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Troponin T expression in trout red muscle correlates with muscle activation

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Summary

Red or aerobic muscle from the anterior of rainbow trout *Oncorhynchus mykiss* activates (generates force) more quickly than that from the posterior. TnT is a component of the troponin complex that modulates muscle activation once Ca^{2+} is bound. Since trout express at least two forms of TnT in their red muscle (S1 and S2), the differential expression of these two forms was predicted to explain variations in contractile properties. TnT isoforms from trout muscle were identified through hydroxyapatite chromatography of purified myofibrillar proteins followed by SDS-PAGE. Western blots employing a mammalian anti-troponin T monoclonal antibody were used to identify TnT isoforms. The relative expression of the two isoforms of TnT was then examined at seven longitudinal positions from each of three fish using SDS-PAGE and densitometry on the silver-stained TnT bands. A significant shift in expression was detected from anterior to posterior in all three fish with TnT S1 becoming more dominant in the posterior red muscle. As predicted, a shift in TnT expression was associated with the decrease in activation rate along the length of the fish.

This study was then extended to include a different species of salmonid, brook trout *Salvelinus fontinalis*, to explore the generality of TnT modulation of muscle activation. Muscle contractile properties were determined from anterior and posterior muscle, and relative expression of S1 and S2 was determined. Unlike rainbow trout, there is no consistent longitudinal pattern of muscle activation in brook trout: some fish have kinetically faster muscle in the anterior, some in the posterior. Similarly, there is no consistent pattern of TnT expression. Individual analysis of muscle activation and TnT expression in brook trout provides insight into the role of TnT in modulating muscle activation in slow fish muscle.

Key words: troponin T, rainbow trout, *Oncorhynchus mykiss*, brook trout, *Salvelinus fontinalis,* muscle activation.

Introduction

The fish myotome is composed of both aerobic and anaerobic muscle. The aerobic or red muscle is used to power slow, steady swimming (Rome et al., 1984). Fishes show a variety of longitudinal or rostral-caudal patterns of variation in the contractile properties and physiological performance of their red muscle. These patterns are associated with differences in body bending kinematics (Coughlin, 2002).

Fish vary in terms of degree of body bending during swimming, from stiff-bodied thunniform and carangiform swimmers to animals that display higher degrees of body curvature along their entire length, such as anguilliform and salmoniform swimmers. Furthermore, these categories of swimmers are loosely associated with different anterior-posterior patterns of muscle contraction kinetics (Coughlin, 2002). For instance, the relatively stiff-bodied scup *Stenotomus chrysops* shows limited variation in muscle kinetics, with only relaxation rate differing from front to back (Rome et al., 1993). Alternatively, rainbow trout (F. Salmonidae, *Oncorhynchus mykiss* Walbaum), which swim with greater body curvature, display a greater level of variation in contractile properties of their red muscle (Fig. 1). In rainbow trout, both relaxation rate and activation rate varies from anterior to posterior (Coughlin, 2000).

This study examines the molecular correlates of longitudinal variation in the contractile properties of the red muscle in fishes. The specific question is, what is the molecular basis for variations in activation rate along the length of the fish? One molecular candidate is the muscle protein troponin T (TnT), because differences in Ca^{2+} sensitivity in isoforms of troponin T result in variations in activation time (Schachat et al., 1987; Fitzhugh and Marden, 1997). An additional line of evidence that TnT is a powerful regulator of muscle activation comes from examination of human muscle diseases such as cardiomyopathy. Mutations in TnT affect Ca^{2+} sensitivity of muscle, leading to certain forms of hypertrophic cardiomyopathy (e.g. Sweeney et al., 1998; Robinson et al., 2002).

The troponin complex consists of three subunits: troponin I (TnI) binds to actin and inhibits the actin-myosin interaction; troponin C (TnC) binds calcium ions and regulates TnI activity

and troponin T (TnT) connects the troponin complex to tropomyosin (Tm). The binding of Ca^{2+} by TnC enhances the bonding of TnC to both TnI and TnT and weakens the TnI-actin interaction (Gordon et al., 2000). This leads to a conformational change in the troponin complex that is transmitted by TnT to the tropomyosin. Changes in tropomyosin position on the actin filament open up myosin binding sites on the actin and, thereby, initiate contraction. TnT has several roles in Ca^{2+} regulation of muscle activity through its complex interactions with Tm, TnI, TnC and actin (Gordon et al., 2000).

The relatively high number of isoforms of TnT (e.g. Breitbart et al., 1985; Smillie et al., 1988; Berchtold et al., 2000) suggest a wide range of contractile properties, including variations in Ca^{2+} sensitivity and rates of activation of the myosin ATPase (Gordon et al., 2000). Rostral-caudal variations in troponin T expression that correlate with variation in activation rate of the anaerobic or white muscle in fishes have been described in cod (Thys et al., 1998) and largemouth bass (Thys et al., 2001). The possible contribution of TnT to the regulation in rainbow trout red muscle activation is suggested by the identification of two isoforms of troponin T in the red muscle (S1 and S2) (Waddleton et al., 1999). Differential expression of these isoforms along the length of the fish could lead to variation in activation time between the anterior and posterior muscle.

In the first part of this study, we test the hypothesis that the longitudinal shift in activation kinetics in rainbow trout aerobic muscle is a function of a shift in TnT isoform expression. Having found a correlation between activation time and TnT expression in rainbow trout, the second part of this study aimed to examine TnT and contraction kinetics in a second species of salmonid, brook trout or charr (F. Salmonidae, *Salvelinus fontinalis* Mitchill). For this species of trout, we measured contractile properties from anterior and posterior red muscle and we examined TnT expression in muscle samples from the same fish. The paired-sample nature of the physiological data and the analysis of protein expression in the sample fish permitted a rigorous examination of connections between muscle activation and troponin T.

