TEMPERATURE-RELATED DIFFERENCES IN THE CALCIUM TRANSIENT BETWEEN ATLANTIC COD (*Gadus morhua*) AND STEELHEAD TROUT (*Oncorhynchus mykiss*) CARDIOMYOCYTES

By

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Abstract

The Atlantic cod, *Gadus morhua*, differs from many teleosts in that its heart does not respond to adrenergic stimulation, and is more capable of maintaining function during acute temperature changes. To examine if differences in intracellular calcium mobilization are associated with these atypical responses, confocal microscopy was used to study the calcium handling of cardiac cells from Atlantic cod vs. steelhead trout at their acclimation temperature (10ºC), or subjected to acute temperature changes (to 4 and 16ºC), while being stimulated across a range of frequencies (10 – 110 min^{-1}). In addition, cells were tested with and without tonic (10 nM) levels of adrenaline at 10ºC, and pharmacological blockers were used to study the relative contributions of the L-type Ca^{2+} channel, sarcoplasmic reticulum and Na^{+}/Ca^{2+} exchanger to the Ca^{2+} transient. Consistent with previous *in vitro* and *in situ* studies, there were few significant effects of adrenaline on the Ca^{2+} transient of cod cardiomyocytes, yet adrenaline had significant positive inotropic effects on trout cardiomyocytes. At 10ºC, peak Ca^{2+} (F/F_0) only differed between the two species at low stimulation frequencies (10, 30 min^{-1}), with trout F/F_0 25-35% higher. In contrast, the time to peak Ca^{2+} and the time to half relaxation were both shorter (by 10 – 35% across frequencies) in cod. Acute temperature changes caused a shift in the Ca^{2+} - frequency relationship in both species, with F/F_0 values higher for trout at low frequencies (< 70 min^{-1}) at 4ºC, whereas this parameter was greater at all frequencies except 10 min^{-1} in cod at 16ºC. Unfortunately, these experiments did not highlight clear species differences in the relative contributions of the L-type Ca^{2+} channels, sarcoplasmic reticulum and Na^{+}/Ca^{2+} exchange to the Ca^{2+} transient.

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List of Abbreviations

AC Adenylate cyclase

AP Action potential

β-ARs Beta-adrenergic receptors

$[Ca^{2+}]_i$ Intracellular calcium concentration

cAMP Cyclic adenosine monophosphate

CCD Charge-coupled device

CICR Calcium-induced calcium release

cMyBP-C Cardiac myosin-binding protein C

cTnC Cardiac troponin C

cTnI Cardiac troponin I

E-C coupling Excitation-contraction coupling

F Fluorescence (peak)

f_H Heart rate

FITC Fluorescein isothiocyanate

F_0 Baseline fluorescence

F_p Post-stimulation fluorescence

Fps Frames per second

F_r Resting fluorescence (pre-stimulation)

G_s Stimulatory G protein

G_i Inhibitory G Protein

I_{Ca} Sarcolemmal calcium current

I_{KI} Background inward rectifier current

I_{KACh} ATP dependent potassium current

I_{Na} Sarcolemmal sodium current

I_{NCX} Sodium-calcium exchanger current

K_D Dissociation constant

MLC-2 Myosin light chain 2

NCX Sodium-calcium exchanger

PKA Protein kinase A

PT Peak tension

Q Cardiac output

Q_{max} Maximum cardiac output

Q_{rest} Resting cardiac output

Q_{10} Temperature coefficient

RyR Ryanodine receptor

RyTh Ryanodine & Thapsigargin treatment

SA Surface area

S.E. Standard error of the mean

SERCA Sarcoplasmic/Endoplasmic reticulum Ca^{2+}-ATPase

SR Sarcoplasmic reticulum

S_V Stroke volume

T_{crit} Critical temperature

T_d Temperature of death

THR Time to half relaxation

T_p Pejus ('getting worse') temperature

TPT Time to peak tension

TM Tropomyosin

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1. Introduction

1.1. The Effects of Temperature

1.1.1. The External Environment

As fish are ectotherms, their body temperature is largely dependent on that of the environment (Fry, 1971; Johnston & Dunn, 1987; Pörtner, 2001). Water covers roughly 71% of the Earth's surface, and contains an estimated 32,000 species of fish (Fish Base). Thus, fish are one of the most widespread and diverse groups of animals on the planet, and show a great deal of plasticity in their evolutionary history, in the ecological niches they occupy, and in the physiological mechanisms that allow them to respond to acute and chronic changes in their environment (Huff et al., 2005; Righton et al., 2010). In the wild, fish encounter both acute (e.g. daily fluctuations in rivers and tidepools, traversing isoclines) and chronic (e.g. seasonal, accelerated climate change) temperature changes. For example, Atlantic cod (*Gadus morhua*) experience temperatures ranging between -1.5°C and 19°C, and in extreme cases daily changes of up to 10°C when foraging (Righton et al., 2010). Species such as the steelhead trout (*Oncorhynchus mykiss*) remain active between 1 and 25°C (Nielsen et al., 1994; Hayes et al., 2011). However, other species can tolerate an even wider range of temperatures. The goldfish (*Carassius carassius*) inhabits waters from 1°C to 37°C (Ferreira et al., 2014), while the killifish (*Fundulus heteroclitus*) is active in waters between 2°C and 35°C (Healy & Schulte, 2012a, b; Fangue et al., 2006). The above species are considered to be eurytherms as they are capable of tolerating a wide range of temperatures. However, many species only inhabit a narrow range of temperatures (i.e. are stenothermal). Despite being widely

distributed throughout the northern hemisphere above 40°N, burbot (*Lota lota*) only inhabit waters cooler than 13°C (Edsall et al., 1993; Carl, 1995; Pääkkönen and Marjomäki, 2000). The icefish (*Pagothenia borchgrevinki*) is restricted to the Antarctic continent and only experiences temperatures ranging from -1.9 to 3°C (Deacon, 1984; Eastman, 1993).

While fish show a great deal of plasticity in their responses to temperature change, there are limits to their performance and ultimately, survival. Figure 1.1 (taken from Wang and Overgaard, 2007) relates thermal tolerance to physiology, and its consequences for aquatic organisms at both the animal and ecosystem levels. While fish may be able to survive over a wide range of temperatures (i.e. between the T_d values; temperatures of death), activity is only possible within a narrower range (i.e. between T_c [critical, T_{crit}] temperatures) and temperatures must not exceed the upper pejus ('getting worse') temperature (T_p) or fall below its lower value if the fish is to maintain its aerobic scope (the difference between resting and maximal rates of oxygen consumption). The latter parameter is critical for important life activities such as growth and reproduction, and if fish remain outside this temperature range for extended periods (for example, as a result of chronic temperature changes caused by global warming), populations are at risk of decline unless they are able to adapt. Accelerated climate change is expected to affect fish populations, not only in terms of their physiology and capability to deal with temperatures outside their optimum range, but also through changes in prey availability, and water salinity, pH, dissolved O_2 and CO_2 (Pörtner and Peck, 2010). Thus, climate change has the potential to have widespread effects on complex ecosystems (Rijnsdorp et al., 2009),

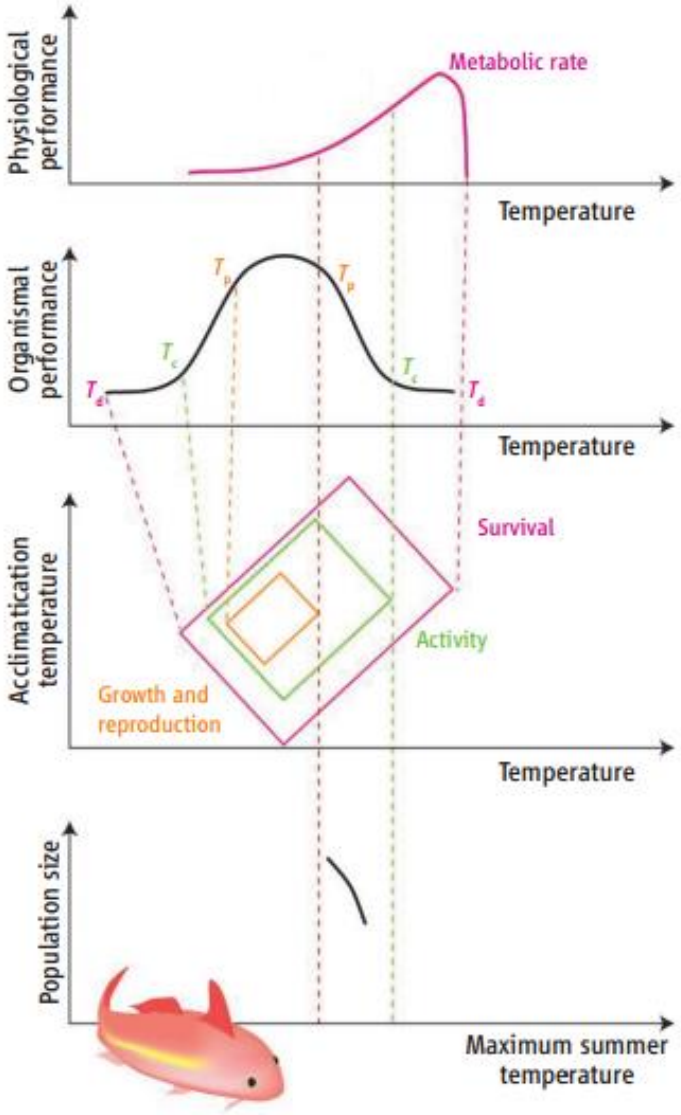

Figure 1.1. The effects of temperature on an aquatic organism's capacity to perform various life activities. Adapted from Wang and Overgaard (2007).

and to impact global fisheries. These factors make the effects of temperature a critical area of research, the findings from which will have significant implications for aquatic ecosystems, commercial fisheries and aquaculture operations.

1.1.2. The Effects of Temperature on the Fish Heart

With regards to a fish's ability to respond to temperature change, the cardiovascular system is critical as it is largely responsible for supplying the oxygen and energy substrates required to fuel metabolic processes. Some temperature-induced cardiovascular responses in fish are morphological in nature. For example, species such as the rainbow trout (Driedzic et al., 1996; Farrell et al., 1996; Aho and Vornanen, 2001), common carp (*Cyprinus carpio*) (Young & Eggington, 2011) and eel (*Anguilla anguilla*) (Methling et al., 2012) normally respond to chronic decreases in temperature by increasing ventricular mass (cardiac hypertrophy). Rainbow trout and zebrafish (*Danio rerio*) increase the proportion of the heart composed of spongy myocardium during cold acclimation (Klaiman et al., 2011; Johnson et al., 2014), whereas the amount of compact myocardium increases with warm acclimation (Klaiman et al., 2011). Finally, the collagen content of the myocardium has been shown to decrease with cold acclimation in zebrafish (Johnson et al., 2014), whereas mitochondrial densities increase (Driedzic et al., 1996; St. Pierre et al., 1998).

However, most of what we know about the response of the cardiovascular system to temperature change is related to cardiac function. For example, a rise in temperature causes concomitant increases in both metabolic demand and cardiac output (Q) (Brett,

1971; Brodeur et al., 2001). The difference between resting cardiac output (Q_{rest}) and maximum cardiac output (Q_{max}) dictates a fish's ability to deal with physiological demands, such as exercise, digestion, growth, etc., and is known as cardiac scope (see Figure 1.2a). As acclimation temperature increases, Q_{rest} increases in a fairly linear fashion to support the elevation in metabolic demand associated with warmer temperatures, and continues to increase until the fish's upper thermal limit is reached. In contrast, Q_{max} becomes limited, and then decreases, well before the upper thermal limit is reached. This response in Q_{max} largely reflects the decrease in stroke volume (S_V) that is observed at high temperatures (Figure 1.2b).

When acutely warmed, salmonids increase cardiac output solely by increasing heart rate (f_H) (Sandblom and Axelsson, 2007; Clark et al., 2008; Steinhausen et al., 2008) (Figure 1.2c) until the critical temperature T_{crit}, where aerobic scope is zero and arrhythmias occur (Anttila et al., 2014). This response is seen not only in whole animal studies, but also in isolated heart preparations (Farrell et al., 1996; Keen and Farrell, 1994), providing further evidence that the heart may be a primary factor influencing fish survival and performance when approaching T_{crit} (Farrell, 2009; Anttila et al., 2014). A key factor in determining heart rate in fishes is the intrinsic pacemaker rhythm, set by spontaneous discharges from pacemaker cells located near the sino-atrial junction (Haverinen & Vornanen, 2007). Pacemaker cell rhythm is modulated in part by temperature (Harper et al., 1995), but is also influenced by thermal history. Acclimation to cold temperatures results in an increase in the intrinsic beating rate of pacemaker cells (Haverinen & Vornanen, 2007), but these fish have a lower thermal tolerance and cannot maintain higher heart rates when

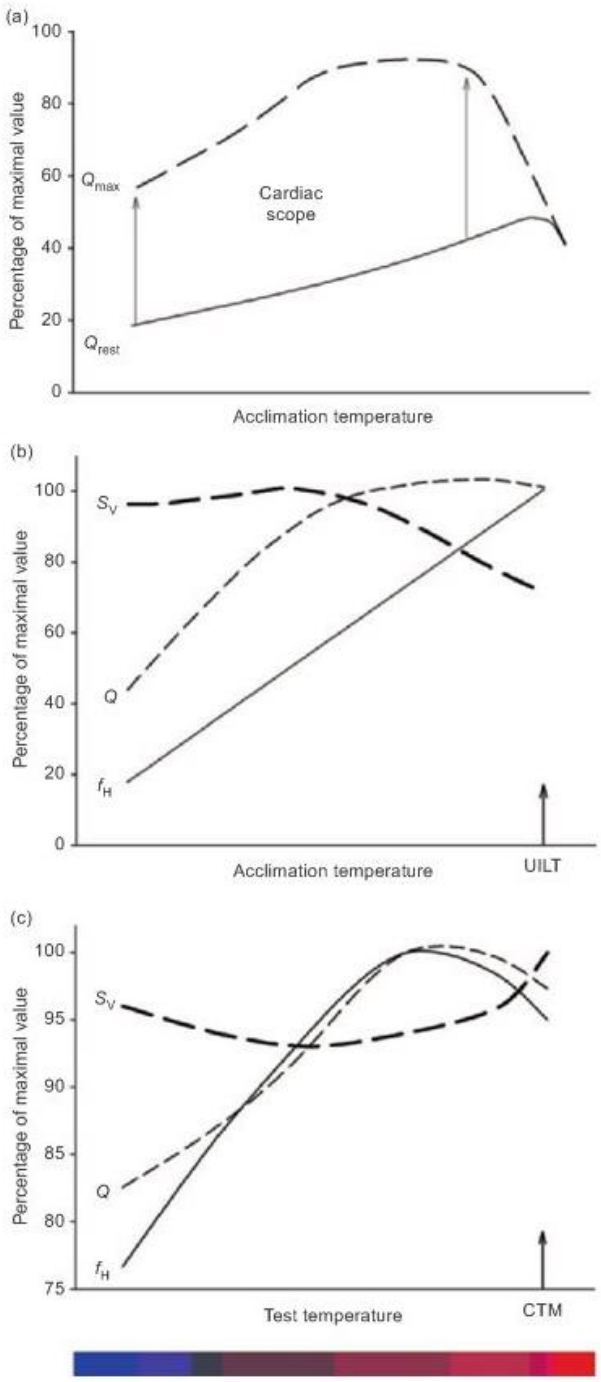

Figure 1.2. Schematic representation of the effects of acclimation temperature on cardiac scope (a), and the effects of acclimation temperature (b) and acute temperature changes (c) on stroke volume (S_V), heart rate (f_H) and cardiac output (Q) (Gamperl, 2011).

temperatures rise (see Figure 1.3). Acclimation to warm temperatures resets the pacemaker to a lower rate (Graham & Farrell, 1989; Haverinen & Vornanen, 2007) (Figure 1.3), and thus, fish are generally able to tolerate a wider thermal range and achieve higher heart rates. For example, Atlantic salmon acclimated to 12°C have a maximum heart rate of 150 beats per minute at their T_{crit} (critical temperature) of 21-23°C, yet when acclimated to 20°C their T_{crit} increases to 27.5°C and they have a maximal heart rate of 200 beats min^{-1} (Anttila et al., 2014).

Why most fish do not increase S_V when faced with a rise in temperature (Figures 1.2b, 1.4) is still being debated. During exercise, which also involves an increase in heart rate of 25 - 30% (Thorarensen et al., 1996; Petersen & Gamperl, 2010), fish are capable of increasing stroke volume by 20 – 50% (Eliason et al., 2013). Further, Keen and Gamperl (2012) showed that fish are indeed capable of increasing stroke volume and Q when acutely warmed if increases in heart rate are limited by the bradycardic pharmacological agent zatebradine. It is possible that the limitation in stroke volume as temperature increases is due to the heart being unable to eject blood effectively. However, it has been shown (in salmonids) that end-systolic volume is essentially zero (Franklin & Davie, 1992b), which makes this unlikely. An alternative theory is that stroke volume may be limited due to an inability to fill the heart, i.e. a lower end-diastolic volume. Initially, it was suggested that this might be due to decreased venous pressure preventing the heart from filling (Farrell, 2007), and/or less time available for filling as f_H increases. However, it appears that the former cannot explain the observed decrease in S_V when temperature increases acutely. Sandblom & Axelsson (2006) showed that venous pressure

actually increases slightly with temperature. More recently it has been suggested that end-diastolic volume may be lower due to increased resting tension of the myocardium (due to elevated cellular Ca^{2+}) that prevents the heart from fully relaxing, and thus, making it more difficult to fill with blood. Indeed, the amount of work required to stretch Atlantic cod muscle strips (termed 'negative work') increases considerably when they are exposed to the combination of high temperatures and pacing frequencies (Syme et al., 2013).

