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# A functional comparison of cardiac troponin C from representatives of three vertebrate taxa: Linking phylogeny and protein function

Elizabeth J. Sears, Todd E. Gillis *

*Department of Integrative Biology, University of Guelph, Guelph, ON N1G 2W1, Canada*
*Cardiovasclar Research Center, University of Guelph, Canada*

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ABSTRACT

The $Ca^{2+}$ affinity of cardiac troponin C (cTnC) from rainbow trout is significantly greater than that of cTnC from mammalian species. This high affinity is thought to enable cardiac function in trout at low physiological temperatures and is due to residues $Asn^{2}$, $Ile^{28}$, $Gln^{29}$, and $Asp^{30}$ (Gillis et al., 2005, Physiol Genomics, 22, 1–7). Interestingly, the cTnC of the African clawed frog *Xenopus laevis* (frog cTnC) contains $Gln^{29}$ and $Asp^{30}$ but the residues at positions 2 and 28 are those found in all mammalian cTnC isoforms ($Asp^{2}$ and $Val^{28}$). The purpose of this study was to determine the $Ca^{2+}$ affinity of frog cTnC, and to determine how these three protein orthologs influence the function of complete troponin complexes. Measurements of $Ca^{2+}$ affinity and the rate of $Ca^{2+}$ dissociation from the cTnC isoforms and cTn complexes were made by monitoring the fluorescence of anilinonapthalenesulfote iodoacetamide (IAANS) engineered into the cTnC isoforms to report changes in protein conformation. The results demonstrate that the $Ca^{2+}$ affinity of frog cTnC is greater than that of trout cTnC and human cTnC. We also found that replacing human cTnC with frog cTnC in a mammalian cTn complex increased the $Ca^{2+}$ affinity of the complex by 5-fold, which is also greater than complexes containing trout cTnC. Together these results suggest that frog cTnC has the potential to increase the $Ca^{2+}$ sensitivity of force generation by the mammalian heart.



## 1. Introduction

Myocyte contraction is initiated when $Ca^{2+}$ binds to troponin C (TnC) and triggers a series of conformational changes through the component proteins of the thin filament that result in the formation of cross-bridges between actin and myosin. Troponin C along with troponin I (TnI) and troponin T (TnT) are the component proteins of the troponin (Tn) complex and it is changes in the interactions between these that lead to the activation of the contractile reaction. Manipulation of the functional characteristics of the troponin complex through either the phosphorylation of specific residues or by manipulation of the amino acid sequence of the component proteins can have a significant influence on the contractile function of striated muscle (Shaffer and Gillis, 2010). For example, the phosphorylation of cardiac TnI (cTnI) by protein kinase A (PKA) following β-adrenergic stimulation decreases the $Ca^{2+}$ affinity of the Tn complex and this enables a faster rate of relaxation between beats (Dong et al., 2007; Fentzke et al., 1999). We have also demonstrated that changes to the amino acid sequence of cardiac TnC (cTnC) that increase its $Ca^{2+}$ affinity, increase the $Ca^{2+}$ sensitivity of force generation by chemically skinned cardiac myocytes (Gillis et al., 2005). More specifically, the $Ca^{2+}$ affinity of rainbow trout cardiac TnC (trout cTnC) is approximately two-fold that of bovine cTnC and the residues responsible for this are $Asn^{2}$, $Ile^{28}$, $Gln^{29}$, and $Asp^{30}$ (Gillis et al., 2005). When bovine cTnC was mutated to contain these four trout residues, its $Ca^{2+}$ affinity was increased two-fold, and when native cTnC in rabbit cardiac myocytes was replaced with this mutant, the $Ca^{2+}$ sensitivity of force generation was increased two-fold compared to controls (Gillis et al., 2005). The comparatively high $Ca^{2+}$ affinity of trout cTnC is thought to be responsible, in part, for the comparatively high $Ca^{2+}$ sensitivity of the trout heart (Gillis et al., 2000). This characteristic is proposed to help enable cardiac function in the trout at low physiological temperatures (Gillis and Tibbits, 2002).

*Abbreviations:* cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; IAANS, anilinonapthalenesulfote iodoacetamide; $k_{off}$, rate of $Ca^{2+}$ dissociation; $k_{on}$, rate of $Ca^{2+}$ association; $cTnC^{T53C}$, cTnC mutant where all native cysteines have been replaced with serines and $Thr^{53}$ has been mutated to a Cys; cTn, cardiac troponin; *n*H, Hill coefficient; $K_{F1/2}$, $Ca^{2+}$ concentration at half-maximum $Ca^{2+}$-dependent fluorescence; $C_HI_KT_R$, cTn complex composed of human cTnC, rat cTnI and rat cTnT; $C_FI_RT_R$, cTn complex composed of frog cTnC, rat cTnI and rat cTnT; $C_TI_RT_R$, cTn complex composed of trout cTnC, rat cTnI and rat cTnT; $C_TI_TT_R$, cTn complex composed of trout cTnC, trout cTnI and rat cTnT.

* Corresponding author.
*E-mail address:* tgillis@uoguelph.ca (T.E. Gillis).
*URL:* http://comparativephys.ca/gillislab/ (T.E. Gillis).

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The studies described above suggest that manipulation of the sequence of cTnC is a mechanism by which evolutionary pressures have altered cardiac contractility. Interestingly, the amino acid sequence of cTnC from the clawed frog, *Xenopus laevis*, is 97% identical to rat cTnC but contains two of the $Ca^{2+}$-sensitizing residues ($Gln^{29}$ and $Asp^{30}$) present in trout cTnC, while the other two ($Asp^{2}$ and $Val^{28}$) are shared with mammalian isoforms of cTnC (Gillis et al., 2007a). Amphibians diverged from the vertebrate lineage after teleost fish but before eutherian mammals. Phylogenetic studies of cTnC from vertebrates demonstrate a similar pattern of divergence for fish cTnC's, frog cTnC and cTnC's from endothermic species (Gillis et al., 2007a). The amino acid sequence of frog cTnC may therefore represent an intermediate step in the evolution of cTnC in the vertebrate lineage. The purpose of this study is to characterize the function of frog cTnC and determine how it influences the functional properties of the cardiac Tn (cTn) complex. We specifically examined the $Ca^{2+}$ binding properties of frog cTnC, trout cTnC and human cTnC in solution, and how this was affected when these proteins were complexed with cTnI and cTnT. The $Ca^{2+}$ affinity of the isolated cTnC isoforms and cardiac troponin (cTn) complexes were determined using a steady state assay, while stopped flow kinetics was used to measure the rate of $Ca^{2+}$ disassociation ($k_{off}$). Both of these assays relied on anilinonapthalenesulfote iodoacetamide (IAANS) engineered into each cTnC at a cysteine at residue 53. We also used circular dichroism measurements to see if the thermal stability of the proteins is reflective of physiological temperature.

## 2. Materials and methods

### 2.1. Cloning of frog cTnC

The University of Guelph Animal Care Committee approved all protocols and the adult, female *X. laevis* used in this study was obtained from Ward's Natural Science (St. Catherines, ON, Canada). The frog was pithed and the heart surgically removed and rinsed with ice-cold physiological saline. The ventricle was then quickly dissected, flash frozen using liquid nitrogen and stored at −80 °C until needed. Total RNA was extracted using TRIzol® Reagent (Invitrogen) according to manufacturer's directions. RNA was 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.

Primers were designed against *X. laevis* (frog) cTnC (GenBank Accession # BC053760.1) with Primer3 software (http://frodo.wi.mit.edu/primer3/) to include the *Nde* I (CA^TATG) or *Xho* I (C^TCGAG) restriction site in the 5′ and 3′ primers, respectively (Table 1). The PCR reactions with the *Xenopus* cDNA were performed using Platinum® *Taq* DNA polymerase (Invitrogen Life Technologies, Frederick, MD, USA) according to manufacturer's directions. The PCR products were subsequently purified and digested with *Nde*I and *Xho*I, then ligated into linearized pET-24a(+) plasmid using T4 DNA ligase (NEB, Ipswich, MA, USA), and transfected into NovaBlue competent cells (Novagen). Liquid cultures were grown overnight and isolated plasmids were sequenced to confirm the presence of the insert. From this point forward *Xenopus* cTnC will be referred to as frog cTnC.

