Evolutionary and Physiological Variation in Cardiac Troponin C in Relation to Thermal Strategies of Fish

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Accepted 8/7/00

ABSTRACT

Striated muscle contraction is initiated when troponin C (TnC) binds Ca^{2+}, which activates actinomyosin ATPase. We investigated (i) the variation between cardiac TnC (cTnC) primary structure within teleost fish and (ii) the pattern of TnC expression in response to temperature acclimation. There were few differences between rainbow trout (*Oncorhynchus mykiss*), yellowfin tuna (*Thunnus albacares*), yellow perch (*Perca flavescens*), goldfish (*Carassius auratus*), white sucker (*Catostomus commersoni*), and icefish (*Chaenocephalus aceratus*) in cTnC amino acid sequence. No variation existed in the regulatory Ca^{2+}-binding site (site 2). The site 3 and 4 substitutions were limited to residues not directly involved in Ca^{2+} coordination. Fish cTnC primary structure was highly conserved between species (93%–98%) and collectively divergent from the highly conserved sequence seen in birds and mammals. Northern blots and polymerase chain reaction showed that thermal acclimation of trout (3°, 18°C) did not alter the TnC isoform pattern. While cardiac and white muscle had the expected isoforms—cTnC and fast troponin C (fTnC), respectively—red muscle unexpectedly expressed primarily ftnC. Cold acclimation did not alter myofibrillar ATPase Ca^{2+} sensitivity, but maximal velocity increased by 60%. We found no evidence that TnC variants, arising between species or in response to thermal acclimation, play a major role in mitigating the effects of temperature on contractility of the adult fish heart.

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Physiological and Biochemical Zoology 73(6):841–849. 2000. 1522-2152/2000/7306-0033$03.00

Introduction

Cardiac myocytes contract when cytosolic Ca^{2+}, which rises after excitation, occupies a single, low-affinity, regulatory Ca^{2+}-binding site on the thin filament protein troponin C (TnC; Fabiato 1983). The conformational changes in TnC accompanying Ca^{2+} binding (Herzberg et al. 1986) lead to changes in relationships between other thin-filament regulatory proteins (TnI, TnT, tropomyosin; Zot and Potter 1987). These conformational changes allow actin to interact with myosin, which stimulates actinomyosin ATPase activity and cardiomyocyte contraction. Striated muscle excitation-contraction coupling is profoundly influenced by factors affecting Ca^{2+} signaling (Bers 1991). In particular, temperature can change the Ca^{2+} sensitivity of myofibrillar ATPase and muscle tension; low temperature reduces Ca^{2+} sensitivity of cardiac muscle but increases that of skeletal muscle (Harrison and Bers 1989, 1990*a*, 1990*b*). In birds and mammals, such temperature effects may be limited to conditions such as hibernation and torpor (e.g., Kondo and Shibata 1984). Thermal sensitivity of cardiac myofibrils would be expected to be more important in ectotherms, where cardiac function must be maintained at extreme or variable temperatures (e.g., Graham and Farrell 1989, 1990). The primary structure of cardiac TnC (cTnC) is highly conserved in birds and mammals, with at most three amino acid substitutions (of 161 total) between any pair of species (Collins 1991). In contrast, salmonid cTnC differs from that of higher vertebrates in 13–14 amino acid residues (Moyes et al. 1996). Our study of the effects of temperature on salmonid myofibrillar ATPase showed that salmon may avoid deleterious effects of low temperature with a higher inherent myofibrillar Ca^{2+} affinity (Churcott et al. 1994). Arguments about the adaptive significance of the structural properties of salmonid cTnC were limited by the paucity of cTnC sequences from lower vertebrates. While the nature of variation in other thin-filament regulatory proteins has been studied in fish skeletal muscles (e.g., tropomyosin, Jackman et al. 1996; TnI, Jackman et al. 1998; TnT, Waddleton et al. 1999), neither TnC nor cardiac muscle has received much attention.

The first goal of this study was to examine cTnC sequence variation within teleost fish. The species chosen in this study differ in many physiological traits (e.g., activity levels and ther-

mal tolerances) and occupy diverse thermal niches (polar, tropical, and temperate waters). The second goal was to evaluate the potential role of TnC isoform shifts in thermal acclimation. Collectively, these investigations could demonstrate no role of physiological and evolutionary variants of TnC in relation to the thermal sensitivity of fish cardiac myofibrils.

