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Effect of cold acclimation on troponin I isoform expression in striated muscle of rainbow trout

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Alderman SL, Klaiman JM, Deck CA, Gillis TE. Effect of cold acclimation on troponin I isoform expression in striated muscle of rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 303: R168–R176, 2012. First published May 16, 2012; doi:10.1152/ajpregu.00127.2012.—In vertebrates each of the three striated muscle types (fast skeletal, slow skeletal, and cardiac) contain distinct isoforms of a number of different contractile proteins including troponin I (TnI). The functional characteristics of these proteins have a significant influence on muscle function and contractility. The purpose of this study was to characterize which TnI gene and protein isoforms are expressed in the different muscle types of rainbow trout (*Oncorhynchus mykiss*) and to determine whether isoform expression changes in response to cold acclimation (4°C). Semiquantitative real-time PCR was used to characterize the expression of seven different TnI genes. The sequence of these genes, cloned from Atlantic salmon (*Salmo salar*) and rainbow trout, were obtained from the National Center for Biotechnology Information databases. One-dimensional gel electrophoresis and tandem mass spectrometry were used to identify the TnI protein isoforms expressed in each muscle type. Interestingly, the results indicate that each muscle type expresses the gene transcripts of up to seven TnI isoforms. There are significant differences, however, in the expression pattern of these genes between muscle types. In addition, cold acclimation was found to increase the expression of specific gene transcripts in each muscle type. The proteomics analysis demonstrates that fast skeletal and cardiac muscle contain three TnI isoforms, whereas slow skeletal muscle contains four. No other vertebrate muscle to date has been found to express as many TnI protein isoforms. Overall this study underscores the complex molecular composition of teleost striated muscle and suggests there is an adaptive value to the unique TnI profiles of each muscle type.

contractile element; thermal acclimation; gene expression; striated muscle; protein isoforms

THE TROPONIN (Tn) complex, composed of troponin C (TnC), troponin I (TnI), and troponin T (TnT), is responsible for making the contractile reaction Ca^{2+} dependent. When a myocyte is relaxed, tropomyosin (Tm) sterically blocks myosin binding sites along the actin filament (14, 19, 26, 30). It is locked in this position by an interaction between TnI and actin (18). After membrane depolarization, cytosolic Ca^{2+} concentration increases and binds to the regulatory domain of TnC. The resultant conformational change triggers the movement of a peptide within the NH_2-terminus of TnI (switch peptide) toward a hydrophobic cleft that has been exposed within the NH_2-terminus of TnC (14, 19, 26, 30). This conformational change of TnI releases its interaction with actin and as a result, Tm is able to move and expose myosin binding sites on actin (8). This allows for the formation of force-generating cross-bridges and ultimately muscle contraction.

Previous work has shown that the three Tn subunits, along with other muscle proteins, have tissue-specific isoforms that have been produced by gene duplication events within vertebrate striated muscle (5, 9). For example, all mammals have distinct cardiac (c), slow skeletal (ss), and fast skeletal (fs) isoforms of TnI and TnT and two isoforms of TnC (fs and c/ss). The isoforms of these proteins have different functional properties that are responsible, at least in part, for the functional differences of the three muscle types (4). For example, the transgenic replacement of cTnI with ssTnI in mouse cardiac myocytes increases the Ca^{2+} sensitivity of force generation and eliminates the effect of protein kinase A (PKA) phosphorylation on Ca^{2+} sensitivity (4). Interestingly, Jackman et al. (12) reported that three TnI isoforms in Atlantic salmon (*Salmo salar*) are expressed in varying numbers in different muscles. This suggests that contraction in salmonid muscle is regulated by the expression of different protein isoforms. This idea is supported by the identification of six different TnI isoforms in the Atlantic salmon genome. It is not known, however, in what tissues these are expressed.

Much is known about the regulation of contractile function in mammals, but limited effort has been spent toward understanding the regulation of contraction in other vertebrates. This is relevant as striated muscle in many non-mammalian species can function over a range of physiological conditions that would impede the function of mammalian muscles. For example, many fish, including the rainbow trout (*Oncorhynchus mykiss*), remain active over a range of environmental temperatures and at temperatures low enough to stop the mammalian heart (6). In addition, we recently demonstrated that a number of key changes occurred in the amino acid sequences of the contractile proteins over evolutionary time, including changes in cTnI (29). It is thought that these reduce the Ca^{2+} sensitivity of the troponin complex and limit its function at low temperatures in endothermic species (29). Therefore, study of the regulatory mechanisms of contractile function in trout muscle will provide insight into the adaptive properties of vertebrate striated muscle and how this has changed over evolutionary time.

To begin studying the regulation of contractility in the different muscle types of trout and how this responds to temperature change, we characterized the TnI isoform profiles of cardiac, slow skeletal, and fast skeletal muscles in control (11°C) and cold acclimated (4°C) rainbow trout. Using a combination of qPCR and tandem mass spectroscopy, we determined that each muscle type expresses the transcripts for up to seven unique isoforms of TnI in varying quantities, and the expression of these can be altered by cold acclimation. This suggests that the thermal tolerance of teleost muscle is intri-

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cately maintained by tissue- and temperature-specific expression of a key regulatory component proteins.

METHODS

Experimental animals and tissue sampling. Adult female rainbow trout (*Oncorhynchus mykiss*) were obtained from the Alma Aquaculture Research Station (Alma, ON). Female fish were used in this study because we wanted to isolate the effect of cold acclimation on contractile function without the confounding response of altered connective tissue content that is seen in the male trout heart with cold acclimation (17). Fish (1567.7 ± 63.5 g) were held at 11 ± 1°C for 2 wk before thermal acclimation. Fish were then acclimated to 4°C and held for a minimum of 2 mo as previously described (17). The control fish were maintained at 11 ± 1°C during the experiment. Water was kept at normoxic levels with a 12-h light:12-h dark photoperiod cycle. All fish were fed Profishent commercial diet (Martin Mills, Alma Ontario) ad libitum during the experiment. To sample muscle tissues, fish were killed by a blow to the head followed by cervical dislocation. Cardiac ventricle, fast muscle, and slow muscle were dissected from the fish, rinsed in physiological saline (in mM: 94 NaCl, 24 $NaCO_3$, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 0.7 $CaCl_2$; pH 7.6 at 15°C), and then stored at −80°C until used. Tissues were taken from seven individuals for both treatment and control. Fast and slow skeletal muscle samples were taken from the same location on each fish. For fast skeletal muscle this was from just behind the head on the dorsal surface and for slow muscle this was from the lateral line just before the caudle fin. All protocols were approved by the University of Guelph Animal Care Committee (protocol no. 06R010).

