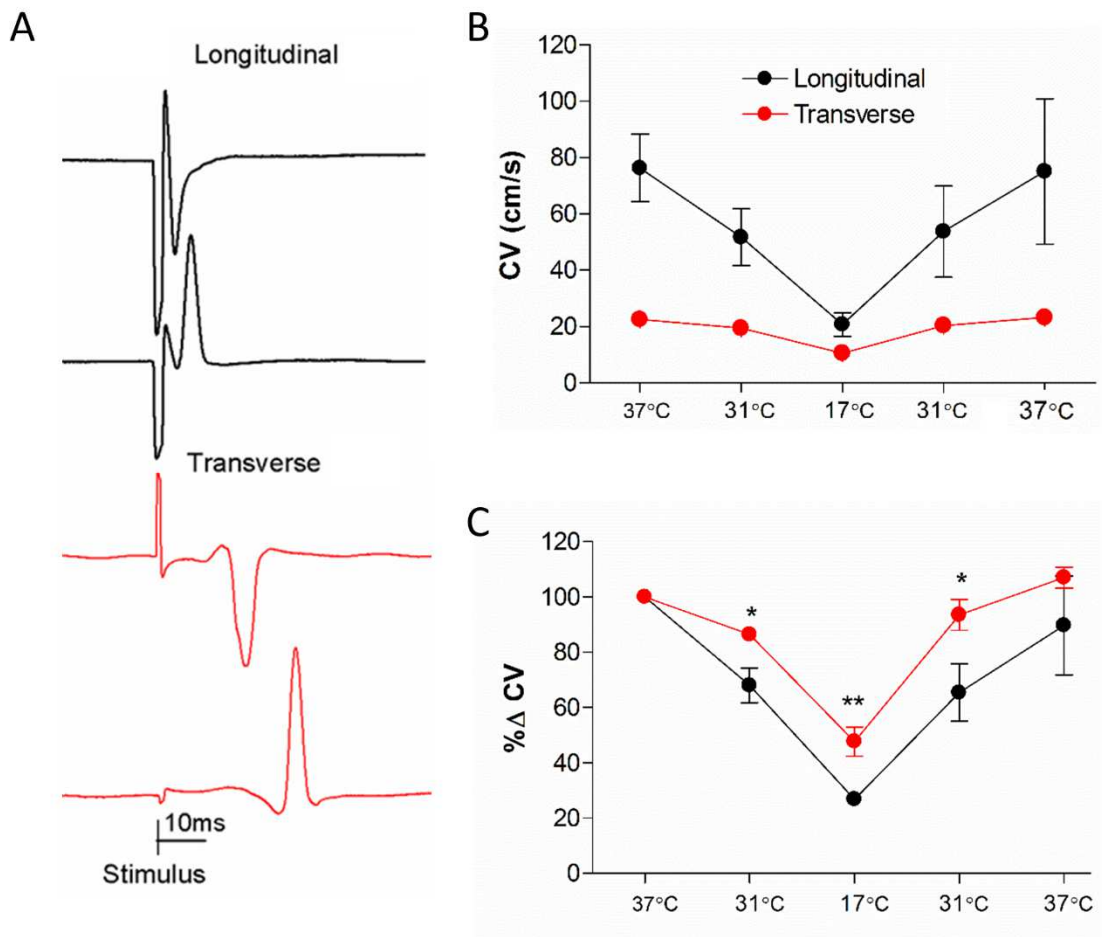


Figure 5.13 Longitudinal vs transverse CV during cooling and rewarming.

A: Example traces showing longitudinal (black) and transverse (red) conduction time. B: Absolute CV values through cooling and rewarming. C: Percentage change CV. Significant differences between longitudinal and transverse at same temperature. \* $p < 0.05$ . \*\* $p < 0.01$

## Discussion

The present results show that cooling to 31°C does not significantly change ventricular activation, but creates repolarisation abnormalities and is pro-arrhythmic. Cooling to 17°C causes parallel changes in ventricular activation and repolarisation and these changes are not pro-arrhythmic. Non-linear effects on ventricular activation can be explained in terms of a low temperature sensitivity of gap junction function. These hypothermia-induced changes in cardiac electrophysiology are observed at temperatures relevant for diverse clinical settings.

Moderate hypothermia ( $\leq 34^\circ\text{C}$ ) has been used therapeutically as a neuro-protectant, particularly in survivors of cardiac arrest (Callaway et al., 2015; Scirica, 2013) although recent meta-analyses have not shown a benefit in terms of survival or neurological outcome (Yu et al., 2015; Huang et al., 2015; Zhang et al., 2015) meaning that routine use of therapeutic hypothermia in cardiac arrest survivors is unlikely to continue. Severe ( $<30^\circ\text{C}$ ) hypothermia is a more extreme situation, yet survival is possible at such temperatures, with 13.7°C being the lowest recorded temperature reported in a patient recovering from accidental hypothermia with a good neurological outcome (Gilbert et al., 2000). The great reduction in metabolic demands induced by severe hypothermia is also utilised for neuroprotection during various surgical procedures (Di Luozzo and Griep, 2012; Dietrichs and Dietrichs, 2015). Nevertheless, treatment of hypothermia-induced arrhythmias remains challenging (Vanden Hoek et al., 2010) and knowledge of underlying mechanisms is of high clinical value.

### Hypothermia and conduction through the heart

Electrical and whole heart conduction timings in the present study indicated differential effects of conduction through the myocardium in moderate and severe hypothermia. At 31°C, only a mild relative delay in atrial and AV nodal conduction was present, while at 17°C conduction was significantly slower (Figure 5.7D, 5.8A). This is consistent with findings in humans, where AV nodal conduction is slowed by direct cooling of the AV node by cold (4°C) isotonic saline (Gould and Reddy, 1976). Similarly, *in vivo* findings have shown that

18.3°C is a critical temperature for the occurrence of AV-block in hypothermia (Hamilton et al., 1936). In contrast, evident from ECG (QR-time) and whole heart conduction timing data, transmural (transverse) ventricular conduction appears relatively insensitive to cooling at 31°C.

