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# Adaptation and acclimation of aerobic exercise physiology in Lake Whitefish ecotypes (*Coregonus clupeaformis*)

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**The physiological mechanisms underlying local adaptation in natural populations of animals, and whether the same mechanisms contribute to adaptation and acclimation, are largely unknown. Therefore, we tested for evolutionary divergence in aerobic exercise physiology in laboratory bred, size-matched crosses of ancestral, benthic, normal Lake Whitefish (*Coregonus clupeaformis*) and derived, limnetic, more actively swimming "dwarf" ecotypes. We acclimated fish to constant swimming (emulating limnetic foraging) and control conditions (emulating normal activity levels) to simultaneously study phenotypic plasticity. We found extensive divergence between ecotypes: dwarf fish generally had constitutively higher values of traits related to oxygen transport (ventricle size) and use by skeletal muscle (percent oxidative muscle, mitochondrial content), and also evolved differential plasticity of mitochondrial function (Complex I activity and flux through Complexes I–IV and IV). The effects of swim training were less pronounced than differences among ecotypes and the traits which had a significant training effect (ventricle protein content, ventricle malate dehydrogenase activity, and muscle Complex V activity) did not differ among ecotypes. Only one trait, ventricle mass, varied in a similar manner with acclimation and adaptation and followed a pattern consistent with genetic accommodation. Overall, the physiological and biochemical mechanisms underlying acclimation and adaptation to swimming activity in Lake Whitefish differ.**

**KEY WORDS:** **Aerobic energy metabolism, adaptation, oxygen transport cascade, phenotypic plasticity, physiology.**

An understanding of the physiological, biochemical, and genetic mechanisms underlying local adaptation in natural populations is needed to better understand the origins and maintenance of biodiversity and predict how populations may respond to future changes in the environment (Hoffmann and Sgro 2011; Savolainen et al. 2013). However, when examining wild populations, it is difficult to distinguish genetically based evolutionary change from environmentally induced phenotypic plasticity (Merila and Hendry 2014). This is especially relevant for physiological traits that commonly vary in response to environmental conditions, a process termed acclimation (i.e., reversible, "physiological" phenotypic plasticity, or flexibility; Piersma and Drent 2003). Although our understanding of the physiological mechanisms contributing to increased tolerance to environmental stressors during acclimation has improved, there is still much to learn about the physiological mechanisms leading to local adaptation in natural populations (reviewed by Hofmann and Todgham 2010; Whitehead 2012; Savolainen et al. 2013). One question of particular interest is whether similar mechanisms are used during local adaptation and acclimation to analogous environmental conditions. This question has particular relevance to our understanding of the role

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of genetic accommodation, a process by which originally environmentally induced phenotypes are selected to become genetically based, in evolutionary change (reviewed by Pfennig et al. 2010; Schlichting and Wund 2014). Yet, little is known about the specific physiological, biochemical, and/or genetic bases of acclimation and adaptation to similar environmental variables among natural populations (but see Scoville and Pfrender 2010; Lee et al. 2011; Whitehead et al. 2011; Cheviron et al. 2014; Morris et al. 2014; Lui et al. 2015).

To study the biochemical and physiological traits underlying local adaptation, and determine if similar traits vary with acclimation and adaptation, we use the well-studied Lake Whitefish model system. The Lake Whitefish is a freshwater fish found in rivers and lakes across North America and much of this species' current distribution was under ice during the Pleistocene glaciation (Bernatchez and Wilson 1998; Scott and Crossmann 1998). At this time (~60,000 years or ~12,000 generations ago), populations of Lake Whitefish were separated into different glacial refugia (Acadian, Atlantic, Mississippian; Bernatchez and Dodson 1991; Jacobsen et al. 2012). At the end of this glaciation, ~12,000 years ago, whitefish recolonized lakes throughout North America and some lineages came back into secondary contact (Bernatchez and Dodson 1990). Since this time, the sympatric glacial lineages have undergone extensive divergence, such that some populations of the Acadian lineage have evolved from an ancestral "normal" epibenthic phenotype into a limnetic, "dwarf" ecotype in response to competition for limited resources and ecological opportunity (Bernatchez 2004; Landry et al. 2007; Landry and Bernatchez 2010), as in other species pairs of postglacial fishes (reviewed by Smith and Skúlason 1996; Taylor 1999). In particular, the dwarf ecotype has evolved morphological specializations to better consume limnetic prey, is a more active swimmer, and has a slower growth rate than the ancestral, normal ecotype (reviewed by Bernatchez et al. 2010). The repeated, independent evolution of the dwarf ecotype from a normal ancestral form strongly suggests that swimming activity, growth rate, and feeding morphology evolved adaptively in dwarf populations and genomic locations associated with these phenotypes display evidence of positive directional selection (Rogers and Bernatchez 2007; Renaut et al. 2011; Gagnaire et al. 2013). In combination with transcriptional studies (e.g., St-Cyr et al. 2008), this work suggests that dwarf fish have evolved to become more active swimmers to increase limnetic foraging and predator avoidance and that the energetic costs of this more active lifestyle result in slower growth rates and fecundity relative to normal whitefish (reviewed by Bernatchez et al. 2010).

In this study, we test if candidate traits related to aerobic capacity (the capacity to uptake, transport and use oxygen, termed the "oxygen transport cascade") may contribute to local adaptation in Lake Whitefish. We focus on the oxygen cascade because variation in this physiological pathway is predicted to underlie adaptive differentiation in swimming activity between ecotypes (Rogers et al. 2002) and is known to influence evolutionary fitness via changes in locomotory and thermoregulatory capacity in other species (Hayes and O'Connor 1999; Irschick et al. 2008). Indeed, past physiological (Evans et al. 2012, 2013) and transcriptomic (Derome et al. 2006, 2008; St-Cyr et al. 2008; Whiteley et al. 2008; Nolte et al. 2009; Renaut et al. 2009; Jeukens et al. 2010; Evans and Bernatchez 2012; Filteau et al. 2013; Dion-Cote et al. 2014) studies in Lake Whitefish suggest that traits related to the oxygen cascade are increased in dwarf fish (reviewed in Table 1; Bernatchez et al. 2010). For example, when corrected for size, wild dwarf whitefish have larger hearts, which should allow for increased cardiac output and oxygen transport (Evans et al. 2013). Wild dwarf whitefish also have lower food conversion efficiencies than normal fish, suggestive of higher standard metabolic rates (Trudel et al. 2001). These physiological changes are associated with over-expression of oxidative phosphorylation (OXPHOS) genes, responsible for mitochondrial ATP production, in the white skeletal muscle of dwarf fish (Derome et al. 2006; Evans and Bernatchez 2012). Furthermore, genes associated with aerobic energy metabolism display evidence of local adaptation (e.g., Whiteley et al. 2008; Renaut et al. 2010, 2011; Jeukens and Bernatchez 2012; Hebert et al. 2013). Together, this work suggests that traits related to aerobic exercise physiology have experienced strong directional selection in the dwarf ecotype and may be key traits underlying local adaptation (reviewed by Bernatchez et al. 2010).

