

Provided for non-commercial research and educational use only.  
Not for reproduction or distribution or commercial use.



This article was originally published in a journal published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues that you know, and providing a copy to your institution's administrator.

All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:

<http://www.elsevier.com/locate/permissionusematerial>

## Parvalbumin expression in trout swimming muscle correlates with relaxation rate

David J. Coughlin\*, Sonia Solomon, Jennifer L. Wilwert

Widener University, Department of Biology, One University Place, Chester, PA 19013, USA

Received 28 January 2007; received in revised form 11 March 2007; accepted 13 March 2007  
Available online 24 March 2007

### Abstract

Rainbow trout (*Oncorhynchus mykiss*) display longitudinal and developmental shifts in muscle relaxation rate. This study aimed to determine the role of variations in parvalbumin content in modulating muscle relaxation. Parvalbumin is a low molecular weight protein that buffers myoplasmic  $\text{Ca}^{2+}$  and enhances muscle relaxation. In some fish, longitudinal variations in muscle relaxation have been linked to variations in the total amount of parvalbumin present in muscle and in the relative expression of two parvalbumin isoforms. We have demonstrated previously that anterior slow-twitch or red myotomal muscle relaxes more rapidly than that from the posterior for both rainbow and brook trout. Further, younger rainbow trout parr have faster red muscle relaxation rates than older smolts. Here we report similar results for fast-twitch or white muscle. We quantified the parvalbumin expression in red and white muscle from different body positions of rainbow trout parr and smolts and for brook trout (*Salvelinus fontinalis*) adults. There was a significant shift in total parvalbumin content of muscle: the faster muscle from the anterior myotome contained greater amounts of parvalbumin. For brook trout, longitudinal variation in relaxation rate was also associated with shifts in the relative expression of the two parvalbumin isoforms. The faster muscle of parr contained more parvalbumin. Lastly, trout white muscle tended to have higher levels of parvalbumin and greater levels of the Parv2 (relative to Parv1) isoform as compared to red muscle. Parvalbumin expression correlated with muscle relaxation rate in trout, although there were species-specific differences in the importance of altering total parvalbumin content versus shifts in relative parvalbumin isoform expression.

© 2007 Elsevier Inc. All rights reserved.

**Keywords:** Rainbow trout; Parvalbumin; *Oncorhynchus mykiss*; Brook trout; *Salvelinus fontinalis*; SDS-PAGE; Protein analysis; Muscle relaxation

### 1. Introduction

Fish display longitudinal variations in their swimming musculature. These variations include shifts along the body in how the muscle is used to power swimming, in the mechanical properties of muscle and in the molecular make-up of the muscle. These longitudinal variations can also differ between the two principle muscle fiber types in the myotome: fast-twitch, anaerobic white muscle and slow-twitch, aerobic red muscle.

In red muscle, the posterior muscle produces more power for swimming than the anterior muscle in a number of fish species, including rainbow trout, scup and bass (Coughlin, 2002). There are anterior–posterior variations in how this muscle is recruited;

the posterior muscle is preferentially recruited at lower speeds in scup (Coughlin and Rome, 1999) and trout (Coughlin et al., 2004). Perhaps because of muscle activity conditions (e.g. muscle strain and muscle activation) that are disadvantageous for power production, anterior muscle typically has faster muscle contractile properties in these fishes (Coughlin, 2002). Although less is known about the white muscle's longitudinal patterns of power production and its recruitment during swimming, white muscle in at least some fishes also shows longitudinal shifts in contractile properties, with faster muscle in the anterior in cod (Davies et al., 1995) and bass (Thys et al., 2002).

The molecular basis for longitudinal variations in contractile properties has been examined for a number of fish species. For instance, in both red and white muscle, there appears to be a role for the myofibrillar protein troponin T in modulating muscle activation rate (Thys et al., 1998, 2002; Coughlin et al., 2005).

\* Corresponding author. Tel.: +1 610 499 4025; fax: +1 610 499 4496.  
E-mail address: [djcoughlin@widener.edu](mailto:djcoughlin@widener.edu) (D.J. Coughlin).

The focus of the present study is the molecular basis for variations in muscle relaxation rate in the swimming muscle. Wilwert et al. (2006) recently showed a role for the myoplasmic protein parvalbumin in modulating relaxation rate in red muscle in sheepshead, *Archosargus probatocephalus*, and kingfish, *Menticirrhus americanus*. In both of these species, the anterior red muscle displays faster rates of relaxation than the posterior muscle. The anterior muscle expresses higher levels of parvalbumin than the posterior. In addition, the anterior muscle expressed a relatively higher proportion of one of the two isoforms of parvalbumin found in the swimming muscle.

Parvalbumin is a low molecular mass protein (9–11 kDa) that binds free  $\text{Ca}^{2+}$ . In muscle, it competes with troponin C for  $\text{Ca}^{2+}$ , leading to a drop in myoplasmic  $[\text{Ca}^{2+}]$  and thereby enhancing relaxation from contraction (Berchtold et al., 2000). Total parvalbumin expression in fish muscle varies widely, from zero to >1.5 mM (Gillis, 1985). Higher amounts of parvalbumin are associated with faster rates of relaxation (Berchtold et al., 2000). Fish muscle also expresses multiple isoforms of parvalbumin. Sheepshead and kingfish express two isoforms in red and white muscle (and pink muscle in sheepshead) (Wilwert et al., 2006), and Huriaux et al. (1996) reported up to five isoforms of parvalbumin in trout muscle throughout development. These different isoforms of parvalbumin appear to differentially affect rates of muscle relaxation (Wilwert et al., 2006), presumably due to differences in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  binding characteristics (Erickson et al., 2005).

Trout display variations in muscle relaxation along their length, as has been reported for both rainbow trout (Coughlin et al., 2001) and brook trout (Coughlin et al., 2005). In addition, there is a developmental slowing of muscle relaxation during the parr–smolt transformation in rainbow trout (Coughlin et al., 2001). We hypothesized that since parvalbumin is a key component in the muscle calcium transport to the sarcoplasmic reticulum, variations in parvalbumin expression are the primary determinant of relaxation in fish swimming muscle. These variations include both total parvalbumin content of the muscle and the relative expression of the two isoforms of parvalbumin typically found in fish muscle.

## 2. Materials and methods

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), and brook trout, *Salvelinus fontinalis* (Mitchill), were obtained from the Huntsdale Fish Culture Station, Carlisle, PA, USA, of the Fish and Boat Commission of the Commonwealth of Pennsylvania. 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 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.