Panoramic optical mapping of hearts during cooling confirmed conduction timings from the ECG as well as electrode-based measurements, and showed unchanged ventricular activation (TAct90, TRise) compared to 37°C. Although ventricular transverse conduction and activation parameters appear insensitive to moderate hypothermic conditions, the timings related to repolarisation (APD90 and TRepol90) show pronounced effects at 31°C that are further enhanced at 17°C (Figure 5.11). Therefore, in moderate hypothermia, there is slowing of conduction through the heart, mostly in the atria and AV node whilst ventricular/transmural activation remain relatively unaffected. However, ventricular repolarisation is significantly prolonged in the presence of unchanged activation, producing a form of acquired long-QT syndrome. Indeed, in the clinical setting, long QT syndrome is a common finding in TH (Kim et al., 2014) although the clinical relevance of this finding is unclear likely due to the confounding influence of pre-existing QT-prolongation, electrolyte derangement and use of anti-arrhythmic drugs in the comatose survivors of VT/VF cardiac arrests. In one meta-analysis, the use of TH was associated with an increased risk of recurrent arrest (Huang et al., 2015) possibly due to QTc prolongation and pro-arrhythmia in these patients. Further cooling to 17°C induces a global activation delay, including a significant delay in transverse ventricular activation, reducing the global activation and repolarisation differences.

### **Ventricular fibrillation threshold during hypothermia**

Whole heart conduction timings showed a markedly greater effect as temperature was lowered from moderate to severe hypothermia. VF threshold showed a different pattern of changes during hypothermia: cooling to 31°C showed a significant reduction in VF threshold (pro-arrhythmic), while further cooling to 17°C showed a substantial increase in VF threshold (anti-arrhythmic) to more than twice that measured at 37°C.

This finding implies that electrophysiological changes within the heart at 31°C provide a more stable substrate for arrhythmias than at 17°C. Indeed, it has been shown in another study of isolated Langendorff perfused rabbit hearts that cooling to 30°C increased the vulnerability of the heart to VF (Hsieh et al., 2009). These findings are in line with results from an *in vivo* study of dogs, progressively cooled from 37°C to 25°C, (Mortensen et al., 1993) which reported that cooling from 31°C to 25°C caused a significant lowering of the number of extra stimuli required to induce VF. The current study is the first to demonstrate experimentally the biphasic relationship in excitability of the ventricle, suggesting that moderate hypothermia (31°C) may make the heart more vulnerable to induction of arrhythmias than deep hypothermia (17°C). Therapeutically, temperatures <28-30°C are thought to potentiate the occurrence of ventricular arrhythmias. Yet 30°C is the lower end of temperatures reported to be used following cardiac arrest in the comatose patient (Polderman and Herold, 2009).

Although we found pro-arrhythmic changes at 31°C, the opposite was the case at 17°C, at which temperature a more stable electrophysiology was observed. The reasons for the difference in VF threshold between moderate and severe hypothermia remain unclear. Based on our data, it is possible that the observed biphasic response to cooling could be linked to the absence of significant effects on ventricular activation times at 31°C while repolarisation values are prolonged, contrasting with 17°C where activation times are substantially prolonged in parallel with repolarisation values. The QR-time of the QRS complex represents ventricular conduction from endocardium to epicardium and is used as a marker of ventricular activation (Perez-Riera et al., 2016). Because this transmural conduction is mainly in the short (i.e. transverse) axis, the question of whether  $CV_t$  and  $CV_l$  are altered differentially in hypothermia merited further study.

### **$CV_l$ vs $CV_t$ in hypothermia**

Separate measurements of CV (Figure 5.13) show that moderate hypothermia affected longitudinal and transverse conduction differently. Although  $CV_l$

decreases consistently throughout cooling,  $CV_t$  was relatively insensitive to cooling to 31 °C, but decreased on cooling further to 17 °C. CV is determined by multiple factors, including tissue excitability and intercellular resistance; the former is linked to inward  $Na^+$  current amplitude, and the latter determined by connexin-mediated gap junctions. For equivalent distances, gap junction resistance has a greater contribution to transverse conduction velocity ( $CV_t$ ) whilst  $Na^+$  channels and intracellular resistance has a greater involvement in longitudinal conduction ( $CV_l$ ). This differential response between  $CV_t$  and  $CV_l$  at moderate hypothermia may suggest that moderate hypothermia has only a small effect on gap-junction resistance relative to the effect on  $Na^+$  current. To explore the role of the two major determinants of CV, propagation along a myocardial fibre of fixed length, measuring activation time at the unstimulated end was modelled computationally (Figure 5.14). This model showed that reducing gap junctional conductance by a quarter (e.g. from 2 to 0.5  $\mu S$ ) caused a 50% reduction in  $CV_t$  but only an 11% reduction in  $CV_l$  (Figure 5.14B). In contrast, reducing intracellular conductance to 25% of control (Figure 5.14C) reduced  $CV_l$  by 34% whereas  $CV_t$  is only reduced by 5%. This modelling suggests a hypothesis to explain the asymmetric changes in  $CV_l$  and  $CV_t$  seen in hypothermia, namely that the intracellular resistance (e.g.  $Na^+$  current) is more sensitive to reduced temperature than gap-junction resistance. Thus, in moderate hypothermia  $CV_t$ , which is dominated by gap-junction resistance, was little affected while  $CV_l$ , which is determined by inward  $Na^+$  current, was reduced by 32%.

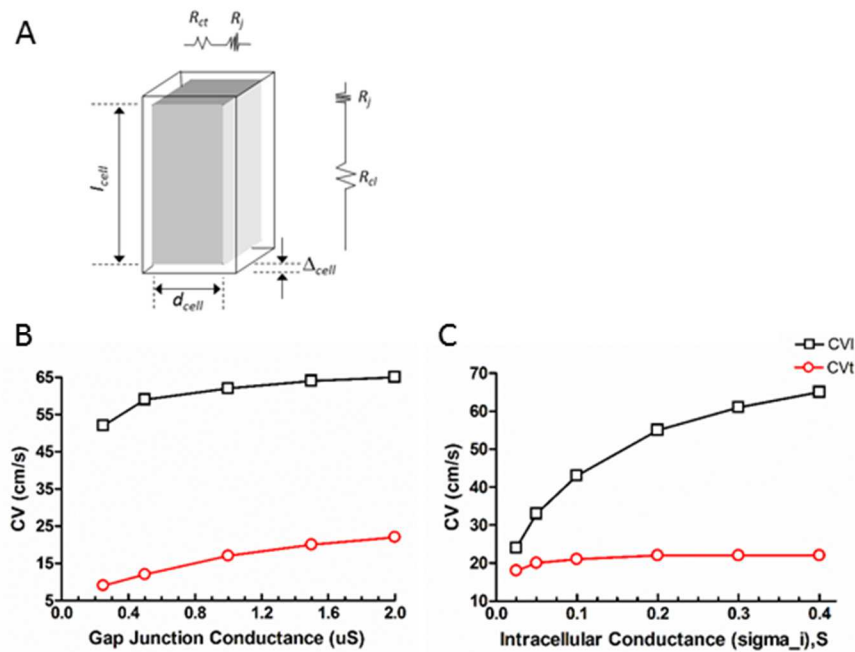


Figure 5.14: Computational modelling of longitudinal and transverse conduction.