Materials and methods

Rainbow trout *Oncorhynchus mykiss* Walbaum and brook trout *Salvelinus fontinalis* Mitchill were obtained from the Huntsdale Fish Culture Station of the Fish and Boat Commission of the Commonwealth of Pennsylvania, Carlisle, PA, USA. The fish were maintained at 10°C in a re-circulating aquarium system and fed a diet of pelleted trout food (Ziegler Trout Grower). All fish used were 18–24 months of age. All handling of experimental animals was reviewed by the Widener University Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Rainbow trout protein analysis

To identify the troponin T isoforms from rainbow trout red muscle, red muscle samples were dissected from three body positions of two adult rainbow trout (total length *TL*=28.5 cm and 23.7 cm). Purified myofibrillar proteins were separated using hydroxy-apatite chromatography, as described by Thys et al. (1998, 2001). The column fractions (ranging from 5 to 200 mmol l^{-1} phosphate) were examined using protein electrophoresis (SDS–PAGE). Protein concentration was determined using a detergent-compatible protein assay (DC Protein Assay; Bio-Rad; Hercules, CA, USA). PAGE samples were made from the column fractions to a final protein concentration of 0.025 mg ml^{-1} in Laemmli buffer. PAGE gels (Bio-Rad Precast Tris-HCl Ready Gels: 16 cm×16 cm× 0.15 cm; 4% stacking gel and 12% resolving gel) were loaded with 50 µl of sample and run for 30 min at 16 mA and then for 3–4 h at 24 mA, all at 4°C in a Tris–glycine–SDS running buffer. Half of each gel was silver stained, and the other half was used for western blot analysis. The sample buffer, running buffer, silver stain kit and electrophoresis cell were supplied by Bio-Rad. Immunoblotting of PAGE gels was carried using a Bio-Rad semi-dry blot transfer system (TransBlot SD), PVDF membrane, Bio-Rad colorimetric blot analysis (GAM-AP) and monoclonal anti-mouse troponin antibody (Sigma, T-6277; St Louis, MO, USA). After pre-incubation in Towbin buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 20% methanol. pH 8.3), blot transfer was achieved using 30 V for 15 min. The membrane was blocked using 3% gelatin in Tris-buffered saline (TBS). Primary antibody was applied in a 1:1000 dilution in 1.5% gelatin in TBS. The membrane was shaken for 1 h, placed in the refrigerator overnight and then shaken for 1 h the next day before continuing with secondary antibody. The secondary antibody (goat anti-mouse; Sigma A-3688) was also diluted 1:1000 in 1.5% gelatin in TBS and was applied for 1 h. Subsequent colour development of the membrane took approximately 1 h. Gels and blots were digitally photographed, and the size of TnT isoforms were estimated using Kodak 1-D Gel Analysis software.

After two isoforms of troponin T had been reliably identified based on size, relative expression patterns of the two forms could be studied. Red muscle samples were dissected from seven body positions (25% to 85% of *TL*) along the length of three fish [*TL*=27.1±2.7 cm, mass=215±22 g (mean ± S.D.)]. Each muscle sample was taken bilaterally from a 10% strip of the fish's length. For instance, the '35%' sample was taken from 30–40% of *TL*. Myofibril isolation was carried out following the method of Lutz et al. (1998), as adapted from Talmadge and Roy (1993). Gels were run as described above, but only silver stain was used. Densitometry (Kodak 1-D Gel Analysis) was used to quantify relative expression of the two isoforms of TnT.

Brook trout muscle physiology

A total of ten brook trout were used for the examination of contractile properties and TnT expression (*TL*=26.5±2.14 cm,

mass=211.2±51.7 g). One of those fish (*TL*=28.8 cm, mass=194.5 g) was also used to identify TnT isoforms. In all fish, contractile properties of the anterior and posterior muscle were measured, as well as relative expression of TnT isoforms in muscle samples from both body positions.

For muscle mechanics experiments, brook trout were killed by spinal transection and pithing. After removing the scales, ~1.0 mm wide strips of red muscle were extracted from just above and below the lateral line of the fish. Muscle preparations were dissected from two longitudinal positions: anterior (ANT, 25–45% of *TL*) and posterior (POST, 65–85% of *TL*). For a few of the fish, muscle from the middle (MID, 45-65% of *TL*) position was also examined. Subsequent dissection was carried out at 4°C with the use of a stereomicroscope in the presence of physiological saline (Altringham and Johnston, 1990). Live bundles were the length of one myomere (~2.5–4 mm) with a muscle fibre cross-sectional area of ~0.25 mm^2. The bundles were tied into a muscle mechanics system comprising a servomotor (Cambridge Technology 300S; Cambridge, MA, USA) and a force transducer (Aurora Scientific 404A; Aurora, Ontario, Canada). Temperature in the apparatus was maintained at 10°C for all experiments; the physiological saline was aerated gently to supply oxygen and to induce circulation. Experimental control and data collection were carried out using a PC, Keithley-Metrabyte DAS-1601 input/output board and custom software.

For each bundle, activation conditions (muscle length, pulse length and amplitude for twitch contractions, stimulus duration and frequency tetanic contractions) were optimised to generate maximal tetanic force. Typical stimulations for tetanus were: stimulus duration of 200–250 ms composed of 2–3 ms pulses at a frequency of 125 Hz. Each pulse had an amplitude of 7–9 V. Isometric force was converted to stress after live fibre area of the muscle bundles was determined. For tetanic contractions, time of activation was defined as the time from 10–90% of maximum isometric stress, and time of relaxation was the time from 90–10% of peak isometric stress. For a limited number of fish (*N*=5), maximum shortening velocity (V_{max}) was also determined for anterior and posterior muscle as previously described (Coughlin et al., 2001).

