Functional Comparison of Cardiac Troponin C from Representatives of Three Vertebrate Taxa: Investigating the Evolution of Cardiac Contractile Function

by

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ABSTRACT

FUNCTIONAL COMPARISON OF CARDIAC TROPONIN C FROM REPRESENTATIVES OF THREE VERTEBRATE TAXA: INVESTIGATING THE EVOLUTION OF CARDIAC CONTRACTILE FUNCTION

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Trout cardiac troponin C (cTnC) contains differences in amino acid sequence that are partially responsible for the disparity in function between trout and mammalian hearts. Interestingly, the physiological temperature and amino acid sequence of cTnC from *Xenopus laevis* are intermediate between trout and mammals. As a result, the study of *Xenopus* cTnC may provide insight into the influence of physiological temperature on the evolution of cardiac function. Circular dichroism demonstrated that the melting temperature of the rat troponin complex was 6°C higher than one containing trout components, with *Xenopus* falling between. Ca²⁺-binding measurements indicated that *Xenopus* and trout troponin components increase the Ca²⁺-affinity of the troponin complex. The Ca²⁺ off-rates (k_{off}) of the complexes containing trout and *Xenopus* components were also lower than the rat complex. This suggests that the Ca²⁺-affinity and thermal stability of the troponin complex are both related to the physiological temperature of the species.
DEDICATION

For my father Paul, and my little brother Eric who will always be with me.
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List of Abbreviations

Ca\(^{2+}\): Calcium ion
CICR: Calcium induced calcium release
cTnC: cardiac troponin C
cTnI: cardiac troponin I
cTnT: cardiac troponin T
DHPR: Dihydropyridine receptors
FHC: Familial hypertrophic cardiomyopathy
HCM: Hypertrophic cardiomyopathy
IAANS: Anilinonaphthalenesulfote iodoacetamide
MCO: Molecular cut off
McTn: Mammalian (rat) cardiac troponin
NCX: Sodium (Na\(^{+}\)) / calcium (Ca\(^{2+}\)) exchange
PKA: protein kinase A
RyR: Ryanodine receptors
ScTn: Salmonid (rainbow trout) cardiac troponin complex (containing mammalian cTnI and cTnT)
SCITn: Salmonid (rainbow trout) cardiac troponin complex (containing mammalian cTnT)
SERCA: sarcoplasmic reticulum Ca\(^{2+}\)-ATPase RyR: Ryodine receptors
SL: Sarcolemma
SR: Sarcoplasmic reticulum
Tn: troponin
T53C: Threonine at position 53 mutated to Cysteine
XcTn: Frog (Xenopus) cardiac troponin complex (containing mammalian cTnI and cTnT)
INTRODUCTION TO CARDIAC CONTRACTILE REGULATION AND THE EVOLUTION OF ENDOTHERMY

1.1 OVERVIEW

This thesis focuses on the molecular interactions that govern myofilament function in the vertebrate heart, and how these regulatory mechanisms have changed over evolutionary time. The evolution of the cardio-pulmonary system within the vertebrate lineage, along with the emergence of endothermy is one of the central points. This is because the function of proteins, and indeed of most cellular processes, is significantly affected by temperature. An increase in body temperature is by definition necessary for endothermy, and can have an astounding effect on many different processes. Most important to this thesis is the effect that a change in temperature can have on muscle contraction, especially in the heart. An increase in cytosolic calcium (Ca$^{2+}$) concentration is the initiating factor for myocyte contraction (Bers, 2000). Thus, the sensitivity of cardiac myocytes to Ca$^{2+}$ is an extremely important factor when considering muscle contraction. Since increasing physiological temperature increases the sensitivity of myocytes to Ca$^{2+}$, it is not surprising that evolution has favoured an overall decrease in myocyte Ca$^{2+}$-sensitivity in endothermic species (Churcott et al., 1994). This is necessary in order to maintain a physiological Ca$^{2+}$ affinity with increased temperatures, as with too high a Ca$^{2+}$-sensitivity, the muscle would become locked in contraction when cytosolic Ca$^{2+}$ levels were even slightly elevated. From a human perspective, it may seem more incredible that ectothermic animals have hearts that can function 20°C below the temperature that our endothermic hearts would fail at. From an evolutionary perspective, however, it is the endothermic specialization to much higher temperatures that is arguably more interesting.
This study investigates the role that the troponin complex, specifically the calcium (Ca\(^{2+}\))-binding protein troponin C (TnC), plays in controlling cardiac contraction. Within, I review how the heart as a whole has evolved within the vertebrates, both on a large anatomical scale as well as on a molecular and biochemical level and how changes in protein sequence and in physiological temperature play into this.

1.2 **Evolution of the Cardio-Pulmonary System**

The evolution of the four-chambered heart in vertebrates is of great importance to our understanding of the requirements of both terrestrial lifestyle, and also of the evolution of endothermy. The hearts of proto-vertebrates were a simple muscular tube, but through evolutionary processes the heart became increasingly complex, both structurally and functionally (Farmer, 1999; Shaffer and Gillis, 2010). Through the formation of valves and septa, the proto-vertebrate muscular tube evolved to a form resembling that of the heart of extant teleosts, consisting of a linear series of four chambers; the sinus venosus, the atrium, ventricle and the bulbous arteriosus (see Figure 1) (Randall, 1968). Of these four chambers, only the atrium and ventricle are contractile. Blood flowing through the fish heart first enters the sinus venosus, then flows into the single atrium, followed by the ventricle. The contraction of the ventricle then pumps the blood through the elastic bulbous arteriosus, which serves a similar function as the mammalian aorta, before re-entering systemic circulation (see Figure 2 A) (Randall, 1968). The bulbous arteriosus is composed of non-contractile smooth muscle as well as elastic fibres, allowing absorption of some of the pulsatile force from the ejection of blood and reducing pressure fluctuations of blood flow to the gills.
Within extant fishes we are able to elucidate not only some of the first major alterations to occur within the vertebrate heart, but also the potential origin of the lung. The origin of lungs in fishes has long been viewed as an adaptation enabling fish to obtain oxygen from the air when their aquatic environments became hypoxic (Farmer, 1999). The possibility exists, however, that the selection pressure for lungs existed not necessarily for increased oxygen supply for the whole body, but for a more direct oxygen supply to the heart (Farmer, 1999). As seen in Figure 2 B, the cardio-pulmonary system of extant lungfish includes a circuit not only to the gills, but also to the lung. The lung circulation system is of utmost importance as it hints at the origins of the separation of pulmonary and systemic circulation – a key adaptation to increase efficiency of the circulatory system. Important to note here, however, is the fact that the heart itself in lungfishes remains relatively unchanged, with no separation of the atrium or ventricle seen and a significant mixing of the pulmonary and systemic blood.

The linear arrangement of cardiac chambers found in extant fishes and also in early vertebrates was altered in the amphibians, with a looping of the heart, and the formation of two separate atria, with one collective ventricle (see Figure 2 C) (Bishopric, 2005; Heinz-Taheny, 2009). Amphibians are also interesting in that gas exchange occurs in multiple locations including both the skin and lungs as well as buccopharyngeal mucosa at different lifestages (Johansen and Hanson, 1968). Although there are multiple gas exchange surfaces, only the pulmonary circuit has a specialized venous return to the heart (Johansen and Hanson, 1968).

The formation of separate ventricles as well as atria for discrete pulmonary and systemic circulation did not occur until evolution of the mammals, birds and crocodilians (see Figure 2 D) (Koshiba-Takeuchi et al., 2009). In endothermic species, the four
contractile chambers achieve complete isolation of pulmonary from systemic circulation. This separation of the circulatory system is extremely important to the efficiency of cardiac and metabolic activity, and is thought to be an important step in the evolution of endothermy (discussed below) (Hillenius and Ruben, 2004). This separation is key in two regards, firstly it allows separation of oxygenated and deoxygenated blood, which avoids mixing and thus higher oxygen concentrations are delivered to the systemic branches overall, and secondly it allows different blood pressures for the two systems. This is very important as in order to deliver sufficient oxygen to systemic systems to maintain the high metabolism necessary for endothermy, blood must be moved quickly and at high pressures (Bennett, 1991). Blood pressure in the pulmonary system however must be kept at relatively low pressures, as tissues are all very thin to allow diffusion of oxygen to the blood. In today’s mammals, blood pressures within the systemic system are therefore often 10 times higher than those in the pulmonary system (~120 mmHg vs ~13 mmHg) (Alpert et al., 1974; Euler and Liljestrand, 1946).
Figure 1: A representation of the chamber arrangement of the teleost heart. Used with permission from Randall, 1968. Blood flow is represented by the arrows, entering first the sinus venosus, then the atrium, flowing into the ventricle and finally through the bulbus arteriosus before re-entering circulation to the gills and other tissues.
Figure 2: Chamber arrangement in extant vertebrate groups. These include A) teleost fishes; B) lungfish; C) amphibians; D) mammals and birds. Within you can see the evolution of the four-chambered heart from a two chambered linear arrangement to the addition of a lung, a looping of the heart and finally full septation of the ventricles. Figure drawings performed by Bruce Darling.
1.3 The Evolution of Endothermy

The maintenance of a high and stable internal body temperature by metabolic means despite fluctuations in external environmental temperatures (endothermy) is an adaptation that is perhaps one of the most well known of extant birds and mammals. In these homeothermic species high internal body temperatures are maintained by both increasing metabolic rate and by minimizing thermal loss with fur, feathers and subcutaneous fat stores. Shivering and non-shivering thermogenesis are also used to increase heat production in response to cold in birds and mammals (Ruben, 1995). Several other phyla have examples of species with heterothermy, including the lamnid sharks, as well as three different families of teleost fish: the makerels, tunas and billfishes (Block et al., 1993). In addition to these fishes, some larger reptiles such as the python can maintain higher than ambient temperatures during brooding seasons using spasmodic contractions of their axial muscles (Ruben, 1995). Although the recurrence of endothermy across multiple phyla indicates some adaptive advantage, the endothermy we see in extant mammals and birds differs from other phyla in the source of metabolic heat. In the fishes and reptiles, the elevated body temperatures are achieved through metabolic heat originating from skeletal muscle. Birds and mammals on the other hand depend not on myogenic heat but on metabolic heat from visceral organs such as the liver and kidneys (Hayes and Garland, 1995). The selective pressure that resulted in the evolution of endothermy in mammals and birds is therefore a subject under much contention. Some proposed factors include: increased brain size, improved postural attributes, increased capacity for thermoregulation and increased aerobic capacity (Koteja, 2004; Ruben, 1995). The final two theories, termed herein the thermoregulatory hypothesis and the aerobic capacity hypothesis, are the two
Theories most widely accepted among researchers today.

The thermoregulatory hypothesis for the evolution of endothermy stipulates that increases in metabolic rate were selected upon because increases in body temperature helped to regulate changes in internal temperature in different environments (Bennett et al., 2000). In brief, according to this hypothesis, the benefits of thermoregulation must have outweighed the energetic costs and were therefore selected for. These benefits include the specialization of enzymes to a single temperature (Somero, 2003) as well as thermal niche expansion (Block et al., 1993). Despite this, experimental tests of the thermoregulatory hypothesis have found evidence against it. A study on highly active lizards (*Varanus exanthematicus*) found that despite increasing metabolic rates by 3-4 fold, body temperature only increased very slightly and decreased in colder environments at the same rate as at lower metabolic rates (Bennett et al., 2000). This suggests that perhaps thermoregulation was not the selection factor for the evolution of endothermy, although it remains a viable contender in the literature.

The aerobic capacity model for the evolution of endothermy stipulates that endothermy evolved due to selection acting to increase maximal aerobic capacity and associated behaviours (Hayes and Garland, 1995; Taigen, 1983). This model states both that selection acted to increase maximal aerobic metabolic rate during activity and also that maximal aerobic rates are linked to resting rates, thus resting metabolic rates increased alongside maximal rates (Hayes and Garland, 1995). Higher maximal aerobic capacity could be advantageous for a myriad of reasons from competition for resources to predator evasion to expanding the size of foraging area (Hayes and Garland, 1995). Several studies have investigated whether resting metabolic rates are linked to maximal rates, and it seems that across vertebrates there is a relatively consistent difference between resting and
maximal metabolic rates (Bennett and Ruben, 1979). This consistent scope can be seen as evidence of a linkage between the two states, and suggests that aerobic capacity is a strong candidate for the evolution of endothermy (Bennett and Ruben, 1979). This is the model that is currently most supported in the literature, and seems logical given the physiological characteristics we see in today’s endotherms, including full separation of pulmonary from systemic circulation and high metabolic activity.

Endothermy has obvious energetic demands for the organism (Schweitzer and Marshall, 2001). Endothermy in birds and mammals requires elevated oxygen consumption and aerobic metabolism compared to ectotherms for heat production to maintain body temperature (Hillenius and Ruben, 2004). High activity is thought to be a strong pressure for air breathing even in today’s lungfishes. In fact, oxygen consumption levels of endothermic vertebrates are on average five to ten-fold higher than the oxygen consumption of a size-matched ectotherm (Bennett and Ruben, 1979). In addition, the energetic demands of the terrestrial lifestyle of many endotherms are also higher than those of an aquatic lifestyle. The energy cost of swimming animals, when compared to that of running and flying animals is significantly lower (Schmidt-Nielsen, 1972). This is in large part because buoyancy in an aquatic environment, alleviates much of the energy costs of gravity. In comparison, terrestrial animals must expend significant energy to lift themselves away from the ground while they move, whether their locomotion is by walking, running or flying (see Figure 3) (Schmidt-Nielsen, 1972). As such, terrestrial endotherms have much higher metabolic costs than aquatic ectotherms, and therefore require high output circulatory systems to maintain their internal temperatures and their locomotory needs.

The higher cost of endothermy is also reflected in the mitochondrial content of
animals’ tissues. Endothermic vertebrates have significantly higher cytochrome oxidase levels in many tissues than ectothermic animals of the same size (Hulbert and Else, 1989). When standardized to total protein content, the cytochrome oxidase activity in heart, lung and skeletal muscle of a mammal is two-fold higher than in a reptile (Hulbert and Else, 1989). As the final enzyme in the mitochondrial electron transport chain, cytochrome oxidase activity is indicative of mitochondrial activity in tissues. This suggests that in these key aerobic tissues, mammals have much higher energetic outputs than reptiles. It is this high energy output and high body temperatures that necessitate changes to both the cardio-pulmonary system as a whole, discussed above, as well as to cardiac muscle structure and function, which I discuss next.
Figure 3: The net energy cost of different types of locomotion. Used with permission from Schmidt-Nielsen (1972), comparing swimming to flying and running. Terrestrial locomotor costs, including running and flying are both much more metabolically expensive than aquatic costs.
1.4 Cardiac Contraction and Regulation

1.4.1 Muscle contraction overview

Although many anatomical and functional changes have occurred to the vertebrate heart over evolutionary time, the basic mechanism of striated muscle contraction has not changed (Shaffer and Gillis, 2010). The sliding filament theory of muscle contraction proposes that muscle fibres undergo contraction in a highly specialized way, dependent upon the contractile proteins found within the muscle. The myofilaments are the contractile elements of each cell, and are primarily composed of thin and thick filaments, along with other associated proteins (Huxley, 1961, 2004; Rayment et al., 1993). The thin filaments are made up of double helically wound actin, while the thick filaments are composed of myosin with the large myosin heads protruding in a rotational manner (Rayment et al., 1993). These myofilaments are arranged into sarcomere units, which are bound at either end by Z-discs - the location at which the actin thin filaments are bound. The thick filaments do not span the entire width of the sarcomere, and are held in the centre of the sarcomere, bound to the Z-discs by the protein titin (Gordon et al., 2000). The sliding of the thick filaments over the thin filaments, pulling the two Z-discs closer together, is what ultimately results in sarcomeric shortening, and muscle contraction (Huxley, 2004).

The troponin complex is a set of regulatory contractile proteins bound to the thin filaments. The complex is composed of three subunits: troponin I (TnI), troponin T (TnT) and troponin C (TnC), which act together to control the calcium (Ca\(^{2+}\)) activation of striated muscle contraction (Barton et al., 2004; Filatov et al., 1999). In short, TnC binds Ca\(^{2+}\), while TnI inhibits the ATPase activity of actomyosin, and TnT binds the inhibitory protein tropomyosin (Barton et al., 2004; Filatov et al., 1999). The release of Ca\(^{2+}\) from the
sarcoplasmic reticulum is followed by the binding of Ca$^{2+}$ to the site II regulatory region of TnC, resulting in a conformational change of the protein (Solaro and Rarick, 1998). This allows the movement of the TnI switch peptide away from the actin myofilament, towards a hydrophobic cleft within the N-terminus of TnC. This pulls the inhibitory peptide of TnI away from tropomyosin. The release of tropomyosin allows it to be shifted across the surface of the actin filament by TnT, exposing myosin binding sites on the thin filament. Myosin heads from the thick myofilaments can then bind to the actin filament, forming cross-bridges. Then, through the hydrolysis of ATP, the myosin heads undergo conformational changes resulting in the movement of the thick filament along the thin filament and ultimately, muscle contraction (Barton et al., 2004).