**A:** Schematic representation of an idealised cuboid cell of length  $l_{cell}$ , width  $d_{cell}$ , showing resistance to current flow along and transverse to the cell's length where  $R_{cl}$  is the cytoplasmic resistance along the cell,  $R_{ct}$  is the cytoplasmic resistance transverse to the cell and  $R_j$  is the resistance of the gap junction. **B:** Variation in CV as gap junction conductivity is varied and **(C)** variation in CV as intracellular conductivity is varied.

## Mechanisms of arrhythmias in hypothermia

These results have shown that at 31 °C there was a prolongation of ventricular repolarisation with minimal change in timing and pattern of ventricular activation. This is accompanied by lower VF threshold at 31 °C, which is not seen at 37 °C or 17 °C. It is known that ventricular arrhythmias can arise from disruptions in the normal sequence of activation and repolarisation (Weiss et al., 2010). The primary change in ventricular electrophysiology at 31 °C is prolongation of the APD and increased heterogeneity of ventricular repolarisation, both of which are considered pro-arrhythmic changes. Interestingly, at 17 °C these changes are more pronounced, but the accompanying decrease in transverse CV appears to raise VF threshold.

## Conclusion

This study suggests that prolonged and heterogeneous repolarisation is only pro-arrhythmic in the context of normal activation times, suggesting that slowing CV and consequently prolonging activation time should be anti-arrhythmic.

## Limitations

All work was carried out on isolated rabbit hearts which were therefore not under normal autonomic modulation that is present *in vivo*. In addition, the recordings were limited to 3 set points so as to allow the heart to physiologically stabilise at each temperature. In future work it would be useful to be able to record electrophysiology during cooling in order to identify the temperature in which hypothermia becomes anti-arrhythmic.



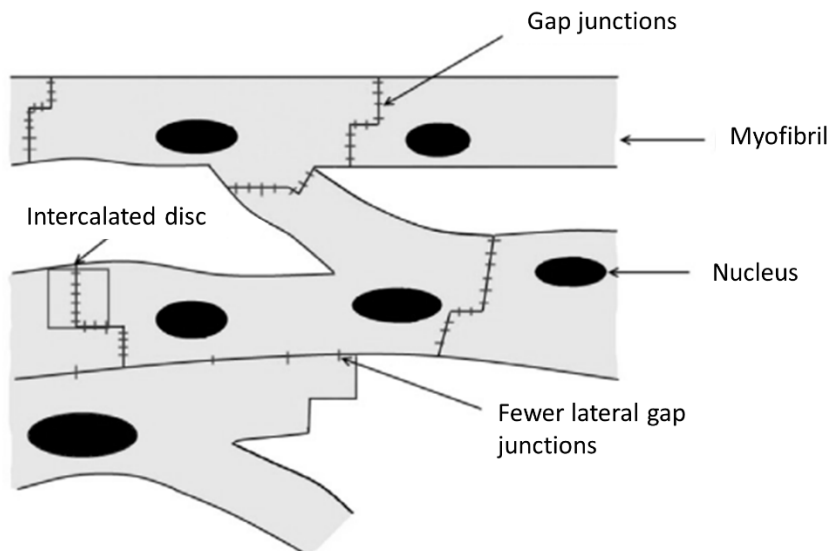
## **Chapter 6: The effect of gap junction uncoupling on cardiac conduction during hypothermia**

## Introduction

The findings from the previous chapter showed that there were differential effects on cardiac conduction when cooling to 31 °C. The primary change in ventricular electrophysiology at 31 °C was prolongation of the APD and increased heterogeneity of ventricular repolarisation, both of which are considered pro-arrhythmic. At 17 °C these changes were more pronounced, but the accompanying decrease in transverse CV appeared to raise VF threshold. This suggests that prolonged and heterogeneous repolarisation is only pro-arrhythmic in the context of normal activation times, suggesting that slowing CV and consequently prolonging activation time in proportion to the other electrophysiological changes should be anti-arrhythmic.

## Intercellular coupling

Under normal circumstances, the heartbeat is initiated in the sinoatrial (SA) node, conducted across the atria, delayed at the AV node, after which the ventricular myocardium is activated via the specialised conduction system. For the heart to function it is essential that there is a rapid conduction of this cardiac impulse, requiring the presence of low resistance connections between cardiac cells (Rohr, 2004). The speed is dictated by the action potential shape, particularly the leading phase, and the electrical properties of the cell, where the conductance of the electrical cable is inversely related to the intercellular (internal) resistance. Low resistance favours fast conduction and the gap junction channels provide low resistance connections between cardiac cells (Spach, 1999). Propagation occurs rapidly through the cytoplasm and slows at the intercellular junctions, so conduction is discontinuous. Due to the arrangement of myocardial fibres, conduction from endo to epicardium is at a direction transverse to the fibre axis. The speed of propagation in this direction is less than half that in the longitudinal axis (Roberts et al., 1979; Valderrabano, 2007). This is thought to be due to the greater number of gap junctions (per unit length) in the transverse direction (Figure 6.1) (Campbell et al., 2014).



**Figure 6.1: Organisation of cardiomyocytes and gap junctions.**

Schematic showing the organisation of cardiomyocytes and their communication via the intercalated disc. Fewer gap junctions (part of intercalated disc) in longitudinal direction vs transverse. Diagram adapted from: (Campbell et al., 2014). In longitudinal propagation the impulse travels through less junctions per unit space than in transverse propagation, therefore the former experiences less delays than the latter.

### Gap junction uncoupling

Under normal circumstances the heart acts as an electrical syncytium via gap junctions. However, if gap junction coupling or gap junctional resistance are altered then an increased heterogeneity of repolarisation can occur, and this exaggerated dispersion may be arrhythmogenic. This cellular uncoupling has been demonstrated in the various forms of injury and in the later phases of ischaemia (Kanno and Saffitz, 2001; Severs et al., 2008; Tse and Yeo, 2015). As cells become progressively uncoupled (gap junctional resistance increases and gap junctional conductance decreases), conduction velocity decreases monotonically (Shaw and Rudy, 1997). This reduction in conduction velocity is due to longer delays in propagation across the gap junctions.

### Heptanol

Gap junctions can be uncoupled pharmacologically using various different agents. For the benefit of this study, two separate gap junction uncouplers were selected. Firstly, the lipophilic agent heptanol. There are several theories

proposed for the mechanism of action, however the most common explanation is that the drug is incorporated into the lipid bilayer which in turn leads to impairment of the gap junction channels. It has been reported that heptanol reduces coupling by reducing the open probability of the gap junction channels. Heptanol has been used to study the effect of gap junction uncoupling and slowed conduction in both the intact heart (Keevil et al., 2000; Tse and Yeo, 2015) and in paired cardiomyocytes (Rüdisüli and Weingart, 1989; Takens-Kwak et al., 1992).

