Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (*Danio rerio*)

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Both exercise training and cold acclimatization induce muscle remodelling in vertebrates, producing a more aerobic phenotype. In ectothermic species exercise training and cold-acclimatization represent distinct stimuli. It is currently unclear if these stimuli act through a common mechanism or if different mechanisms lead to a common phenotype. The goal of this study was to survey responses that represent potential mechanisms responsible for contraction- and temperature-induced muscle remodelling, using an ectothermic vertebrate. Separate groups of adult zebrafish (*Danio rerio*) were either swim trained or cold acclimatized for 4 weeks. We found that the mitochondrial marker enzyme citrate synthase (CS) was increased by $1.5\times$ in cold and by $1.3\times$ with exercise ($P < 0.05$). Cytochrome c oxidase (COx) was increased by $1.2\times$ following exercise training ($P < 0.05$) and $1.2\times$ ($P = 0.07$) with cold acclimatization. However, only cold acclimatization increased β-hydroxyacyl-CoA dehydrogenase (HOAD) compared to exercise-trained (by $1.3\times$) and pyruvate kinase (PK) relative to control zebrafish. We assessed the whole-animal performance outcomes of these treatments. Maximum absolute sustained swimming speed (U_{crit}) was increased in the exercise trained group but not in the cold acclimatized group. Real-time PCR analysis indicated that increases in CS are primarily transcriptionally regulated with exercise but not with cold treatments. Both treatments showed increases in nuclear respiratory factor (NRF)-1 mRNA which was increased by $2.3\times$ in cold-acclimatized and $4\times$ in exercise-trained zebrafish above controls. In contrast, peroxisome proliferator-activated receptor (PPAR)-α mRNA levels were decreased in both experimental groups while PPAR-β_1 declined in exercise training only. Moreover, PPAR-γ coactivator (PGC)-1α mRNA was not changed by either treatment. In zebrafish, both temperature and exercise produce a more aerobic phenotype, but there are stimulus-dependent responses (i.e. HOAD and PK activities). While similar changes in NRF-1 mRNA suggest that common responses might underlie aerobic muscle remodelling there are distinct changes (i.e. CS and PPAR-β_1 mRNA) that contribute to specific temperature- and exercise-induced phenotypes.

(Resubmitted 10 August 2006; accepted after revision 19 September 2006; first published online 21 September 2006)

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Skeletal muscle plays important roles not only in locomotion but also in whole-body metabolic homeostasis. Within a species, skeletal muscle shows a high degree of phenotypic plasticity, both developmentally and in response to energetic and environmental stress. This plasticity may involve remodelling the muscle into a more aerobic phenotype through changes in mitochondrial mass and the upregulation of metabolic pathways involved in aerobic metabolism. Whether there is remodelling of either the anaerobic or aerobic machinery of muscle depends on the type and duration of the stress. Moreover, the molecular mechanisms involved in remodelling may differ, depending on many factors including the energetic status of the animal. It has been known for decades that repeated contractions associated with endurance exercise training can lead to a more aerobic phenotype of skeletal muscle in mammals (e.g. Gollnick & King, 1969; Gollnick *et al.* 1972). Indeed, in mammals, contraction-induced muscle metabolic remodelling and mitochondrial biogenesis are thought to occur through a series of cellular events that begin with the activation of various signalling pathways (reviewed in Moyes & Hood, 2003). Calcium signalling appears to play a major role in contraction-induced gene expression acting through

the Ca^{2+}/calmodulin-dependent kinase (CaMK) pathway (Chin, 2005), but multiple signals including changes in ATP turnover and reactive oxygen species (ROS) are likely to also be involved (Adhihetty et al. 2003; Moyes & Hood, 2003). The corresponding increased expression of transcription factors eventually elevates mRNA for genes encoding enzymes of various metabolic pathways and of mitochondrial proteins (Hood, 2001). Transcription factors shown to be important in mammals for differentiation of muscle fibres to a more aerobic phenotype include the nuclear respiratory factors (NRF)-1 and -2, peroxisome proliferators-activated receptors (PPAR)-α, -β/δ, -γ and PPAR-γ cofactor (PGC)-1α (Lin et al. 2002). Along with chronic exercise there are other stimuli known to induce muscle remodelling in mammals, including shivering thermogenesis, chronic electrical stimulation, and hyperthyroidism (reviewed in Leary & Moyes, 2000). Although there are some differences in how remodelling is induced (Weitzel et al. 2003), a common stimulus (hypermetabolism) leads to similar muscle phenotypes. In non-mammalian ectothermic vertebrates, quite different stimuli can produce similar changes in muscle phenotype. For example, in muscle a more aerobic phenotype can occur in response to chronic cold temperature exposure, yet this can coincide with a decrease in metabolic rate. Many fish species have been found to increase muscle mitochondrial content to compensate for the deceleration effect of low temperature on metabolism to maintain suitable levels of muscular activity for feeding and reproducing. In fact, in teleost fishes, cold-induced skeletal muscle remodelling (Battersby & Moyes, 1998) occurs to the same extent as exercise training (Johnston & Moon, 1980; Farrell et al. 1991). Relatively few studies have investigated the potential molecular mechanisms responsible for temperature-induced muscle remodelling in ectotherms (e.g. Battersby & Moyes, 1998; Lucassen et al. 2003; Malek et al. 2004). No studies have investigated these mechanisms in response to exercise. It is possible that there are conserved mechanisms that lead to a more aerobic muscle phenotype regardless of the initial stimuli. Alternatively, different stimuli may rely on unique molecular mechanisms but lead to the same phenotype. In addition, it is unclear how these different stimuli affect qualitative aspects of mitochondrial metabolism. Specifically, which stimuli trigger increases in mitochondrial volume density *versus* the ability of mitochondria to preferentially oxidize fatty acids over pyruvate, amino acids or lactate? Current evidence suggests that the temperature-induced muscle remodelling in fish may occur through a separate set of molecular mechanisms than contraction-induced remodelling and different from those seen in mammals (Battersby & Moyes, 1998; Malek et al. 2004). Moreover, adult zebrafish (*Danio rerio*) represent a potentially powerful model organism for making these comparisons. The zebrafish holds many advantages for the study of muscle physiology, having a comprehensive genomic database, homologous muscle fibre masses, ability to exercise in large groups and amenability to large-scale screening for examination of heritable aspects of muscle performance.

The goals for this study were to exploit the many advantages that already exist for zebrafish as a model organism and to extend this to the study of adult vertebrate muscle phenotypic plasticity, to determine: (i) the changes in key metabolic and mitochondrial enzymes used as indices of skeletal muscle remodelling and whether they are similar with cold acclimatization and exercise training; (ii) the changes in transcription for genes that encode these enzymes and in the transcription factors implicated in muscle remodelling in mammals; and (iii) the whole-organism performance using a standardized critical swimming speed test after exercise training and cold acclimatization. We hypothesize that contraction and cold-induced muscle remodelling will produce a similar phenotype, but the patterns of gene expression will differ due to the divergent nature of the initial stimulus.