1.4.2 Calcium Cycling in Mammalian Cardiomyocytes

The initiation of cardiac contraction in mammals occurs following membrane depolarization, when voltage-gated Ca$^{2+}$ channels open on the sarcolemma (SL), allowing the entrance of extracellular Ca$^{2+}$ into the cell. This small rise in intracellular [Ca$^{2+}$] then triggers the release of Ca$^{2+}$ from intracellular stores within the sarcoplasmic reticulum (SR) (Bassani et al., 1995). This stepwise increase in cytosolic Ca$^{2+}$ is known as Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) and results in the activation of the Tn complex via troponin C. The end result is the formation of cross-bridges between actin and myosin and the contraction of the myocyte. Following contraction, relaxation of the muscle must occur, requiring a decrease in intracellular [Ca$^{2+}$], and therefore Ca$^{2+}$ must again be sequestered away from the myofilaments. This occurs by the movement of Ca$^{2+}$ out of the cell, as well as by a sequestration of Ca$^{2+}$ by the SR. The movement of Ca$^{2+}$ out of the cytosol occurs via four different pathways, primarily involving the sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA)
and sarcolema \( \text{Na}^{+}/\text{Ca}^{2+} \) exchange (NCX), but also including the sarcolemmal \( \text{Ca}^{2+} \)-ATPase and the mitochondrial \( \text{Ca}^{2+} \) uniport system (Bers, 2000, 2002). This whole process, known as excitation-contraction, is the basic mechanism by which contraction of muscle is triggered (Bers, 2002; Fabiato and Fabiato, 1972; Langer, 1973). An overview of the key movements of \( \text{Ca}^{2+} \) in and out of the cytosol outlined briefly above can be seen in Figure 4, taken from Bers (2002).

The SL is a system of membranes responsible for separating and facilitating solute transfer between the intracellular and extracellular environments. Included within the SL system of mammalian cardiomyocytes is a network of invaginations called T-tubules that protrude into the cell. These T-tubules are the primary regions through which \( \text{Ca}^{2+} \) enters the cell via voltage-gated L-type \( \text{Ca}^{2+} \) channels called dihydropyridine receptors (DHPR) (Bers, 2002). The T-tubules allow the uniform release of \( \text{Ca}^{2+} \) throughout the cell, allowing faster cycling rates. The increase in intracellular \([\text{Ca}^{2+}]_c\) from the SL triggers the release of \( \text{Ca}^{2+} \) from the SR through the SR release channels (or ryanodine receptors; RyR). The SR plays the role of intracellular \( \text{Ca}^{2+} \) store in myocytes, responsible for sequestering \( \text{Ca}^{2+} \) during relaxation and releasing it to trigger contraction. It has been found that the SR of mammalian cardiomyocytes primarily interact with the T-tubules in a region known as the terminal cistae, where junctions between the DHPR and RyR occur (Scriven et al., 2000). These junctions consist of clusters of RyR in close proximity to DHPR known as “couplons” (Franzini-Armstrong et al., 1999), with the space between them being referred to as the diadic cleft (Langer and Peskoff, 1996). The influx of \( \text{Ca}^{2+} \) into the dyadic cleft from the DHPR results in a subsequent release from the SR through RyR, resulting in a very quick increase in the local \([\text{Ca}^{2+}]_c\) of the dyadic cleft (Langer and Peskoff, 1996). A
Ca\(^{2+}\)-binding protein called calsequestrin situated on the junctional SR membrane is responsible for buffering the local Ca\(^{2+}\) amounts (Beard et al., 2004). The buffering of Ca\(^{2+}\) levels in and around the dyadic cleft aids in facilitating activation of RyR, and thus the release of Ca\(^{2+}\) from the SR. This orchestration therefore allows an increased rate of CICR from the SR and faster Ca\(^{2+}\) cycling on the whole.

The Ca\(^{2+}\) released from the SR then goes on to interact with troponin C on the actin thin filament, resulting in myofilament interaction and ultimately contraction of the myocyte. Following contraction, relaxation of the muscle necessitates another orchestrated movement of Ca\(^{2+}\), this time out of the cytosol and away from the myofilaments. This reduction in cytosolic [Ca\(^{2+}\)] results from the movement of Ca\(^{2+}\) both out of the cell as well as back into the SR. The primary pathways are described above, lacking only the role of the protein phospholamban (PLB). PLB is a pentameric protein situated on the SR membrane is close association with SERCA (Tada and Toyofuku, 1998). Its role is to slow SERCA and thus, the uptake of Ca\(^{2+}\) into the SR during contraction. Its propensity to inhibit SERCA is changed by phosphorylation state and has a role in determining the rate of contraction as well as contractility (Frank and Kranias, 2000; Koss and Kranias, 1996; MacLennan and Kranias, 2003).
Figure 4: Movement of Ca$^{2+}$ within mammalian cardiomyocytes. Used with permission from Bers (2002). The movement of Ca$^{2+}$ into the cell primarily occurs via dihydropyridine receptors (DHPR) Ca$^{2+}$ channels within the T-tubules, initiating a larger Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR). This Ca$^{2+}$ then interacts with the myofilaments of the cell, resulting in cell contraction. For relaxation to occur, the Ca$^{2+}$ must be re-sequestered into the SR via sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) (mediated in turn, by phospholamban [PLB]), as well as pumped back out through the sarcolemma (SL) via Ca$^{2+}$-ATPase and Na$^+$/Ca$^{2+}$ exchange (NCX), and finally sequestered in the mitochondrion using a Ca$^{2+}$ uniport system.
1.4.3 The Role of Phosphorylation

Many different factors influence myocyte contractility, including Ca\(^{2+}\) sensitivity, the rate of cross-bridge cycling, the rate of ATP hydrolysis and the strength of interactions between contractile proteins (Gordon et al., 2000). The β-adrenergic system is an important regulator of cardiac output in vertebrates, affecting both the rate and strength of muscle contraction (Rapundalo, 1998). Binding of β-agonists (such as epinephrine and norepinephrine) to receptors activates adenylylate cyclase via G-proteins, resulting in a rise of cyclic-AMP in the myocyte (Hausdorff et al., 1990). This results in the activation of cyclic-AMP dependent protein kinase A (PKA), which can then phosphorylate specific amino acid residues on proteins (Mersmann, 1998). β-adrenergic agonists can affect cardiac muscle both by stimulating the pacemaker cells and altering heart rate, as well as by causing the phosphorylation of the contractile proteins within myocytes, affecting the strength of muscle contraction (Clarke et al., 1995; Rapundalo, 1998). Only some proteins can be phosphorylated, due to both their amino acid sequence and their tertiary structure, which governs the amino acid residues being exposed to phosphorylating agents (Rapundalo, 1998). Only certain amino acid residues are capable of being phosphorylated (tyrosine, serine and threonine), meaning that small changes in residues may significantly affect whole protein phosphorylation state (Rapundalo, 1998). Phosphorylation of a number of regulatory proteins have been found to affect contractility, including myosin, TnT, TnI, myosin light chain, myosin binding protein C, titin and phospholamban among others (Adelstein, 1983; Gruen et al., 1999; MacLennan and Kranias, 2003; Robertson et al., 1982; Tada and Toyofuku, 1998; Westfall and Metzger, 2007; Yamasaki et al., 2002; Yuan et al., 2006). For review, see Adelstein (1983) and Stull et al. (1980).
1.5 **DIFFERENCES IN CARDIAC PERFORMANCE BETWEEN ECTOTHERMS AND ENDOTHERMS**

1.5.1  **Cardiac Output and Stretch Response**

One of the major measures of cardiac performance is cardiac output, which is defined as the volume of blood ejected from the heart per minute, and can be described by the equation:

\[ Q = SV \times HR \]

wherein Q represents cardiac output (in volume of blood per minute), SV represents stroke volume (in volume of blood per beat) and HR represents heart rate (in beats per minute, bpm). Fish primarily use stroke volume to control cardiac output by regulating the ejection fraction from the ventricle as well as the stretching of the atrium (Shaffer and Gillis, 2010).

A distinct difference exists in how extant endothermic mammals and birds manage cardiac output, however, as they modulate not stroke volume but instead their heart rate to control cardiac output. Although amphibians and reptiles can also modulate their cardiac output by heart rate, the degree to which heart rate can increase in ectotherms does not reach that of endotherms (Farrell, 1991). There appears to be an upper limit of 120 bpm in a wide variety of ectotherms, across fishes, amphibians and reptiles (see Figure 5). This upper limit in no way holds true for endotherms, however, where hummingbirds can have a heart rate of ~1200 bpm and rats have a heart rate of nearly 600 bpm (Farrell, 1991). It is thought that as ectotherms, it is simply unnecessary to increase metabolic rates to the point that has occurred in endotherms (Bennett and Ruben, 1979). The maintenance of such a high internal body temperature in endotherms can often necessitate extreme increases in heart rate. Increased body temperature and high heart rates are therefore thought to have
evolved together. From this it can be discerned that not only anatomical changes to the heart have occurred over evolutionary time, but also regulatory changes to allow such drastic changes in the heart rates of endotherms.

The discrepancy in control of cardiac output along with large anatomical differences at the whole heart level leads to a discussion of other key differences in muscle function between ectotherms and endotherms. One difference that can be related back to control of cardiac output is the response of cardiac muscle to stretch. In general, the stretch response of cardiac muscle is defined by the Frank-Starling mechanism, which stipulates that a stretch of the muscle will result in an increase in the force of contraction (Shiels and White, 2008a; Shiels et al., 2006). The Frank-Starling response can therefore be described as a curve, with increasing tension development over increasing sarcomere lengths, with tension eventually reaching an asymptote, and force of contraction decreasing at extremely long sarcomeric lengths (Shiels and White, 2008a). Interestingly, fish cardiac myocytes have been found to develop increasing tensions at larger sarcomere lengths than mammalian myocytes (Shiels et al., 2006). It is thought that this difference in stretch response is key to changing cardiac output by stroke volume. By allowing more stretch in each myocyte, the fish heart can then have a greater modulation of heart volume than a mammalian heart can undergo. Some reports suggest that amphibian cardiomyocytes are also capable of maintaining active tension over longer sarcomeric lengths than mammals, but few studies have focused on this exclusively (Allen and Blinks, 1978; Tarr et al., 1981). It has been suggested that there is especially high interspecific variability in the amphibians, due to large differences in dessication tolerance, known cause changes in blood volume due to dehydration. This may mean that amphibians have variable ability to alter cardiac output depending on their lifestyle (Burggren et al., 2011; Shiels and White, 2008a)
Figure 5: Relative heart rate ranges of different vertebrate species. Modified from Farrell (1991). Heart rates of endothermic species seem to be consistently higher than those of ectothermic species, the division between species shown by the red line. Tuna, as a heterothermic teleost appear to be an exception to this, with higher heart rates than any other ectotherms. Key for ectotherms and experimental temperatures is: 1) Scaphiopus, 25°C; 2) Bufo, 25°C; 3) Xenopus, 25°C; 4) Rana, 25°C; 5) Natrix, 25°C; 6) Myxine, 11°C; 7) Bufo, 20°C; 8) Salmo, 20°C; 9) Ophiosaurus, 25°C; 10) Rana, 20°C; 11) Salmo, 10°C; 12) Testudo, 25°C; 13) Gadus, 10°C; 14) Iguana, 35°C; 15) Varanus, 35°C; 16) Hemitripterus, 10°C; 17) Ophiodon, 10°C.
1.5.2 Comparison of Different Species Cardiac Myocytes

Temperature affects heart function at multiple levels of organization, including whole organ, cellular and molecular levels. Considering these effects from a physiological standpoint helps us to elucidate some of the key factors that evolutionary pressures may have selected for. Decreased body temperatures results in an increase in blood viscosity (Eckmann et al., 2000) which in turn translates to a higher work load for the heart. Decreased temperature also decreases the functional rates of enzymes, which results in a myriad of changes to the function of the heart. Ectothermic animals that live at different environmental temperatures therefore must have muscles that are adapted to function properly at their physiological temperature. With this in mind, comparing cardiac myocytes from different species reveals a series of differences both in morphology as well as in function (see Table 1). Perhaps one of the most obvious differences between mammalian cardiomyocytes and all other species is the presence of T-tubules. It is thought that it is the low surface area to volume ratio of mammalian cells that necessitates the presence of T-tubules. The relatively low volume of other species cardiomyocytes (and thus, high surface area to volume ratio) is in large part due to their long, thin shape which allows them to have interactions between the RyR and DHPR at the surface sarcollemma instead of within tubules (Shiels and White, 2008a).
Table 1: Comparison of cardiac myocyte morphology in extant vertebrates. Representative fish, amphibian, reptile, bird and mammal are shown. Adapted from Table 1 in Shiels and White (2008a).

<table>
<thead>
<tr>
<th></th>
<th>Trout</th>
<th>Frog</th>
<th>Turtle⁵</th>
<th>Turkey⁶</th>
<th>Rat⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell length (µm)</td>
<td>159.8a</td>
<td>300d</td>
<td>189.1</td>
<td>136</td>
<td>141.9</td>
</tr>
<tr>
<td>Cell width (µm)</td>
<td>9.9a</td>
<td>5d</td>
<td>7.2</td>
<td>8.7</td>
<td>32</td>
</tr>
<tr>
<td>Cell depth (µm)</td>
<td>5.7a</td>
<td>n/a</td>
<td>5.4</td>
<td>n/a</td>
<td>13.3</td>
</tr>
<tr>
<td>Cell capacitance (pF)</td>
<td>46b</td>
<td>75e</td>
<td>42.4</td>
<td>25.9</td>
<td>170.2</td>
</tr>
<tr>
<td>Volume (pL)</td>
<td>6.5b</td>
<td>2.9f</td>
<td>2.3</td>
<td>4.0</td>
<td>34.4</td>
</tr>
<tr>
<td>SA/V ratio (pF/pL)</td>
<td>18.2b</td>
<td>25.8f</td>
<td>18.3</td>
<td>6.5</td>
<td>4.9</td>
</tr>
<tr>
<td>T-tubules</td>
<td>Nonec</td>
<td>Nonee</td>
<td>None</td>
<td>None</td>
<td>Present</td>
</tr>
</tbody>
</table>

Note: SA/V is surface area (determined by cell capacitance)/volume. Data are means, s.e.m. has been left out. ⁵(Galli et al., 2006); ⁶(Vornanen, 1998); ⁷(Shiels and White, 2005); ⁸(Goaillard et al., 2001); ⁹(Bean et al., 1984); ¹⁰[derived from Goaillard et al. (2001) assuming elliptical cross sectional area]; ¹¹(Kim et al., 2000); ¹²(Satoh et al., 1996)
Chapter 1.5.3  Comparison of Response to Temperature Changes

The response of a mammal to a decrease in external temperature is to maintain a steady internal body temperature. This occurs by sequestering heat internally within the vital organs, reducing blood flow to the extremities and by adjustments of metabolic rate. When the external temperature of an ectotherm changes, however, their internal body temperature changes as well. These two thermal lifestyles are therefore remarkably different at an organismal as well as at a cellular level. Where an endotherm’s heart becomes dysfunctional, with ventricular fibrillations occurring after an acute drop in temperature (Julian et al., 1956), many eurythermal fishes regularly experience seasonal changes in temperature of up to 20 °C without any apparent loss of cardiac function, given appropriate acclimation (Aho and Vornanen, 2001; Threader and Houston, 1983).

It is known that changes in temperature naturally result in changes in blood pH in ectotherms, with pH decreasing as temperature increases by approximately -0.0175 units/°C (Malan et al., 1976; Reeves, 1972). The protection of protein charge states is carried out via alpha-stat regulation, wherein imidazole groups from histidine-containing proteins dissociate into the bloodstream. This dissociation, along with changes in the partial pressure of CO₂ are what are responsible for the relatively small change in blood pH over changes in temperature (Malan et al., 1976). Alpha-imidazole, or alpha-stat regulation thus maintains the acid-base balance in ectotherms despite acute changes in temperature, and allows proper protein function over a range of temperatures. Endothermic vertebrates, by maintaining a constant internal body temperature, avoid the changes in pH associated with changing temperature. This means that endothermic proteins (enzymatic, structural, etc.) are often specialized to function best within a very narrow temperature and pH range.