The fibre area of the live muscle bundles was determined at the end of each experiment (Coughlin, 2000). Bundles were stained with Trypan Blue to identify dead tissue, embedded in gelatin and frozen with liquid nitrogen. The frozen bundles were sectioned at 16 µm and stained with succinic dehydrogenase (SDH) for mitochondrial content. Darkly stained areas indicated aerobic muscle fibres. The cross section of live muscle fibres within the experimental muscle bundles could be determined by excluding both dead fibres and connective tissue. This cross-sectional area was used to calculate isometric tension for twitch and tetanic contractions.

Brook trout protein analysis

Protein analysis on brook trout followed the same methods as for rainbow trout. PAGE along with hydroxy-apatite chromatography was used to identify the apparent size of two TnT isoforms. The relative expression of those isoforms was quantified using PAGE and densitometry for three body positions: ANT, POST and MID (45–65% *TL*) for the same ten fish used in the physiology experiments. In these experiments, silver staining was used to determine the sizes of TnT isoforms in brook trout, while Sypro Ruby (BioRad) was used to assess expression.

Results

Rainbow trout TnT expression

Two isoforms of troponin T were identified in the red or aerobic muscle of rainbow trout (Fig. 2A). The molecular masses estimated from the silver-stained gels (Fig. 2B) were 26.7 kDa for TnT S1 and 25.4 kDa for TnT S2. Troponin was found in the highest concentration in the 75 mmol l^{-1} phosphate concentration hydoxyapatite column fraction (data not shown). Analysis of expression along the length of trout revealed a shift in the relative amount of each TnT isoform. In the anterior red myotome, both forms of slow TnT are found, although TnT S1 dominates. In the posterior myotome, TnT S1 constitutes nearly 100% of the TnT (Figs 3 and 4). This shift is statistically significant (Kruskal–Wallace test, H=17.2, P<0.01). The

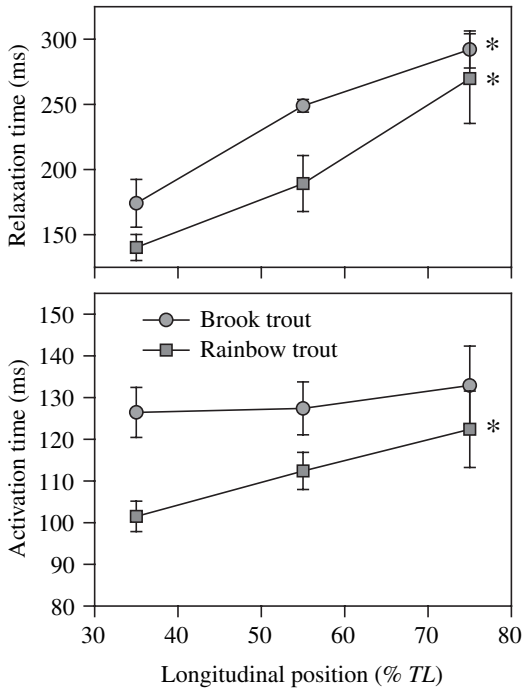

Fig. 1. Contraction kinetics of red muscle in rainbow and brook trout. For rainbow trout, activation and relaxation time increased significantly from anterior to posterior (data are from Coughlin, 2000). For brook trout, new data on muscle activation and relaxation are plotted for three body positions (*N*=10 for anterior and posterior, *N*=3 for middle). Brook trout show a significant increase in relaxation time from anterior to posterior, but there is no difference in activation rate along the length of these fish. Asterisks indicate statistically significant variation along the length of the fish (P<0.05).

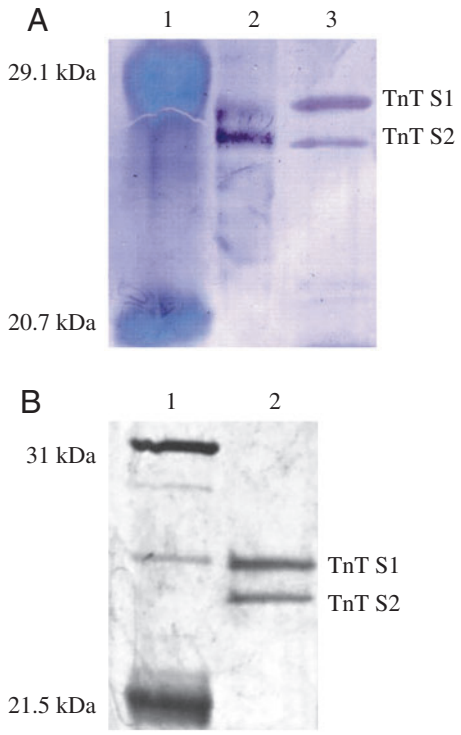

Fig. 2. (A) Identification of troponin T (TnT) S1 and S2 isoforms in rainbow trout using western blotting. Lane 1 is a pre-stained ladder of protein standards (BioRad), and lane 2 is authentic mammalian troponin used as a control (Sigma T-3515). Lane 3 is the 75 mmol l^{-1} phosphate hydroxy-apatite column fraction that contains two isoforms of purified rainbow trout red muscle TnT, identified as TnT S1 and S2. (B) Determination of sizes of TnT isoforms using a silver-stained PAGE gel. Lane 1 is a protein ladder (BioRad), and lane 2 is the purified TnT.

statistical variation in TnT expression correlates with previous research showing a statistically significant longitudinal variation in rate of muscle activation (Coughlin, 2000).

Brook trout contraction kinetics

Red muscle bundles from the two body positions of brook trout did not differ in terms of force production, ratio of twitch force to tetanic force production or V_{max} (Table 1). Brook trout red muscle displays a longitudinal shift in muscle relaxation, but no shift in muscle activation (Fig. 1). The posterior red muscle displayed significantly longer relaxation times than the anterior red muscle (Paired sample *t*-test, t=2.90, df=9, P=0.018). All ten fish showed this same pattern (see below). However, there was no rostral-caudal shift in muscle activation of brook trout red muscle (Paired sample *t*-test, t=0.35, d.f.=9, P=0.73). Furthermore, some fish showed a rostral-caudal increase in activation time, as observed in rainbow trout, and others displayed a rostral-caudal decrease in activation time.