Material and Methods

Comparative Analyses of Fish cTnC Sequences

Animals and Tissue Preparation. A polymerase chain reaction (PCR) approach was used to determine the extent of cTnC sequence variation in representatives from three orders of teleost fish. White suckers (*Catostomus commersoni*) were obtained from a local bait shop in early summer. Goldfish (*Carassius auratus*) were obtained from a local pet store. Yellow perch (*Perca flavescens*) were donated by local fishermen after being caught by hook and line from Lake Ontario in summer. Yellowfin tuna (*Thunnus albacares*) hearts were kindly provided by R. Brill (National Marine Fisheries, Kewalo Laboratories, Honolulu; flash frozen hearts were shipped on dry ice). Total RNA was extracted from heart tissue of these species and used for reverse transcriptase–polymerase chain reaction (RT–PCR). Icefish (*Chaenocephalus aceratus*) cTnC was amplified from genomic DNA derived from gill tissue kindly provided by B. Sidell (University of Maine, Orono) via B. Tufts (Queen's University, Kingston, Ont.). Rat tissues were collected from animals killed by carbon dioxide overdose. Ventricles and skeletal muscles were frozen and extracted as described for trout tissues. The white muscle sample was lateral gastrocnemius (37% fast oxidative, 58% fast glycolytic, 5% slow oxidative fibers); red muscle was soleus (16% fast oxidative, 0% fast glycolytic, 84% slow oxidative fibers; Ariano et al. 1973).

PCR Strategy for Amplifying Fish cTnC. The forward and reverse PCR primers were constructed for the cTnC coding region based on the nucleotide sequence of salmonid cTnC (Moyes et al. 1996). The N-terminal (MNDIYKA) and C-terminal (EFMKGVE) regions of the protein are highly conserved across species (see Moyes et al. 1996). Primers were designed from these regions and designated cTnC-F (5′-ATG AAC GAC ATC TAC AAA GC-3′) and cTnC-R (5′-TTA TTC TAC TCC TTT CAT GA-3′). Total RNA was purified using guanidinium thiocyanate as previously described in Leary et al. (1998). Total RNA from each sample was reverse transcribed in the following reaction mix: 5 μg of total RNA, 0.2 μg of random primers (Promega), and nuclease-free water up to 11 μL. The mixture was incubated at 70°C for 5 min and cooled rapidly on ice. Other components of the reaction mixture were added in the following order: 4 μL of 5 × M-MuLV (moloney murine leukemia virus) reverse transcriptase reaction buffer (Fermentas), 2 μL of 10 mM 4dNTPs mix, 20 units of RNase inhibitor (Pharmacia Biotech), nuclease-free water up to 20 μL, and 40 units of M-MuLV reverse transcriptase (Fermentas). The mixture was held at room temperature for 10 min, incubated at 37°C for 60 min, heated to 70°C for 10 min, chilled rapidly on ice, and then stored at −20°C until further use.

First-strand cDNA was the template in a PCR reaction consisting of the following: 34.7 μL water, 5 μL 10 × polymerase reaction buffer (Pharmacia), 200 ng of each primer (cTnC-F and cTnC-R), 2 μL of 10 mM dNTPs, 4 μL of 25 mM $MgCl_2$, 1.5 units of Taq polymerase, and 1 μL of cDNA sample. After an initial denaturation (95°C for 5 min), cDNA was amplified by 30 cycles of denaturation (95°C for 30 s), annealing (47°C for 90 s), and extension (72°C for 90 s), followed by a final extension (72°C for 10 min) to ensure that all PCR products were full length. The PCR reaction was electrophoresed on a 1% agarose gel (90 V for 1 h). The PCR product, which appeared as a single band, was excised, purified (QIAEX II, Qiagen), quantified at 260 nm, and then sequenced in both directions (MOBIX Central Facility, McMaster University, Hamilton, Ont.).

Myofibrillar ATPase in Relation to Acclimation

Animals and Thermal Acclimation. Rainbow trout (*Oncorhynchus mykiss*) were obtained from Pure Springs Trout Farms (Belleville, Ont.). Fish were approximately 500 g in size and randomly divided into two tanks. The two groups of 20 fish each were held in covered tanks (2.1 × 0.6 × 0.6 m) and provided with continuous dechlorinated water and aeration. One tank of fish was provided with water from a header tank in which the water temperature was cooled down with a Ranco water chiller. The water temperature in the other tank was warmed with a Thermal Compact aquarium heater. Acclimation temperatures (2°–4°, 18°C) were achieved by slowly changing inflow temperature from 12°C over 2 wk and held for 2 mo.

Trout were anesthetized in dechlorinated water containing 250 mg L^{-1} 3-aminobenzoic acid ethyl ester (MS-222, Sigma) buffered with 500 mg L^{-1} $NaHCO_3$ and then killed by decapitation. The hearts were rapidly excised and trimmed of nonventricular tissue. Fresh tissue was used for myofibrillar ATPase measurements. Frozen tissue was used for RNA analyses (see "Myofibrillar Isolation and ATPase Measurements").