While many species exhibit similar responses to increased temperature, there is significant variation in how fish cardiac function responds to colder temperatures. One example is within the carp family; the crucian carp *Carassius carassius* is a cold-active species capable of maintaining cardiac output during periods of cold (8°C) and anoxia, whereas its relative the common carp *Cyprinus carpio* (a cold-dormant species) survives just 24 hours under similar conditions (Stecyk et al., 2004). Further, a recent study by Lurman et al. (2012) showed that *in situ* Atlantic cod heart preparations are surprisingly effective at maintaining function at cold temperatures. When Atlantic cod were acclimated to temperatures of 10, 4 and 0°C, there was no significant difference in maximum *in situ* cardiac power output whether they were tested at their acclimation temperature or acutely cooled from 10 to 4°C or 4 to 0°C. In addition, when acutely warmed from 4 to 10°C maximum cardiac output and maximum power output were twice as high as values measured in 10°C acclimated fish. This study suggests that cod hearts do not suffer the negative effects of cooling in the same way as other teleosts, and show adaptations to cold temperatures. For example, the sea raven *Hemitripterus americanus* experiences a 40-45% decrease in Q_{max} when faced with an acute decrease in temperature

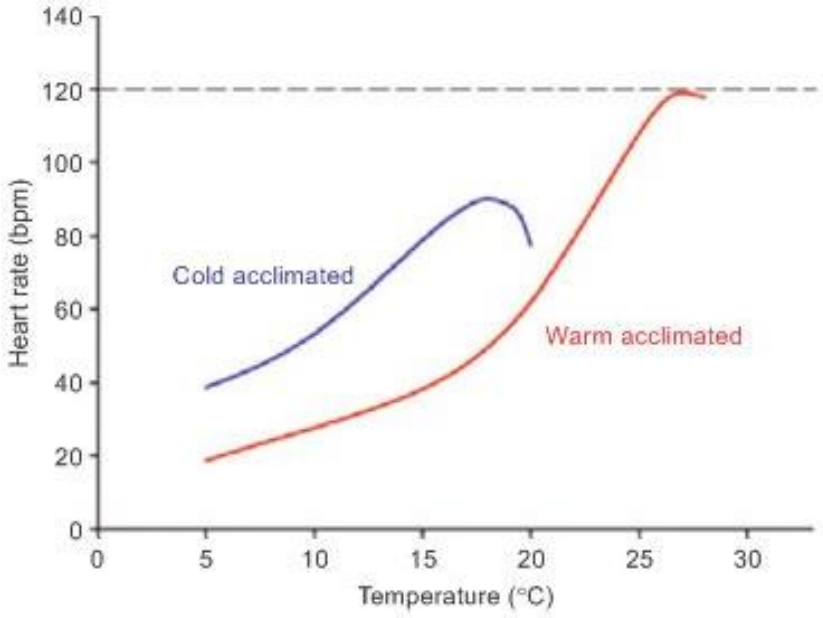

Figure 1.3. The effects of thermal acclimation on heart rate in fish experiencing an acute temperature increase (Gamperl, 2011).

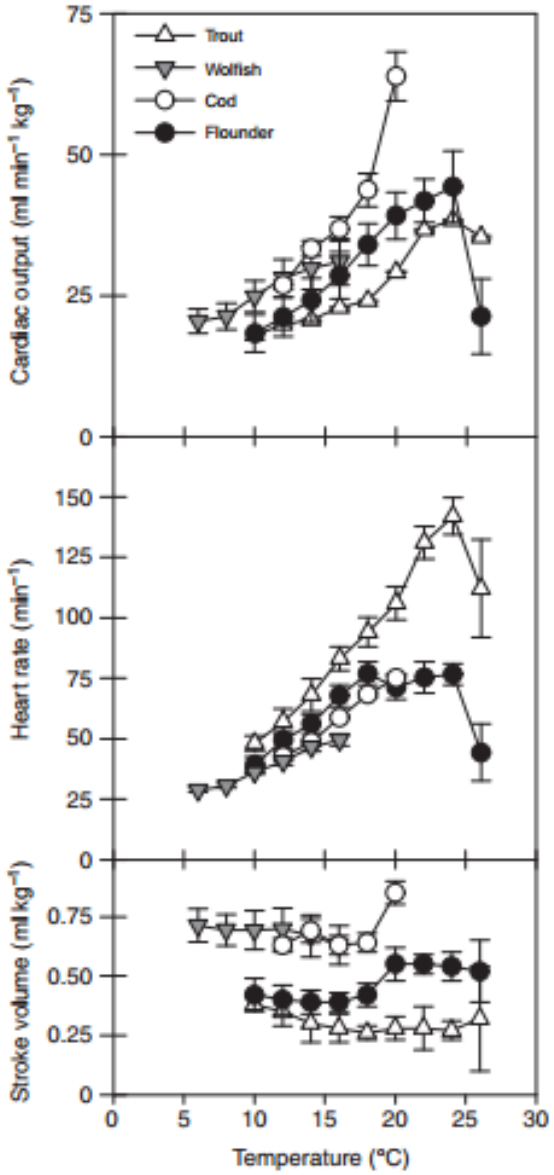

Figure 1.4. Cardiorespiratory variables in resting fish when exposed to an acute increase in temperature. Taken from Farrell (2009), data provided by Dr. Kurt Gamperl.

(from 13°C to 3.3°C) (Graham & Farrell, 1985), 4°C acclimated rainbow trout hearts experience a 60% decrease in maximum contractile force when acutely warmed to 10°C (Aho & Vornanen, 2001), and even the burbot experiences a decrease in maximum isometric tension when acutely warmed (Tiitu & Vornanen, 2002). It has been suggested that changes in calcium handling allow some species to deal with colder temperatures more effectively than other teleosts. Studies at the cellular level have shown both longer action potentials (Haverinen & Vornanen, 2009a; Galli et al., 2009) and an augmentation of I_{Na} (sarcolemmal Na^+ current) (Haverinen & Vornanen, 2004) in cold-tolerant species, both of which increase the contributions of the NCX (sodium-calcium exchanger) to calcium entry into the myocyte. In addition, the cold-active burbot is capable of maintaining cardiac function due to high Ca^{2+} contributions from the sarcoplasmic reticulum and NCX at cold temperatures (Tiitu & Vornanen, 2002), and the Pacific bluefin tuna (*Thunnus orientalis*) is able to retain pumping ability during cold dives across thermoclines due to high SR Ca^{2+}-ATPase activity (Blank et al., 2004). It is possible that Atlantic cod also rely on SR-Ca^{2+} contributions, as ryanodine (a blocker of SR-Ca^{2+} release) causes a shift from a flat or positive force-frequency relationship to a negative force frequency relationship (Driedzic & Gesser, 1988).

1.2. Calcium's Role in Myocardial Contraction

1.2.1. The Calcium Transient

Excitation-contraction coupling (E-C coupling) begins with depolarization of the sarcolemmal membrane due to the arrival of an action potential (AP), and results in the rise in cytosolic Ca^{2+}. When Ca^{2+} rises during contraction, it binds to troponin C (cTnC)

pulling troponin I (cTnI) away from the actin filament, and this shifts tropomyosin (TM) away from its binding site on the actin filament allowing cross bridges to form. This process ultimately leads to the shortening of the myofilaments (Sandow, 1952), and thus, muscle contraction. For the muscle to relax, however, this same Ca^{2+} must now be removed from the cytoplasm. This transient rise and fall of Ca^{2+} is known as the calcium transient, the duration and amplitude of which determines the speed and force of muscle contraction, respectively, and the magnitude of the transient is used as an index of force development (Yue, 1987).

1.2.2. Routes of Calcium Entry/Exit and the Effects of Temperature

In fish, there are three major routes by which calcium may enter and/or exit the cardiomyocyte (Figure 1.5). L-type Ca^{2+} channels are voltage-dependent calcium channels located on the sarcolemma that open shortly after the action potential reaches the cell's surface, allowing entry of Ca^{2+} into the myocyte. Ca^{2+} is then released from the sarcoplasmic reticulum via calcium-induced calcium release (CICR), whereby increased levels of Ca^{2+} in the cytosol activate ryanodine receptors on the SR to release Ca^{2+} into the cytoplasm (Fabiato, 1983; Bers, 2002).

The SR is also involved in the removal of Ca^{2+} during relaxation as it has a high density of SR-Ca^{2+} ATPase pumps. Finally, although the NCX normally removes Ca^{2+} from the myocyte in exchange for Na^{+}, increased action potential duration can allow the NCX to operate in 'reverse mode', whereby it brings Ca^{2+} into the myocyte in exchange

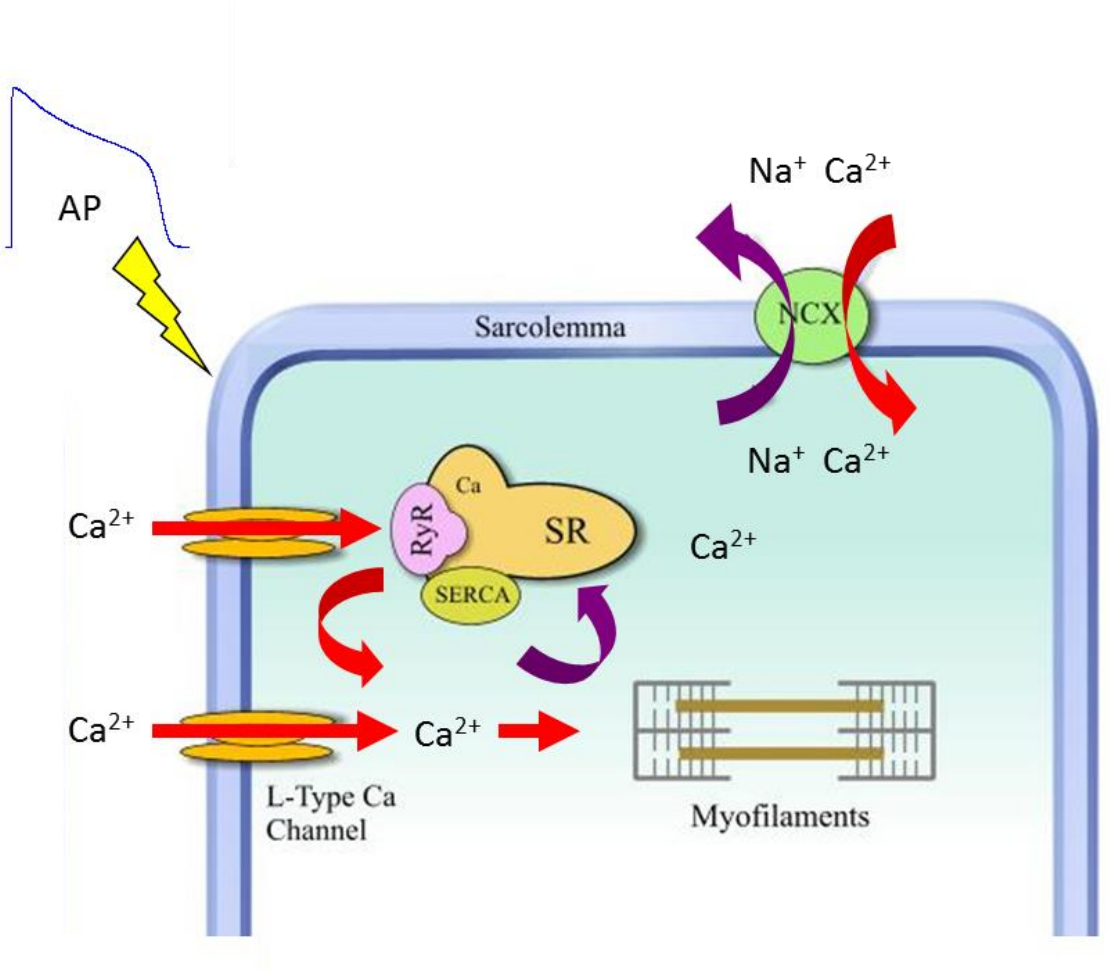

Figure 1.5. Schematic showing routes of calcium entry/exit in the fish cardiomyocyte including the roles of L-type Ca^{2+} channels, the sarcoplasmic reticulum (SR) the ryanodine receptor (RyR), SERCA (Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase) and sodium-calcium exchanger (NCX). Figure modified from Gamperl & Shiels, 2014.

for Na^+. While L-type Ca^{2+} channels, SR and NCX are found in all species of fish, there are considerable inter-specific differences in the relative contributions of these three Ca^{2+} sources to the overall calcium transient (Aho and Vornanen, 1999; Tiitu and Vornanen, 2001).

Due to the large surface area to volume ratio of fish cardiomyocytes (see Figure 1.6), sarcolemmal Ca^{2+} entry is generally thought to be more important for myocardial contraction in fishes (Tibbits et al., 1992; Shiels & White, 2005). While L-type Ca^{2+} channels are thought to be the major source of sarcolemmal Ca^{2+} in fish, binding studies have shown a lower L-type Ca^{2+} channel density in burbot (*Lota lota*) than in rainbow trout or crucian carp (Tiitu and Vornanen, 2003). This has led to the suggestion that burbot may rely more upon Ca^{2+} contributions from the SR or NCX than other species.

Of the species studied, tuna appear to have the largest SR contributions to intracellular Ca^{2+} levels. In this taxa, the SR contribution is approx. 35% of the increase in cytosolic Ca^{2+} (Keen et al., 1992; Shiels et al., 1999; Shiels and Farrell, 2000; Landeira-Fernandez et al., 2007) compared to other species such as the rainbow trout and crucian carp where it is approx. 13% and < 5 % of the Ca^{2+} increase, respectively (Haverinen & Vornanen, 2009b). The amount of Ca^{2+} contributed by the SR is also affected by temperature (Keen et al., 1994; Shiels and Farrell, 1997; Shiels et al., 2002a; Galli et al., 2009). For example, cold-acclimated rainbow trout rely less upon sarcolemmal Ca^{2+} transport via L-type Ca^{2+} channels, and more upon SR Ca^{2+} release, than warm-acclimated rainbow trout (Vornanen, 1998). Further, bigeye tuna *Thunnus obesus* and mahi mahi *Coryphaena hippurus* rely more on SR-based Ca^{2+} when faced

with an acute decrease in temperature, allowing them to maintain contractility when traversing isoclines (Galli et al., 2009). In many species, SERCA (sarco/endoplasmic reticulum Ca^{2+} ATP-ase) plays a major role in Ca^{2+} removal, and has also been shown to be temperature dependent, with increased contributions of the SR to Ca^{2+} removal at low temperatures (Coyne et al., 2000; Galli et al., 2009; Tiitu and Vornanen, 2001). An exception to this appears to be the crucian carp, a cold-dormant species, whose rate of SR-Ca^{2+} uptake decreases after cold acclimation (Aho and Vornanen, 1998).

There are a number of reasons for the temperature dependence of SR function, and varying SR contributions to the Ca^{2+} transient between species. The first relates to differences in SR load (the amount of Ca^{2+} stored within the SR). Cold acclimation increases SR load in burbot (although its load remains low compared to other species), however, it has no effect on rainbow trout or crucian carp SR load (Haverinen and Vornanen, 2009b). Second, while most fish have a relatively high SR load (Hove-Madsen et al., 1999; Shiels et al., 2002a), they do not utilise it under normal conditions, making differences in SR load a questionable explanation for variations in SR-Ca^{2+} contributions (Moller-Nielson & Gesser, 1992; Aho & Vornanen, 1999; Harwood et al., 2000; Shiels & White, 2005). For example, an eight-fold increase in contractile strength is seen in rainbow trout when exposed to caffeine (Coyne et al., 2000), which opens RyRs (ryanodine receptors) and releases all Ca^{2+} from the SR. Another potential explanation is differences in RyR density – more RyRs on the SR allow it to be activated via CICR more easily and cytoplasmic [Ca^{2+}] to rise more quickly. Indeed, rainbow trout have been shown to have a higher RyR density than both burbot and crucian carp (Haverinen &

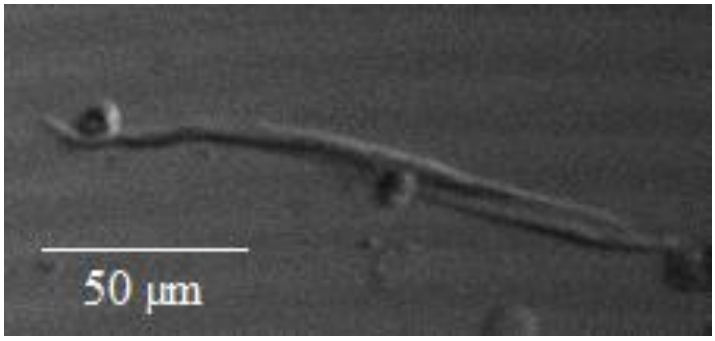

Figure 1.6. Photograph of a steelhead trout cardiomyocyte in 2 mM Ca^{2+} solution at 10°C, taken with an Olympus confocal microscope at 100x magnification. Note the long, thin, spindle shape giving the myocyte a high surface area to volume ratio (present study).

Vornanen, 2009b), and cold acclimation increases RyR density in rainbow trout, but not crucian carp (Haverinen & Vornanen, 2009b). Taking burbot as an example, one might ask the question: if cardiomyocytes of this species have lower SR Ca^{2+} load, and lower RyR densities than other species, how can the burbot still rely on the SR more than other species? The answer may be RyR Ca^{2+} sensitivity, in that less cytosolic Ca^{2+} is required to stimulate CICR. The burbot has been shown to have a higher RyR Ca^{2+} sensitivity than rainbow trout or crucian carp (Vornanen; 2006; Haverinen & Vornanen, 2009b).

In the case of the burbot, the low L-type Ca^{2+} channel density (Tiitu & Vornanen, 2003) has also lead researchers to believe that alternative routes are used for calcium entry. Aside from the SR, the NCX may also play a role in this process. Thus, studies are beginning to explore the importance of the NCX's contributions to the calcium transient. Under normal conditions, the NCX is particularly important in Ca^{2+} removal from the cardiomyocyte, where it is the major efflux pathway (Hume and Uehara, 1986). However, increased AP duration allows for the influx of Ca^{2+} when operating in "reverse-mode", and blocking the NCX with the drug KB-R7943 caused a decrease in peak tension (~ 50% at 10°C) in ventricle tissue strips from the eel (*Anguilla anguilla*) (Methling et al., 2012). This latter research suggests that, in this species, the NCX operates in reverse mode, contributing to calcium influx. This is also seen in crucian carp, where approximately one-third to one-half of total Ca^{2+} influx is through the NCX (Vornanen, 1999), and it has been proposed that a lower peak NCX current at cold temperatures (4ºC) is compensated for by an extended action potential, allowing more time for Ca^{2+} entry (Vornanen, 1999). Finally, the NCX is the primary source of Ca^{2+} at 4°C in the

cold, stenothermic, burbot (Shiels et al., 2006), with I_{NCX} approximately double in burbot as compared to crucian carp (Vornanen, 1999; Shiels et al., 2006). Interestingly, while the burbot I_{NCX} exhibits a high Q_{10} (close to mammalian) of 2.47 between 4 and 11°C (Shiels et al., 2006), its activity is still higher than other species at low temperatures. This latter point suggests that the NCX is particularly important in cold conditions.