Methods

Animals

The present study was approved by the McMaster University Animal Research Ethics Board (AREB). Adult zebrafish (*Danio rerio*) were obtained from a local supplier (PetsMart, Ancaster, ON, Canada) and control fish were housed in 3 l tanks (Aquatic Habitats, Apopka, FL, USA) in recirculating dechlorinated Hamilton tap water. Fish were fed commercial tropical fish food (Top Fin, Phoenix, AZ, USA) 1–2 times per day. Unless otherwise noted, water temperature was maintained at 28°C and the room was kept on a 12 h : 12 h light : dark cycle. Water quality was monitored weekly. These experiments were carried out over a 14-month period using two separate groups of zebrafish. Group 1 was used for the enzyme activities and gene expression measurements and group 2 for the swimming performance (U_{crit}) and metabolite measurements. Since body mass can affect metabolism (Hochachka, 1987) and body length can affect swimming power output, we assessed both in all groups after treatments. Physical characteristics of these two groups at the time of tissue sampling (see below) appear in Table 1. Condition factor (k) is a measure of the condition of fish based on the cubes of weight and length, because growth in weight are proportional to growth in volume (Fulton, 1902). If $k < 1.0$ a fish is in poor condition and if $k > 1.4$, a fish is in good to excellent condition. This was calculated using the following equation:

$$k = 100 \, {}^* \, \text{mass} \, (\text{length}^{-1})^3 \qquad (1)$$

where mass was measured in g and length in cm.

Table 1. Physical characteristics of all adult zebrafish from Group 1 used for enzyme activity and gene expression measurements and Group 2 used for swimming performance and muscle metabolite measurements

All fish	Group	Weight (g)	Length (cm)	Condition factor, k
Control	1	0.55 ± 0.03 (36)	3.31 ± 0.06 (31)	1.52 ± 0.05 (31)
	2	0.41 ± 0.01 (29)‡	2.84 ± 0.04 (29)‡	1.74 ± 0.09 (29)
Cold acclimatized	1	0.58 ± 0.02 (33)	3.29 ± 0.04 (33)	1.61 ± 0.04 (33)
	2	0.52 ± 0.02 (30)*	3.07 ± 0.06 (30)*‡	1.79 ± 0.06 (30)
Exercise trained	1	0.48 ± 0.02 (32)†	3.00 ± 0.04 (32)*†	1.77 ± 0.05 (32)
	2	0.54 ± 0.03 (26)*	3.13 ± 0.05 (26)*‡	1.81 ± 0.09 (26)

The total number of individuals in each category appears in parentheses. Values are means $\pm$ S.E.M. *Significantly different from controls from the same group, †significantly different from cold acclimatized from the same group, ‡significantly different from the same treatment in group 1 ($P < 0.05$).

Exercise training

Approximately 60 fish (group 1) were transferred to a Brett-style recirculating swim flume (Brett, 1964) kept at 28°C, and were allowed to acclimatize to the tunnel for 1 week. Several litres of water were drained from the flume daily and were replaced with fresh 28°C dechlorinated water. Fish were exercised twice daily for 3 h, 6 days a week with a 2 h rest period when the flow was turned off and the fish were fed. Starting at a speed of 6.0 cm s^{-1} (equivalent to 2 BL s^{-1}, where BL is body length), speed was increased by 1 BL s^{-1} each week to a final speed of 15.0 cm s^{-1} (5 BL s^{-1}) and training lasted for a total of 4 weeks. The water current was kept at a speed of 3 cm s^{-1} ($\sim$1 BL s^{-1}) at all other times (i.e. non-training and overnight). Based on previous measures of maximal sustained swimming speed (Plaut, 2001), these training speeds represent low to moderate exercise intensity and aerobic training for zebrafish ($\sim$25–30% maximal sustainable swimming speed). We experienced $\sim$30% mortality in the trained fish (compared to $\sim$15% for controls) as a result of a single occurrence of a dislodged air stone in the swim tunnel at 3 weeks. In the first set of experiments, the fish were removed from the swim flume and killed by flick of the finger to the head; length and weight were recorded, and fish were then decapitated. The skin, tail, and gut were removed, leaving the muscle mass which was quickly immersed in liquid nitrogen and then stored at −80°C for future analysis.

Temperature acclimatization

Approximately 50 fish were transferred to two 40 l aquaria kept at 28°C in a 10°C cold room. The temperature was lowered 2°C day^{-1} over 1 week to 18°C, and fish were kept at this temperature for a further 3 weeks. At the end of the 4 week period, muscle samples were taken as described above. Control fish were maintained at 28°C.

Enzyme activities

Muscles from 10 fish in each group were powdered using a liquid nitrogen-cooled mortar and pestle. Maximal enzyme activities were measured on individual fish by taking approximately 50 mg from each individual and homogenizing in 20 volumes of extraction buffer consisting of 20 mM Hepes (pH 7.0), 1 mM EDTA, and 0.1% Triton X-100, using a glass homogenizer. The activities (in U g^{-1}) of cytochrome c oxidase (COx), citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (HOAD), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were assayed in these crude homogenates at 28°C as previously described (McClelland *et al.* 2005). Analysis was carried out in 96-well format on either a Spectramax 340pc or a Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Optical path lengths were determined for each machine according to Brooks (1994).

Total genomic DNA per gram of muscle tissue was determined on enzyme homogenates as previously described (McClelland *et al.* 2005). Briefly, aliquots of homogenates were digested overnight at 55°C using digestion buffer consisting of (mM): 100 NaCl, 10 Tris-HCl (pH 8.0), 25 EDTA (pH 8.0) 0.5% SDS and proteinase K (0.2 mg ml^{-1}). DNA was quantified using PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR, USA), using a standard curve constructed from a known amount of zebrafish DNA isolated from additional fish as described above. Samples and standards were loaded on a 96-well microplate, and fluorescence was measured on a fluorescence spectrophotometer (excitation at 485 nm and emission at 538 nm on a Spectramax Gemini Spectrofluorometer, Molecular Devices, Sunnyvale, CA, USA). Total protein was measured by the Bradford method (Bio-Rad, Mississauga, ON, Canada) on the same homogenates used for enzyme activity and DNA measurement.