Myofibrillar Isolation and ATPase Measurements. Cardiac myofibrils from both cold- and warm-acclimated trout were prepared as previously described in Churcott et al. (1994). The final myofibrillar pellet was resuspended to a concentration of 3-mg protein mL^{-1}. Ca^{2+}-buffered solutions for the myofibrillar ATPase assay were prepared exactly as described by Churcott et al. (1994). Reactions were performed in 650 μL, which contained 65 μL myofibrils (3 mg mL^{-1}), 6.5 μL 130 mM ATP, 325 μL of appropriate 2 × pCa buffer, and balance deionized water. This myofibrillar concentration resulted in maximal phosphate production within the linear range for colorimetric reaction but did

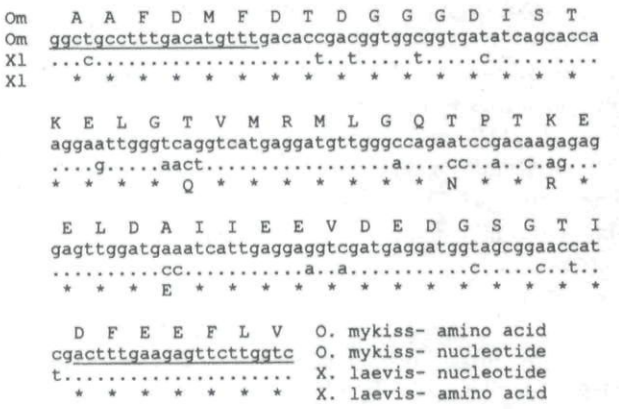

Figure 1. Comparison of the nucleotide and amino acid sequences of the trout and *Xenopus* fast troponin C (f TnC). Polymerase chain reaction product amplified by f TnC-F and f TnC-R primers is homologous with the f TnC beta isoform from *Xenopus* (GenBank AB003079). A dot in the *Xenopus* nucleotide sequence indicates identity with the corresponding nucleotide of the trout sequence. An asterisk in the *Xenopus* amino acid sequence indicates identity with the corresponding residue of the trout sequence.

not deplete ATP by more than 10%. The assay solutions were incubated at the intermediate test temperature of 10°C for 5 min before the addition of myofibrillar protein. Previous studies (Churcott et al. 1994) showed that a 1-min period of incubation led to a linear rate of phosphate production, whereas longer incubations resulted in significant decreases in calculated myofibrillar ATPase rates. After a 1-min incubation, reactions were terminated by the addition of 65 μL of 55% tricholoroacetic acid (TCA) and then put on ice. Contaminating phosphate was assessed using a sample prepared and incubated in the same manner but with myofibrils added after TCA. Samples were spun for 2 min at maximum speed with a bench-top centrifuge in order to obtain a protein-free supernatant. Acid hydrolysis of ATP was minimized by keeping all samples on ice and proceeding with the phosphate determination step as quickly as possible.

Table 1: Comparison of nucleotide/amino acid identity in cTnC

	Sucker	Goldfish	Perch	Tuna	Icefish	Lamprey
Trout	83/94	83/93	87/95	88/95	87/94	78/78
Sucker		91/98	85/95	84/95	84/93	76/81
Goldfish			85/95	85/94	84/93	76/81
Perch				94/98	94/96	77/80
Tuna					99/98	76/79
Icefish						76/78

Note. Species sequenced in this study were white sucker (*Catostomus commersoni*), goldfish (*Carassius auratus*), yellow perch (*Perca flavescens*), yellowfin tuna (*Thunnus albacares*), and icefish (*Chaenocephalus aceratus*). Literature data for rainbow trout (*Oncorhynchus mykiss*) were from Moyes et al. (1996), and data for lamprey (*Entosphenus japonicus*) were from Yuasa et al. (1998). Each value was generated from the multiple alignments of sequences by CLUSTAL W (Thompson et al. 1994).

The amount of phosphate in the protein-free supernatant was determined using the Fiske-Subbarow method, adapted from Churcott et al. (1994) for use in microplates. Triplicate 200 μL samples and standards were added to a microplate well before addition of 30 mL Reagent C, prepared by combining 1.47 mL Reagent A (5 mL of 5% ammonium molybdate, 5 mL of 2.86 M NaOH, 0.1 mL of 7.84 mM $CuSO_4$) with 630 mL Reagent B (100 mg of Fiske-Subbarow reducer in 630 μL water). Absorbance was determined after 30 s of shaking and a 5-min incubation at room temperature.

Curve Fitting and Analyses. Rates of phosphate production in relation to pCa were subjected to a nonlinear curve fitting to a logistic regression function derived from the Hill equation given below (Churcott et al. 1994):

$$V = V_{max}/\{1 + \exp[\eta(\mathrm{pCa} - K_{0.5})]\} + V_{min}.$$

The logistic fit equation returned values of the pCa at which 50% of maximum myofibrillar ATPase activity was generated ($K_{0.5}$) as well as the slope at the point of inflection or Hill coefficient (η) value for the data set. The rate at the lowest Ca^{2+} concentration can be interpreted as Ca^{2+}-independent ATPase activity.