Brook trout TnT expression

The molecular mass of brook trout TnT estimated from the silver-stained gels were 29.2 kDa for TnT S1 and 28.0 kDa for TnT S2 (Fig. 5A). As with rainbow trout, troponin was found in the highest concentration in the 75 mmol l^{-1} phosphate concentration hydoxy-apatite column fraction (data not shown). Brook trout display no consistent rostral-caudal pattern of the relative expression of the two isoforms of TnT. Some fish show a shift of increasing relative expression of TnT S2 (Fig. 5C), while others show the opposite pattern. Overall, there was no significant relationship between body position and the relative expression of TnT S1 and S2 (Kruskal–Wallis test, H=0.258, P>0.05; Fig. 6).

Individual examination of TnT expression and muscle activation revealed intra-specific variation. All ten brook trout displayed the same pattern of increasing relaxation time from anterior to posterior (Fig. 7C). However, only six showed a rostral-caudal increase in activation time, while four showed a decrease (Fig. 7B). For eight of the ten fish, there is agreement in the predicted relationship of TnT isoform expression and activation time. Four of those fish showed the pattern observed in rainbow trout: slower activation in the posterior is associated

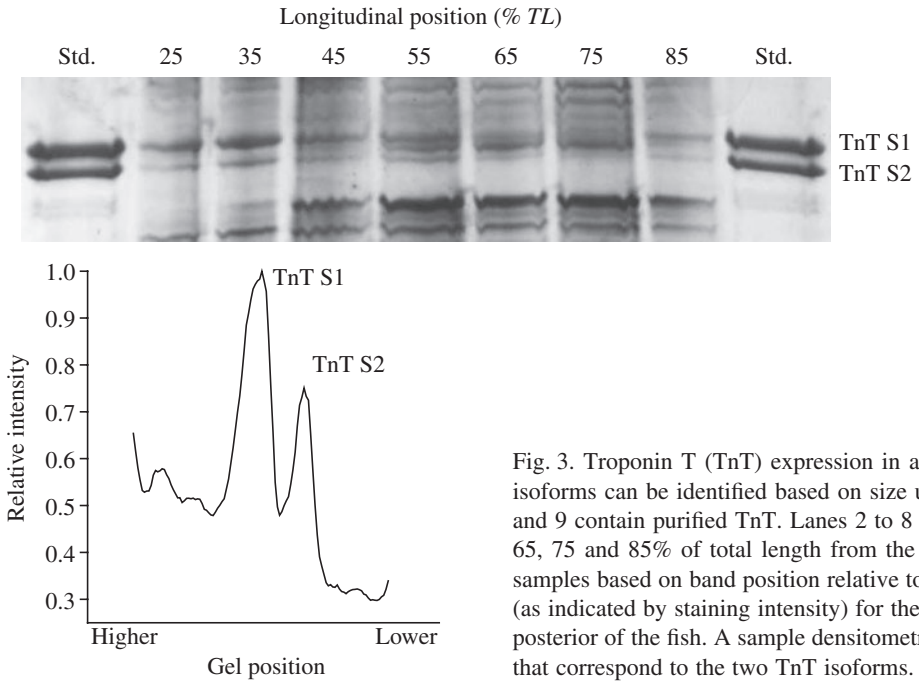

Fig. 3. Troponin T (TnT) expression in a representative rainbow trout. Red muscle TnT isoforms can be identified based on size using a silver-stained PAGE gel (Top). Lanes 1 and 9 contain purified TnT. Lanes 2 to 8 contain purified myofibrils from 25, 35, 45, 55, 65, 75 and 85% of total length from the snout. TnT isoforms can be identified in these samples based on band position relative to standards (Std.). The relative expression levels (as indicated by staining intensity) for the lower band (TnT S2) decrease from anterior to posterior of the fish. A sample densitometry profile is shown at the bottom with two peaks that correspond to the two TnT isoforms.

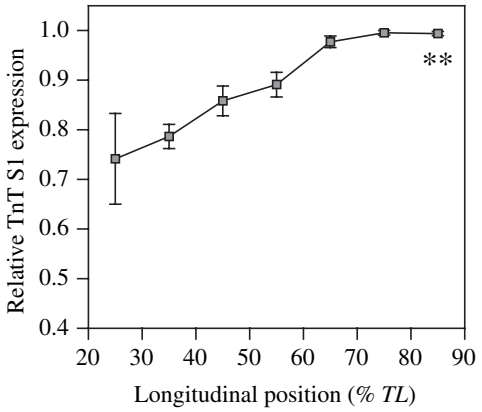

Fig. 4. Relative expression of troponin T (TnT) isoforms along the length of rainbow trout. There is a significant shift in the percentage expression of TnT S1 isoform relative to TnT S2 from anterior to posterior. The average expression of TnT S1 increases from ~75% in the anterior to 100% in the posterior myotome. Double asterisks indicate statistically significant variation along the length of the fish (P<0.01).

with higher relative expression of TnT S1 (e.g. brook trout 10, Fig. 7). Four other fish showed the opposite but equivalent pattern: faster activation in the posterior is associated with relatively higher expression of TnT S2 (e.g. brook trout 8, Fig. 7). For two fish, the physiological and protein expression results do not agree with patterns observed in rainbow trout (e.g. brook trout 9, Fig. 7). In these two fish, the anterior muscle had faster rates of activation but also had relatively higher expression of the TnT S1 isoform. The binomial probability of two or fewer mismatches out of ten is 0.055. This indicates that the results observed (8 out of 10 fish matching the prediction) would occur by chance in at most 5.5% of repetitions of this experiment.