The main goal of this project was to compare muscle relaxation times to patterns of parvalbumin expression in the red and white myotomal swimming muscle of rainbow trout parr and smolt and brook trout adults. Red muscle contractile properties have been described previously for rainbow trout of both

developmental stages (Coughlin et al., 2001) and for brook trout adults (McGlinchey et al., 2001). In the present study, contractile properties of white muscle were determined for both fish species. For brook trout, the data for white muscle are from the same set of experiments in which red muscle contractile properties were previously characterized (Coughlin et al., 2005). For rainbow trout, data for white muscle were collected from three body positions of both parr and smolts. Total parvalbumin expression and the relative expression of two isoforms of parvalbumin were then quantified for red and white muscle of brook trout adults and both developmental stages of rainbow trout.

### 2.1. Physiology experiments

White muscle bundles of rainbow trout and brook trout were used to examine longitudinal patterns of contractile properties. The muscle bundles were extracted from a total of seven rainbow trout parr (Mean  $\pm$  SD, TL = 13.9  $\pm$  1.3 cm, Mass 26.6  $\pm$  8.0 g), six rainbow trout smolts (TL = 26.1  $\pm$  1.7 cm, Mass 180.4  $\pm$  38.7 g) and five brook trout (TL 26.6  $\pm$  1.8 cm, Mass 202.2  $\pm$  33.0 g). The rainbow trout smolts were similar in size to the brook trout used in this study.

To perform mechanics experiments, the fish were killed by spinal transection and pithing. The scales were removed and strips of white muscle (~1.0 mm wide) were removed from just above and below the lateral line of the fish. Muscle preparations were dissected from three longitudinal body positions: anterior (ANT, 35% TL); middle (MID, 55% TL); and posterior (POST, 75% TL). Subsequent dissection was carried out in physiological saline at 4 °C with the use of stereomicroscope (Coughlin et al., 2005). Live muscle bundles were the length of one myomere (4–5 mm) with a cross-sectional area of 0.25–1.0 mm<sup>2</sup>. The muscle mechanics system was comprised of a servomotor (Cambridge Technology 300 S) and a force transducer (Cambridge Technology 404A). The muscle bundles were tied into the system and maintained at a temperature of 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, a National Instruments input/output board and customized Lab-View software (National Instruments).

Activation conditions (muscle length, pulse length and amplitude for twitch contractions, stimulus duration and frequency for tetanic contractions) for each bundle were optimized to generate maximal tetanic force. The duration of the stimulus period was typically 150–200 ms and was composed of 2.0–2.5 ms pulses at a frequency of 200 Hz. Pulses were square waves with an amplitude of 7–9 V. For tetanic contractions, time of activation (TA) was defined as the time from 10–90% of maximum isometric stress. Time of relaxation (TR) was the time from 90–10% of peak isometric stress. Twitch time (TW 90) was defined as the time from stimulation to 90% recovery (10% of peak isometric stress) in twitch contractions.

At the end of each experiment, the cross-sectional area of the muscle bundle that contained live fibers was estimated based on the width and depth of bundles as measured in the muscle

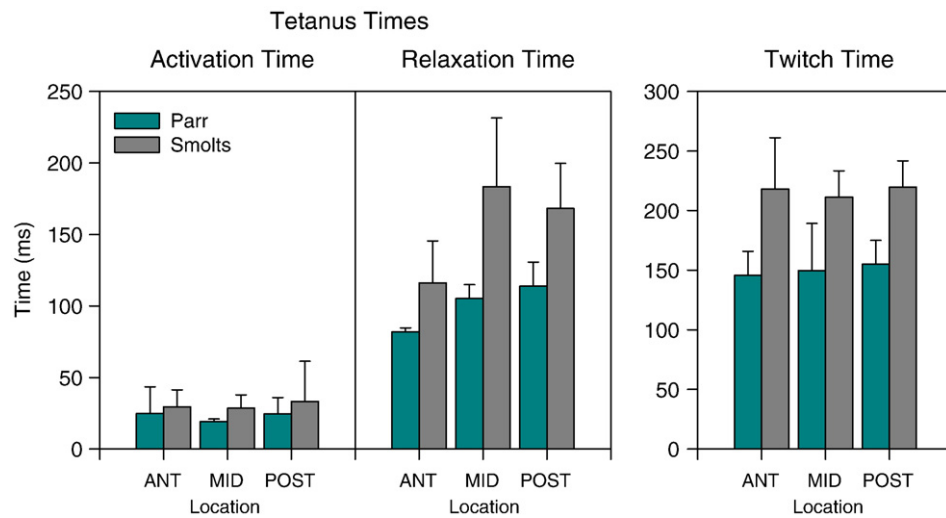


Fig. 1. Tetanic activation and relaxation time and twitch time for isometric contractions in rainbow trout white muscle. Definitions of variables and statistical results found in the text. Sample sizes were  $n=4$  to  $n=7$  for anterior and posterior body positions and  $n=3$  for the middle body position for each age of fish. The younger trout parr had faster contractile properties than older smolts. For relaxation time, the anterior muscle was faster than the posterior muscle.

mechanics apparatus. Live fiber area was estimated from bundle cross-section area by multiplying by 0.63, accounting for  $\sim 30\%$  dead fiber area and  $\sim 10\%$  connective tissue in white muscle. These relatively conservative factors are based on prior experience with histological analysis of muscle (e.g. Coughlin, 2000; Thys et al., 2002). Tension (force per unit area) calculated from measures of force production and the estimated live muscle bundle area ranged from  $100\text{--}150\text{ kN m}^{-2}$ . No additional analysis of force production between muscle samples was carried out.

## 2.2. Protein analysis

### 2.2.1. Parvalbumin identification

Prior to analysis of parvalbumin expression, parvalbumin isoforms were identified using SDS-PAGE and Western Blots.

Representative muscle samples were extracted from the white muscle of rainbow trout and brook trout. The muscle fibers were homogenized using a protocol adapted from Lutz et al. (1998) upon the advice of Dr. Fred Schachat, Duke University. Muscle samples were weighed, and a homogenization solution (250 mM sucrose, 100 mM KCl, 20 mM Tris-base, 5 mM EDTA, 1 mM PMSF, 10 ng/ $\mu\text{L}$  leupeptin, and 10 ng/ $\mu\text{L}$  pepstatin.) was added to the sample in a 1:1 volume/weight ratio. Homogenization was performed using 7.0 ml glass-in-glass grinders. Samples were spun at 12,000 g for 10 min. The parvalbumin-rich supernatant was removed. The supernatant was partially purified for parvalbumin by raising the temperature to  $95\text{ }^\circ\text{C}$  for 5 min, after which it was placed on ice and then centrifuged for 10 min at  $10,000\times g$ . The resulting supernatant contained parvalbumin and little other protein (F. Schachat, per. coms.). SDS-PAGE running samples were prepared using Tricine Buffer (BioRad).