1.3. Adrenergic Stimulation of the Heart

Contractility of the heart is modulated by adrenergic stimulation - the binding of circulating or neuronally-released catecholamines (adrenaline and noradrenaline) to adrenoreceptors on the cardiomyocyte cell surface (Chen-Izu et al., 2000). The major group of cardiac adrenoreceptors found in fish are β-adrenoreceptors (β-ARs), of which there are three subtypes: β_1, β_2 and β_3. β_1 and β_2-ARs are coupled to G-stimulatory (G_s) proteins, and activate adenylyl cyclase (AC). This leads to increased levels of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA), which in turn phosphorylates L-type Ca^{2+} channels and results in greater calcium entry into the cell (Vornanen, 1998; Chen-Izu et al., 2000; Ballesta et al., 2012) by increasing the peak L-type Ca^{2+} channel current (I_{Ca}) (Shiels et al., 2003). This adrenaline-mediated increase in contractility is observed in species such as rainbow trout (Farrell et al., 1986; Keen et al., 1993; Farrell et al., 1996) and tuna (Galli et al., 2009). However, the responses to adrenaline vary between species.

The rainbow trout heart shows more reliance on β-adrenergic stimulation than that of the crucian carp (Vornanen, 1998), and the hearts of flounder (*Pleuronectes*

americanus; Mendonca & Gamperl., 2009), sea bass (*Dicentrarchus labrax*; Farrell et al., 2007), tilapia (*Oreochromis* sp.; Lague et al., 2012) and Atlantic cod (Lurman et al., 2012) exhibit little to no response to adrenaline. There are several explanations for these species' lack of response to adrenaline. β-adrenergic receptor density, and affinity for adrenaline, varies between species (Olsson et al., 2000), and there may be fewer cardiac adrenergic receptors in these species. Alternatively, they may have a lower affinity for adrenaline, or differences in the G-protein regulated biochemical cascade. In addition, there may be differences in the abundance of β3 adrenergic receptors, which play a 'protective' role against β1- and β2- overstimulation in rainbow trout (Nikinmaa, 2003; Nickerson et al., 2003), and have also been identified in the eel (Imbrogno et al., 2006). Another alternate explanation for this observation may be differences in calcium handling. These latter species may be more reliant upon Ca^{2+} influx via the NCX than adrenaline-sensitive channels such as L-type Ca^{2+} channels and the SR (Lurman et al., 2012). This would explain why perfused heart preparations from fish such as Atlantic cod can pump at resting and maximum levels without adrenergic stimulation (Lurman et al., 2012), while rainbow trout require a tonic level of adrenergic stimulation (in the nM range) to even generate resting cardiac output (Graham & Farrell, 1989; Shiels et al., 2003).

Adrenaline is particularly important at cold temperatures (Keen et al., 1993; Shiels et al., 2003), as its positive inotropic effects allow fish to counter the negative impacts of cold on myocardial contractility. For instance, while the South American lungfish, *Lepidosiren paradoxa*, suffers from limited Ca^{2+} availability at the lower end of its

thermal range (15°C vs 25°C), adrenaline is able to restore cardiac performance (Costa et al., 2004). Rainbow trout acclimated to 4°C show a greater contractile response to adrenaline than those acclimated to 17°C (Aho & Vornanen, 2001), and those acclimated to 8°C vs. 18°C are approximately 10 times more sensitive to adrenaline (Keen et al., 1993). In the Keen study (1993), increased adrenergic sensitivity was explained by an up-regulation of β-adrenergic receptor density with cold acclimation, whereas acute temperature changes had no effect on adrenergic sensitivity.

In contrast to its inotropic effects, adrenergic stimulation is not thought to have time-dependent effects on the calcium transient in fishes. In a study of two Antarctic teleosts, the icefish *Chaenocephalus aceratus* which lacks haemoglobin and the red-blooded *Notothenia coriiceps*, there was an increase in contractile force with adrenaline, however no change in contractile kinetics (time to peak / time to half relaxation) was observed (Skov et al., 2008). Similarly, adrenaline appears to have no chronotropic effects on rainbow trout at physiological heart rates (Graham & Farrell, 1989; Shiels & Farrell, 1997).

1.4. Rationale for Study

A great deal of research into the effects of chronic and acute temperature change has been carried out in the rainbow trout (Farrell et al., 1996; Shiels et al., 2002a, b; Klaiman et al., 2011; Klaiman et al., 2014), and as such, this species provides a good model for studying the cardiomyocyte's response to changes in this important environmental variable. However, as previously mentioned, there are large differences between fish species in

their cardiac responses to acute temperature changes and adrenergic stimulation (Driedzic and Gesser, 1988; Lurman et al., 2012). It is quite possible that variations in the source of [Ca^{2+}_i] (e.g. NCX, SR, L-type Ca^{2+} channels) contribute to, and/or, underlie these differences. For example, Lurman et al. (2012) suggested that an enhancement of sarcolemmal Na^+ current (I_{Na}) may augment Ca^{2+} influx through Na^+/Ca^{2+} exchange (Haverinen and Vornanen, 2004), or that aspects of sarcoplasmic reticulum (SR) function may play a major role in enabling the *in situ* Atlantic cod heart to maintain performance over a range of temperatures and to elevate performance when exposed to an acute increase in temperature. Investigating the effects of temperature, contraction frequency, and adrenergic stimulation at the cellular level will provide detailed information about the calcium transient and whether it differs between the two species. In this thesis, steelhead trout were used to compare the Ca^{2+} transient and other parameters with Atlantic cod. These fish are rainbow trout that have been acclimated to seawater.

1.5. Research Objectives

Compare the morphology of myocytes from the spongy myocardium of Atlantic cod and steelhead trout ventricles. The spindle shape of fish myocytes creates a large surface area to volume ratio, and generally allows trans-sarcolemmal Ca^{2+} entry to be the primary source of calcium for contraction in fishes. Morphological differences in steelhead trout vs. Atlantic cod myocytes could influence the potential contribution of extracellular calcium to the [Ca^{2+}_i] transient, and thus, mediate differences in cardiac

function between the two species. Thus, I will measure the length, width, surface area and volume of cardiomyocytes from the ventricle of the steelhead trout and Atlantic cod.

Examine the contribution of adrenergic stimulation to the [Ca^{2+}_i] transient in both species, and how it varies with respect to contraction frequency (10 to 110 min^{-1}; see below). Such work is required to further establish that changes in the [Ca^{2+}_i] transient in Atlantic cod are not dependent on adrenergic stimulation, and that the lack of adrenergic sensitivity of the Atlantic cod myocardium / heart is not frequency dependent. At present, all fish studies that show a lack of or a diminished cardiac response to adrenaline have been conducted at resting (intrinsic) heart rates (Vornanen, 1998; Farrell et al., 2007; Lague et al., 2012; Lurman et al., 2012), and it is possible that the importance of adrenergic stimulation to cardiac function (the calcium transient) in Atlantic cod may become evident at high contraction frequencies (i.e. those typical of elevated cardiac performance associated with exercise or exposure to elevated temperatures) (Farrell et al., 1996).

Compare the magnitude and dynamics of the calcium transient in steelhead trout vs. Atlantic cod at 4, 10 and 16°C. This will allow for a cellular level analysis of the possible contributions of calcium to cardiac performance at the acclimation temperature of 10°C vs. those after acute decreases and increases in temperature. Cells will be stimulated at contraction frequencies of 10, 30, 50, 70, 90 and 110 min^{-1} [i.e. those that span the range of *in vivo* heart rates for both species: rainbow trout max. approx. 120

beats min^{-1} (Keen & Gamperl, 2012); Atlantic cod ~ 70 beats min^{-1} (Gollock et al., 2006)], and thus, I will be able to examine the relationship between stimulation frequency and characteristics of the Ca^{2+} transient (i.e. time to peak calcium and time to half relaxation) at each temperature.

Examine the relative contributions of the NCX, SR and L-type Ca^{2+} channels to the overall calcium transient in both species. After the calcium-frequency relationships are established for each species, the potential contributions of these mechanisms of Ca^{2+} entry into the cell will be investigated using pharmacological blockers (ryanodine and thapsigargin to block SR-Ca^{2+} release and re-uptake; verapamil to block L-type Ca^{2+} channels; and KB-R7943 to block reverse-mode NCX). This work was conducted at the fish's acclimation temperature (10°C) and at 50 stimulation min^{-1} where clear species differences in the $[Ca^{2+}_i]$ transient exist (see Results).

2. Materials and Methods

All procedures involving the fish were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol 12-40-KG) and performed in accordance with the guidelines of the Canadian Council on Animal Care.

2.1. Fish Husbandry

Steelhead trout (*Oncorhynchus mykiss*) (653.4 $\pm$ 67.7 g) and Atlantic cod (*Gadus morhua*) (512.9 $\pm$ 34.4 g) were housed in 3000 - 6000 L fibreglass tanks supplied with

aerated seawater at 10°C and a 12h light: 12h dark photoperiod, and fed commercial pellets (Atlantic cod, Skretting, Europa; steelhead trout, Skretting Optiline) at a ration of 1.5% body weight every 2 days. The fish were acclimated to these conditions for over 2 months before experiments began.

2.2. Myocyte Isolation

After killing the fish with a blow to the head (cerebral percussion), hearts from both species were immediately removed, weighed and placed in nominally Ca^{2+}-free isolation solution containing (in mM): 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 30 Taurine, 20 glucose, 10 HEPES.

To obtain cardiomyocytes, whole hearts were initially digested using retrograde perfusion on a modified Langendorf apparatus at 10°C. Hearts were then perfused with nominally Ca^{2+}-free isolation solution for 8 min., followed by 12-15 min. (for Atlantic cod) or 12 min. (for steelhead trout) of enzymatic digestion using 0.75 mg ml^{-1} collagenase Type IA, 0.75 mg ml^{-1} BSA, and 0.5 mg ml^{-1} Trypsin Type IX.S (Sigma-Aldrich, Co., Oakville, ON, Canada). At this point, the Atlantic cod ventricle was removed, placed in fresh isolation solution and cut into small (< 1 mm) pieces. In contrast, the steelhead trout ventricle was cut in half and the compact myocardial layer removed by careful dissection. The spongy myocardium was then placed in fresh isolation solution and cut into small (<1 mm) pieces. Finally, both preparations were gently agitated to increase the yield of free myocytes, and a sample of cells from each preparation was stained with Trypan blue to confirm that cell viability was high (greater

than 90%). Cells were kept on ice prior to experimentation, and used within a maximum of 8 hours after isolation.

2.3. Research Methods

2.3.1. Morphology of Steelhead Trout and Atlantic Cod Myocytes

Still images were taken during measurements of intracellular calcium levels using a motorized, inverted, Olympus IX81 microscope (Olympus Canada Inc. Richmond Hill, ON) fitted with 10x and 60x objectives, a high sensitivity CCD camera (Rolera MGI plus, Q Imaging Systems, Surrey, BC) and a desktop computer running Mag Biosystems (Santa Fe, USA) Advanced Capture software. Myocyte length, and width at 5 locations, were measured for cells of both species at 10^{o}C using Image J software (National Institutes of Health, USA), and then cell volume and surface area were calculated as described in Vornanen (1996). Cell volume was calculated from the length and width using the equation:

$$V = \pi * a * b * l$$

Where a and b are the short and long radii of an ellipse, and l is the length of the cell. a and b are derived from the width measurements of the cell, where a = average width / 6 and b = average width / 2.

Cell surface area was calculated as the sum of the surface area of a cylinder (A_1) and its ends (A_2).

Surface area of the cylinder was calculated as:

$$A_1 = P * l$$

While the perimeter of the ellipse (P) was approximated from the equation,

$$P = 2\pi * \sqrt{1/2(a^2 + b^2)}$$

and the surface area of the ends was calculated as:

$$A_2 = \pi * a * b$$

2.3.2. The Effect of Temperature and Adrenaline on Calcium Transients

The isolated cells were diluted by a factor of 10 [100 µl of cell suspension added to 900 µl of working solution containing (in mM): 155 NaCl, 5 KCl, 2 $CaCl_2.2H_2O$, 2 $MgSO_4.7H_2O$, 10 HEPES, 1 NaH_2PO_4, 5 glucose, 5 pyruvate]. The cells were then loaded with 4 µM Fluo-4-AM (Life Technologies, NY, USA) for a period of 15 min. in darkness at room temperature, and placed into the cell bath on the microscope for a further 5 min. to allow the cells to settle to the bottom of the bath (total loading time 20 min.). Cells were then bathed and rinsed with working solution (at 4°C, 10°C or 16°C) for a further 15 min. to: 1) rinse away excess Fluo-4 AM, and to allow for intracellular de-esterification of the Fluo-4-AM into the active probe Fluo-4; and 2) for adjustment of the cells to the test temperature.

Ca^{2+} fluorescence measurements were made using a confocal microscope [a motorized, inverted, Olympus IX81 microscope combined with a spinning disk unit (Yokogawa, Tokyo, Japan), and high sensitivity CCD camera (Rolera MGI plus)]. The Fluo-4 fluorescence was excited at 488 nm by a laser diode (Mag Biosystems) and emission was collected at 512 nm using a FITC set of filters. Full resolution (512 x 512

pixels) images were sampled at 30 frames per second (fps) and stored in a Metamorph operated PC (Figure 2.1).

During measurements, cells were continuously perfused with oxygenated working solution at 4°C, 10°C or 16°C, while they were subjected to field stimulation frequencies of 10, 30, 50, 70, 90 and 110 events min^{-1} (one frequency per cell). Stimulating electrodes were placed on either side of each cell using a micromanipulator, ensuring that only one cell was stimulated during each recording. This ensured that recordings performed on subsequent cells were not influenced by prior stimulation. The stimulation was delivered as square pulses of 2 ms duration, and with amplitude set at 10% above the cell activation threshold (15-30 V in all experiments). Cells were stimulated 10 times before recordings to allow for optimal Ca^{2+} loading in the selected cell, and to obtain uniform initial Ca^{2+} conditions among cells used in the experiment. Then a series of 300 frames (at 30 fps) were sampled to record local variations in Ca^{2+} dependent fluorescence in the cell (Figure 2.2). One series was collected per cell and one frequency was tested per cell. This protocol was used to minimize the known photo-oxidation effects associated with long illumination times. Each cell provided one video for analysis, with 1-15 stimulations per video depending on stimulation frequency. Stacks of 300 frames were converted into ratio images (F/F_0) by dividing the stack (F values) by a reference image (F_0) The area of interest within the myocyte that was used to extract numerical Ca^{2+} transient data was selected based on a number of criteria: 1) it was as an area with little intracellular variation in fluorescence (avoiding aggregations of Ca^{2+} within the cell); 2) it was

contracting freely; and 3) remained within the plane of focus during contraction (Figure 2.3).

Additional experiments were carried out for each species at 10°C, both in the presence of adrenaline hydrochloride (10 nM; Sigma) and in its absence. Adrenaline was added to the working solution perfusing the bath, and replenished after 20 min if required to avoid photo-oxidation of adrenaline in the solution. Finally, in some experiments, Ca^{2+} measurements were also taken prior to myocyte stimulation (i.e. to estimate resting Ca^{2+} levels; F_r), for 30 frames after the first 10 contractions (as above), and 7 seconds after stimulation had stopped; F_p). This allowed me to assess how baseline Ca^{2+} levels changed from resting conditions vs. between stimulations (i.e resting vs. F_0) in the two species, and how resting levels pre- and post-stimulation/excitation compared at the various frequencies (Figure 2.2).

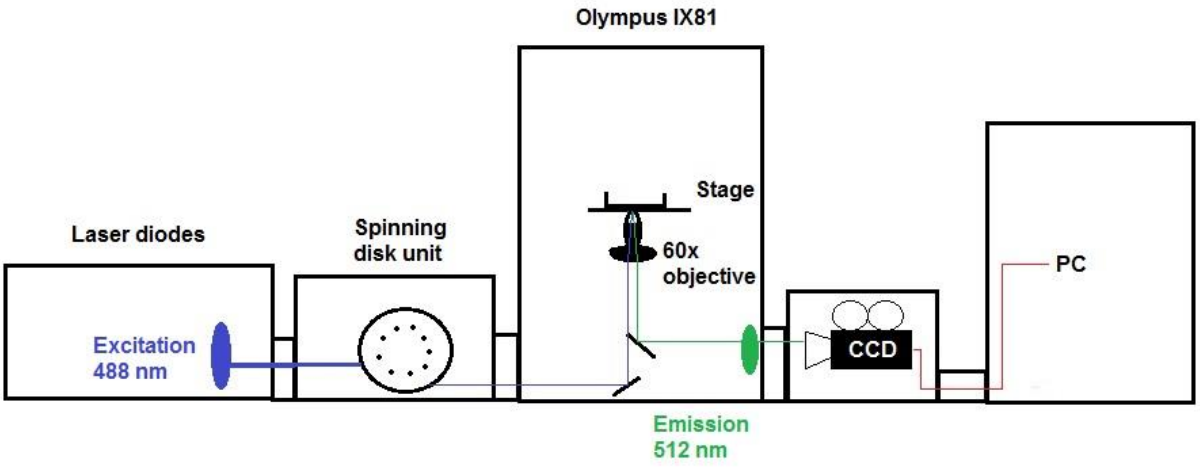

Figure 2.1. Diagram of confocal microscope setup used for fluorescent calcium imaging of fish cardiomyocytes. Fluo-4 fluorescence was excited at 488 nm by a laser diode (blue) and emission was collected at 512 nm using a FITC set of filters (green). Images were recorded with a high-sensitivity CCD camera and stored in a Metamorph operated PC (see red line).