However, there remain uncertainties: patterns of gene expression among individual proteins within OXPHOS complexes, among complexes, and among replicate populations of Lake Whitefish are not consistent (Derome et al. 2006, 2008; Evans and Bernatchez 2012). The activities of many OXPHOS enzyme complexes are also posttranscriptionally regulated, making it difficult to predict the effects of transcriptional variation on mitochondrial function (Suarez and Moyes 2012; Bremer et al. 2014). Next, not all physiological traits match predictions of increased aerobic capacity in dwarf whitefish, as gill surface area is larger in normal fish (Evans et al. 2013). Most importantly, all prior physiological studies in Lake Whitefish (Evans et al. 2012, 2013), and most studies of muscle gene expression, have been conducted on wild fish (Derome et al. 2006; Evans and Bernatchez 2012; but see Derome et al. 2008), that differ in adult size and activity levels. Therefore, previous studies could not rule out the possibility that observed differences solely result from allometric variation and/or phenotypic plasticity (e.g., Davison 1997; Burness et al. 1999). The influence of phenotypic plasticity is particularly important because many traits that differ between wild whitefish ecotypes are responsive to swim training (summarized in Table 1). Therefore, the more active lifestyle of the dwarf fish, and not

**Table 1. Predictions for the evolution of exercise physiology in the more active, derived dwarf Lake Whitefish form from a normal-like ancestral form (reviewed by Bernatchez et al. 2010) and the effects of swim training at sustainable swimming speeds in fish (reviewed by Davison 1997; McClelland 2012).**

| | Predictions: trait evolution in dwarf (athletic) fish | Predictions: effect of swim training |
|---|---|---|
| Oxygen transport capacity: | | |
| (1) Hematocrit (Hct; Fig. 1) | **Data**: Not known, but see Evans et al. (2012) for data on hemoglobin isoforms. Often higher in athletic and hypoxia-tolerant fish (reviewed by Gallaugher and Farrell 1998).<br>**Predictions**: Unknown. Dwarf fish are more active (Rogers et al. 2002), but normal fish are predicted to be more hypoxia-tolerant (Landry et al. 2007). | **Data**: Swim training increases (Davison 1997), or has no effect (e.g., Davie et al. 1986; Skov et al. 2011).<br>**Predictions**: ↑ or = |
| (2) Ventricle size (Fig. 1) | **Data**: Wild dwarf fish have slightly, but not significantly, larger ventricles (Evans et al. 2013). Athletic species often have larger ventricles (e.g., Dickson 1995; Eliason et al. 2011; Dalziel et al. 2012).<br>**Predictions**: ↑ in dwarf fish. | **Data**: Swim training either increases or has no effect on ventricle size (reviewed by Davison 1997).<br>**Predictions**: ↑ or = |
| (3) Ventricle metabolic capacity: activities of enzymes involved in energy metabolism (Fig. 2) | **Data**: Not known. Athletic species often have higher activities (e.g., Dickson 1995; Moyes 1996; Bernal et al. 2003; but see Dalziel et al. 2012).<br>**Predictions**: ↑ or = in dwarf fish. | **Data**: Swim training leads to intensity-dependent increases in enzyme activities (Farrell et al. 1990, 1991; Castro et al. 2013).<br>**Predictions**: ↑ or = |
| Capacity for oxygen uptake by muscle: | | |
| (4) Percentage of red, oxidative skeletal muscle (Fig. 3) | **Data**: Not known. Athletic species tend to have a higher proportion (reviewed by Langerhans 2008).<br>**Predictions**: ↑ in dwarf fish. | **Data**: Swim training either increases (reviewed by Davison 1997) or has no effect (e.g., Lemoine et al. 2010).<br>**Predictions**: ↑ or = |
| (5) Muscle capillary density (Fig. 3) | **Data**: Not known. Athletic species may have higher densities (e.g., Dickson 1995).<br>**Predictions**: ↑ in dwarf fish. | **Data**: Swim training either leads to increases (reviewed by Davison 1997) or no change (e.g., Johnston and Moon 1980).<br>**Predictions**: ↑ or = |
| Capacity for oxygen use by skeletal muscle: | | |
| (6) Muscle mitochondrial content (quantity): amount per gram muscle (Fig. 4) | **Data:** Dwarf whitefish populations generally have higher expression of OXPHOS genes (Derome et al. 2006; Evans et al. 2012). Athletic species tend to have higher muscle mitochondrial content (Dickson 1995).<br>**Predictions**: ↑ in dwarf fish. | **Data**: Swim training generally increases (Davison 1997; Anttila et al. 2006), but may have no effect (e.g., Lemoine et al. 2010; Martin-Perez et al. 2012; McClelland 2012; He et al. 2013; Magnoni et al. 2013). Dependent on muscle fiber-type (reviewed by Davison 1997).<br>**Predictions**: ↑ or = |
| (7) Muscle mitochondrial function (quality): respiratory capacity per milligram mitochondrial protein (Fig. 5) | **Data**: Not known. Some athletic species have higher respiratory capacity (e.g., Moyes et al. 1992).<br>**Predictions**: ↑ in dwarf fish. | **Data**: Not known in fishes.<br>**Predictions**: ? |
| (8) Muscle mitochondrial function (quality): electron transport chain enzyme activity per milligram mitochondrial protein (Fig. 6) | **Data**: Not known in fishes.<br>**Predictions**: ? | **Data**: Not known in fishes.<br>**Predictions**: ? |

**Upward arrows (↑) indicate predicted increases in trait values, downward arrows (↓) indicate predicted decreases in trait values, equal signs ( = ) indicate no predicted changes among groups, and question marks (?) indicate that there is not sufficient data to predict how traits may vary. In this study, we focus on steps in the oxygen cascade related to oxygen transport, uptake, and use by the skeletal muscle and not on oxygen extraction from the environment. As well, the effects of swim training in fish are often dependent upon training intensity and the species studied.**

genetically based differences, could lead to the observed patterns in wild populations.

The goals of this study were to: (1) to test the hypothesis that dwarf whitefish have evolved increased capacities for oxygen transport (hematocrit, ventricle size, and metabolic potential), uptake at the muscle (percentage red muscle and capillary density), and use (muscle mitochondrial content, enzyme activity, and cellular respiration) in size-matched, laboratory-bred and raised fish, (2) determine if mitochondrial enzyme activity and function changes as predicted from shifts in metabolic gene expression in wild fish (Derome et al. 2006; Evans and Bernatchez 2012), (3) test for evolutionary divergence in additional traits

related to related to oxygen transport and use, and (4) compare phenotypic plasticity of dwarf and normal fish in response to conditions emulating the high-activity limnetic foraging of dwarf whitefish (high water flow, leading to swim training) and the less active, benthic lifestyle of normal whitefish (low water flow, fish not required to actively swim to maintain their position in the current). In particular, we test if phenotypic plasticity in the ancestral normal form is associated with trait evolution in the derived dwarf form, a pattern consistent with genetic accommodation (Ghalambor et al. 2007; Schlichting and Wund 2014).

## Materials and Methods

### EXPERIMENTAL FAMILIES

Crosses were produced from parents from Témiscouata (dwarf whitefish, Acadian lineage, 47°36′N, 68°45′W) and Aylmer Lake (normal whitefish, Atlantic lineage, 45°50′N, 71°26′W) in Québec, Canada, caught in November 2011. Gametes were collected in the field and brought to the Laboratoire de Recherche en Sciences Aquatiques (LARSA, Université Laval) for artificial fertilization following Nolte et al. (2009). Gametes from multiple females and males were mixed: the dwarf cross was produced from seven females and seven males and the normal cross from nine females and 14 males. We reared crosses in a freshwater, flow through system under identical temperatures, lighting schedules, water flow velocities, and feeding regimes for the first 16 months. The major difference from previous studies is that our fish continued to eat *Artemia* napulli enriched with Selco and algae (to provide essential fatty acids), as they would not switch to dry pellets. This led to a reduced growth rate, but there was no morbidity and fish of both forms were healthy during the experiment. Normal fish were also kept at a slightly higher density (0.78 kg/m$^3$) than dwarf fish (0.56 kg/m$^3$) to minimize genetically based differences in growth, but in all cases fish were kept at densities far below typical aquaculture conditions (15–40 kg/m$^3$; Ellis et al. 2002). At the start of our experiment, dwarf fish were slightly larger (average mass of 1.84 $\pm$ 0.60 g, and fork length of 5.98 $\pm$ 0.87 cm) than normal fish (1.56 $\pm$ 0.83 g, 5.19 $\pm$ 0.86 cm; $P = 0.026$ for mass; $P < 0.001$ for length). By the end of the experiment there was no significant difference in mass among ecotypes (dwarf 4.89 $\pm$ 0.16, normal 5.06 $\pm$ 0.21, $P = 0.893$), but dwarf fish were still longer than normal fish (dwarf 8.57 $\pm$ 0.10, normal 8.06 $\pm$ 0.12, $P = 0.0171$), as expected from genetically based differences in body shape (Laporte et al. 2015). There was no effect of swim training on final mass or length (data not shown, $P = 0.731$, $P = 0.995$, respectively).