Identification of TnI isoforms. (Please note that gene names are in italic type, whereas their protein products are in Roman type.) A search was performed to identify all available TnI gene sequences from both *O. mykiss* and *Salmo salar* using the National Center for Biotechnology Information (Bethesda, MD) nonredundant protein database (http://www.ncbi.nlm.nih.gov/guide/proteins/), the Ensembl Genome Browser (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK) (http://uswest.ensembl.org/index.html), and GenBank (http://www.ncbi.nlm.nih.gov/genbank/). A total of eight TnI isoforms were identified, two from rainbow trout (RT) and six from Atlantic salmon (AS). Seven of these genes were identified as muscle-specific isoforms (GenBank protein/*gene*): *1*) *RT cardiac* (c) *TnI* (*RTcTnI*, NP_001171957.1/*NM_001185028*); *2*) *AS fast skeletal (fs) TnI* (*ASfsTnI*, ACI67113.1/*BT047312*); *3*) *AS slow skeletal* TnI_1 (*ASssTnI*$_1$, ACM09409.1/*BT057537*); *4*) *ASssTnI*$_2$ (ACM09762.1/*BT057890*); *5*) *ASssTnI*$_3$ (ACM08558/*BT049066*); *6*) *AScTnI*$_1$ (AAB53386.1/*U84394*); and *7*) *AScTnI*$_2$ (ACM09452.1/*BT057580*). The eighth was cloned from an unspecified muscle type of rainbow trout (NP_001123462.1/*NM_001129990*). We therefore designated this isoform as *RT undetermined TnI* (*RTudTnI*). Nucleotide (coding sequence) and amino acid homology between the eight identified isoforms was determined using the multiple sequence alignment tool ClustalW2.

Semiquantitative real-time PCR. Muscle samples were powdered on dry ice, and total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY) according to manufacturer's instructions. After DNase treatment (Life Technologies), cDNA was synthesized from 500 ng RNA using the High Capacity cDNA Synthesis Kit (Life Technologies) according to manufacturers' instructions. A subset of semiquantitative real-time PCR (qPCR) samples was included in duplicate cDNA reactions that omitted the Multiscribe RT enzyme and served as nonreverse transcribed (non-RT) controls. Transcript abundance was measured in duplicate by qPCR using a StepOne Plus. Each 15-μl reaction contained 1xSYBR Green Master Mix (Life Technologies), 200 nM each gene-specific primer (Table 1), and 1:15 vol:vol cDNA. Gene-specific primers were designed using Primer Express 3.0. The uniqueness of each primer was confirmed using pair-wise alignments against all TnI isoforms (ClustalW2), and the maximum identity for a primer ranged from 52% to 71% (Table 1). Default cycling conditions were used for the qPCR reactions followed by a dissociation cycle. Only reactions that generated a single-peaked dissociation curve at the predicted amplicon melting temperature were included in analysis. The mRNA abundance of each isoform was quantified by fitting the threshold cycle to the antilog of standard curves prepared from serially diluted cDNA. Isoform transcript abundance was standardized to the mRNA abundance of *elongation factor 1α* (*EF1α;* GenBank Accession Number: AF498320; forward primer: GGGCAAGGGCTCTTTCAAGT; reverse primer: CGCAATCAGCCTGAGAGGT), which was expressed at constant levels across muscle types and treatments. All non-RT controls failed to amplify.

Identification of TnI protein isoforms in muscle samples. Samples (~400 mg) of cardiac muscle, slow skeletal muscle, and fast skeletal were ground into a fine powder using liquid nitrogen and a mortar and pestle. The powder was suspended in physiological saline containing 0.1 mmol PMSF and 1% vol/vol Triton X and then incubated on ice for 15 min. This mixture was sonicated with a Vibra Cell sonicator (Sonics and Materials 148, Danbury) for 3 × 15 s then centrifuged at 1,500 *g* for 10 min. Proteins in the pellet were fully solubilized by utilizing a resuspension buffer containing 7 M urea, 2 M thiourea, 30 mmol/l Tris, and 0.1 mmol PMSF, pH 8.5. Aliquots of this solution from each muscle sample were separated using SDS-PAGE. The gels also contained a weight ladder and a protein standard containing

Table 1. *Forward and reverse primer sequences used to amplify seven different salmonid isoforms of TnI using qPCR*

Gene	Primer Sequence	Maximum Primer Identity	Amplicon Size, bp	GenBank
AScTnI$_1$	F GAAAGAAGCAGCAGACTTGATAACG	64% (*ASssTnI*$_2$)	65	U84394
	R CCGGAGCAGGGCAGTTT	70% (*ASfsTnI*)		
AScTnI$_2$	F ACAATATTATCCTCAACTGCCTGATG	58% (*RTcTnI*)	68	BT057580
	R TGGGTGGAAGCAACGTTCTC	65% (*RTudTnI*)		
ASfsTnI	F CATTGCTAAAGGCTTACTGGAAGAC	68% (*AScTnI*$_1$)	66	BT047312
	R CGCCACCCATCTTATTCTCTCT	68% (*ASssTnI*$_2$, *RTcTnI*)		
ASssTnI$_2$	F CACTCCAAAGGTCGAACACA	65% (*RTcTnI*)	149	BT057890
	R CCCCCTAGGTAGCGAACCTT	60% (*RTcTnI*)		
ASssTnI$_3$	F GTCCAGGAGCAAAGCAACAAG	62% (RTcTnI, ASssTnI$_2$)	63	BT049066
	R CGGTGGTACTCTCTCGTTTAAGG	52% (ASfsTnI, AScTnI$_2$)		
RTcTnI	F TGCCAGAGCAAGTACAAGAGA	71% (*AScTnI*$_2$)	169	BT057537
	R GGTCTGTAAGGTGGGTGCTC	65% (*ASssTnI*$_2$)		
RTudTnI	R GGTCTGTAAGGTGGGTGCTC	55% (*AScTnI*$_2$, *RTcTnI*)	78	NM_001185028
	R CGGAGATCTTAGCCATGAAATTG	65% (*ASssTnI*$_2$)		

The maximum percent identity of a primer to any of the six other isoforms is listed, and the most similar isoform(s) is indicated. The amplicon size amplified by each primer set, as well as the GenBank accession numbers are provided for each isoform. RT, rainbow trout; c, cardiac; ss, slow skeletal; fs, fast skeletal; TnI, troponin I; AS, Atlantic salmon; ud, undetermined TnI, troponin I; qPCR, semiquantitative PCR; F, fast primer; R, reverse primer.