1.6 Structure of Troponin C

Troponin C is a 18 kDa protein that can be classed within the family of Ca\(^{2+}\)-binding proteins (a multigene group including calmodulin, myosin light chains 1 and 2, parvalbumin, and the intestinal Ca\(^{2+}\)-binding protein). All of these proteins include at least one structure called an EF-hand, or a helix-loop-helix Ca\(^{2+}\) binding site characterized by a binding loop (a 12 amino-acid long sequence) contained between a pair of \(\alpha\)-helices (Parmacek and Leiden, 1991). The structure and function of TnC (both skeletal and cardiac) has been described multiple times using different techniques. Skeletal TnC was the first of the TnC isoform to have its structure determined using X-ray crystallography in 1985 (Herzberg and James, 1985, 1988; Satyshur et al., 1994; Sundaralingam et al., 1985a, 1985b), following which nuclear magnetic resonance (NMR) techniques were used to reinforce the determined structure (Li et al., 1995, 1997; Spyracopoulos et al., 1997). TnC is said to have a dumbbell-like structure, with two globular terminal domains connected by a long central helix (see Figure 6) (Filatov et al., 1999). The N-terminal domain contains five \(\alpha\)-helices, (termed helix N, A, B, C and D), while the C-terminal domain contains four (helix E, F, G and H). Each of the terminal globular domains contains two EF-hand Ca\(^{2+}\) binding sites, termed sites I-IV (sites I and II in the N-terminal domain, and sites III and IV in the C-terminal domain). The C-terminal binding sites (III and IV) are able to bind both Mg\(^{2+}\) and Ca\(^{2+}\) with a very high affinity, and as such are always occupied under physiological conditions (Parmacek and Leiden, 1991). It is thought that these high-affinity C-terminal sites have a structural role in the protein, helping it remain anchored to the rest of the troponin complex (Gillis et al., 2007). The N-terminal binding sites I and II, however, are Ca\(^{2+}\)-specific, with much lower affinities for divalent cations than their C-
terminal counterparts. Two mutations in binding site I of the vertebrate cardiac TnC isoform (D29L and D31A) as well as an insertion (Val-28) have resulted in the inactivation of this site, leaving only site II as a low-affinity binding site for Ca\(^{2+}\) (Van Eerd and Takahashi, 1975; Gillis et al., 2007). This means that the cTnC isoform contains one active binding site, while the skeletal (sTnC) isoform contains two. This difference in the number of active binding sites has an impact on the ability to regulate activation of the muscle. The second Ca\(^{2+}\) binding site on sTnC functionally “opens” the N-terminal structure of the protein more, exposing a larger hydrophobic surface area between apo and Ca\(^{2+}\)-saturated protein states (Spyracopoulos et al., 1997). This in turn is thought to translate functionally to more distinct activated and deactivated states in sTnC, where modulation of activation in the cTnC isoform is more dependent on interactions with other proteins.

The binding of Ca\(^{2+}\) to TnC and the interaction between the components of the Tn complex during this activation has also been studied by a variety of techniques including fluorescence spectrometry, circular dichroism (CD), multidimensional NMR, as well as direct binding measurements such as calorimetry (Campbell and Sykes, 1991; Hincke et al., 1978; Hinkle and Tobacman, 2003; Horwitz et al., 1979; Ingraham and Swenson, 1983; Lin and Cassim, 1978; Mani et al., 1975; McCubbin et al., 1974; Slupsky and Sykes, 1995; Yamada, 1999).
Figure 6: Ribbon diagram of cTnC based on NMR structure by Sia et al. (1997) (PDB#1AJ4). N-terminal low affinity domain is shown in top right with helices N, A, B, C and D labelled. C-terminal high affinity domain is shown in bottom left with helices E, F, G and H also depicted. Ca$^{2+}$-binding sites indicated by blue spheres, and flexible linker shown in centre between helices D and E.
1.7 Evolution of Troponin C

The recurrence of the EF-hand structure in Ca\(^{2+}\) binding proteins across multiple genetic loci is interesting in an evolutionary perspective, with 45 subfamilies of EF-hand proteins identified. Many of these subfamilies are thought to have evolved from a single common domain, with gene duplication events and speciation (Kawasaki et al., 1998). With this in consideration, the importance of proper cTnC function is underscored by the fact that over 250 million years of evolution, the protein sequence has been highly conserved (Parmacek and Leiden, 1991). Across vertebrates, over 80% conservation of cTnC sequence has been maintained between lamprey, teleost fish, amphibians and endotherms, with over 90% conservation within jawed vertebrates. Despite this fact, some sequence changes have occurred that have had significant impacts on the function of the protein. As reviewed by Gillis et al. (2007), TnC has an interesting evolutionary history, with all animals from amphioxus to beetles to vertebrates containing some ortholog of the protein. Interestingly, it is only vertebrates that have undergone a TnC duplication resulting in separate cardiac and skeletal TnCs (cTnC and sTnC, respectively). Within these two isoforms, there remains greater sequence variability within the sTnCs than within the cTnCs. When comparing TnC sequences from endotherms (mammalian and avian), for instance, the skeletal isoforms are 90% identical (14 sequence differences), while the cardiac isoforms are between 96.8%-99.4% identical (1-6 sequence differences) (Gillis et al., 2007). Along with the consideration of phylogenetic branch length differences between the vertebrate cTnCs and sTnCs, it has been suggested that the cardiac isoform has not undergone divergence to the same extent as its skeletal counterpart. This may mean that skeletal muscle in general has undergone more adaptive changes to different conditions.
(such as temperature, pH, etc) than cardiac muscle (Gillis et al., 2007). This in turn suggests that heart muscle may be under greater selection pressures to maintain a physiological optimum while skeletal muscles from different species may have a greater range of physiological capabilities (Gillis et al., 2007).

The potential implications of the evolution of TnC when considered alongside the evolution of endothermy are also intriguing. Alignments of the N-terminal region of cTnCs from ectothermic and endothermic vertebrates show some striking differences between these two thermal strategies (see Figure 7). Within the non-functional EF-Hand Site I of cTnC are some differences in amino acid residues that align with ectothermic and endothermic species respectively. These differences have been found to have significant impact on the Ca\(^{2+}\)-activation of the protein, a difference further explained in Section 1.10. It has been found that Asn\(^2\), Ile\(^{28}\), Gln\(^{29}\) and Asp\(^{30}\) are responsible for maintaining the higher sensitivity to Ca\(^{2+}\) that is thought to be necessary for muscle contraction at the low temperatures experienced by teleost fishes (Gillis et al., 2003). Replacing the corresponding mammalian cTnC residues with these teleost residues have been found to be responsible for a two-fold increase in Ca\(^{2+}\) sensitivity of mammalian cardiac myocytes (Gillis et al., 2005).
Figure 7: Protein sequence alignment of N-terminal region of cTnC from ectothermic and endothermic species. Modified from Gillis et al. (2007). Highlighted in blue are four residues, found in trout cTnC, that increase the Ca\(^{2+}\) affinity of mammalian cardiac myocytes two-fold (Gillis et al., 2005). Highlighted in red are the corresponding residues found in endothermic species.
1.8 Structure and Evolution of Troponin I

TnI’s ability to inhibit the interaction between myosin and actin myofilaments was what earned TnI its name. Although some parts of the TnI structure have been solved multiple times, some uncertainty still exists about the structure as a whole. The sequence of cTnI can be subdivided into six different regions based on their functional roles (Kobayashi and Solaro, 2005). The human cTnI isoform contains a 35-residue N-terminal extension that contains two serines at residue numbers 23 and 24 that are phosphorylation targets for protein kinase A (PKA). This extension is what accounts for the difference in size between cardiac and skeletal isoforms of TnI (cTnI ~ 24kDa; sTnI ~ 21kDa). Four of the other five regions are responsible for binding other proteins, while the last is the triggering region. Starting from the N-terminus, the region order is a) N-terminal extension; b) region binding the C-terminal lobe of cTnC; c) region binding the C-terminal region of cTnT; d) the inhibitory peptide that binds actin; e) the regulatory peptide or “trigger”; and f) a second actin binding region (Kobayashi and Solaro, 2005). This binding of the actin filament occurs in low Ca$^{2+}$ concentrations in the relaxation phase, and is responsible for inhibiting ATPase activity.

In extant mammals and birds, troponin I has three different isoforms expressed in cardiac, fast and slow muscle. Studies have found that the duplication event which led to the presence of a cardiac isoform within the vertebrates occurred between 440-500 million years ago (mya) (Shaffer and Gillis, 2010). The ancestral ascidians appear to have only one TnI gene, while tetrapods have multiple, suggesting that the gene duplication occurred after vertebrates diverged (Hastings, 1997). A phylogenetic construction of the evolution of TnI reveals that the addition of an 32 amino acid N-terminal extension to the cardiac isoform of
TnI occurred after the divergence of the fishes (Shaffer and Gillis, 2010). This N-terminal extension is known to contain two serine residues (Ser$^{22}$ and Ser$^{24}$) which have been shown to be targets for PKA phosphorylation (Westfall and Metzger, 2001). As targets for phosphorylation, these changes in the amino acid structure can have direct effects on the degree of regulation afforded to β-adrenergic stimulation. PKA phosphorylation of mammalian cTnI at these sites decreases the whole complex Ca$^{2+}$ affinity, and increase the rate of Ca$^{2+}$ dissociation from cTnC (Robertson et al., 1982). Interestingly, it has also been found that both of these serines must be phosphorylated in order for this effect to be seen (Zhang et al., 1995a). By increasing the rate of Ca$^{2+}$ dissociation, the phosphorylation of these N-terminal extension sites can cause the muscle to relax more quickly, allowing greater regulatory control over cardiac performance (Zhang et al., 1995b). When considering which vertebrates have evolved this N-terminal extension, we find an interesting correlation between the presence of the extension and the evolution of endothermy. This is an important insight when considering that regulatory control over the heart has improved in endotherms, allowing them to control cardiac output not through stroke volume, but through heart rate (Shaffer and Gillis, 2010). This allows endothermic species to exercise greater control over their circulatory system using the β-adrenergic system, making them more sensitive to the “fight or flight” instinct. This also allows them to match heart rate with aerobic requirements, improving the overall efficiency of their energy expenditure (Shaffer and Gillis, 2010).
1.9 Structure of the Troponin Complex as a Whole

With the establishment of the general role that troponin plays in muscle contraction, as well as the structure of its components, we can now discuss how these three proteins interact with one another as well as with associated proteins and with Ca\(^{2+}\). The structure of the core domain of the human cTn complex together with tropomyosin has been solved, allowing us to identify key interacting residues in the different proteins (Takeda, 2005; Takeda et al., 2003). The troponin complex can be subdivided into two parts: the IT arm and the regulatory head (see Figure 8). The IT arm includes TnC residues 93-161, TnI residues 42-136 and TnT residues 203-271, while the regulatory head includes TnC residues 3-84 and TnI residues 150-159 (Takeda et al., 2003). The regulatory head includes the portion of TnC which actively binds Ca\(^{2+}\) as well as the amphiphilic helix of TnI, H3 which binds to the hydrophobic patch revealed in the N-terminal lobe of cTnC upon Ca\(^{2+}\) binding. Ca\(^{2+}\) dissociation from TnC, however, results in a release of H3 from the hydrophobic patch, allowing the two actin/tropomyosin binding sites of TnI (the inhibitory region and the C-terminal region) to bind to actin/tropomyosin and inhibit cross-bridge cycling (see Figure 9) (for review see Takeda, 2005; Takeda et al., 2003).
Figure 8: Cardiac troponin complex core domain structure, used with permission from Li et al. (2004). Circled are the two main functional components: the IT arm (circled on the bottom of the diagram), which includes most of the cTnT protein, the C-terminal lobe of cTnC and the N-terminal region of cTnI; and the regulatory head (circled on the top right), which includes the N-terminal lobe of cTnC and the C-terminal region (the inhibitory region) of cTnI.
Figure 9: Representative of Ca$^{2+}$-activation of the core domain of the cardiac troponin complex, used with permission from Li et al. (2004). Bound Ca$^{2+}$ ions are represented by grey spheres, while cTnT is shown in yellow, cTnI is shown in green and cTnC is shown in its structural form in grey. Binding of Ca$^{2+}$ to the N-terminal region of cTnC is represented in the top image (contraction state), shown by the interaction of TnT and tropomyosin, while in a relaxed state, represented in the bottom image, the C-terminal region of TnI binds actin-tropomyosin (shown in cyan and yellow, respectively), inhibiting contraction.
Calcium (Ca\(^{2+}\)) plays a critical role in the activation of striated muscle. The sensitivity of muscle to Ca\(^{2+}\) can be quantified using the concentration of Ca\(^{2+}\) required to reach half-maximal activation of the myofilaments (Rüegg, 1998). In this way, if a given set of conditions causes the myofilaments to activate at a lower concentration of Ca\(^{2+}\), then these conditions have increased the Ca\(^{2+}\)-sensitivity of the muscle (Rüegg, 1998).

Several factors are known to alter the Ca\(^{2+}\)-sensitivity of muscle, including temperature. Previous work on rabbit ventricular muscle has revealed that as temperature was decreased from physiological 36°C to 1°C, Ca\(^{2+}\)-sensitivity also decreased (Harrison and Bers, 1989). This temperature dependent decrease in Ca\(^{2+}\) sensitivity in cardiac muscle appears to be true across a wide array of species, including rabbit, rat, guinea pig, frog and trout (Churcott et al., 1994; Harrison and Bers, 1989, 1990). Although all of these species exhibit decreased Ca\(^{2+}\)-sensitivity with decreased temperature, there still remain interspecies differences in Ca\(^{2+}\)-sensitivity (Churcott et al., 1994). Furthermore, when compared at a given temperature, major differences in cardiac Ca\(^{2+}\)-sensitivity exist among the vertebrates. For example, rat (Rattus norvegicus) cardiac myofibrils are 10 times less sensitive to Ca\(^{2+}\) than those of the rainbow trout (Oncorhynchus mykiss) when compared at 7°C (see Figure 10) (Churcott et al., 1994). This is thought to be a compensatory mechanism for the natural increase in Ca\(^{2+}\)-sensitivity with increased temperatures, as mammals have an internal body temperature of 37°C, an increase of 22°C compared to trout (which live in environments between 5 and 15°C) (Gillis et al., 2007). This is an incredible feat, considering that a mammalian heart exposed to these temperatures would enter cardioplegia (arrest). Survival at this range of low temperatures is only possible for
rainbow trout because over acclamatory changes in temperature, their hearts are able to remain responsive to changes in intracellular \([\text{Ca}^{2+}]\) and contract. Thus, the \(\text{Ca}^{2+}\) sensitivities of ectotherms and endotherms at their normal physiological temperatures (i.e: the trout heart at around 7°C, and the mammalian heart at 37°C) are similar, even though when compared at the same experimental temperature the \(\text{Ca}^{2+}\) sensitivity of the trout heart is 10 fold that of the rat heart (Gillis and Tibbits, 2002).

The impressive interspecies differences in \(\text{Ca}^{2+}\) sensitivity can in part be explained by structural differences in cardiac troponin C (cTnC). TnC is responsible for the binding of \(\text{Ca}^{2+}\) and triggering muscle contraction, as was described above. As such, it is the most likely contractile protein to be responsible for changes in \(\text{Ca}^{2+}\) sensitivity through amino acid changes. Gillis et al. (2005) found that four amino acid residues found in the rainbow trout cTnC (Asn\(^2\), Ile\(^{28}\), Gln\(^{29}\) and Asp\(^{30}\)) were responsible for approximately a twofold increase in \(\text{Ca}^{2+}\) sensitivity when inserted into rabbit cardiac myocytes. When mammalian cTnC was mutated to contain the four residues above and inserted into rabbit cardiomyocytes, there was a twofold increase in the \(\text{Ca}^{2+}\) sensitivity of force generation of skinned rabbit cardiac myocytes (Gillis et al., 2005). See again Figure 7, comparing amino acid sequences of rat, trout and clawed frog cTnC, with the four \(\text{Ca}^{2+}\) sensitizing residues highlighted. These four differences in amino acid sequence are found to some degree in cTnCs of ectothermic species, but not in any endothermic species (Gillis et al., 2007). However, an exception to this has been found in a human patient with familial hypertrophic cardiomyopathy (FHC). FHC is caused by mutations to more than ten different sarcomeric protein genes, and results in fibrosis of cardiac tissue, and asymmetrical enlargement of the ventricles (Redwood et al., 1999). This demonstrates again how important single amino acid substitutions can be and how critical having an appropriate \(\text{Ca}^{2+}\) sensitivity level can
be. Increasing the Ca\textsuperscript{2+} sensitivity of the mammalian heart beyond the physiological norm causes problems, as the muscle contracts under much lower concentrations of Ca\textsuperscript{2+}, resulting in arrhythmias, and potentially cardiac arrest (Gillis and Tibbits, 2002).
Figure 10: Calcium sensitivity of cardiac muscle preparations from three different species, modified from Churcott et al. (1994). Circled in blue, at a common temperature, trout muscle preparations are ten times more Ca$^{2+}$-sensitive than rat preparations. Circled in red however, at physiological temperatures of the animals, trout, frog and rat muscle preparations have very similar Ca$^{2+}$ sensitivities.
1.11 **Protein Stability and Temperature**

In order for a protein to have optimal function, it must maintain a state of what has been termed “marginal stability” (Somero, 2003). This term refers to the balance of stabilizing and destabilizing forces within the protein structure (such as ionic interactions, hydrogen bonds and entropy). Most proteins, to attain their optimal function must maintain the folded tertiary structure that allows them to be active in their biological role. For instance, in the case of a substrate-binding protein, a balance of lability vs stability must be attained to allow the needed rapid change in conformation that will inevitably accompany any interactions with other molecules (Somero, 1995). Important to consider is that in actuality a single protein does not maintain a single conformation, but will have a natural fluctuation among a range of microstates. These microstates vary in their conformation, and thus in the case of a ligand-binding protein, can vary in their ability or affinity for their specific ligand (Somero, 2003).

The interaction of protein stability with temperature is a topic of interest to many different fields of study, including physiologists, microbiologists, physicists and evolutionists. Mechanistic studies of species-specific temperature tolerances has led to the discovery of antifreeze and ice-nucleating molecules, heat shock proteins, and evolutionary changes in structural proteins as well as in enzymes (Somero, 1995). Most interesting to this review are the evolutionary changes to many different proteins that have been found to allow vertebrate adaptation to different temperatures. Studies of muscle enzymes, for instance, have shown that the structural stability of myofibrillar ATPase from different fish is strongly correlated with their habitat temperature (Johnston and Walesby, 1977).