### SWIM TRAINING

Swim training began on 8 April 2013 using 127 fish (63 dwarf and 64 normal) approximately 17 months of age. At this time, we weighed and measured the fish, and distributed fish into eight circular tanks (50 cm in diameter, 1 m depth; approximately eight dwarf and eight normal fish per tank, and approximately equal biomass). All tanks were part of a flow through system and the inner portion of each tank was fitted with a 16 cm diameter circular ring to block the center of the tank and form a "swimming track." Water speed was set by adjusting water inflow through vertical pipes that ran the length of the tank, with holes drilled to keep flow similar at all depths. We used a current meter (Swoffer Model 3000) to set the speed to 7.5 cm/s for 6 h/day in the "swim training" tanks. This current velocity corresponds to 1.25 body length per second (BL/s) for dwarf fish and 1.45 BL/s for normal fish based upon initial fork lengths. Minimal flow (<0.5 cm/s) was set in "control" tanks. By May 1, the speed was increased to 10 cm/s to account for fish growth. Sampling began on 23 July 2013 (approximately three months and one week of training) and continued until 16 October 2013 (six months training). At this time, the fish were swimming at an average of 1.2 BL/s (1.17 BL/s for dwarf fish and 1.24 BL/s for normal fish). As Bernatchez and Dodson (1985) found that Lake Whitefish have a lower prolonged swimming capacity than other salmonids and Anttila et al. (2008) found reduced training effects at high speeds, we chose experimental speeds that would match predicted natural activity levels (constant swimming during limnetic foraging in dwarf fish), in which fish could swim aerobically.

### SAMPLE COLLECTION

Between 9 a.m. to noon, we sacrificed experimental fish with cervical dislocation using a needle followed by pithing to avoid respiratory changes caused by anesthetic overdose. We blotted fish dry and measured and weighed each individual. Next, we severed the caudal peduncle with a razor blade ~6 mm posterior to the cloaca and collected blood in heparinized microhematocrit (hct) capillary tubes (Fisher Scientific, Nepean, ON, Canada). Hct samples were kept on ice and measured within 30 min. We made a second cut ~3 mm posterior to the cloaca to collect a muscle "steak" for histology. This steak was coated in mounting medium (Fisher Histoprep, Nepean, ON, Canada), placed in a plastic base mold (Fisher Scientific, Nepean, ON, Canada), rapidly frozen in 2-methylbutane cooled in liquid $N_2$, and stored at –80°C. A second muscle steak was taken for enzyme assays by making a third cut at the cloaca and was snap frozen at −80°C. Internal organs were then dissected, weighed, and snap frozen in liquid nitrogen. Prior to weighing hearts we separated the *bulbus arteriosis* and *atria* from the ventricle and blotted away remaining blood. White muscle samples from both sides of the animal were collected, skinned, freed of red muscle and used to isolate mitochondria. To ensure blind sampling, we identified ecotypes with a diagnostic mitochondrial RFLP assay after sampling (sequence data from Jacobsen et al. 2012). Cytochrome b was amplified from 20 ng of DNA and also 2 μL

of a 100× dilution of our isolated mitochondrial samples (to double check identification; materials and methods "Measurement of mitochondrial oxidative capacities") in a polymerase chain reaction (PCR) reaction (5 min 95°C, 40 cycles of 1 min at 95°C, 1 min at 51°C, 1 min at 72°C, followed by 90 s at 72°C) using the following forward (5′-CATAATTCCTGCCCGGACTCTAA-3′) and (5′-TTTAACCTCCGATCTCCGGATTA-3′) reverse primers. We digested our PCR product with SnaBI which cut the normal haplotype once and did not cut the dwarf haplotype. All protocols were approved by Université Laval's animal care committee (Protocol 82178).

## MEASUREMENT OF MITOCHONDRIAL OXIDATIVE CAPACITIES

We isolated mitochondria from white muscle following Martin et al. (2009) and re-suspended the final mitochondrial pellet in 0.1 volumes of reaction buffer with Bovine Serum Albumin (BSA) to maintain mitochondrial quality (Guderley et al. 1997). Three aliquots were taken to: (1) measure mitochondrial respiration, (2) freeze at −80°C for later enzyme analyses (see "Activities of oxidative phosphorylation enzymes in isolated mitochondria"), and (3) wash away BSA and measure mitochondrial protein content. Mitochondrial protein was measured on frozen samples with the Bradford Reagent following the manufacturer's instructions (Sigma-Aldrich, Oakville, ON, Canada).

We measured mitochondrial oxygen consumption on fresh preparations (~0.2 mg protein $mL^{-1}$) at 12°C using a temperature-controlled polarographic $O_2$ monitoring system (Martin et al. 2009). All concentrations are those in the assay chamber. We sparked the Krebs cycle by adding 0.1 mM malate and then added 1.5 mM pyruvate to measure maximal substrate-oxidation rate (state 2). We then added 0.6 mM ADP to measure maximal phosphorylating respiration rate (state 3) and measured nonphosphorylating respiration rate (state 4) after the depletion of ADP. We only included preparations with respiratory control ratios (RCR = state 3/state 4) > 2. These RCRs are lower than those obtained for red muscle mitochondria (Martin et al. 2009) as contaminating myofibrils regenerate ADP from the ATP produced by oxidative phosphorylation (e.g., Burpee et al. 2010). However, acceptor control ratios (state 3/state 2) between 5.7 and 7.5 indicated that mitochondrial respiration was strongly stimulated by ADP and mitochondria were of good quality (e.g., Scott et al. 2009a). After state 4 stabilized, we measured oxygen consumption due to flux through Complexes I–IV by adding 1.15 mM pyruvate + 0.1 mM malate and 2.85 mM ADP. We corrected for residual rates after inhibition of Complex I by adding 1 μg $mL^{-1}$ rotenone and 12 mM succinate to stimulate flux through Complexes II–IV. After steady rates were reached, we added 5 μg $mL^{-1}$ antimycin A to inhibit Complex III and 8 mM ascorbate and 0.8 mM TMPD to stimulate flux through Complex IV (cytochrome c oxidase [COX]). We corrected rates for auto-oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) in the presence of ascorbate after inhibition of COX by 100 μM potassium cyanide.

## MEASUREMENT OF ENZYME ACTIVITIES

All enzyme activities were measured at 26°C with a 96-well plate spectrophotometer (Spectramax 190, Molecular Devices). Assays were optimized to ensure substrates, cofactors, and linking enzymes were not limiting. Samples were assayed in triplicate and either background rates (no substrate present) or nonspecific activity (activity present when the target enzyme is inhibited) was subtracted. To calculate activities, we used millimolar extinction coefficients of 6.22 $mM^{-1}$ $cm^{-1}$ for NADH (340 nm), 13.6 $mM^{-1}$ $cm^{-1}$ for DTNB (412 nm), 19.1 $mM^{-1}$ $cm^{-1}$ for DCIP (600 nm), and 29.5 $mM^{-1}$ $cm^{-1}$ for cytochrome c (550 nm). Tissue protein content was measured in quadruplicate.