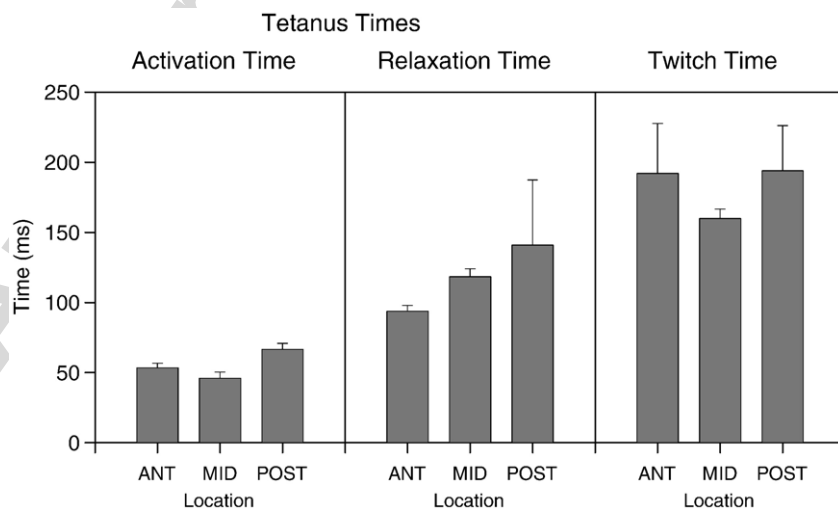


Fig. 2. Tetanic activation and relaxation time and twitch time for isometric contractions in brook trout white muscle. Definitions of variables and statistical results found in the text. Sample size was  $n=5$  for all body positions. Relaxation rate varied along the length of the fish, with the anterior displaying faster relaxation than the posterior muscle.

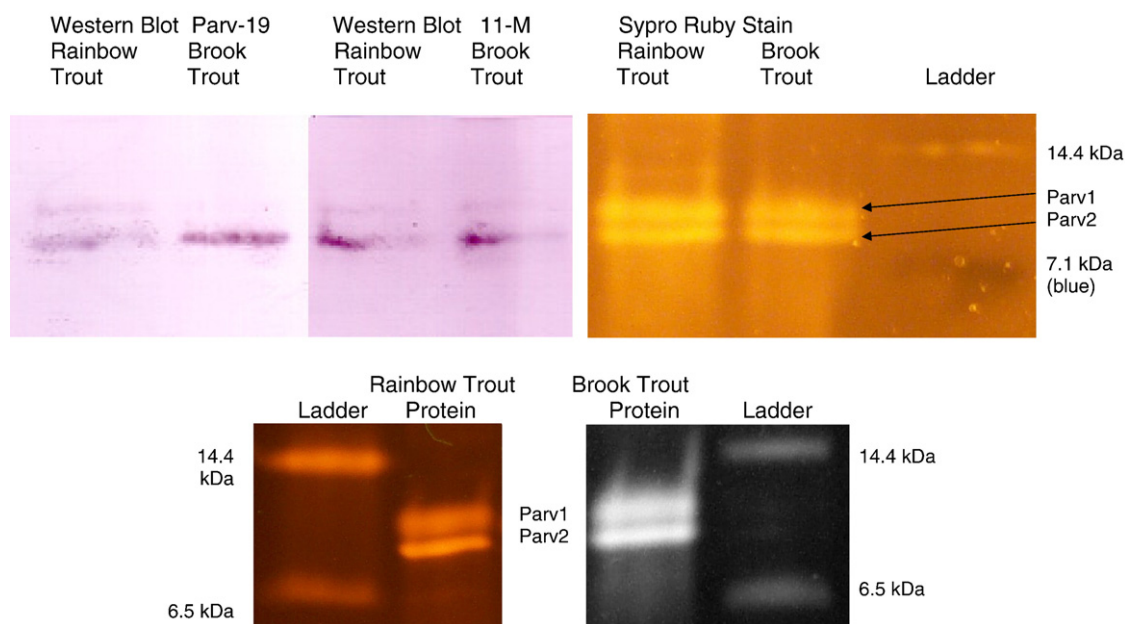


Fig. 3. Identification of parvalbumin in rainbow trout and brook trout. Western blots confirmed the identification of parvalbumin in trout muscle. White muscle samples with relatively high parvalbumin content were probed with two anti-parvalbumin antibodies, Parv 19 and 11-M. Both antibodies had high affinity for the smaller parvalbumin isoform, Parv2, and low affinity for the larger isoform, Parv1. Sypro Ruby stained SDS-PAGE gels were analyzed to estimate the sizes of the two isoforms. For rainbow trout, Parv1 was 10.7 kDa and Parv2 was 9.2 kDa, while for brook trout, Parv1 was 11.1 kDa and Parv2 was 9.9 kDa.

For Western Blots, 25  $\mu$ l of sample were loaded onto a 16.5% Tris-Tricine/Peptide precast gel (BioRad). The gel was kept at 4  $^{\circ}$ C and was run at 50 V for one hour and 100–125 V for 2 to 3 h. Parvalbumin from the SDS-PAGE gel was transferred to the PVDF membrane using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad) in the presence of Towbin buffer. After the application of the parvalbumin, the PVDF membrane was blocked using 3% gelatin in Tris-Buffered Saline (TBS) and rinsed in Tween-20 Tris Buffered Saline (TTBS). An antibody solution (1:1000 dilution of anti-parvalbumin antibody in buffer (1% gelatin in TTBS)) was applied. Two primary monoclonal antibodies were employed, Parv-19 (Sigma, P3088) and 11-M (Alpha Diagnostic). After 2 h of incubation with gentle

agitation, the membrane was washed with TTBS, and the secondary antibody solution was added for 1 h (1:1000 dilution of goat anti-mouse IgG (Sigma, A-3688) in antibody buffer). The membrane was washed in TTBS and then TBS, and an Alkaline Phosphatase color development solution (AP Color Development, BioRad) was used to detect the presence of parvalbumin. The membrane was allowed to incubate until bands were fully visible. Membranes were scanned for further analysis using Kodak 1-D gel analysis software. Subsequent to the transfer of protein to the membrane, the gel was stained with Sypro Ruby Stain (BioRad). This permitted determination of the apparent molecular weight (in Daltons) of parvalbumin identified by Western Blot.