The question to consider is therefore how can the stability of a protein be adapted to a
certain temperature? Amino acid substitution sites termed “adaptive hot spots” in the amino acid sequence of proteins may be the answer. Specific amino acid substitution sites in lactate dehydrogenase-A (LDH-A) have been found to have large impacts on the amount of energy required for conformational changes (Fields and Somero, 1998). Alterations in the amino acid sequence of a mobile helix region that is known to undergo displacements during ligand binding were found to have large changes in the energy costs of the conformational change (Fields and Somero, 1998; Somero, 1995). It is through these single residue changes in key regions of the protein that Antarctic notothenioid fish LDH-A can remain functional at extremely low temperatures (Fields and Somero, 1998).

It is through structural changes such as these that differences in function between trout and mammalian cTnC exist. Studies have found that the conformation of the regulatory domain of trout cTnC at 7 °C is similar to that of the mammalian cTnC at 30°C, indicating that conformation is dependent on physiological temperatures (Blumenschein et al., 2004). Thus, the thermal stability of key regulatory proteins may help determine the specific structure/function relationship for the protein and also be dependent on the physiological conditions they are evolved within.

1.12 EVOLUTION OF THE HEART: WHERE AMPHIBIANS FIT IN

The evolution of the vertebrate heart has not only occurred at the general anatomical level, but also at the level of individual proteins. Subtle changes in amino acid sequence of the cardiac troponin complex have been shown to result in large differences in Ca$^{2+}$ sensitivity as well as in sensitivity to protein phosphorylation between cold-adapted ectothermic species and endothermic species. The current knowledge of Ca$^{2+}$ sensitivity
and phosphorylation effects mainly compares a temperate fish (the rainbow trout) and a mammal (the rat), but does not consider intermediate taxa. When considering the evolution of the cardiopulmonary system and of endothermy, investigating a cardiac system such as that of the African clawed frog (*Xenopus laevis*) could offer great insights. The African clawed frog is an amphibian, and as such has a heart anatomically intermediate to fish and mammals, with two separate atria, and one ventricle (Bishopric, 2005). As an amphibian, the African clawed frog is also an ectotherm, but it lives at temperatures significantly higher than the rainbow trout (15-25°C; Jackson and Tinsley, 2002), making their hearts potentially more specialized to higher temperatures than the temperate fish. As well, as a semi-terrestrial taxon, amphibians generally have a larger locomotory cost than fish, and would therefore require a circulatory system suited to higher cardiac output, due to higher rate of contraction and higher blood pressure (Schmidt-Nielsen, 1972). The genome of *X. laevis* has been sequenced, and as such we can compare the amino acid sequence of this amphibian’s cTnC to that of both the rat and of the rainbow trout. *X. laevis* cTnC has an amino acid sequence that is intermediate to the rat and the trout, containing two of the four residues identified in Gillis et al. (2005) as $\mathrm{Ca}^{2+}$-sensitizing in the trout (see Figure 7). With this interesting mix of peptides in mind, the following question was formed.
2 Research Question, Hypotheses and Predictions

Question: How do the functional properties of a cTn complex containing amphibian (Xenopus laevis) cTnC compare to that of representative mammalian (Rattus norvegicus) and fish (Oncorhynchus mykiss) species?

Hypothesis: Troponin complex calcium binding has evolved in ectotherms and in endotherms to maintain consistent function at physiological temperatures.

Prediction 1: If troponin is structurally adapted to physiological temperatures, then cTnC and cTn complexes from trout, frog and rat exposed to increasing temperatures will lose stability in an order that correspond to their physiological temperatures.

Prediction 2: If troponin is functionally adapted to physiological temperatures, then the Ca\(^{2+}\) affinity of cTnCs and cTn complexes should increase with decreasing natural physiological temperatures of the animal in question.

In order to test these predictions, I cloned the cTnC genes from Rattus norvegicus, Xenopus laevis and Oncorhynchus mykiss. I then expressed the proteins recombinantly, and assembled them into complexes containing Rattus cTnT and either Rattus or Oncorhynchus cTnI. To test the first prediction I performed circular dichroism measures of thermal stability of the individual proteins and of the complexes. To test the second prediction I performed both steady state Ca\(^{2+}\) affinity measures as well as stopped-flow kinetics studies of Ca\(^{2+}\) off rate. A fluorescent probe was used to measure the conformational change in cTnC associated with Ca\(^{2+}\)-binding.
3 MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Two Xenopus laevis were obtained from Ward’s Natural Science (St. Catherines, ON, Canada). Upon arrival, the animals were sacrificed by pithing and then the atria and ventricle of the heart surgically removed. All tissue was then flash frozen in liquid Nitrogen and stored at -80 °C until further methods were performed.

3.2 cDNA PREPARATION:

In order to clone the full length of the X. laevis cardiac troponin C, total RNA was first extracted from X. laevis heart tissue using TRIzol® Reagent (Invitrogen Life Technologies, Frederick, MD, USA) according to manufacturer’s directions. RNA was then treated with DNase I, Amplification Grade (Sigma-Aldrich, Oakville, ON, Canada) to eliminate any genomic DNA contamination. After DNase treatment, the reverse transcription reaction was conducted using the First-Strand cDNA Synthesis Kit (Applied Biosystems Inc., Streetsville, ON, Canada) according to manufacturer’s directions.

3.3 POLYMERASE CHAIN REACTION (PCR):

PCR primers were designed using Primer3 online software (http://frodo.wi.mit.edu/primer3/) for X. laevis and O. mykiss cTnC from the known nucleotide sequences found on the NCBI website (accession numbers NM_001090295.1 and AY281129.1, respectively). Forward primers were designed to include the Nde I restriction digest enzyme sequence (CA\^TATG), while reverse primers included the Xho I enzyme sequence (C\^TCGAG). Length of the primers varies from 30 to 37 nucleotides (see Table 2). Primer pairs were designed to have similar melting temperatures (T_m) and
with as high a GC% as possible. The primer design was limited, however, as both the
digest sites and the C and N-terminus of the proteins had to be included as the beginnings
of the forward and reverse primers. This meant that optimal primer regions could not be
utilized, as they would not result in the full protein as a product.

The PCR reactions were performed using cDNA from *Xenopus laevis* heart tissue for
XcTnC, and from a previously ligated plasmid containing ScTnC for the trout cTnC. This
template DNA was used with the primers listed in Table 2, and Platinum® *Taq* DNA
polymerase (Invitrogen Life Technologies, Frederick, MD, USA) according to
manufacturer’s directions. PCR amplification was performed under the conditions:
denature at 94 °C for 2 min; 2 cycles of: (denature at 94 °C for 30 sec, anneal at 60 °C,
extend at 72 °C for 1 min); 3 cycles of: (94 °C for 30 sec, anneal at 62.5 °C, 72 °C for 1
min); 30 cycles of: (94 °C for 30 sec, anneal at 65 °C, 72 °C for 1 min); maintain at 4 °C.
PCR products were then loaded on a 1% agarose gel containing GelRed™ Nucleic Acid
Gel Stain (Biotium Inc., Hayward, CA, USA) along with the 1 Kb Plus DNA Ladder
(Invitrogen Life Technologies, Frederick, MD, USA) and run out at 120 V for ~30 min.
Size of the product was confirmed using UV illumination on a ChemiDoc™ XRS+ System
(Bio-Rad Hercules, CA, USA). After ensuring the product size, the PCR product was
column purified using the EZ-10 spin column PCR Products Purification Kit according to
manufacturer’s directions (Bio Basic Inc., Markham, ON, Canada). Following purification,
the PCR product was double digested with Nde I and Xho I enzymes (New England
Biolabs, Ipswich, MA, USA), according to manufacturer’s directions and then column
purified once more. The concentration of product was then determined using an ND-1000
spectrophotometer (NanoDrop, Wilmington, DE, USA).
Table 2: *Xenopus laevis* and *Oncorhynchus mykiss* cardiac troponin C PCR primers designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and nucleotide sequences obtained from the US National Center for Biotechnology Information (NCBI at http://www.ncbi.nlm.nih.gov/).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XcTnC forward (NdeI)</td>
<td>GCGTGCCATATGGATGATATTTACAAAGCAGCGGTTG</td>
<td>37</td>
<td>80.3</td>
</tr>
<tr>
<td>XcTnC reverse (XhoI)</td>
<td>TCGCGCTCGAGTTATTCAACTCCCTTCATGAATTCC</td>
<td>36</td>
<td>80.5</td>
</tr>
<tr>
<td>ScTnC forward (NdeI)</td>
<td>GCGTGCCATATGAACGACATCTACAAAGCA</td>
<td>30</td>
<td>75.9</td>
</tr>
<tr>
<td>ScTnC reverse (XhoI)</td>
<td>TCGGCCTCGAGTTATTCTACTCTTCATGAACTC</td>
<td>35</td>
<td>75.5</td>
</tr>
</tbody>
</table>
The PCR product was then ligated into the pET-24a(+) expression vector which had been digested using the same restriction enzymes as the PCR products. The ligations were accomplished using T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA), according to the manufacturer’s directions. The plasmid was then transformed into NovaBlue competent *E. coli* cells (Novagen, San Diego, CA, USA), and grown on a lysogeny broth (LB) agar plate with 50 µg/mL Kanamycin according to the manufacturer’s directions. The plasmid was then mini-prepped in 3 mL aliquots of LB with 50 µg/mL Kanamycin using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Indianapolis, IN, USA). Mini-prepped samples were checked for inserts via double digest and visualization on an agarose gel. Those that contained the proper sized insert were then sent to the University of Guelph College of Biological Science Genomics Facility (CBS-GF) for sequencing to ensure that the gene was properly inserted in the plasmid. Sequences were then compared to the *Xenopus laevis* cTnC nucleotide sequence available on NCBI (Accession NM_001090295.1).

### 3.4 Mutation of cTnCs

In order to facilitate the labeling of the cTnCs with the fluorescent probe anilinonaphthalenesulfote iodoacetamide (IAANS), the proteins were mutated to remove all native cysteines (replacing them with serines) while inserting a cysteine at position 53. This position allows good labeling efficiency while maintaining native function of the protein (Hazard et al., 1998; Liu et al., 2012). The removal of the native cysteines is necessary, as previous studies have shown that attachment of IAANS to these positions alter the natural function of the protein (Putkey et al., 1997). The mutations necessary for
each of the three cTnCs are illustrated in Figure 11. The triple mutant McTnC$^{C35S, T53C, C84S}$ in the pET24a(+) plasmid was provided to us by Dr. J. P. Davis at Ohio University. The mutations for the ScTnC and XcTnC were performed in one PCR reaction using the QuickChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer’s directions. For the XcTnC mutations, the XcTnC in pET24a(+) was used as the template DNA while primers for each of the mutations were designed according to the manufacturer’s directions (sequences shown in Table 3). After the PCR reaction, the 25 µL product was treated with DpnI in order to cleave all methylated template DNA leaving only the mutated form. This product was then transformed into XL-10 competent cells according to manufacturer’s directions and grown up overnight on LB Kanamycin plates. These plasmids were again mini-prepped in 3 mL aliquots of LB with 50 µg/mL Kanamycin using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Indianapolis, IN, USA). Those confirmed to contain an insert were again sent to CBS-GF for sequencing in order to compare to the native sequence to check that the mutation reaction was successful (see Figure 11). For the ScTnC, the four mutations necessary had previously been performed in our lab, but in a plasmid inappropriate for protein expression. As such, the mutated plasmid was used as the template DNA and cloned into the pET24a(+) plasmid. After mini-prepping, as with the XcTnC, the ScTnC clone was also sent to CBS-GF for sequencing to check the mutation remains in the pET24a(+) plasmid (see also Figure 11).
Figure 11: Mutations to cTnCs necessary for successful IAANS labeling. Shown in bolded black, residue 53 was mutated from a threonine to a cysteine, this is the residue the IAANS was bound to. Residues 35 and 84 (shown in bolded red) of all three species were mutated from cysteine to serine to ensure that IAANS would not bind at these sites. Site 102 in the trout was also mutated from a cysteine to a serine, a mutation that was not necessary in the other two species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wild Type Sequence</th>
<th>Mutated Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>MDDIYKAAVEQLTEEQKNEFKAAFDIFVGLAAPDECGCISTKELGKVMRMLGQ</td>
<td><strong>MDDIYKAAVEQLTEEQKNEFKAAFDIFVGLAAPDECGCISTKELGKVMRMLGQ</strong></td>
</tr>
<tr>
<td>Xenopus</td>
<td>MDDIYKAAVEQLTEEQKNEFRAGDAFDIFVGLAAPDECGCISTKELGKVMRMLGQ</td>
<td><strong>MDDIYKAAVEQLTEEQKNEFRAGDAFDIFVGLAAPDECGCISTKELGKVMRMLGQ</strong></td>
</tr>
<tr>
<td>Trout</td>
<td>MDDIYKAAVEQLTEEQKNEFKAAFDIFVGLAAPDECGCISTKELGKVMRMLGQ</td>
<td><strong>MDDIYKAAVEQLTEEQKNEFRAGDAFDIFVGLAAPDECGCISTKELGKVMRMLGQ</strong></td>
</tr>
</tbody>
</table>

| Rat      | NPTPEELQEMIDEVIDGQSGTVDPEFLVMMVR**CMKDDSKGKSEELSDL** | NPTPEELQEMIDEVIDGQSGTVDPEFLVMMVRCMKDDSKGKSEELSDL |
| Xenopus  | NPTPEELQEMIDEVIDGQSGTVDPEFLVMMVR**CMKDDSKGKSEELSDL** | NPTPEELQEMIDEVIDGQSGTVDPEFLVMMVRCMKDDSKGKSEELSDL |
| Trout    | NPTPEELQEMIDEVIDGQSGTVDPEFLVMMVR**CMKDDSKGKSEELSDL** | NPTPEELQEMIDEVIDGQSGTVDPEFLVMMVRCMKDDSKGKSEELSDL |

| Rat      | FRMFDKNADGYIDLDELKLMMLQATGETITEDDIEELMKDGDNNDGRIDY | **FRMFDKNADGYIDLDELKLMLEATGETITEDDIEELMRDGDNNDGRIDY** |
| Xenopus  | FRMFDKNADGYIDLDELKLMLEATGETITEDDIEELMRDGDNNDGRIDY | **FRMFDKNADGYIDLDELKLMLEATGETITEDDIEELMRDGDNNDGRIDY** |
| Trout    | FRMFDKNADGYIDLDELKLMLEATGETITEDDIEELMRDGDNNDGRIDY | **FRMFDKNADGYIDLDELKLMLEATGETITEDDIEELMRDGDNNDGRIDY** |

| Rat      | DEFLEFMKGVE | DEFLEFMKGVE |
| Xenopus  | DEFLEFMKGVE | DEFLEFMKGVE |
| Trout    | DEFLEFMKGVE | DEFLEFMKGVE |
Table 3: *Xenopus laevis* cardiac troponin C mutation PCR primers designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and nucleotide sequences obtained from the US National Center for Biotechnology Information (NCBI at http://www.ncbi.nlm.nih.gov/). Mutations include the changing of all native cysteines to serines (positions 35 and 84) and the changing of one native threonine to cysteine (position 53). Primers were designed according to the specifications of the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
<th>Tm</th>
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</thead>
<tbody>
<tr>
<td>XcTnC C35S</td>
<td>ACGCTGAAGATGGCAGCATTTAGCACAAG</td>
<td>29</td>
<td>77.2</td>
</tr>
<tr>
<td></td>
<td>forward</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XcTnC C35S</td>
<td>TTTGGTGCTAATGCTGCCATCTTCAGCGT</td>
<td>29</td>
<td>77.2</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XcTnC T53C</td>
<td>GGGGCAGAATCCCTGTCTCGAGGAGTTA</td>
<td>28</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>forward</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XcTnC T53C</td>
<td>TAACTCTCAGGACAGGGATTTCTGCCCC</td>
<td>28</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
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<tr>
<td>XcTnC C84S</td>
<td>GGTATGATGTCAGCTGCTGCCGATTGGAACGACAG</td>
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<td>79.8</td>
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<tr>
<td></td>
<td>forward</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XcTnC C84S</td>
<td>CTGTCGTCTTTCAATGCTGCGGACCACATCATAACC</td>
<td>33</td>
<td>79.8</td>
</tr>
</tbody>
</table>
3.5 Protein Expression – cTnCs, McTnT and McTnI

Protein expression methods are as in Kirkpatrick et al. (2011), who used a modified version of the protocol from Guo et al. (1994). After confirming insertion direction of the gene, the plasmid was transformed into Novagen BL21 Gold (DE3) competent E. coli cells (Novagen, San Diego, CA, USA) according to the manufacturer’s directions, and grown overnight on a (LB) agar plate with 50 µg/mL of the appropriate antibiotic (kanamycin for XcTnC, ScTnC, McTnT and McTnI, ampicillin for McTnC). Ten to twelve colonies from the plate were then used to inoculate 100mL of LB plus 50 µg/mL kanamycin, and grown overnight at 37 °C with shaking. Six flasks containing 1L of broth were then inoculated with 15 mL each of the 100 mL overnight growth, and grown at 37 °C until the optical density (OD) at 600 nm was between 0.6-0.8. Once this OD had been reached, 500 µL of 1M isopropyl b-D1-thiogalactopyranoside (IPTG) was added to each flask. This allowed the induction of protein expression via the lac promoter. The samples were then grown at 37 °C for another 4-5 hours, following which the cells were harvested by spinning at 6000 rpm and 4 °C for 5 min. The pellet was then stored overnight at -20 °C until protein purification could be completed.