### *Activities of oxidative phosphorylation (OXPHOS) enzyme complexes in isolated mitochondria*

To quantify OXPHOS enzyme activities, we followed the protocols of Kirby et al. (2007) modified for fish white muscle by Davies et al. (2012). We thawed frozen mitochondrial samples on ice and centrifuged samples for 10 min at 20,000 × *g* at 4°C. Mitochondrial pellets were re-suspended in either isotonic (Complexes III and IV; 100 mM KCl, 25 mM $K_2HPO_4$, 5 mM $MgCl_2$, pH 7.4) or hypotonic solution (Complexes I, II, V; 25 mM $K_2HPO_4$, 5 mM $MgCl_2$, pH 7.4) and frozen/thawed three times. Final concentrations for each assay were as follows: Complex I (CI, 25 mM $K_2HPO_4$, 25 μM dichlorophenol indophenol [DCPIP], 65 μM ubiquinone-2, 0.2 mM NADH, 2 μg/mL antimycin, 2 mM KCN, and 2 μg/mL rotenone). Complex II (CII, 25 mM $K_2HPO_4$, pH 7.2, 25 μM DCPIP, 65 μM ubiquinone-2, 20 mM succinate 2 mM KCN, and 2 μg/mL antimycin), Complex III (CIII, 25 mM $K_2HPO_4$, pH 7.2, 5 mM $MgCl_2$, 2.5 mg/mL BSA, 0.6 mM lauryl maltoside, 35 μM reduced ubiquinol-2, and 50 μM oxidized cytochrome c, 2 μg/mL rotenone and 2 mM KCN, and 2 μg/mL antimycin A), Complex IV (CIV, 25 mM $K_2HPO_4$, pH 7.2, 5 mM $MgCl_2$, 2.5 mg/mL BSA, 0.6 mM lauryl maltoside, 50 μM reduced cytochrome c), and Complex V (CV, 5 mM ATP, 1 mM PEP, 0.2 mM NADH, 1 U/mL pyruvate kinase [PK], 1 U/mL lactate dehydrogenase [LDH], and 0.5 μg/mL oligomycin). CI, III, and V activities were calculated by subtracting residual activity in the presence of specific inhibitors (rotenone, antimycin A, and oligomycin, respectively) from total activities.

### *Activities of metabolic enzymes in frozen tissues*

We dissected red and white muscle from frozen steaks and used full frozen ventricles to measure activities of "key metabolic enzymes" (Egan and Zierath 2013) in whole tissues (Moyes et al.

1997). We weighed tissues and immediately homogenized in 20 volumes (white muscle) or 60 volumes (red muscle, ventricles) of chilled homogenization buffer (50 mM Hepes, 1 mM EDTA, and 0.1% Triton X-100, pH 7.4) in 4 mL Wheaton glass homogenizers on ice. We assayed the following enzymes using these final concentrations: (1) COX (EC 1.9.3.1, 50 μM reduced cytochrome c, 0.5% Tween 20, in 50 mM Tris, pH 8), (2) citrate synthase (CS; EC 2.3.3.1, 0.5 mM oxaloacetate, 0.15 mM DTNB, and 0.15 mM acetyl CoA in 50 mM Tris, pH 8), (3) malate dehydrogenase (MDH; EC 1.1.1.37, 0.5 mM oxaloacetate 10 mM KCl, and 0.2 mM NADH in 100 mM Hepes, pH 8), (4) 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35, 0.15 mM aceto-acetyl CoA, 0.5 mM NADH, 0.2 mM DTT, 0.1% Triton X-100 in 50 mM Tris, pH 8), (5) hexokinase (HK; EC 2.7.1.1, 1 mM ATP, 10 mM KCl, 5 mM $MgCl_2$, 5 mM glucose, 0.5 mM β-NADP, and 2 U/mL glucose 6 phosphate dehydrogenase (G6PDH) in 100 mM Hepes, pH 7.4), (6) phosphoglucose isomerase ( EC 5.3.1.9, 4 mM fructose-6-phosphate, 10 mM KCl, 2.5 mM NADP, and 1 U/mL G6PDH in 100 mM Hepes, pH 7.4), (7) phosphofructokinase (EC 2.7.1.11, 10 mM fructose-6-phosphate, 10 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM ATP, 5 mM AMP, 0.2 mM NADH, 1 U/mL aldolase, 29 U/mL triose phosphate isomerase, and 10 U/mL glycerol-3-phosphate dehydrogenase in 100 mM Hepes, pH 8.2), (8) PK (EC 2.7.1.40, 5 mM phosphoenol pyruvate, 0.15 mM NADH, 5 mM ADP, 10 mM KCl, 10 mM $MgCl_2$, 10 μM fructose 1,6-bisphosphate, and1 U/mL LDH in 100 mM Hepes, pH 7.4), (9) LDH (EC 1.1.1.27, 25 mM pyruvate and 0.5 mM NADH in 50 mM Tris, pH 7.4), and (10) creatine phosphokinase (EC 2.7.3.2, 50 mM creatine phosphate, 3 mM ADP, 1.5 mM NADP, 20 mM glucose, 12 mM AMP, 25 mM $MgCl_2$, 2 U/mL HK, and 1.5 U/mL G6PDH in 100 mM Hepes, pH 7.4). Background rates were calculated from wells lacking a specific substrate (first chemical listed), with the exception of COX.

### MUSCLE HISTOLOGY

Frozen muscle samples were sectioned (16 μm) transverse to fiber length in a –19°C cryostat (Leica Microsystems, GmbH, Wetzlar, Germany), placed on glass slides, and stored at –80°C. Sections were stained for succinate dehydrogenase activity to distinguish mitochondria-rich red muscle from mitochondria-poor white muscle and alkaline phosphatase to determine capillary density in red muscle (too low to reliably estimate in white muscle) following the protocols of Scott et al. (2009b). Total red and white muscle cross-sectional area was measured in at least two sections for each sample and capillary density in at least two different areas of the red muscle for each individual. All counts and measurements were conducted with ImageJ (Rasband 2014).

### STATISITICAL TESTS

Statistical analyses were conducted using R version 3.1.1 (R Development Core Team 2014). Although we were able to control for variation in average size among groups, differences among individuals within a group were still present so we used the residuals from a best-fit least-squared linear regression against mass in subsequent analyses when this effect was significant. To test the effects of ecotype (fixed effect, dwarf, or normal) and treatment (fixed effect, swim trained, or control), we ran mixed effects linear models using the nlme package in R with tank nested as a random effect and individual fish nested within tanks (two-way nested ANOVAs; Pinheiro et al. 2015). "Plasticity" was defined as a significant effect of treatment (both ecotypes display a similar direction and extent of plasticity) and/or a significant interaction effect (only one ecotype is plastic or ecotypes display different directions and/or extents of plasticity) and "evolution" was defined by similar criteria (an effect of "ecotype" and/or a significant interaction indicating evolutionary variation in plasticity).

We conducted multivariate analyses to determine if the traits that best differentiate ecotypes (dwarf vs. normal) are the same as those that respond to treatment (control vs. swim trained). To do this we performed three linear discriminant (ld) function analysis (DFA) on mean tank values for all traits which showed any significant effects in our univariate analyses. We conducted analyses with the MASS package in R and standardized all variables to have a mean of zero and variance of one (Venables and Ripley 2002). Plots were created with ggplot2 (Wickham 2009).

## *Results*

### OXYGEN TRANSPORT CAPACITY: HEMATOCRIT, VENTRICLE MASS, AND VENTRICLE ENZYME ACTIVITIES

There were significant differences between ecotypes in three oxygen transport-related traits: hematocrit (Fig. 1A), ventricle size (Fig. 1B), and ventricle PK activity per milligram protein (Fig. 2J). In particular, normal whitefish had higher hematocrits than dwarf whitefish (Fig. 1A), and dwarf whitefish had larger size-adjusted ventricles (Fig. 1B), with higher PK activity per milligram ventricle protein and per gram ventricle (Fig. 2J, Table S1).