recombinant RTcTnI and bovine serum albumen (BSA). Protein bands were cut from lanes generated for each muscle type for control and cold fish. Every band with a weight ±8 kDa of the band for the RTcTnI standard was cut from the gel and prepared for trypsin digestion as previously described (16). These samples were then subjected to tandem mass spectrometry at the Advanced Protein Analysis Center at Sick Kids Hospital (Toronto, ON) using an Applied Biosystems/MDS Sciex API QSTAR XL Pulsar MALDI QTOF mass spectrometor. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search the NCBInr 20111113 database (selected for Chordata, 1556210 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 3.0 Da. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Pyro-glu from E of the NH_2-terminus, *S*-carbamoylmethylcysteine cyclization of the NH_2-terminus, deamidation of asparagine and glutamine, oxidation of methionine and acetylation of the NH_2-terminus were specified in Mascot as variable modifications.

Criteria for protein identification. Scaffold (version Scaffold 3.3.1, Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (13). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (24). Table 2 lists the number of peptides unique to the identified isoform detected in the sample as well as the percentage of the protein sequence covered by those peptides.

Statistics. For each TnI isoform, differences in mRNA abundance between the three muscle types in control and acclimated fish were determined using a two-way ANOVA followed by a Holm-Sidak test for multiple comparisons (n = 3–5; $P < 0.05$). To meet the assumption of normality, all gene expression data were log transformed before analysis, except for *EF1α*.

RESULTS

Identification and comparison of salmonid TnI isoforms. Of the eight isoforms identified, one homologous pair was identified from rainbow trout and Atlantic salmon based on their high nucleotide (93%) and amino acid (91%) identities (*RTcTnI* and *ASssTnI*$_1$). It should also be noted that a number of the differences in amino acid sequence between these two isoforms were conservative substitutions (Fig. 1). The *ASssTnI*$_1$ isoform was excluded from further analyses. The remaining pairwise identities of nucleotide sequences between the seven isoforms ranged from 59% to 82% (average 64%), and amino acid identities ranged from 46% to 76% (average 55%). A summary of the nucleotide and amino acid identities between the eight isoforms is presented in Table 3.

TnI isoform transcript expression patterns. Quantification of the seven different TnI transcripts in rainbow trout cardiac, slow skeletal, and fast skeletal muscle showed a unique TnI expression pattern for each muscle type as summarized in Table 4. Although all seven transcripts were detected in each muscle type, one or two isoforms were expressed at 10-fold greater abundance than any other isoform. In slow skeletal muscle *AScTnI*$_2$ had the highest expression; in fast skeletal muscle *RTudTnI* and *AScTnI*$_1$ were the dominant transcripts; and in cardiac muscle *RTcTnI* had the greatest expression. Also, each TnI isoform was detected at significantly higher levels ($P < 0.05$) in one of the three muscle types (Fig. 2), with the exception of *ASfsTnI* and *ASssTnI*$_3$. *ASfsTnI* was expressed at very low levels with no significant differences in expression between muscle types (Fig. 2*C*). *ASssTnI*$_3$ was expressed at moderately high levels in slow skeletal muscle but with considerable variation between samples (control and cold acclimated, 1.09 + 0.95 and 1.59 + 0.78 arbitrary units, respectively; n = 3–4). In control acclimated fast and cardiac muscle, amplification of *ASssTnI*$_3$ produced a multipeaked dissociation curve, indicating nonspecific amplification of multiple genes, so the data could not be utilized. In cold-acclimated cardiac muscle, 3 of 5 samples also had multipeaked dissociation curves and the other two samples had very low levels of *ASssTnI*$_3$. A unimodal dissociation curve resulted from amplification of *ASssTnI*$_3$ in cold-acclimated fast skeletal muscle, and average expression was very low (0.10 + 0.02 arbitrary units; n = 5).

Cold acclimation significantly increased the expression of one TnI transcript in both slow and fast skeletal and four in cardiac muscle (Fig. 2). In fast skeletal muscle, *AScTnI*$_2$ increased 7.4-fold over controls (Fig. 2*B*; $P < 0.05$). In cardiac muscle, *AScTnI*$_1$ increased 377-fold (Fig. 2*A*), *AScTnI*$_2$ increased 7.7-fold (Fig. 2*B*), *ASssTnI*$_2$ increased 12.1-fold (Fig. 2*D*), and *RTudTnI* increased 333-fold (Fig. 2*F*) relative to controls ($P < 0.05$). Of note, the significant changes observed in cardiac muscle gene expression following cold acclimation were for the four TnI isoforms with the lowest abundance in this muscle type, and they remain in very low abundance despite the large increases in expression. The mRNA abundance of *ASfsTnI* and *RTcTnI* did not change in any muscle type in response to cold acclimation.

Protein expression patterns in striated muscle types. Multiple proteins and isoforms of multiple proteins, identified using the presence of unique peptide sequences, were found in most protein bands (Fig. 3, Tables 2 and 4). This result is not surprising as two-dimensional gels of muscle tissue contain multiple protein spots at similar weights but different isoelectric points. In addition there were a number of proteins that were found in multiple bands. This indicates that there was incomplete reduction of the protein sample before running and/or that there were proteolytic fragments detected. The proteolysis may have occurred within the muscle sample while the animal was still alive or during sample preparation. Evidence that proteolytic fragments were detected is that peptides corresponding to the NH_2- and COOH-termini of $ASTnT_2$ were found in bands two and four while only peptides from the NH_2-terminus of $ASTnT_2$ were detected in bands eight and nine (data not shown). On the gel, bands two and four had a higher molecular weight than bands eight and nine.