### **Carbenoxolone**

Carbenoxolone (CBX) is a mineralocorticoid that is used to promote healing in the treatment of gastric and mouth ulcers. It is thought to work by stimulating the production of mucus that forms a protective coating over the stomachs lining. Initially, experimental work with carbenoxolone was restricted to neurological investigations where it was found to reversibly abolish gap junction mediated intercellular coupling, however in recent years there have been more studies examining the cardiac effect. One study by deGroot (2003) concluded that carbenoxolone was an effective agent that delayed conduction and caused localised conduction slowing which indicated gap junction uncoupling. They also reported no adverse effects on action potential characteristics, whilst calcium, sodium and potassium currents remained unaffected. In a separate study it has also been shown in humans to reduce wavefront propagation by 27% whilst not affecting refractoriness (Kojodjojo et al., 2006). The slowing of myocardial conduction by carbenoxolone in this study demonstrated that it is a useful agent for investigating the effects of gap junction uncoupling on human arrhythmogenesis.

### **Aims**

In the previous chapter slowing of conduction was associated with a pro-arrhythmic state at 31°C but not at 17°C. The temperature sensitivity of the gap junction was a proposed mechanism which could be modulated. Therefore, the aims of this chapter were:

i) to assess the effect of pharmacological gap junction uncoupling on  $CV_l$  and  $CV_t$  during moderate hypothermia.

ii) to examine whether any alterations in CV had a subsequent effect on VF threshold.

## Methods

Male New Zealand White Rabbits (~3-3.5kg) were used for all experimental work in this chapter. Hearts were isolated and Langendorff perfused (as described in Chapter 2). All hearts were perfused with Blebbistatin (10 $\mu$ M) and allowed a settling period of approx. 20 mins to physiologically stabilise before any recordings were made. CV<sub>i</sub>, CV<sub>t</sub> and VF threshold was recorded (see Chapter 5 for full description) at 37°C, 31°C and following pharmacological gap junction uncoupling.

### Gap junction uncoupling with CBX

Previous work in our lab studied a concentration range of 10-100  $\mu$ M (CBX) on whole heart electrophysiology. It was found that <50  $\mu$ M had little effect on electrophysiology, however >60  $\mu$ M had dramatic and toxic effects on the heart, including an increase in perfusion pressure as well as an increase in end systolic and end diastolic pressures. Based on these findings, concentrations of 3  $\mu$ M, 10  $\mu$ M and 50  $\mu$ M were used for this study. CBX (Sigma, UK) was dissolved in de-ionised water and was prepared immediately prior to each experiment.

Following CV and VF threshold measurements at 31°C hearts were allowed approx. 15 min recovery time. CBX (3  $\mu$ M) was delivered to the heart over a 5 min period via syringe driver (Figure 6.2). Following recordings, the heart was allowed a 25 min washout period with Tyrode's solution (de Groot et al., 2003) before increasing CBX concentration to 10  $\mu$ M. The process was repeated for 50  $\mu$ M.

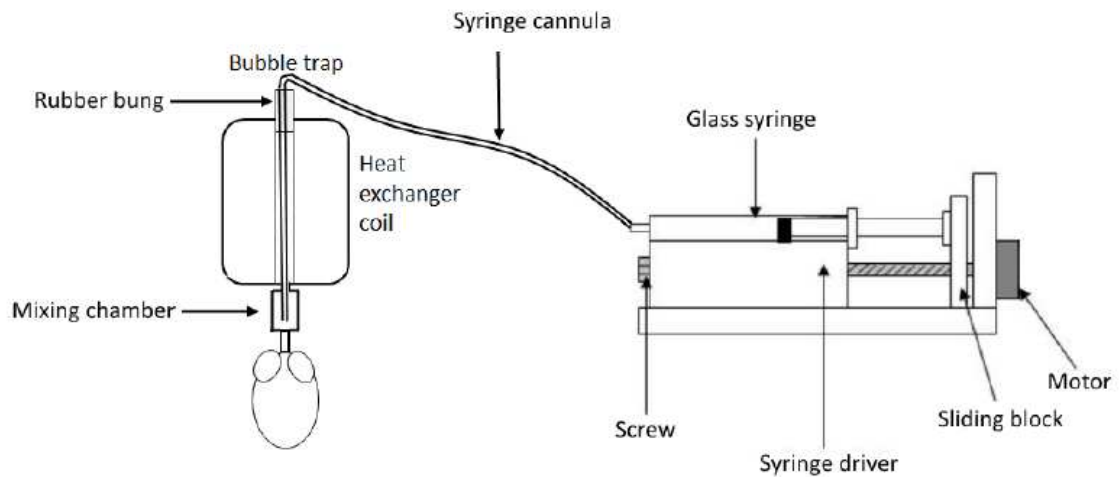


Figure 6.2: Diagram of syringe driver.

Diagram showing the setup of syringe driver used to deliver CBX to the heart. Perfusion was maintained at a constant rate, to ensure concentration remained the same throughout. A syringe cannula was used to allow the CBX to be delivered as close to the coronary arteries as possible and minimise 'dead space' in the system. CBX was perfused at 0.4ml per min and mixed with Tyrode's solution (31 °C) before reaching the aorta.

### Gap junction uncoupling with heptanol

Separate experiments pharmacologically inhibited gap junctions using heptanol (0.3mM). This concentration was based on previous work within our lab which showed significant effects on CV in isolated rabbit hearts. Due to the lipophilic nature of heptanol it is insoluble in water so it was dissolved in ethanol before adding to Tyrode's solution. The final concentration of ethanol in the solution was less than 0.005%.

Following CV and VF threshold measurements at 31 °C, the heart was allowed approx. 15 min to recover following which perfusion was changed to heptanol via a 2-way switch incorporated in the perfusion lines. After 5 min perfusion CV and VF threshold measurements were taken. Due to the reversible nature of heptanol (Takens-Kwak et al., 1992), perfusion was continued until CV and VF measurements were complete.

### Statistical analysis

Data are expressed as mean  $\pm$  SEM. CV and VF threshold measurements were assessed by one-way ANOVA for repeated measures. When significant differences were found, data were post-hoc analysed using Tukey's test. All statistical tests were performed using GraphPad Prism software.