3.6 Protein Purification – cTnCs and McTnT

The pellet was first re-suspended in 250 mL of 10 mM Tris (pH = 7.4), and then centrifuged down once again at 6000 rpm and 4 °C for 5 min. The pellet from this was then resuspended in 250 mL of 50 mM Tris (pH = 7.4), 0.1% Triton X-100, 1mM PMSF. While stirring, 25 mg of lysozyme was added dropwise to this mixture in a glass beaker. The lysozyme and protein pellet mixture was stirred for 3-4 hours at room temperature,
following which it was sonicated in 2 min bursts using a Vibra-Cell™ ultrasonic processor (Sonics & Materials Inc., Danbury CT, USA) until the solution was homogeneous and watery in appearance. The sample was then spun at 14000 rpm for 25 min, following which the pellet was discarded. The supernatant was then mixed with 15.4 g/100 mL of ammonium sulfate and again centrifuged at 14000 rpm for 20 min. The supernatant was saved again, and dialyzed in 6000-8000 Molecular Cut Off (MCO) tubing against DEAE Column Running Buffer (see Appendix for list of buffers), with the solution changed 2-3 times. Before running on the chromatography column, all solutions were filtered through 0.22 µm filter paper (Millipore Corporation, Bedford, MA, USA).

Protein purification for cTnC and McTnT were performed using a Fast Protein Liquid Chromatography (FPLC) Diethylaminoethyl Sepharose (DEAE) column. The sample was applied to the DEAE column in DEAE Column Running Buffer and eluted using a DEAE High Salt Elution Buffer. The elutions were performed in a linear gradient. Peaks observed in the UV output from the FPLC were collected in fractions (see Figure 12 for cTnC and Figure 13 for McTnT) and then run via SDS-PAGE to determine the elution location and relative purity of the proteins (see Figures 14 and 15). It was determined that cTnC was eluted in a relatively pure form in the second peak after the beginning of the high salt run (peak “B”). McTnT was eluted toward the end of the column run (see Figure 15).
Figure 12: Representative output of FPLC column purification of cTnC from *Xenopus laevis* on a DEAE column. The pink line at 0 mL indicates the injection of the sample onto the column. The initial peak contains flow-through proteins that were not bound to the column. The green slope indicates the gradient injection of the High Salt Elution Buffer while the red numbered lines indicate fraction collection of the elute. The eluted peaks A, B and C were run on SDS-PAGE to test for cTnC. Fractions collected from peak B were found to contain relatively pure cTnC (see Figure 14).

Figure 13: Representative output of FPLC column purification of McTnT (cardiac troponin T from *Rattus norvegicus*) on a DEAE column. The eluted peak A as well as representative fractions after the peak were run on SDS-PAGE to test for the presence of McTnT. The fractions at the end of the run were found to contain relatively pure McTnT (see Figure 15).
Figure 14: Representative SDS-PAGE run of peaks from FPLC DEAE *X. laevis* cTnC purification. Peak B was split up into 4 sections to test what section of the peak had the highest quantity and the purest cTnC. Smearing of the sample occurred due to the high concentration of protein. Gel is 12% acrylamide, stained with Coomassie Blue stain, imaged using a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA).
Figure 15: Representative SDS-PAGE run of FPLC DEAE McTnT (cardiac troponin T from *Rattus norvegicus*) purification. The only peak in the output did not contain TnT, but post-peak fractions near the end of the elution contained relatively pure McTnT (post-peak elution fractions 5-10). Gel is 12% acrylamide, stained with Coomassie Blue stain, imaged using a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA).
After purification, the fractions determined to contain pure cTnC were dialyzed in 6000-8000 MCO dialysis tubing against ddH$_2$O, with 3 changes of the water. The fractions of McTnT were also dialyzed against ddH$_2$O, but in 12000-14000 MCO dialysis tubing. The samples were then frozen at -80 °C and lyophilized using a Virtis Advantage Bench Top Freeze Dryer (SP Scientific, Gardiner, NY) until all water had been removed.

3.7** PROTEIN PURIFICATION - McTnI**

The pellet was resuspended into 100mL of Sonication Buffer and then sonicated 10-15 times for 1 min with 2-3 min resting on ice between sonication steps. After sonication, the sample was centrifuged for 1 hour at 10 000 RPM. The supernatant of the centrifuge step underwent an ammonium sulfate cut of 15 g/100mL and centrifuged once more. The supernatant was then dialyzed in 12000-14000 MCO tubing against CM Column Buffer with the solution changed 2-3 times. This was then run on the FPLC CM-Sepharose chromatography column with the CM Column Buffer and eluted using the CM High Salt Elution Buffer. The elution was performed in a linear gradient with a UV output determining the peak fractionation (see Figure 16). SDS-PAGE was again run to determine elution location and purity (see Figure 17). Purity of each protein was then double checked by SDS-PAGE before progressing to labeling and complexing (see Figure 18).
Figure 16: Representative output of FPLC column purification of McTnI (cardiac troponin I from *Rattus norvegicus*) on a CM column. The eluted peak A as well as representative fractions after the peak were run on SDS-PAGE to test for the presence of McTnI. Post-peak fractions 2-4 were found to contain relatively pure McTnT (see Figure 17).
Figure 17: Representative SDS-PAGE run of FPLC CM-Sepharose McTnI (cardiac troponin I from *Rattus norvegicus*) purification. The only peak in the output did not contain TnI, but post-peak fractions near the end of the elution contained relatively pure McTnI (post-peak elution fractions 2-4). Gel is 12% acrylamide, stained with Coomassie Blue stain, imaged using a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA).
Figure 18: SDS-PAGE run of all cloned cTnCs (McTnC being mammalian, *Rattus norvegicus* cardiac troponin C, XcTnC being *Xenopus laevis* cTnC, ScTnC being *Oncorhynchus mykiss* cTnC) to check relative protein purity. All three cloned cTnCs were of a high enough purity to continue to be complexed together with McTnT and McTnI (or ScTnI) to form Tn complexes. Gel is 12% acrylamide, stained with Coomassie Blue stain, imaged using a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA).
3.8 LABELING cTnC

The purified and lyophilized cTnC was labeled with the fluorescent probe IAANS in order to provide a fluorescent measure of Ca\(^{2+}\) binding to cTnC following the protocol described in Davis et al. (2007). In short, the purified cTnC was suspended in, and then dialyzed into IAANS Labeling Buffer overnight, and then dialyzed into the same buffer without DTT. This sample was then incubated at 4 °C in the dark for 8 hours with a 5x Molar excess of IAANS, following which the incubation was halted by addition of 2 mM DTT. The mixture was then dialyzed three times against IAANS Labelling Buffer 2. The efficiency of the IAANS probe labeling was then checked with a Bradford assay for protein concentration and a spectrofluorometer for the probe (labeling efficiencies were found to be 88% for ScTnC, 89% for XcTnC and 102% for McTnC).

3.9 RECONSTITUTION OF CARDIAC TROPNIN COMPLEX

The cardiac troponin complexes were reconstituted according to a protocol described by Davis et al. (2007). In short, troponin subunits were dialyzed separately against Complexing Buffer 1, following which the subunits were mixed in a Molar ratio of TnT:TnC:TnI = 1.5:1.0:1.5. Each mixture was allowed to mix at room temperature for 20 min, following which each was then dialyzed through Complexing Buffer 2-4, with the final buffer repeated three times. Each dialysis step was performed for 12 hours, at 4 °C, and in the dark. Excess TnI and TnT that precipitated during the reaction were removed by centrifugation (14 000 rpm for 20 min at 4 °C). The protein concentrations of the final complexed sample were then determined with a Bradford assay. Complexes were also run on SDS-PAGE gel to ensure that complexing was successful (see Figure 19).
Figure 19: SDS-PAGE run of all cTnCs and Tn complexes to check relative protein purity as well as complexing. McTnC denotes cardiac troponin C (cTnC) cloned from mammalian Rattus, XcTnC denotes cTnC from Xenopus, ScTnC denotes cTnC from salmonid Oncorhynchus. MTn denotes cardiac troponin complex containing all mammalian Rattus components (cTnI, cTnT and cTnC), XTn denotes mammalian cTn containing Xenopus cTnC, STn denotes mammalian cTn containing salmonid cTnC and SCITn denotes mammalian cTn containing salmonid cTnC and cTnI. All three cTnCs are relatively pure, and Tn complexes appeared to be successfully complexed (based on relative band intensities). Gel is 12% acrylamide, stained with Coomassie Blue stain, imaged using a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA).
3.10 **CIRCULAR DICHROISM**

The thermal stability of the different Tn complexes and cTnCs were determined using circular dichroism (CD) spectrometry. For this method, the proteins were not labelled with the IAANS probe in order to remove any confounding effects the fluorescent probe would have on circular dichroism absorption measures. Samples were first dialyzed in Complexing Buffer 4 with at least 3 buffer exchanges. The concentrations of the protein samples were then determined via Bradford Assay, using the Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA). The proteins were then diluted to 0.16 mg/mL in the same Complexing Buffer 4. Using crystal cuvettes with a 1mm wavelength, 200 µL of each sample was used for each measure. The negative ellipticity of the proteins was measured at 222 nm (a measure of the folded state of α-helices) with an increasing thermal gradient of 1 °C / min from 5 – 80 °C, with measurements taken every 5 °C, using a JASCO J-815 Chiro-optical spectrometer (JASCO, Easton, MD). The relative rate of change of negative ellipticity at 222 nm for each sample run was then plotted as a function of temperature and fitted with a Weibull fit. The melting temperature (T_m) of each protein was determined as the minimum value of the Weibull fit, or as the point at which the protein unfolding was occurring at the greatest rate. This method of determining the T_m of proteins has previously been validated (Mundia et al., 2012; Pereteanu and Dawson, 2008). The average values for each protein were then compared between frog, rat and trout cTnCs and cTns using a one-way ANOVA with Holm-Sidak post-hoc testing. Students’ t-tests were used to compare averages of complexes to their corresponding cTnC in isolation. All fittings and statistical analyses were performed using using SigmaPlot 12.5 (Systat Software, San Jose, CA).
3.11 **Steady State Ca\(^{2+}\) Binding Titrations**

Reconstituted cTn units and individual cTnCs were titrated with increasing concentrations of Ca\(^{2+}\) according to the protocol in Davis et al. (2007). All steady-state fluorescence measures were taken using a LS55 Fluorescence Spectrometer (Perkin Elmer, Waltham, MA) at 15°C. IAANS fluorescence was excited at 330 nm, with emission monitored at 450 nm as microlitre amounts of CaCl\(_2\) were titrated into 2 mL of sample. Each sample consisted of the cTn units and cTnCs diluted to 0.15 µM in Titration Buffer. Free Ca\(^{2+}\) concentrations were calculated through MaxChelator online software (http://www.stanford.edu/~cpatton/webmaxcS.htm) in order to determine the pCa (-log[Ca\(^{2+}\)]) of each sample point. Fluorescence data was normalized and then fitted with a 4-parameter Hill equation. The pCa\(_{50}\) (-log of the Ca\(^{2+}\) concentration required for 50% binding of cTnC or Tn complex), a measure of binding affinity, and the Hill coefficient, a measure of cooperativity of binding sites within a protein were then calculated from the fittings of each individual run. The average values for each protein were then compared between the different cTnCs as well as between the cTns using one-way ANOVAs with Holm-Sidak post-hoc testing. Students’ t-tests were also used to compare averages of complexes to their corresponding cTnC in isolation. All fittings and statistical analyses were performed using using SigmaPlot 12.5 (Systat Software, San Jose, CA).
3.12 **STOPPED FLOW KINETICS**

The affinity of a binding site for its ligand is described by the following equation:

\[ K_a = \frac{k_{on}}{k_{off}} \]

in which \( K_a \) is the affinity of the binding site for the ligand, \( k_{on} \) is the rate at which the ligand binds to the site and \( k_{off} \) is the rate at which the ligand dissociates from the site. While the \( \text{Ca}^{2+} \)-binding measurements determined the \( K_a \) of the cTnCs and cTns, the stopped-flow measures determine the \( k_{off} \) values of both the N-terminal and C-terminal binding domains of the cTnC both in isolation and in complex. These values can then be used to determine the \( k_{on} \), or the rate at which the ligand (\( \text{Ca}^{2+} \)) is bound to the low-affinity binding site of cTnC. Lower \( k_{off} \) values when taken into consideration of the affinity equation above equate to a higher \( \text{Ca}^{2+} \) affinity of the complex.

Stopped flow kinetics experiments were conducted using an SX20 Stopped-Flow Spectrometer (Applied Photophysics, Leatherhead, Surrey, UK). Tn complexes were first dialyzed into Stopped Flow Buffer then diluted in this same buffer to 0.1 µM. After protein dilution, \( \text{CaCl}_2 \) was added to the protein samples to a concentration of 0.2 mM. The rates of \( \text{Ca}^{2+} \)-dissociation were measured for both the N and C-termini of the cTnCs both in isolation as well as within the Tn complexes using EGTA as a \( \text{Ca}^{2+} \) chelator. The N-terminal region of cTnC contains a low-affinity \( \text{Ca}^{2+} \) binding region, and thus has \( \text{Ca}^{2+} \) dissociation over 0.2 sec when in complex, while the C-terminal region’s high-affinity region has \( \text{Ca}^{2+} \) dissociation over 20 sec. When in isolation, \( \text{Ca}^{2+} \) dissociation from the N-terminal region of cTnC occurs at an extremely high rate that cannot be accurately measured via stopped flow (see Appendix, Figure 29 for measures taken over 0.025 sec).
Measurements of Ca\(^{2+}\) dissociation were taken over these time frames in order to determine both N-terminal and C-terminal Ca\(^{2+}\) off-rates (\(k_{\text{off}}\)). The IAANS fluorescent reporter was excited at 330 nm, with emission measured at 450 nm. The data obtained was then fit to an exponential decay, single 3 parameter equation and a \(k_{\text{off}}\) value calculated from each fit equation. The \(k_{\text{off}}\) values were then compared between the cTnCs as well as between the Tn complexes using one-way ANOVAs with Holm-Sidak post-hoc testing. Students’ t-tests were also used to compare average C-terminal \(k_{\text{off}}\) values of complexes to their corresponding cTnC in isolation. All fittings and statistical analyses were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA).
4 RESULTS

4.1 CIRCULAR DICHROISM: THERMAL STABILITY

The melting behaviour of the cTnCs was significantly different between species. Differences were evident when viewing melt curves by a left-ward or right-ward shift of the curves relative to each other (see Figure 20). Interestingly, it was XcTnC \( (n=3) \) that had the highest \( T_m \) of \( 57.31 \, ^\circ C \pm 0.34 \), followed by McTnC \( (n=3) \) at \( 55.85 \, ^\circ C \pm 0.41 \) and then ScTnC \( (n=3) \) at \( 52.01 \, ^\circ C \pm 0.29 \). All three differences were significant when compared with a one-way ANOVA (see Table 4).