Three isoforms of TnI were found in fast skeletal and cardiac muscle, whereas four were found in slow skeletal muscle (Table 4). In fast skeletal muscle these were $AScTnI_1$, ASfsTnI, and RTudTnI; in cardiac muscle these were $AScTnI_1$, RTcTnI, and RTudTnI; and in slow skeletal muscle these were $AScTnI_2$, $ASssTnI_2$, $ASssTnI_3$, and RTudTnI. There was at least one protein isoform found exclusively in each muscle type. For fast skeletal muscle this was ASfsTnI, for cardiac muscle this was RTcTnI, and for slow skeletal muscle these were $AScTnI_2$, $ASssTnI_2$, and $ASssTnI_3$. As there are multiple TnI isoforms detected in single protein bands, it is not known, therefore, if there were differences in the basal protein levels of different isoforms within a given muscle type nor if there were

Table 2. *Contractile regulatory proteins identified in protein bands labeled in Fig. 3*

Band Number	Proteins Identified	Unique Peptides	Coverage
1	**RTcTnI (NP_001171957.1)**	12	40%
2	$ASfsTnT_2$ (ACH71020.1)	4	20%
	ASfs tropomyosin (NP_001117128.1)	2	9%
	$ASfsTnT_1$ (ACH71024.1)	1	10%
3	ASfs tropomyosin (NP_001117128.1)	4	23%
4	$ASfsTnT_2$ (ACH71020.1)	6	26%
	AS myosin light chain-1 (NP_001133218.1)	5	31%
	AS slow myosin light chain-1 (NP_001117085.1)	4	34%
	$ASfsTnT_1$ (ACH71024.1)	3	23%
5	AS myosin light chain-1 (NP_001133218.1)	10	44%
6	AS myosin light chain-1 (NP_001133218.1)	6	28%
7	AS myosin light chain-1 (NP_001133218.1)	9	38%
8	$ASfsTnC_1$ (ACM09304.1)	10	61%
	RT fast myosin light chain-2 (NP_001118151.1)	5	28%
	$AScTnI_1$ (AAB53386.1)	4	22%
	$ASfsTnC_2$ (ACH70760.1)	3	58%
	RTudTnI (NP_001123462.1)	2	22%
	$ASfsTnT_2$ (ACH71020.1)	2	14%
	ASfs tropomyosin (NP_001117128.1)	1	3%
9	**$AScTnI_1$ (AAB53386.1)**	11	58%
	RT fast myosin light chain-2 (NP_001118151.1)	10	66%
	$ASfsTnC_1$ (ACM09304.1)	7	50%
	RTudTnI (NP_001123462.1)	6	49%
	$ASfsTnC_2$ (ACH70760.1)	3	47%
	$ASfsTnT_2$ (ACH71020.1)	2	13%
	$ASfsTnT_1$ (ACH71024.1)	2	20%
	ASfsTnI (ACI67113.1)	2	47%
	O. kisutch myosin light chain-2 (AAF71271.1)	2	55%
10	RT fast myosin light chain-2 (NP_001118151.1)	13	76%
	RTudTnI (NP_001123462.1)	3	25%
	ASfs tropomyosin (NP_001117128.1)	3	13%
	O. kisutch myosin light chain-2 (AAF71271.1)	2	61%
	$AScTnI_1$ (AAB53386.1)	2	19%
11	AS myosin light polypeptide-4 (NP_001134936)	4	38%
	$AScTnI_1$ (AAB53386.1)	4	22%
	RTudTnI (NP_001123462.1)	3	29%
	AS myosin light chain-1 (NP_001133218.1)	2	8%
	RT fast myosin light chain-2 (NP_001118151.1)	2	11%
	RTcTnI (NP_001171957.1)	1	6%
	$ASfsTnC_1$ (ACM09304.1)	1	11%
12	AS myosin light chain-1 (NP_001133218.1)	1	7%
13	RTcTnC (ACI67223.1)	2	17%
	RT fast myosin light chain-2 (NP_001118151.1)	2	12%
14	RTcTnC (ACI67223.1)	6	35%
15	AS slow myosin light chain-1 (NP_001117085.1)	10	22%
	AS myosin light chain-1 (NP_001133218.1)	5	53%
	$AScTnI_2$ (ACM09452.1)	2	14%
	RT slow myosin light chain-2 (NP_001118150.1)	2	15%
	RTudTnI (NP_001123462.1)	1	8%
	$ASssTnI_2$ (ACM09762.1)	1	5%
16	RTcTnC (ACI67223.1)	8	55%
	RT fast myosin light chain-2 (NP_001118151.1)	7	49%
	RT slow myosin light chain-2 (NP_001118150.1)	7	34%
	O kisutch myosin light chain-2 (AAF71271.1)	2	36%
	AS slow myosin light chain-1 (NP_001117085.1)	2	12%
	$ASssTnI_3$ (ACM08558)	1	8%
	RTudTnI (NP_001123462.1)	1	7%
17	AS slow myosin light chain-1 (NP_001117085.1)	8	37%
	AS myosin light chain-1 (NP_001133218.1)	7	36%
	$ASssTnI_2$ (ACM09762.1)	4	20%
	$AScTnI_2$ (ACM09452.1)	1	7%
	RT slow myosin light chain-2 (NP_001118150.1)	1	8%
	ASfs tropomyosin (NP_001117128.1)	1	3%
18	**$ASssTnI_3$ (ACM08558)**	4	33%
	RTcTnC (ACI67223.1)	4	34%
	$AScTnI_2$ (ACM09452.1)	3	19%
	RT slow myosin light chain-2 (NP_001118150.1)	3	21%
	RT fast myosin light chain-2 (NP_001118151.1)	3	18%
	RTudTnI (NP_001123462.1)	2	15%
	$ASfsTnC_1$ (ACM09304.1)	1	11%
19	**$ASssTnI_3$ (ACM08558)**	5	33%
	RTcTnC (ACI67223.1)	5	34%
	RT slow myosin light chain-2 (NP_001118150.1)	4	28%
	$ASfsTnC_1$ (ACM09304.1)	3	23%
	RT fast myosin light chain-2 (NP_001118151.1)	3	18%
	RTudTnI (NP_001123462.1)	3	25%
	$AScTnI_2$ (ACM09452.1)	2	12%
	S. Trutta slow muscle troponin T (AAB58912.1)	2	9%

O. kisutch, Oncorhynchus kisutch. S. Trutta, Salmo Trutta. Numbers in parentheses are protein accession numbers. All TnI isoforms are indicated in bold. Unique peptides represents the number of peptides unique to the identified isoform detected in the sample; Coverage indicates the percentage of the protein sequence covered by those peptides.

changes in the abundance of a specific protein isoform as a result of cold acclimation. We did not try using Western blotting to delineate between isoforms as previous efforts, with multiple TnI antibodies, only identified a single band in all trout muscle types (data not shown). Finally, it should be noted that $ASssTnI_1$ was not detected using mass spectroscopy in any muscle type.