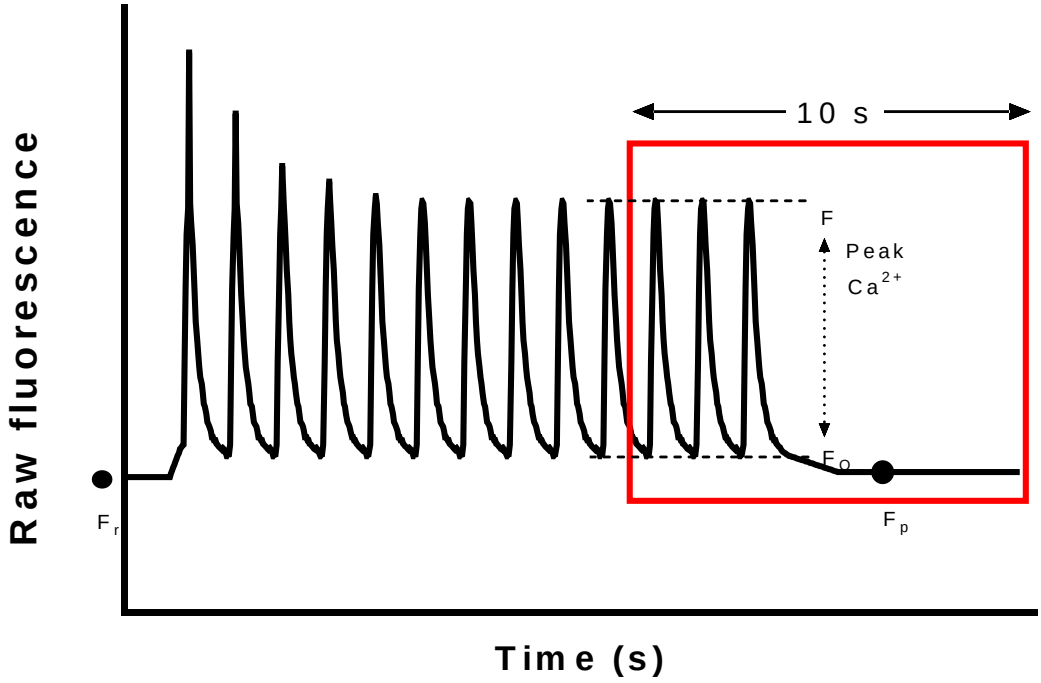

Figure 2.2. Schematic representation of the train of stimulations given to each cell to allow Ca^{2+} levels to reach a steady state, followed by a recording period of 10s. In this example, the recording period is shown in red, and the cell is stimulated 3 times, the stimulation is stopped, and the cell is allowed to recover. Abbreviations given are: F_r – fluorescence at rest, F – highest/peak fluorescence during stimulation, F_0 – lowest fluorescence point during stimulation, F_p –fluorescence after stimulation when cell has recovered to a constant baseline value (this baseline may or may not return to F_r levels depending on treatment of myocyte).

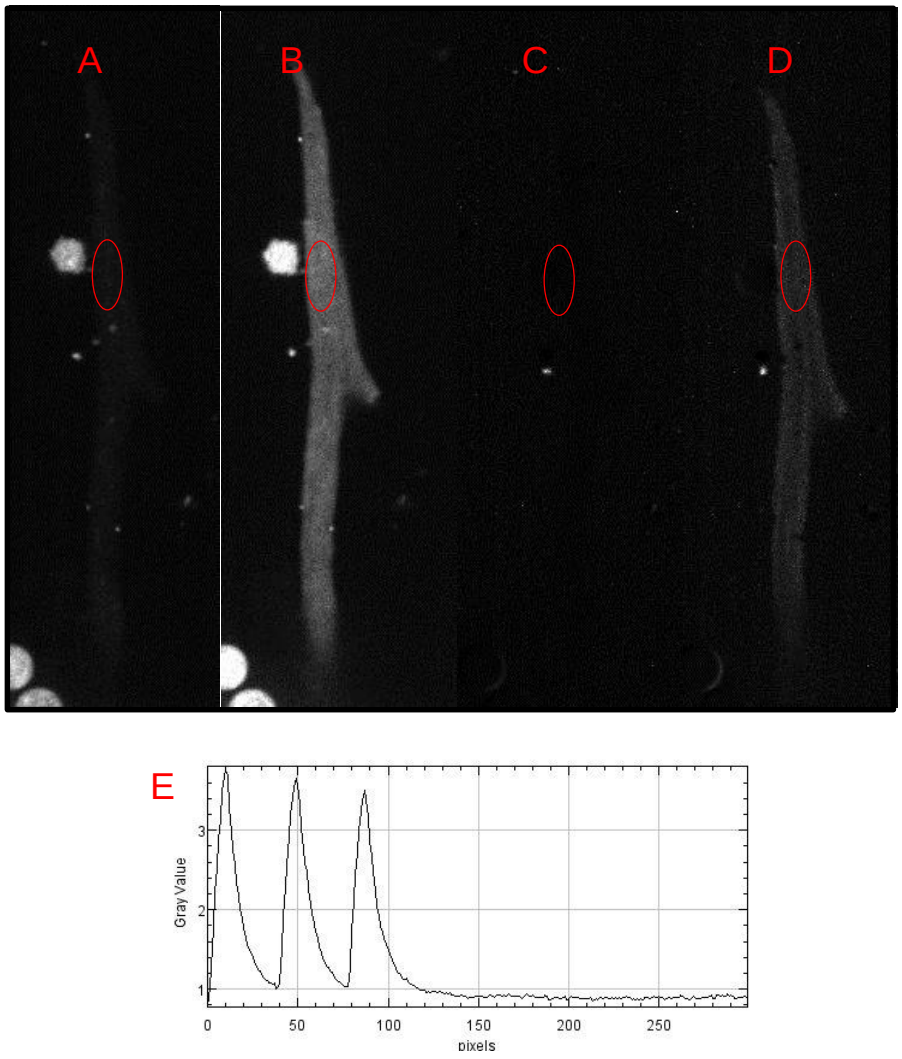

Figure 2.3. Images of an Atlantic cod myocyte being analysed using ImageJ software. Still images show the myocyte at F_0 (A, C) and at the peak of the transient (B, D). Images A and B show the original myocyte recording, while C and D show the myocyte image after being converted into ratio images in order to determine F/F_0 values. The red area provided transient data (E) and was positioned so that the myocyte remained in focus during contraction, and aggregations of Ca^{2+} within the cell were avoided. The representative trace shows the 3 Ca^{2+} transients following a train of 10 stimulations, and calcium levels as they fell following stimulation (i.e. so that F_p could be calculated).

2.3.3. Role of the SR, L-type Ca^{2+} channels and the NCX

In order to block SR-Ca^{2+} release and re-uptake, ryanodine (10 µM) and thapsigargin (1 µM) were added to the working solution. L-type Ca^{2+} channels were blocked using verapamil (10 µM). The NCX "reverse-mode" was inhibited using KB-R7943 (10 µM) (all pharmacological blockers from Tocris Bioscience, Bristol, UK). Cells were initially perfused with working solution as per the original protocol to allow de-esterification, before perfusion was switched to a temperature controlled, recirculating closed system containing a total of 30 ml of working solution as a control, which was then supplemented with either ryanodine/thapsigargin, verapamil or KB-R7943. Cells were perfused with this solution for 10 min before recording. These studies were conducted at 10^{o}C (the fish's acclimation temperature) and at 50 stimulations min^{-1}. This a physiological (*in vivo*) rate of contraction at 10°C (Keen and Gamperl, 2012; Lurman et al., 2011; Gollock et al., 2006), and where clear species differences in the Ca^{2+} transient existed in my previous experiments (Figure 3.1).

A pharmacological approach to studying function / importance of the NCX has, thus far, proved difficult in fish. Blockers such as KB-R7943 were developed based on mammalian studies (Iwamoto et al., 1996; Watano et al., 1996), and thus it is questionable how efficient these blockers are in fish. Recent research (Shiels, unpublished) suggests that these compounds are not effective for studies on fishes, however, other studies have used KB-R7943 as an effective blocker of reverse-mode NCX in the eel (*Anguilla anguilla*) (Methling et al., 2012), and more recently KB-R7943 has been shown to block I_{K1} and I_{KACh}

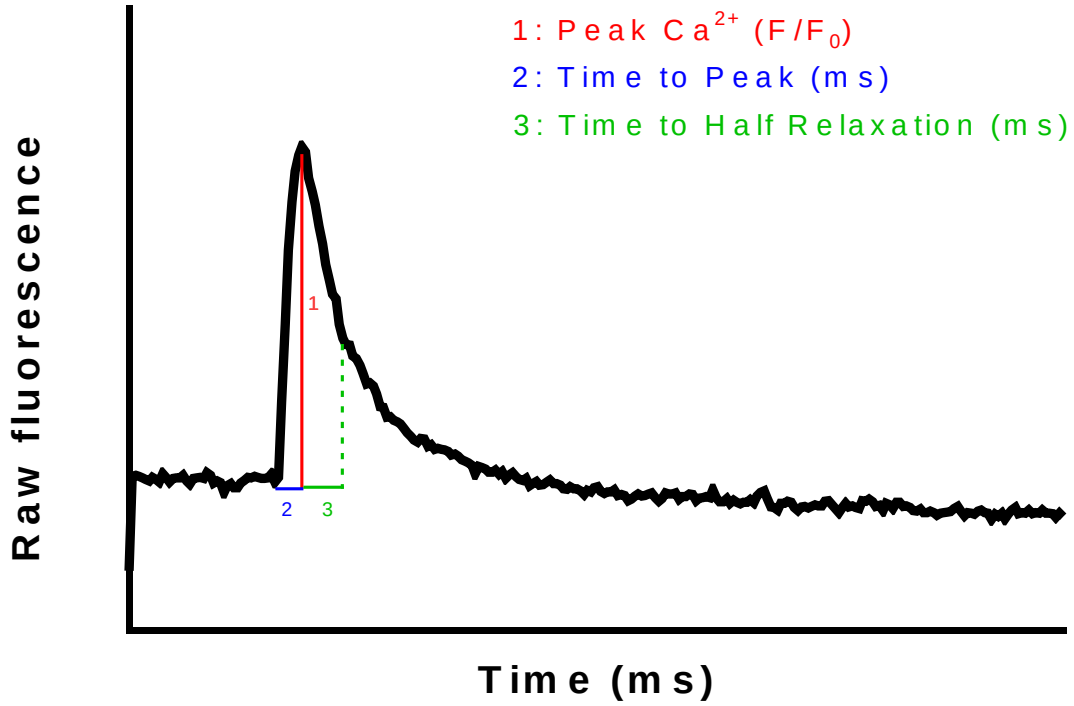

Figure 2.4. Schematic diagram showing aspects of the Ca^{2+} transient that were calculated/analyzed during the experiment.

as well as I_{NCX} in crucian carp (Abramochkin et al., 2013). Thus its effects in fish are still being investigated.

2.4. Data Analyses

Recordings were converted into .AVI files using the Mag Biosystem software, then analysed using ImageJ software to obtain F/F_0 (standardized fluorescence) ratio images. Transients were characterized by peak Ca^{2+} level (F/F_0), time to peak $[Ca^{2+}]_i$, and half-time to relaxation (Figure 2.4).

Statistical analyses were performed using SigmaPlot (Systat Software, San Jose, CA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA) and consisted of the following:

1. Differences in cell morphology (length, width, cell volume and surface area) were compared between the two species using a two-tailed Student's t-test.

2. The effect of temperature on peak Ca^{2+} (F/F_0) (Figure 3.1) was not analyzed as temperature-dependent effects on the K_d of fluo-4-AM (Woodruff et al., 2002) were not known under my experimental conditions. Thus, two-way ANOVAs, followed by Tukey's post-hoc tests at each temperature were used to examine the effects of species and stimulation frequency. In contrast, effects of temperature on time to peak Ca^{2+} and time to half relaxation could be examined, and thus, these parameters were initially analysed used a three-way ANOVA with temperature (4, 10, 16°C), species and stimulation frequency (10 – 110 min^{-1}) as main effects.

There were significant interactions between the main effects, and thus, one-way ANOVAs followed by Tukey's post-hoc tests were used to examine the effect of frequency at each temperature within each species, while Student's t-tests were used to compare between species at each frequency and temperature.

3. The effect of adrenaline on F/F_0, time to peak, and time to half relaxation were initially analysed using a three-way ANOVA with adrenaline (0 vs 10 nM), species and stimulation frequency (10 – 110 min^{-1}) as main effects. Again, significant interactions were present between the main effects, thus, further analyses used Student's t-tests to compare between adrenaline treatments at each frequency within a species, and one-way ANOVAs (followed by Tukey's post-hoc tests) were used to analyse the effect of frequency on the myocyte transients of each species.

4. Two-way ANOVAs were used to analyse the effects of species and blockers on F/F_0, time to peak Ca^{2+}, time to half relaxation, as well as F_0/F_r and F_p/F_r. One-way ANOVAs (with Dunnett's post-hoc tests) were used to examine the effects of the blockers ryanodine/thapsigargin and verapamil, while a paired Student's t-test was used to compare KB-R7943 to its control.

Statistical analyses were carried out using data from individual cells, and this resulted in the number of recordings (15-30) being much greater than the number of fish used for each experiment (4-6). In order to avoid issues of pseudoreplication, the results of the

ANOVA analyses were adjusted to obtain F and p values based on 'n' referring to the number of fish, rather than number of cells recorded (Millar & Anderson, 2004).

3. Results

3.1. Cell Morphology

Atlantic cod cardiomyocytes were significantly shorter and narrower than those of the steelhead trout (Table 3.1). This resulted in much lower values for surface area and cell volume (by 50.6 and 65%, respectively), but a 44% greater surface area to volume ratio, for the Atlantic cod cells.

3.2. Effects of Temperature and Stimulation Frequency on the Calcium Transient

3.2.1. Temperature

At the acclimation and test temperature of 10ºC, increasing the stimulation rate from 10 to 30 min^{-1} had little effect on F/F_0 in either species. However, this parameter was higher in cells from the steelhead trout vs. Atlantic cod at these two stimulation rates, this difference (24 %) at 30 min^{-1} being significant ($p < 0.05$) (Fig. 3.1 B). In contrast, F/F_0 was similar in both species and declined as stimulation rate was increased further. F/F_0 was approx. 2.70 at 50 min^{-1}, but only 1.80 at 110 min^{-1}. At this test/acclimation temperature, time to peak Ca^{2+} and time to half relaxation were consistently longer (by approx. 25 and 45 % respectively) in the steelhead trout as compared to the Atlantic cod (Figs. 3.1 E and H). In both species, these parameters decreased slightly (but

Table 3.1. Morphology of isolated myocytes from Atlantic cod and steelhead trout hearts. The values represent the average of 4 cells from 4 fish per group. (Total = 16 cells per value). The cells were viewed at 10°C in 2 mM Ca^{2+} buffer solution at 10x and 60x magnification. * denotes a significant ($p < 0.05$) difference between the species as determined using unpaired Student's t-tests. SA = surface area, V = cell volume. Values are means $\pm$ 1 S.E.

	Length (µm)	Width (µm)	Cell surface area (μm^2)	Cell volume (μm^3)	SA:V
Atlantic Cod	117.5* ± 3.0	5.8* ± 0.3	1436.8* ± 106.6	870.4* ± 116.5	1.83:1*
Steelhead Trout	163.7 ± 14.1	7.4 ± 0.6	2970.4 ± 469.7	2693 ± 659.6	1.27:1

significantly, $p < 0.05$) as stimulation rate was increased, and this difference was approximately 50-90 ms for time to peak Ca^{2+} and 75-100 ms for time to half relaxation between 10 and 110 stimulations min^{-1}.

Acutely decreasing the test temperature to 4°C accentuated the species' difference in F/F_0 observed at 10°C at the lower stimulation frequencies (this difference significant from 10 to 50 min^{-1}; by approx. 20%), and resulted in a difference in the overall pattern of change in this parameter with stimulation rate. F/F_0 declined steadily in the Atlantic cod from 10 (2.51 ± 0.42) to 110 min^{-1} (1.75 ± 0.09), whereas there was an abrupt drop observed for the steelhead trout cells between 50 (2.73 ± 0.29) and 70 (1.87 ± 0.21) min^{-1} (Fig. 3A). At 4°C, both time to peak Ca^{2+} and time to half relaxation were longer at 10 min^{-1} than measured at 10°C, but not at 100 stimulations min^{-1} (Fig. 3.1 D and G). This resulted in a greater decrease in these parameters with stimulation frequency (from 421 – 197 ms and 408 – 183 ms, respectively) as compared with that measured at 10°C (from 255 – 160 ms and 280 – 180 ms, respectively). Time to peak Ca^{2+} was also significantly longer in the steelhead trout vs. the Atlantic cod at all stimulation frequencies at 4°C by approx. 50 %, and this was greater than that observed at 10°C. In contrast, time to half relaxation was only greater in steelhead trout as compared to Atlantic cod (by approx. 45 %) at the lower frequencies. No difference was seen between species at stimulation rates of 70 – 100 min^{-1} (Fig. 1G).

Increasing the test temperature to 16°C had two main effects on the measured parameters. First, while F/F_0 decreased steadily with stimulation rate in steelhead trout, there was no significant difference in this parameter from 10 to 50 stimulations min^{-1} in

the Atlantic cod cells. This resulted in the Atlantic cod having significantly (at $0.05 \leq p \leq 0.1$) higher values for F/F_0 at stimulation rates from 30 – 100 min^{-1} (Fig. 3.1 C). The direction of this difference was opposite to what was observed at both 10 and 4°C (Fig. 3.1 A and B respectively), and is related to the disparate effect that test temperature had on F/F_0 in the two species. Second, although the difference in time to peak Ca^{2+} was similar between the two species at 16°C as compared to 4 and 10°C (Fig. 3.1F), the difference in time to half relaxation was greater at the lowest stimulation rates at 16°C. For example, at 10 and 30 stimulations per minute, this difference was approx. 150 ms at 16°C (Fig. 3.1 I) whereas it was only approx. 100 ms at 10 and 4°C (Fig. 3.1 G and H).

3.2.2. Effects of Stimulation Rate on Baseline Ca^{2+}

F_0/F_r in Atlantic cod myocytes was low (1.38 ± 0.11 to 2.12 ± 0.24), and independent of stimulation frequency. The same was true for the steelhead trout cardiomyocytes at frequencies from 10 – 70 min^{-1}. In contrast, at stimulation frequencies of 90 and 110 min^{-1}, the baseline Ca^{2+} in stimulated steelhead trout cardiomyocytes increased dramatically, and was 10-fold higher, respectively, as compared to that for Atlantic cod cells (Figure 3.2). This suggests that Atlantic cod cells at 10°C are more capable of removing intracellular Ca^{2+} at high contraction frequencies than those of the steelhead trout.

3.3. Adrenaline

Tonic levels of adrenaline (10 nM) had limited effects on calcium transient dynamics in Atlantic cod (Fig. 3.3 B, D, F) cardiomyocytes. The only significant effects were a small

(20 %) increase in F/F_0 at the lowest frequency tested (10 min^{-1}) (Fig. 3.3. B), and an increase in time to peak Ca^{2+} at 10 and 20 stimulations per min^{-1} (from 186 to 276 ms and 197 to 241 ms, respectively) (Fig. 3.3. D). Conversely, adrenaline caused substantial increases in peak Ca^{2+} in steelhead trout at all stimulation rates. This increase was approximately 30% at 70 and 100 stimulations per minute, 50% at 10 and 50 min^{-1}, and almost 100% at 30 min^{-1} (Fig. 3.3. A). Time to peak Ca^{2+} was also significantly longer in the presence of adrenaline (an increase of approx. 60 ms at frequencies from 10 to 90 min^{-1}) (Fig. 3.3 C). However, there were only minor effects of adrenergic stimulation on time to half relaxation (15% decrease) (Fig. 3.3.E).