Three traits displayed evidence of plasticity: Ventricle protein concentration decreased with swim training (Fig. 2A) and MDH activity was upregulated per milligram protein (Fig. 2C), but not per gram of ventricle (Table S1) in both ecotypes. Only one trait, residual ventricle size, displayed a near-significant interaction between training and ecotype, increasing with training in normal, but not dwarf fish ($P = 0.06$; Fig. 1B).

### CAPACITY FOR OXYGEN UPTAKE: PERCENTAGE OF OXIDATIVE MUSCLE AND CAPILLARY DENSITY

Dwarf whitefish had significantly more red muscle than normal fish (Fig. 3A), but there were no differences among ecotypes in capillary density, expressed per square micrometer or per

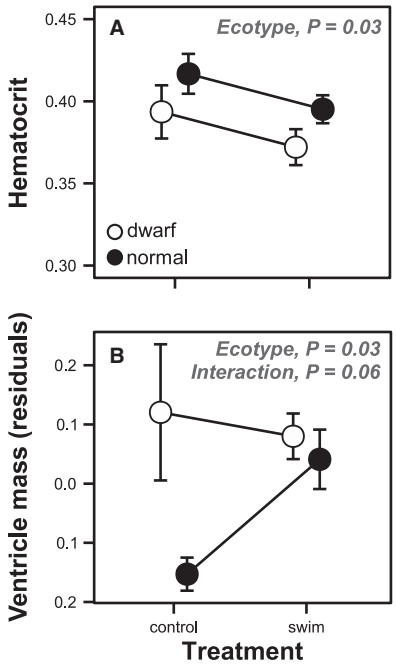


**Figure 1.** Traits associated with oxygen transport capacity in swim-trained and control dwarf (white circles) and normal (black circles) whitefish ecotypes. (A) Percentage of red blood cells per total blood volume (hematocrit; ecotype: $P = 0.03$, $F_{1,93} = 5.05$, treatment: $P = 0.24$, $F_{1,6} = 1.73$, interaction: $P = 0.99$, $F_{1,93} < 0.01$). (B) Ventricle mass (residuals from a regression against body mass, ecotype: $P = 0.03$, $F_{1,90} = 5.14$, treatment: $P = 0.24$, $F_{1,6} = 1.73$, interaction: $P = 0.06$, $F_{1,90} = 3.59$). Ventricle mass as a percentage of body mass is presented in Table S1. All data are presented as the grand means ± SEM of all tank means for each group, but statistical analyses included all individuals in a mixed-effects, nested, two-way ANOVA model. Only *P*-values ≤ 0.10 are noted in the figure panels.

muscle fiber (Fig. 3C, E). There were also no effects of training or interactions among training regime and ecotype.

## CAPACITY FOR OXYGEN USE AT THE SKELETAL MUSCLE: MITOCHONDRIAL CONTENT AND FUNCTION

We assessed muscle mitochondrial content by measuring the amount of mitochondrial protein isolated per gram of white muscle tissue and measuring the activities of mitochondrial enzymes that act as "biomarkers" for overall mitochondrial content in both red and white muscle (Larsen et al. 2012; Fig. 4). Generally, we found that dwarf fish have a higher muscle mitochondrial content than normal fish (Fig. 4). For example, in white muscle, dwarf whitefish have more mitochondrial protein (Fig. 4C) and higher activities of CS, COX, and MDH per gram white muscle (Fig. 4D, F, H). Similarly, in red muscle, dwarf whitefish had higher activities of COX, CS, and MDH per gram (Fig. 4E, G, I). Because dwarf whitefish have a higher concentration of muscle protein than normal fish (Fig. 4A, B), we also expressed mitochondrial enzyme activities per milligram muscle protein. Dwarf white muscle enzyme activities remained significantly higher when expressed per milligram protein, with the exception of COX (Table S2). However, when expressed per milligram protein enzyme activities were no longer significantly higher in dwarf red muscle than in normal fish (Table S3). There were no significant treatment effects or interactions for traits associated with muscle mitochondrial content.

We tested for qualitative differences in mitochondrial function by measuring maximal rates of mitochondrial respiration (Fig. 5) and the activities of each of the oxidative phosphorylation (OXPHOS) complexes per milligram mitochondria (Fig. 6) in white muscle. All preparations had Activator Control Ratios (ACRs) > 4, indicating that mitochondrial respiration was well coupled (Scott et al. 2009a; Table S4). Dwarf fish had higher activities of Complex IV per milligram mitochondrial protein (Fig. 6D), and tended to have increased flux through Complexes II–IV (Fig. 5E, $P = 0.06$, $F_{1,33} = 3.79$). However, other measures of mitochondrial function (Fig. 5, Table S4) and the activities of other OXPHOS complexes (Fig. 6) did not have a significant effect of ecotype. There was no effect of treatment on mitochondrial respiration (Fig. 5, Table S5), but swim training did increase Complex V activity (Fig. 6E) and decrease Complex II activity per milligram mitochondrial protein (Fig. 6B) in both ecotypes.

Finally, three traits had significant interactions between ecotype and treatment, indicating plasticity in at least one ecotype: flux through Complexes I–IV and IV (Fig. 5C, G) and Complex I activity per milligram mitochondrial protein (Fig. 6A). In all cases, traits increased after swim training in dwarf fish and either decreased (Fig. 5C, G; flux through Complexes I–IV and IV) or remained the same after training in normal fish (Fig. 6A, Complex I activity). Together, these interactions indicate ecotype-specific responses to swim training.

## SUMMARY OF CHANGES IN EXERCISE PHYSIOLOGY—MULTIVARIATE ANALYSIS

To summarize the variation in candidate traits related to aerobic metabolism and determine which traits best discriminate among our experimental treatments, we ran a series of DFAs. Discriminating by experimental group produced a first ld that clearly differentiated groups by ecotype and accounted for 83.3% of the variation whereas the second ld mainly differentiated fish by treatment and explained 10.6% of the variation (Fig. 7A). We then conducted two more DFAs discriminating by either ecotype (Fig. 7B) or treatment (Fig. 7C), to determine which variables have strongest correlations with these factors (i.e., factor loadings; Fig. 7D, E). When groups were differentiated by ecotype, the five traits with the highest factor loadings were COX, MDH, and CS