**Table 1**
Sequence of primers utilized to engineer restriction sites for Nde1 and Xho1 into cDNA for frog cTnC and trout cTnC.

| Primer | Sequence |
|---|---|
| frog cTnC forward (NdeI) | GCGTGCCATATGGATGATATTTACAAAGCAGCGGTTG |
| frog cTnC reverse (XhoI) | TCGCGCTCGAGTTATTCAACTCCCTTCATGAATTCC |
| trout cTnC forward (NdeI) | GCGTGCCATATGAACGACATCTACAAAGCA |
| trout cTnC reverse (XhoI) | TCGGCCTCGAGTTATTCTACTCCTTTCATGAACTC |

### 2.2. Mutation of the cDNA for frog cTnC and trout cTnC

Previous studies have measured $Ca^{2+}$ binding to the N-terminus of cTnC when it is in complex with cTnI and cTnT through the use of the fluorescent probe IAANS. To attach the IAANS, the cDNA for these proteins were mutated to replace native cysteine residues at positions 35 and 84 with serine residues and to replace the threonine at residue 53 with a cysteine. IAANS is then attached to $Cys^{53}$ via a disulphide bond. This mutation and subsequent labeling of the cTnC molecules has been demonstrated to not affect the $Ca^{2+}$ binding properties of mammalian isoforms of cTnC in isolation or when complexed with cTnI and cTnT (Hazard et al., 1998; Liu et al., 2012). While the amino acid sequences of the frog cTnC and trout cTnC differ from that of mammalian cTnCs, it is not expected that IAANS will alter the function of these proteins as it is attached in a region of the protein that does not influence the $Ca^{2+}$ activation of the molecule or its interaction with cTnI or cTnT (Davis et al., 2007; Kirkpatrick et al., 2011). This method was therefore followed in the current study for frog cTnC and trout cTnC. The cDNA for trout cTnC was a gift from Dr. Glen Tibbits (Simon Fraser University). There is an additional cysteine in trout cTnC at position 103. This was also, therefore, mutated to serine. This sequence manipulation is not expected to influence the $Ca^{2+}$ binding properties of the molecule as position 103, in the C-terminal domain, does not interact with the N-terminus of the protein (Takeda et al., 2003) nor do changes to the C-terminus affect $Ca^{2+}$ binding to site II (Gillis et al., 2005; Gillis et al., 2003b). The primers utilized to complete the above mutations to frog cTnC and trout cTnC are listed in Table 2. These mutations were performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's directions, and subsequently sequence-verified. The two mutant proteins will be denoted as frog cTnC and trout cTnC.

### 2.3. Expression of component proteins

Frog $cTnC^{T53C}$, trout $cTnC^{T53C}$, human cTnC (human $cTnC^{T53C}$), rat cTnI, rat cTnT, and trout cTnI were expressed as previously described using BL21 Gold (DE3) competent *E. coli* cells (Novagen, San Diego, CA) (Kirkpatrick et al., 2011). The expressed proteins were then purified using an AKTA FPLC (GE Healthcare) as previously described (Kirkpatrick et al., 2011). The purity of all proteins was confirmed using SDS-PAGE. Each cTn component protein was then lyophilized and stored at −20 °C.

### 2.4. IAANS labeling of the $cTnC^{T53C}$ mutants

Purified and lyophilized human $cTnC^{T53C}$, trout $cTnC^{T53C}$, and frog $cTnC^{T53C}$ were labeled with the fluorescent probe IAANS as previously described (Kirkpatrick et al., 2011). In brief, the three proteins were dialyzed into (in mmol $l^{-1}$): 50 Tris, 6000 urea, 90 KCl, 1 EGTA, pH 7.5. Protein concentration was determined using a Bradford assay and then a 5-fold molar excess of IAANS was added to the solution. The labeling reaction proceeded for 8 h at 5 °C in the dark, and was then stopped with 2 mmol $l^{-1}$ DTT. The IAANS labeled $cTnC^{T53C}$ isoforms

**Table 2**
Sequence of primers used to mutate the sequences of frog cardiac troponin C (cTnC) and trout cTnC to enable the attachment of IAANS to $Cys^{53}$. The sequence of the forward primer of each primer set is shown.

| Primer | Sequence |
|---|---|
| frog cTnC C35S | ACGCTGAAGATGGCAGCATTAGCACCAAG |
| frog cTnC T53C | GGGGCAGAATCCCTGTCCTGAGGAGTTA |
| frog cTnC C84S | GGTTATGATGGTCCGCAGCATGAAAGACGACAG |
| trout cTnC C35S | ATGCGGAGGACGGCAGCATCAGTACCAAG |
| trout cTnC T53C | GGGGCAGAACCCCTGCCCCGAGGAGCTG |
| trout cTnC C84S | GGTGATGATGGTGAGGAGCATGAAGGACGACAG |
| trout cTnC C102S | GAACTGGCAGATCTCTTCAGCATGTTTGACAAGAATG |

were then dialyzed against (in mM): 10 MOPS (pH 7.0), 4600 urea, 1 DTT, with 0.01% $NaN_3$. IAANS concentration was calculated from its absorbance at 325 nm using its extinction coefficient of 24,900 l $mol^{-1}$ $cm^{-1}$. The efficiency of binding was calculated by dividing the concentration of the labeled protein by the IAANS concentration. The efficiency of the labeling was determined with a Bradford assay for protein concentration and a spectrofluorometer for the probe (labeling efficiencies were 88% for trout $cTnC^{T53C}$, 89% for frog $cTnC^{T53C}$ and 100% for human $cTnC^{T53C}$). To simplify the following descriptions and discussions the IAANS labeled $cTnC^{T53C}$ isoforms will be herein designated as trout cTnC, frog cTnC and human cTnC.

### 2.5. cTn complex formation

The cTn complexes used in this study were as follows: $C_HI_RT_R$ (human cTnC, rat cTnI and rat cTnT); $C_FI_RT_R$ (frog cTnC, rat cTnI and rat cTnT); $C_TI_RT_R$ (trout cTnC, rat cTnI and rat cTnT); and $C_TI_TT_R$ (trout cTnC, trout cTnI and rat cTnT). The only sequence difference between rat cTnC and human cTnC is a conservative substitution of methionine for isoleucine at residue 119 that should not alter the function of the protein. Therefore, the proteins in $C_HI_RT_R$ complex would be interacting with each other within a mammalian conformation. The cTn complexes were prepared as previously described (Kirkpatrick et al., 2011). In brief, cTn subunits were dialyzed separately into (in mM) 10 MOPS (pH 7.0), 4600 urea, 1 DTT, and 0.01% $NaN_3$, and then mixed for 20 min at a molar ratio of 3:2:3 (cTnI:cTnC:cTnT). The complexes were dialyzed against a series of buffers containing (in mM) 10 MOPS (pH 7.0), 0.5 DTT, 3 $MgCl_2$, and 0.01% $NaN_3$. The first buffer contained 2 M urea and 1 M KCl; the second contained 1 M KCl; and the third contained 0.15 M KCl (repeated three times). The complexes were then dialyzed against a titration buffer containing (in mM) 200 MOPS (pH 7.0), 150 KCl, 3 $MgCl_2$, 1 DTT, 0.02% Tween-20, and 2 EGTA. Fig. 1 is a protein gel illustrating the composition of the Tn complexes.