When the activation time is plotted against the expression level of TnT S1 for each fish, the same eight fish show a positive correlation – higher levels of TnT S1 are associated with longer activation times (Fig. 8). The same two fish show the opposite pattern. An ANOVA, with activation time as the dependent variable, fish identity as a random effects independent variable and the expression level of TnT S1 as covariate, was carried out for all ten fish. The expression data, which are proportions, were log-transformed to compensate for the skewed nature of percentages. However, neither fish identity nor TnT expression level significantly affect activation time (for TnT S1 expression, F=2.110, P=0.172; for fish identity, F=1.781, P=0.174). Eighteen of 23 data points fall are clustered along a positive correlation between TnT S1 expression level and activation time (Fig. 8).

Discussion

Rainbow trout and brook trout

In rainbow and brook trout, troponin T appears to modulate muscle activation. In rainbow trout, a statistically significant increase in muscle activation time from the anterior to posterior red myotome is associated with a significant shift in troponin T expression. The relatively higher expression level of TnT S2 in the physiologically faster anterior muscle suggests that TnT S2 is the kinetically faster isoform of trout red muscle TnT. TnT S2 constitutes 15–25% of TnT in the physiologically faster anterior red muscle and 0% of the TnT in the physiologically slower posterior red muscle. This agrees with similar results observed in the white muscle of largemouth bass *Micropterus salmoides* (Thys et al., 2001). In trout red muscle and bass white muscle, the smaller sized isoform is associated with faster muscle activation.

In brook trout, there is no consistent shift in muscle activation, the mean value for muscle activation time is the same for both anterior and posterior red muscle. Similarly, there is no shift in TnT expression. However, the story is complicated by intra-specific variations in the patterns of muscle physiology and TnT expression in brook trout. Although mean values show no consistent longitudinal pattern of variation in activation time and TnT expression, individuals show a variety of patterns of activation and TnT expression. Some fish show a 30–40% increase in muscle activation time from anterior to posterior and a similar increase in the relative expression of TnT S1 in the same muscle (e.g. brook trout 10, Fig. 7). Others show >40% decrease in muscle activation time from anterior to posterior and

Table 1. *Contractile properties of brook trout red muscle*

Position	Maximum tentanic tension (kN m^{-2})	Ratio of twitch to tetanic tension	Maximum shortening velocity (V_{max}, muscle lengths s^{-1})
Anterior	171.0±22.1	0.41±0.04	2.01±0.51
Posterior	162.7±36.1	0.51±0.03	2.06±0.22
Paired sample *t*-test			
N	10	10	5
t	0.11	2.23	0.14
P	0.917	0.053	0.892
Power	<0.50	<0.50	<0.50

Maximum tension, twitch tension and shortening velocity do not vary between anterior and posterior red muscle in these fish, although power is low for all three comparisons.

N=10 means that anterior and posterior muscle samples were used from each of 10 fish.

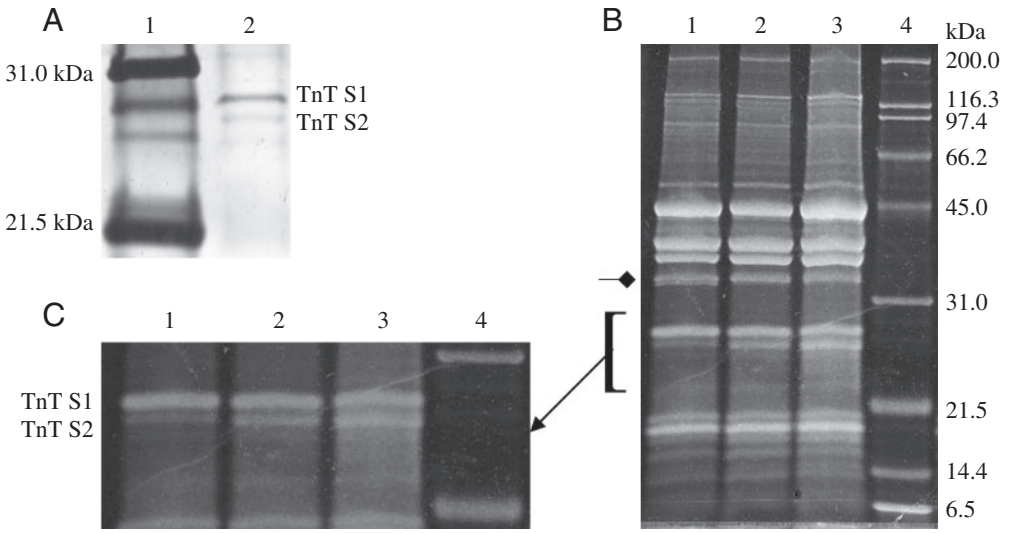

Fig. 5. (A) Identification of troponin T (TnT) S1 and S2 isoforms in brook trout based on hydroxy-apatite chromatography and a silver-stained PAGE gel. Lane 1 is a ladder of protein standards (BioRad), and lane 2 is the 75 mmol l^{-1} phosphate hydroxy-apatite column fraction that contains two isoforms of purified rainbow trout red muscle TnT, identified as TnT S1 and S2. (B) Myofibrillar proteins in a representative brook trout. Partially purified myofibrillar proteins ranging in size from myosin heavy chain at the top of the gel (largest protein visible) to putative parvalbumin at the bottom of the gel (smallest protein visible). Lanes 1–3 contain purified myofibrils from 35, 55 and 75% of total length from the snout. Lane 4 contains a ladder of protein standards. There is some indication of longitudinal variation in a protein at 33–34 kDa (tropomyosin?, see diamond-head arrow). At the anterior position (35% *TL*) there is a doublet, but the middle and posterior positions show only a single band. No other obvious longitudinal variations are observed here or in other gels except for variations in TnT. (C) TnT expression in a representative brook trout. Magnification of the bands from B. Red muscle TnT isoforms were identified based on size using a Sypro Ruby-stained PAGE gel. The two visible bands are the brook trout red muscle TnT isoforms. In this individual, relative expression levels (as indicated by staining intensity) for the lower band (TnT 2S) increase from anterior to posterior of the fish.

a similar decrease in the relative expression of TnT S1 in the same muscle (e.g. brook trout 8, Fig. 7).