Table 2. Primer sequences used for RT-PCR and real-time PCR analysis of mRNA expression in zebrafish

Gene	Forward primer	Reverse primer	Amplicon size (bp)
(Ascension no.)	(5′–3′)	(5′–3′)	
CS (BC045362)	agcgctgctatgaatggtct	ctgaggaagacagaccctcg	199
CYC-1* (AY996924)	acttagccaaccaggagcac	gggtggaagaagtcagaagc	163
β-actin* (AF057040)	ttctggtcggtactactggtattgtg	atcttcatcaggtagtctgtcaggt	170
HOAD (ZGI TC279717)	ccacaggacattcagtggtg	gtcagtgccatgaacgacag	200
LDH-A* (NM131246)	cacagttgaagatggcctca	tgtgcgtcttgagaaacagg	201
Dlat† (AY188775)	tggaactacctgccttgtcc	ctccaatgggcacatctctt	201
PGC-1α (XM678107)	tgaggaaaatgaggccaact	agcttcttcagcagggaagg	200
PPAR-α (U93473)	gttcttcaggcggacgatt	agggggaaacacttctgaaa	121
PPAR-β1 (AF342937)	gcgtaagctagtcgcaggtc	tgcaccagagagtccatgtc	204
NRF-1 (NM131680)	aggccctgaggactatcgtt	gctccagtgccaacctgtat	202
sMyHC (AY333451)	agcccagaagcaactcaaga	gttcctgctcagccagtttc	203
fMyHC (AF165817)	aaagcaaaggccaatcttga	tgttttcctcgtgtgagctg	200

CS, citrate synthase; CYC-1, cytochrome c oxidase subunit 1; HOAD, β-hydroxyacyl-CoA dehydrogenase; LDH-A, lactate dehydrogenase A subunit; dLat, pyruvate dehydrogenase E_2 subunit; PPAR, peroxisome proliferator-activated receptor; PGC, PPAR-γ cofactor; sMyHC, myosin heavy chain slow isoform; fMyHC, myosin heavy chain fast isoform; NRF, nuclear respiratory factor. *Ton *et al.* (2003); †Taylor *et al.* (2004).

mRNA analysis by real-time PCR

Total RNA was extracted from ∼50 mg of skeletal muscle from additional fish in group 1 using Trizol reagent (Invitrogen Life Technologies, Calsbad, CA, USA). Muscle was first powdered using a liquid nitrogen-cooled mortar and pestle, and placed in Trizol and homogenized using a motorized homogenizer (Fisher Scientific). RNA was then further purified and DNase treated (Qiagen DNase I) using RNeasy columns (Qiagen Sciences, Mississauga, ON, Canada). Total RNA was quantified at 260 nm, and purity was verified by absorbance $(A)_{260/280}$ ratios greater than 1.6 (normally 1.8–2.0). Random RNA samples were also denatured and separated on 1% agarose-formaldehyde gels, and ribosomal RNA stained with ethidium bromide to determine if any degradation occurred during tissue storage and processing. First-strand cDNA was synthesized from 2 μg of total RNA using Stratascript reverse transcriptase (RT, Stratagene, La Jolla, CA, USA), RNase inhibitor (Amersham Biosciences, Piscataway, NJ, USA) and random primers (Promega Corp., Madison, WI, USA) for 5–6 individuals from each group. mRNA expression for genes of interest was measured in duplicate with a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as a reference dye (either Bio-Rad iTaq SYBR green supermix (Bio-Rad Laboratories Inc., Mississauga, ON, Canada), or Stratagene SYBR green supermix (Stratagene, La Jolla, CA, USA)). Primers (Table 2) were designed using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) or using previously published primer sequences (Ton *et al.* 2003; Taylor *et al.* 2004). Specificity of all primer pairs was tested by RT-PCR and gel electrophoresis using DNA size standards (Fermentas GeneRuler). In addition a SYBR green dissociation curve was run to ensure a single fluorescence peak at the appropriate temperature. Expression levels are relative to a randomly selected control sample (calibrator), and are calculated by the delta-delta threshold cycle (C_t) method (Livak & Schmittgen, 2001) using the Stratagene software:

$$2^{-\Delta\Delta C_t} \tag{2}$$

where $\Delta\Delta C_t$ is the ΔC_t for the unknown $-\Delta C_t$ for the calibrator sample. ΔC_t is the difference between the C_t for the target gene and the housekeeping gene β-actin. A value of 2 is used for 100% efficiency, but was modified to reflect true efficiencies determined from standard curves. Negative controls included RNA processed for cDNA in the absence of RT (no RT) and reactions run with RNase-, DNase-free H_2O in place of cDNA template (no template).

Total RNA per gram of muscle was quantified on a separate set of five fish from control and treatment groups in group 1 by extracting RNA from 40–100 mg of tissue in 1 ml of Trizol, and sampling the aqueous phase without further purification. Quantification of 2 μl of each sample was via Ribogreen (Molecular Probes, Eugene, OR,

USA) compared to an RNA standard curve (excitation at 485 nm and emission at 520 nm on a Spectramax Gemini Spectrofluorometer, Molecular Devices, Sunnyvale, CA, USA).

Critical swimming tests (U_{crit})

The critical swimming speed test in fish involves a stepwise increase in swimming speed until fatigue. This test is used to assess prolonged swimming capacity, and to estimate maximum sustained swimming speed. Fish in group 2 underwent the same training and cold acclimatization protocols as described above. Critical swimming tests were carried out in a 30 cm × 8 cm section of a Brett-style swim tunnel at either 18°C or 28°C as previously described for zebrafish (Plaut, 2000). Fish were placed in the swim tunnel in groups of 10–15 and allowed to acclimatize to the tunnel for 48 h. The exercise training groups were tested 48 h after their last training session. Swimming speed was progressively increased from 1 BL s^{-1} (3 cm s^{-1}) every 5 min (as used by other studies on small fish; Plaut, 2000; Hammill *et al.* 2004) until individuals could not hold their position in the current and rested on a grid at the back of the swimming chamber. The final swimming speed achieved prior to fatigue was defined as the maximal sustainable critical swimming speed (U_{crit}) and calculated by the following formula:

$$U_{crit} \ V_{1s} + (t_s/t_i) \times V_i \qquad (3)$$

where V_{ls} is the final speed (cm s^{-1}) completed, t_s is the time spent at the final speed, t_i is the time increment (min), and V_i is the speed increment (Plaut, 2001; Richards *et al.* 2002). At fatigue, each individual fish was siphoned out of the swim flume, and their skeletal muscle quickly dissected and frozen using liquid nitrogen-cooled tongs. These samples were used to assess post-exercise (U_{crit}) metabolite levels at fatigue. Separate control individuals were sampled for resting baseline metabolite levels after anaesthetic (MS222) overdose, then muscle quickly frozen using liquid nitrogen-cooled tongs. All samples were placed at −80°C for future metabolite analysis (see below).

Metabolites

Muscle samples were powdered using a liquid nitrogen-cooled mortar and pestle. Metabolites were extracted and quantified according to standard methods (Bergmeyer, 1974) adapted to 96-well format for the Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Briefly, samples were homogenized in 600 μl of cold 8% perchloric acid, and homogenized using a power homogenizer. The homogenate was centrifuged at 10 000 g for 10min at 4°C, and an aliquot of the supernatant was removed and stored

at −80°C for lactate determination. Final assay conditions for lactate were 2 mM NAD$^+$ in glycine/hydrazine buffer pH 9.2 (Sigma-Aldrich, Oakville, ON, USA) and the assay was started with 5 U LDH (Sigma from bovine muscle). A separate aliquot was neutralized with 3 M K$_2$CO$_3$ and centrifuged at 10 000 g for 10 min at 4°C. The supernatant was assayed for creatine phosphate (CrP) and ATP on the same day as homogenization. Final assay conditions for CrP and ATP were (mM) 5 MgCl$_2$, 20 Tris (pH 8.0), 5 glucose, 2 NAD$^+$, 1 U ml^{-1} glucose-6-phophate dehydrogenase (G6PDH, Roche, from *L. mesenteriodes*), and for CrP only 1 mM ADP and 1.5 U ml^{-1} hexokinase (HK, Roche, from yeast). For ATP the reaction was started with 1 U of HK, and for CrP the reaction was started with 2.5 U of creatine phosphokinase (CPK, Sigma, from rabbit muscle).