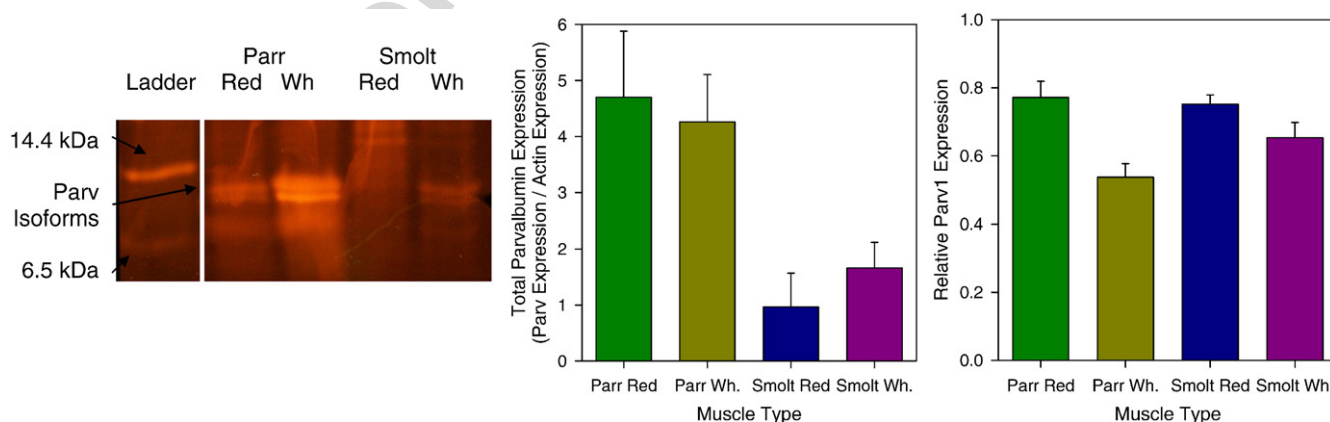


Fig. 4. SDS-PAGE gel of parvalbumin expression in rainbow trout muscle — development study. A sample gel for muscle samples red and white muscle of a trout parr and a smolt is shown, demonstrating higher parvalbumin expression in the red and white muscle of parr (left). Mean ( $\pm$ SE) values from the analysis of five MID samples of each muscle type in each age of fish confirm significantly higher levels of parvalbumin in the younger fish, although there were no significant differences between fiber type within an age group (middle). The relative expression of the two isoforms (defined as a Parv1 expression/total parvalbumin expression) did differ between fiber type but not with development (right). The parvalbumin of red muscle was composed of a higher proportion of the Parv1 antibody.

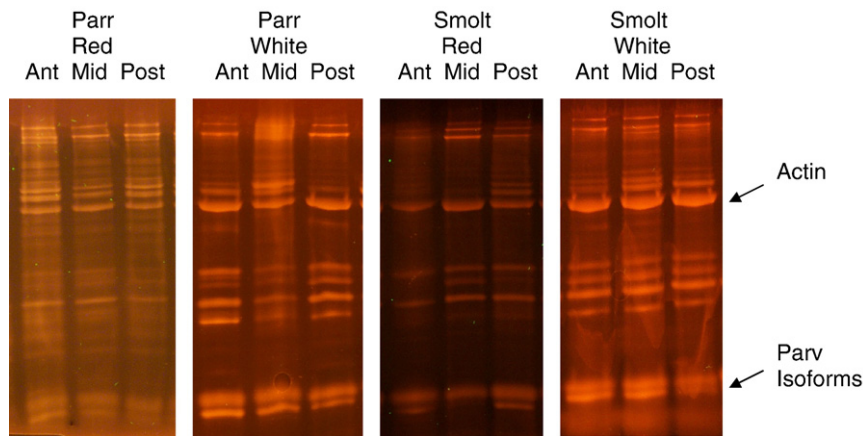


Fig. 5. SDS-PAGE gel of parvalbumin expression in rainbow trout muscle — longitudinal study. Four representative gels are shown for red and white muscle of a trout parr and a smolt. The actin band, which was used as a control for total protein content, is indicated. In all muscle samples, there was lower parvalbumin expression in the posterior compared to the anterior. The relative expression of the two isoforms did not vary with body position.

### 2.2.2. Analysis of parvalbumin expression

Muscle samples were extracted for analysis of parvalbumin expression from red and white muscle of brook trout and rainbow trout. For brook trout, the same five fish used for physiology experiments were used for parvalbumin analysis, allowing for the correlation of parvalbumin expression with relaxation rate for individual bundles. For rainbow trout parr and smolts, parvalbumin expression data are for a set of fish distinct from those used in the white muscle physiology experiments and the earlier red muscle physiology experiments. Five trout parr (TL =  $12.4 \pm 0.7$  cm, Mass =  $25.0 \pm 5.4$  g) and five trout smolts (TL =  $27.0 \pm 1.5$  cm, Mass =  $225.7 \pm 51.1$  g) were used in the analysis of parvalbumin expression and development. For the study of the effect of body position on parvalbumin expression, again five trout parr (TL =  $13.7 \pm 1.2$  cm, Mass =  $27.5 \pm 6.5$  g) and five trout smolts (TL =  $26.5 \pm 1.9$  cm, Mass =  $181.7 \pm 41.2$  g) were used.