Two of the eight fish show a distinctly different relationship between TnT S1 expression and activation time, with a negative correlation between TnT S1 and activation time (e.g. brook trout 1 and 9, Fig. 8). We cannot explain this observation with our current hypothesis but can only suggest that the variation in expression in various other muscle proteins may underlie observed variations in muscle contractile properties. Our laboratory is currently examining other muscle proteins in rainbow and brook trout, including myosin light chain 2 and parvalbumin, to assess intra-individual, intra-specific and inter-specific variations in these proteins.

Besides variations in longitudinal patterns of muscle activation and TnT expression, brook and rainbow trout display other rostral-caudal variations in muscle properties. At least for the hatchery strains of fish examined in this and previous research in this laboratory, there are inter-specific differences in muscle recruitment. At low and moderate steady swimming speeds, rainbow trout recruit primarily their posterior red myotome, while brook trout recruit their entire myotome (anterior to posterior) with an even level of recruitment intensity (Coughlin et al., 2004). Differences in muscle recruitment correlated with another inter-specific variation in trout muscle. During steady swimming, rainbow trout generate significantly more power in their posterior myotome than in the anterior (Coughlin, 2000), which correlates with the preferential recruitment of muscle that produces greater mechanical power at lower swimming speeds when not all of the red myotome is needed (Coughlin et al., 2004). Alternatively, brook trout display no longitudinal variation in red muscle power production during steady swimming (McGlinchey et al., 2001) and correspondingly display no longitudinal variation in muscle recruitment. The addition of inter-specific variation in longitudinal patterns of red muscle protein expression is perhaps expected, as some underlying variation in muscle composition (i.e. form) should be predicted to underlie variations in muscle function.

Troponin T

A number of studies have demonstrated a role for troponin T in the modulation of muscle activation, particularly in fishes. Cod *Gadus morhua* show longitudinal variation in the

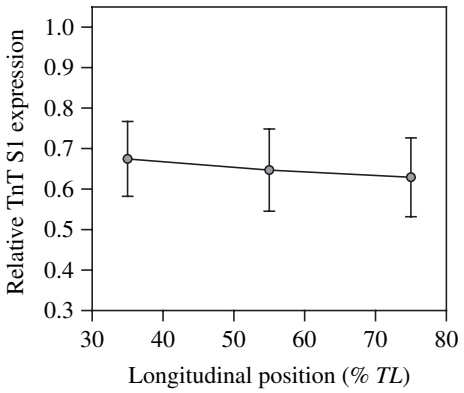

Fig. 6. Relative expression of troponin T (TnT) isoforms along the length of brook trout. There is no significant variation in the percentage expression of TnT S1 isoform relative to TnT S2 from anterior to posterior. The mean average expression of TnT S1 is ~65% at all body positions.

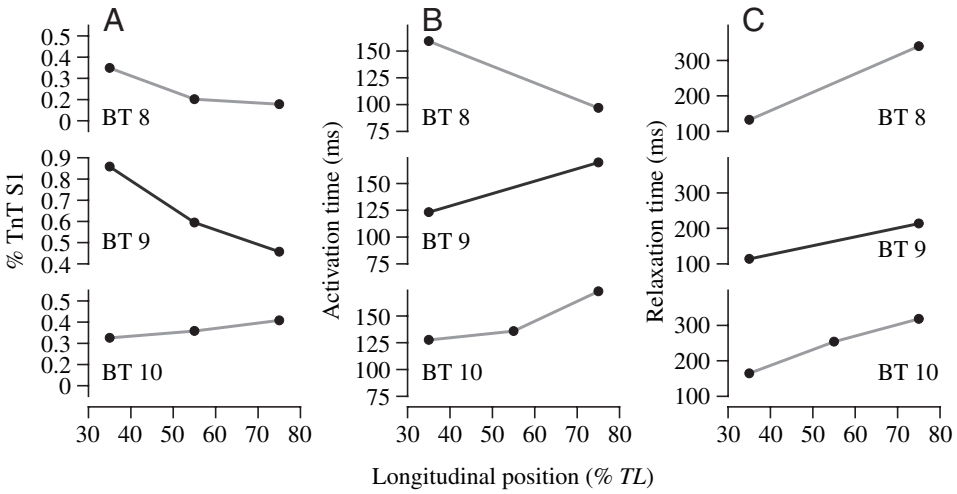

Fig. 7. Individual analysis of brook trout (BT) troponin T (TnT) expression (A), muscle activation (B) and muscle relaxation (C). Three representative analyses are shown, but of the ten fish analysed, six showed a decrease in the relative TnT S1 expression from anterior to posterior (e.g. BT 8 and BT 9). The other four fish showed an increase (e.g. BT 10). For eight of the fish, the pattern of variation in TnT S1 agrees with the longitudinal pattern of muscle activation (e.g. BT 8 and BT 10). All fish showed the identical pattern of increasing muscle relaxation time from anterior to posterior.

activation of their white muscle (Davies et al., 1995). Thys et al. (1998) demonstrated that this shift in muscle contractile properties correlates with a longitudinal pattern of expression of two isoforms of TnT. Similarly, Thys et al. (2001) showed a significant rostral–caudal shift in both muscle activation and TnT expression in the white muscle of largemouth bass. In both cod and bass, the anterior muscle is kinetically faster and expresses relatively greater amounts of an isoform of TnT that migrates faster on PAGE gels. (TnT-2; Thys et al., 1998, 2002). For both white (cod and bass) and red (rainbow and brook trout) muscle in fishes, the kinetically faster isoform of TnT appears to be the physically smaller isoform found in each type of muscle. Other fish, such as saithe *Pollachius virens* (Altringham et al., 1993), show rostral-caudal variation in the activation time of swimming muscle and can be predicted to show variations in troponin T isoforms.