### 2.6. $Ca^{2+}$ titrations

The $Ca^{2+}$ affinities of each of the IAANS labeled cTnC mutants and cTn complexes were measured as previously described (Kirkpatrick et al., 2011) using a spectrofluorometer (model LS 55; Perkin Elmer, Waltham, MA). Briefly, 2 ml of 0.15 $\mu M^{-1}$ cTnC or cTn were titrated with 1μl volumes of $CaCl_2$ stock solutions into quartz cuvettes. Temperature was maintained at 15 °C and a stir bar provided constant mixing throughout the experiment. This temperature was chosen as it has been used in multiple other studies examining the $Ca^{2+}$ binding

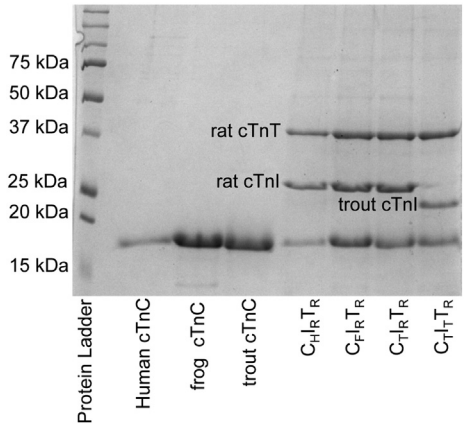


**Fig. 1.** Image of SDS-PAGE gel all cTnCs and cTn complexes to check relative protein purity as well as complexing. Gel is 12% acrylamide, stained with Coomassie Blue stain. Lane 1 is the standard weight ladder. $C_HI_RT_R$ (human cTnC, rat cTnI, and rat cTnT); $C_FI_RT_R$, (frog cTnC, rat cTnI, and rat cTnT); $C_TI_RT_R$ (trout cTnC, rat cTnI, and rat cTnT); and $C_TI_TT_R$ (trout cTnC, trout cTnI, and rat cTnT).

characteristics of cTnC isoforms and cTn complexes (Davis et al., 2007; Kirkpatrick et al., 2011). In addition, previous work by Gillis et al. (2000) demonstrated that the $Ca^{2+}$ affinity of trout cTnC and bovine cTnC are affected identically by the same change in temperature. This means that the difference in function is maintained between the two proteins when measured across a range of temperatures. An excitation wavelength of 330 nm was used with fluorescence being monitored at 450 nm. The free $Ca^{2+}$ concentrations were determined as previously described (Gillis et al., 2000) using MaxChelator (Bers et al., 1994), following which the –log of these concentrations (pCa) were plotted against relative fluorescence of IAANS. The $Ca^{2+}$ affinity data for $C_HI_RT_R$ is that from (Kirkpatrick et al., 2011) generated using the same protein complex, identical buffers and methods as described here. All curves were fitted with a 4-parameter Hill equation using SigmaPlot 12.5 (Systat Software, San Jose, CA). The free $Ca^{2+}$ concentrations were plotted against the percentage maximal fluorescence (relative fluorescence) of IAANS. The resultant curves were then fitted to the Hill equation:

$$Y = F_{max}\left[x^{nH}/\left(K_{F1/2}{}^{nH} + x^{nH}\right)\right], \quad (1)$$

where Y is the relative fluorescence, $F_{max}$ is the maximum $Ca^{2+}$-dependent fluorescence equal to 1, x is the free [$Ca^{2+}$] (M), *n*H is the Hill coefficient and $K_{F1/2}$ is the $Ca^{2+}$ concentration at half-maximum $Ca^{2+}$-dependent fluorescence. $K_{F1/2}$ was used as a measure of $Ca^{2+}$ affinity. Each fitting generated a goodness of fit value ($R^2$) and this value was between 0.89 and 0.99 for all fits.

### 2.7. $Ca^{2+}$ off-rates

Stopped-flow kinetics experiments of the IAANS labeled cTnC isoforms and cTn complexes were conducted as previously described (Gillis et al., 2007b) using an SX20 Stopped-Flow Spectrometer (Applied Photophysics, Leatherhead, Surrey, UK) to measure the rates of $Ca^{2+}$-dissociation ($k_{off}$) from both the N- and C-terminus using EGTA as a $Ca^{2+}$ chelator. These measurements were completed at 15 °C as this is the same temperature as the $Ca^{2+}$ affinity measurements. 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 three-parameter equation and a $k_{off}$ value calculated from each fit equation. The $k_{off}$ from the N-terminus of the isolated cTnC isoforms and cTn complexes was measured over a period of 0.2 s (Gillis et al., 2007b). The $k_{off}$ values presented for the N-terminus of each complex were calculated by summing and fitting the data from three reactions and then repeating this at least four more times with 12 other reactions. The rate presented for the C-terminus represents the second rate from a double exponential fit of data collected over a 20 s period (Gillis et al., 2007b).

### 2.8. Determination of thermal stability

The melting temperature of the three cTnC isoforms and four cTn complexes were measured as in (Mundia et al., 2012; Perieteanu et al., 2008). In brief, the proteins and protein complexes were dialyzed into a buffer containing (in mM) 200 MOPS (pH 7.0), 150 KCl, 3 $MgCl_2$, 1 DTT, 0.02% Tween-20, and 2 EGTA, then diluted to 0.16 mg $ml^{-1}$. Measurements were made using a 200 μl quartz cuvette and a JASCO J-815 Chiro-optical spectrometer (JASCO, Easton, MD). The negative ellipticity of the protein samples was measured every 5 °C at 222 nm with an increasing thermal gradient of 1 °C $min^{-1}$ from 5 to 80 °C. The relative rate of change of negative ellipticity was then plotted as a function of temperature and Weibull fitted using SigmaPlot 12.5 (Systat Software, San Jose, CA). The melting temperature ($T_m$) of each protein was the minimum value of the Weibull fit (Mundia et al., 2012; Perieteanu et al., 2008).

### 2.9. Data and statistical analysis

Differences between the $K_{½}$ values, the Hill coefficients (*n*H), the $k_{off}$ values and the $T_m$ values were analyzed using one-way ANOVAs followed by Holm-Sidak post-hoc testing since each experimental repetition was independent. Students' *t*-tests were also used to compare each average value of the cTn complexes to their corresponding cTnC. All data was plotted and fit using SigmaPlot 12.5 (Systat Software, San Jose, CA).

## 3. Results

### 3.1. $Ca^{2+}$ binding properties of cTnC isoforms

The IAANS fluorescence of all three cTnC's increased during the $Ca^{2+}$ titration and reached an asymptote upon saturation (Fig. 2A). The $Ca^{2+}$ affinity of frog cTnC, as determined by the $Ca^{2+}$ concentration at half maximal fluorescence ($K_{½}$), was 2.1- and 1.4-fold that of human cTnC and trout cTnC, respectively, and that of trout cTnC was 1.4-fold that of human cTnC (Table 3). The Hill coefficient (*n*H) is typically used to describe the co-operativity of $Ca^{2+}$ binding to a single molecule with multiple binding sites, or of $Ca^{2+}$-activated tension in a functioning myofibril. However, as this study examined $Ca^{2+}$ binding of cTnC, which has a single activation site due to mutations in the putative $Ca^{2+}$ binding site I, co-operativity is highly unlikely. For this reason, the *n*H is used only as a parameter of curve fitting, and a physiological interpretation of these differences has been avoided. The *n*H for frog cTnC and trout cTnC were greater than that of human cTnC, reflecting that the slopes of the $Ca^{2+}$ binding curves were steeper, indicative of a greater increase in fluorescence per unit $Ca^{2+}$ (Table 3). The $Ca^{2+}$ off-rates ($k_{off}$) from the N-terminal domain of the uncomplexed cTnC isoforms were too fast to be measured. Previous studies have reported $k_{off}$ values for both isolated human and trout cTnC mutants, but these contained mutations that reduced $Ca^{2+}$ affinity, including the F27W mutation (Liang et al., 2008; Tikunova and Davis, 2004). The C-terminus $k_{off}$ of trout cTnC was 0.64 $s^{-1}$ ± 0.001. This was the fastest $k_{off}$ of the isolated isoforms followed by frog cTnC (0.54 $s^{-1}$ ± 0.001) and human cTnC (0.54 $s^{-1}$ ± 0.002) (Table 3).