Statistical analysis

Data presented are means ± S.E.M. Data were analysed using a combination of ANOVA, *post hoc* tests (Tukey's) and *t* tests with Bonferoni correction. Data with unequal variance were analysed using a Mann–Whitney rank sum test.

Results

Muscle total RNA, protein and DNA

Changes in total RNA, DNA and protein per gram of muscle were determined in response to 4 weeks of exercise training or 4 weeks of cold-acclimatization in group 1 fish. There was no significant difference in total RNA between the groups (Fig. 1*A*). However, both cold acclimatization (0.34 ± 0.02 mg g^{-1}) and exercise training (0.33 ± 0.02 mg g^{-1}) showed significant increases in total DNA content relative to controls (0.18 ± 0.02 mg g^{-1}; Fig. 1*B*). Total protein content was greater in cold-acclimatized (103 ± 4 mg g^{-1}) than either exercise-trained (75 ± 7 mg g^{-1}) or control (73 ± 6 mg g^{-1}) fish muscle (Fig. 1*C*). Exercise-trained fish from group 1 were significantly lighter and shorter than cold-exposed fish. Exercised fish were also shorter but did not differ in weight from controls. Exercise-trained and cold-acclimatized fish from group 2 were heavier and longer than controls. All fish in both groups 1 and 2 were in good physical condition with *k* values all over 1.5. (Table 1).

Enzyme activities

Activities for several enzymes in muscle metabolism were measured at 28°C (Fig. 2). Zebrafish muscle responded to prolonged cold exposure and exercise training with

increases in CS by 1.5× and 1.3×, respectively. Exercise increased COx activity 1.2× ($P < 0.05$), while cold acclimatization showed a non-significant rise ($P = 0.07$) of 1.2× above controls. Only cold exposure increased the activity of the β-oxidation enzyme HOAD used as an index of fatty acid oxidation potential, by 1.3× above that measured in trained fish muscle ($P < 0.05$; Fig. 2C). Both cold acclimatization and exercise increased the activity of LDH (Fig. 2D). Cold also increased the activity of PK, but exercise training did not affect this enzyme (Fig. 2E).
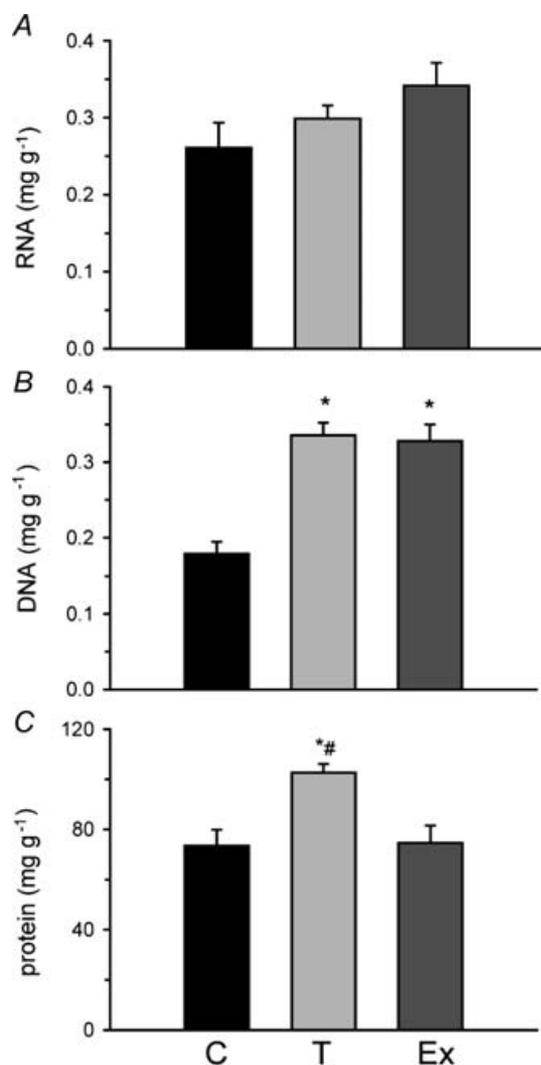
Figure 1. Total skeletal muscle RNA (A) DNA (B) and protein (C) from zebrafish cold acclimatized (T) to 18°C for 4 weeks, exercise-trained (Ex) for 4 weeks at 28°C compared to 28°C controls (C) from group 1 fish

$N = 9$—10 for DNA and protein measured on the same samples; $n = 5$ for RNA from separate fish. *Significantly different from controls, #significantly different from exercise-trained fish. Error bars represents S.E.M.

Gene expression

mRNA expression was assessed using real-time PCR for genes encoding mitochondrial enzymes involved in electron transport (CYC-1), the Kreb's cycle (CS), carbohydrate oxidation (pyruvate dehydrogenase E_2 subunit, dLat), β-oxidation of fatty acids (HOAD) and glycolysis (LDH) plus contractile elements (MyHC). Only mRNA levels for *CS* in exercise-trained individuals increased significantly (Fig. 3A). All other mRNA levels examined did not change significantly with exercise training or cold exposure relative to controls. mRNA expression was also measured for transcription factors implicated in muscle aerobic remodelling in mammals. There was a significant increase in NRF-1 expression relative to controls by 4× with exercise and 2.3× with cold acclimatization ($P < 0.05$). There were also significant decreases in PPAR-α in both experimental groups and PPAR-β_1 in exercise-trained zebrafish (Fig 4B and C). Levels of mRNA for PGC-1α did not change significantly after 4 weeks' training or cold exposure (Fig. 4D).

Critical swimming speed (U_{crit})

Critical swimming tests revealed that only exercise training resulted in a significant increase in absolute maximum sustainable swimming speed (U_{crit}; Fig. 5A) at 28°C. Exercise-trained zebrafish reached an average sustainable swimming speed at 28°C of 63 ± 2 cm s^{-1} compared to 57 ± 2 cm s^{-1} for controls at the same temperature. Both controls and cold-acclimatized zebrafish had significantly decreased swimming performance at 18°C. Moreover, prolonged exposure to 18°C in the cold acclimatized group did not improve swimming performance at 28°C relative to controls or exercise-trained fish (Fig. 5). Group 2 cold acclimatized (T) and exercise-trained (Ex) zebrafish were heavier and longer than control fish (Table 2). This resulted in similar relative U_{crit} values to controls when standardized to individual body length (as BL s^{-1}, Fig. 5B).