The present study and those cited above for white muscle do not definitively establish the role of TnT in modulating muscle activation in fishes, but strong evidence exists in the literature that variations in TnT isoform do affect contractile properties of muscle (Schachat et al., 1987; Fitzhugh and Marden, 1997). Several studies from the laboratory of James Marden have elucidated the effects of subtle shifts in the relative expression TnT isoforms in dragonfly *Libellula pulchella* flight muscle on Ca^{2+} sensitivity and flight performance (Fitzhugh and Marden, 1997; Marden et al., 1999, 2001). Up to six TnT isoforms, identified by Marden et al. (1999) as splice variants, are found in the flight muscle of dragonflies. Differences between individuals in terms of the relative expression of these isoforms leads to an order of magnitude variation in Ca^{2+} sensitivity in skinned muscle fibres and significant variation in the maximum oscillatory power output of muscle (Marden et al., 2001). Furthermore, there are developmental shifts in TnT isoform expression that correlate with changes in flight performance between newly emerged adult and mature adult dragonflies (Fitzhugh and Marden, 1997), suggesting that TnT expression has a direct relationship to behavioural performance.

Other animals show developmental shifts in TnT expression that may also correlate with muscle function. For instance, there is a developmental shift in TnT expression from an embryonic to adult form in rat hearts (Jin and Lin, 1988). In rainbow trout and brook trout, variations in TnT may well affect performance, but that has not yet been clearly established. For a start, as mentioned above, previous research has shown longitudinal variation in oscillatory power output during swimming in rainbow trout (Coughlin, 2000) but not brook trout (McGlinchey et al., 2001). In corresponding fashion, TnT expression varies along the length of rainbow trout but not brook trout, suggesting that TnT expression relates to function of muscle during swimming.

The present work cannot rule out that variation in TnT S1 merely correlates with muscle kinetics and that other muscle proteins are responsible for observed variations in muscle activation time in both rainbow and brook trout. A superficial examination of all the contractile proteins from these fish suggest

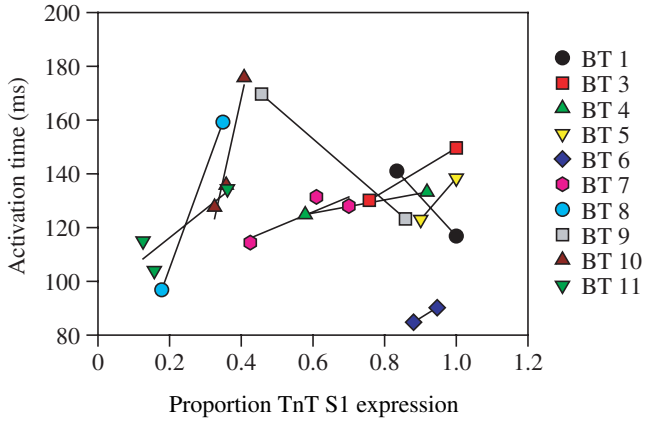

Fig. 8. Individual patterns of the relationship of troponin T (TnT) expression and activation time in brook trout (BT). Individual points are the proportion of TnT S1 and the activation time for 2–3 muscle samples per fish (ANT and POST or ANT, MID and POST). For each fish, a line of best fit is included to indicate the relationship of the two variables. For eight of the fish, the evident trend is a higher proportion of TnT S1 is associated with a longer time of activation (slower muscle). There were two exceptions (BT 1 and BT 9).

that other proteins do not show marked longitudinal patterns, although there may be some variation in another, unidentified protein (Fig. 5B). In addition, previous work using protein analysis has shown no longitudinal variation in myosin heavy chain along the length of the red muscle in trout (Coughlin et al., 2001). Current work in our lab using quantitative real-time PCR to examine developmental shifts in myosin light chain 2 expression have not shown any longitudinal patterns of expression of this regulatory light chain in rainbow trout. The absence of observations of longitudinal variation in other muscle proteins and prior research demonstrating the functional role of TnT in regulating activation in other species (e.g. dragonflies) taken together strongly suggest that the relative expression of TnT S1 and S2 in trout red muscle does have a regulatory effect on muscle activation.

Trout in the wild

A future direction of this work is the examination of protein expression in wild populations of rainbow and brook trout. The present work was carried out with hatchery strains raised under identical conditions at a trout hatchery operated by the Commonwealth of Pennsylvania. Little is know about the physiological properties and less about variations in muscle composition in wild populations in these fish species. Marden et al. (2001) reported 'broad intraspecific' variation in TnT expression and muscle contractile properties in flight muscle from two distinct populations of dragonflies, similar to the results reported here for the hatchery brook trout. Do wild populations of brook trout also show such intraspecific variation in TnT expression? Given that adult brook trout vary widely in size and body morphology in the extensive variety of natural habitats in which they are found (Karas, 1997), such a prediction can be readily made.

More important than simply finding variation in TnT expression is determining whether or not patterns of TnT expression affect muscle contractile properties. Is there a relationship between TnT expression and muscle activation in wild-run fish? Do differences between rainbow trout and brook trout described here and in previous studies in this lab (e.g. Coughlin, 2000; McGlinchey et al., 2001; Coughlin et al., 2004) have any bearing on fitness in the wild? To answer these questions will require a comparative study of diverse populations of trout using muscle mechanics experiments combined with analysis of TnT expression patterns to assess the protein correlates of variations in muscle contractile properties.

Conclusions

In this study, variations in troponin T expression was described for the first time in the red muscle of fish. Rainbow trout display a significant longitudinal shift in the relative expression of two isoforms of TnT, and this shift correlates with rostral-caudal differences in muscle activation. Another species of salmonid, brook trout, was shown to not display the same pattern of TnT expression and muscle activation. Instead, this species showed considerable intraspecific variation in the role of TnT in modulating muscle properties.

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References

Altringham, J. D. and Johnston, I. A. (1990). Scaling effects on muscle function: power output of isolated fish muscle fibres performing oscillatory work. *J. Exp. Biol.* **151**, 453-467.