The melting of the complexed Tn units were also investigated using circular dichroism, and again significant differences were found. Differences in melt curves were quite evident (see Figure 21), however results were slightly different from the cTnCs in isolation. When considering the \( T_m \)\( s \) of each of the complexes, the order of highest to lowest changes slightly from the cTnCs alone. When in complex, it is MTn \( (n=3) \) that has the highest \( T_m \) of \( 49.85 \, ^\circ C \pm 1.06 \), followed by XTn \( (n=3) \) at \( 48.46 \, ^\circ C \pm 0.43 \), then STn \( (n=3) \) at \( 47.45 \, ^\circ C \pm 1.19 \) and finally SCITn \( (n=3) \) at \( 43.79 \, ^\circ C \pm 0.91 \). Only the SCITn \( T_m \) was found to be significantly different from the MTn and the XTn \( T_m \)\( s \) when compared with a one-way ANOVA. Each Tn complex was also compared to its corresponding cTnC unit to determine whether complexing also contributed to differences in melting temperature. Student’s t-tests revealed that all complexed Tn units had significantly lower \( T_m \) than their isolated cTnC counterpart (see Table 4).
Figure 20: Thermal stability melting curves of cTnCs from three different species. The average relative change in negative ellipticity at 222 nm was measured over increasing temperatures using circular dichroism. Melt curves of mammalian *Rattus norvegicus* cTnC (McTnC, shown in red, n=3), *Xenopus laevis* cTnC (XcTnC, shown in green, n=3) and salmonid *Oncorhynchus mykiss* cTnC (ScTnC, shown in blue, n=3) with 5 parameter Weibull fittings revealed significant differences in melting temperature of the three different cTnCs.
Figure 21: Thermal stability melting curves of Tn complexes containing cTnCs from three different species. MTn denotes cardiac troponin complex containing all mammalian *Rattus norvegicus* components (cTnI, cTnT and cTnC), XTn denotes mammlian cTn containing *Xenopus laevis* cTnC, STn denotes mammalian cTn containing salmonid *Oncorhynchus mykiss* cTnC and SCITn denotes mammalian cTn containing salmonid *Oncorhynchus mykiss* cTnC and cTnI. The average relative change in negative ellipticity at 222 nm was measured over increasing temperatures using circular dichroism. Melt curves of MTn (shown in red, n=3), XTn (shown in green, n=3) STn (shown in blue, n=3) and SCITn (shown in cyan, n=3) with 5 parameter Weibull fittings revealed some significant differences in melting temperature of the four different Tn complexes.
Table 4: Melting temperatures of cTnCs from three different species in isolation and in complex.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Melting Temperature (Tₘ, °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McTnC</td>
<td>55.85 ± 0.41 A</td>
</tr>
<tr>
<td>XcTnC</td>
<td>57.31 ± 0.34 B</td>
</tr>
<tr>
<td>ScTnC</td>
<td>52.01 ± 0.30 C</td>
</tr>
<tr>
<td>MTn</td>
<td>49.85 ± 1.06 a*</td>
</tr>
<tr>
<td>XTn</td>
<td>48.46 ± 0.43 ab*</td>
</tr>
<tr>
<td>STn</td>
<td>47.45 ± 1.19 ab*</td>
</tr>
<tr>
<td>SCITn</td>
<td>43.79 ± 0.91 b*</td>
</tr>
</tbody>
</table>

Note: McTnC denotes cardiac troponin C (cTnC) cloned from mammalian *Rattus norvegicus*, XcTnC denotes cTnC from *Xenopus laevis*, ScTnC denotes cTnC from salmonid *Oncorhynchus mykiss*. MTn denotes cardiac troponin complex containing all mammalian *Rattus* components (cTnI, cTnT and cTnC), XTn denotes mammalian cTn containing *Xenopus* cTnC, STn denotes mammalian cTn containing salmonid cTnC and SCITn denotes mammalian cTn containing salmonid cTnC and cTnI. Values represent averages ± SEM (n = 3 for each). Differences in capital letters indicate significant differences between cTnCs determined by one-way ANOVA. Differences in lower case letters indicate significant differences between Tn complexes determined by one-way ANOVA. Asterisks indicate significant differences between complexes and their corresponding cTnC determined by Student’s t-test (p<0.01).
4.2 Steady State Measurements of Ca\(^{2+}\) Affinity

The Ca\(^{2+}\) affinity \((K_a)\) of cTnCs in isolation when investigated with fluorescence spectrometry revealed several significant differences between species. Again, differences were evident from viewing of the titration curves by leftward or rightward shift of the curve, quantified by the pCa\(_{50}\) (-log[Ca\(^{2+}\)] required for \(\frac{1}{2}\) maximal fluorescence) (see Figure 22). The 4-parameter Hill equation fittings revealed significant differences in pCa\(_{50}\) between the three cTnCs. ScTnC \((n=3)\) was found to have the highest pCa\(_{50}\) of 7.00 ± 0.0046, followed by McTnC \((6.95 ± 0.020, n=4)\) and then XcTnC \((n=3)\) had the lowest pCa\(_{50}\) of 6.89 ± 0.01 (see Figure 22). This indicates ScTnC having a 1.12-fold increase in Ca\(^{2+}\) affinity over McTnC and a 1.29-fold increase in Ca\(^{2+}\) affinity over XcTnC. When compared with a one-way ANOVA, all of these differences were found to be significant (see Table 5).

The Ca\(^{2+}\) affinities \((K_a)\) of complexed Tn units were found to be not only different between species, but also different from the cTnCs in isolation. Titration curves of the Tn complexes revealed differences in pCa\(_{50}\) by left or rightward shift of the curves (see Figure 23). The 4-parameter Hill equation fittings revealed significant differences in pCa\(_{50}\)s between the four Tn complexes. XTn \((n=7)\) was found to have the highest pCa\(_{50}\) of 6.30 ± 0.09, followed by SCITn \((6.12 ± 0.05, n=8)\), with MTn \((5.88 ± 0.12, n=7)\) and STn \((5.87 ± 0.04, n=7)\) having the lowest pCa\(_{50}\)s. This indicates XTn having a 2.6-fold increase in Ca\(^{2+}\) affinity over MTn and SCITn having a 1.7-fold increase over MTn. When compared with a one-way ANOVA, XTn and SCITn were found to be significantly different from all others, while MTn and STn did not differ significantly in their pCa\(_{50}\)s (see Table 5).
When comparing between cTnCs and Tn complexes, two-tailed Student’s t-tests found significant differences between all pairs, with the cTnCs having significantly higher pCa$_{50}$s than their complexed counterparts (see Table 5).

The Hill coefficients of the Ca$^{2+}$ titration curves also revealed differences between species, observed by the slope of each curve (see Figures 22 and 23). Hill equation fittings found significant differences between the three cTnCs as well as between the four Tn complexes. The McTnC had the highest Hill coefficient of the three cTnCs at 28.59 ± 1.54, followed by XcTnC (26.00 ± 0.67), with ScTnC (23.89 ± 0.30) having the lowest Hill coefficient. When compared with a one-way ANOVA, all of these differences were found to be significant (see Table 5). Of the Tn complexes, SCITn had the highest Hill coefficient at 35.68 ± 3.23 followed by XTn (16.74 ± 0.62) and STn (15.66 ± 0.81) with MTn (8.14 ± 0.81) having the lowest Hill coefficient. When compared with a one-way ANOVA, MTn had a significantly lower Hill coefficient than the other Tn complexes, while SCITn had a significantly higher Hill coefficient than the other Tn complexes (see Table 5). XTn and STn Hill coefficients did not differ significantly from each other (see Table 5).

When comparing between the cTnCs and their corresponding Tn complexes (for instance: McTnC vs MTn), two-tailed Student’s t-tests found significant differences between all pairs of Hill coefficients, except the pair including SCITn. Each of the significant differences revealed that the Tn complexes had a lower Hill coefficient than their cTnC counterpart (see Table 5).
Figure 22: Ca$^{2+}$ titration curves for cTnCs from three different species. Relative fluorescence of IAANS fluorescence probe over a range of pCa (−log[Ca$^{2+}$]) are shown for *Rattus norvegicus* cTnC (McTnC shown in red, n = 4), *Xenopus laevis* cTnC (XcTnC shown in green, n = 3) and *Oncorhynchus mykiss* cTnC (ScTnC shown in blue, n = 3). Plots were fitted with 3-parameter Hill equations using SigmaPlot in order to determine pCa$_{50}$ values and Hill coefficients for each of the curves.
Figure 23: Ca^{2+} titration curves for Tn complexes containing cTnCs from three different species. Relative fluorescence of IAANS fluorescence probe over a range of pCa are shown for a Tn complex containing *Rattus norvegicus* cTnC (MTn shown in red, n = 7), *Xenopus laevis* cTnC (XTn shown in green, n = 7), *Oncorhynchus mykiss* cTnC (STn shown in dark blue, n = 7) and containing both salmonid cTnC and cTnI (SCITn shown in light blue, n = 8). Plots were fitted with 3-parameter Hill equations using SigmaPlot in order to determine pCa_{50} values and Hill coefficients for each of the curves.
4.3 Stopped-Flow: Ca\(^{2+}\) Off Rates

The high and low-affinity binding sites of both the cTnCs in isolation and of the Tn complexes were investigated separately by monitoring the change in fluorescence of IAANS over different time frames. The N-terminal (low affinity) \(k_{\text{off}}\) of the Tn complexes were determined over a 0.2 second time frame by a fit with a 3-parameter exponential decay equation (see Figure 24). STn had the highest N-terminal \(k_{\text{off}}\) of 51.36 s\(^{-1}\) ± 2.88, followed by MTn (36.32 s\(^{-1}\) ± 1.39), then XTn (30.07 s\(^{-1}\) ± 1.29), and finally SCITn had the lowest N-terminal \(k_{\text{off}}\) of 21.78 s\(^{-1}\) ± 0.97 (see Figure 24). All of the differences found between complexes were significant when compared by one-way ANOVA (p<0.05 for each difference). N-terminal \(k_{\text{off}}\) values for the cTnCs in isolation were measured over a 0.025 sec time frame at 5°C from Ca\(^{2+}\) dissociation curves but dissociation rates were too fast to measure accurately (curves shown in Appendix, Figure 29). As such, dissociation rates of cTnCs and Tn complexes could not be compared statistically. The fact that N-terminal \(k_{\text{off}}\) values could be determined for Tn complexes over a 0.2 second time frame at 15°C, while even 0.025 seconds at 5°C was too long a time frame for the cTnCs indicate that the \(k_{\text{off}}\) of cTnCs in isolation is much faster than that of the complexed Tn units.

The C-terminal (high affinity) \(k_{\text{off}}\) of the cTnCs were determined over a 20 second time frame (see Figure 25). ScTnC was found to have the highest C-terminal \(k_{\text{off}}\) of 0.64 s\(^{-1}\) ± 0.00, followed by XcTnC (0.54 s\(^{-1}\) ± 0.00) and McTnC (0.54 s\(^{-1}\) ± 0.00). ScTnC had a significantly higher \(k_{\text{off}}\) than both XcTnC and McTnC, but there was no significant difference between XcTnC and McTnC \(k_{\text{off}}\) when compared by one-way ANOVA (see Table 5). C-terminal \(k_{\text{off}}\) values of the Tn complexes were also determined over a 20 second time frame (see Figure 26). SCITn was found to have the highest C-terminal \(k_{\text{off}}\) of
0.64 s\(^{-1}\) ± 0.00, followed by STn (0.32 s\(^{-1}\) ± 0.01), then MTn (0.21 s\(^{-1}\) ± 0.03) and XTn with the lowest \(k_{\text{off}}\) value of 0.11 s\(^{-1}\) ± 0.00. All differences between C-terminal \(k_{\text{off}}\) of the four Tn complexes were found to be significant when compared by one-way ANOVA (see Table 5).

When comparing the C-terminal \(k_{\text{off}}\) of the cTnCs to their corresponding Tn complexes, two-tailed Student’s t-tests found significant differences in all off-rates, except the pair including SCITn. In significant pairings, the cTnCs had higher \(k_{\text{off}}\) rates than their Tn complex counterparts (see Table 5).
Figure 24: Average N-terminal Ca\(^{2+}\) dissociation curves of Tn complexes containing cTnCs from three different species (n = 10 for each complex). MTn denotes cardiac troponin complex containing all mammalian *Rattus norvegicus* components (cTnI, cTnT and cTnC), XTn denotes mammalian cTn containing *Xenopus laevis* cTnC, STn denotes mammalian cTn containing salmonid *Oncorhynchus mykiss* cTnC and SCITn denotes mammalian cTn containing salmonid *Oncorhynchus mykiss* cTnC and cTnI. Relative fluorescence of IAANS fluorescence probe are shown over 0.20s period after introduction of chelator EGTA.
Figure 25: Average C-terminal Ca$^{2+}$ dissociation curves for cTnCs from three different species (n = 10 for each cTnC). McTnC denotes cardiac troponin C (cTnC) cloned from mammalian Rattus norvegicus, XcTnC denotes cTnC from Xenopus laevis, ScTnC denotes cTnC from salmonid Oncorhynchus mykiss. Relative fluorescence of IAANS fluorescence probe are shown over 20s period after introduction of chelator EGTA.
Figure 26: Average C-terminal Ca\(^{2+}\) dissociation curves for Tn complexes containing cTnCs from three different species (n = 10 for each complex). MTn denotes cardiac troponin complex containing all mammalian *Rattus norvegicus* components (cTnI, cTnT and cTnC), XTn denotes mammalian cTn containing *Xenopus laevis* cTnC, STn denotes mammalian cTn containing salmonid *Oncorhynchus mykiss* cTnC and SCITn denotes mammalian cTn containing salmonid *Oncorhynchus mykiss* cTnC and cTnI. Relative fluorescence of IAANS fluorescence probe are shown over 20s period after introduction of chelator EGTA.
Table 5: Comparison of Ca^{2+} binding properties of cTnCs and Tn complexes containing cTnCs from three different species.

<table>
<thead>
<tr>
<th>Protein</th>
<th>pCa_{50}</th>
<th>Hill Coefficient</th>
<th>k_{off} N-terminal (s^{-1})</th>
<th>k_{on} N-terminal (µM s^{-1})</th>
<th>k_{off} C-terminal (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>McTnC</td>
<td>6.95 ± 0.02 A</td>
<td>28.59 ± 1.54 A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.54 ± 0.00 A</td>
</tr>
<tr>
<td>XcTnC</td>
<td>6.89 ± 0.01 B</td>
<td>26.00 ± 0.67 B</td>
<td>N/A</td>
<td>N/A</td>
<td>0.54 ± 0.00 A</td>
</tr>
<tr>
<td>ScTnC</td>
<td>7.00 ± 0.01 C</td>
<td>23.89 ± 0.30 C</td>
<td>N/A</td>
<td>N/A</td>
<td>0.64 ± 0.00 B</td>
</tr>
<tr>
<td>MTn</td>
<td>5.88 ± 0.12 a*</td>
<td>8.14 ± 0.81 a*</td>
<td>36.32 ± 1.39 a</td>
<td>47.87 ± 1.05</td>
<td>0.21 ± 0.025 a*</td>
</tr>
<tr>
<td>XTn</td>
<td>6.30 ± 0.09 b*</td>
<td>16.74 ± 0.62 b*</td>
<td>30.36 ± 1.29 b</td>
<td>15.22 ± 1.04</td>
<td>0.11 ± 0.00 b*</td>
</tr>
<tr>
<td>STn</td>
<td>5.87 ± 0.04 a*</td>
<td>15.66 ± 0.81 b*</td>
<td>51.36 ± 2.88 c</td>
<td>69.28 ± 2.63</td>
<td>0.32 ± 0.01 c*</td>
</tr>
<tr>
<td>SCITn</td>
<td>6.12 ± 0.05 c*</td>
<td>35.68 ± 3.23 c</td>
<td>21.77 ± 0.97 d</td>
<td>16.51 ± 0.86</td>
<td>0.64 ± 0.00 d</td>
</tr>
</tbody>
</table>

Note: McTnC denotes cardiac troponin C (cTnC) cloned from Rattus, XcTnC denotes cTnC from Xenopus, ScTnC denotes cTnC from salmonid Oncorhynchus. MTn denotes cardiac troponin complex containing all mammalian Rattus components (cTnI, cTnT and cTnC), XTn denotes mammalian cTn containing Xenopus cTnC, STn denotes mammalian cTn containing salmonid cTnC and SCITn denotes mammalian cTn containing salmonid cTnC and cTnI. Values represent averages ± SEM (n varies from 3 to 10 depending on samples). Differences in capital letters indicate significant differences between cTnCs determined by one-way ANOVA (p<0.05). Differences in lower case letters indicate significant differences between Tn complexes determined by one-way ANOVA. Asterisks indicate significant differences between complexes and their corresponding cTnC determined by a two-tailed Student’s t-test (p<0.001). The k_{on} rates were calculated using the K_a values in µM (calculated using pCa_{50} values) and the k_{off} rates of each complex using Equation 1.
5 DISCUSSION

5.1 SUMMARY

This thesis has advanced the understanding of cardiac muscle function as well as the evolution of the contractile protein complex troponin. The importance of considering the complex as a whole was demonstrated several times with different methodologies. Across protein thermal stability as well as Ca\(^{2+}\) affinity measurements, physiological relevance may be dependent on investigating troponin as a whole complex, rather than in individual subunits. The interaction of the three components of the troponin complex is of utmost importance to the function of cardiac muscle and differences in protein function between ectothermic and endothermic species may have evolutionary significance.

Using circular dichroism, I have determined that the troponin complex of animals may have differences in stability that allow their proteins to remain optimally functional at different physiological temperatures. A difference in melting temperature of 5 °C was found between a troponin complex made up of all mammalian components and one including cTnI and cTnC from a rainbow trout. This change in T_m is indicative of a large change in activation energy between the proteins of these species, possibly signifying that the rainbow trout troponin complex can be more easily activated at lower temperatures than that of the rat. This is supported by previous research which found that cTnC in rainbow trout had a similar regulatory domain structure at 7 °C as a mammalian cTnC did at 30 °C (Blumenschein et al., 2004). Along similar lines, previous work on enzymatic structure and rates in cold-adapted vertebrates have also found adaptive changes in protein stability allowing enzymatic function at low temperatures (Fields, 2001; Fields and Somero, 1998). Circular dichroism has never before been used to investigate differences in cardiac
contractile protein stability between ectothermic and endothermic species. The success demonstrated in this study suggests that this method could potentially be used in future studies as a test for thermal adaptations of proteins in animals with different thermal lifestyles, such as has been performed on hydrothermal vent bacterial proteins (Razvi and Scholtz, 2006).

