# Thermal acclimation in rainbow smelt, *Osmerus mordax*, leads to faster myotomal muscle contractile properties and improved swimming performance

**John R. Woytanowski and David J. Coughlin***

Department of Biology, Widener University, Chester, PA 19013, USA
*Author for correspondence (djcoughlin@widener.edu)

## Summary

**Rainbow smelt (*Osmerus mordax*) display an impressive ability to acclimate to very cold water temperatures. These fish express both anti-freeze proteins and glycerol in their plasma, liver, muscle and other tissues to avoid freezing at sub-zero temperatures. Maintenance of glycerol levels requires active feeding in very cold water. To understand how these fish can maintain activity at cold temperatures, we explored thermal acclimation by the myotomal muscle of smelt exposed to cold water. We hypothesized that cold-acclimated fish would show enhanced swimming ability due to shifts in muscle contractile properties. We also predicted that shifts in swimming performance would be associated with changes in the expression patterns of muscle proteins such as parvalbumin (PV) and myosin heavy chain (MyHC). Swimming studies show significantly faster swimming by smelt acclimated to 5℃ compared to fish acclimated to 20℃ when tested at a common test temperature of 10℃. The cold-acclimated fish also had faster muscle contractile properties, such as a maximum shortening velocity ($V_{max}$) almost double that of warm-acclimated fish at the same test temperature. Cold-acclimation is associated with a modest increase in PV levels in the swimming muscle. Fluorescence microscopy using anti-MyHC antibodies suggests that MyHC expression in the myotomal muscle may shift in response to exposure to cold water. The complex set of physiological responses that comprise cold-acclimation in smelt includes modifications in muscle function to permit active locomotion in cold water.**

## Introduction

Rainbow smelt (*Osmerus mordax*, Mitchill 1814) (hereafter referred to as smelt) inhabit a wide range of aquatic ecological conditions, including broad variation in salinity and temperature. Some populations encounter temperatures below 0℃, while others face temperatures above 20℃ (Nellbring, 1989). Smelts encountering cold temperatures display thermal acclimation responses related to prevention of freezing in sub-zero temperatures (Driedzic and Ewart, 2004). Remarkably, the fish are very active in sub-zero temperature water, capable of active feeding at −1.8℃ (Raymond, 1995).

To prevent freezing and permit high levels of activity, smelts produce glycerol and antifreeze proteins (AFP) when exposed to cold temperatures (both in a laboratory setting and naturally during the winter months). Both glycerol and AFP act to reduce the freezing point of their serum, enabling them to swim in sub-zero temperatures (Liebscher et al., 2006). Glycerol acts in a colligative manner, while AFP is non-colligative in inducing thermal hysteresis. In addition, glycerol acts as a protein chaperon that enhances AFP activity (Gong et al., 2011). Driedzic and Short found that the natural production of glycerol and AFP requires a significantly higher energy and

nutrient intake than under non-cold acclimation condition (Driedzic and Short, 2007). One complication is that glycerol is freely lost to the environment across the gills and other exposed surfaces of the fish (Driedzic and Ewart, 2004). Cold-exposed smelt must consume considerable food to support a continuous need for glycerol through the winter months as well as for the production of AFP.

High levels of glycerol production require active feeding and the conversion of glucose from the diet and glycogen stores. Specific to glycerol, a suite of enzymes, including glycerol phosphate dehydrogenase, are upregulated to insert a branch point in glycolysis and gluconeogenesis to convert glucose into glycerol (Hall et al., 2011; Hall et al., 2012). In addition to glycerol and AFP, the muscle of cold exposed smelt shows increased levels of urea, trimethylamine oxide (TMAO) and other compounds (Raymond, 1998; Treberg et al., 2002).

To achieve an active lifestyle in an environment at or below the freezing point of pure water, smelt must overcome the effects of temperature and various osmolytes on muscle function. Low temperature will have a strong effect on muscle physiology and reduce swimming performance (Johnston et al., 1990; Coughlin and Rome, 1996; Coughlin et al., 1996; Rome et al., 2000). The

focus of this paper is thermal acclimation of smelt muscle to permit high activity levels at cold temperatures. For isometric contractions, $Q_{10}$ values of muscle physiology variables such as activation and relaxation times are typically greater than 2 – muscle takes twice as long to generate force or to relax from contraction when temperature drops by 10°C (e.g. Coughlin et al., 1996; Schoenman et al., 2010). Temperature also leads to a slower shortening velocity. Maximum shortening velocity ($V_{max}$) of muscle has $Q_{10}$ values of ~1.5 (e.g. Coughlin et al., 1996). Power output by muscle bundles stimulated with a cyclical stimulation pattern that matches *in vivo* muscle activity had high values of $Q_{10}$ – in some cases $Q_{10}$ for power output >10 (Rome et al., 2000). Muscle at low temperatures takes longer to activate, shortens more slowly and produces less power than muscle at warm temperatures. To the whole animal, this results in a reduction in swimming performance (e.g. Coughlin and Rome, 1996; Yan et al., 2012; many others).