Altringham, J. D., Wardle, C. S. and Smith, C. I. (1993). Myotomal muscle function at different locations in the body of a swimming fish. *J. Exp. Biol.* **182**, 191-206.

Berchtold, M. W., Brinkmeier, H. and Müntener, M. (2000). Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity and disease. *Physiol. Rev.* **80**, 1215-1265.

Breitbart, R. E., Nguyen, H. T., Medford, R. M., Destree, A. T., Mahdavi, V. and Nadal-Ginard, B. (1985). Intricate combinatorial patterns of exon splicing generate multiple regulated troponin T isoforms from a single gene. *Cell* **41**, 67-82.

Coughlin, D. J. (2000). Power production during steady swimming in largemouth bass and rainbow trout. *J. Exp. Biol.* **203**, 617-629.

Coughlin, D. J. (2002). Aerobic muscle function during steady swimming in fishes. *Fish Fisheries* **3**, 1-16.

Coughlin, D., Burdick J., Stauffer K. A. and Weaver, F. E. (2001). Rainbow trout display a developmental shift in red muscle kinetics, swimming kinematics and myosin heavy chain isoform. *J. Fish Biol.* **58**, 701-715.

Coughlin, D., Spiecker, A. and Schiavi, J. M. (2004). Red muscle recruitment during steady swimming correlates with rostral–caudal patterns of power production in trout. *Comp. Biochem. Physiol.* **137A**, 151-160.

Davies, M. L. F., Johnston, I. A. and Van de Wal, J. (1995). Muscle fibers in rostral and caudal myotomes of the Atlantic cod (*Gadus morhua* L.) have difference mechanical properties. *Physiol. Zool*. **68**, 673-697.

Fitzhugh G. H. and Marden, J. H. (1997). Maturational changes in troponin T expression, Ca^{2+}-sensitivity and twitch contraction kinetics in dragonfly flight muscle. *J. Exp. Biol.* **200**, 1473-1482.

Gordon, A. M., Homsher, E. and Regnier, M. (2000). Regulation of contraction in striated muscle. *Physiol. Rev.* **80**, 853-924.

Jin, J.-P. and Lin, J. J.-C. (1988). Rapid purification of mammalian cardiac troponin T and its isoform switching in rat hearts during development. *J. Biol. Chem.* **263**, 7309-7315.

Karas, N. (1997). *Brook Trout,* 371 pp. New York, NY, USA: The Lyons Press.

Lutz, G. J., Cuizon, D. B., Ryan, A. F. and Lieber, R. L. (1998). Four novel myosin heavy chain transcripts define a molecular basis for muscle fiber types in *Rana pipiens*. *J. Physiol.* **508.3**, 667-680.

Marden, J. H., Fitzhugh, G. H., Wolf, M. R. and Rowan, B. (1999). Alternative splicing, muscle calcium sensitivity, and the modulation of dragonfly flight performance. *Proc. Natl. Acad. Sci. USA* **96**, 15304-15309.

Marden, J. H., Fitzhugh, G. H., Girgenrath, M., Wolf, M. R. and Girgenrath, S. (2001). Alternative splicing, muscle contraction and intraspecific variation: associations between troponin T transcripts, Ca^{2+} sensitivity and the force and power output of dragonfly flight muscles during oscillatory contraction. *J. Exp. Biol.* **204**, 3457-3470.

McGlinchey, S. M., Saporetti, K. A., Forry, J. A., Pohronezny, J. A. and Coughlin, D. J. (2001). Red muscle function during steady swimming in brook trout, *Salvelinus fontinalis*. *Comp. Biochem. Physiol.* **129A**, 727-738.

Robinson, P., Mirza, M., Knott, A., Abdulrazzak, H., Willott, R., Marston, S., Watkins, H. and Redwood, C. (2002). Alterations in thin filament regulation induced by a human cardiac troponin T mutant that causes dilated cardiomyopathy are distinct from those induced by troponin T mutants that cause hypertropic cardiomyopathy. *J. Biol. Chem.* **277**, 40710-40716.

Rome, L. C., Loughna, P. T. and Goldspink, G. (1984). Muscle fiber recruitment as a function of swim speed and muscle temperature in carp. *Am. J. Physiol.* **247**, R270-R272.

Rome, L. C., Swank D. and Corda, D. (1993). How fish power swimming. *Science* **261,** 340-343.

Schachat, F. H., Diamond, M. S. and Brandt, P. W. (1987). Effect of different troponin T-tropomyosin combinations on this filament activation. *J. Mol. Biol.* **198**, 551-554.

Smillie, L. B., Golosinska, K. and Reinach, F. C. (1988). Sequences of complete cDNAs encoding four variants of chicken skeletal muscle troponin T. *J. Biol. Chem.* **263**, 18816-18820.

Sweeney, H. L., Feng, H. S., Yang, Z. and Watkins, H. (1998). Functional analyses of troponin T mutations that cause hypertrophic cardiomyopathy: Insights into disease pathogenesis and troponin function. *Proc. Natl. Acad. Sci. USA* **95**, 14406-14410.

Talmadge, R. J. and Roy, R. R. (1993). Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J. Appl. Physiol*. **75**, 2337-2340.

Thys, T. M., Blank, J. M. and Schachat, F. H. (1998). Rostral-caudal variation in troponin T and parvalbumin correlates with differences in relaxation rates of cod axial muscle. *J. Exp. Biol.* **201**, 2993-3001.

Thys, T. M., Blank, J. M., Coughlin, D. J. and Schachat, F. H. (2001). Longitudinal variation in muscle protein expression and contraction kinetics of largemouth bass axial muscle. *J. Exp. Biol.* **204**, 4249-4257

Waddleton, D. M., Jackman, D. M., Bieger, T. and Heeley, D. H. (1999). Characterisation of troponin-T from salmonid fish. *J. Musc. Res. Cell. Motil.* **20**, 315-324.