Metabolites

Depletions of metabolites when fatigue was reached were compared to resting control fish at 28°C. All swimming tests in all groups resulted in large accumulations of lactate in the muscle. The highest levels at 15 ± 1 mM were seen in the exercise-trained fish, with resting levels in controls of 0.9 ± 0.3 mM (Fig. 6A). Significant depletions in CrP and ATP were seen in all trials. There was no significant difference in the ATP levels between the groups after exercise, but CrP reached a lower level in exercise-trained fish relative to cold acclimatized fish at 28°C ($P < 0.05$, t test) (Fig. 6B and C).

Discussion

Adult zebrafish muscle remodelling

Adult zebrafish muscle demonstrates considerable phenotypic plasticity to stresses that produce a more aerobic phenotype. Both cold-induced and contraction-induced muscle remodelling show similar increases in biochemical markers (Fig. 2) of mitochondrial density (CS) and pyruvate to lactate flux capacity (LDH). However, only in the exercise-trained fish did mRNA levels for CS increase significantly (Fig. 3). The transcription factor NRF-1 was also elevated in cold-acclimatized ($2.3 \times$) but to a greater extent in trained ($4 \times$) fish compared to controls (Fig. 4). Important differences exist with muscle response to temperature (Fig. 2), stimulating the β-oxidation machinery (HOAD) as well as potential for glycolytic flux (PK) not observed in the exercise-trained fish. Muscle mRNA levels for PPAR-α, a transcription factor important for the regulation of

the fatty acid pathway in mammals (Gulick *et al.* 1994; Vredendaal *et al.* 1998), were downregulated with both experimental treatments compared to controls. Another isoform of this factor, PPAR-β_1 had mRNA levels that were downregulated in the exercise-trained but not in the cold-acclimatized group (Fig. 4). Surprisingly, the cofactor PGC-1α, important for enhancing DNA binding of PPARs (Lemberger *et al.* 1996), for mitochondrial biogenesis and for producing an aerobic muscle phenotype in mammals (Koves *et al.* 2005), did not change in expression after either treatment (Fig. 4*D*). Chronic cold temperature and exercise both produce a more aerobic phenotype in adult zebrafish muscle. Interestingly, similar changes in NRF-1 mRNA suggest this as a common response that might underlie aerobic muscle remodelling because similar changes are seen in endurance-trained mammalian muscle. However, there are stimulus-dependent changes in enzyme profiles, transcription factors and other gene-expression profiles, as well as absolute sustained
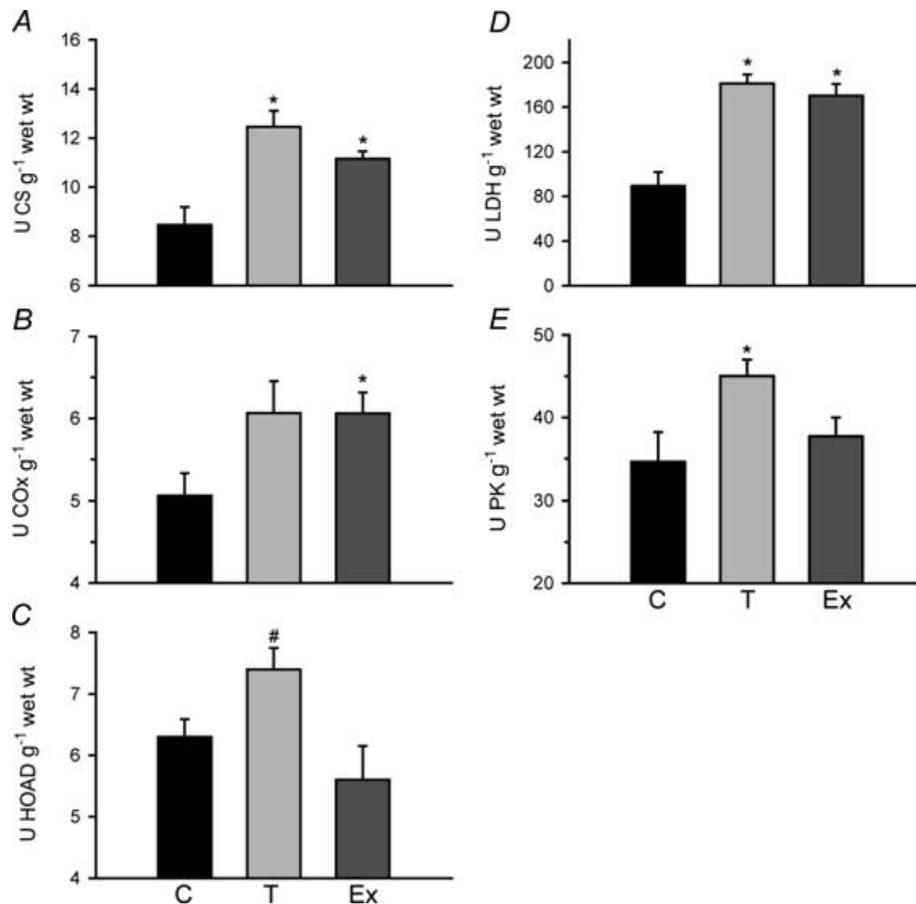
Figure 2. Skeletal muscle enzyme activities (assayed at 28°C) in adult zebrafish (*Danio rerio*) after exposure to cold (18°C, T) for 2 and 4 weeks and after 4 weeks of exercise training (Ex) at 28°C, compared to 28°C controls (C)
Values are presented for *A*, citrate synthase (CS) *B*, cytochrome oxidase (COx), *C*, β-hydroxyacyl-CoA dehydrogenase (HOAD), *D*, lactate dehydrogenase (LDH) and *E*, pyruvate kinase (PK) All measurements are from group 1 $n = 10$, except HOAD from group 2, $n = 5$. *Significantly different from controls ($P < 0.05$). U, μmol min^{-1}. Error bars represent S.E.M.

swimming speeds, which suggest distinct responses contribute to the development of temperature- and exercise-induced muscle phenotypes.