The combination of increased energy requirements but reduced swimming ability with cold temperature suggests that rainbow smelt will show a strong thermal acclimation in their myotomal or swimming muscle. Thermal acclimation of myosin expression in fish muscle has been observed in a number of fish species. The myosin hexamer is the primary determinant of muscle performance, and the myosin heavy chain (MyHC) component is central to the modulation of muscle contractile properties such as $V_{max}$ (Moss et al., 1995). Common carp (*Cyprinus carpio*) alter MyHC expression with temperature of acclimation; exposure to cold water leads to an upregulation in the expression of a fast isoform of MyHC (Watabe et al., 1992). This fast isoform confers faster contractile properties on cold-acclimated carp myotomal muscle (Watabe, 2002); and cold-acclimated carp show improved swimming performance compared to warm acclimated fish (Wakeling et al., 2000). Japanese medaka (*Oryzias latipes*) are very sensitive to cold acclimation. The transcripts of MyHC detected in myotomal muscle in these fish shift significantly with cold acclimation (Liang et al., 2007). Similarly, the prevalent isoforms of MyHC in grass carp (*Ctenopharyngodon idella*) myotomal muscle is regulated by temperature of acclimation (Tao et al., 2004; Fukushima et al., 2009). Shifts in myosin ATPase activity and thermal compensation in swimming performance after exposure to cold temperatures have been observed in other fish species as well (Guderley and Blier, 1988).

Another muscle protein that might permit thermal acclimation in smelt is parvalbumin (PV). This myoplasmic protein binds calcium in competition with troponin and enhances muscle relaxation from contraction, effectively shortening the calcium transient (Berchtold et al., 2000). Variations in PV content correlate with differences in muscle contractile properties in a variety of fishes (Wilwert et al., 2006; Coughlin et al., 2007; Schoenman et al., 2010; Campion et al., 2012). To enhance muscle function in cold water, smelt may increase PV expression during periods of thermal acclimation.

In the present study, we explored the effects of thermal acclimation on swimming performance, muscle contractile properties and MyHC expression in rainbow smelt. We predict that cold-acclimated (CA) smelts will outperform warm-acclimated (WA) smelts in swimming performance, and we expect to see differences in $V_{max}$ among the groups that correlate with the swimming performance. If $V_{max}$ is higher in CA smelt, we predict that shifts in MyHC will explain this difference.

Specifically, CA smelt myotomal muscle will great expression of fast MyHC isoforms compared to the myotomal muscle. In addition, we predict that cold-acclimation will also include an upregulation in PV expression in swimming muscle.

## Materials and Methods

### Animal handling and swimming experiments

The smelts were obtained from the Harmon Brook Farm and Maine Smelt Hatchery in Canaan, Maine, and kept in recirculating aquaria at 10°C for two weeks, then randomly assigned to thermal acclimation groups at 5°C (CA), 10°C (Control) and 20°C (WA). The fish were fed bloodworms and were allowed to acclimate at these temperatures for six weeks prior to any experiments. All handling of the smelts 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. Fish were received in Fall 2010 for experiments carried out in January–February 2011 (2011 fish) and in Fall 2011 for experiments carried out in January–February 2012 (2012 fish). The 2011 fish ($n=13$) had a mean ($\pm$ s.d.) mass of 3.16±0.77 g and mean total length of 8.7±0.5 cm; the 2012 fish ($n=14$) had a mean mass of 3.94±1.1 g and total length 9.2±0.8 cm. Although the 2012 fish were slightly larger, this difference was not statistically significant (t=1.67, $P=0.11$, df=19 for mass; t=1.65, $P=0.11$, df=19 for total length).

After six weeks of acclimation, maximum steady swimming speed and maximum tailbeat frequency were determined for three fish from each acclimation group. Three fish of a given acclimation group were placed into a temperature controlled Loligo Systems respirometry swim tunnel (185 l, purchased from Qubit Systems, http://www.qubitsystems.com) at 10°C. After allowing them to acclimate in the flume for 2 hours, the fish were swum at increasing flow speeds. Starting at 1.5 body lengths per second (BL s⁻¹), the speed was increased every 10 minutes by 0.5 BL s⁻¹, until a speed was reached in which the fish were unable to swim. Swimming experiments were filmed from above. Tailbeat frequency was determined for each fish at each speed using PC based video software (Windows Movie Maker). Sample sizes were $n=3$ for CA, control and WA in 2011 and CA and WA in 2012.

### Muscle physiology

Isometric, isovelocity and workloop muscle mechanics experiments were carried out on smelt from each thermal acclimation group from each year. Isometric experiments were used to characterize muscle activation and relaxation, isovelocity experiments were used to measure muscle maximum shortening velocity ($V_{max}$), and workloop experiments permitted comparison of power output under oscillatory activity. For muscle mechanics experiments, smelt from each group were sacrificed. Individual muscle bundles were dissected from the hypaxial muscle from approximately directly under the dorsal fin; dissection was carried out with the muscle in physiological saline (Coughlin and Carroll, 2006). All muscle examined was white, fast-twitch myotomal muscle. The bundles included two myomeres with an active muscle length of ~4 mm and ~0.5 mm² cross-section. Responsive muscle bundles were tied into a muscle mechanics apparatus composed a servomotor (Aurora Scientific 318B), force transducer (Aurora Scientific 404A), temperature controlled chamber of recirculating physiological saline, platinum stimulating electrodes and PC control via custom Lab-View software and input/output computer board (National Instruments).

At the start of isometric contraction experiments, muscle length and stimulation conditions were optimized for each bundle to maximum tetanic force output. Muscle bundles that generated low force (<50 mN mm⁻²), reflecting low tissue quality, were eliminated from the dataset. For tetanic contractions, time of activation (TA) was defined as the time from 10–90% of maximum peak isometric stress, and time of relaxation (TR) was the time from 90–10% of peak isometric stress. In addition, twitch time (TW90) was defined as the time from stimulation to 90% recovery (10% of peak isometric stress) in twitch contractions. For each bundle in 2011, isometric measurements were made at 5, 10 and 20°C, while in 2012, measurements were made at 10°C. Sample sizes were $n=6$ for CA and $n=5$ for WA in 2011 and $n=7$ for CA and $n=5$ for WA in 2012.