Influence of Thermal Acclimation on TnC Expression

Analyses of TnC Isoforms. Heart, red, and white muscle samples were frozen in liquid nitrogen, pulverized, and then stored at −80°C. Total RNA was purified using acid phenol, denatured using glyoxal/DMSO, electrophoresed, and transferred to nylon membranes as previously described in Leary et al. (1998). Northern analyses were conducted using a cTnC probe that encoded the full-length (486 bp), open-reading frame of rainbow trout cTnC (Moyes et al. 1996). An antisense probe was labeled using Klenow fragment and the reverse primer. Template, 75 ng of gel-purified trout cTnC cDNA, was combined with 200 ng of the reverse (primer cTnC-R) in 14 μL, boiled for 3 min, and then set on ice for 2 min. Reaction mix (11 μL) containing 2.5 μL of 0.5 mM dNTPs (-dCTP; Fermentas), 2.5 μL of 10 × reaction buffer, 5 units of Klenow fragment (Promega), and 50 μCi of α-^{32}P dCTP (New England Nuclear) was added to the denatured DNA and incubated at room temperature for 4 h. After the incubation, the labeled DNA was separated from the unincorporated radioactive precursors by a G-50 Nick Translation column (Pharmacia). Typically, the incorporated radioactive probes had a specific activity around 10^8 cpm μg^{-1} DNA (or above 70% incorporation). The membrane was prehybridized in 20 mL of hybridization solution (10% dextran sulfate, 50% formamide, 1% SDS, 5 × Denhardt's reagent, 1 M NaCl, and 2 mg of denatured and sheared salmon

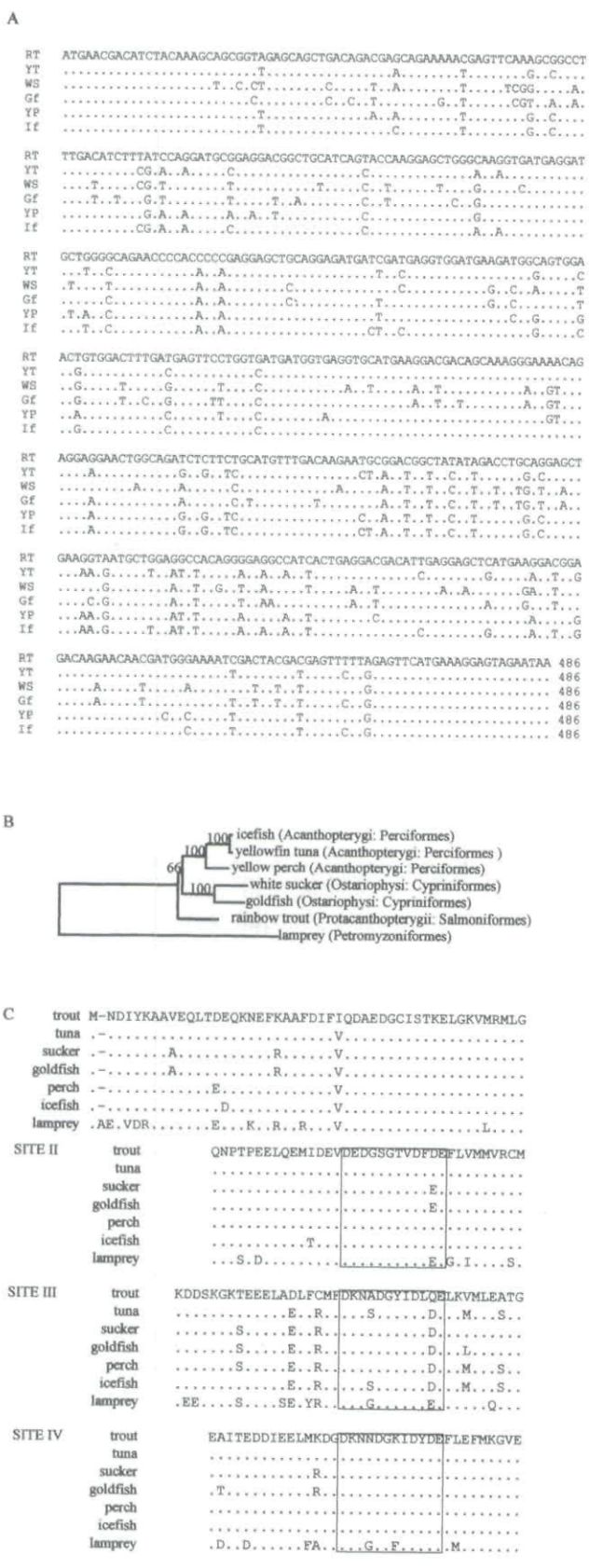

Figure 2. Comparison of nucleotide and amino acid sequences of fish cardiac troponin C (cTnC). *A*, Nucleotide sequence of the cTnC coding region of rainbow trout (*RT*, *Oncorhynchus mykiss*; Moyes et al. 1996). Species sequenced in this study include yellowfin tuna (*YT*, *Thunnus albacares*), white sucker (*WS*, *Catostomus commersoni*), goldfish (*Gf*, *Carassius auratus*), yellow perch (*YP*, *Perca flavescens*), and icefish (*If*, *Chaenocephalus aceratus*). *B*, Cladogram developed from nucleotide sequences of cTnC. All fish are Teleosteii, except the out-group lamprey. Unrooted tree was produced with MEGA (Kumar et al. 1993) using the maximum likelihood method. Values indicate the percentage of 100 bootstrap resamplings that support these topological elements. *C*, Derived amino acid sequences of the cTnC coding region, including lamprey (*Entosphenus japonicus*; Yuasa et al. 1998). Squares indicate the Ca^{2+}-binding loops of sites 2, 3, and 4. In *A* and *C*, dots indicate identity with the salmon sequence.