## Results

### Effect of CBX during moderate hypothermia

In this study, initial experiments (n=2) were carried out to examine the response of CV with different concentrations of the gap junction uncoupler CBX. Hearts were perfused with 3 $\mu$ M, 10 $\mu$ M and 50 $\mu$ M CBX at 31 $^{\circ}$ C, with a washout period of 25 mins between each concentration. For these initial experiments, continuous CV<sub>t</sub> recording was carried out to assess the response of the drug. Due to limitations with equipment, both CV<sub>l</sub> and CV<sub>t</sub> could not be recorded simultaneously. Cooling to 31 $^{\circ}$ C reduced CV<sub>t</sub> by 12.6% compared to 37 $^{\circ}$ C. However, the addition of CBX at 3 $\mu$ M showed a reduction in CV<sub>t</sub> of only 10.9% compared to baseline. In comparison to this, 10 $\mu$ M and 50 $\mu$ M CBX decreased CV<sub>t</sub> by 16.5% and 24.5% respectively. Based on these findings, further experiments (n=4) were carried out using only 10 $\mu$ M and 50 $\mu$ M CBX.

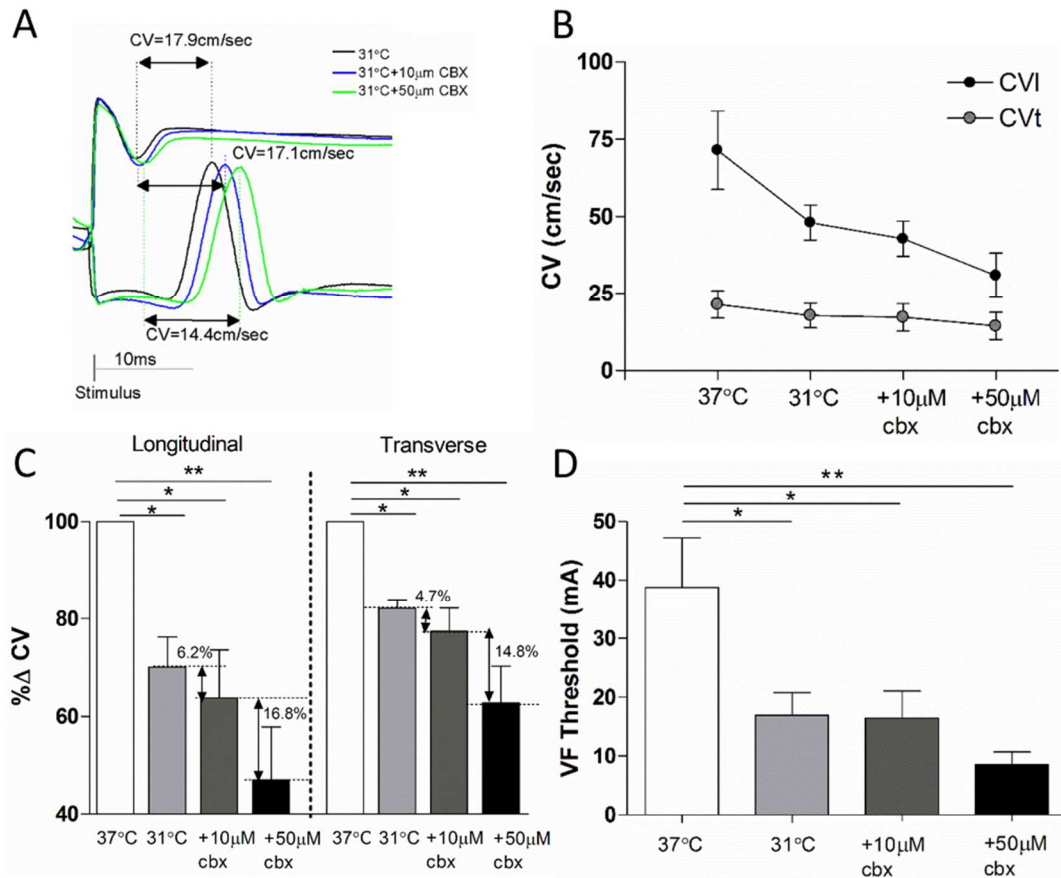
The effect of CBX perfusion on CV and VF threshold can be seen in Figure 6.3. Example traces show that both 10  $\mu$ M and 50  $\mu$ M slowed conduction. The difference between CV<sub>l</sub> and CV<sub>t</sub> when cooling to 31 $^{\circ}$ C can be clearly seen in Figure 6.3B, where there appears to be a greater effect on CV<sub>l</sub> than CV<sub>t</sub>. Moderate hypothermia decreased CV<sub>l</sub> by  $29.9 \pm 6.2\%$  compared to 37 $^{\circ}$ C ( $p < 0.05$ ). The addition of 10  $\mu$ M CBX decreased CV<sub>l</sub> by  $36.3 \pm 9.8\%$  ( $p < 0.05$ ) and by  $52.9 \pm 10.8\%$  at 50  $\mu$ M ( $p < 0.01$ ). Conduction in the transverse axis (Figure 6.3 A-C) was reduced by  $17.9 \pm 1.8\%$  at 31 $^{\circ}$ C when compared to 37 $^{\circ}$ C ( $p < 0.05$ ). Further decreases in CV<sub>t</sub> of  $22.6 \pm 4.8\%$  ( $p < 0.05$ ) and  $37.4 \pm 7.5\%$  ( $p < 0.01$ ) compared to baseline (37 $^{\circ}$ C) were seen at 10 $\mu$ M and 50 $\mu$ M respectively.

Figure 6.3D shows VF threshold measurements in the presence of CBX. Cooling to 31 $^{\circ}$ C significantly decreased VF threshold ( $38.8 \pm 8.5$  vs  $16.9 \pm 3.9$  mA,  $p < 0.05$ ).

The addition of 10 $\mu$ M and 50 $\mu$ M CBX further *decreased* VF threshold to  $16.4 \pm 4.6$  mA ( $p < 0.05$ ) and  $8.6 \pm 2.1$  mA ( $p < 0.01$ ) respectively. At both 10  $\mu$ M and 50  $\mu$ M all hearts required cardioversion using KCl to return to sinus rhythm following VF.

These findings indicate that gap junction uncoupling by CBX had a greater effect on CV<sub>l</sub> than CV<sub>t</sub> at 31 $^{\circ}$ C. This was accompanied by a reduction in VF threshold suggesting that CBX is pro-arrhythmic in hypothermic conditions.