Isovelocity experiments were used to measure $V_{max}$ in muscle bundles at 10°C. Force–velocity curves were constructed and $V_{max}$, maximum steady state power output (steady state $W_{max}$) and optimal shortening velocity ($V_{opt}$) were determined as described previously (Coughlin et al., 1996; Carroll et al., 2009). A series of isovelocity ramps were imposed on muscle bundles of increasing velocity. Muscle tension was determined during each ramp, generating a list of force *versus* velocity points to be plotted as a force–velocity curve (Coughlin et al., 1996). After correcting for passive tension, $V_{max}$ was found by fitting the Hill muscle model, and $V_{opt}$ and steady state $W_{max}$ were derived using the model and muscle bundle mass. Sample sizes were $n=4$ for CA and WA for each year and $n=2$ for control fish for each year.

Oscillatory power output was measured using workloop experiments (Coughlin et al., 1996; Coughlin, 2000). Oscillatory stimulation conditions were developed in

pilot experiments across a range of stimulation frequencies (2–10 Hz) that spanned observed tailbeat frequencies in swimming fish. The stimulation conditions did not replicate *in vivo* muscle activity but were developed to allow comparison of the relative power output by muscle from CA and WA 2012 smelt. Maximum oscillatory power output (oscillatory $W_{max}$) was determined for each acclimation group. Sample sizes were $n=4$ for CA and WA in 2012.

### Protein analysis

Muscle parvalbumin content and myosin heavy chain expression were assessed in muscle samples from CA and WA fish in 2012. For PV, a 0.5 g block of fast-twitch, white myotomal muscle dissected from just anterior to the dorsal fin. Protein extraction was carried out as previously described (Schoenman et al., 2010; Campion et al., 2012), and running samples were prepared from muscle extracts semi-purified for PV using tris-tricine based buffer system. Proteins were separated on 16.5% Tris-Tricine/Peptide SDS-PAGE precast gel (Bio-Rad). As described in more detail (Campion et al., 2012), gels were probed via Western blots using PVDF membrane (BioRad), an anti-PV antibody (Parv 19, Sigma) and an Alkaline Phosphatase Color Development kit (BioRad). Gels were stained with Sypro Ruby Stain (BioRad) and analyzed using UV translumination on a Kodak Gel Logic 212 imaging system. The apparent molecular weight (in Daltons) of the PV band identified by Western Blot was determined, and total PV level for each protein sample was determined and normalized to total actin content. Although actual PV content of the muscle was not determined, the relative levels of PV in the muscle of fish from each acclimation group could be quantified. Sample sizes were $n=4$ for CA and WA in 2012.

To tentatively examine MyHC expression, cross-sections ("steaks") of the smelt were dissected from immediately behind the dorsal fin. The histochemical procedure followed Hernandez et al., as modified by Campion et al. (Hernandez et al., 2005; Campion et al., 2012). The tissue samples placed in Carnoy's buffer for 1 week. Subsequently, they were dehydrated into 95% ethanol in PBS and rehydrated into PBS. The samples were embedded in 1% agar solution, placed in 30% sucrose solution over night at $4^{\circ}C$ and then frozen in dry ice-cooled hexanes. The blocks were sectioned at 12 μm using a Miles Tissue Tek II Cryostat and mounted on Fisher Superfrost Plus Slides. The slides were dried for 2 hours on a slide warmer, and stored at $-20^{\circ}C$.

Processing of slides occurred at room temperature. Sets of slides ($n=4$ of CA and WA fish) were stained with monoclonal antibodies S58 and EB165. The staining procedure followed the protocol described by Campion et al. (Campion et al., 2012). S58 binds slow MyHC (Miller et al., 1985; Hernandez et al., 2005), while EB165 binds fast MyHC (Tee et al., 2009). Slides were also probed with MF20, which binds to all MyHC (Hernandez et al., 2005; Campion et al., 2012), as a positive control, although those results are not shown. S58, EB165 and MF20 were acquired from the Developmental Studies Hybridoma Bank at the University of Iowa. The sectioned muscles were rehydrated in PBT (PBS + 0.1% Tween) and then incubated in blocking solution (PBT + 1% BSA). The primary antibody solution was diluted (1:5) in blocking solution containing 5% normal goat serum (NGS). After slides were incubated for 90 minutes in primary antibody solution in a humid chamber, sections were washed with PBT and blocked again before application of secondary antibody. Alexa Fluor 488 goat anti-mouse IgG secondary antibody was diluted (1:250) in blocking solution containing 5% NGS. Slides were incubated for 30 minutes in a humid chamber with secondary antibody. The slides were then washed and stained with 2 g ml$^{-1}$ Hoechst 33342 dye diluted in PBT for DNA for 10 minutes at room temperature. After three 5-minute washes with PBT, samples were mounted in 50% glycerol in 1× PBS. Coverslips were sealed using clear nail polish and stored at $-20^{\circ}C$. Slides were analyzed using a Nikon Eclipse 80i compound microscope system with Nikon Plan Fluor objective lenses. Images were captured using a Nikon DS-Ri1 digital camera. Full details of the procedure can be found in Campion et al. (Campion et al., 2012).

### Statistical analysis

Comparisons of maximum tailbeat frequencies and muscle contractile properties between CA and WA fish groups were made using *t*-tests for 2011 and 2012 fish. $V_{max}$, steady state $W_{max}$ and $V_{opt}$ were compared between CA, WA and control groups using ANOVA for 2011 and 2012 fish. Oscillatory $W_{max}$ and total PV content were compared between 2012 CA and WA smelt groups via *t*-tests.