sperm DNA) in a Hybaid minihybridization oven at 42°C for 2 h. After the radioactive probe was added, the membrane was left to hybridize at the same temperature overnight. The membrane was washed twice with 2 × standard saline citrate (SSC), 1% SDS for 15 min (25°C); once with 1 × SSC, 0.1% SDS for 15 min (42°C); and once with 0.1 × SSC, 0.1% SDS for 5 min (42°C). The membrane was then exposed to autoradiography film at room temperature overnight.

Semiquantitative PCR Analyses of Isoform Expression. Initially, a fast troponin C (fTnC) probe was constructed based on consensus sequence from higher vertebrates. The forward and reverse degenerate primers were constructed for the fTnC coding region based on published fTnC nucleotide sequences from mammals, birds, and frogs. Primers were chosen from highly conserved sequences, which flank a 173-nucleotide region containing Ca^{2+}-binding sites 1 and 2 of fTnC. The isoform specificity of the primers was due to the sequence divergence at site 1 (fTnC-F: 5′-GGC TGC CTT TGA CAT GTT T-3′; fTnC-R: 5′-GAC CAA GAA CTC TTC AAA GT-3′).

A salmon fTnC probe was constructed by using the same PCR protocol and reaction mix as previously described (see "PCR Strategy for Amplifying Fish cTnC") but with an annealing temperature of 51°C using rainbow trout white muscle cDNA as template. The resulting cDNA product showed strong homology with the *Xenopus* fTnC sequence (85% at nucleotide level, 92% at amino acid level; Fig. 1). While northern analyses proved effective for showing cTnC expression, blots using this fTnC probe had unacceptable levels of background. As an alternative, we developed a semiquantitative PCR approach using these same PCR primers to amplify specific TnC isoforms. Samples of RNA were collected and reverse transcribed as previously described (see "PCR Strategy for Amplifying Fish cTnC"). The resulting cDNA was diluted for use as PCR template. In all cases, only single bands of the correct size were seen. This approach can be considered semiquantitative at best;

although the amount of product depended on the amount of template, the relationship was not linear.

Results

Comparative cTnC Sequences

In each case, the PCR product obtained from fish heart cDNA (or icefish genomic DNA) was 486 nucleotides that would encode a protein of 161 amino acid residues. It is important to recognize that the first and last seven amino acids would be determined by the primers. The sequence comparison among those fish reveals a remarkable conservation of cTnC primary structure. At the nucleotide level of the coding region, the teleosts showed between 83% and 99% identity (Table 1; Fig. 2*A*). At the amino acid level, there was no less than 93% identity between teleosts, although lamprey had much lower sequence identity with the teleosts (Table 1). Only three amino acid variations occurred in the Ca^{2+}-binding loops of sites 2 and 3. No differences occur in either site 1 or 4 (Fig. 2*C*).

The cladogram developed from cTnC sequences is consistent with the accepted phylogenies of these fish. In particular, the similarities between Antarctic icefish, tropical tuna, and temperate perch reflect their membership in a well-supported monophyletic clade.

Ca^{2+} Sensitivity of Myofibrillar ATPase

Table 2 and Figure 3 summarize the influence of acclimation temperature on the Ca^{2+} sensitivity of myofibrillar ATPase. Data are presented both as "Total ATPase" and "Ca^{2+}-dependent ATPase." $K_{0.5}$ and the Hill coefficients were calculated using Ca^{2+}-dependent rates. There was no significant effect of acclimation temperature on either the Hill coefficient or $K_{0.5}$. However, cold acclimation led to a 60% increase in the maximal Ca^{2+}-dependent activity.

Influence of Acclimation of TnC Expression

The specificity of the trout cTnC probe was established using a blot with the three fish muscle types along with reference rat tissues. The results of the northern blot probed with trout cTnC revealed a single band in the heart muscle, but no bands of the expected size were detected in either red muscle or white muscle (Fig. 4). While it was expected that the probe would hybridize with heart muscle, the lack of hybridization with red muscle was surprising and prompted further investigation. However, the reciprocal experiment, probing the same blot with a trout fTnC probe, was less satisfactory (data not shown). An alternative approach for studying TnC isoform tissue distribution pattern was developed to confirm the pattern of TnC expression and better evaluate the effects of acclimation. As in northern analyses, the cTnC dominates in heart muscle; the fTnC dominates in white muscle (Fig. 5). Both heart muscle and white muscle showed faint bands corresponding to the other isoform. As suggested by northern analyses, red muscle was dominated by fTnC, although cTnC was expressed at a low level. In all three muscle types, thermal acclimation had no gross effect on the relative levels of each TnC isoform.