For the fish used in the study of the effect of development on parvalbumin expression in rainbow trout, red and white muscle

samples were extracted from the MID position of five parr and five smolts. For all of the fish used in the study of longitudinal body position on parvalbumin expression, red and white muscle samples were dissected from the ANT, MID and POST position of five fish from each group: rainbow trout parr and smolts and brook trout adults. Parvalbumin was extracted as above, and 16.5% Tris-Tricine SDS-PAGE gels run in the same manner. After staining with Sypro Ruby, Kodak 1-D gel analysis software was used to quantify total parvalbumin expression and the relative expression of the two observed isoforms. Total parvalbumin content of the muscle sample was expressed as the summed staining intensity of the two bands (i.e. the two isoforms) of parvalbumin on the gel divided by the staining intensity of the actin band. This controlled for differences (usually slight) in loading of the lanes (Wilwert et al., 2006). Relative expression of the two parvalbumin isoforms (Parv1 and Parv2) observed in muscle samples at each body position along the length of the fish was expressed as the proportion of Parv 1 (larger sized isoforms) out of total parvalbumin

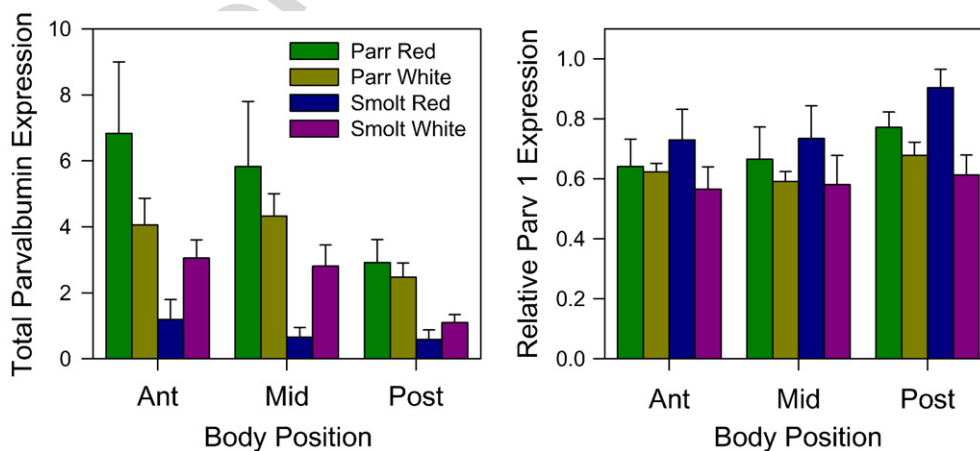


Fig. 6. Parvalbumin expression in rainbow trout muscle — longitudinal study analysis. Mean ( $\pm$ SE) values from the analysis of five samples for each body position of each muscle type in each age of fish are shown. There was a significant decrease in total parvalbumin with body position for all four muscle fiber type/fish age combination (left). The red muscle samples expressed higher levels of the Parv1 isoform (right). Each body position shows the same basic pattern with regard to total parvalbumin expression and relative Parv1 expression as the development study (Fig. 4), although the comparison is most apt for the posterior position.

expression. For each sample, the background was subtracted using the Kodak software, and a Gaussian algorithm was used to curve-fit the absorption pattern of the peaks for the two parvalbumin isoforms. In practice, the two peaks could be readily resolved.

### 2.3. Statistical analysis

All physiological data and protein analysis data are presented as mean  $\pm$  SE. White muscle contractile properties were analyzed via *t*-test and ANOVA. For comparison between ages of rainbow trout, contractile property variables such as time of relaxation and twitch time were examined for differences between parr and smolt for posterior position muscle. For comparisons of contractile properties along the length of rainbow trout parr and smolt and brook trout adults, single factor ANOVA was used with body position (ANT vs. MID vs. POST) as the independent variable. Since the relative parvalbumin isoform expression data were proportions, they were arcsine-transformed to permit their analysis with parametric statistics. For the study of development and parvalbumin expression in rainbow trout, *t*-tests were used to compare total parvalbumin expression and the relative expression of parvalbumin isoforms between parr and smolts for each fiber type. For the study of longitudinal body position and parvalbumin, total parvalbumin expression and the relative expression of parvalbumin isoforms were examined relative to body position using factorial ANOVA for rainbow trout and brook trout. Total parvalbumin and relative isoform expression in rainbow trout were analyzed with factorial ANOVA with three factors: development, body position and fiber type. Similarly, total parvalbumin and relative isoform expression in brook trout were analyzed with factorial ANOVA with two factors: body position and fiber type. For the factorial ANOVAs, the interactive effects were all non-significant ( $p > 0.05$ ) except for one relationship. The one significant interactive effect is indicated below. Multiple regression was used to relate the parvalbumin expression variables to relaxation time in brook trout.

## 3. Results

### 3.1. Muscle physiology

The white muscle of rainbow trout shows both developmental and longitudinal variations in contraction kinetics (Fig. 1). Slower contractile properties were observed in older trout smolts

and at more posterior body positions. In terms of development, time of activation, time of relaxation and twitch time were all longer in the older smolts compared to parr. However, this effect was only significant for TR ( $t(10)=3.585$ ,  $p=0.005$ ) and TW90 ( $t(7)=4.06$ ,  $p=0.005$ ) but not TA ( $t(9)=0.676$ ,  $p=0.516$ ). There was a significant shift in TR with body position in parr ( $F(2,12)=8.010$ ,  $p=0.006$ ) but not smolts ( $F(2,12)=1.537$ ,  $p=0.279$ ). Neither TW90 nor TA were affected by body position in either developmental stage (TW90 — parr,  $F(2,12)=0.190$ ,  $p=0.829$ ; TW90 — smolt,  $F(2,8)=0.021$ ,  $p=0.979$ ; TA — parr,  $F(2,12)=0.167$ ,  $p=0.847$ ; TA — smolt,  $F(2,8)=0.048$ ,  $p=0.995$ ). Brook trout white muscle also displayed longitudinal variations in contractile properties, with slower kinetics in the posterior (Fig. 2). There were significant effects of body position on TA ( $F(2,9)=40.2$ ,  $p < 0.0001$ ) and TR ( $F(2,9)=86.1$ ,  $p < 0.00001$ ) but not TW90 ( $F(2,9)=0.182$ ,  $p=0.835$ ).

Previous research has shown longitudinal and developmental variations in the red muscle of rainbow trout and longitudinal variations in brook trout. The posterior muscle activates and relaxes more slowly than the anterior muscle in rainbow trout (Coughlin et al., 2001) species. The posterior muscle relaxes more slowly than the anterior muscle in brook trout (McGlinchey et al., 2001; Coughlin et al., 2005).

### 3.2. Parvalbumin analysis

Two isoforms of parvalbumin were consistently identified in the myotomal muscle of rainbow trout and brook trout (Fig. 3). For the two parvalbumin antibodies, the Parv1 isoform showed a much lower level of reactivity than the Parv2 isoform. However, the western blot allowed for positive identification of both isoforms in both species of trout.