Detection of other regulatory proteins. The mass spectrometry results demonstrate the presence of other contractile proteins in the bands that were analyzed for TnI content (Table 2). This includes multiple protein isoforms for TnC, TnT, and different myosin light chains in each tissue type. For example, fast skeletal muscle contained two TnT isoforms, two TnC isoforms, and five different myosin light chains. As we were specifically targeting TnI, only bands with a weight ±8 kDa of the RTcTnI standard were cut from the gel. Therefore, it is most likely that we did not detect all of the different isoforms of these proteins as they vary in size from TnI. This does, however, demonstrate that the different muscle types express multiple isoforms of other regulatory proteins besides TnI.

DISCUSSION

The results of this study suggest that contractile function in trout muscle is controlled by a complex molecular system that varies with muscle type and physiological condition. We demonstrate that each of the three muscle types in trout transcribes seven TnI isoforms and translates a unique complement of these into protein. Changes in select TnI transcript levels in cardiac and fast skeletal muscle following cold acclimation suggests that the specific complement of TnI isoforms in the muscle types is important to maintain optimal contractile function in varied physiological conditions. Differences in key amino acid residues further supports functional differences between the salmonid TnI isoforms.

TnI expression in striated muscle types. The transcripts for seven distinct TnI isoforms were identified in rainbow trout cardiac, slow skeletal, and fast skeletal muscle types. This relatively high number compared with mammalian species is

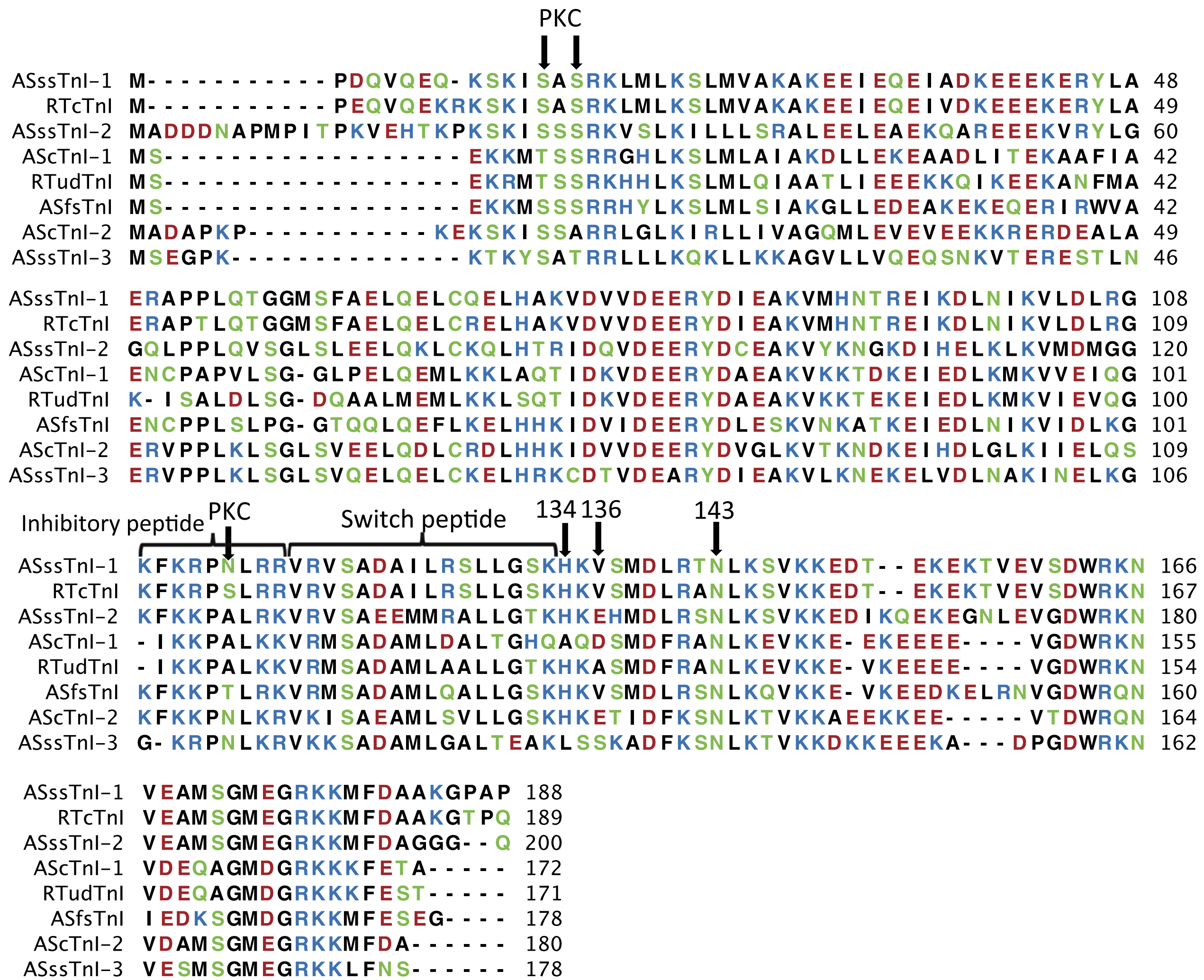

Fig. 1. Sequence alignment of Atlantic salmon (AS) (*Salmo salar*) cardiac troponin I (TnI) 1 ($AScTnI_1$), Atlantic salmon cardiac TnI 2 ($AScTnI_2$), Atlantic salmon fast skeletal (fs) TnI (ASfsTnI), Atlantic salmon slow skeletal (ss) TnI 1 ($ASssTnI_1$), Atlantic salmon slow skeletal TnI 2 ($ASssTnI_2$), Atlantic salmon slow skeletal TnI 3 ($ASssTnI_3$), rainbow trout (RT) (*Oncorhynchus mykiss*) cardiac TnI (RTcTnI), and rainbow trout undetermined TnI (RTudTnI). Putative protein kinase C (PKC) targets in mouse cardiac TnI are indicated, as are the position equivalents of residues 131, 133, and 139. Location of the inhibitory and switch peptides are also indicated on the sequence. Color of single letters represents the charge and polarity of the represented amino acid. Red indicates amino acids that are negatively charged and polar; blue indicates positively charged and polar; green indicates neutral and polar; and black indicates neutral and nonpolar.

likely the result of two genome duplications. The first occurring in the teleost linage during the Devonian period ~440 million years ago (22) and the second occurring in salmonid fishes 25–100 million years ago (23). The maintenance of seven unique genes for TnI in the trout genome and their continued expression in the tissue suggests that each is functionally significant to the species. This is further supported by the low amino acid identity between the seven isoforms, which ranges from 46% to 76% (Table 3).