Another interesting finding included in this thesis is that differences in Ca$^{2+}$ affinities exist between troponin complexes of different animals. The differences found do not entirely support the hypothesis that troponin function has been maintained at different physiological temperatures, but I believe that this is due to a lack of data including the cTnI and cTnT from the respective species. In this thesis I have found that the frog cTnC increases the Ca$^{2+}$ affinity of the mammalian Tn complex by approximately 2.6-fold, supporting the idea that certain residues found in ectothermic cTnC increase the sensitivity of the heart to Ca$^{2+}$. Another very interesting finding was that it was only with the addition of both cTnC and cTnI of the teleost fish that an increase in Ca$^{2+}$ affinity of the mammalian Tn complex was found. The addition of trout cTnI to the complex increased the Ca$^{2+}$ affinity of the complex by 1.8-fold, directly comparable to previous findings on trout cTnI (Kirkpatrick et al., 2011). The N-terminal $k_{\text{off}}$ rates also indicate an increasing Ca$^{2+}$ affinity with ectothermic species, as $k_{\text{off}}$ rates were lowest in the complex containing both trout cTnC and cTnI, followed by the complex containing frog cTnC and then the complex containing mammal cTnC. As a higher $k_{\text{off}}$ rate indicates a lower Ca$^{2+}$ affinity, this order of seems to indicate that the ectothermic proteins may be increasing the affinity of the complex.
The importance of the interaction of the three component proteins of the troponin complex has become increasingly clear through my studies. Not only did differences in cTnC cause differences in function, trout cTnI also had a significant impact on the function of the complex. With this knowledge, one must consider that cTnT differences (a relatively unstudied area) may also have significant impacts on whole muscle functional differences, as could other myofilament proteins (such as myosin binding protein C, tropomyosin, etc.). Investigating the interactions that the complex as well as associated proteins have, along with proteins from other representative species would be areas that future studies should focus upon to further illucidate contractile functional differences across species.

5.2 THERMAL STABILITY OF TROPONIN

This study has found that the sequence differences between the trout, mammalian and Xenopus cTnCs have a significant effect on the thermal stability of the protein. This is the first time that CD has been used on Tn components from different animals to investigate the possibility of adaptations to physiological temperatures. Not only does it appear that sequence differences within the cTnCs of these animals are affecting the stability of the protein, it also appears that the interplay between the subunits of the Tn complex is also key to the stability of the complex as a whole. When considering the cTnCs in isolation, I found that the largest difference in $T_m$ was between the trout and the frog. Although this may seem surprising, the fact is that the frog and mammalian $T_m$s were relatively similar compared to the trout, which had a far lower $T_m$ ($\sim 4^\circ C$) than the other two species. This supports the hypothesis that changes in the contractile proteins of the heart have been made in order to remain functional at the natural physiological temperatures of the animals, as the trout lives at significantly lower temperatures than the other two study species.
Interestingly, I also found that when in complex, it was only the combination of ScTnC and ScTnI within a single complex that resulted in the anticipated reduction in Tm. This difference in Tm of nearly 6°C is an extremely large functional difference between these proteins, and one that could also be considered adaptive to the species lifestyle. The fact that changing cTnC alone was inadequate to see any significant change in complex Tm indicates that the interaction between the complex proteins is of extreme importance to the stability of the whole complex. This could indicate the importance of cTnT and cTnI to the function of the whole Tn unit. Not only does complexing significantly change the thermal stability of the proteins, it was also only when both the trout cTnC and cTnI were present in the complex that functional differences could be discerned within the complexes.

These differences in melting temperatures between these species may signify that the rainbow trout troponin complex can be more easily activated at lower temperatures than that of the rat. This is supported by previous research which found that the structure of the regulatory domain of rainbow trout cTnC at 7 °C was similar to that of mammalian cTnC at 30 °C (Blumenschein et al., 2004). Studies of Ca²⁺ binding to these same cTnC isoforms also indicate a similar difference in structural stability between salmonid and mammalian isoforms, with an alteration in binding properties. While at lower temperatures (7 °C and 21 °C) Ca²⁺ binding experiments resulted in typical sigmoidal curves, at 37 °C this curve shape becomes near-linear (Gillis et al., 2000). This is thought to be indicative of a loss of function at these higher temperatures, perhaps due to denaturation of the protein. This is not entirely surprising, as temperatures above 25 °C are lethal for salmonids, thus it is logical that their proteins may not remain stable and/or functional at these higher temperatures.
The stability of a protein is a characteristic of intrinsic importance to its function. It has been determined several times that thermally extreme animals often have protein mutations which allow those proteins to function properly despite the extreme temperatures they are exposed to. Antarctic ice fish, for instance have alterations to key enzymes in order to maintain optimal function of tissues at extremely cold temperatures (Fields and Somero, 1998). Thermal stability measures have also been used to determine the effects that disease mutations have on proteins. In these cases, it is not necessarily a measure of temperature effects, but instead a measure of whether a disease mutation has a significant effect on the natural balance of lability and stability in the protein. This balance is what dictates whether or not a protein can undergo its physiological function or remains inactivated, and thus can have dire consequences. It has been found, for instance, that an actin mutation seen in hypertrophic cardiomyopathy (HCM) patients (R312H) can have a significant impact on the thermal stability of the protein (Mundia et al., 2012). This underscores the potential implications even a single residue change can have to the function of the whole protein. Thus, despite the fact that relatively few amino acid changes have occurred in the evolution of TnC, it is known that even single residue changes can significantly impact the function of proteins. It would thus be of great interest to discern what residues may be causing these observed differences in thermal stability.

5.3 Calcium Affinities

Within this thesis I have investigated the Ca\textsuperscript{2+} affinities of both cTnCs in isolation as well as Tn complexes. This data has demonstrated not only the importance that different cTnCs play in the Ca\textsuperscript{2+} sensitivity of whole muscle, but perhaps more importantly that interactions within the Tn complex may have very large effects on the Ca\textsuperscript{2+} sensitivity.
When considering the cTnCs in isolation, it is interesting to note that the order of affinities is trout > mammal > frog, with trout having 1.12-fold the Ca\(^{2+}\) affinity of the mammalian isoform. This is a smaller increase in affinity than has previously been found with trout cTnC. When compared at 21 °C, ScTnC was found to have a 2.29-fold higher Ca\(^{2+}\) affinity than the mammalian (bovine) BcTnC (Gillis et al., 2000). This discrepancy could be due to some of the methodological differences between these studies. My study used a different species for the mammalian cTnC (rat instead of bovine), which has 2 amino acid residue differences (positions 115 and 119). It is unlikely that these differences in residues would have any effect on Ca\(^{2+}\) affinity of the cTnCs, since they reside in the C-terminal end of the protein, which is known to have little effect on Ca\(^{2+}\) binding (Gillis et al., 2003).

Another important consideration that may contribute to the difference between previous findings and this study is the use of the fluorescent probe IAANS instead of using the F27W mutation method. Most important to this difference are the mutations to the protein necessary to attach the fluorescent probe IAANS. Unlike previous work on mammalian cTnC, which involves three mutations (two of which are to remove native cysteine residues), labeling trout cTnC with IAANS necessitates a fourth mutation as it contains three native cysteine residues instead of two. Previous studies have routinely performed the mutations necessary for the mammalian cTnC and have been shown to conserve function of the protein, unlike using the native cysteines at position 84 and 35 (Davis et al., 2007; Putkey et al., 1997). However, no studies have ever investigated the effect of removing the extra cysteine residue found in trout cTnC (residue 102). Replacing this cysteine residue should not have a drastic effect on the function of the protein due to its location within the protein. This residue corresponds to EF-Hand Site III in the C-terminal
domain of the protein. Previous work has found that the C-terminal region of cTnC has little effect on the Ca\(^{2+}\) affinity of the protein. Gillis et al. (2003) found that despite the removal of the C-terminus from ScTnC and McTnC, the trout truncated cTnC still maintained an approximately two-fold higher Ca\(^{2+}\) affinity than the mammalian truncated cTnC. It should therefore be noted that amino acid residue changes in the C-terminal domain will most likely not have a significant effect on Ca\(^{2+}\) binding activities.

In contrast to previous findings, this study did not find an increase in Ca\(^{2+}\) sensitivity with the addition of ScTnC to the mammalian complex. This is inconsistent with previous studies considering the cTnC isoforms in isolation wherein a 2.29-fold sensitivity increase was found between ScTnC and bovine cTnC (BcTnC) (Gillis et al., 2000). It also is inconsistent with studies of whole myocytes, wherein insertion of a mutated cTnC with trout equivalent residues at positions 2, 28, 29 and 30 (NIQD McTnC) into rabbit cardiomyocytes resulted in a 2-fold increase in Ca\(^{2+}\) sensitivity (Gillis et al., 2005). It is important to note, however, that my study considers a different level of organization from either of these previous studies. My study looked at the whole troponin complex, where previous work looked at cTnCs in isolation or whole cardiomyocyte function. Interestingly, one study investigating the impact the L29Q mutation caused in human cTnC also found a discrepancy between isolated cTnC and cTn complex data (Li et al., 2013). They found that although L29Q produced an increase in Ca\(^{2+}\) binding affinity in isolated cTnC, no difference in Ca\(^{2+}\) affinity was found at the level of the cTn complex when combined with a phosphomimetic cTnI (Li et al., 2013). This aligns with my finding that trout cTnC did not significantly change the Ca\(^{2+}\) affinity of the Tn complex even though at the individual cTnC level it did have a difference in Ca\(^{2+}\) affinity. Perhaps of more interest
to our study is the fact that replacement of McTnC with the frog XcTnC resulted in a 2.6-fold increase in Ca$^{2+}$ affinity of the complex. This is a very significant increase in affinity and can be considered indicative of the importance of an increased Ca$^{2+}$ affinity to the ectothermic Tn complex. This is especially interesting considering frog cTnC contains the same L29Q residue, suggesting that both the interactions between complex proteins as well as individual residue differences may both be important factors to determining Ca$^{2+}$ affinity.

It is not only cTnC differences that I found to affect Ca$^{2+}$ affinities, the addition of trout cTnI to the complex also resulted in an increase in Ca$^{2+}$ affinity of the complex. This study revealed a 1.8-fold increase in affinity associated with the addition of trout cTnI to the complex also containing trout cTnC. This result is directly comparable to previous findings where the replacement of rat cTnI with trout cTnI was previously found to increase the Ca$^{2+}$ affinity of the complex 1.8-fold (Kirkpatrick et al., 2011). Not only has trout cTnI been found to increase the Ca$^{2+}$ affinity of the Tn complex, it also affects the Ca$^{2+}$ sensitivity of myocyte contraction. The C-terminus of the trout cTnI contains three amino acid residues (His$^{163}$, Val$^{165}$, and Asn$^{171}$ respectively, see Figure 28) that are known to increase the Ca$^{2+}$ sensitivity of the heart (Westfall and Metzger, 2007). It is therefore not surprising that with the addition of trout cTnI into the complex that a change in Ca$^{2+}$ sensitivity is observed, as there are residues known to cause this. The insertion of any of the mammalian residues (Ala, Glu and His respectively) into alternate TnI isoforms at the same relative position has been found to decrease the Ca$^{2+}$ sensitivity of myocyte contraction (Westfall and Metzger, 2007). It has been suggested that this is due to the need for the mammalian heart to decrease its Ca$^{2+}$ sensitivity in response to increased body
temperatures, to avoid Ca\(^{2+}\) remaining bound to TnC and the muscle being locked in contraction (Shaffer and Gillis, 2010). Interestingly, the histidine residue found at position 163 of the trout cTnI can also be found in the mammalian fetal cTnI isoform, a fact that will be discussed further below.

Although the pCa\(_{50}\)s found did not entirely follow the predicted pattern of trout > frog > rat, when considered in conjunction with the Hill coefficients, some interesting conclusions can be made. The Hill coefficients increased from all mammal components to 2/3 fish components, suggesting that a Tn complex containing more trout proteins responds to much smaller changes in [Ca\(^{2+}\)] than a complex with mammalian counterparts. With this in consideration, the fact that the Tn complex containing frog cTnC had a higher pCa\(_{50}\) than any other complex may not be indicative of the Ca\(^{2+}\)-binding activity a complete frog Tn complex. It must also be noted, however that Hill coefficients are a measure of cooperativity of a system. As the cardiac troponin isoform contains only one binding site, these measures therefore may not have much physiological relevance. Along the same lines, even when combined with cTnI and cTnT, each troponin complex acts in isolation when in solution and thus cooperativity measures may not be entirely meaningful without the presence of other elements of the contractile system. It is known that Ca\(^{2+}\) binding in whole muscle is a cooperative system. Interactions between different component proteins (mainly troponin and tropomyosin) increase the likelihood of Ca\(^{2+}\) binding to a site in proximity to one that has already bound Ca\(^{2+}\) (Tobacman and Sawyer, 1990). In studies such as this one, however, with no other contractile proteins included in the system, Hill coefficients are mainly used as a measure of the curve-fitting and not necessarily as a measure of cooperativity.
5.4 Calcium Off and On Rates

The N-terminal Ca\(^{2+}\) off-rates \((k_{off})\) from the troponin complexes reveal an interesting pattern, with the complex containing both trout cTnI and cTnC had the lowest off-rate, followed by the complex containing the frog cTnC and then the all mammalian protein complex. This order is logical, as the higher affinity afforded by the ectothermic cTnCs and trout cTnI seems to increase the N-terminal affinity for Ca\(^{2+}\), which would translate to a lower off-rate. Previous work has only looked at the off-rates from a mammalian complex in interaction with different proteins, but these values are comparable to those found in my study. Davis et al. (2007) found that the cTn complex had an off-rate of 41.9/s, while Li et al. (2013) found an off-rate of 33.1/s. The off-rate that I determined in this study (36.3/s) falls directly between these previously reported values, making the off-rates highly comparable with known values. No other studies have investigated off-rates from either trout or frog cTnCs, and thus I can only suggest that my results are indicative of differences in Ca\(^{2+}\) binding function between the proteins of these species.

The N-terminal Ca\(^{2+}\) on-rates \((k_{on})\) from the troponin complexes provide more interesting evidence for differences in Ca\(^{2+}\) binding between different species. The fact that my numbers are calculated from mean \(K_a\) and \(k_{off}\) values determined from two separate experiments makes it difficult to discern significance. In addition to this, very few other studies have looked at on-rates, making comparisons equally difficult, especially as some rely on computer modeling techniques and/or contain differences in methodology (Davis et al., 1999; Tikunova and Davis, 2004). Despite this, it is interesting to note that the N-terminal \(k_{on}\) of the troponin complex containing trout cTnC was higher than the mammalian complex, suggesting that Ca\(^{2+}\) binds more quickly to this complex than any of the others.
This is unsurprising, given that previous work has found that the structure of this protein may allow it to become activated more easily (Blumenschein et al., 2004). This again points to differences in ligand binding function between species, suggesting that conservative substitutions in the amino acid sequence can have large effects on function.

5.5 **Evaluation of Methods**

In order to determine whether the troponin complexes were in fact complexed, I both ran SDS-PAGE gels to visualize relative protein levels of each dialysed sample as well as checked the fluorescence of the complexes. The fluorescence levels of TnCs in isolation increased with the addition of Ca\(^{2+}\), while fluorescent levels of complexed Tn decrease with the addition of Ca\(^{2+}\) due to the interaction of TnC with TnI and TnT (previously reported in Davis et al. (2007); Kirkpatrick et al. (2011)). While this indicates that the proteins are interacting, it does not tell us whether the proteins are in their proper conformation. Additional evidence indicating that the proteins are interacting when in complex is the fact that N-terminal off-rates from complexed Tn units were significantly slower than off-rates from cTnCs in isolation. This effect of Tn complexing on \(k_{\text{off}}\) rates has been previously reported by Davis et al. (2007), where both formation of a complex, as well as the addition of other associated proteins (tropomyosin in this case) to the Tn complex slowed the rate of Ca\(^{2+}\) dissociation (\(k_{\text{off}}\)) from the complex. Dissociation rates from the N-terminal domain of cTnC in isolation are therefore usually too high to be measured accurately. In this study for instance, despite lowering the temperature of the reaction to 5 °C and shortening the timeframe to 0.025 s, \(k_{\text{off}}\) values were too inconsistent and high to be considered. Complexing of TnI and TnT with TnC, however, slowed the
dissociation reaction to the point that not only was it detectable, but it was detectable over a longer time frame and at a higher temperatures (0.2 s, 15 °C). Since the C-terminal domain under physiological conditions never undergoes Ca\(^{2+}\) (or Mg\(^{2+}\)) dissociation, the values I have determined for C-\(k_{\text{off}}\) rates may not be of great importance in the large scheme. Nonetheless, the fact that the off-rates determined for cTnCs in isolation were significantly faster than those of their corresponding complex indicates yet again that the cTnCs in isolation are more sensitive to changes in Ca\(^{2+}\) concentration than the Tn complex in whole. This slowing of the Ca\(^{2+}\) off-rate again is indicative of an interaction between the three complex proteins, but does not necessarily determine whether all proteins are in their correct orientation. In order to determine this, future work should include a method for determining the physical structure of either parts of the complex using nuclear magnetic resonance (NMR) as in Blumenschein et al. (2004), or of the entire complex using X-ray crystallography techniques such as multi-wavelength anomalous dispersion (MAD), as in Takeda et al. (2003).

The high Hill coefficient values calculated for the complexes may be due to differences in complexing between the four samples. It would be important, therefore to check the structures are fully complexed and that the subunits from different species interact in the appropriate way. Previous studies have indicated that troponin subunits from different species are complementary enough to complex appropriately, but it would be interesting to investigate structural differences between complexes made from multiple species’ subunits.