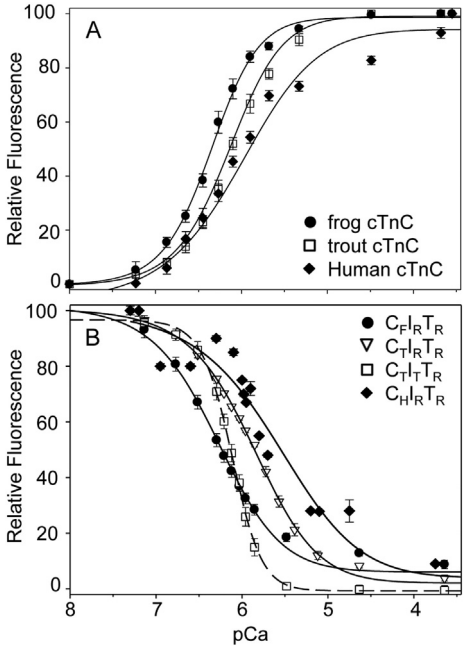


**Fig. 2.** $Ca^{2+}$ titration curves of human cTnC, frog cTnC, trout cTnC, $C_HI_RT_R$, $C_FI_RT_R$, $C_TI_RT_R$, and $C_TI_TT_R$. A) $Ca^{2+}$ titration curves frog cTnC, human cTnC and trout cTnC. B) $Ca^{2+}$ titration curves of $C_HI_RT_R$ (human cTnC, rat cTnI, and rat cTnT), $C_FI_RT_R$ (frog cTnC, rat cTnI, and rat cTnT), $C_TI_RT_R$ (trout cTnC, rat cTnI, and rat cTnT) and $C_TI_TT_R$ (trout cTnC, trout cTnI, and rat cTnT). Values shown on each figure are means ± standard error. N for each data set is 6–7 $Ca^{2+}$ titration runs of separate samples a given protein or complex.

### 3.2. $Ca^{2+}$ binding properties and $Ca^{2+}$ dissociation rates of the cTn complexes

As in previous studies (Davis et al., 2007; Kirkpatrick et al., 2011), the fluorescence of all cTn complexes decreased during the $Ca^{2+}$ titration and reached an asymptote upon saturation (Fig. 2B). This change in fluorescence with the addition of $Ca^{2+}$ is opposite to when the cTnC's are functioning in isolation and therefore is indicative of the cTnC isoforms working in complex with cTnI and cTnT. Comparison of the $K_{½}$'s of the cTn complexes reveals that the $Ca^{2+}$ affinity of $C_FI_RT_R$ was 5.2-, 2.7-, and 1.5-fold that of $C_HI_RT_R$, $C_TI_RT_R$ and $C_TI_TT_R$, respectively (Table 3). The $Ca^{2+}$ affinity of $C_TI_TT_R$ was 3.5- and 1.8-fold that of $C_HI_RT_R$ and $C_TI_RT_R$, respectively (Table 3). Finally, the $Ca^{2+}$ affinity of $C_TI_RT_R$ was 1.9-fold that $C_HI_RT_R$ (Table 3). The mean *n*H for $C_HI_RT_R$ was 0.77 ± 0.04. This is the lowest *n*H of all the cTn complexes. The replacement of human cTnC in the $C_HI_RT_R$ with either trout cTnC or frog cTnC increased the *n*H by approximately 0.4 and the replacement of rat cTnI in $C_TI_RT_R$ with trout cTnI increased the *n*H by a further 1.39 (Table 3).

The $Ca^{2+}$ dissociation from all cTn complexes caused an increase in IAANS fluorescence (Fig. 3). A similar result has been reported by others using similar methods to measure the $k_{off}$ of cTn complexes (Davis et al., 2007). The $k_{off}$ of the $C_HI_RT_R$ complex (36.32 ± 1.39) is quite similar to that previously reported (41.9 ± 0.4) for this cTn complex by others using the same method (Davis et al., 2007). In addition, as mentioned above, the $k_{off}$ of cTnC isoforms in isolation is much faster than that of cTnC isoforms in complex. The $k_{off}$'s measured in the current study are therefore indicative of cTnC isoforms functioning in a cTn complex $k_{off}$. The $k_{off}$'s of $C_TI_TT_R$ and $C_FI_RT_R$ were significantly slower than that of $C_HI_RT_R$, as would be expected of cTn complexes with higher $Ca^{2+}$ affinities (Table 3). The $k_{off}$ of $C_TI_RT_R$ was 1.4-, 1.7- and 2.4-fold faster than that of $C_HI_RT_R$, $C_FI_RT_R$ and $C_TI_TT_R$, respectively (Table 3). An estimate of the $Ca^{2+}$ on-rate ($k_{on}$) for each of the cTn complexes was calculated using the relationship $k_{on} = K_{F1/2} * k_{off}$ where $K_{F1/2}$ represents the binding affinity of the N-domain of cTnC for a single $Ca^{2+}$ ion and $k_{off}$ represents the release of a single $Ca^{2+}$ ion (Tikunova and Davis, 2004). These calculations indicate that $C_FI_RT_R$ has the fastest $k_{on}$ followed by that of $C_TI_RT_R$, $C_TI_TT_R$ and $C_HI_RT_R$ (Table 3). The C-terminal $k_{off}$ of $C_TI_TT_R$ was 0.64 $s^{-1}$ ± 0.002. This was the fastest of all of the cTn complexes, followed by $C_TI_RT_R$ (0.32 $s^{-1}$ ± 0.006), $C_HI_RT_R$ (0.21 $s^{-1}$ ± 0.025) and $C_FI_RT_R$ (0.11 $s^{-1}$ ± 0.003). All differences between the C-terminal $k_{off}$'s of the four cTn complexes were statistically significant.

### 3.3. Thermal stability

An increase in assay temperature caused the relative negative ellipticity, of the cTnC and cTn complexes to increase (Fig. 4). As this method characterized global tertiary structure the results demonstrated that the melting point of trout cTnC was significantly lower than that of frog cTnC and human cTnC (Table 3). When these three cTnC isoforms were complexed with rat cTnT and rat cTnI there were no differences in melting points between the three complexes ($C_HI_RT_R$, $C_FI_RT_R$ and $C_TI_RT_R$) (Table 3). However, the replacement of rat cTnI in $C_TI_RT_R$ with trout cTnI ($C_TI_TT_R$) caused a significant decrease in the melting point of the complex (Table 3).

## 4. Discussion

The results of this study demonstrate that the $Ca^{2+}$ affinity of frog cTnC is significantly higher than that of trout cTnC and human cTnC, that frog cTnC increases the $Ca^{2+}$ affinity of the mammalian cTn complex ($C_HI_RT_R$) and that the melting temperature of frog cTnC is higher than that of human cTnC and trout cTnC. These results therefore demonstrate that the sequence differences between frog cTnC and that of

**Table 3**
Comparison of $Ca^{2+}$ binding properties and melting temperatures of human cTnC, frog cTnC, trout cTnC, $C_HI_RT_R$, $C_FI_RT_R$, $C_TI_RT_R$, and $C_TI_TT_R$.