In order to compare time – dependent effects of adrenaline with other studies, the data were also expressed relative to Peak Ca^{2+} levels as in Skov et al. (2008) (Figure 3.4), i.e. Peak Ca^{2+} divided by TPT, and Peak Ca^{2+} divided by THR. There was no change in Peak Ca^{2+}/TPT or Peak Ca^{2+}/THR in steelhead trout in the absence of adrenaline as stimulation rate increased ($p < 0.05$) (Figure 3.4. A, C). However, Peak Ca^{2+}/TPT was significantly higher in steelhead trout with adrenaline at 30 and 50 min^{-1} and at the highest frequency (by approx. 50% at 50 and 110 min^{-1}, 60% at 30 min^{-1}) (Figure 3.4. A). Similarly, Peak Ca^{2+} /THR was significantly greater with adrenaline across the range of frequencies (approx. 10% higher at 90 min^{-1}, 50 - 60% higher at 10, 50 and 70 min^{-1}, and approx. 100% higher at 30 and 110 min^{-1}) (Figure 3.4. C). Adrenaline generally had minimal effects on Peak Ca^{2+}/TPT in Atlantic cod. However, small, yet significant, decreases were seen at 10 and 30 min^{-1} (20% lower with adrenaline) and at 90 min^{-1} (35% lower with adrenaline) (Figure 3.4. B). Peak Ca^{2+}/THR was not frequency dependent in Atlantic cod,

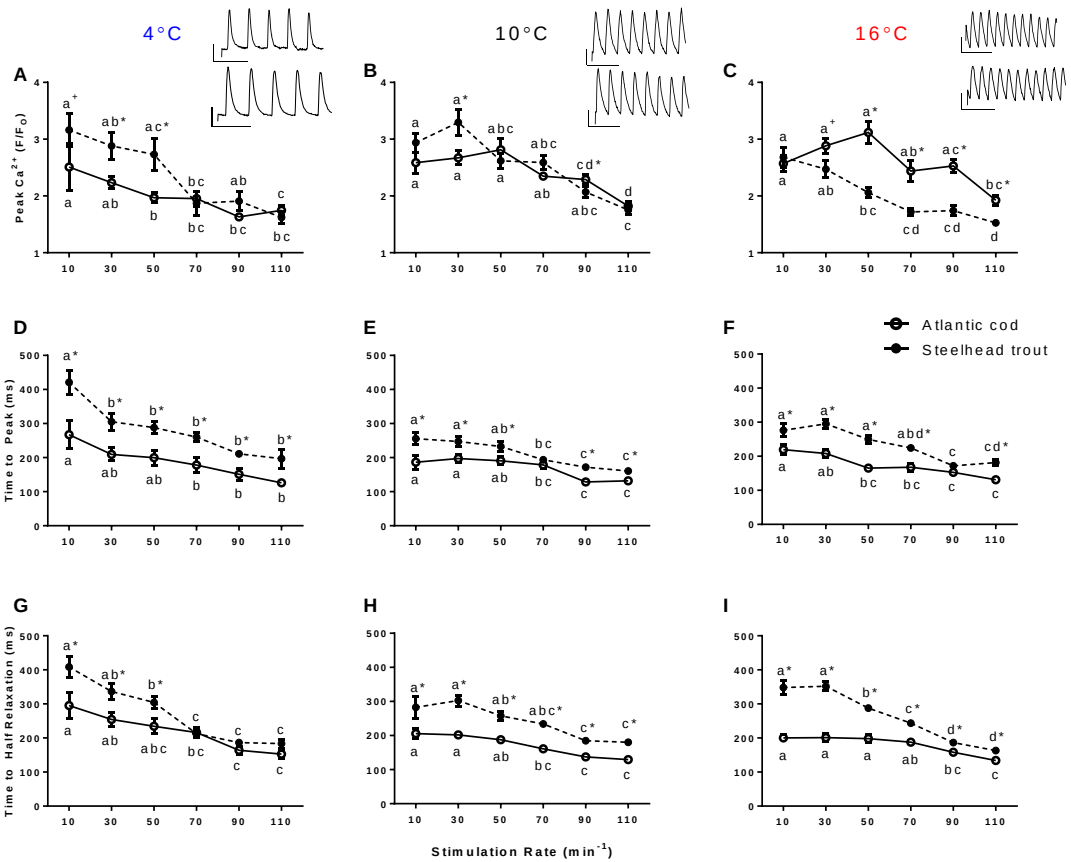

Figure 3.1. Effects of an acute temperature change (from acclimation temperature of 10°C to 4°C or 16°C) and stimulation rate on peak Ca^{2+} (A, B, C), time to peak Ca^{2+} (D, E, F) and time to half relaxation (G, H, I) in Atlantic cod and steelhead trout cardiomyocytes. Stimulation rates without a letter in common are significantly different within a species (ANOVAs, $p < 0.05$). * and $^{+}$ denote a significant difference between species at a given stimulation rate (Student's t-tests, $p < 0.05$ and $p < 0.10$, respectively). Values are means $\pm$ 1 SEM. Sample transients show representative traces at physiologically relevant pacing rates for each temperature (30, 50 and 70 min^{-1} respectively) for Atlantic cod (upper transient) and steelhead trout (lower transient).

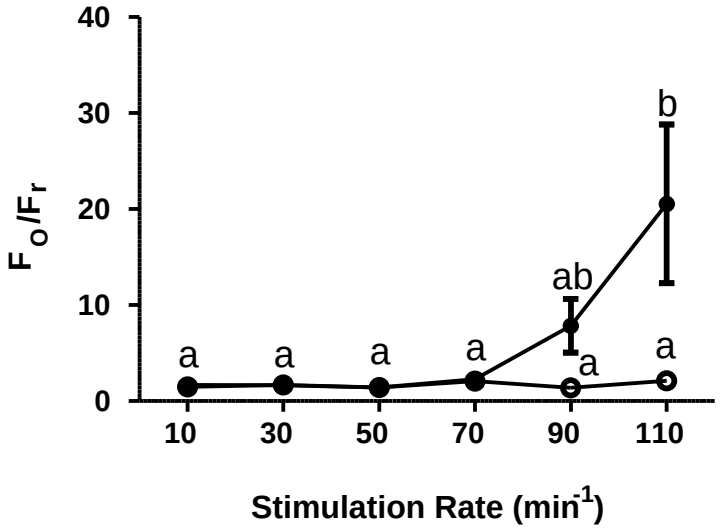

Figure 3.2. Effect of increasing stimulation rate (min^{-1}) on diastolic (baseline; F_0) relative to resting (pre-stimulation; F_r) Ca^{2+} fluorescence in steelhead trout (black circles) and Atlantic cod (open circles) cardiomyocytes. Stimulation rates without a letter in common are significantly different within a species ($p < 0.05$; one-way ANOVAs). Stimulation rate had no effect on F_0/F_r in Atlantic cod. Data are means $\pm$ 1 SEM.

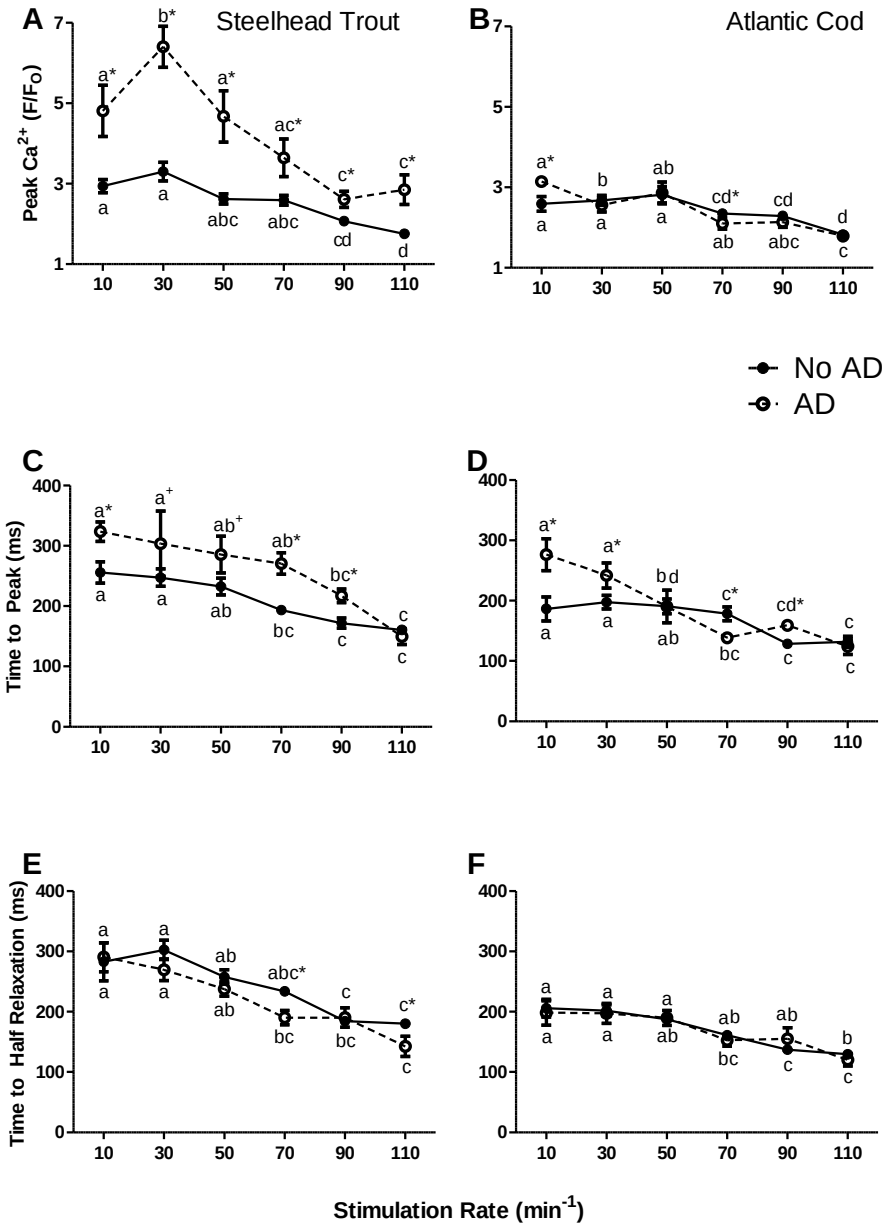

Figure 3.3. Effects of adrenaline (10 nM) and stimulation rate on peak Ca^{2+} (A, B), time to peak Ca^{2+} (C, D) and time to half relaxation (E, F) in Atlantic cod and steelhead trout cardiomyocytes at 10°C. Stimulation rates without a letter in common are significantly different within a species (one-way ANOVAs, $p < 0.05$). * Denotes a significant difference between species at a particular stimulation rate (Student's t-tests, $p < 0.05$). Values are means $\pm$ 1 SEM**.**

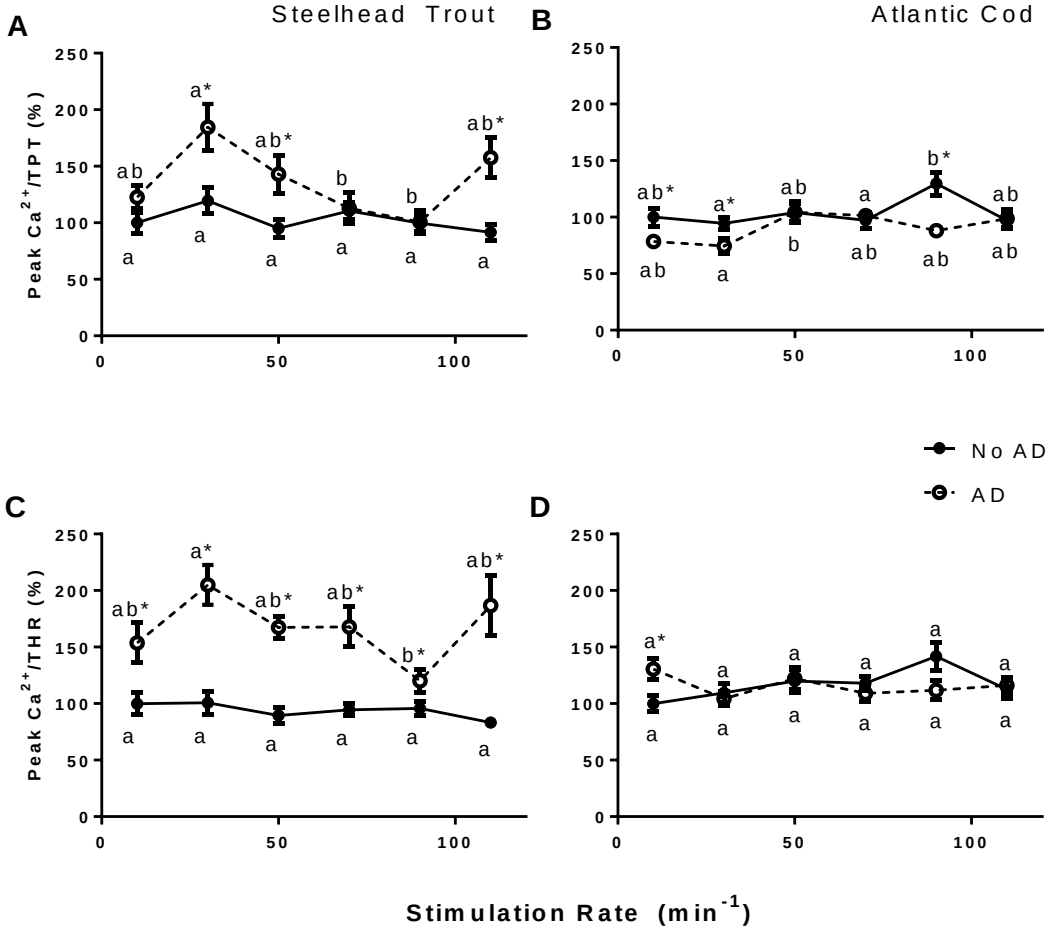

Figure 3.4. Effects of adrenaline (10 nM) and stimulation rate on time to peak tension (TPT) (A, B) and time to half relaxation (THR) (C, D) in Atlantic cod and steelhead trout cardiomyocytes when values are expressed relative to Peak Ca^{2+} (F/F_0) values as per Skov et al., (2008). Stimulation rates without a letter in common are significantly different within a species (one-way ANOVAs, $p < 0.05$). * Denotes a significant difference between species at a particular stimulation rate (Student's t-tests, $p < 0.05$). Values are means $\pm$ 1 SEM.

and the only significant effect of adrenaline was an increase of approx. 30% at 10 min^{-1} (Figure 3.4. D).

3.4. Contributions of NCX, SR and L-Type Ca^{2+} Channels

3.4.1. NCX

The effects of KB-R7943 (used to block reverse-mode NCX) on Ca^{2+} dynamics were quite variable between the 3 groups (Fig. 3.5). KB-R7943 had no significant effect on the F/F_0 of steelhead trout myocytes in the absence of adrenaline, or on that of Atlantic cod myocytes. However, this compound caused a significant (55%; $p < 0.05$) decrease in the F/F_0 of steelhead trout myocytes in the presence of adrenaline (Fig. 3.5.A). KB-R7943 did not affect F_0/F_r in Atlantic cod cardiomyocytes. However, it resulted in an increase in this parameter in steelhead trout cells with no adrenaline (by approx. 30%, $p < 0.01$) and a 50% decrease in F_0/F_r ($p < 0.01$) in the presence of adrenaline. F_p/F_r increased in both Atlantic cod (from 1.2 ± 0.06 to 1.47 ± 0.11) and steelhead trout myocytes (from 1.27 ± 0.06 to 1.82 ± 0.14) in the absence of adrenaline, yet no effect was seen in steelhead trout myocytes when adrenaline was used (Fig. 3.5 C). There was a 25% decrease in the time to peak Ca^{2+} of Atlantic cod cardiomyocytes when using KB-R7943, however, the opposite was seen in steelhead trout (a 30% increase) (Fig. 3.5. D). Finally, KB-R7943 had no significant effect on time to half relaxation in either species, with or without adrenaline (Fig. 3.5 E).

3.4.2. Sarcoplasmic Reticulum

Blocking the SR with a combination of ryanodine and thapsigargin had no effect on the F/F_0 of Atlantic cod or steelhead trout calcium transients in the absence of adrenaline. However, when adrenaline was used, there was a 30% decrease in F/F_0 in the steelhead trout cardiomyocytes (Fig. 3.6 A). Similarly, there was no significant effect of ryanodine and thapsigargin on F_0/F_r or F_p/F_r in either species without adrenaline, but when adrenaline was present the F_0/F_r of the steelhead trout cells decreased from 2.58 to 1.80 (Fig. 3.6 B and C). Interestingly, there was no evidence that ryanodine and thapsigargin affected the timing of the calcium transient, as neither time to peak Ca^{2+} or time to relaxation were influenced by exposure to these drugs (Fig. 3.6 D and E).

3.4.3. L-Type Ca^{2+} Channels

Verapamil only had one significant effect on cardiomyocyte calcium dynamics in the 3 groups. This was a decrease in F_p/F_r (from 1.37 to 1.08) in steelhead trout myocytes when adrenaline was used (Fig. 3.6 C).