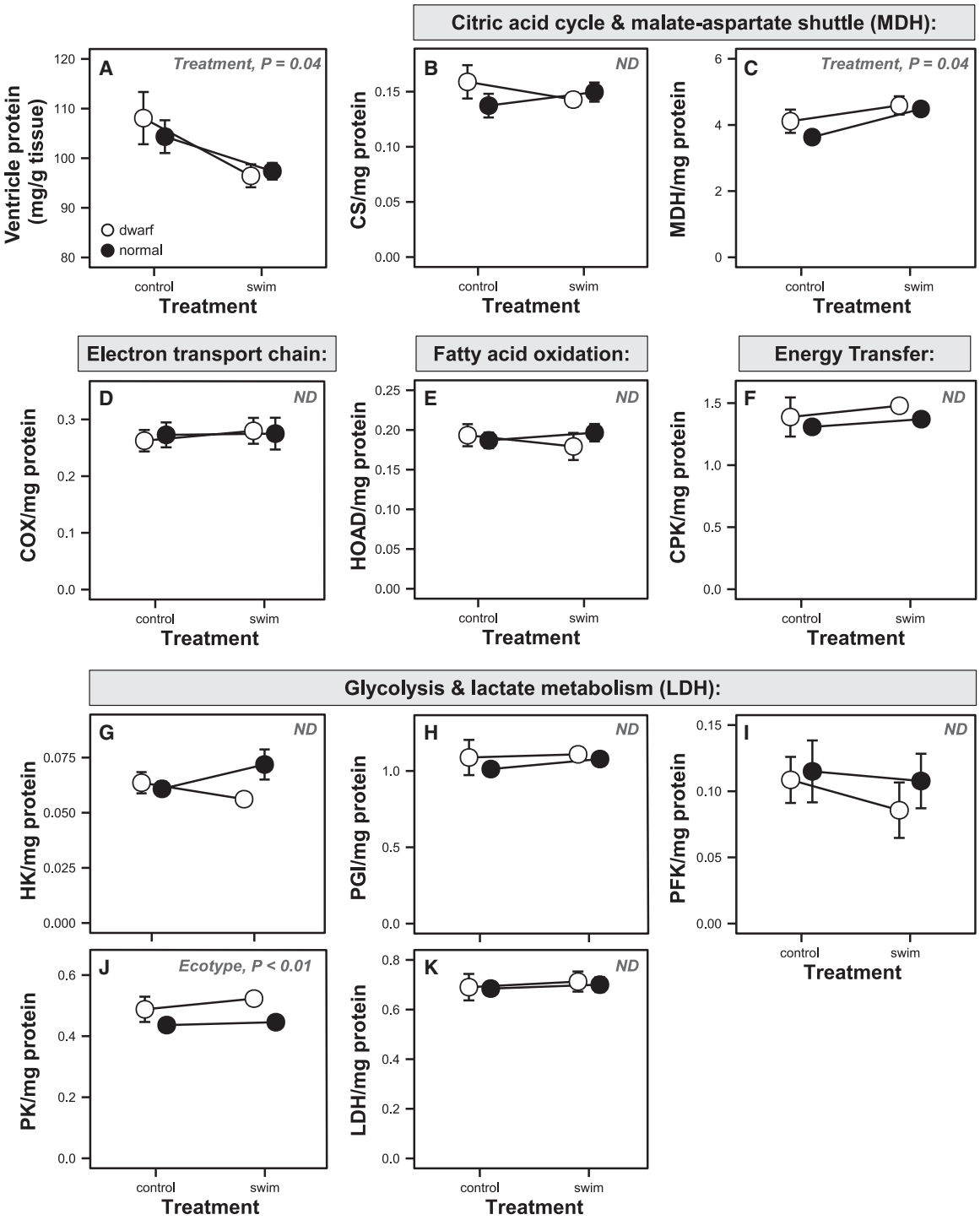


**Figure 2.** Activities of metabolic enzymes in the ventricles of swim-trained and control dwarf (white circles) and normal (black circles) whitefish. (A) Total protein concentration per gram ventricle. (B, C) Activities of citrate synthase (CS) and malate dehydrogenase (MDH), two citric acid cycle enzymes. Note that MDH is also involved in the malate-aspartate shuttle and that activity represents a combination of cytosolic and mitochondrial isoforms. (D) Activity of cytochrome c oxidase (COX), Complex IV in the mitochondrial electron transport chain. (E) Activity of β-hydroxyacyl CoA dehydrogenase (HOAD), part of the mitochondrial fatty acid β-oxidation pathway. (F) Activity of creatine phosphokinase (CPK), an enzyme involved in energy transfer and storage. (G–K) Activities of the glycolytic enzymes hexokinase (HK), phosphoglucose isomerase (PGI), phosphoructokinase (PFK), pyruvate kinase (PK), and lactate dehydrogenase (LDH). LDH is reversible so may inter-11 convert pyruvate to lactate. All data are expressed as $\mu mol \cdot min^{-1} \cdot$ mg of total ventricle $protein^{-1}$ and follow the format used in Figure 1. Any *P*-values $\leq 0.10$ are noted in the figure panels and if no factors have *P*-values $\leq 0.10$ "ND" is included to denote no significant differences. Full statistical results and enzyme activities, also expressed as $\mu mol \cdot min^{-1} \cdot g$ ventricle, are included in Table S1.

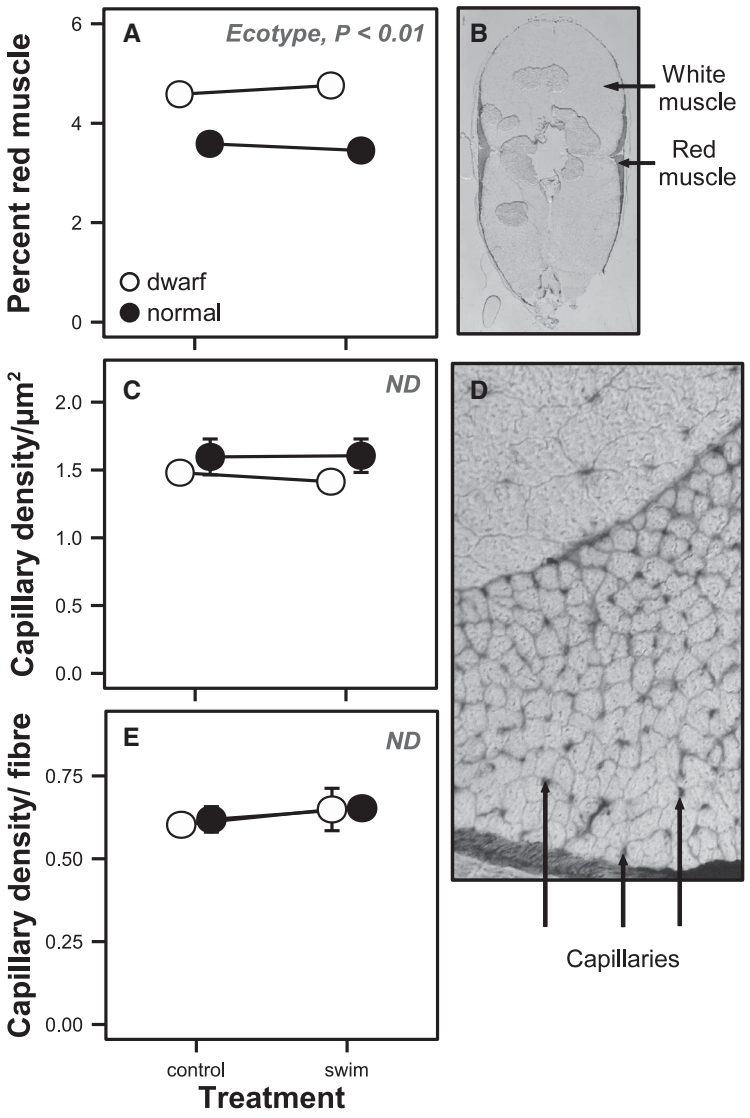


**Figure 3.** Traits related to oxygen uptake capacity in the skeletal muscle of swim-trained and control dwarf (white circles) and normal (black circles) whitefish. (A) Percentage of red, oxidative muscle fibers in a cross-section of fish muscle (ecotype: $P < 0.01$, $F_{1,18} = 61.81$, treatment: $P = 0.99$, $F_{1,6} < 0.01$, interaction: $P = 0.29$, $F_{1,18} = 1.20$), calculated from (B) histochemical staining of succinate dehydrogenase activity (Complex II in the mitochondrial ETC) as a marker for oxidative capacity. (C) Density of capillaries in the red skeletal muscle expressed as the number of capillaries per micrometer (ecotype: $P = 0.35$, $F_{1,17} = 0.91$, treatment: $P = 0.52$, $F_{1,6} = 0.47$, interaction: $P = 0.83$, $F_{1,17} = 0.05$) or (E) per red muscle fiber (ecotype: $P = 0.56$, $F_{1,20} = 0.35$, treatment: $P = 0.53$, $F_{1,6} = 0.44$, interaction: $P = 0.82$, $F_{1,20} = 0.05$), calculated from (D) histochemical staining of muscle sections cut transverse to fiber length for capillary alkaline phosphatase activity. Data are presented as in Figure 1.

activities in white muscle, MDH activity in red muscle and hematocrit (Fig. 7D). When groups were differentiated by treatment, Complex V activity, ventricle protein concentration, hematocrit, COX activity in red muscle, and Complex I activity had the highest factor loadings. Thus, only one of the top five traits discriminated both ecotypes and treatments (hematocrit). The classification success for all DFAs was 100%, and assignment was calculated from all experimental data and not from a random subset to train the model due to limited sample size. The patterns of variation in all traits are summarized in Table 2.