Enzyme activities in cold *versus* exercise

Enzyme activities can be used as biochemical indices of mitochondrial density (CS; Reichmann *et al.* 1985), the electron transport chain (COx), fatty acid oxidation capacity (HOAD) and capacity of glycolytic flux (PK, LDH). Previous research on other fish models has studied muscle biochemical and structural changes in separate studies either with cold-acclimatization or with prolonged exercise. These studies have demonstrated increases in mitochondrial and cytosolic enzymes in muscle, following cold acclimatization (mainly trout: Cordiner & Egginton, 1997; Battersby & Moyes, 1998; Bouchard & Guderley, 2003; McClelland *et al.* 2005) and exercise training (coalfish *Pallachius virens*: Johnston & Moon, 1980; and rainbow trout: Farrell *et al.* 1991). Proliferation of the mitochondria results in increased surface area over

which diffusion between the mitochondria and cytosolic compartments would occur (Guderley & St-Pierre, 2002), and a reduction in intermitochondrial diffusion distances (Portner, 2002). This is beneficial for both life in the cold to compensate for the deceleration effect on metabolism, and for the increased muscle aerobic capability important for exercise. Here we directly compare these stressors at the level of both muscle biochemistry and gene expression changes. The significant increase in maximal CS activities seen in cold-acclimatized and exercise-trained zebrafish (Fig. 2*A*) confirms that both stimuli increase muscle mitochondrial mass in fish muscle. Seasonal acclimatization to cold in trout also increases LDH in white muscle (Cordiner & Egginton, 1997) as observed here for mixed muscle in zebrafish (Fig. 2). Previous studies have found that exercise training also increases HOAD in both red and white muscle (Johnston & Moon, 1980; Farrell *et al.* 1991). Perhaps this is explained by the greater training intensity ($\sim$60% U_{crit}) and duration used in these studies compared to the 25–30% U_{crit} used in the current study. Moreover, muscle
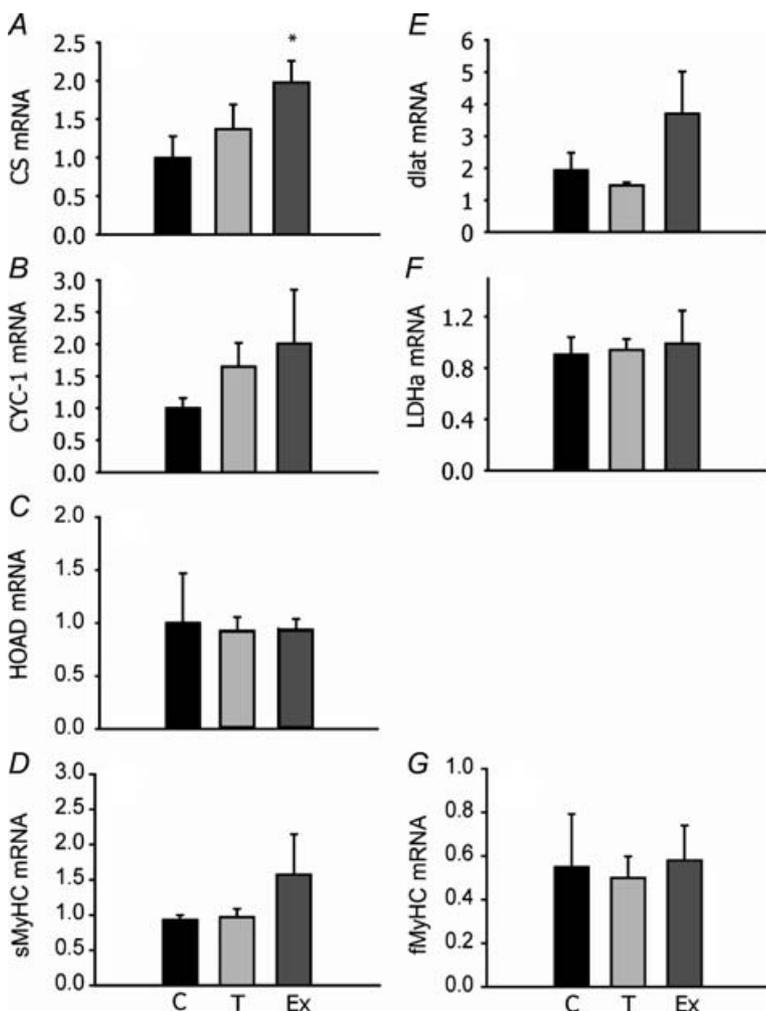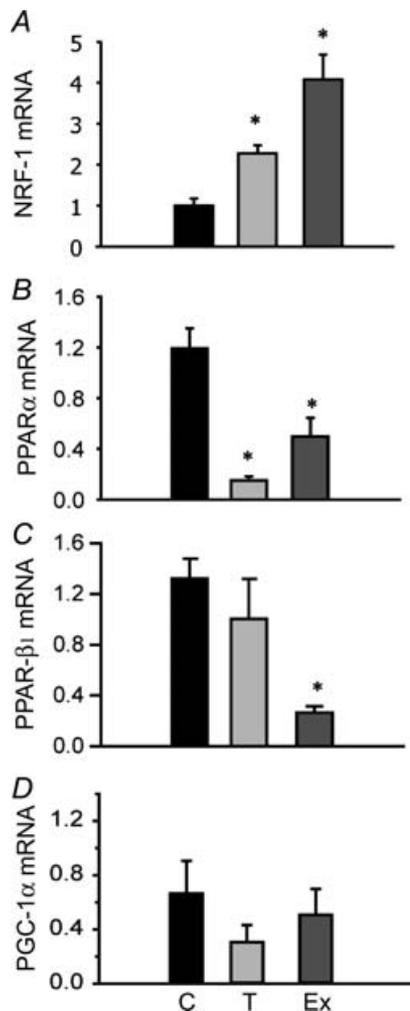

Figure 3. Relative gene expression levels (mRNA) for citrate synthase (CS: *A*), cytochrome c oxidase subunit I (CYC-1; *B*), β-hydroxyacyl-CoA dehydrogenase (HOAD; *C*), myosin heavy chain slow isoform (sMyHC; *D*), pyruvate dehydrogenase E$_2$ subunit (dLat; *E*), lactate dehydrogenase (LDH; *F*) and fast (fMyHC; *G*) isoform in adult zebrafish exposed to cold temperatures (18°C, T) for 4 weeks and exercise trained for 4 weeks (Ex), compared to 28°C controls (C)
Expression levels were measured by real-time PCR using SYBR green. Results are relative to a randomly selected control sample and normalized to β-actin. $n = 5$ except for MyHC ($n = 3$). *Significantly different from controls ($P < 0.05$). Error bars represent S.E.M.

remodelling in zebrafish with cold and exercise either produce qualitatively different mitochondria (specialized for fat oxidation in cold-acclimatization) or there is a fibre-specific response to each treatment.

Gene expression

As a window into some of the potential molecular mechanisms that govern cold-induced and contraction-induced muscle remodelling, expression changes of genes encoding metabolic enzymes and key transcription factors were quantified. We used 4 week endpoints for both our cold-acclimatization and exercise-training treatments to maximize phenotypic changes. This sampling protocol may have missed earlier changes in gene expression. For instance, cold-acclimatization did not change levels of mRNA of genes for key enzymes in metabolism (Fig. 3). However, mRNA levels for genes of interest may have their own distinct patterns of expression over the course of our treatments. For example, hepatic mRNA levels for CS peak at approximately 5 days of cold acclimatization while CS activity peaks at 12 days and remains elevated in a eurythermic eelpout (*Zoarces viviparus*) (Lucassen *et al.* 2003). Although mRNA levels with exercise training have not been measured before in fish, elevated CS mRNA seen after 4 weeks of training in this study suggests that either expression of this gene peaks late in training or, more likely, it remains elevated throughout the training period. Future studies may choose to examine the temporal patterns in mRNA levels with these