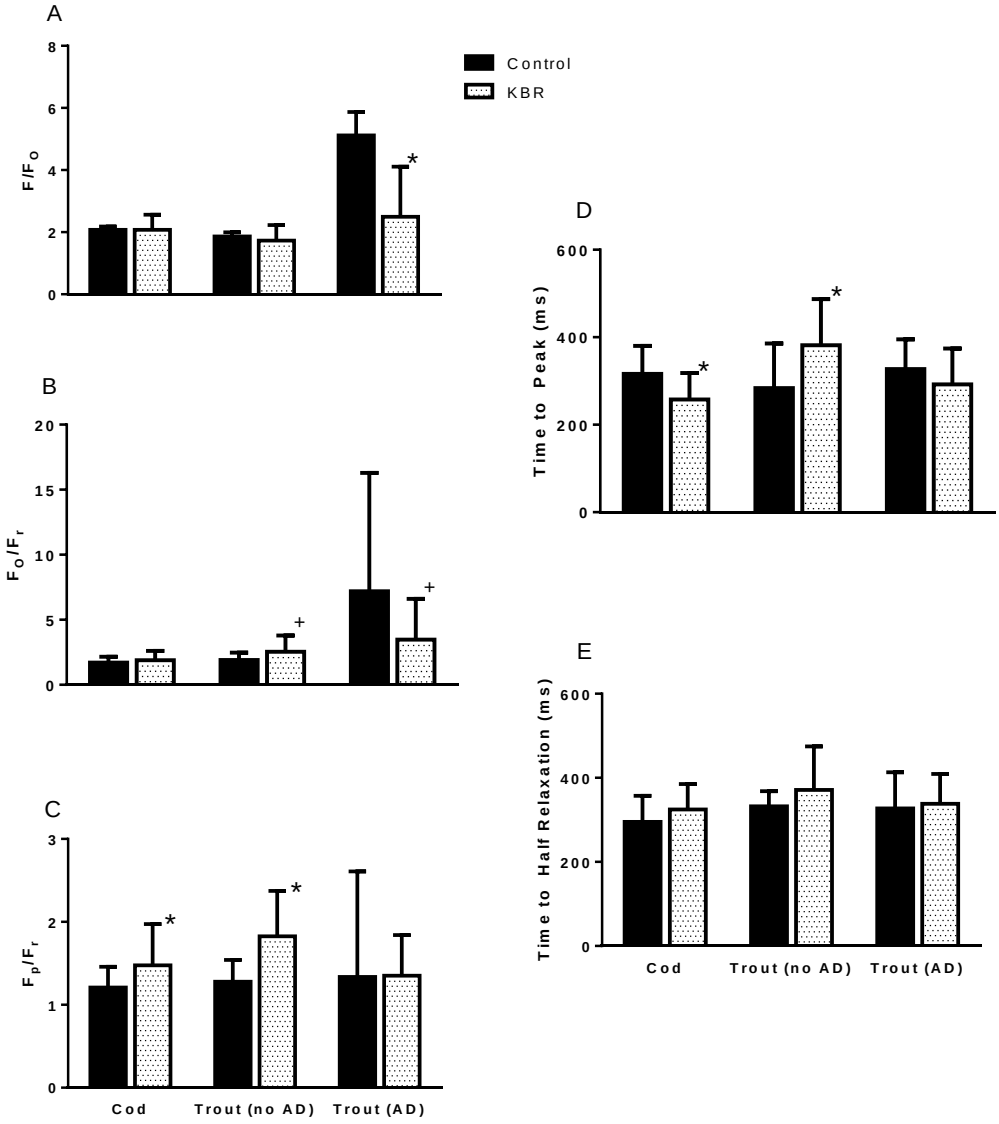

Figure 3.5. Effects of the NCX blocker KB-R7943 on Ca^{2+} dynamics in steelhead trout and Atlantic cod cardiomyocytes. * denotes a significant difference ($p < 0.05$) from the control values, and $^{+}$ denotes a difference at $0.05 < p < 0.1$. (Student's t-tests). Values are means $\pm$ 1 SEM.

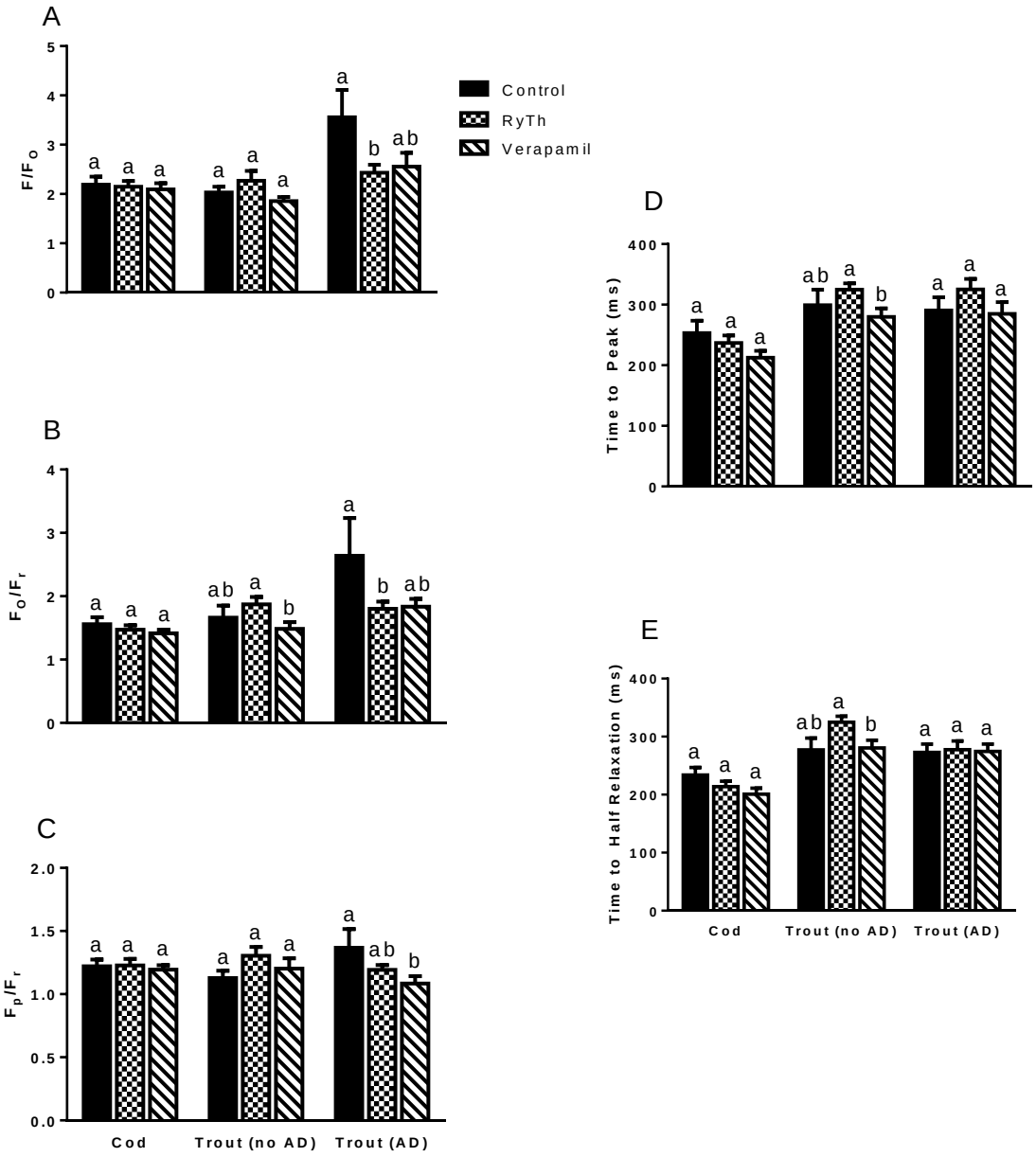

Figure 3.6. Effects of SR and L-type Ca^{2+} channel blockers [ryanodine/thapsigargin (RyTh), and verapamil, respectively] on Ca^{2+} dynamics in steelhead trout and Atlantic cod cardiomyocytes. Dissimilar letters indicate significant differences between parameters (one-way ANOVAs, $p < 0.05$).

4. Discussion

4.1. Myocyte Morphology

In this study, I made morphometric measurements of myocytes from the Atlantic cod ventricle (length 117.5 µm, width 5.8 µm) and from the spongy myocardium of the steelhead trout ventricle (length 163.7 µm, width 7.4 µm). I am unaware of previous measurements of cardiomyocyte dimensions in Atlantic cod. However, our values for the steelhead trout are in line with those reported by Vornanen (1998; length 196.7 µm, width 7.42 µm) and Shiels & White (2005; length 159.8 µm, width 9.9 µm).

Research has shown that fish cardiomyocyte size does not vary tremendously, when one considers the large difference in heart mass that exists between fishes. For example, myocytes of the bluefin tuna (*Thunnus orientalis*) heart are approx. 185 µm long x 7.6 µm wide (Shiels et al., 2004), whereas the zebrafish heart has cells that are approx. 100 µm long x 4.6 µm wide (Brette et al., 2008). Nonetheless, a significant difference in these values was observed between the two species used in this study, despite their similar cardiac masses (Atlantic cod, 0.609 g; steelhead trout, 0.470 g), and this resulted in a much greater surface area to volume ratio (by approx. 70%) for the Atlantic cod myocytes (Table 3.1). One might predict that this would have functional implications as the potential for sarcolemmal calcium entry (via L-type calcium channels) and entry/exit (via NCX) to play a predominant role in Ca^{2+} handling would be greater in cells with a larger surface to volume ratio. Interestingly, however, there was little evidence of this in the current study (see Section 4.4).

4.2. Temperature

4.2.1. The Calcium Transient at Acclimation Temperature

When tested at their acclimation temperature (10ºC), F/F_0 initially increased at lower stimulation frequencies in both species before falling, and there were few inter-specific differences in F/F_0 values (Figure 3.1B). The largest difference between the two species was at 30 min^{-1} where steelhead trout had a 25% higher F/F_0 value than Atlantic cod. Isometric force development by the myocardium of steelhead trout and most teleosts generally displays a negative force-frequency relationship (Driedzic & Gesser, 1988; Hove-Madsen & Gesser, 1989; Hove-Madsen, 1992). From 30 – 110 stimulations min^{-1} there is also a clear negative F/F_O-frequency relationship for the steelhead trout used in this study, similar to that reported by other authors (e.g. Hove-Madsen & Gesser, 1989; Hove-Madsen, 1992). However, when data for 10 stimulations min^{-1} are included, the relationship appears to be bell-shaped. This may be explained by the wider range of frequencies tested in this study compared to the above studies. Some species such as skipjack tuna, *Katsuwonus pelamis* (Keen et al., 1992) and Atlantic cod (Driedzic & Gesser, 1988; Syme et al., 2013) do not exhibit a negative force-frequency effect, but a distinctly bell-shaped force-frequency relationship. A similar relationship was observed for the F/F_0– frequency relationship for Atlantic cod in this study. The decrease in F/F_0 noted in both the steelhead trout and Atlantic cod at 10ºC as stimulation frequency increased is indicative of a decrease in developed force during each contraction (Yue, 1987). F/F_0 values, however, do not show the total amount of Ca^{2+} in the myocyte during contraction (Figure 2.1), and F_0/F_r actually increased significantly at high stimulation

frequencies in steelhead trout, but not in Atlantic cod (Figure 3.2). The elevated F_0/F_r values strongly suggest that resting tension is elevated at high frequencies in steelhead trout, and this is consistent with data on cycling salmon muscle strips. Thomas, Syme & Gamperl (unpublished) found that lengthening power increased with increasing stimulation frequency and Shiels & Farrell (1997) reported that isometrically contracting rainbow trout muscle strips had an elevated resting tension when paced at elevated frequencies without adrenaline. Adrenaline was not used in this study, with the exception of the data presented in Figures 3.3 and 3.6, and rainbow trout may need adrenergic stimulation to improve/activate Ca^{2+} resequestration in the SR (Gesser, 1996; Shiels & Farrell, 1997; Shiels et al., 1998) and avoid elevated Ca^{2+} levels and values for resting tension at fast stimulation frequencies. According to Figure 3.2, the Atlantic cod myocardium should not experience myocardial stiffness at high stimulation frequencies. However, these data do not agree with Syme et al. (2013) who showed that net power falls at high stimulation frequencies due to an increase in lengthening work and power (i.e. the muscle is difficult to stretch). Thus, it would appear that the myocardial stiffness observed by Syme et al. (2013) is not due to elevated Ca^{2+} levels, but possibly mechanical aspects of contraction. The rate at which muscle is stretched (strain rate) affects the muscle's passive force, and faster strain rates have been shown to result in increased visco-elastic resistance in rat cardiac (de Tombe & Ter Keurs, 1992; Stuyvers et al., 1997) and diaphragm (Syme, 1990) muscle. A substantial amount of this increase in visco-elastic resistance could be due to the functional characteristics of titin (Horowits, 1992; Granzier & Irving, 1995; Linke & Fernandez, 2003). Titin has been shown to become stiffer when normal cross-bridge binding is lacking or insufficient (Horowits &

Podolsky, 1987; Stuyvers et al., 1997; Joumaa et al., 2008), and thus, could be contributing to the muscle stiffness (elastic resistance) at the high stimulation frequencies observed by Syme et al. (2013). Strain rate and the role of titin in frequency-dependent muscle stiffness would make interesting topics for further study, as this area has not been fully investigated in fish cardiac muscle.

Both time to peak Ca^{2+} and time to half relaxation were faster in Atlantic cod myocytes at all stimulation frequencies (Figure 3.1 D-I). There are several possible explanations for this observation. First, a faster time to peak Ca^{2+} in Atlantic cod could imply higher densities of L-type Ca^{2+} channels, or larger L-type Ca^{2+} current densities (I_{Ca}). Crucian carp have a larger I_{Ca} than rainbow trout (Vornanen, 1998), and like Atlantic cod, crucian carp show a diminished response to adrenaline in comparison to rainbow trout (Vornanen, 1998). A second contributor to rising Ca^{2+} in the myocyte could be the SR, as the SR's contribution to the Ca^{2+} transient is known to vary between species (Galli et al., 2009; Haverinen & Vornanen, 2009b). If Atlantic cod had a higher density of RyRs, or these receptors had a higher Ca^{2+} sensitivity than those in rainbow trout (for example, like the burbot; Vornanen; 2006; Haverinen & Vornanen, 2009b), it may account for the faster accumulation of Ca^{2+} in the cytoplasm. Since neither I_{Ca} nor the SR contribution to the Ca^{2+} transient have been confirmed in Atlantic cod, these questions require further investigation. Finally, due to the Atlantic cod's lack of response to adrenaline, Lurman et al. (2012) suggested a more prevalent role of the NCX in Atlantic cod cardiomyocytes. However, pharmacological data from this study did not provide evidence for significant contributions from the NCX in either species when cells are

given a train of stimulations (Figure 3.6). The faster times to half relaxation in Atlantic cod indicate higher rates of Ca^{2+} resequestration by the SR, or higher densities of NCX (operating in forward-mode). Again, pharmacological data from this study did not provide evidence of significant differences between the two species in terms of relative contributions of L-type Ca^{2+} channels, SR or NCX (Figure 3.6; see Sections 4.4 and 4.5.1 for full discussion).

In addition to time to peak Ca^{2+} and time to half relaxation both being faster in the Atlantic cod as compared to the steelhead trout, the effect of stimulation frequency on both of these parameters was much greater in the latter species. My data for steelhead trout support other studies that report a decrease in time to peak Ca^{2+} and time to half relaxation with increased stimulation frequencies in this species (Shiels & Farrell, 1997) and other fish such as the icefish *C. aceratus* (Skov et al., 2008). Why time to peak Ca^{2+} and time to half relaxation are less sensitive to stimulation/contraction frequency in Atlantic cod is not known, but species differences in the effects of frequency on time to peak tension and time to half relaxation were also reported by Skov et al. (2008). Unlike *Chaenocephalus aceratus*, these parameters of the Ca^{2+} transient were not frequency dependent in another icefish species, *Notothenia coriiceps*.

4.2.2. Effects of Acute Temperature Change on the Calcium Transient

When acutely cooled to 4ºC, steelhead trout F/F_0 values were similar to those measured at 10ºC across the physiological range of stimulation frequencies (10 – 50 min^{-1}), whereas those for the Atlantic cod were lower (Figure 3.1). These results suggest that steelhead

trout cardiomyocytes are capable of maintaining adequate calcium cycling better than Atlantic cod when faced with an acute drop in temperature. However, this does not agree with Lurman et al. (2012), who reported that Atlantic cod are better than teleosts such as rainbow trout (Aho & Vornanen, 2001) and sea raven (Graham & Farrell, 1985) at maintaining cardiac function when faced with an acute decrease in temperature. One potential explanation for this discrepancy is differences in myofilament Ca^{2+} sensitivity between species.

Myofilament Ca^{2+} sensitivity is determined by cardiac troponin C (cTnC), a thin filament protein which initiates myocyte contraction when bound to Ca^{2+} (Gillis et al., 2002). Rainbow trout myofilament Ca^{2+} sensitivity decreases at cold temperatures (Gillis et al., 2002), which could explain why steelhead trout hearts perform poorly when exposed to acute decreases in temperature despite the maintenance of F/F_0 levels. There is evidence for species differences in the amino acid sequence of cTnC (Yuasa et al., 1998; Yang et al., 2000).While most of the amino acid substitutions reported are located in areas outside of the Ca^{2+} binding domains (Yuasa et al., 1998; Yang et al., 2000), Ca^{2+} sensitivity has been shown to increase in mammalian cardiac troponin C (McTnC) mutated with trout amino acid sequences outside the major Ca^{2+} binding domain due to allosteric changes to the binding domain (Gillis et al., 2005). At this time there is no information regarding amino acid sequences of cTnC in Atlantic cod, however species differences in amino acid sequences may provide an explanation for the discrepancy seen between F/F_0 and *in situ* studies with respect to the effects of an acute drop in temperature, or species differences in Ca^{2+} sensitivity.

An alternate explanation for the ability of Atlantic cod to maintain *in situ* cardiac function at cold temperatures, despite lower F/F_0 values than steelhead trout, is that the phosphorylation state of key contractile proteins changes following an acute drop in temperature, and that the magnitude / importance of this effect, varies between the Atlantic cod and steelhead trout. This hypothesis is supported by data which show that temperature acclimation can affect the phosphorylation state / rate of key contractile proteins (Gillis et al., 2000; Klaiman et al., 2014) and that phosphorylation rates of cTnI and cardiac myosin binding protein C (cMyBP-C) change rapidly in both mammals and fish in response to varying Ca^{2+} levels and PKA pre-treatment (Stelzer et al., 2007; Gillis & Klaiman, 2011). In rainbow trout, MLC-2 (myosin light chain 2) is phosphorylated by PKA (Patrick et al., 2010), and this changes the flexibility of the myosin head, and in turn, its ability to bind to actin and the kinetics of cross-bridge formation (Buck et al., 1999; Olsson et al. 2004). There are no data on the effects of acute temperature change on phosphorylation state / rates of MLC-2 in fishes, and thus, at this point it is unknown whether differences in the phosphorylation of MLC-2 or other proteins might be associated with the better *in situ* cardiac function of Atlantic cod hearts at cold temperatures.