## Discussion

The physiological and biochemical mechanisms underlying local adaptation in natural populations of animals are largely unknown, and a question of particular interest is whether the same mechanisms contribute to acclimation and adaptation to similar environmental factors. If so, do common mechanisms lead to constraints or facilitations in evolutionary potential and under what circumstances (Schlichting and Wund 2014)? To address these questions, we conducted a common garden study and swim-training

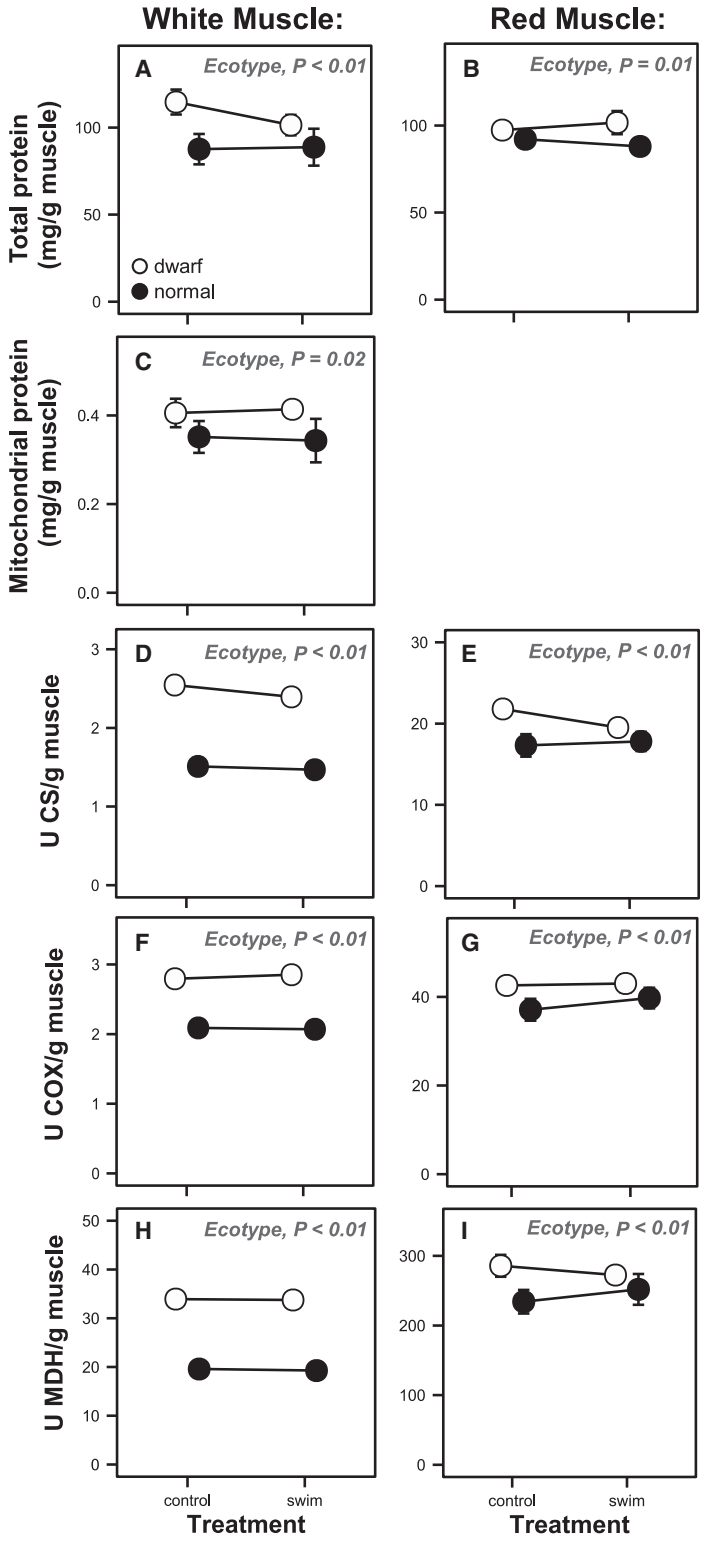


**Figure 4.** Quantitative differences in mitochondrial content (per gram skeletal muscle). Mitochondrial protein and enzyme activities in the skeletal muscles of control and swim-trained dwarf (white circles) and normal (black circles) whitefish. (A, B) Mg total protein per gram of white and red muscle. (C) Mg of total mitochondrial protein isolated per gram of white muscle. (D–I) Activities of mitochondrial enzymes, expressed as $\mu mol \cdot min^{-1} \cdot$ g muscle; citrate synthase (CS, citric acid cycle enzyme found in the mitochondrial matrix), cytochrome c oxidase (COX, Complex IV in the ETC and located on the inner mitochondrial membrane), and malate dehydrogenase (MDH, a citric acid cycle enzyme with cytosolic and mitochondrial isoforms, not distinguished in this assay) per gram of white (D, F, H) and red (E, G, I) muscle. Data are presented as in Figure 2. Full statistical results, and enzyme activities expressed as $\mu mol \cdot min^{-1} \cdot$mg muscle protein, are included in Tables S2 and S3.