In rainbow trout development experiment, both development (parr vs. smolt) and fiber type affected parvalbumin expression in trout muscle (Fig. 4, left). Higher levels of parvalbumin are found in the muscle (both red and white) of the younger trout parr relative to smolts. Less obvious to the naked eye is a subtle shift in the relative expression of the two isoforms between fiber type. Development significantly affected total parvalbumin expression (Fig. 4, middle). Parr expressed higher levels of total parvalbumin than smolts for both muscle fiber types (red muscle,  $t(8)=2.82$ ,  $p=0.022$ ; white muscle,  $t(8)=2.70$ ,  $p=0.027$ ). Development did not affect the relative expression of the two isoforms of parvalbumin for either muscle fiber type (Fig. 4, right; red muscle,  $t(8)=0.42$ ,  $p=0.688$ ; white muscle,

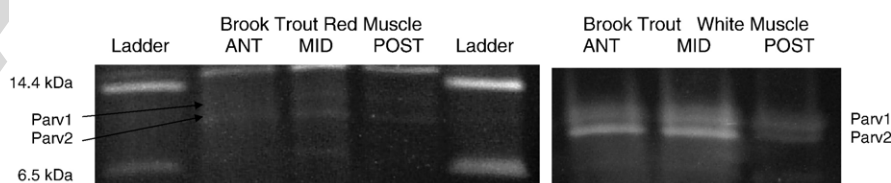


Fig. 7. SDS-PAGE gel of parvalbumin expression in brook trout muscle — longitudinal study. Two representative gels are displayed for red and white muscle. Total parvalbumin expression was higher in white muscle than red muscle (left). The red muscle expressed very little parvalbumin. A shift in the relative expression of the two parvalbumin isoforms was observed. For instance, in white muscle gel shown, the Parv1 isoform was expressed in relatively higher proportion in the posterior compared to the anterior.

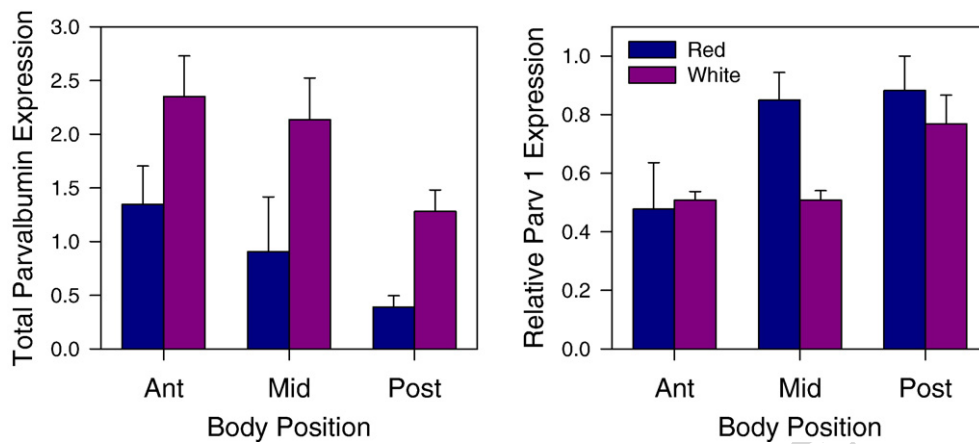


Fig. 8. Parvalbumin expression in brook trout muscle — longitudinal study analysis. Mean ( $\pm$ SE) values from the analysis of five samples for each body position of each muscle type are shown. There was a significant drop in total parvalbumin expression from anterior to posterior (left). In addition, there was a significant shift in the relative expression of the two isoforms with body position (right). Higher levels of Parv1 were observed in the posterior.

$t(8)=1.96$ ,  $p=0.085$ ). When the data for each muscle fiber type are pooled, there is a significant affect of muscle fiber type on the relative expression of parvalbumin isoforms, with the red muscle expressing a higher proportion of the Parv1 isoform ( $t(17)=3.77$ ,  $p=0.0015$ ).

In the rainbow trout longitudinal study, there were anterior vs. posterior differences in total parvalbumin expression (Figs. 5 and 6 left), with greater parvalbumin expression in the anterior. The relative expression of the parvalbumin isoforms showed little or no variation with body position (Figs. 5 and 6 right). Total parvalbumin was significantly affected by body position ( $F(2,45)=5.54$ ,  $p=0.007$ ) and development ( $F(1,45)=29.9$ ,  $p<0.001$ ) but not fiber type ( $F(1,45)=0.178$ ,  $p=0.675$ ). There was a significant interactive effect between fiber type and development with regard to total parvalbumin expression ( $F(1,45)=9.385$ ,  $p=0.004$ ). Relative expression was significantly affected by fiber type ( $F(1,45)=11.50$ ,  $p=0.001$ ) but not development

( $F(1,45)=1.57$ ,  $p=0.216$ ) or body position ( $F(2,45)=2.90$ ,  $p=0.065$ ). These results from the longitudinal data agree with those from the development study. For instance, when longitudinal data were examined for the effects of development, the patterns observed at any one body position (e.g. Fig. 6, left) were similar to those observed in the original development study (Fig. 4). Younger fish expressed higher levels of total parvalbumin. In terms of relative parvalbumin isoform expression, red muscle expresses a relatively high proportion of Parv1 (Figs. 4 and 6, right).

In brook trout red and white muscle, there were variations in parvalbumin expression with body position (Figs. 7 and 8). The anterior muscle expresses more parvalbumin and relatively more of the Parv1 isoform. Further, there were fiber type differences, with white muscle expressing greater amounts of parvalbumin and relatively more of the Parv2 isoform. Total parvalbumin was

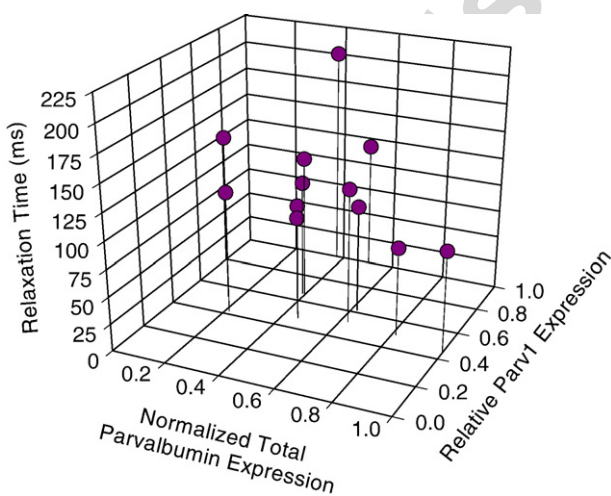


Fig. 9. Relaxation time as a function of both normalized total parvalbumin and relative Parv1 expression in brook trout white muscle. There was a significant multiple regression ( $F(2,9)=5.258$ ,  $p=0.031$ ) for the regression of TR against both total parvalbumin content and the relative expression of the Parv1 isoform. Faster muscle (shorter time of relaxation) had more parvalbumin and relatively more of the Parv2 isoform (less of the Parv1 isoform).