## Results

### Swimming experiments and muscle physiology

Thermal acclimation affected swimming performance (Fig. 1). In the 2011 dataset, the CA fish had a maximum swimming speed of 4 BL s$^{-1}$, and the WA had a maximum speed of 2 BL s$^{-1}$. The tailbeat frequency at maximum swimming speed was greater than 8 Hz for CA and less than 4 Hz for WA. This difference was statistically significant (*t*-test, $P<0.05$). The control fish fell
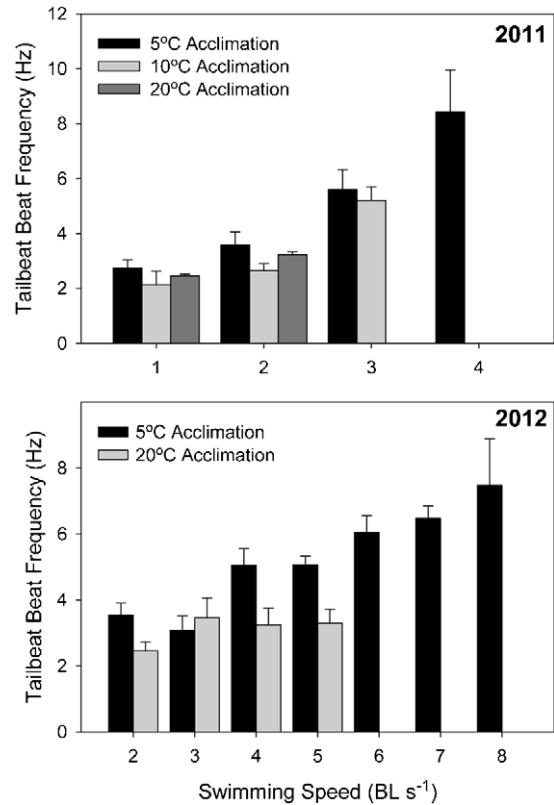


**Fig. 1. Tailbeat frequencies during steady swimming in smelt (mean + s.e.).** Data are shown for 2011 and 2012. Three thermal acclimation groups were examined in 2011, CA ($5^{\circ}C$ acclimation), WA ($20^{\circ}C$ acclimation) and control ($10^{\circ}C$ acclimation), while only the CA and WA groups were examined in 2012. The highest speed for each acclimation group represents the highest swimming speed for all fish of that group. There was no variation in maximum steady swimming speed in our study. CA fish swam at faster maximum speeds and with higher tailbeat frequencies than WA fish. The 2012 fish swam faster than the 2011 fish. $n=3$ for each bar.

between CA and WA fish for both maximum swimming speed and tailbeat frequency. In the 2012 dataset, swimming speeds were higher than in 2011, with CA fish reaching a maximum steady swimming speed of 8 BL s$^{-1}$, while WA fish had a maximum steady swimming speed of 5 BL s$^{-1}$. Similar to 2011 dataset, the 2012 CA fish employed a significantly higher tailbeat frequency than the WA fish (*t*-test, $P<0.05$).

Isometric contractile properties show modest variations with thermal acclimation (Fig. 2). For instance, although TR was shorter for CA fish at all experimental temperatures in 2011 and at $10^{\circ}C$ in 2012, these differences were significant for 10 and $20^{\circ}C$ in 2011 (*t*-tests, $P<0.05$ for 10 and $20^{\circ}C$ in 2011, $P>0.05$ for $5^{\circ}C$ in 2011 and $10^{\circ}C$ in 2012, Fig. 2, second row). TW90 was typically shorter for CA fish for each experimental temperature, but these differences were not significant for any of the comparisons (*t*-tests, $P>0.05$ for all four comparisons, Fig. 2, third row). TA showed some variations with thermal acclimation – WA fish had shorter TA values than CA fish at 5 and $10^{\circ}C$ experimental conditions in 2011 (*t*-tests, $P<0.05$ for 5 and $10^{\circ}C$ in 2011, $P>0.05$ for $20^{\circ}C$ in 2011 and $10^{\circ}C$ in 2012). The ratio of twitch force to tetanic force did not vary with acclimation and was near 0.8 for all groups.
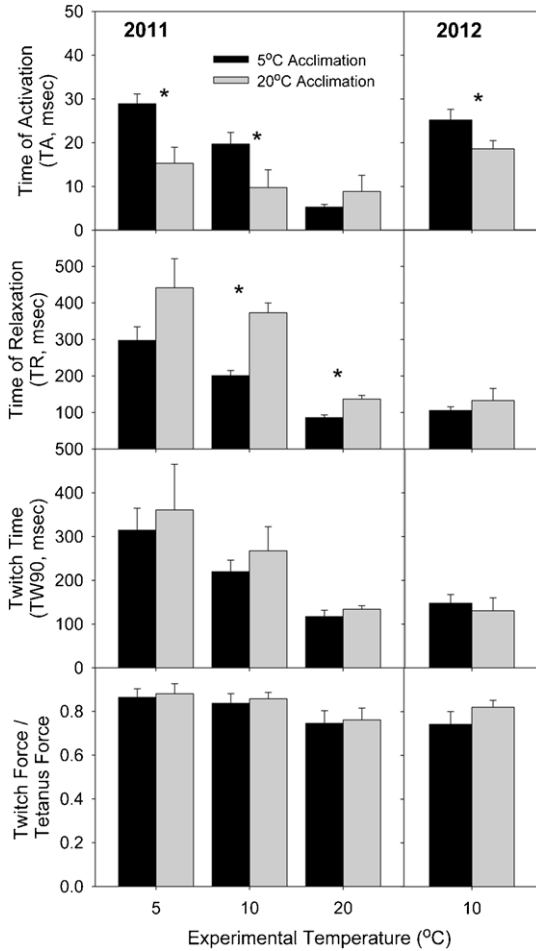
**Fig. 2. Thermal acclimation and isometric contractile properties of smelt myotomal muscle.** TR, TA, TW90 and twitch to tetanic force ratio (mean + s.e., all defined in Materials and Methods) were determined for muscle bundles stimulated isometric conditions optimized for muscle length and stimulus intensity. Individual muscle bundles from CA and WA fish were tested at three temperatures in 2011 (5, 10 and 20°C) and at 10°C in 2012. Sample sizes were $n=6$ for CA and $n=5$ for WA in 2011, and $n=7$ for CA and $n=5$ for WA in 2012. Statistically significant differences are indicated by an asterisk ($t$-test, $P<0.05$).