| Protein | $K_{F½}$ (μM) | *n*H | $k_{off}$ N-terminal ($s^{-1}$) | $k_{on}$ N-terminal ($\times 10^7\ M^{-1}\ s^{-1}$) | Melting temperature (°C) |
|---|---|---|---|---|---|
| Human cTnC | 0.98 ± 0.08 A | 1.09 ± 0.07 A | N/A | N/A | 55.9 ± 0.4 A |
| Frog cTnC | 0.46 ± 0.03 B | 1.60 ± 0.53 B | N/A | N/A | 57.3 ± 0.3 B |
| Trout cTnC | 0.71 ± 0.04 C | 1.57 ± 0.14 B | N/A | N/A | 52.0.3C |
| $C_HI_RT_R$ | 2.6 ± 0.12 a* | 0.77 ± 0.04 a* | 36.32 ± 1.39 a | 1.40 | 50.0 ± 1.1 a* |
| $C_FI_RT_R$ | 0.50 ± 0.09 b | 1.15 ± 0.04 b* | 30.36 ± 1.29 b | 6.07 | 48.6 ± 0.4 ab* |
| $C_TI_RT_R$ | 1.35 ± 0.04 c* | 1.16 ± 0.06 b* | 51.36 ± 2.88 c | 3.81 | 47.5 ± 1.2 ab* |
| $C_TI_TT_R$ | 0.76 ± 0.05 d | 2.55 ± 0.23 c | 21.77 ± 0.97 d | 2.90 | 43.9 ± 0.9 b* |

$K_{F½}$, the $Ca^{2+}$ concentration at half maximal fluorescence; *n*H, Hill coefficient calculated using the Hill equation; $k_{off}$ N-terminal, the rate of $Ca^{2+}$ dissociation from the N-terminus; $k_{on}$ N-terminal, the rate of $Ca^{2+}$ binding the N-terminus calculated using the $K_a$ and $k_{off}$ of each complex. Melting temperatures of cTnCs and cTn complexes were determined from Weibull fittings of circular dichroism ellipticity measures at 222 nm. $C_HI_RT_R$, complex of human cTnC, rat cTnI, and rat cTnT; $C_FI_RT_R$, complex of frog cTnC, rat cTnI and rat cTnT; $C_TI_RT_R$, complex of trout cTnC, rat cTnI and rat cTnT; and $C_TI_TT_R$, complex of trout cTnC, trout cTnI and rat cTnT. Values in the same column indicated by different uppercase or lowercase letters are different from each other. Asterisks indicate significant differences between complexes and their corresponding cTnC. N/A indicates measurements that are not applicable to the system studied. Values represent averages ± SEM. $N = 6$–7 for $Ca^{2+}$ binding measurements, 10 for the $k_{off}$ measurements, and 3 for the melting temperature measurements.

human cTnC and trout cTnC have functional consequence. We also found that the replacement of rat cTnI with trout cTnI in a cTn complex containing trout cTnC and rat cTnT, increases the $Ca^{2+}$ affinity of the complex.

### 4.1. The $Ca^{2+}$ affinities of frog cTnC, trout cTnC and human cTnC

As predicted by previous studies (Gillis et al., 2005; Gillis et al., 2000; Gillis et al., 2003b), the $Ca^{2+}$ affinity of trout cTnC was greater than that of human cTnC. The residues responsible for this functional difference are $Asn^2$, $Ile^{28}$, $Gln^{29}$ and $Asp^{30}$ (Gillis et al., 2005). These four residues appear to be relatively conserved in fish cTnC's with at least three of the residues present in cTnC's cloned from 26 species of fish (Fig. 5). In all mammalian cTnC's these residues correspond to $Asp^2$, $Val^{28}$, $Leu^{29}$ and $Gly^{30}$ (Gillis et al., 2007a) and in frog cTnC these are $Asp^2$, $Val^{28}$, $Gln^{29}$ and $Asp^{30}$ (Fig. 6). The frog sequence therefore appears to be intermediate between fish cTnC isoforms and mammalian cTnC isoforms as it contains $Gln^{29}$ and $Asp^{30}$ present in all fish cTnC isoforms and $Asp^2$ and $Val^{28}$, present in all mammalian cTnC isoforms (Fig. 6). However, the results of this study indicate that the function of frog cTnC is not intermediate to that of trout cTnC and human cTnC in that its $Ca^{2+}$ affinity was significantly greater than both of these two isoforms. Previous studies have demonstrated that the amino acid sequences of cTnCs from mammalian species (humans, mice, porcine, bovine, rat, mouse) are highly conserved. At the level of the complete protein, there is 99% conservation, while the N-terminal region of the proteins is 100% conserved (Gillis et al., 2007a). This is also true of cTnC sequences from multiple bird species (common quail, Japanese quail, and chicken), suggesting that variation in the functional regions of cTnC is extremely low in endothermic animals (Gillis et al., 2007a).

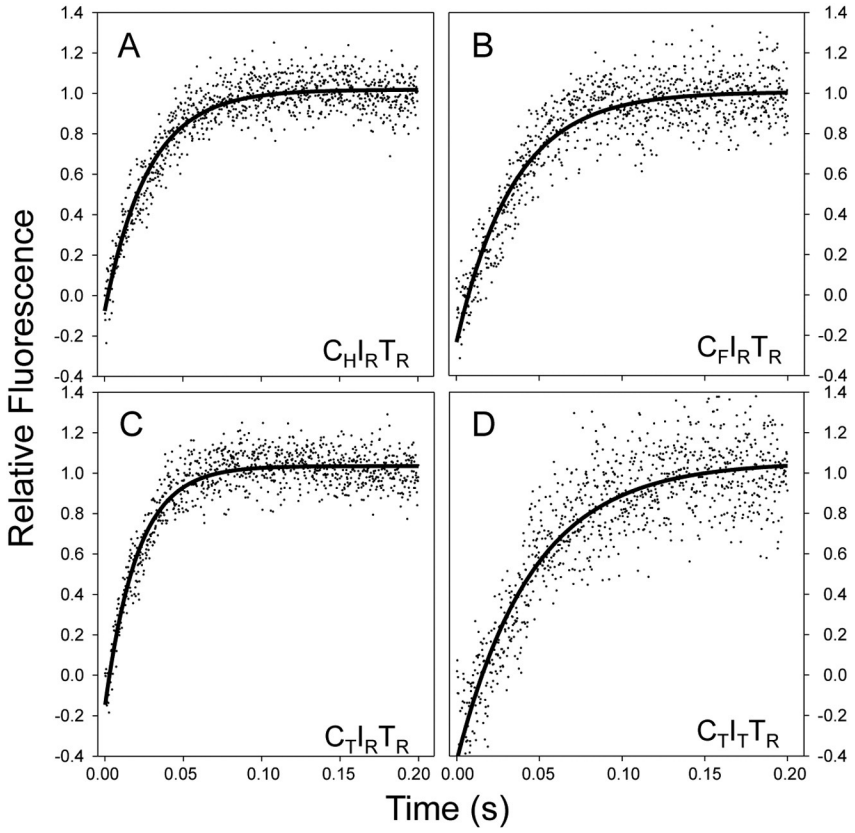


**Fig. 3.** N-terminal $Ca^{2+}$ dissociation curves of $C_HI_RT_R$, $C_FI_RT_R$, $C_TI_RT_R$, and $C_TI_TT_R$. $C_HI_RT_R$ (human cTnC, rat cTnI, and rat cTnT), $C_FI_RT_R$ (frog cTnC, rat cTnI, and rat cTnT), $C_TI_RT_R$ (trout cTnC, rat cTnI, and rat cTnT) and $C_TI_TT_R$ (trout cTnC, trout cTnI, and rat cTnT). Relative fluorescence is over a 0.20 s period after introduction of the chelator EGTA. N for each data set is 10 $Ca^{2+}$ dissociation runs of separate samples of a given complex.