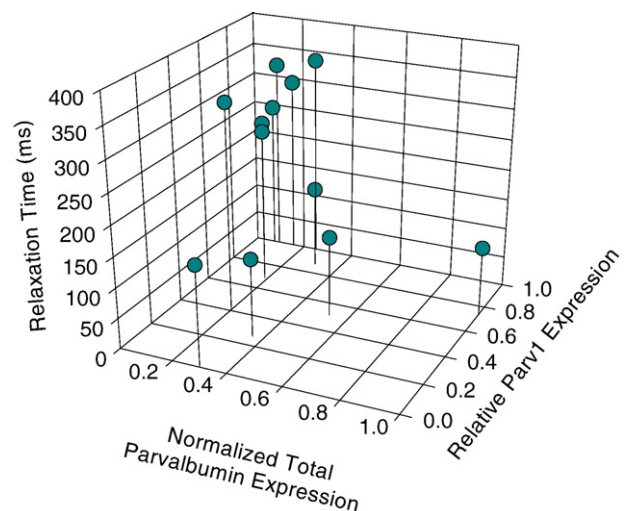


Fig. 10. Relaxation time as a function of both normalized total parvalbumin and relative Parv1 expression in brook trout red muscle. The trend was similar to white muscle, with faster relaxation associated with higher total parvalbumin content and a relatively higher contribution of the Parv2 isoforms (lower relative Parv1 expression). However, the multiple regression was not significant ( $F(2,10)=1.392$ ,  $p=0.293$ ). Physiology data are from Coughlin et al. (2005).

significantly affected by both body position ( $F(2,19)=4.108$ ,  $p=0.033$ ) and fiber type ( $F(1,19)=38.9$ ,  $p<0.001$ ). The relative expression of the two isoforms of parvalbumin was also significantly affected by both body position ( $F(2,19)=5.093$ ,  $p=0.017$ ) and fiber type ( $F(1,19)=102.9$ ,  $p<0.001$ ). For brook trout white muscle, there was a significant multiple regression for the effects of total parvalbumin content and relative parvalbumin isoform expression on time of relaxation (Fig. 9). This relationship was not significant for red muscle (Fig. 10).

## 4. Discussion

### 4.1. Relaxation rate and parvalbumin expression in trout

Both rainbow and brook trout display longitudinal variations in muscle relaxation: the anterior muscle relaxes more rapidly than the posterior muscle. We have reported this previously for the slow-twitch or red muscle of parr and smolt rainbow trout (Coughlin et al., 2001) and for red muscle of adult brook trout (Coughlin et al., 2005). In the present study, we have demonstrated the same result for fast-twitch or white muscle of rainbow trout parr and smolts and for adult brook trout. For rainbow trout, we have also shown a developmental dimension to changes in relaxation time, with faster rates of relaxation found in younger fish for both red muscle (Coughlin et al., 2001) and white muscle (present study). Lastly, the values reported previously for red muscle relaxation were considerably longer than those reported here for white muscle — for brook trout adults and for both developmental stages in rainbow trout, the white muscle has significantly faster rates of relaxation.

The mechanisms by which parvalbumin expression may be modulated by fishes to generate longitudinal and developmental shifts in muscle relaxation appears to differ between the two species of trout. In brook trout, longitudinal variations in relaxation rate were correlated with both differences in the total amount of parvalbumin expressed and the relative expression of the two isoforms detected in the skeletal muscle. For both red and white muscles, the anterior muscle contained more parvalbumin, and more of the Parv2 isoform (Fig. 8). The importance of both total parvalbumin and relative expression of parvalbumin isoforms was demonstrated by the significant multiple regression of these two parvalbumin expression variables on muscle relaxation (Fig. 9). However, this result was not significant in the red muscle (Fig. 10). This result bears further study, as the present result is associated with a low level of statistical power ( $\text{Power}<0.80$ ). The results for brook trout were similar to those observed in sheephead and kingfish, where both total parvalbumin and relative isoform expression affect muscle relaxation (Wilwert et al., 2006).

Rainbow trout appeared to modulate relaxation rate along the length of the fish by manipulating total parvalbumin expression. As with brook trout, sheephead and kingfish, there were significantly higher amounts of parvalbumin in the anterior muscle for red and white muscle of both developmental stages (Fig. 6, left). However, there was little longitudinal variation in the relative expression of the two isoforms. There appeared to be

a modest shift in the relative amount of the isoforms in red muscle (Fig. 6, right), but this effect was not significant. Alternatively, developmental shifts in muscle relaxation in the red and white muscles were related to changes in total parvalbumin content of the swimming muscle (Fig. 4, middle), but not the relative expression of the two isoforms (Fig. 4, right).

Fiber type differences in parvalbumin content were consistent for the older rainbow trout smolts and the similar-sized brook trout adults. For both of these fish, the white muscle expressed higher amounts of parvalbumin and significantly higher amounts of the Parv2 isoform (Figs. 4, 6 and 8; for rainbow trout, compare smolt red to smolt white). For the smaller rainbow trout parr, there were fiber type differences in the relative expression of the two isoforms but not for total parvalbumin (Figs. 4 and 6). The faster relaxation rates observed in the muscle of smaller rainbow trout appear to be achieved with relatively high levels of parvalbumin for both red and white muscles. As indicated above, both fiber types in the small fish do express significantly higher levels of parvalbumin than those observed in the red or white muscle of older fish of either fish species. The fiber type differences in these animals appear to be achieved by varying the relative expression of the two isoforms.

### 4.2. Parvalbumin isoforms in fish muscle

Both our previous work on sheephead and kingfish (Wilwert et al., 2006) and the present study on trout indicate that the smaller (Parv2) of the two isoforms of parvalbumin in fish muscle may be kinetically faster. Relatively higher expression of this isoform is associated with faster rates of relaxation. We are interested in determining the biochemical differences in the two isoforms that are the basis of differing abilities to buffer  $\text{Ca}^{2+}$  and, thereby, affect relaxation rate. Parvalbumin binds  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . During relaxation from contraction in a muscle (when  $\text{Ca}^{2+}$  is sequestered into the sarcoplasmic reticulum), myoplasmic parvalbumin binds  $\text{Mg}^{2+}$ . Hou et al. (1991, 1993) demonstrated that the dissociation rate for  $\text{Mg}^{2+}$  determines the physiological properties of parvalbumin and, therefore, establishes its contribution to relaxation rate.