**Figure 6.3: Effect of CBX on CV and VF threshold**

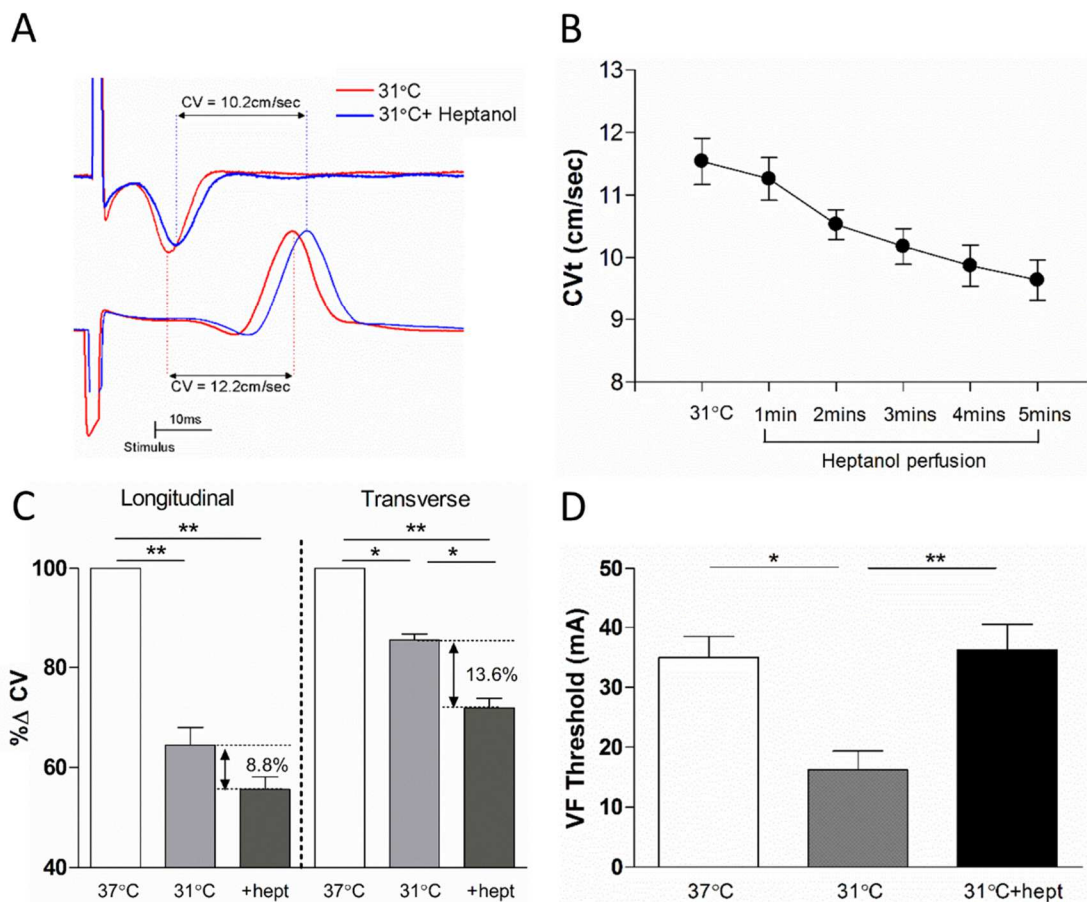
**A:** Example traces of CV (transverse) at 31 °C (black) and following perfusion of 10µM (blue) and 50µM (green) CBX. **B:** Mean longitudinal (black circles) and transverse (grey circles) CV values following hypothermia and CBX perfusion. **C:** Percentage change of CV. **D:** VF threshold measurements. \* $p < 0.05$ , \*\* $p < 0.01$

### Effect of heptanol during moderate hypothermia

Separate experiments ( $n=4$ ) examined CV and VF threshold following 5 mins of 0.3mM heptanol perfusion. Figure 6.4 shows the effect of heptanol on CV and VF threshold. Cooling to 31 °C slowed CV<sub>l</sub> by  $35.5 \pm 3.6$  %, whilst heptanol reduced it by  $44.3 \pm 2.5$  % when compared to 37 °C ( $p < 0.01$ ). CV<sub>t</sub> was reduced by  $14.5 \pm 1.2$  % at 31 °C ( $p < 0.05$ ) and  $28.1 \pm 1.9$  % following heptanol ( $p < 0.01$ ), when compared to 37 °C baseline. There was a significant difference of 13.6% between CV<sub>t</sub> at 31 °C and following perfusion with heptanol.

Figure 6.4D shows VF threshold measurements in the presence of heptanol. Cooling to 31 °C significantly decreased VF threshold ( $35.0 \pm 3.5$  vs  $16.3 \pm 3.1$  mA,  $p < 0.05$ ). Following perfusion with heptanol during hypothermia VF

threshold *increased* back to baseline values. In 3 out of 4 experiments hearts converted to sinus rhythm spontaneously without the need for KCl.



**Figure 6.4: Effect of heptanol on CV and VF threshold**

**A:** Example traces of CV (transverse) at 31 °C (red) and following perfusion of 0.3mM (blue) heptanol. **B:** Mean transverse conduction at 31 °C and throughout 5 mins of heptanol perfusion. **C:** Percentage change of CV. Shown are the differences between 31 °C and 0.3mM heptanol. **D:** VF threshold measurements. \* $p < 0.05$ , \*\* $p < 0.01$

### Gap junction uncoupling and anisotropy

Figure 6.5 shows absolute CV values following hypothermia and gap junction uncoupling alongside anisotropic ratio ( $CV_l/CV_t$ ). Ratio was calculated between 37 °C and 31 °C and between 31 °C and pharmacological intervention. No change in anisotropy was detected between hypothermic hearts and following CBX at either 10 $\mu$ M or 50  $\mu$ M. In contrast to this, the addition of heptanol showed a strong trend towards an increase in ratio ( $p=0.07$ ).

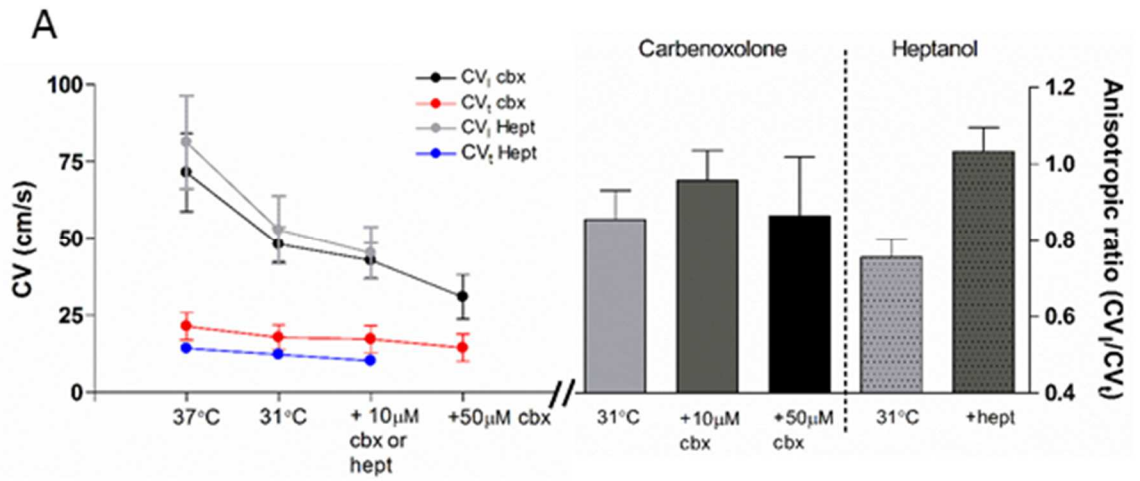


Figure 6.5: Gap junction uncoupling and anisotropy.