When tested at 4ºC and stimulated at higher frequencies (70 – 110 min^{-1}), steelhead trout F/F_0 dropped considerably, whereas changes in F/F_0 in Atlantic cod cardiomyocytes over this stimulation range were consistent with that observed at lower frequencies (Figure 3.1 A). While AP duration is longer when fish are acutely cooled (allowing more time for Ca^{2+} entry/removal), the amplitude of I_{Ca} is lower, and this has

been shown to reduce $[Ca^{2+}]_i$ (Shiels et al., 2015). It could be that the amplitude of I_{Ca} is not large enough to bring sufficient amounts of Ca^{2+} into steelhead trout cardiomyocytes given that these high stimulation frequencies are not normally experienced *in vivo* at 4ºC. In contrast, time to peak Ca^{2+} and time to half relaxation values were much faster in Atlantic cod at all stimulation frequencies and all temperatures, and these parameters showed less temperature sensitivity when acutely cooled/warmed. Augmented SR function at cold temperatures has been noted in rainbow trout (Aho & Vornanen, 1998; Aho & Vornanen, 1999) and burbot (Tiitu & Vornanen, 2002; Shiels et al., 2006), and thus, it could be that improved SR function in Atlantic cod allows them to maintain faster times to peak Ca^{2+} and times to half relaxation following an acute temperature change as compared to steelhead trout. Indeed, a decrease in force was observed in Atlantic cod ventricular strips treated with ryanodine (Driedzic & Gesser, 1988), however, at this time there is little other information available regarding SR function in Atlantic cod.

When acutely warmed to 16ºC, steelhead trout F/F_0 values decreased from those at 10ºC, and the Ca^{2+}-frequency relationship changed from being bell-shaped to entirely negative (Figure 3.1 C). These data are in agreement with previous studies on rainbow trout where acute warming was shown to decrease contractile force (Aho & Vornanen, 2001) and $[Ca^{2+}]_i$ (Shiels et al., 2002b). The decreased $[Ca^{2+}]_i$ observed with acute warming in Pacific bluefin tuna is associated with a shortening of the AP (Shiels et al., 2015), and this phenomenon may reduce the time available for Ca^{2+} entry into steelhead trout cells at 16ºC. Although this would decrease myocardial contractility, it is thought that higher *in vivo* heart rates at warmer temperatures compensate for the negative effects

of warming on the heart (Blank et al., 2004; Clark et al., 2013). Atlantic cod F/F_0 values showed the opposite response to elevated temperature, and increased considerably from values observed at 10ºC. At this point, I have no plausible explanation for this phenomenon, as it was predicted that F/F_0 values would be highest at their acclimation temperature, as other studies have shown a decrease in force development with acute increases in temperature in burbot (Tiitu & Vornanen, 2002).

Similar to what was observed at 4 and 10ºC, time to peak $Ca^{2+}$and time to half relaxation in Atlantic cod were faster than in steelhead trout at 16ºC across the range of stimulation frequencies. As mentioned previously, I hypothesized that this might be due to differing contributions of L-type Ca^{2+} channels, the SR and NCX to the Ca^{2+} transient. However pharmacological data from this studied failed to highlight / identify clear species differences at the fish's acclimation temperature (10ºC) (Figures 3.5, 3.6; see and Sections 4.4 and 4.5.1 for full discussion).

4.3. Effects of Adrenaline

4.3.1. Effects on Peak Ca^{2+}

In this study, I show that tonic (10 nM) levels of adrenaline had few/no effects on the F/F_0 of the Atlantic cod Ca^{2+} transient, but caused significant increases in this parameter (by up to 2-fold) in steelhead trout (Figure 3.3 A & B). The first finding supports evidence from previous studies which showed that adrenaline has little/no effect on myocardial contractility in Atlantic cod (Axelsson, 1988; Lurman et al., 2012), and is consistent with that observed for other species such as winter flounder (Mendonca & Gamperl, 2009), sea

bass (Farrell et al., 2007) and tilapia (Lague et al., 2012). Likewise our findings for the steelhead trout cardiomyocyte Ca^{2+} transient reflect the positive inotropic effects of adrenaline (increased peak tension and I_{Ca} peak current) observed in the rainbow trout heart in other studies (Shiels & Farrell, 1997; Shiels et al., 2003).

There are three potential explanations for the lack of adrenergic response observed in Atlantic cod. First, the NCX was shown to be the primary mechanism of Ca^{2+} entry into cardiomyocytes of the burbot (Shiels et al., 2006). Based on these data, and that the Atlantic cod is a close relative of the burbot (both members of the family Gadidae), Lurman et al. (2012) proposed that that Atlantic cod may predominantly utilize the NCX for calcium entry rather than adrenaline-sensitive L-type Ca^{2+} channels or the SR. However, this study did not provide evidence for the NCX as a major pathway for Ca^{2+} in Atlantic cod (see Figure 3.5 and Sections 4.4 and 4.5.1 for full discussion). β-adrenergic receptor densities are known to vary between species (Olsson et al., 2000), thus a second explanation could be that the Atlantic cod myocardium has either a low ß-adrenoreceptor density or different proportions of β_2 vs. β_3 adrenergic receptors than adrenaline-responsive species such as rainbow trout. Recent work by Petersen et al. (unpublished) has shown that Atlantic cod ventricular myocytes have a ß-receptor density that is actually higher that of the rainbow trout as determined with the ligand ^{3}H CGP, but that the affinity of the receptors is similar (K_D values of 0.16 and 0.24, respectively). These data indicate that the Atlantic cod myocardium has a sufficient population of ß-receptors to provide a robust adrenergic response. Nonetheless, these authors do not provide any information on the presence or proportion of $ß_3$-adrenoreceptors. β_3 adrenergic receptors

have been documented in hearts of species including the eel (Imbrogno et al., 2006), zebrafish (Wang et al., 2009), fathead minnow (*Pimephales promelas*) (Giltrow et al., 2011) and rainbow trout (Nickerson et al., 2003; Nikinmaa, 2003). While information is still limited in fish, the function of β_3 adrenergic receptors has been studied in both eel (Imbrogno et al., 2006) and rainbow trout (Petersen et al., 2013). Stimulation of β_3 adrenergic receptors causes a negative inotropic response, which results in a decrease in stroke volume in both species (Imbrogno et al., 2006; Petersen et al., 2013). This decrease in contractility results because β_3 adrenergic receptors are coupled with G_i proteins, and this association activates the NO-cGMP-PKG signal transduction pathway (Imbrogno et al., 2006). Finally, β_1/β_2 adrenergic receptors can become uncoupled from G_s proteins leading to changes in the signal transduction pathway and cAMP production after prolonged periods of elevated circulating catecholamine levels (Vatner et al., 1989). Thus, it is possible that the coupling between β_2-adrenoreceptors and G_s in rainbow trout vs. Atlantic cod is fundamentally different, or that there is less amplification through the signal transduction pathway. Clearly, the β-adrenoreceptor-G-protein signal transduction pathway in fish that do not respond robustly to adrenergic stimulation is an important area for further study.

The effects of adrenaline on F/F_0 were frequency dependent in both species at 10°C. In Atlantic cod there was no difference in F/F_0 at physiological pacing frequencies (approx. 30 to 50 min^{-1} at 10°C; Webber et al., 1998; Gollock et al., 2006; Lurman et al., 2012), but a significant enhancement at 10 min^{-1}. It could be that at such low stimulation frequencies adrenaline may play a role in maintaining contractility in Atlantic cod.

However, this stimulation frequency is not physiological and is unlikely to be seen *in vivo* (Figure 1.4). Shiels and Farrell (1997) suggest that at low pacing frequencies, Ca^{2+} leaks from the SR due to prolonged periods between depolarizations, and that this unpredictability at unphysiological pacing frequencies may lead to unreliable data. In contrast, the largest differences were seen at physiological pacing frequencies in steelhead trout (30 – 50 min^{-1}) with minimal effects at higher frequencies. While other studies have shown an increase in contractility in the presence of adrenaline (Farrell et al., 1986; Keen et al., 1993; Farrell et al., 1996), this frequency-dependent relationship has not been widely noted. For example, although Shiels et al. (1998) reported a negative force-frequency relationship in rainbow trout ventricular trabeculae stimulated between 0.4 – 1.4 Hz (approx. 24 – 84 stimulation min^{-1}), increasing adrenaline from 10 nM to 10 µM did not change the shape of the force-frequency relationship (Shiels et al., 1998).

4.3.2. Effects on Time Course of the Calcium Transient

In this study, tonic levels of adrenaline had variable time-dependent effects on the calcium transient. There were no significant differences in time to half relaxation in Atlantic cod when adrenaline was applied (Figure 3.3 F), and the only significant effects observed in steelhead trout were at 70 and 110 stimulation min^{-1} (Figure 3.3 E). In contrast, both species showed an increase in the time to peak $F/F_{0,}$ between 10 – 90 stimulation min^{-1} in steelhead trout and at 10, 30 and 90 stimulation min^{-1} in Atlantic cod. Faster rates of contraction and relaxation were reported by Shiels et al. (1998) for rainbow trout ventricular trabeculae stimulated between 0.4 – 1.4 Hz (24 – 84 stimulation

min^{-1}) when adrenaline was increased from 10 nM to 10 µM. However, Shiels and Farrell (1997) found that adrenaline had no effect on the time to peak, and that the only time dependent effect of adrenaline was a slower time to half relaxation at a low, unphysiological, frequency (0.2 Hz). A study on two Antarctic icefish species, *Chaenocephalus aceratus* and *Notothenia coriiceps*, also observed some time-dependent effects of adrenaline on myocardial contractility (Skov et al., 2008). Similar to Shiels & Farrell (1997), that study's only time-dependent effect was a decrease in time to half relaxation at a very low frequency of 0.1 Hz (6 stimulation min^{-1}). However, the interpretation of the data was different when time to peak and time to half relaxation were expressed relative to the peak tension (PT/TPT and PT/THR, respectively). When this was done, Skov et al. (2008) reported an increase in the relative rates of contraction and relaxation with increasing adrenaline concentrations. When data from the present study for steelhead trout are expressed in a similar manner (Peak Ca^{2+}/TPT and Peak Ca^{2+}/THR) (Figure 3.4), two things become apparent: 1) although time to peak Ca^{2+} in steelhead trout did increase with adrenaline (by approx. 25%) this change was much less than the increase in Peak Ca^{2+} (~ 40 to 100% depending on stimulation frequency) (Figures 3.3 and 3.4); i.e. the increase in time to peak Ca^{2+} relative to F/F_0 was lower; and 2) despite the large increase in peak Ca^{2+}, there was no change (or even a slight decrease) in THR (Figures 3.3 and 3.4). Thus, adrenaline has a greater effect on F/F_0 adjusted THR than TPT in steelhead trout. This is in agreement with other studies that report relative changes in rates of contraction rather than absolute contraction times (Shiels & Farrell, 1997; Shiels et al., 1998; Skov et al., 2008). These Peak Ca^{2+}/TPT and Peak Ca^{2+}/THR responses were highly frequency dependent in steelhead trout (Figure 3.4 A, C), which

was also seen in PT/TPT and PT/THR of ventricular trabeculae from this species when tested at 12ºC (Shiels & Farrell, 1997). Small frequency and adrenaline effects were also seen in Atlantic cod Peak Ca^{2+} /TPT and Peak Ca^{2+} /THR, but only at the lowest two frequencies (10 and 30 min^{-1}). However, they were of a much smaller magnitude than in steelhead trout (Figure 3.4 B, D), and this agrees with the data presented for both species of icefish (*C. aceratus* or *N. coriiceps*) where there was little to no frequency-dependent effects on these parameters (Skov et al., 2008). It is entirely possible that adrenergic stimulation is having much greater effects on L-type Ca^{2+} channels and/or the SR in steelhead trout, and this resulted in augmented Ca^{2+} entry into and removal from the myocyte (see Section 4.4 for full discussion).

4.4. Routes of Calcium Entry/Exit

4.4.1. NCX

Myocytes were treated with KB-R7943, a pharmacological blocker of reverse-mode NCX. KB-R7943 had little / no effect on F/F_0, F_0/F_r or time to half relaxation in Atlantic cod or steelhead trout (Figure 3.5 A, B, E). However, an increase was seen in Atlantic cod and steelhead trout F_p/F_r (Figure 3.5 C) suggesting that the myocytes were unable to recover to resting Ca^{2+} levels after the train of stimulations. This effect was not seen in steelhead trout with adrenaline, suggesting that adrenaline may be able to compensate for the effects of KB-R7943. Atlantic cod myocytes did show a decrease in time to peak Ca^{2+} with KB-R7943 (Figure 3.5 D), whereas this parameter increased in steelhead trout. It was expected that blocking reverse-mode NCX would cause a decrease in F/F_0, as less

Ca^{2+} would be entering the myocyte through reverse mode Na^{+}/Ca^{2+} exchange. Such an effect was reported in eel, where KB-R7943 caused a 50% decrease in peak tension at 10ºC (Methling et al., 2012). However, a decrease in F/F_0 with KB-R7943 was only seen in steelhead trout cardiomyocytes exposed to tonic adrenaline (Figure 3.5 A), which is surprising as adrenaline is only known to augment Ca^{2+} entry via L-type Ca^{2+} channels. Nonetheless, KB-R7943 has recently been shown to block more than just reverse-mode NCX [i.e. I_{KATP}, I_{K1} and I_{KACh}, as well as I_{NCX}, are all blocked by this drug in crucian carp (Abramochkin et al., 2013)] making it difficult to interpret the effects of KB-R7943 on NCX activity in this study. The unexpected results from this study suggest that either KB-R7943 is not an effective blocker of reverse-mode NCX in Atlantic cod or steelhead trout, or that under these experimental conditions myocytes were able to compensate for the blocking of the NCX by increased Ca^{2+} influx through the L-type Ca^{2+} channels or SR (also see Section 4.5.1). Previous studies have shown the NCX to be a contributor to Ca^{2+} entry in rainbow trout (Hove-Madsen et al., 2003), and NCX activity has been noted in many other species including eel (Methling et al., 2012), crucian carp (Vornanen, 1999) and burbot (Shiels et al., 2006). Lurman et al (2012) suggested that the NCX may be a key contributor to Ca^{2+} entry in Atlantic cod cardiomyocytes given that Atlantic cod hearts are unresponsive to adrenaline. In order to better investigate the role of NCX in Atlantic cod myocytes, an electrophysiological approach should be used similar to Abramochkin et al. (2013) to control membrane potential.

4.4.2. Sarcoplasmic Reticulum

In these experiments, ryanodine and thapsigargin were used to block SR-Ca^{2+} release and reuptake, respectively. The only effects of ryanodine and thapsigargin observed in this study were in steelhead trout myocytes exposed to tonic levels of adrenaline, where decreases were seen in both F/F_0 (~ 30% decrease) and F_0/F_r (~20 % decrease). The decrease in F/F_0 is consistent with Harwood et al. (2000), who also noted a 15% decrease in amplitude of the Ca^{2+} transient, and *in situ* studies that show a decrease in contractile force after the application of ryanodine / thapsigargin (Driedzic & Gesser, 1988; Aho & Vornanen, 1999). The proportional decrease in F_0/F_r can be explained by the smaller F/F_0, as F_0 was not increasing greatly as myocytes were stimulated. It was somewhat unexpected that F/F_0 would decrease following ryanodine / thapsigargin treatment in cardiomyocytes exposed to tonic adrenaline, but not in cells bathed in adrenaline-free saline. However, Cros et al. (2015) suggest that the SR is only needed during emergency/stressful situations, not for basal function. Thus, it may not contribute to the calcium transient when there is a lack of adrenergic stimulation or when cells are treated with physiologically relevant, unstressful conditions (10ºC, 50 stimulations min^{-1}). Blocking the SR had no effects on any aspects of the Ca^{2+} transient in Atlantic cod. It is possible that the SR is not an important contributor of Ca^{2+} in Atlantic cod, given their lack of response to adrenaline (Lurman et al., 2012) and that SR contributions are known to vary greatly between species (Keen et al., 1992; Aho and Vornanen, 1999; Shiels et al., 1999; Shiels and Farrell, 2000; Tiitu and Vornanen, 2001; Castilho et al., 2007; Haverinen & Vornanen, 2009b).

4.4.3. L-Type Ca^{2+} Channels

Blocking L-type Ca^{2+} channels with verapamil also had little / no effect on the Ca^{2+} transient in either species (Figure 3.6). The only effect seen was a decrease in F_p/F_r in steelhead trout in the presence of adrenaline. Of the three routes of Ca^{2+} entry, L-type Ca^{2+} channels are thought to be the primary route of Ca^{2+} entry into fish cardiomyocytes (Tibbits et al., 1992; Tiitu & Vornanen, 2003; Shiels & White, 2005), making the lack of an effect of verapamil on the dynamics of the calcium transient very surprising. As previously mentioned, however, it is possible that other routes of Ca^{2+} entry may compensate to maintain contractility when one route of Ca^{2+} entry / exit was blocked (also see next Section).

4.5. Further Study and Conclusions

4.5.1. Further Study

Adrenaline has been shown to be important in maintaining fish myocardial contractility during acute temperature changes (Franklin & Davie, 1992a; Keen et al., 1993; Aho & Vornanen, 2001; Shiels et al., 2003; Galli et al., 2009; Shiels et al., 2015), thus, additional experiments should be carried out across a range of temperatures to investigate if changes in the Ca^{2+} transient occur when faced with such thermal challenges. In particular, studies should focus on acute cooling where adrenaline is most important in rainbow trout (Ask et al. 1981; Ask 1983; Shiels and Farrell 1997; Aho and Vornanen 2001; Shiels et al., 2003). Studies should also cover a range of adrenaline concentrations (from tonic to

maximal), as rainbow trout exhibit a dose-dependent response to adrenaline (Shiels & Farrell, 1997; Shiels et al., 1998; Shiels et al., 2003) and many other studies provide data comparing myocardial function when exposed to tonic vs. maximal adrenaline concentrations (Shiels & Farrell, 1997; Shiels et al., 1998; Skov et al., 2008; Lurman et al., 2012).

The experiments involving pharmacological blockade (see Figures 3.5, 3.6) did not identify any major effects of the blockers in the absence of adrenaline, or any clear species differences in Ca^{2+} handling via the NCX, SR or L-type Ca^{2+} channels. This was very surprising given that prior to carrying out the current set of experiments the efficacy of the blockers were confirmed in cardiomyocytes given a single stimulation (see Appendix B). Previous studies have only shown the effects of these blockers at low (unphysiological; i.e. 6 – 12 min^{-1}) stimulation frequencies or following single stimulations (Hove-Madsen, 1992; Shiels et al., 1997; Shiels et al., 1998; Vornanen, 1997; Vornanen, 1998). Thus, it is possible that at physiologically relevant rates of stimulation (i.e. 50 min^{-1} as used in this study) the impact of a blocking a particular route of Ca^{2+} entry/exit/uptake is reduced as others pathways compensate to maintain contractility. Additional studies should expand on my experiments by blocking more than one channel at a time, for example blocking the SR and L-type Ca^{2+} channels to isolate contributions of the NCX to the Ca^{2+} transient at physiologically relevant pacing frequencies. Experiments should also be carried out across a range of acclimation temperatures, and following acute changes in temperature. This study only examined the effect of these blockers on cardiomyocytes at the fish's acclimation temperature, and

failed to highlight any inter-specific variation. It is possible that species differences would be seen when subjected to acute changes in temperature, as previous studies have shown that the relative contributions of the SR and L-type Ca^{2+} channels vary with temperature (Keen et al., 1994; Shiels and Farrell, 1997; Vornanen, 1998; Shiels et al., 2002a; Galli et al., 2009; Silva et al., 2011), and highlighted NCX as an important source of Ca^{2+} in the cold (Shiels et al., 2006).