While all three striated muscle types in trout express transcripts for each of the seven salmonid TnI isoforms (with the possible exception of $ASssTnI_3$ in cardiac muscle), the relative abundance of each transcript varies between muscle types (Table 4, Fig. 2). This indicates that there are muscle-specific expression profiles. Contrary to the gene expression data, only three or four protein isoforms of TnI were identified in each muscle type. In many cases the proteins that were identified corresponded to highly expressed transcripts. For example, a relatively high transcript level for $AScTnI_1$ was found in fast skeletal muscle, and the protein product was detected in the muscle. The same can be said for $AScTnI_2$, $ASssTnI_2$, and $ASssTnI_3$ in slow skeletal muscle and *RTcTnI* in cardiac muscle. In other cases, however, the corresponding proteins of lowly expressed transcripts were identified, such as $AScTnI_1$ in cardiac muscle and *ASfsTnI* in fast skeletal muscle; and in other cases no protein was detected despite the relatively high expression of its transcript, such as $AScTnI_2$ in fast skeletal muscle. These results are difficult to interpret without quantifying the abundance of each protein isoform. One method that would allow for protein abundance to be quantified is two-dimensional difference gel electrophoresis (2D-DIGE). This method was not used due to the significant cost of identifying the seven TnI isoforms, using mass spectroscopy, from the constellation of 1,000 plus proteins present on a two-dimensional muscle gel (17).

Given that there are multiple TnI isoforms expressed in each type of the three striated muscle types in trout (Table 4), the convention of designating a muscle type for each TnI isoform

Table 3. *Percent identity between the amino acid and nucleotide sequences of the translated region of the different isoforms of Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) TnI*

	AScTnI1	AScTnI2	ASfsTnI	ASssTnI1	ASssTnI2	ASssTnI3	RTcTnI
AScTnI1	X						
MM 19,390							
AScTnI2	47%	X					
MM 20,600	*60%*						
ASfsTnI	61%	51%	X				
MM 20,694	*71%*	*60%*					
ASssTnI1	50%	54%	57%	X			
MM 21,578	*62%*	*65%*	*63%*				
ASssTnI2	49%	56%	53%	60%	X		
MM 22,797	*60%*	*63%*	*60%*	*66%*			
ASssTnI3	47%	52%	49%	52%	46%	X	
MM 20,166	*68%*	*68%*	*67%*	*69%*	*65%*		
RTcTnI1	50%	55%	57%	91%	60%	51%	X
MM21,813	*60%*	*65%*	*62%*	*93%*	*66%*	*70%*	
RTudTnI	76%	46%	61%	49%	51%	42%	49%
MM 19,568	*82%*	*60%*	*70%*	*59%*	*62%*	*72%*	*60%*

MM, molar mass in Da. Nucleotide sequence identity is indicated in italic font. Please note that ASssTnI1 was not found in any trout striated muscle.

is not appropriate. For example, the supposed cardiac isoform *AScTnI2* is transcribed at relatively high levels in all three muscle types, and the protein RTudTnI is found in all muscle types. It may therefore be useful to stop using the name of the muscle type in the protein name but instead include the protein accession number. The finding of multiple protein isoforms in trout muscle is supported, in part, by Jackman et al. (12) where two TnI isoforms were found in Atlantic salmon slow muscle using Western blot. These authors also report that three TnI isoform were found in Atlantic salmon cheek muscle. It is likely, however, that this muscle is a mix of fast and slow muscle fibers. In contrast to the presence of multiple TnI isoforms in each type of striated muscle in trout (present study), healthy adult mammals contain only a single TnI isoform unique to each striated muscle type. The exception to this is changes in the expression of TnI that occur in the heart during early development (28) and with the onset of a number of stress-induced pathologies (15, 34).

Changes in TnI expression with thermal acclimation. The increase in the expression of one TnI isoform transcript in fast muscle and four in cardiac muscle with cold acclimation indicates that these muscle types are responding to the change in physiological conditions. While these increases likely correspond to changes in protein abundance, the magnitude of change is not expected to be equal given that transcription and translation are differentially regulated by multiple factors. Previous studies have demonstrated that warm and cold acclimation of eurythermal species can cause an increase in gene expression. This includes an increase in the expression of genes associated with protein biosynthesis and transport in the longjaw mudsucker *Gillichthys mirabilis* with warm acclimation (20), the transcript for citrate synthase in the eelpout *Zoarces viviparous* with cold acclimation (21), and multiple transcripts associated with protein biosynthesis in the trout heart with cold acclimation (35). In addition, we have recently demonstrated that cold acclimation causes cardiac hypertrophy in trout indicating an increase in the expression of gene transcripts associated with cellular growth and protein expression (17). The increased expression of select TnI transcripts in the three tissue types in the current study with cold acclimation suggests a specific strategy for each muscle type and not a generalized response. A reduction in temperature does impair muscle contractility, but there are potential differences in how each muscle type responds. For example, a reduction in temperature reduces the Ca^{2+} sensitivity and maximum Ca^{2+}-activated force of cardiac muscle from the cat, rabbit, rat, guinea pig, and frog (2, 10, 11). However, cooling mammalian skeletal muscle increases myofilament Ca^{2+} sensitivity but decreases maximal Ca^{2}-activated force (7, 31). This difference in the affect of temperature on contractility reflects the net effect of the temperature change on the function of the proteins that compose the muscle types. Because each muscle type composed of different mixtures of protein isoforms, it is likely that the temperature change is influencing function differently in each tissue type. Therefore, each tissue responds in a specific manner to maintain function. However, because we were not able to quantify changes in protein expression, it is not possible to say if cold acclimation resulted in changes in the relative amounts of the different protein isoforms expressed in each muscle type.

We have recently demonstrated that cold acclimation of trout results in a 50% increase in the maximal rate of actin-

Table 4. *Comparison of transcript abundance of seven isoforms of troponin I (AScTnI1, AScTnI2, ASfsTnI, ASssTnI2, ASssTnI3, RTcTnI and RTudTnI) in slow skeletal, fast skeletal, and cardiac muscles of rainbow trout maintained at 11°C*

Gene	Slow Skeletal	Fast Skeletal	Cardiac
AScTnI1	+++	+++++*	+*
AScTnI2	+++++*	++++	+++
ASfsTnI	+	+*	++
ASssTnI2	++++*	+++	++
ASssTnI3	+++*	+	+
RTcTnI	+	++	++++*
RTudTnI	+++*	+++++*	++*

Expression of each gene was quantified by qPCR and normalized to the expression of elongation factor 1α (*n* = 3-5). Relative abundance is indicated by the number of +, with each + representing a 10-fold higher expression. *Corresponding protein was found in the muscle using tandem mass spectrometry.