**Fig. 3. Maximum shortening velocity and thermal acclimation of smelt myotomal muscle at 10°C.** $V_{max}$, $V_{opt}$ and steady state $W_{max}$ (mean + s.e., all defined in Materials and Methods) were determined for muscle bundles stimulated under optimized conditions. Isovelocity ramps were used to construct force–velocity curves and solve for the three variables. $V_{opt}$ was expressed as a proportion of $V_{max}$ for each bundle. $V_{max}$ was significantly affected by both thermal acclimation and year, with CA fish having faster maximum shortening velocities compared to WA fish, and 2012 fish being faster than 2011 fish (ANOVA, see text for details). Sample sizes were $n=4$ for CA and WA fish for each year and $n=2$ for control fish in each year.

Maximum shortening velocity showed a strong dependence on thermal acclimation and varied by year (Fig. 3). In 2011, the CA group had the fastest mean $V_{max}$, $\sim 9$ ML s$^{-1}$, while the WA group had the slowest $V_{max}$, $\sim 5$ ML s$^{-1}$. The same pattern was observed in 2012, except that muscle shortening velocities were higher for all groups. In 2012, CA fish had a mean $V_{max}$ above 18 ML s$^{-1}$, while WA had a mean $V_{max}$ of $\sim 12$ ML s$^{-1}$. In both years, the effect of thermal acclimation on $V_{max}$ was significant (ANOVA, df=2,7 for each year; $F=14.2$, $P<0.01$ in 2011; $F=6.9$, $P<0.05$ in 2012). Optimal shortening velocity, expressed as a ratio of $V_{opt}$ to $V_{max}$, was not affected by thermal acclimation and ranged from 0.34 to 0.39 (Fig. 3). Maximum steady state $W_{max}$ was highest in CA fish and lowest in WA fish in 2012, although this effect was not significant (Fig. 3; ANOVA, df=2,7, $F=1.6$, $P>0.05$).

Oscillatory or workloop experiments revealed significant differences in power output as a result of thermal acclimation (Fig. 4, top). Across a rang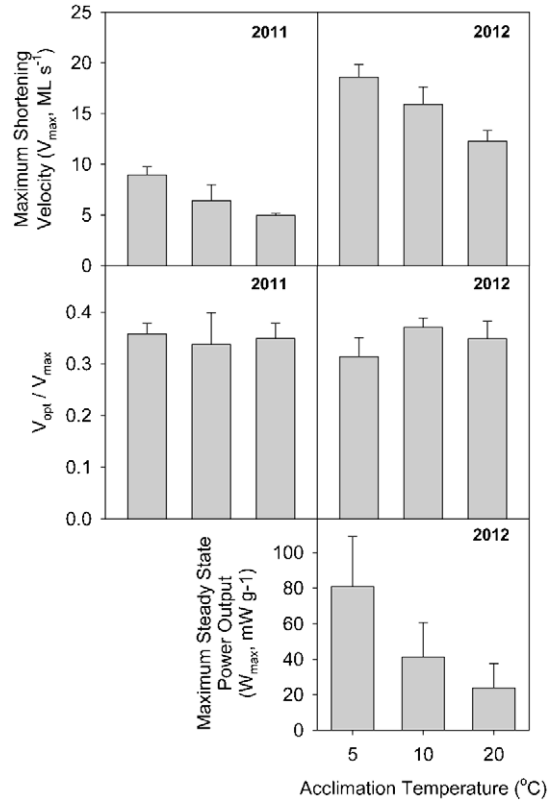e of stimulus oscillatory frequencies (2–10 Hz), muscle bundles from CA fish produced more power than those from WA fish using standardized stimulation conditions. The difference is significant at each frequency ($t$-test, $P<0.05$ for the comparison between CA and WA at each frequency. Work per cycle is greater in muscle bundles from CA fish due to apparent higher levels of activation during shortening, while muscle relaxation and stretch activation appear similar between bundles from CA and WA fish (Fig. 4, bottom).