4.5.2. Conclusions

Some key species differences in cardiomyocyte morphology/physiology were highlighted in this thesis, and the data contribute significantly to our understanding of how temperature affects fish cardiac physiology. For example, steelhead trout and Atlantic cod cardiomyocytes are morphologically different, with the latter being shorter, wider, and having a larger surface area (Table 3.1). Ca^{2+} transients from Atlantic cod cardiomyocytes were consistently faster in terms of time to peak Ca^{2+} and time to half relaxation across the range of stimulation frequencies tested (10 – 110 min^{-1}), and cardiomyocytes from Atlantic cod were more sensitive to acute temperature change than those from steelhead trout. In addition, my data provides additional evidence that Atlantic cod do not require adrenergic stimulation in order to maintain contractility (Axelsson et al., 1988; Lurman et al., 2012).

However, this thesis also provides some results that are not easy to explain or interpret. For example, the final experiments where L-type Ca^{2+} channels, the SR and the NCX were blocked only revealed a minor contribution of these modes of Ca^{2+}

entry/exit/uptake in the presence of 10 nM adrenaline in steelhead trout, but not in steelhead trout myocytes in adrenaline–free solution. This could possibly be explained by the fact that steelhead trout cardiomyocytes require tonic concentrations of adrenaline in order to function normally *in vivo* and under experimental conditions. However, this argument cannot be applied to Atlantic cod myocytes, as this study provided further evidence that adrenaline has no/minimal effects on the cardiac function of this species. It is possible that at low levels of performance that are physiologically relevant *in vivo*, it is difficult to detect antagonistic effects of the blockers used in these experiments. This hypothesis is based on the fact that I was able to demonstrate that KB-R7943 and ryanodine / thapsigargin were indeed having an effect on the Ca^{2+} transients of myocytes given a single stimulation (Appendix B). This discrepancy questions the use of single stimulations versus trains of stimulations at physiologically-relevant frequencies for studying / understanding the role of L-type Ca^{2+} channels, SR and NCX in fish cardiomyocyte Ca^{2+} transients (i.e. function) under various conditions.

Appendix A

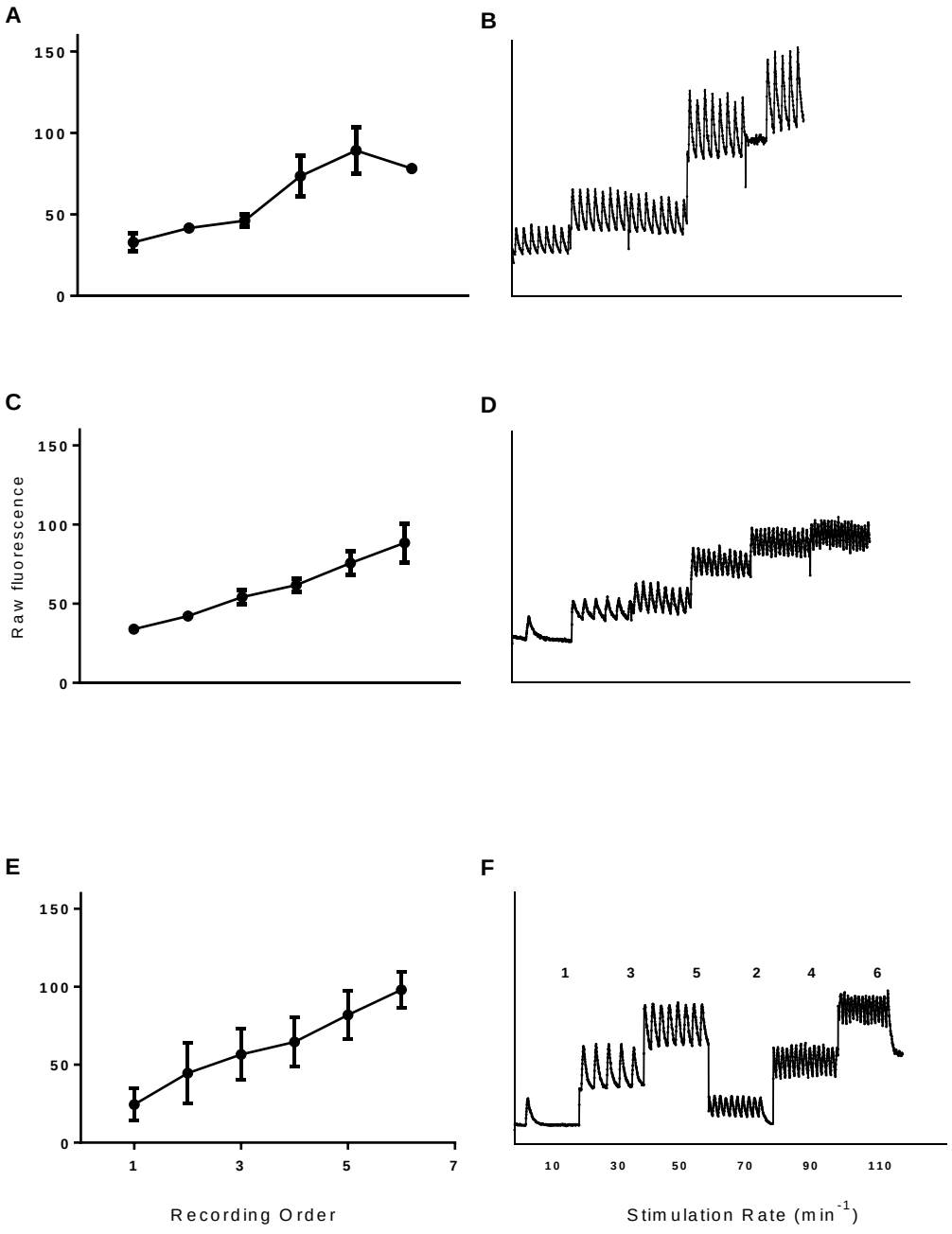

Figure A1. Preliminary experiments in which individual Atlantic cod myocytes were given repeated stimulations at 50 min^{-1} (A, B), multiple stimulation frequencies in ascending order (C, D) or these same frequencies in randomized order (E, F).

In preliminary experiments, individual cardiomyocytes from steelhead trout and Atlantic cod were repeatedly exposed to trains of stimulation to determine plausibility of making repeated measures of the Ca^{2+} transient on one individual myocyte (Figure A1). Cells were treated in the same manner as outlined in the method section, but were left for 5 minutes after each recording to allow the cells to recover from stimulation and exposure to the laser.

Cells were given three different protocols: 6 repeated measures at 50 stimulation min^{-1} (Figure A1 A, B), 6 measures with increasing stimulation frequency in order from 10 – 110 min^{-1} (Figure A1 C, D), and 6 measures between 10 – 110 min^{-1} where frequency was randomly ordered (Figure A1 E, F). Recording order is given in F from 1 – 6. Figures A1 A, C and E show average baseline values (F_0 in other experiments) ± SE. Figures A1 B, D and F show representative transients from individual Atlantic cod myocytes. Values are given in raw fluorescence (taken straight from video files) rather than F/F_0 in other experiments in order to compare fluorescence levels from each recording to the first video. Each set of experiments used the same settings on the Mag Biosystem video software and the same laser intensity throughout.

Whether recordings were carried out at 50 min^{-1}, in order of increasing frequency, or with frequency randomized, it is clear that baseline Ca^{2+} levels increased as the number of measurements increased. This was most likely due to irreversible damage caused by prolonged exposure to the laser, rather than the cardiomyocytes being subjected to high stimulation frequencies that are (particularly in Atlantic cod) not normally obtainable in muscle strip preparations. Based on the above data (Figure A1), I determined that it

would not be possible to take multiple recordings from one myocyte and maintain proper cell function. Thus, each cell was only given one train of stimulations in subsequent experiments.

Appendix B

In preliminary experiments, individual cardiomyocytes were given a single stimulation and recorded under control conditions (10ºC, 2 mM Ca^{2+}, no pharmacological blockers). The cardiomyocytes were then given 5 min to recover, and treated with KB-R7943 (10 µM) and given another single stimulation. The cardiomyocytes were then allowed to recover again, and treated with ryanodine (1 µM) and thapsigargin (10 µM) (RyTh), before being stimulated one final time.

This procedure was carried out in Atlantic cod (no adrenaline) and steelhead trout (with and without adrenaline), and the results (Figures A2 – A4) clearly show that these blockers had significant, and large, effects on the magnitude and the timing of single Ca^{2+} transients.

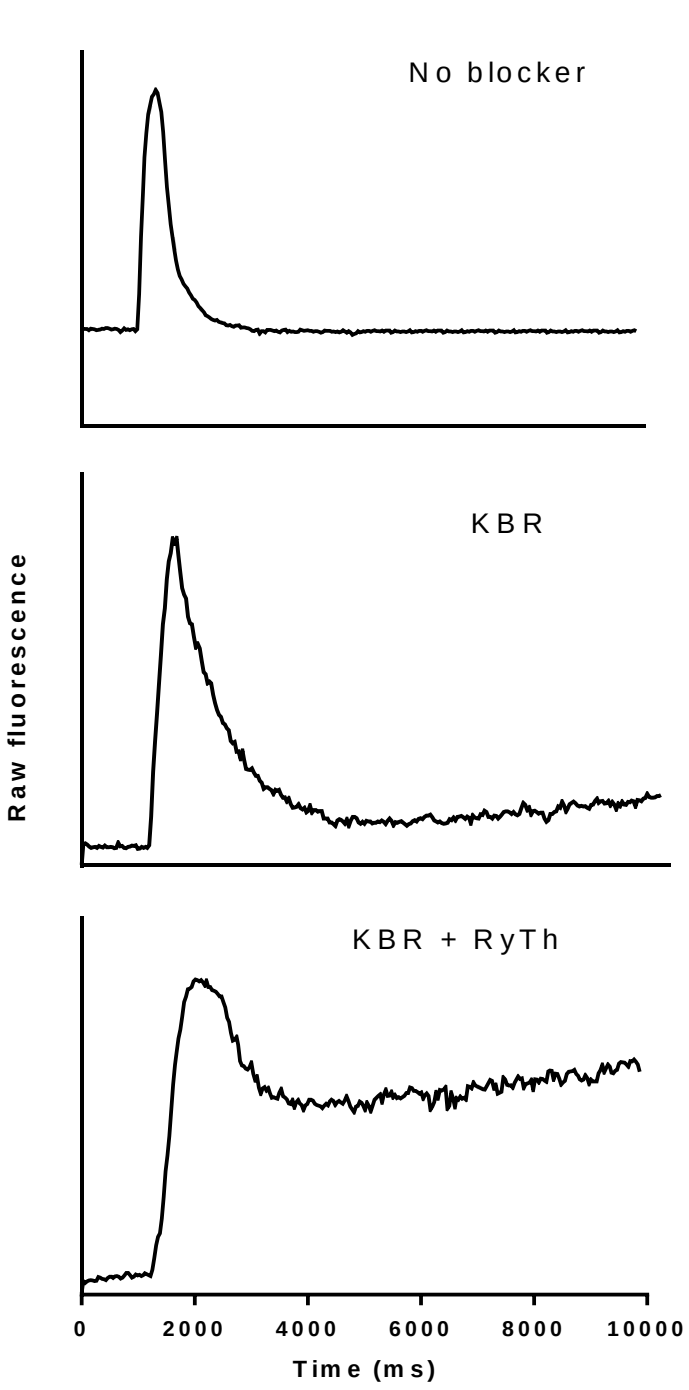

Figure A2. Sample transients from individual Atlantic cod cardiomyocyte stimulated once in control buffer, with KB-R7943 (10 µM) in the bath, and with KB-R7943 (10 µM), ryanodine (1 µM) and thapsigargin (10 µM) in the bath.

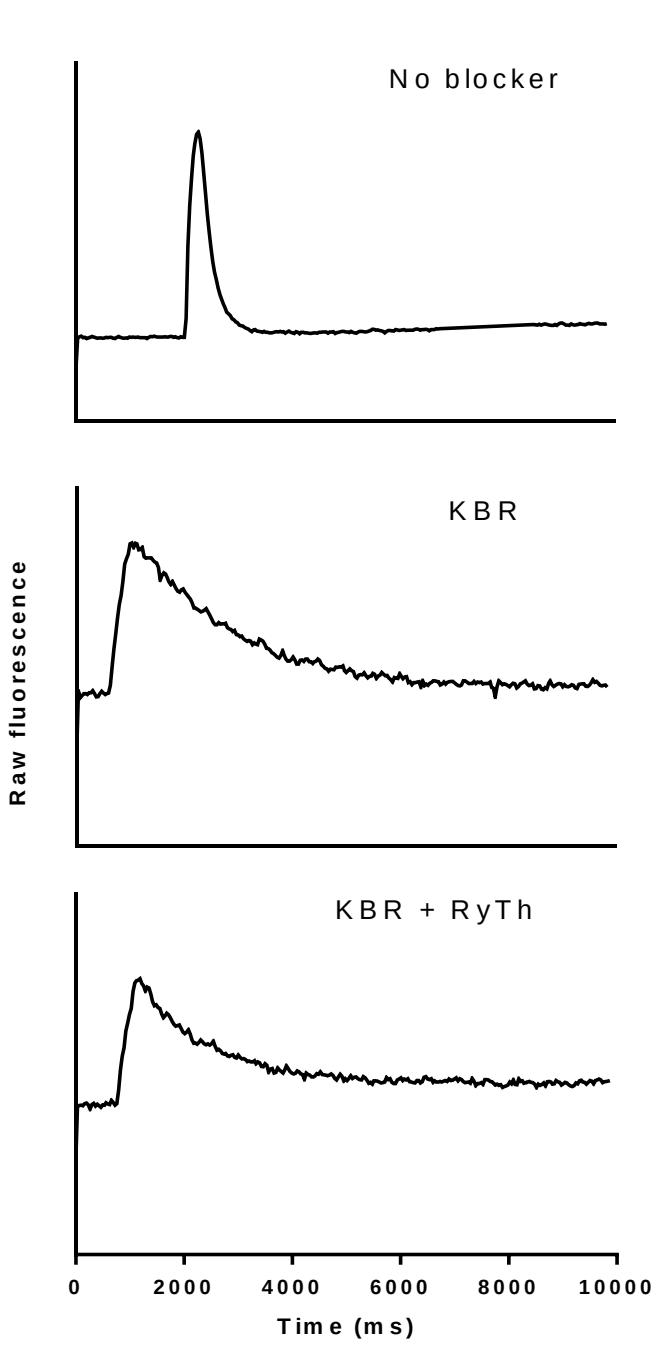

Figure A3. Sample transients from individual steelhead trout cardiomyocyte (no adrenaline in the bath) stimulated once in control buffer, with KB-R7943 (10 µM) in the bath, and with KB-R7943 (10 µM), ryanodine (1 µM) and thapsigargin (10 µM) in the bath.

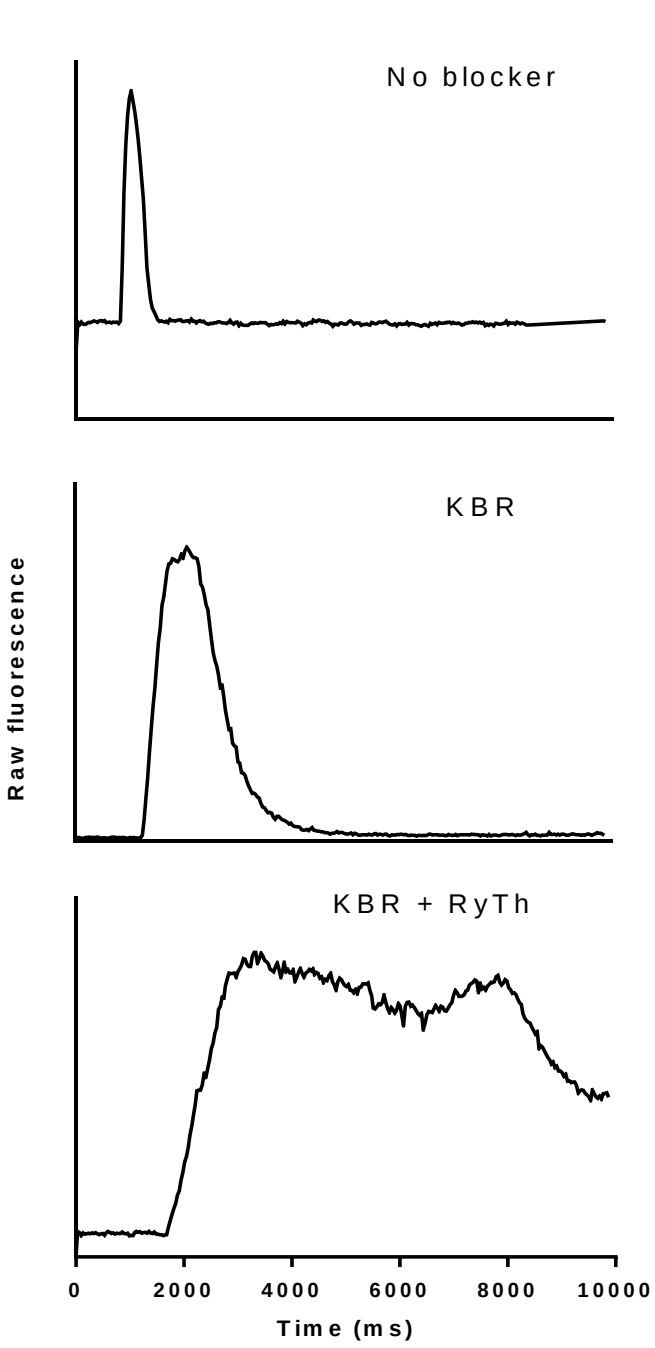

Figure A4. Sample transients from individual steelhead trout cardiomyocyte (with 10 nM adrenaline in the bath) stimulated once in control buffer, with KB-R7943 (10 µM) in the bath, and with KB-R7943 (10 µM), ryanodine (1 µM) and thapsigargin (10 µM) in the bath.

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