A: Left panel shows absolute CV values. Black and red lines represent CV<sub>i</sub> and CV<sub>t</sub> following CBX, whilst grey and blue lines represent the addition of heptanol. Left panel shows anisotropic ratio (CV<sub>i</sub>/CV<sub>t</sub>). Light grey bars show ratio between 37 °C and 31 °C. Dark grey/black represent ratio between 31 °C and addition of CBX/heptanol.

## Discussion

Following on from the results of Chapter 5, this study described the effect of gap junction uncoupling at 31 °C alongside parallel measurements of VF threshold. To test the hypothesis that maintained  $CV_t$  when cooling from 37 °C to 31 °C is required for a pro-arrhythmic state induced by cooling to 31 °C, VF threshold was examined at moderate hypothermia (31 °C) before and after  $CV_t$  was reduced pharmacologically. As anticipated from the computational model, heptanol, a gap junction uncoupler, lowered  $CV_t$  proportionally more than  $CV_l$  and when heptanol was applied to hearts at 31 °C it increased VF threshold to within normothermic values. The dose of heptanol used reduced the relative  $CV_t$  to the same extent as the hypothermia-induced delays in other parameters of cardiac conduction. The use of CBX as a gap junction uncoupler did not show the same effects and led to increased arrhythmias. There are several findings from this work that are important to mention:

- i. The slowing of conduction at 31 °C supports evidence that reducing temperature decreases gap junctional conductance (Bukauskas and Weingart, 1993; Chen and DeHaan, 1993).
- ii. Gap junctional uncoupling by CBX showed a greater effect on  $CV_l$  than  $CV_t$  which was accompanied by a significant reduction in VF threshold. The reason for the difference in  $CV_l$  and  $CV_t$  is not clear as previous reports have shown that infusion resulted in decreased transverse but not longitudinal CV. However there are several reports about the non-specificity of CBX in neuronal gap junctions (Connors, 2012) and side effects as a result of electrolyte imbalances (Davies et al., 1974). In cardiac preparations it has been shown to reduce gap junction permeability, however it has been associated with either no change in VF threshold or an increased incidence of arrhythmias (de Groot et al., 2003; Miura et al., 2016).
- iii. Gap junctional uncoupling by heptanol showed a greater effect on  $CV_t$  than  $CV_l$ . The efficacy of heptanol as a gap junction uncoupler is well

described (Delmar et al., 1987; Balke et al., 1988; Rüdüsüli and Weingart, 1989; Takens-Kwak et al., 1992; Keevil et al., 2000; Tse et al., 2016). However there have been differing reports on the effect of heptanol and the incidence of arrhythmias. Evidence has suggested that regional perfusion with heptanol may decrease CV but could also induce atrial (Ohara et al., 2002) and ventricular (Tse et al., 2016) arrhythmias. In contrast to this, and in line with our findings, Sun (2014) showed that there was a decreased incidence of VF after heptanol infusion.

Gap junction uncoupling and the subsequent slowing of conduction (particularly in the transverse direction) abolished the pro-arrhythmic state that was induced by moderate hypothermia. One possible explanation for this finding is the safety factor principle of conduction. Shaw and Rudy (1997) described the safety factor as a dimensionless parameter that indicates the margin of safety with which the action potential propagates relative to the minimum requirements for sustained conduction. When an action potential propagates, the excited cell serves as a source of electric charge for neighbouring unexcited cells towards their excitation threshold. For propagation to succeed, the excited cell must provide sufficient charge to the unexcited cells to bring their membrane to excitation threshold. Once the threshold is reached and the action potential is generated, the load on the excited cell is removed and the newly excited cell becomes the source for downstream tissue, which in turn perpetuates the action potential propagation. This has been described as a source-sink relationship whereby the source is the electric charge and the sink is the unexcited cell. (Kléber and Rudy, 2004).

There will be a lowered safety factor for conduction when there is reduced excitability. This means that the propagating action potential can slow or stop, leading to conduction block or re-entry. As well as reduced membrane excitability, gap junction uncoupling also decreases the velocity of the propagating action potential. Both theoretical (Joyner, 1982; Rudy and Quan, 1987) and experimental (Spach et al., 1981; de Bakker et al., 1988; Delgado et al., 1990) studies have suggested that there is a greater slowing through gap junction uncoupling compared to reductions in excitability. Despite the slowed

conduction, evidence suggests that propagation across cells with reduced coupling has a high safety factor (Leon and Roberge, 1991; Rohr, 2004).

Based on this model, moderate hypothermia (31°C) produces an environment where there is a low safety factor, and as a result there is an increased risk of arrhythmia. However, the use of heptanol to uncouple gap junctions and further slow  $CV_t$  provides a more stabilised cellular environment whereby the action potential is able to fully propagate. This suggests that the relative difference in  $CV_l$  and  $CV_t$  in moderate hypothermia is an important aspect of the safety factor.

## Conclusions

These findings propose that the low temperature sensitivity of gap-junction conductance is an important pro-arrhythmic factor during moderate hypothermia. This suggests that modulation of gap junction function is a potential therapeutic target to protect patients in moderate hypothermia from arrhythmias during therapeutic as well as during accidental hypothermia.

## Limitations

The limitations described here also apply to chapter 5. There seemed to be variation between baseline CV values in different hearts, ranging from ~65-90cm/sec in the longitudinal direction. This is likely due to the differences in position of the electrode on each heart. Although every effort was made to keep the angle consistent, every heart has a different fibre architecture. The different effects of CBX and heptanol was also limiting and it would be useful if there was a specific gap junction blocker that could be applied to both research and clinical practice.

## Chapter 7: General Discussion

The general aims of this thesis were to assess cardiac electrophysiology and mechanical function at temperatures occurring in therapeutic and accidental hypothermia.