Discussion

Interspecies Differences in Cardiac TnC

Although the primary structure of cardiac TnC is accepted to be highly conserved among homeotherms (Collins 1991), there are very few sequences known for lower vertebrates. Salmon cTnC shares general cTnC features with the other higher-vertebrate isoforms but has much lower homology, differing in amino acid sequence in 13–14 positions in comparison to bird/mammal cTnC isoforms (Moyes et al. 1996). It was unclear whether these differences were specific to salmonids or more generalized in fish. In this study, we compared primary structures of cTnC from different species of fish. These species were not chosen based on phylogenetic relatedness; they collectively represent diverse taxa with vastly different activity levels as well as thermal preferences and tolerances. Perch, sucker, and goldfish are all temperate eurytherms and show moderate levels of activity. Trout are active, cold-water stenotherms. Tuna are tropical stenotherms and very active with high cardiac outputs (Farrell et al. 1992). Icefish are extreme stenotherms that live in subzero temperatures and are relatively inactive. The se-

Table 2: Influence of acclimation temperature on $K_{0.5}$ and Hill coefficient

Acclimation Temperature (°C)	V_{max} (nmol Pi min^{-1} mg^{-1})	$K_{0.5}$ (pCa)	Hill Coefficient
Total ATPase:			
3	156.7 ± 14.8	...	...
18	126.8 ± 17.6	...	...
Ca^{2+} dependent:			
3	74.7 ± 6.3*	6.71 ± .14	2.02 ± .47
18	47.5 ± 7.8*	6.83 ± .14	2.67 ± .89

Note. Values are expressed as mean (n = 7) ± SE. All assays were performed at 10°C. Asterisks indicate values significantly different from each other ($P < 0.05$).

quence analysis of cTnC among these wild fish was thought to provide some insight into the sort of variation in cTnC structure that has arisen over evolutionary time. Although tuna and icefish have marked differences in their thermal physiology, their cTnC primary structures are strikingly similar. The cladogram derived from cTnC sequence agrees well with accepted phylogenies and therefore does not reveal any evolutionary adaptations in TnC structure within fish. However, the impact of the differences between fish (collectively) and homeotherm cTnC remains unknown.

The consequences of the cTnC variants seen in this study can be assessed using TnC structural (Herzberg et al. 1986) and functional models (e.g., Farah and Reinach 1995) developed from the bird and mammal proteins. TnC demonstrates the helix-loop-helix motif of a canonical Ca^{2+}-binding protein composed of 12 amino acids contributing six coordinating residues (Herzberg et al. 1986). The fish cTnC amino acid sequences are remarkably conserved, particularly in the Ca^{2+}-binding sites (Table 1; Fig. 2).

Site 2 is the sole regulatory Ca^{2+}-binding site in the cardiac isoform. In this study, only one amino acid variation was found in site 2. The residue between $-x$ and $-z$ is occupied by glutamate (sucker, goldfish, and lamprey) or aspartate (salmon, tuna, perch, and icefish). This is a noncoordinating position, and both amino acids are hydrophilic and acidic, which suggests that the interspecies differences will have a limited effect on Ca^{2+} binding.

The impact of variation in sites 3 and 4 is more difficult to determine with certainty because the sites contribute primarily to associations between TnC and TnI. Two amino acid variations exist in site 3, and neither is at coordinating position. The position between y and z is occupied by uncharged residues, either glycine (lamprey), serine (tuna, icefish), or alanine. The residue between $-x$ and $-z$ is either aspartate (tuna, sucker, perch, goldfish, and icefish), glutamate (lamprey), or glutamine (salmon). While glutamine and the acidic amino acids differ in charge, all three are highly polar residues. Babu et al. (1993) showed that glutamine could readily replace glutamate in a Ca^{2+}-binding loop.

Two amino acid substitutions also occur in the Ca^{2+}-binding loop of site 4. The position between y and z is asparagine in all fish (Fig. 2) and homeotherms (Collins 1991) except for lamprey. At position $-y$ of site 4, lamprey has phenylalanine instead of the lysine found in all fish (Fig. 2) or the arginine found in mammals and birds (Collins 1991).

The conclusion from the comparative sequence analyses is that the primary structures of the cTnC are fundamentally similar among teleost fish.