Figure 4. Gene expression levels (mRNA) for transcription factors: nuclear respiratory factor (NRF)-1 (*A*), peroxisome proliferator activator receptor (PPAR)-α (*B*), PPAR-β_1 (*C*) and PPAR-γ cofactor (PGC)-1α (*D*) in adult zebrafish exposed to cold temperatures (18°C, T) for 4 weeks and exercise trained for 4 weeks (Ex), compared to 28°C controls
Expression levels were measured by real-time PCR using SYBR green. Results are relative to a randomly selected control sample and normalized to $\b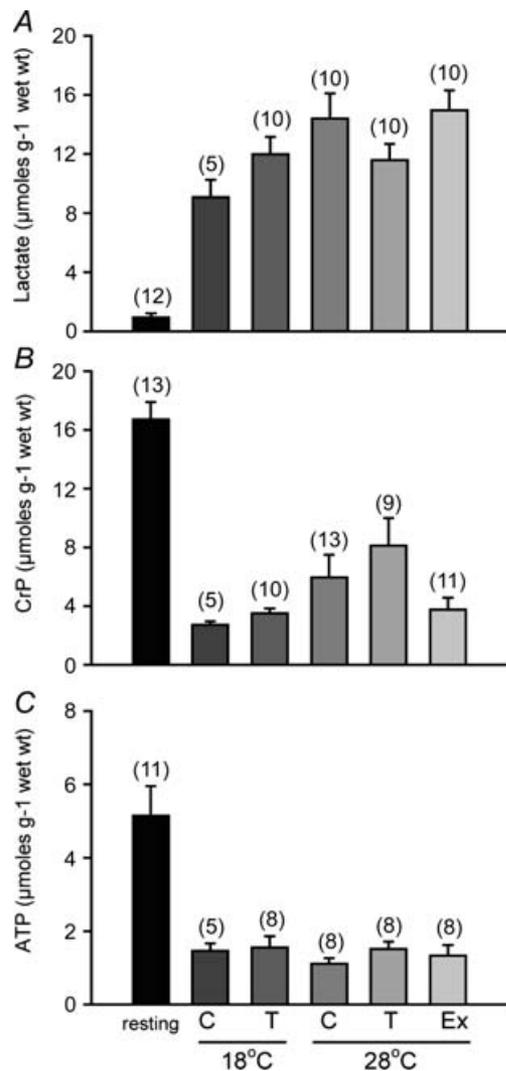eta$-actin. $n = 5$ for all except PCG-1α ($n = 6$). *Significantly different from control ($P < 0.05$). Error bars represent S.E.M.
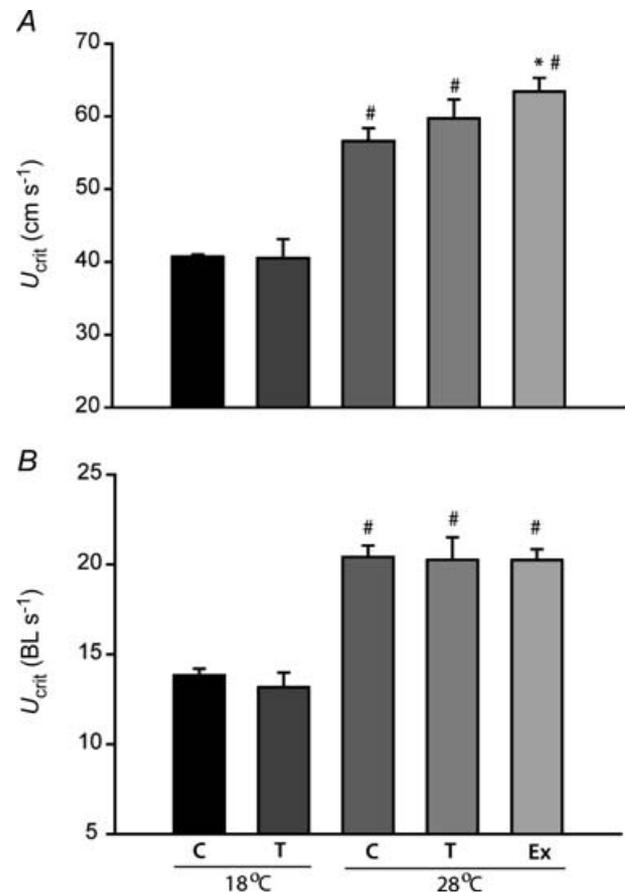
Figure 5. Critical swimming speeds (U_{crit}) in absolute speeds (cm s^{-1}; *A*) or relative U_{crit} in body lengths per second (BL s^{-1}; *B*) at 18°C and 28°C in adult zebrafish exposed to cold temperatures (18°C, T) for 4 weeks and exercise trained for 4 weeks (Ex), compared to controls (C)
*Significantly different from 28°C controls, #significantly different from 18°C. $n = 15$ (C, T and Ex at 28°C) and $n = 10$ (C, T at 18°C). Error bars represent S.E.M.

treatments to elucidate times of peak expression. We assessed changes in total muscle RNA with our treatments to determine estimates of protein synthesis capacity (>98% of total RNA being ribosomal). Storch *et al.* (2005) found cold-acclimatizaton results in increased RNA : protein ratios indicative of increased protein synthesis capacities. In contrast, our cold-acclimatization data suggest a decrease in RNA : protein ratios and therefore a decrease in protein synthesis capacity. It is possible that translational efficiency is modified in cold-acclimatized zebrafish, but this was not directly measured. Interestingly, the half-life of some genes with exercise training in mammals is thought to increase (reviewed in Fluck & Hoppeler, 2003). Whether this occurs in zebrafish is unknown, but our data do suggest that RNA : protein ratios and protein synthesis capacity increase after training. After the removal of a single outlier (0.91 mg g^{-1}) from the control fish data, cold acclimatization and exercise -training showed significant increases in DNA content per gram of tissue. Although this suggests gene dosage as a potential form of regulation, cold acclimatization and exercise training increased DNA content equally, while the mRNA expression for genes of interest showed distinct patterns between these groups. Higher myonuclear content in muscle fibres has also been seen after training in the common carp, with nuclei increasing in relation to fibre diameter (Johnston, 2006). However, our own data (K. Dhekney and G. B. McClelland, unpublished observations) are beginning to show that total red and white muscle area is unchanged with either exercise or cold in zebrafish.

There is very little information available for the regulation of transcription factors in fish muscle but increases in NRF-1 seen in this study suggest a conserved response between cold and exercise in fish and exercise in mammals (Adhihetty *et al.* 2003). However, mammalian studies also find increases in gene expression for all three PPAR (α, γ and β/δ) isoforms and PGC-1α after endurance exercise (60–85% $\dot{V}_{O_2max}$, Mahoney *et al.* 2005) not seen here for cold or exercise in zebrafish (Fig. 4). Again, it is possible that differences in training intensity and duration might explain some of the differences in exercise-trained zebrafish compared to the training response in mammals. It is important to note that many transcription factors can be regulated by kinase pathways such as the p38-MAPK and ERK-MAPK pathways, at least in mammals (e.g. Barger *et al.* 2000, 2001). Future studies using fish should determine changes in DNA binding of these and other transcription factors with different stressors. It is also important to note that tetraploid zebrafish have more copies of genes than mammals (e.g. Amores *et al.* 1998). For example zebrafish have at least two isoforms of PPAR-β (β_1 and β_2; Robinson-Rechavi *et al.* 2001) but it is unclear if they respond in the same way to exercise or cold.