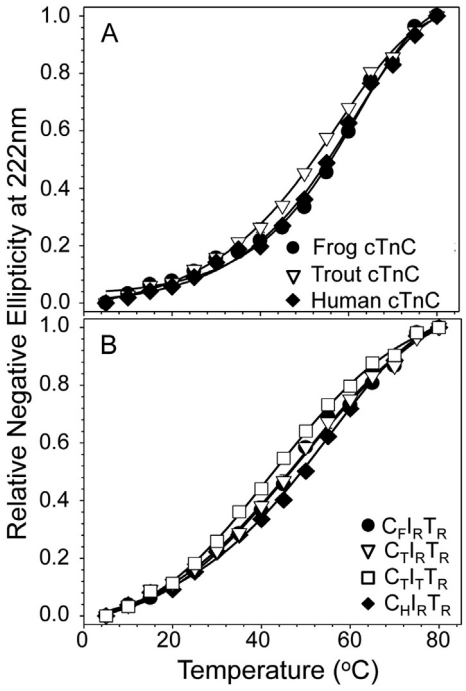


**Fig. 4.** Thermal stability melt curves of human cTnC, frog cTnC, trout cTnC, $C_HI_RT_R$, $C_FI_RT_R$, $C_TI_RT_R$, and $C_TI_TT_R$. The average relative change in negative ellipticity at 222 nm was measured over increasing temperatures using circular dichroism. A) Melt curves of human cTnC, frog cTnC and trout cTnC. B) Melt curves $C_HI_RT_R$ (human cTnC, rat cTnI, and rat cTnT), $C_FI_RT_R$ (frog cTnC, rat cTnI, and rat cTnT), $C_TI_RT_R$ (trout cTnC, rat cTnI, and rat cTnT) and $C_TI_TT_R$ (trout cTnC, trout cTnI, and rat cTnT). Each data set was fit with a 5 parameter Weibull fitting to calculate melting point. N for each data set is 3 melt runs of separate samples of a given protein or complex.

For further discussion of the evolution of cTnC please refer to Gillis et al. (2007a).

There are five sequence differences between frog cTnC and human cTnC. These are $Arg^{21}$, $Gln^{29}$, $Asp^{30}$, $Glu^{122}$ and $Arg^{138}$ in frog cTnC (Fig. 6). We have previously demonstrated that it is sequence differences in the N-terminus of cTnC (1-89) that influence the $Ca^{2+}$ affinity of Site II (Gillis et al., 2005; Gillis et al., 2003b). This suggests, therefore, that the residue most likely responsible for the comparatively high $Ca^{2+}$ of frog cTnC, in addition to $Gln^{29}$ and $Asp^{30}$, is $Arg^{21}$. The fact that the $Ca^{2+}$ affinity of frog cTnC is also greater than that of trout cTnC; and the trout isoform, like human cTnC, has a lysine at position 21 instead of an arginine, supports this idea. At physiological pH, lysine and arginine are both charged, aliphatic amino acids therefore a switch from one to another is considered a conservative substitution. However, we have demonstrated that relatively subtle changes in the N-terminus of cTnC can have significant consequences on the ability of the molecule to be activated by $Ca^{2+}$ (Gillis et al., 2005; Gillis et al., 2003b). One reason is that residues in this region of the molecule, especially those surrounding the non-functional $Ca^{2+}$ binding site I in cTnC, undergo a significant shift in position within the tertiary structure of the cTnC molecule during $Ca^{2+}$ activation (Blumenschein et al., 2004; Gillis et al., 2003a). This includes the amino acids at positions 21, 29, and 30 (Blumenschein et al., 2004; Gillis et al., 2003a). As sequence changes to a protein can affect intermolecular interactions as well as how the protein interacts with the solvent, manipulations to this highly mobile region of cTnC have significant potential to affect the ability of the protein to be activated by $Ca^{2+}$ (Blumenschein et al., 2004; Gillis et al., 2003a). In addition, changes to residues in this region have the potential to affect the $Ca^{2+}$ affinity of site II, the activation site, through an allosteric interaction (Blumenschein et al., 2004; Gillis et al., 2003a). Further study is required to determine how the Lys/Arg substitution at residue 21 in combination with the sequence differences at positions 29 and 30 influences the function of frog cTnC.

### *4.2. The $Ca^{2+}$ binding properties of the cTn complexes*

The $Ca^{2+}$ affinities of $C_FI_RT_R$ and $C_TI_RT_R$ were 5.2-fold and 1.9-fold that of $C_HI_RT_R$ indicating that the replacement of human cTnC with either frog cTnC or trout cTnC increases the $Ca^{2+}$ affinity of the cTn complex. This suggests that the comparatively high $Ca^{2+}$ affinity of frog cTnC and trout cTnC, measured in isolation, are maintained when the proteins are functioning with common components of the troponin complex (rat cTnI and rat cTnT). Interestingly, the difference in $Ca^{2+}$ affinity between $C_FI_RT_R$ and $C_HI_RT_R$ is even greater than that between the frog cTnC and human cTnC in isolation. This must be due to differences in the interactions between the two, cTnC isoforms and the rat cTnI and rat cTnT in the cTn complex influencing the $Ca^{2+}$ affinity of the $Ca^{2+}$ binding site. Previous work has demonstrated that the $Ca^{2+}$ affinity of the cTn complex or of the $Ca^{2+}$ sensitivity of a cardiac muscle

```
rainbow trout          MNDIYKAAVEQLTDEQKNEFKAAFDIFIQDAEDGCISTKELGKVMRMLGQNPTPEELQEM 60
Atlantic salmon        ............................................................ 60
green puffer           ..........N..E.............................................. 60
zebrafish              .........A..........R.....V.................................. 60
sablefish              ............E.............V.................................. 60
yellow croaker         ............................V.................................. 60
rainbow smelt          ............................V.................................. 60
turquoise killifish    ............................V.................................. 60
Northern pike          ............................V.................................. 60
Victorian cichlid      ............................V.................................. 60
mummichog              ............................V.................................. 60
Japanese pufferfish    ............................V.................................. 60
tongue sole            ............................V.................................. 60
Atlantic herring       ............NE......R...EV.V.................................. 60
Mexican cavefish       ..............S..R.....V.................................. 60
Senegal bircher        ..........N..E.....V.....L.................................. 60
zebra mbuna            .D.V......N..E.............................................. 60
sheepshead minnow      .D.V......N..E.............................................. 60
Southern platyfish     .D.V......N..E.............................................. 60
African cichlid        .D.V......N..E.............................................. 60
annual killifish       .D.V......N..E.............................................. 60
black rockcod          .D.V......N..E.............................................. 60
bicolour damselfish    .D.V......N..E.............................................. 60
fairy cichlid          .D.V......N..E.............................................. 60
sailfin molly          .D.V......N..E...........V.................................. 60
coelacanth             .S..........E...A.........V.....................S..A..... 60
African clawed frog    .D..........E......R.....V.................................. 60
Western clawed frog    .D..........E......R.....V.................................. 60
Human                  .D..........E............VLG................................. 60
```

**Fig. 5.** Comparison of the first 60 amino acids of cTnC isoforms cloned from 26 fish species, two frog species and humans. Residues in blue in rainbow trout cTnC sequence are the residues responsible for its comparatively high $Ca^{2+}$ affinity. Single letters in other sequences indicate the residue difference compared to the rainbow trout sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