Role of TnC Isoforms in Thermal Acclimation of Trout

Although few differences in cTnC sequence are evident across fish species, the role of intraindividual changes has not been assessed. In mammalian heart muscle, it is known that switching cTnC with fTnC dramatically alters many features of the myofibrillar Ca^{2+} dependency, including sensitivity of the myofibrillar ATPase to temperature. Cooling decreases myofilament Ca^{2+} sensitivity in various cardiac preparations, whereas it increases skeletal muscle myofilament Ca^{2+} sensitivity (Godt and Lindley 1982; Stephenson and Williams 1985). The desensitizing effect of temperature on Ca^{2+} affinity of cardiac myofilaments is reduced when the native cTnC is replaced by fTnC (Harrison and Bers 1990*a*). The TnC isoforms also exert influence on the length-induced autoregulation of myocardial

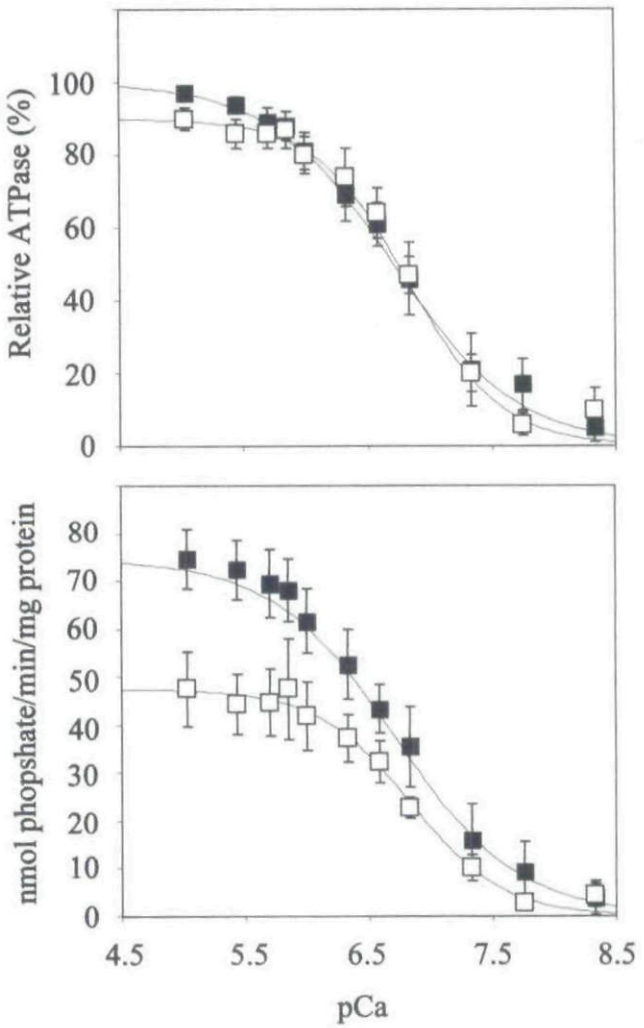

Figure 3. Effects of acclimation temperature on Ca^{2+} sensitivity of myofibrillar ATPase. Ca^{2+} sensitivity of myofibrillar ATPase was measured in heart myofibrils of rainbow trout acclimated 2 mo to either 2°–4° (*filled squares*) or 18°C (*open squares*). ATPase rates are expressed relative to maximal rates (*top*) and as absolute rates (*bottom*), with both corrected for the Ca^{2+}-independent ATPase activity. All assays were performed at the intermediate temperature of 10°C.

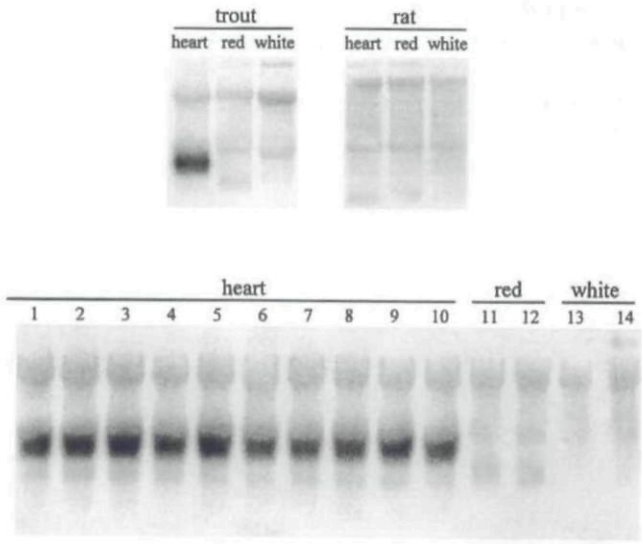

Figure 4. Northern blots probed with rainbow trout cardiac troponin C (cTnC). Top panel demonstrates that the rainbow trout cTnC probe is both species and isoform specific. Bottom panel shows the effects of temperature acclimation on cTnC expression in heart and skeletal muscle of trout acclimated to warm or cold temperatures. Odd lanes are from trout acclimated to 2°–4°C. Even lanes are total RNA from trout acclimated to 18°C. Rat tissues were included to show species specificity of the salmonid cTnC probe. Red muscle was soleus. White muscle was gastrocnemius.

performance (Moss et al. 1986; Babu et al. 1988). The ectopic expression of the fTnC in the heart of transgenic mice reduces the depressant effect of acidic pH on the Ca^{2+} sensitivity of cardiac muscle. The extraction of endogenous fTnC from fast skeletal skinned fibers and reconstitution with purified cTnC reciprocally increases the depressant effect of acidic pH on Ca^{2+} sensitivity (Metzger et al. 1993). Each of these studies suggests that TnC isoforms play an important role in determining the different sensitivities of cardiac muscle and fast skeletal muscle to physiological conditions. While experimentally induced shifts in TnC have profound effects on heart myofibrils and developmental shifts in TnC isoform expression are well established (Toyota and Shimada 1983; Obinata 1985; Gahlmann et al. 1988), TnC isoform shifts have not been shown to occur in adult mammalian myocardium. Based on these mammalian studies, we investigated the possibility that one compensatory mechanism during thermal acclimation in fish might involve the expression of fTnC in heart muscle.