The two binding sites on parvalbumin molecules have higher affinity for  $\text{Ca}^{2+}$  than for  $\text{Mg}^{2+}$ . During muscle contraction, parvalbumin binds  $\text{Ca}^{2+}$  in competition with troponin C. However, parvalbumin must first lose its  $\text{Mg}^{2+}$  to permit  $\text{Ca}^{2+}$  binding. This may be the rate limiting step in the role of parvalbumin to play a role in decreasing myoplasmic  $[\text{Ca}^{2+}]$ . Ultimately, SR  $\text{Ca}^{2+}$  ATPase pumps move  $\text{Ca}^{2+}$  into the sarcoplasmic reticulum (Berchtold et al., 2000). Erickson et al. (2005) and Erickson and Moerland (2006) have demonstrated that parvalbumin from different fish vary in terms of their  $\text{Ca}^{2+}$  binding properties. Although, they specifically examined  $\text{Ca}^{2+}$  dissociation constants ( $K_D$ ), their results do support the suggestion that different isoforms of parvalbumin within one animal may vary in terms of other binding characteristics, such as the  $\text{Mg}^{2+}$  dissociation constant or  $K_D$ .

Our work indicates that parvalbumin is present at physiologically relevant concentrations in red and white swimming

muscle of fishes. There is an association between muscle relaxation and the expression of parvalbumin in both muscle fiber types. Parvalbumin has been reported as only abundant in white or fast-twitch muscle in vertebrates (Berchtold et al., 2000; Chauvigné et al., 2005). Wilwert et al. (2006) and the present work demonstrates that red or slow-twitch muscle of fishes expresses high enough levels of parvalbumin to affect relaxation. This is particularly true for young rainbow trout parr that express relatively higher levels of parvalbumin than those observed in the white muscle of older trout (Fig. 4). It seems clear that the amount of parvalbumin present (regardless of isoform) may affect muscle relaxation. We are interested in detailing how the two isoforms of parvalbumin present in the swimming muscle may differentially affect muscle relaxation. Our present objectives are two fold. We are purifying the two parvalbumin isoforms to determine their binding characteristics, such as the  $Mg^{2+}$  off-rate, in rainbow trout and sheepshead. In addition we will determine the protein sequence of the two isoforms in trout muscle to investigate how the sequences vary relative to the known cation binding sites on the parvalbumin molecule.

### Acknowledgements

This research was supported by the National Science Foundation, Research in Undergraduate Institutions Program (NSF RUI-IBN 0111112) and by Widener University.

### References

- Berchtold, M., Brinkmeier, H., Muntener, M., 2000. Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol. Rev.* 80, 1216–1265.
- Chauvigné, F., Cauty, C., Rallièrre, C., Rescan, P.Y., 2005. Muscle fiber differentiation in fish embryos as shown by *in situ* hybridisation of a large repertoire of muscle-specific transcripts. *Dev. Dyn.* 233, 659–666.
- 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.J., Rome, L., 1999. Recruitment of pink and red muscle in swimming scup varies with temperature and swimming speed. *Biol. Bull.* 196, 145–152.
- Coughlin, D.J., Burdick, J., Stauffer, K.A., Weaver, F.E., 2001. The parr–smolt transformation in rainbow trout (*Oncorhynchus mykiss*) involves a transition in red muscle kinetics, swimming kinematics and myosin heavy chain isoform. *J. Fish Biol.* 58, 701–715.
- Coughlin, D., Spiecker, A., Schiavi, J.M., 2004. Red muscle recruitment during steady swimming correlates with rostral–caudal patterns of power production in trout. *Comp. Biochem. Physiol. A* 137, 151–160.
- Coughlin, D.J., Caputo, N.D., Bohnert, K.L., Weaver, F.E., 2005. Troponin T expression in trout red muscle correlates with muscle activation. *J. Exp. Biol.* 208, 409–417.
- Davies, M.L.F., Johnston, I.A., 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.
- Erickson, J.R., Moerland, T.S., 2006. Functional characterization of parvalbumin from the Arctic cod (*Boreogadus saida*): similarity in calcium affinity among parvalbumin from polar teleosts. *Comp. Biochem. Physiol. A* 143, 228–233.
- Erickson, J.R., Sidell, B.D., Moerland, T.S., 2005. Temperature sensitivity of calcium binding for parvalbumins from Antarctic and temperate zone teleost fishes. *Comp. Biochem. Physiol. A* 140, 179–185.
- Gillis, J.M., 1985. Relaxation of vertebrate skeletal muscle. A synthesis of the biochemical and physiological approaches. *Biochim. Biophys. Acta* 811, 97–145.
- Hou, T., Johnson, J.D., Rall, J.A., 1991. Parvalbumin content and  $Ca^{2+}$  and  $Mg^{2+}$  dissociation rates correlated with changes in relaxation rate of frog muscle fiber. *J. Physiol.* 441, 285–304.
- Hou, T., Johnson, J.D., Rall, J.A., 1993. Role of parvalbumin in relaxation of frog skeletal muscle. In: Sugi, H., Pollack, G.H. (Eds.), *Mechanism of Myofibril Sliding in Muscle Contraction*. Plenum Press, New York, pp. 141–153.
- Huriaux, F., Melot, F., Vandewalle, P., Collin, S., Focant, B., 1996. Parvalbumin isoforms in white muscle from three teleost fish: characterization and their expression during development. *Comp. Biochem. Physiol. B* 113, 475–484.
- Lutz, G.J., Cuizon, D.B., Ryan, A.F., 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.
- McGlinchey, S.M., Saporetti, K.A., Forry, J., Pohronezny, J.A., Coughlin, D.J., 2001. Red muscle function during steady swimming in brook trout, *Salvelinus fontinalis*. *Comp. Biochem. Physiol. A* 129, 727–738.
- Thys, T.M., Blank, J.M., 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., Schachat, F.H., 2002. Longitudinal variation in muscle protein expression and contraction kinetics of largemouth bass axial muscle. *J. Exp. Biol.* 204, 4249–4257.
- Wilwert, J.L., Madhoun, N., Coughlin, D.J., 2006. Parvalbumin correlates with relaxation rate in the swimming muscle of sheepshead and kingfish. *J. Exp. Biol.* 209, 227–237.