Figure 6. Mixed muscle concentrations of *A*, lactate (*A*), phosphocreatine (*B*) and ATP in μmol (g wet weight)$^{-1}$ (*C*)
Resting values from 28°C controls are compared to fatigue values at the end of the critical swimming (U_{crit}) tests at 18°C and 28°C in adult zebrafish exposed to cold temperatures (18°C, T) for 4 weeks and exercise trained for 4 weeks (Ex), compared to controls (C). Samples sizes appear above each bar. All post-exercise data are significantly different from resting controls. #Significantly different from T exercised at 28°C. Error bars represent s.e.m.

Swimming performance

To assess any potential whole-animal performance consequences of these treatments, we employed a standard swimming trial used extensively in fish research. Critical swimming speed (U_{crit}) is used as a way to assess swimming capacity of fish by increasing water speeds until the fish can no longer keep its position in the current and fatigues (Brett, 1964). Oxygen consumption increases up to fatigue (Brett, 1964), and it is assumed that maximum

oxygen consumption occurs at the critical swimming speed (Hammer, 1995). Moreover, absolute critical swimming speed increases with size while relative (to body length, BL) critical swimming speed decreases with size. Previously, it has been found that zebrafish are capable of unusually high swimming speeds compared to most other fish species for which this measure is documented (Plaut & Gordon, 1994; Plaut, 2000). Zebrafish in the current study had maximum sustained swimming speeds at 28°C of 56–63 cm s^{-1} or 20–22 BL s^{-1} in agreement with previous measurements in this species (Plaut & Gordon, 1994; Plaut, 2000). Only exercise-trained zebrafish showed significant increases in absolute maximum sustained swimming speeds at 28°C used as an indicator of whole-body swimming capacity (Fig. 5*A*). When standardized relative to fish length, relative critical swimming speeds were the same between all fish since training stimulated growth in group 2 fish (Table 2). Zebrafish also show a typical decrease in maximum sustained swimming speeds with temperature (Fig. 5*A* and *B*) due to declines in muscle power output associated with a drop in temperature (Day & Butler, 2005). Previously, muscle HOAD activities and maximum sustained swimming speeds were found to be highly correlated in trout (Farrell *et al.* 1991), and increased with training (Farrell *et al.* 1990). In contrast, trained zebrafish had a greater maximum absolute sustained swimming speed (Fig. 5) despite lower HOAD activities (Fig. 2) compared to cold-acclimatized and control fish. In fish, anaerobic metabolism is initiated at approximately 70–80% U_{crit} and at 100% U_{crit}, pronounced changes in muscle metabolites can occur as fish fatigue (Burgetz *et al.* 1998). We assessed post-exercise metabolite levels and found significant increases in lactate and significant decreases in CrP and ATP in all groups with exercise compared to non-exercised controls (Fig. 6), in agreement with previous studies (Burgetz *et al.* 1998). Interestingly, training in coalfish increased the activity of the enzyme creatine phosphokinase (CPK) (Johnston & Moon, 1980), and this may explain why trained zebrafish are able to deplete muscle CrP levels below the levels in cold-acclimatized fish muscle (Fig. 6).

Muscle morphology

A major advantage of using fish for muscle research is that they have anatomically separated slow (red) and fast (white) muscle masses, which allows the measurement of fibre-type-specific responses to stress. However, due to their small size, mixed muscle samples were used for all of the biochemical and gene expression measurements in this initial study. Past studies on fish have found that cold acclimatization (Johnston & Lucking, 1978) and exercise training (Johnston & Moon, 1980; Hammill *et al.* 2004) can alter the distribution of muscle fibre types, with both stimuli increasing the proportion of red muscle mass.

Moreover, exercise training in trout has been found to stimulate somatic growth and muscle fibre hypertrophy of fast fibres (Martin & Johnston, 2005). No significant differences in the mRNA levels for fast or slow myosin heavy chains were detected with either cold or exercise (Fig. 3*C* and *F*), but it is unclear if this translates to different protein levels. Our own measurement using myosin-ATPase histochemistry are beginning to show that red muscle mass does not increase either in absolute area or in the proportion of total cross-sectional area with either cold exposure or exercise training (K. Dhekney and G. B. McClelland, unpublished observations). This is supported by protein : DNA ratios (Fig. 1*B* and *C*) that are not increased with either treatment. It is possible that muscle fibre types respond in a similar way or perhaps to different degrees to stress. For example, in a continuous 3 week training experiment, coalfish showed similar changes in metabolic enzyme activities in both red and white muscle (Johnston & Moon, 1980). In contrast, rainbow trout exercised 18 h a day at 60% of their maximal sustainable swimming speed showed a greater plasticity in enzyme activities in white *versus* red muscles (Farrell *et al.* 1991). Cold acclimatization in rainbow trout has been found to induce similar metabolic changes in red and white muscle (McClelland *et al.* 2005). Experiments under way will determine fibre-specific changes in gene expression to cold and to exercise in zebrafish.

Conclusions and perspectives

Cold acclimatization and exercise training represent different stimuli that induce aerobic phenotypes in zebrafish muscle. The present data suggest that there may be different mechanisms responsible for muscle remodelling between fish and mammals and between different modes of aerobic phenotype induction in zebrafish. However, certain responses (NRF-1 mRNA) could be classified as essential for the generation of an aerobic phenotype, a common response to different stimuli, and evolutionarily conserved. Moreover cold acclimatization may represent a reliable and straightforward surrogate to exercise-training protocols for the study of conserved muscle remodelling events. Other responses (PPAR-β_1) may be more specialized to different stimuli and result in the development of distinct phenotypes. Direct causation between these responses and changes in muscle phenotype remain to be established. It is still unclear what mechanisms ultimately trigger this remodelling in response to cold or exercise. There is currently some controversy regarding the importance of calcium (Adhihetty *et al.* 2003; Martin & Johnston, 2005) and reactive oxygen species (ROS) signalling mechanisms (Abele *et al.* 2002) in other systems, and so a comprehensive picture of the mechanisms involved is currently lacking.

The zebrafish has been used extensively as a model of vertebrate development, including the formation of different muscle fibre types (e.g. Bryson-Richardson *et al.* 2005). We show here that zebrafish can also be used as a model of adult vertebrate muscle plasticity. They offer the added advantages of the ability to perform genetic screens or the use of molecular techniques to establish direct connections between mechanisms of remodelling and muscle phenotypes (e.g. morpholinos and iRNA). Clearly many fundamental aspects regarding the mechanisms of muscle plasticity can be readily assessed using the adult zebrafish system.

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Acknowledgements