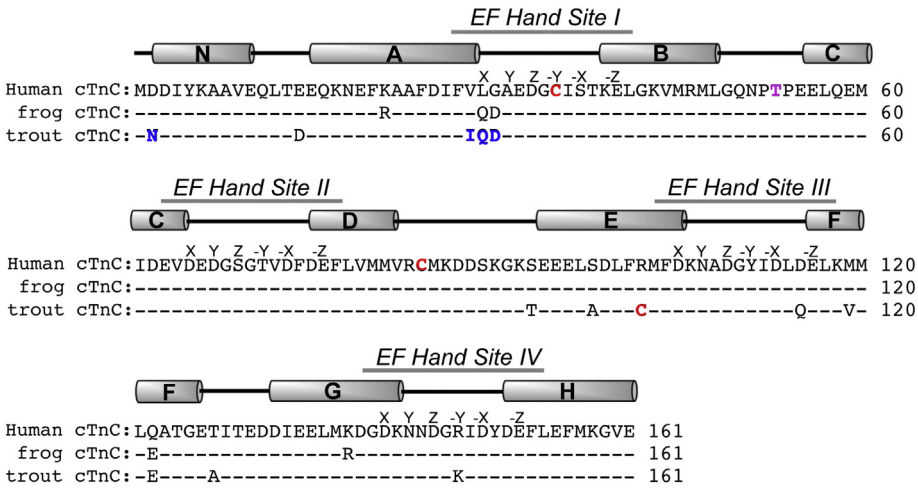


**Fig. 6.** The amino acid sequence alignment of human cTnC, frog cTnC and trout cTnC. Differences responsible for the high $Ca^{2+}$ affinity of trout cTnC are indicated at positions 2, 27, 28, and 29 in blue. The threonine that was mutated to cysteine at residue 53 in all cTnC isoforms to allow the attachment of IAANS is in bolded violet. The cysteines that were mutated to serines are in bolded red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

preparation can be altered through manipulation of either cTnI or cTnT through mutation, phosphorylation or replacement (Chandra et al., 1997; Gillis and Klaiman, 2011; Kirkpatrick et al., 2011; Sumandea et al., 2003). The 1.8-fold increase in the $Ca^{2+}$ affinity of $C_TI_RT_R$ caused by the replacement of human cTnI with trout cTnI in the current study further illustrates this point. This replacement was completed to determine how trout cTnC and trout cTnI, working together influences the $Ca^{2+}$ affinity of the cTn complex. These results reiterate the importance of the inter-component interactions to the $Ca^{2+}$ affinity of the complex. For example, the amino acid sequence of frog cTnI is 70% identical to rat cTnI and frog cTnT is 67% identical to rat cTnT. This suggests then that the $Ca^{2+}$ affinity of frog cTnC could be quite different when it is in complex with its native cTnI and cTnT then when in complex with mammalian components. Future studies should examine how Arg21 in frog cTnC influences the interaction between the Tn components in complex.

The $Ca^{2+}$ affinity of $C_FI_RT_R$ was 5-fold that of $C_HI_RT_R$ but its $k_{off}$ was only 16% slower that of $C_HI_RT_R$. It is thus likely that the difference in affinity is due to a difference in the $k_{on}$ as the affinity of a $Ca^{2+}$ binding site is determined by the rate of $Ca^{2+}$ association ($k_{on}$) divided by the rate of $Ca^{2+}$ disassociation ($k_{off}$). Indeed, the calculated $k_{on}$ of $C_FI_RT_R$ was 4.3-fold that of $C_HI_RT_R$. This suggests that the sequence differences between these two cTnC isoforms are increasing the rate at which $Ca^{2+}$ binds to the protein complex. This result is supported by previous studies that have demonstrated that some cTnC mutants with higher $Ca^{2+}$ affinities also had a higher $k_{on}$ (Tikunova and Davis, 2004).

We have previously demonstrated that the replacement of rat cTnI in $C_HI_RT_R$ with the trout isoform increases the $Ca^{2+}$ affinity of the cTn complex by 2-fold (Kirkpatrick et al., 2011). Here we show that the replacement of human cTnC in $C_HI_RT_R$ with trout cTnC increases the $Ca^{2+}$ affinity of the complex by 1.8-fold and that the replacement of rat cTnI in $C_TI_RT_R$ with trout cTnI increases the $Ca^{2+}$ affinity by a further 1.9-fold. Together, these results indicate that there is an additive effect of trout cTnC and trout cTnI on the $Ca^{2+}$ affinity of the cTn complex and that these proteins are partially responsible for trout cardiac muscle being 10-fold more sensitive to $Ca^{2+}$ than preparations from rats and rabbits when compared at the same temperature (Churcott et al., 1994). The replacement of rat cTnI in $C_TI_RT_R$ with trout cTnI decreased the $k_{off}$ of the complex by 57%. This indicates that $Ca^{2+}$ is staying bound to the complex longer and this change is responsible for the higher $Ca^{2+}$ affinity of $C_TI_TT_R$. Similarly, the $Ca^{2+}$ affinity of a mammalian cTn was increased when human cTnI was replaced with mammalian slow skeletal TnI (ssTnI) (Davis et al., 2007). This result is consistent with previous experiments demonstrating that ssTnI increases the $Ca^{2+}$ sensitivity of force generation in mammalian cardiac tissue (Dong et al., 2007; Fentzke et al., 1999; Metzger et al., 2003; Westfall and Metzger, 2007). The results of the current study therefore suggest that trout cTnI does play a role in the comparatively high $Ca^{2+}$ sensitivity of the trout heart.

### 4.3. Thermal stability

The melting temperatures of the cTnC isoforms and cTn complexes can also be described as the temperature at which the proteins lose their tertiary structure. The comparatively low melting temperature of trout cTnC therefore suggests that it is the least thermally stable. This low thermal stability may reflect the low physiological temperature of trout compared to frog and mammals, as previous work has demonstrated that proteins from cold-adapted species denature at lower temperatures than those from animals adapted to higher temperatures (Davies et al., 1994; Feller et al., 1992; Genicot et al., 1996; Maes et al., 1999). A lower structural stability, or higher molecular flexibility, is thought to enable cold adapted proteins to maintain a functional conformation at lower temperatures (Blumenschein et al., 2004; Fields and Somero, 1998). Related to this, we have demonstrated that the solution NMR structure of the N-terminus of bovine cTnC solved at 30 °C is more similar to that of trout cTnC solved at 7 °C than at 30 °C (Blumenschein et al., 2004). This suggests that these molecules have a similar tertiary structure when functioning at temperatures close to their respective physiological temperatures (Blumenschein et al., 2004). While the relationship between physiological temperature and melting temperature does not hold for frog cTnC relative to human cTnC, this may reflect functional differences. The lack of difference in melting temperatures between $C_HI_RT_R$, $C_FI_RT_R$, and $C_TI_RT_R$ suggests that complexing the cTnC isoforms with rat cTnI and rat cTnT diminishes the differences in molecular stability between cTnC isoforms. However, the reduction in melting temperature of the complex with the replacement of rat cTnI with trout cTnI suggests that the presence of more trout proteins further reduces the structural stability of the protein complex. This change may reflect the fact that trout cTnI has also evolved to function at a lower temperature than rat cTnI.

#### 4.3.1. Conclusions and perspective

The results of this study demonstrate that frog cTnC has a higher $Ca^{2+}$ affinity than human cTnC and trout cTnC and that this protein increases the $Ca^{2+}$ affinity of a troponin complex composed of mammalian subunits. This result was not predicted based on initial sequence comparisons of the three isoforms. However, it is also clear that the

influence of the other components of the cTn complex on the $Ca^{2+}$ affinity of cTnC must be taken into account when considering how thin filament function has evolved in the vertebrate heart. In addition, while the $Ca^{2+}$ affinity of $C_FI_RT_R$ is more than 5-fold that of $C_HI_RT_R$ its $k_{off}$ is only 16% slower than that of $C_HI_RT_R$. This is relevant as Davis et al. (Davis et al., 2007) have suggested that the rate of $Ca^{2+}$ disassociation from cTn influences the rate of cardiac muscle relaxation. This suggests that the insertion of frog cTnC into a mammalian muscle would increase the $Ca^{2+}$ sensitivity of the muscle but have a relatively small impact on diastolic function. The next step of this study is therefore to determine how the contractility of mammalian cardiac tissue is affected by the replacement of native cTnC with frog cTnC. These studies will be important to determine if frog cTnC can increase the $Ca^{2+}$ sensitivity of cardiac muscle without causing diastolic dysfunction. By using frog cTnC as a research tool to manipulate muscle function we will gain greater insight into the molecular interactions that regulate contractile function in the vertebrate heart.

## Author contributions

EJS and TEG designed the study. EJS and TEG completed the experiments and analyzed the data. EJS and TEG wrote the paper. Both authors approved the submitted manuscript.