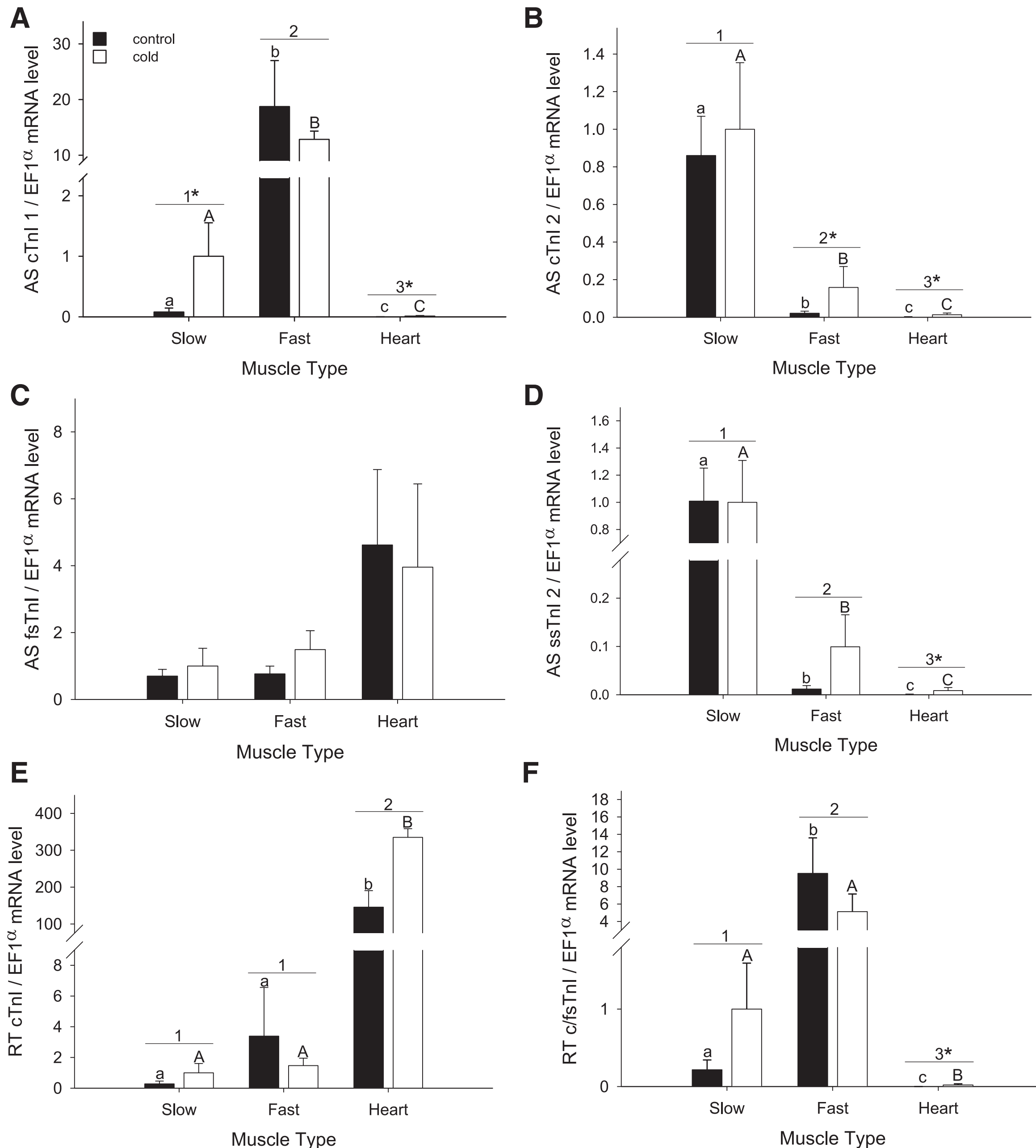

Fig. 2. The influence of cold acclimation (4°C) on the expression of gene transcripts for Atlantic salmon (*Salmo salar*) cardiac TnI 1 (*AScTnI₁*, *A*), Atlantic salmon cardiac TnI 2 (*AScTnI₂*, *B*), Atlantic salmon fast skeletal TnI (*ASfsTnI*, *C*), Atlantic salmon slow skeletal TnI 2 (*ASssTnI₂*, *D*), rainbow trout (*Oncorhynchus mykiss*) cardiac TnI (*RTcTnI*, *E*), and rainbow trout undetermined TnI (*RTudTnI*, *F*), in fast skeletal, slow skeletal, and cardiac muscle of rainbow trout. The expression of each transcript is expressed relative to the amount of that transcript in the slow muscle of the control fish. This value is set to one on each panel. Statistical differences, calculated by a two-way ANOVA, are indicated on each panel. Numbers indicate overall differences between tissue types. Upper case letters indicate differences between tissues within the control fish. Lower case letters indicate differences between tissues within the cold acclimated fish. *An effect of cold acclimation on gene expression within the specific tissue type.

myosin ATPase in the heart (17). In that study we used 2D-DIGE followed by mass spectroscopy to characterize the contractile proteins present in the trout heart as well as changes in phosphorylation state. This work indicated that cold acclimation causes subtle increases in the phosphorylation of TnT and myosin binding protein C. However, only a single isoform of TnI was detected. In the current study we have identified three TnI isoforms in the heart. The advantage of the methods used in the current study is that multiple proteins are identified in each protein band.