Protein analysis
Western blotting led to the detection of one PV band on SDS-PAGE gels (Fig. 5). The band corresponds to a protein size of $\sim 9.7$ kD. The band may contain multiple PV isoforms but it does represent total PV content. Total PV content, when normalized to actin level, was higher in mytomal muscle from CA fish compared to WA fish (Fig. 6). This difference was significant ($t$-test, $P<0.01$). The relative expression of slow versus fast MyHC within the myotomal muscle could be observed using the antibodies S58 and EB165 (Fig. 7). Comparison of myotomal muscle from CA versus WA smelt suggests a shift in MyHC expression with thermal acclimation; cold acclimation is associated with lower levels of slow MyHC and higher levels of fast MyHC in the white or fast myotomal muscle (Fig. 8).
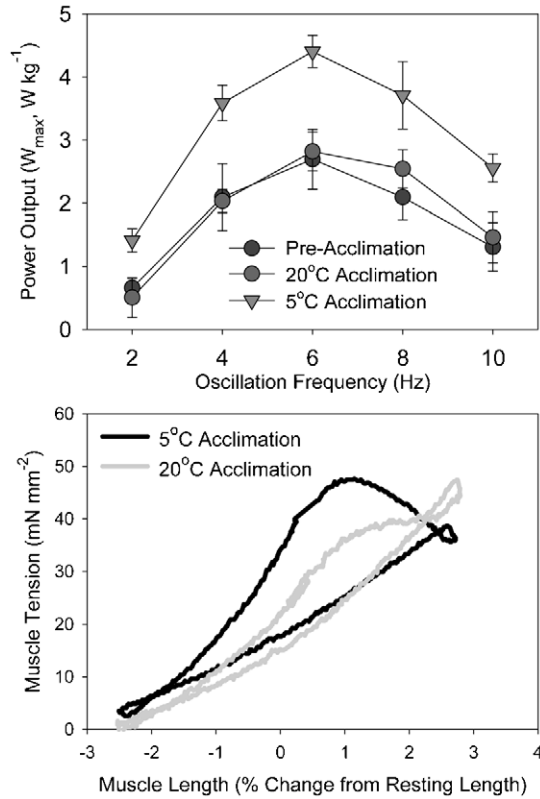
Fig. 4. Oscillatory work and thermal acclimation in smelt myotomal muscle from 2012 fish. Oscillatory power output, expressed in mass specific units of W kg$^{-1}$ (mean ± s.e.), was determined across a range of frequencies using the workloop technique for CA, WA and pre-acclimation smelt (top panel). Cold acclimation led to increased power output at each oscillation frequency. Sample workloops from CA and WA fishes at the oscillation frequency that gave the highest power output (6 Hz) showed a greater level of activation in the muscle bundle from a CA fish. Sample sizes were $n=4$ for CA and WA fish.

## Discussion

### Thermal acclimation and swimming performance

Rainbow smelt are remarkable due to the wide variety of habitats in which they thrive. The mechanism of their cold tolerance and freeze resistance has been well documented. Smelts exposed to cold water express anti-freeze proteins and glycerol at relatively high levels to prevent freezing but with a costly energy expense (Driedzic and Short, 2007). In the present study we showed that cold-acclimated fish demonstrate substantially faster sustained swimming speeds compared to warm acclimated fish. We suggest that this elevated swimming performance is a requirement for maintenance of glycerol content in the blood, muscle and other tissues. The higher energetic costs of remaining in water that is at and below the freezing point of water requires active feeding throughout winter. To make active feeding possible, an acclimation response in the myotomal muscle permits high level of muscle performance in very cold muscle. This is the first study to demonstrate that the cold acclimation response on smelt includes shifts in swimming performance and muscle physiology.

From a muscle mechanics perspective, the swimming performance of cold-acclimated smelt is associated with modest shifts in isometric contractile properties. The cold-acclimated fish show somewhat faster muscle relaxation, but a larger sample size
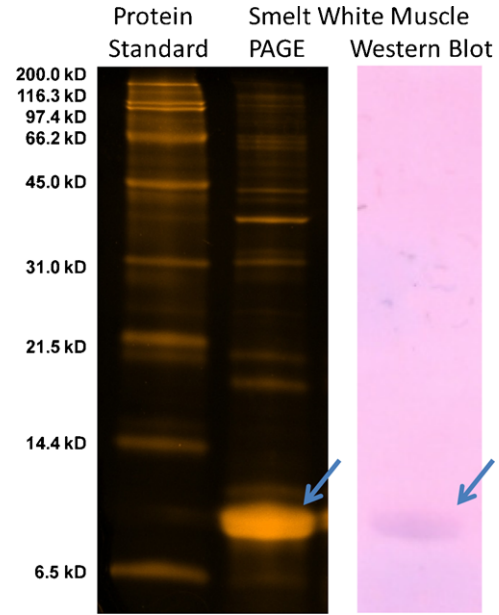


Fig. 5. Western blotting of parvalbumin (PV) in myotomal muscle from smelt. The band containing PV (indicated by arrows) was identified via blotting with an anti-PV antibody. The protein standards permitted estimation of the size of PV in smelt as 9.7 kD.

is needed for confidence in this relationship. However, the cold-acclimated fish do show a significant change in maximum shortening velocity. The white myotomal muscle of cold-acclimated smelt has a higher $V_{max}$ than muscle from warm acclimated fish. The difference is almost twofold for both of the years examined in this study. Similarly, the optimal shortening velocity, the muscle shortening velocity that generates the highest power, also increases with cold acclimation. $V_{opt}$ remains about 35% of $V_{max}$ under all condition, meaning that a doubling of $V_{max}$ with cold acclimation leads to a doubling of $V_{opt}$. The physiological relevance is that a higher $V_{opt}$ means the fish can employ a higher tailbeat frequency for a given tailbeat amplitude.
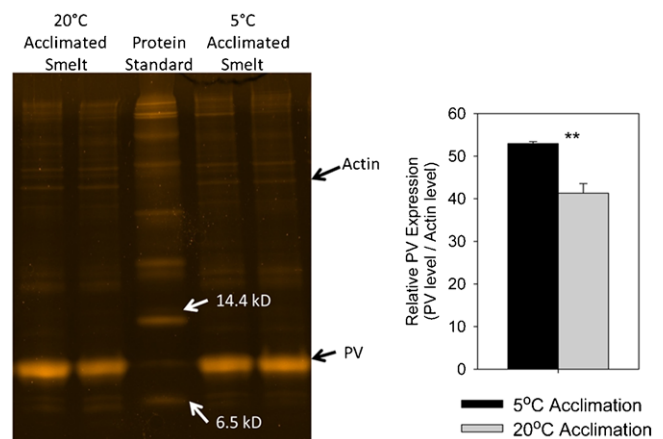