The use of the fluorescent probe IAANS to determine changes in conformation of cTnC is a method that has been performed previously with success (Davis et al., 2007;
Johnson et al., 1980; Putkey et al., 1989, 1997). This method is considered both accurate and fairly convenient as it covalently binds to cysteine within the protein. Also, unlike the labeling of native cysteine residues, the use of a T53C mutation allows for minimal alteration of function of cTnC as well as excellent reporting of structural changes (Davis et al., 2007). However, as a fairly large molecule (504 Da), IAANS does have the potential to alter the natural function of the protein it is bound to. Alternative methods to consider include different fluorescent probes (such as DAANZ) and the use of tryptophan fluorescence (F29W mutant protein), along with the use of a fluorescent Ca$^{2+}$ chelator such as Quin-2 (Tikunova and Davis, 2004). In solution experiments such as those performed herein using tryptophan as a fluorescent marker would be a useful comparative method, but for experiments using whole muscle preparation, background fluorescence from different myocyte proteins naturally containing tryptophan make this method unsuitable. To allow direct comparison to whole muscle experiments, it is thus ideal to use non-native fluorescence such as the IAANS probe. In addition to this, the fluorescent chelator Quin-2 is useful for measuring Ca$^{2+}$ off-rates, but cannot help determine Ca$^{2+}$-affinities, nor whole muscle sensitivity.

6  **Future Directions**

6.1 **Sequence of Trout Cardiac Troponin T, Complete Complexes**

Despite multiple attempts to clone troponin T from trout cardiac muscle, no sequence has yet been determined for this protein. PCR primers designed based on the Atlantic salmon cTnT sequence found in the NCBI database (Reference: NM_001146662.1), have obtained product fragments using trout cardiac cDNA. The full ScTnT sequence is still
unknown however, and thus complexing a complete trout Tn complex remains to be accomplished.

A similar situation exists for *Xenopus laevis* cTnI and cTnT, although the sequences for each can be found (GI: 410050 and GI: 148233663, respectively). This project was limited to cloning cTnC from the *Xenopus laevis*, but an important future step would be to clone the entire complex in order to obtain more complete data. This would be especially important for cTnI, as increasingly more data is indicating that this inhibitory protein plays a key role in the Ca$^{2+}$-sensitivity of the muscle. In addition to this, data on complete troponin complexes from multiple species could potentially give a more physiologically relevant picture than cTnCs in isolation, since the complex functions as a unit within the cardiomyocyte.

Although this study provided significant insight into the roles of both cTnC and cTnI within the complex, we cannot discern what role cTnT has until studies can be performed comparing the mammalian cTnT with that of another species. The cTnT sequences available for rat and frog reveal several amino acid residue differences, but without a functional comparison as well as a trout sequence, much is conjecture. Similarly, mutational studies would be necessary to reveal the role that single amino acid residues play within the complex. Such studies have previously been performed to investigate pathological mutations such as the L29Q mutation in cTnC of hypertrophic cardiomyopathy patients. As such, the methodologies are well known to allow the isolation of single residue changes, but such studies have rarely been focused on the evolutionary perspective before.
6.2 **Protein Isoform Expression**

The expression of multiple, or of different isoforms of a protein within one tissue is one method of quickly adapting, or compensating for large changes in a physiological condition. This has been seen in both different species as well as under different conditions. Rainbow trout (*Oncorhynchus mykiss*) have been found to express seven different isoforms of TnI in their hearts, and acclimation to cold waters has been shown to change gene expression of these isoforms significantly (Alderman et al., 2012). This unprecedented number of gene transcripts found in a single tissue is most likely due to the fact that salmonids have undergone several genome duplication events over evolutionary time, and are now mostly tetraploids (Meyer and Schartl, 1999). These duplicated copies also seem to have been retained and expressed, suggesting that they serve an important purpose, as otherwise such duplicates would be lost over evolutionary time. However, despite the number of gene transcripts found in the tissue, only three of the isoforms of TnI were found to be expressed as a protein when investigated with mass spectrometry (Alderman et al., 2012). Despite the finding of multiple TnI isoforms in different thermal conditions, research has shown that there is remarkably little variation in TnC sequences between fishes, even between those with remarkably different lifestyles (Yang et al., 2000). It has also recently been found that two separate isoforms of TnC can be expressed in the teleost heart: both a cardiac (cTnC) and a slow skeletal isoform (ssTnC) (Genge et al., 2013). This finding is interesting in that it opens the possibility of multiple isoforms of my protein of interest being expressed within the heart. However, this same study found that cTnC was almost exclusively expressed within the ventricle of the heart expressed with the ssTnC isoform only being found at low levels in the atrium (Genge et al., 2013). Despite
this, the fact that teleost fish have retained such a diversity of isoforms of these key proteins must still be taken into consideration.

Isoform switching is also known to occur during development as well, with many animals switching from a “fetal” isoform to an “adult” isoform after some stage of development (species dependent). Such fetal isoform switching occurs for both TnT and TnI, with a slow skeletal isoform of TnI in fetal mammals switching to the cardiac isoform after birth, and with multiple TnT isoforms switching over to one in a similar pattern (Gorza et al., 1993; Saggin et al., 1989; Schiaffino et al., 1993). The fetal isoform of mammalian cTnI is known to contain a “histidine button” (at position 164 in mammals) that also happens to be found in adult trout cTnI. It has been found that this histidine is responsible for protecting contractile function during acidosis due to ischemic heart failure (Day et al., 2006). Acidic conditions are known to reduce force production by reducing the Ca$^{2+}$ sensitivity of myofilaments (Lee and Allen, 1991). It is the charge differences in TnI isoforms, brought about by the presence of histidine residues that results in the differences in pH-sensitivity of the proteins. This may be related to the ability of ectotherms to maintain protein function over a wider range of temperatures, and also to their ability to regulate pH changes with temperature ($\alpha$-stat regulation). Knowing that these isoform switches occur in many animals, as well as knowing that multiple isoforms can be expressed at any one time in one tissue leads to a consideration of whether multiple isoforms of different troponin subunits are being expressed in the animals considered in this study. If this is the case, multiple different protein isoforms may need to be characterized in order to investigate the true physiological condition occurring within the heart of these species.
6.3 Interactions of Troponin Complex Proteins

The interacting regions of the troponin complex, as highlighted in Takeda et al. (2003) are informative of what amino acid residue changes may be important to functional changes. Figure 27 illustrates a linear depiction of the three proteins of the troponin complex, alongside tropomyosin and actin. Highlighted with arrows are the key regions of interaction between proteins, with colours differentiating those interactions that are Ca\(^{2+}\)-dependent. Using this type of alignment, we can predict what residues may be contributing to the functional differences I observed, especially considering the large changes in Ca\(^{2+}\) sensitivity associated with changing the TnI within the complex. As seen in Figure 27, cTnI interacts with cTnC and actin. The interaction between cTnI helix 3 (H3) and the cTnC N-terminal hydrophobic cleft is of particular interest, as it is dependent on Ca\(^{2+}\), and thus could have implications for the Ca\(^{2+}\) affinity of the troponin unit as well as the Ca\(^{2+}\) sensitivity of the whole muscle. The C-terminal region of TnI also is thought to interact with cTnC, though the degree of this interaction has yet to be determined, due to the unknown nature of the complete TnI structure. Not only is this interaction dependent on Ca\(^{2+}\), it is also one of the primary drivers of the interaction between the inhibitory region (IR) of TnI and actin – the interaction responsible for inhibition of acto-myosin-ATPase activity. Thus, understanding not only the full structure of TnI, but also how differences in residues can change interactions of these proteins could provide much greater understanding of how contraction is regulated.

With these important interaction sites in mind, we can then consider changes in amino acid sequence of these key domains across different species (see Figure 28). This alignment of cTnI clarifies the intermediate structure of the frog cTnI, which contains an
“ectothermic” C-terminus containing residues in common with the trout, but the N-terminal extension and PKA sites commonly associated with endotherms. The C-terminal region of trout cTnI contains three residues (shown in blue in Figure 28) that have been found to increase the Ca\(^{2+}\) sensitivity of cardiomyocytes (Westfall and Metzger, 2007). The N-terminal extension found in the cardiac isoform of some species (shown in green in Figure 28) is known to affect muscle response to adrenergic stimulation through the addition of two phosphorylation sites (Ser\(^{23}\) and Ser\(^{24}\) in the mammalian isoform). This study did not include any phosphorylation experiments, but previous work has found that trout cTnI is less affected by PKA phosphorylation than rat cTnI (Kirkpatrick et al., 2011). Treatment of cTn complexes containing either rat or trout cTnI with PKA resulted in decreased Ca\(^{2+}\) affinity of both complexes, with a 2.2-fold larger difference in affinity in the complex containing rat cTnI (Kirkpatrick et al., 2011). This suggests that the difference in PKA sites in different species cTnI has a large effect when it comes to functional responses to adrenergic stimulation. Thus, future studies should also consider differences in phosphorylation states between species, as adrenergic stimulation of the heart plays an important role in the regulation of cardiac output. Differences in key residues can have implications for cardiac contraction, as well as for sensitivity to pH and Ca\(^{2+}\) signaling (Day et al., 2006; Layland et al., 2004; Li et al., 2004; Westfall and Metzger, 2001, 2007).

When one considers the highlighted sections of the cTnI sequence (shown in Figure 28), several differences in amino acid sequence between the mammal, frog and trout can be seen. Although the functional implications of some of these differences have been investigated (such as N-terminal serines affecting PKA phosphorylation and C-terminal residues affecting Ca\(^{2+}\) affinity), no studies have ever investigated these differences in
conjunction with each other as well as in an evolutionary perspective. These differences in sequence of key regions known to interact directly with other proteins in the complex would be of great interest to study in future work, as they could provide the answer to those differences in Ca$^{2+}$ affinity I have seen that cannot be fully explained by cTnC alone.
Figure 27: Linear depiction of troponin complex proteins with regions of protein interaction shown. The orange arrow indicates that the interaction between TnI N-terminal helical region and the C-terminal hydrophobic cleft of TnC is strong under physiological conditions due to the fact that EF-Hand Sites III and IV are permanently occupied with ions. Green, double-ended arrows indicate interactions that are dependent on the state of Ca\(^{2+}\) binding of the complex. IR stands for inhibitory region of TnI.
Figure 28: Alignment of cTnI from the three study species, rat (Rattus norvegicus), frog (Xenopus laevis) and trout (Oncorhynchus mykiss). Highlighted in green is the N-terminal extension containing Ser^23 and Ser^24 (shown in bold), known targets for phosphorylation by PKA. Highlighted in blue are residues known to increase Ca^{2+} sensitivity in the trout (His^163, Val^165, and Asn^171, respectively). Highlighted in red is the inhibitory region (IR) and in orange is helix 3 (H3) of TnI. Yellow highlight indicates the C-terminal region known to also undergo some interaction with cTnC.
6.4 COMPLEXING AND WHOLE MUSCLE PREPARATIONS

Another important consideration for future studies is the interaction not only between the troponin subunits and Ca\(^{2+}\), but also the interactions between the troponin complex and the rest of the contractile unit. Performing troponin exchange into whole cardiac trabeculae would provide insight into how changes in cTnC and other Tn subunits can affect the muscle as a whole. An important consideration when comparing heart and muscle function from different species is their differences not only in protein sequence, but also in contractile regulation. The Frank-Starling, or stretch response is one of the differences in mechanism we can observe between ectothermic and endothermic species. It is known that ectothermic cardiomyocytes maintain an active response over sarcomeric lengths above and beyond those that mammals can successfully create tension over (Shiels et al., 2006). This difference is thought to be one of the contributing factors in the method employed to change cardiac output, with ectotherms modulating mainly stroke volume, while endotherms modulate heart rate. The experiments I performed herein, while adding knowledge at the protein level, does not give us data on sarcomeric interactions. Future work should include studies exchanging troponin subunits within whole cardiomyocytes to determine how each subunit can affect not only Ca\(^{2+}\) affinity of the complex, but also of the whole muscle preparation (Gillis et al., 2005).

6.5 OTHER SPECIES

At the scale of the whole cardio-pulmonary system, it is also important to consider the relative activity level of these species. It is possible that as a relatively low-activity species of frog, which remains aquatic throughout its life, the African clawed frog (*Xenopus laevis*) may be a difficult species to compare with relatively active species such
as the rainbow trout and the rat. *Xenopus laevis* despite inhabiting permanent bodies of water, are capable of traveling over land, occasionally migrating between adjacent bodies of water (Wilson et al., 2000). Unfortunately, relatively few studies have been performed on the *Xenopus* heart, despite the fact that it is often used as a model species for developmental pathways (Kay and Peng, 1992). What studies have been done focus less on the relative pressure load of the heart and more on the distribution of oxygenated vs deoxygenated blood (Graaf, 1957). Thus, it is difficult to characterize whether this amphibian species has what would be characterized as a “pressure pump” or a “volume pump” heart.

Future studies should also investigate the differences in function of other species, as little work has been done on the Tn complexes of other amphibians, reptiles or birds. Investigating the functional characteristics of the cTnC animals such as the alligator (*Alligator mississippiensis*) would give us more data to consider and add to our understanding of the evolution of endothermy and of the cardiac system. These two species are fairly well studied and thus have sequence data readily available for future study. Another interesting possibility would be to focus more on the physiological temperatures that species are adapted to live at, and from there choose study species which can unveil some of the largest differences. Antarctic icefish would be an interesting species to consider from a thermal adaptation point of view, as despite some differences in cTnC amino acid sequence from the trout (see Figure 7 again), no significant difference in Ca$^{2+}$ affinity between the cTnCs of these species exist (Gillis et al., 2003). Obtaining sequences for the other troponin subunits for this species might reveal how cardiac muscle remains functional at extremely low temperatures. In addition to this, but one must also consider
that this study was only performed at the level of the N-terminal domain of the cTnC in isolation. Scaling this up to the level of the whole complex as well as including studies with filament proteins and whole trabeculae would give us a more complete picture of the interactions at play.

7 **SIGNIFICANCE**

This project has provided novel insight into how cardiac contractile function has changed during the evolution of endothermy in vertebrates. By examining how sequence differences in *X. laevis* cTn alters its functional properties, we have increased our understanding of the molecular interactions that regulate cardiac muscle contraction. A greater understanding at a molecular level is of particular academic interest, as it is at this most basal level that evolution occurs. Single nucleotide substitutions due to random mutations can lead to large changes in protein function by altering the amino acid sequence. This change in protein function may lend itself better to a particular condition or environment than the unchanged protein (ie: lower Ca$^{2+}$ binding affinity at high temperatures), and allow greater survival of individuals with the mutation. The nucleotide substitution will then be selected for in the new condition, leading to evolution of proteins with altered function. By investigating the function of a cardiac protein from an animal intermediate both evolutionarily and in internal body temperature, we can perhaps learn which nucleotide changes are key in the evolution of cardiac function and endothermy.

This project has potential implications for human health as well as for the evolution of troponin proteins. Some of the sequence differences that have been found in the frog and trout cTn can also be found in the human pathological condition hypertrophic cardiomyopathy (HCM). The first human mutation of cTnC found (L29Q) has been
subject to many studies and interestingly is one of the known N-terminal residues found in trout cTnC that increases Ca\(^{2+}\) affinity of the cTnC (Gillis et al., 2005; Hoffmann et al., 2001; Li et al., 2013). In human pathological conditions, it has been found to cause a similar change in Ca\(^{2+}\) sensitivity, and thus results in contractile dysfunction (Li et al., 2013). One of the known HCM pathological mutations to cTnI (K183E) is also an ancestral residue found in trout cTnI (Gomes and Potter, 2004; Harada and Morimoto, 2004). This residue falls within the C-terminal domain of cTnI, the region known to interact with the N-terminal hydrophobic cleft in cTnC (Takeda et al., 2003). It therefore has the potential to also affect Ca\(^{2+}\) sensitivity of the complex. Finally, another HCM mutation found in the C-terminus of cTnT (S69F) can also be found in cTnT of lower vertebrates such as teleosts (Gomes and Potter, 2004). Thus, this study is another step to providing insight into the relative importance specific amino acid sequence changes have on regulatory function of the heart. Knowing what sequence changes have the largest effect on the Ca\(^{2+}\) sensitivity of the heart (a detrimental condition in humans) could potentially provide avenues for screening of pathological conditions such as HCM.

The importance of this thesis to our understanding of cardiac contractile function and how it has changed across vertebrates can therefore be seen not only from an evolutionary perspective, but also from a human health perspective. We must consider organisms from multiple levels of biological organization in order to understand the function of not only individual proteins but also how they interact. An understanding of differences in Ca\(^{2+}\) binding properties within the hearts of vertebrates thus scales upwards, and must be considered of significant importance to our understanding of the basics of muscle function and to the evolution of the heart.


Herzberg, O., and James, M.N.G. (1988). Refined crystal structure of troponin C from turkey skeletal muscle at 2·0 Å resolution. J. Mol. Biol. 203, 761–779.


### APPENDIX

#### 9.1 Buffers

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<td></td>
<td>1 mM EDTA</td>
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<td></td>
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<td></td>
<td>90 mM KCl</td>
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<td>pH 7.0</td>
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1% SDS

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<th>Complexing Buffer 3</th>
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Figure 29: N-terminal Ca$^{2+}$ dissociation curves for cardiac troponin Cs (cTnCs) from three different species (n=10 for each species). McTnC denotes cTnC cloned from Rattus norvegicus, XcTnC denotes cTnC from Xenopus laevis, ScTnC denotes cTnC from salmonid Oncorhynchus mykiss. Relative fluorescence of IAANS fluorescence probe are shown over 0.025s period after introduction of chelator EGTA.
9.3 Permissions

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