Functional implications of sequence differences between isoforms. Comparison of the amino acid sequence of the seven TnI isoforms found in trout muscle reveals considerable differences (Fig. 1 and Table 3). One region where such variation occurs is in the NH_2-terminus of the different isoforms (Fig. 1). This region of TnI interacts with TnC within the tertiary structure of the troponin complex, and mutations here influence the Ca^{2+} activation of the contractile reaction (1, 4, 33). Significantly, there are variations between isoforms in the number and distribution of glutamate and aspartate residues in

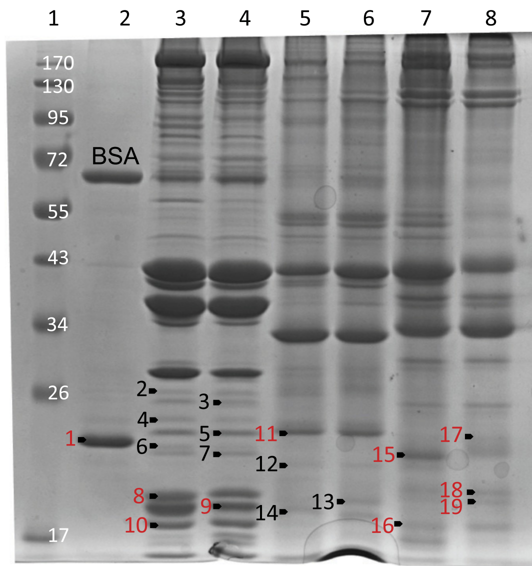

Fig. 3. Identification of contractile proteins in fast skeletal muscle, cardiac muscle, and slow skeletal muscle from rainbow trout (*Oncorhynchus mykiss*) using polyacrylamide gel electrophoresis. *Lane 1* contains the weight ladder; *lane 2* contains bovine serum albumin (BSA; molecular weight 66.5 kDa) and recombinant RTcTnI (molecular weight 21.8 kDa). *Lanes 3* and *4* contain myofilament proteins from fast skeletal muscle of control (11°C) and cold-acclimated (4°C) rainbow trout, respectively. *Lanes 5* and *6* contain myofilament proteins from cardiac ventricle of control and cold-acclimated rainbow trout, respectively. *Lanes 7* and *8* contain myofilament proteins from slow skeletal muscle of control and cold-acclimated rainbow trout, respectively. The protein gel was stained with Coomassie R250. Numbers (1–19) indicate the bands that were dissected from the gel and analyzed using tandem mass spectroscopy. The results of this analysis are in Table 2. The bands where one or more TnI isoform were detected are numbered in red font.

the NH_2-terminus. For example, RTcTnI and $ASssTnI_2$ have eleven aspartate/glutamate residues here while RTudTnI and ASfsTnI have only six within the same region. Each of these residues adds an additional charge to the protein. This can have a significant influence on tertiary structure and protein/protein interactions. In fact, the insertion of a glutamate or aspartate via mutation is used to mimic the effect of protein phosphorylation on troponin function (27, 32). The end result of the additional glutamate and aspartate residues in the NH_2-terminus could, therefore, be differences in the Ca^{2+} sensitivity of force generation. There are also two putative phosphorylation targets in the NH_2-terminus of the TnI isoforms at residues that correspond to 13 and 15 in $ASssTnI_1$ (Fig. 1). These are present in all trout isoforms except for $AScTnI_2$ where there is a single phosphorylatable target (Fig. 1). The phosphorylation of the corresponding residues in mouse cTnI (Ser 42 and Ser 44) causes a decrease in maximum tension generation and in maximal MgATPase activity (25).

There are other potentially functionally important sequence differences between isoforms at residues that correspond to 114, 134, and 136 in $AScTnI_2$ (Fig. 1). In cTnI from eutherian mammals, the residue that corresponds to residue 114 in $AScTnI_2$ is a target for PKC phosphorylation following α-adrenergic stimulation. Of the seven isoforms found in the trout, only RTcTnI and ASfsTnI contain phosphorylatable residues at the corresponding location (Fig. 1). The phosphorylation of the corresponding residue in mouse cTnI (Tyr^{144}) reduces the Ca^{2+} sensitivity of the contractile reaction (3). When this functional change is coupled with an increase in the rate and levels of Ca^{2+} cycling through the cell caused by the phosphorylation of the Ca^{2+} handling proteins, the end result is an increase in the rate and strength of cardiac contraction. The presence of a TnI isoform that is phosphorylatable at this residue in both fast skeletal and cardiac muscle may make these tissues more responsive to α-adrenergic stimulation. Experiments testing this possibility are required. Residues at positions that correspond to 134 and 136 in $ASssTnI_1$ have been demonstrated to regulate the Ca^{2+} sensitivity of the contractile reaction in mouse ssTnI and cTnI (36). We have suggested that the presence of His and Val at positions that correspond to 134 and 136, respectively, in ASfsTnI is the ancestral state and that Ala, Glu, at the same positions in cTnI from all eutherian mammals is the derived state (29). Interestingly, $AScTnI_1$ contains Ala and Asp at these positions and $ASssTnI_2$ and $AScTnI_2$ contain a Glu at the residue that corresponds to 136. Westfall and Metzger (36) have shown that the insertion of either Ala or Glu in mouse ssTnI at positions that correspond to 134 and 136, respectively, reduce the Ca^{2+} sensitivity of myocyte contraction.

The variation in sequence between the seven TnI isoforms found in the different muscle types of the trout have the potential therefore to influence the Ca^{2+} activation of the contractile reaction. Changes in the expression of these could be responsible, at least in part, for the increase in the rate of cardiac AM Mg^{2+}-ATPase we have previously found in cold-acclimated trout over a range of Ca^{2+} concentrations (17). In addition, the ability of fast and slow muscle to each express multiple TnI transcripts may enable different groups of skeletal muscle within the same animal (bodywall vs. caudal fin) to have unique functional characteristics. This would be the result of differential expression. As we sampled fast and slow muscle from the same region of each fish in the current study, further work is therefore required to test this idea.

Perspectives and Significance

The results of this study indicate that up to seven different TnI transcripts can be expressed in each of the striated muscle types of rainbow trout. No other vertebrate has been found to express this many TnI transcripts in any muscle. Because of the influence of TnI on contractile function, this compliment of isoforms has significant potential to manipulate phenotypic plasticity of the different muscle types. Changes in the expression of these multiple isoforms may be partially responsible for the functional changes in cardiac AM Mg^{2+}-ATPase previously characterized in rainbow trout following cold acclimation. Characterizing changes in the absolute protein isoform expression with thermal acclimation will aid in understanding this remodelling response. Determining how the sequence differences between the different TnI isoforms impact the Ca^{2+} activation of the trout Tn complex and whether or not this translates into a change in muscle function is the next objective of this work.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.L.A., J.M.K., C.A.D., and T.E.G. conception and design of research; S.L.A., J.M.K., and C.A.D. performed experiments; S.L.A., J.M.K., and T.E.G. analyzed data; S.L.A., J.M.K., and T.E.G. interpreted results of experiments; S.L.A., J.M.K., and T.E.G. prepared figures; S.L.A., J.M.K., C.A.D., and T.E.G. drafted manuscript; S.L.A., J.M.K., and T.E.G. edited and revised manuscript; S.L.A., J.M.K., and T.E.G. approved final version of manuscript.

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