### **Inotropic response to hypothermia**

This work aimed to better understand hypothermia/rewarming induced contractile dysfunction. The first aim was to address whether the inotropic response following hypothermia and rewarming that is evident *in vivo* could be recapitulated in an *in vitro* model. The findings showed that following 3 hours at 16 °C (stimulated at 1Hz), upon rewarming there was a greater than 50% reduction in shortening. These findings were similar to a recently published study using a similar cell model by Schaible *et al* (2016). This was not accompanied by a change in cytosolic Ca<sup>2+</sup> transients therefore suggesting a lowered myofilament response to Ca<sup>2+</sup> was the basis of the reduced inotropy. Several mechanisms were proposed to explain this change in sensitivity including; metabolic dysfunction, possibly as a result of increased mitochondrial Ca<sup>2+</sup> (Wold *et al.*, 2013), cAMP/PKA mediated phosphorylation of cTnI (Han *et al.*, 2010), or due to an increase in reactive oxygen species and subsequent modification of the myofilaments. Whilst the underlying mechanism of this myofilament sensitivity was not identified within this study, it has provided a reproducible model of rewarming induced contractile dysfunction in isolated myocytes that can be used in future work.

The second aim of this work was to assess electrophysiology in isolated hearts following *in vivo* hypothermia and rewarming. This work showed that 3 hours of stable hypothermia followed by rewarming led to a significant reduction in CO when compared to baseline. Ventricular epicardial AP measurements showed that these hearts also displayed a shorter APD. This may be as a result of metabolic disruptions in the myocyte leading to opening of the K<sub>ATP</sub> channel. Assessment of transient hypothermia in separate experiments showed that APD was prolonged following rewarming. These findings showed that following *in vivo* hypothermia/rewarming there was a contractile dysfunction alongside a chronically shorter APD, suggesting that a sustained electrophysiological effect



that would manifest as a shortened QT interval. In contrast to this, a period of transient hypothermia had alternative detrimental effects on the cardiac APD when compared to prolonged hypothermia, an effect that could predispose to the induction of long QT related arrhythmias and ventricular tachycardia.

A better understanding of the mechanisms involved in this hypothermia/rewarming contractile dysfunction may indicate therapies to address the issue. Future work could utilise the *in vitro* cell model to look at the possible mechanisms for myofilament sensitivity. A more detailed study looking to better understand E-C coupling factors could be carried out on myocytes, e.g. modulation of adrenoreceptors and PDE function could be assessed *in vitro* by using isoproterenol and selective PKA and PDE inhibitors. This could potentially fit well with recent work using the rat model which has examined the effect of pharmacological modulation of the PKA pathway (Dietrichs et al., 2014a; Dietrichs et al., 2014b) and has shown promising results, with improvements in CO and SV after rewarming. Future work using the optrode should also look to assess whether there is a change in  $Ca^{2+}$  alongside the APD shortening.

### **Cardiac conduction and hypothermia**

The second part of this thesis focused on the electrical aspects of cooling and rewarming. Accidental hypothermia is complicated by risk of ventricular arrhythmias, however there is a lack of understanding regarding the underlying mechanisms and the optimal treatment of the hypothermic heart.

Chapters 5 and 6 of this thesis focused on cardiac conduction and excitability during hypothermia and rewarming. The aim was to assess conduction and electrical activity at temperatures seen in therapeutic (31°C) and also accidental hypothermia (17°C). Findings from this study showed that cooling to 31°C prolonged the PR and QT interval, whilst at 17°C all aspects of the ECG were prolonged. Panoramic optical mapping of the LV showed that cooling to 31°C does not significantly change ventricular activation, but creates repolarisation abnormalities (TRepol and APD90 prolongation) and is pro-arrhythmic. Cooling to 17°C causes parallel changes in ventricular activation and

repolarisation and these changes are not pro-arrhythmic. This finding implies that electrophysiological changes within the heart at 31°C provide a more stable substrate for arrhythmias than at 17°C. The current study is the first to demonstrate experimentally the biphasic relationship in excitability of the ventricle, suggesting that moderate hypothermia (31°C) may be more vulnerable to induction of arrhythmias than deep hypothermia (17°C). Therapeutically, temperatures <28-30°C are thought to potentiate the occurrence of ventricular arrhythmias. Yet 30°C is the lower end of temperatures reported to be used following cardiac arrest in the comatose patient (Polderman and Herold, 2009). The experimental work showed that  $CV_l$  decreased consistently throughout cooling, however,  $CV_t$  was relatively insensitive to cooling to 31°C, but decreased on cooling further to 17°C. Therefore, this study suggested that non-linear effects on ventricular activation can be explained in terms of a low temperature sensitivity of gap junction function. To assess this, the effect of pharmacological gap junction uncoupling on  $CV_l$  and  $CV_t$  during moderate hypothermia was studied. Gap junctional uncoupling by CBX showed a greater effect on  $CV_l$  than  $CV_t$  which was not associated with a change in VF threshold. Uncoupling by heptanol showed a greater effect on  $CV_t$  than  $CV_l$  and was associated with VF threshold that had returned to baseline (37°C). These findings propose that the low temperature sensitivity of gap-junction conductance is an important pro-arrhythmic factor during moderate hypothermia. This suggests that modulation of gap junction function is a potential therapeutic target to protect patients in moderate hypothermia from arrhythmias during therapeutic as well as during accidental hypothermia.

Future work should examine this finding further. The next step would be to identify a clinically relevant inhibitor and test it *in vivo*, to assess its efficacy as a short term treatment of hypothermia. However, identification of that suitable drug is as yet unclear. CBX was selected for this work because it is an approved drug that is readily available, however it did not show the anti-arrhythmic effect of heptanol. Currently there is work examining the effect of Rotigaptide, a novel anti arrhythmic peptide which is being investigated as a potential connexin mediated pharmacological treatment (Su et al., 2015). It has been shown to prevent ischaemia-induced ventricular tachycardia (Liu et al., 2014) by

altering cell to cell coupling. However further work is required to understand whether this is an appropriate choice for the hypothermic heart.

### **Animal models**

Both rat and rabbit cardiac measurements were examined within these studies. This cross species work was due to the different nature of the experimental objectives. The studies on the inotropic response of hypothermia were based on replicating the rat model of rewarming shock developed by Tveita *et al* (University of Tromso). Therefore, it was logical to develop an *in vitro* model using the same animal. However, for the conduction studies it was more practical to use a rabbit model. The scale of these experiments, and techniques used (e.g. conduction timings, panoramic imaging) were designed for a larger heart and also the rabbit electrophysiology shares more similarities with the human heart (e.g. AP plateau phase is present in rabbits but not small rodents).

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