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## References

Bers, D.M., Patton, C.W., Nuccitelli, R., 1994. A practical guide to the preparation of $Ca^{2+}$ buffers. In: Nuccitella, R. (Ed.), A Practicle Guide to the Study of Calcium in Living Cells. Academic Press, New York.

Blumenschein, T.M., Gillis, T.E., Tibbits, G.F., Sykes, B.D., 2004. Effect of temperature on the structure of trout troponin C. Biochemistry 43, 4955–4963.

Chandra, M., Dong, W.J., Pan, B.S., Cheung, H.C., Solaro, R.J., 1997. Effects of protein kinase A phosphorylation on signaling between cardiac troponin I and the N-terminal domain of cardiac troponin C. Biochemistry 36, 13305–13311.

Churcott, C.S., Moyes, C.D., Bressler, B.H., Baldwin, K.M., Tibbits, G.F., 1994. Temperature and pH effects on $Ca^{2+}$ sensitivity of cardiac myofibrils: a comparison of trout with mammals. Am. J. Phys. 267, R62–R70.

Davies, J.R., Ledward, D.A., Bardsley, R.G., Poulter, R.G., 1994. Species dependence of fish myosin stabilities to heat and frozen storage. Int. J. Food Sci. Technol. 29, 287–301.

Davis, J.P., Norman, C., Kobayashi, T., Solaro, R.J., Swartz, D.R., Tikunova, S.B., 2007. Effects of thin and thick filament proteins on calcium binding and exchange with cardiac troponin C. Biophys. J. 92, 3195–3206.

Dong, W.J., Jayasundar, J.J., An, J., Xing, J., Cheung, H.C., 2007. Effects of PKA phosphorylation of cardiac troponin I and strong crossbridge on conformational transitions of the N-domain of cardiac troponin C in regulated thin filaments. Biochemistry 46, 9752–9761.

Feller, G., Lonhienne, T., Deroanne, C., Libioulle, C., Van Beeumen, J., Gerday, C., 1992. Purification, characterization, and nucleotide sequence of the thermolabile alpha-amylase from the antarctic psychrotroph *Alteromonas haloplanctis* A23. J. Biol. Chem. 267, 5217–5221.

Fentzke, R.C., Buck, S.H., Patel, J.R., Lin, H., Wolska, B.M., Stojanovic, M.O., Martin, A.F., Solaro, R.J., Moss, R.L., Leiden, J.M., 1999. Impaired cardiomyocyte relaxation and diastolic function in transgenic mice expressing slow skeletal troponin I in the heart. J. Physiol. 517, 143–157.

Fields, P.A., Somero, G.N., 1998. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. Proc. Natl. Acad. Sci. U. S. A. 95, 11476–11481.

Genicot, S., Rentier-Delrue, F., Edwards, D., VanBeeumen, J., Gerday, C., 1996. Trypsin and trypsinogen from an Antarctic fish: molecular basis of cold adaptation. Biochim. Biophys. Acta 1298, 45–57.

Gillis, T.E., Klaiman, J.M., 2011. The influence of PKA treatment on the $Ca^{2+}$ activation of force generation by trout cardiac muscle. J. Exp. Biol. 214, 1989–1996.

Gillis, T.E., Tibbits, G.F., 2002. Beating the cold: the functional evolution of troponin C in teleost fish. Comp. Biochem. Physiol. A 132, 763–772.

Gillis, T.E., Marshall, C.R., Xue, X.H., Borgford, T.J., Tibbits, G.F., 2000. $Ca^{2+}$ binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. Am. J. Phys. 279, R1707–R1715.

Gillis, T.E., Blumenschein, T.M., Sykes, B.D., Tibbits, G.F., 2003a. Effect of temperature and the F27W mutation on the $Ca^{2+}$ activated structural transition of trout cardiac troponin C. Biochemistry 42, 6418–6426.

Gillis, T.E., Moyes, C.D., Tibbits, G.F., 2003b. Sequence mutations in teleost cardiac troponin C that are permissive of high $Ca^{2+}$ affinity of site II. Am. J. Phys. 284, C1176–C1184.

Gillis, T.E., Liang, B., Chung, F., Tibbits, G.F., 2005. Increasing mammalian cardiomyocyte contractility with residues identified in trout troponin C. Physiol. Genomics 22, 1–7.

Gillis, T.E., Marshall, C.R., Tibbits, G.F., 2007a. Functional and evolutionary relationships of troponin C. Physiol. Genomics 32, 16–27.

Gillis, T.E., Martyn, D.A., Rivera, A.J., Regnier, M., 2007b. Investigation of thin filament near-neighbour regulatory unit interactions during force development in skinned cardiac and skeletal muscle. J. Physiol. 580, 561–576.

Hazard, A.L., Kohout, S.C., Stricker, N.L., Putkey, J.A., Falke, J.J., 1998. The kinetic cycle of cardiac troponin C: calcium binding and dissociation at site II trigger slow conformational rearrangements. Protein Sci. 7, 2451–2459.

Kirkpatrick, K.P., Robertson, A.S., Klaiman, J.M., Gillis, T.E., 2011. The influence of trout cardiac troponin I and PKA phosphorylation on the $Ca^{2+}$ affinity of the cardiac troponin complex. J. Exp. Biol. 214, 1981–1988.

Liang, B., Chung, F., Qu, Y., Pavlov, D., Gillis, T.E., Tikunova, S.B., Davis, J.P., Tibbits, G.F., 2008. Familial hypertrophic cardiomyopathy-related cardiac troponin C mutation L29Q affects $Ca^{2+}$ binding and myofilament contractility. Physiol. Genomics 33, 257–266.

Liu, B., Tikunova, S.B., Kline, K.P., Siddiqui, J.K., Davis, J.P., 2012. Disease-related cardiac troponins alter thin filament $Ca^{2+}$ association and dissociation rates. PLoS One 7, e38259.

Maes, D., Zeelen, J.P., Thanki, N., Beaucamp, N., Alvarez, M., Thi, M.H., Backmann, J., Martial, J.A., Wyns, L., Jaenicke, R., Wierenga, R.K., 1999. The crystal structure of triosephosphate isomerase (TIM) from *Thermotoga maritima*: a comparative thermostability structural analysis of ten different TIM structures. Proteins 37, 441–453.

Metzger, J.M., Michele, D.E., Rust, E.M., Borton, A.R., Westfall, M.V., 2003. Sarcomere thin filament regulatory isoforms. Evidence of a dominant effect of slow skeletal troponin I on cardiac contraction. J. Biol. Chem. 278, 13118–13123.

Mundia, M.M., Demers, R.W., Chow, M.L., Perieteanu, A.A., Dawson, J.F., 2012. Subdomain location of mutations in cardiac actin correlate with type of functional change. PLoS One 7, e36821.

Perieteanu, A.A., Sweeting, B., Dawson, J.F., 2008. The real-time monitoring of the thermal unfolding of tetramethylrhodamine-labeled actin. Biochemistry 47, 9688–9696.

Shaffer, J.F., Gillis, T.E., 2010. Evolution of the regulatory control of vertebrate striated muscle: the roles of troponin I and myosin binding protein-C. Physiol. Genomics 42, 406–419.

Sumandea, M.P., Pyle, W.G., Kobayashi, T., de Tombe, P.P., Solaro, R.J., 2003. Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T. J. Biol. Chem. 278, 35135–35144.

Takeda, S., Yamashita, A., Maeda, K., Maeda, Y., 2003. Structure of the core domain of human cardiac troponin in the $Ca^{2+}$-saturated form. Nature 424, 35–41.

Tikunova, S.B., Davis, J.P., 2004. Designing calcium-sensitizing mutations in the regulatory domain of cardiac troponin C. J. Biol. Chem. 279, 35341–35352.

Westfall, M.V., Metzger, J.M., 2007. Single amino acid substitutions define isoform-specific effects of troponin I on myofilament $Ca^{2+}$ and pH sensitivity. J. Mol. Cell. Cardiol. 43, 107–118.