We examined the changes in myofibrillar Ca^{2+} sensitivity with temperature and in doing so also examined the pattern of TnC isoform expression in rainbow trout heart and skeletal muscles. Both northern blot and RT–PCR analyses suggest that TnC isoform switching does not contribute to the thermal acclimation strategy. TnC is an important determinant of myofibrillar Ca^{2+} sensitivity, but other regulatory proteins (e.g., myosin light-chain kinase, TnI, TnT) can also alter myofibrillar Ca^{2+} sensitivity (see Solaro and Rarick 1998). Our myofibrillar ATPase analyses provide no evidence for shifts in Ca^{2+} sensitivity with acclimation, although an increase in maximal Ca^{2+}-dependent myofibrillar ATPase was evident. The biochemical basis for this increase in V_{max} is not known, but cyanogen bromide peptide mapping did not reveal changes in myosin heavy chain proteins (data not shown). Regardless of the mechanism behind the seasonal increase in V_{max}, the changes would be expected to help mitigate the debilitating effects of temperature on cardiac function; at any physiological Ca^{2+} concentration, greater force would be achieved in cold-acclimated fish. It is possible that the increase in catalytic capacity adequately offsets the reductions in temperature, possibly augmented by changes in Ca^{2+} delivery.

Interestingly, the pattern of TnC expression in trout striated muscle was different from that seen in mammals and birds. The PCR approach showed the expected isoforms to be expressed in both cardiac and white muscles. Surprisingly, red muscle predominately expressed fTnC, unlike the situation in mammals and birds. In homeotherms, neural stimulation can influence expression of skeletal Tn isoforms (Toyota and Shimada 1983; Phillips and Bennett 1984). At the cellular level,

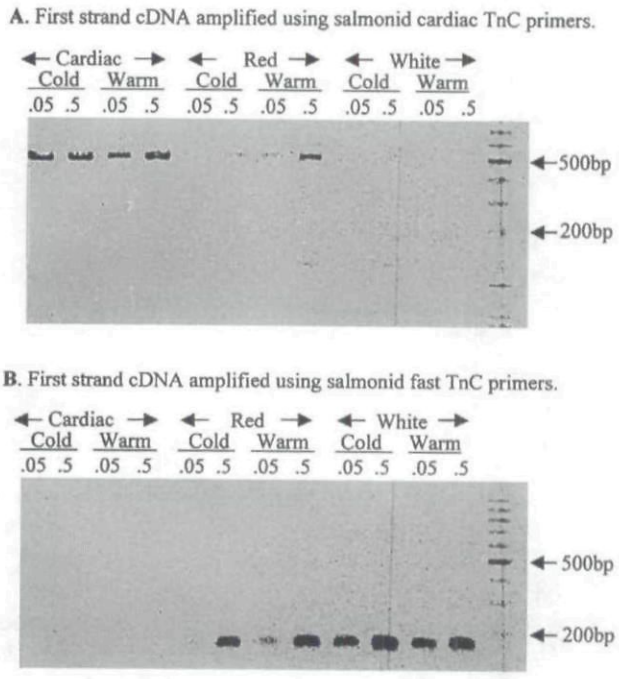

Figure 5. Semiquantitative reverse transcriptase–polymerase chain reaction using isoform-specific primers for cardiac and fast skeletal troponin C. Samples were collected from cold- (3°C) and warm- (18°C) acclimated fish. First-strand cDNA reaction was used directly (0.5 mL) or diluted 10-fold (0.05 mL) as template in polymerase chain reactions (PCR) with isoform-specific PCR primers.

phenotypic and developmental changes in Tn isoform expression appear to be transcriptionally regulated through isoform-specific elements such as SURE (slow upstream response element) and FIRE (fast intronic response element; Calvo et al. 1999). The genetic mechanisms underlying the unusual isoform distribution in fish are unknown.

In summary, we found little evidence that TnC plays an important role in either evolutionary adaptation or physiological acclimation to temperature in fish. It is likely that other proteins such as TnI, TnT, and thick filament may play more important roles in both evolutionary adaptation and physiological variation in myocardial excitation-contraction coupling in fish as well as in vertebrate skeletal muscle (Goldspink et al. 1992; Goldspink 1998) and mammalian cardiac muscle (Martin et al. 1991; Morimoto et al. 1999).

Acknowledgments

Funding for this study was provided by the National Sciences and Engineering Council of Canada (C.D.M.). We wish to thank Thor Borgford for his many helpful discussions and Dr. B. Tufts for access to his fish holding facilities.

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