Fig. 6. PV content and thermal acclimation of mytomal muscle from 2012 smelt. Myotomal muscle PV content from was quantified from SDS-PAGE gels (left) as Sypro Ruby staining intensity of the PV band divided by the staining intensity of the actin band to normalize for variations in loading (which were modest). Myotomal muscle from CA fish had significantly higher PV content than that from WA fish (right) ($n=4$ for CA and WA fish).
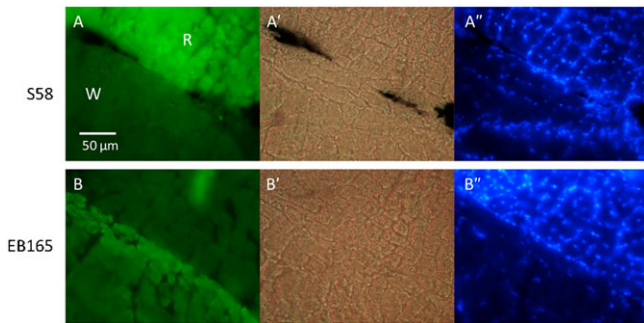
**Fig. 7. Anti-myosin heavy chain antibodies in smelt myotomal muscle.** Images of antibody stained muscle cross-sections are given for green fluorescence for anti-MyHC activity (**A,B**), white light (**A′,B′**) and blue fluorescence for Hoechst stain (**A″,B″**). In the top row, S58 binds preferentially to slow myosin, demonstrated by the green fluorescence of the slow or red muscle region (A). The slow muscle fibers are smaller in diameter in this region, as indicated by the staining of the peripheral nuclei of muscle bundles in cross-section (A″). In the bottom row, EB165 binds preferentially to fast myosin, as indicated by the green fluorescence of the fast or white muscle region (B). These faster fibers have larger diameter than the slow fibers (B″). The sections shown for S58 and EB165 are serial sections from approximately the same location in the red and white muscle border, but separated by ∼100 μm along the length of the fish. Scale bar: 50 μm.

As tailbeat amplitude tends to show little variation with speed in fishes (e.g. Coughlin et al., 2001), higher frequencies will increase swimming speed.

The workloop experiments support this conclusion. These experiments generate estimates of oscillatory power output – that is the power output that corresponds to activity of muscle during repeated cyclical contractions made during active swimming. The muscle of cold-acclimated fish produced twice the mass-specific power as that of warm-acclimated fish. This difference correlates with the shift in $V_{max}$ and provides a physiological mechanism
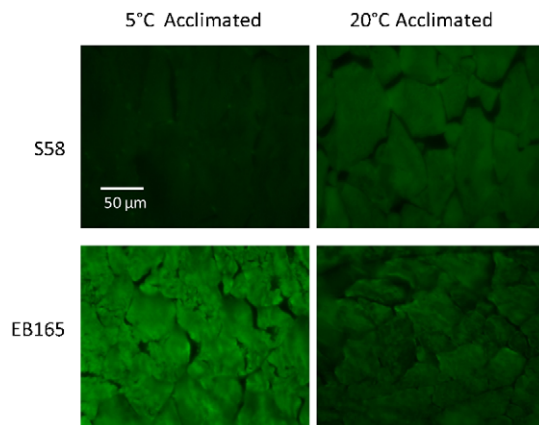


**Fig. 8. Shifts in MyHC expression with thermal acclimation of smelt myotomal muscle.** Representative images are presented for white mytomal muscle form CA and WA fish (*n*=4 for each) for the two experimental anti-MyHC antibodies. Staining intensity of S58 is higher in myotomal muscle from WA smelt (top right) compared to CA smelt (top left). Conversely, staining intensity of EB165 is higher in the muscle from CA smelt (bottom left) compared to WA smelt (bottom right). These results were observed in all four fish examined histologically. These results suggest a shift in MyHC expression with thermal acclimation. Cold acclimation is associated with a downregulation of slow MyHC and an upregulation of fast MyHC within the fast white myotomal muscle. Scale bar: 50 μm.

for understanding the improved swimming performance of cold-acclimated smelt. Exposure to cold temperature leads to muscle that produces more power during steady swimming across a wide range of tailbeat frequencies. This same result has been observed in other fish species, such as scup (Swank and Rome, 2001).

There was evident year-to-year variation in the rainbow smelt in this study. The $V_{max}$ values of the smelt in 2012 were nearly double those of the 2011 fish for both cold- and warm-acclimated smelt. In addition, the swimming speeds of the 2012 fish were nearly double those of the 2011 fish. The reason for the year-to-year variation is not known, but the hatchery from which the fish came was aware of differences in performance between the different year classes (John Whalen, Harmon Brook Farm and Maine Smelt Hatchery, personal communication). This area merits further study. The smelt in this study were raised in a hatchery from wild-caught fish that were entering freshwater for breeding. Variations in the health status and genetics of the parental generation presumably influence the performance of the generation under study, but twofold differences in maximum swimming speed and $V_{max}$ in the two year classes of fish raised under identical conditions is remarkable. Year-to-year variation in the successful recruitment of year classes of fishes has been well documented, with variables such as food availability, water temperature and winter storm activity affecting young-of-the-year success (and presumably health status) (e.g. Paxton et al., 2009).

### Swimming efficiency and muscle contractile properties

The differences in $V_{max}$ values and putative variation in MyHC content of cold- *versus* warm-acclimated suggest differences in energy consumption during swimming. The substantial differences in $V_{max}$ between thermal acclimation conditions is strong indicator of differences in MyHC content of the muscle, although that conclusion must be confirmed via molecular techniques (see below). If cold-acclimated fish express higher levels of fast MyHC in their myotomal muscle, ATPase activity would likely be higher in these fish. This should be reflected in metabolic rate during swimming. Specifically, the cold-acclimated fish may consume more oxygen during swimming than warm-acclimated fish.

Glycerol production as a thermal acclimation response appears to create a paradox for smelt. To remain active in cold water, these fish express elevated levels of anti-freeze proteins and glycerol and accumulate relatively high concentrations of several osmolytes in their blood and tissues, including muscle. To supply the energy needed to maintain glycerol production, however, requires changes in muscle proteins that increase the energetic cost of the muscle. This further increases the need for dietary intake of energy in a seeming positive feedback loop. What is the selective advantage for this complex thermal acclimation response in smelt? Small size limits migratory potential (Hein et al., 2012), so staying in place may be the only option for rainbow smelt. Enduring winter without freezing therefore requires a series of physiological changes that last almost half a year each winter season.

### Gene expression and thermal acclimation of smelt muscle

A fuller understanding of the thermal acclimation response in smelt muscle would be possible through physiological genomics (Whitehead, 2012). Gene array and subsequent qPCR could help elucidate the mechanisms of shifting myosin expression and other

muscle proteins that are likely changing (e.g. Hall et al., 2011; Hall et al., 2012). For instance, the regulatory myosin light chain (MyLC2) is thought to influence MyHC activity (Gordon et al., 2000). Further, variations in MHC protein expression are known to be associated with variations in other muscle proteins, such as MyLC2 (Schiaffino and Reggiani, 1996). MyLC2 may modulate or fine-tune cross-bridge kinetics (Andruchov et al., 2006). Shifts in the relative contribution of fast *versus* slow isoforms of MyLC2 may affect $V_{max}$ (Bottinelli and Reggiani, 2000), and phosphorylation of MyLC2 influences force production at sub-maximal $Ca^{2+}$ levels (e.g. Wang et al., 2006). We suggest it may contribute to the thermal acclimation response in smelt.

Sarco/endoplasmic retituculum $Ca^{2+}$ ATPase (SERCA) is another muscle protein that may be influenced by thermal acclimation in smelt. SERCA expression was recently shown to vary with thermal acclimation in rainbow trout (*Oncorhyncus mykiss*) (Korajoki and Vornanen, 2012). SERCA levels were increased in cold-acclimated trout hearts, presumably enhancing SR $Ca^{2+}$ activity. Shifts in SERCA levels in the myotomal muscle of smelt could explain observed changes in isometric contractile properties. Seebacher et al. demonstrated that SERCA activity enhances fatigue resistance in swimming carp (*Cyprinus carpio*) (Seebacher et al., 2012), suggesting that enhanced SERCA levels in cold-acclimated smelt would enhance swimming performance. Troponin I has also been recently shown to vary in its expression under the influence of thermal acclimation. Alderman et al. used semi-quantitative real-time PCR to demonstrate that cold acclimation affects the relative expression of seven different isoforms of TnI in skeletal, smooth and cardiac muscle in rainbow trout (Alderman et al., 2012).

The list of muscle proteins that modulate muscle activity and are labile to thermal acclimation in other species is long, including MyHC, MyLC2, SERCA and Troponin I, as discussed above. Additional muscle proteins, such as ryanodine receptors, MyLC1, other elements of the troponin complex and tropomyosin may also contribute to thermal acclimation in rainbow smelt. Hall et al. demonstrated the efficacy of a salmonid cDNA array for rainbow smelt gene expression (Hall et al., 2011) (trout and smelt are both salmoniforms). Hall and colleagues focused on genes associated with glycerol production (Hall et al., 2011). Thermal acclimation to the swimming muscle myotome in rainbow smelt should be an interesting target for the analysis of gene expression of muscle proteins such as MyHC, MyLC2, MyLC1, SERCA and MANY others.

## Conclusions

Smelt show a comprehensive thermal acclimation response, with many labile elements in their response to prolonged exposure to cold water. In addition to well documented increases in anti-freeze protein, glycerol and other osmolytes in their plasma and other tissues, these fish show substantial changes in muscle function. Cold-acclimation leads to dramatic increases in swimming performance along with corresponding changes in muscle contractile properties and the protein composition of the myotomal muscle. Importantly, we observed thermal acclimation effects after six weeks at $5°C$. Smelt in the North Atlantic encounter temperatures below $0°C$ for extended portions of the winter; their thermal acclimation response may be even more impressive. One thing that is known about smelt exposed to extremely cold water is that their thermal acclimation response includes a significant increase in plasma and muscle tissue content of glycerol, urea and other osmolytes. How a doubling of osmolarity in the tissue may affect muscle function remains an intriguing question.

## Acknowledgements

## Competing Interests

The authors have no competing interests to declare.

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