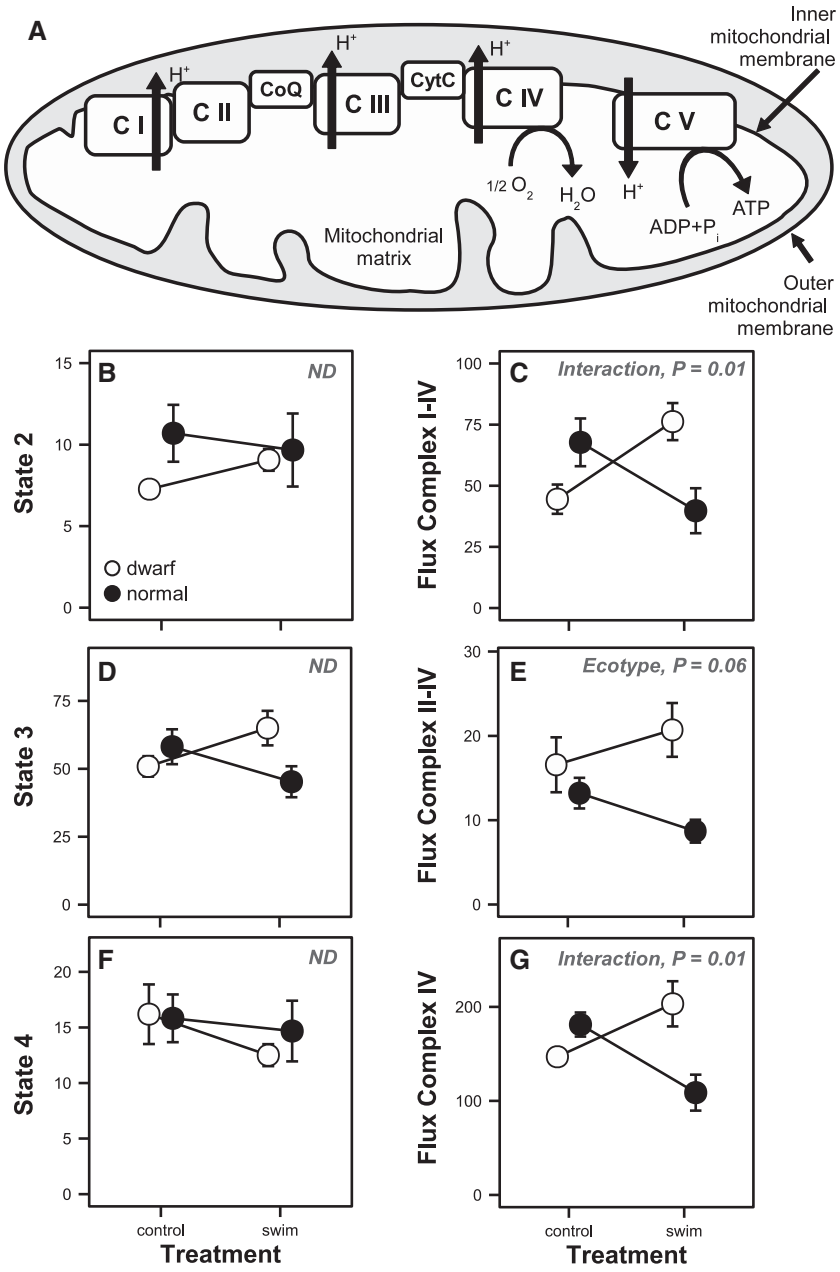


**Figure 5.** Qualitative differences in mitochondrial function (per milligram mitochondrial protein). Respiration rates of mitochondria isolated from the white skeletal muscle of control and swim-trained dwarf (white circles) and normal (black circles) whitefish. All oxygen consumption data are expressed as natoms oxygen·min$^{-1}$·mg$^{-1}$ mitochondrial protein. (A) Cartoon depicting mitochondrial oxidative phosphorylation (OXPHOS) including the five OXPHOS enzyme complexes (labeled CI–CV), electron carriers (coenzyme Q [CoQ] and cytochrome c [CytC]), arrows indicating which complexes pump $H^+$, reduction of oxygen by CIV and the formation of ATP by CV. (B) State 2, background respiration rate with substrates added (pyruvate and malate) but not ADP, (D) state 3, maximal rate of substrate oxidation stimulated by the addition of ADP, (E) state 4, nonphosphorylating respiration measured after depletion of ADP. Oxygen uptake due to flux through electron transport chain (ETC) Complexes (C) I–IV, (E) II–IV, (G) and IV, cytochrome c oxidase. Data are presented as in Figure 2. Full statistical results and mitochondrial respiration expressed as natoms oxygen·min$^{-1}$·mL$^{-1}$ of isolated mitochondria are included in Table S4.

experiment to study the evolution of, and plasticity in, traits related to exercise physiology and aerobic energy metabolism in normal and dwarf ecotypes of Lake Whitefish (*Coregonus clupeaformis*). We found extensive evolutionary divergence in traits associated with oxygen transport and use, revealing that the more active dwarf whitefish have evolved a higher capacity for oxygen transport and use. However, traits related to oxygen uptake show mixed results, indicating that additional selective pressures

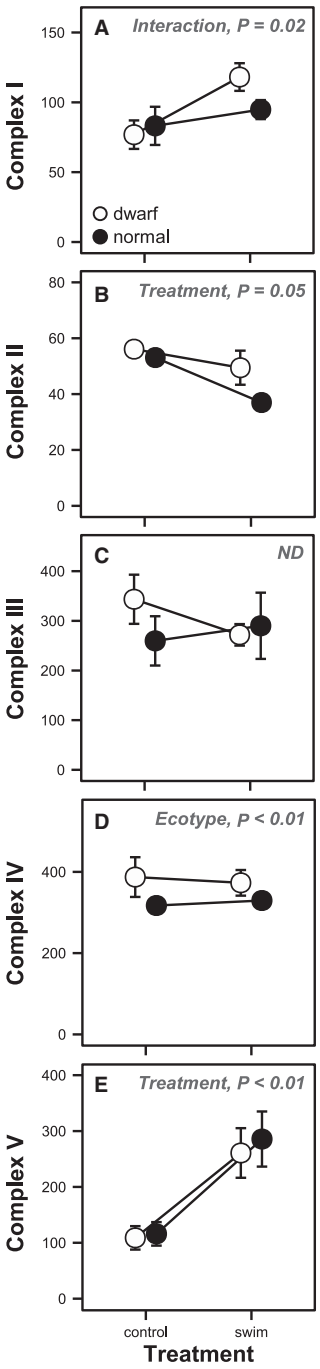


**Figure 6.** **Qualitative differences in mitochondrial enzyme activities (per milligram mitochondrial protein). Activities of oxidative phosphorylation (OXPHOS) enzymes per milligram white muscle mitochondria from swim-trained and control dwarf (white circles) and normal (black circles) whitefish. Activities of the electron transport chain enzymes are expressed as nmoles $min^{-1}$ $mg^{-1}$ mitochondrial protein. (A) Complex I, NADH dehydrogenase, (B) Complex II, succinate dehydrogenase, (C) Complex III, quinol-cytochrome c reductase, (D) Complex IV, cytochrome c oxidase, and the phosphorylating enzyme (E) Complex V, $F_0F_1$-ATPase, which uses the proton gradient created by the electron transport chain to drive ATP synthesis. Data are presented as in Figure 2. Full statistical results are included in Table S5.**

(e.g., hypoxia and temperature) or stochastic evolutionary processes (e.g., genetic drift), may influence the evolution of oxygen extraction capacity. We also found significant, but less pervasive, effects of swim training. Finally, the specific steps of the oxygen transport cascade that varied among ecotypes differed from those that responded to swim training, and only one of the measured traits displayed a pattern of evolution consistent with genetic accommodation.

## DIFFERENCES IN EXERCISE PHYSIOLOGY BETWEEN ECOTYPES

Dwarf whitefish had higher values than normal fish for a suite of traits within the oxygen transport cascade, as predicted (Table 1). In particular, dwarf whitefish have significantly larger ventricles, which could allow for increased cardiac output and delivery of oxygenated blood (Gamperl and Farrell 2004). Furthermore, our finding that normal fish, but not dwarf fish, increase ventricle size after swim training suggests that wild fishes, which should be quite active (e.g., Anttila and Manttari 2009), may display little variation in ventricle size. Indeed, Evans et al. (2013) found that size-corrected ventricle size was slightly, but not significantly larger in wild dwarf fish. Ventricle metabolic capacity was similar among ecotypes; only PK (higher in dwarf whitefish per gram tissue and per milligram protein), which catalyzes the last step of glycolysis, varied among ecotypes. It is possible that increased cardiac PK activity could enhance glycolytic capacity, but the shared control of pathway flux among the 10 glycolytic enzymes suggests this is unlikely (Eanes et al. 2006; Suarez and Moyes 2012). Therefore, differences in cardiac performance among ecotypes, if present, would most likely be due to changes in ventricle mass.

We also measured the activities of aerobic metabolism enzymes in the white skeletal muscle to clarify the impacts of variation in OXPHOS transcript levels in wild fish (Derome et al. 2006; Evans and Bernatchez 2012) and to link molecular variation to differences in whole-animal swimming behavior (Rogers et al. 2002). Wild dwarf fish generally express OXPHOS genes in white muscle at higher levels than normal fish (Derome et al. 2006; Evans and Bernatchez 2012; but see Derome et al. 2008), but different subunits of the same enzyme complexes often had different expression patterns, making it difficult to predict the effects of transcriptional divergence on enzyme activity and mitochondrial function. We found consistent increases in the activity of mitochondrial enzymes in dwarf white muscle, which argues that "master regulators" of mitochondrial biogenesis may vary among ecotypes and suggests candidates for future studies (reviewed by Bremer et al. 2014). However, variation in individual protein coding genes may also be important; there is strong evidence for selection on the proximal promoter of the cytoplasmic MDH (Jeukens and Bernatchez 2012) and the coding

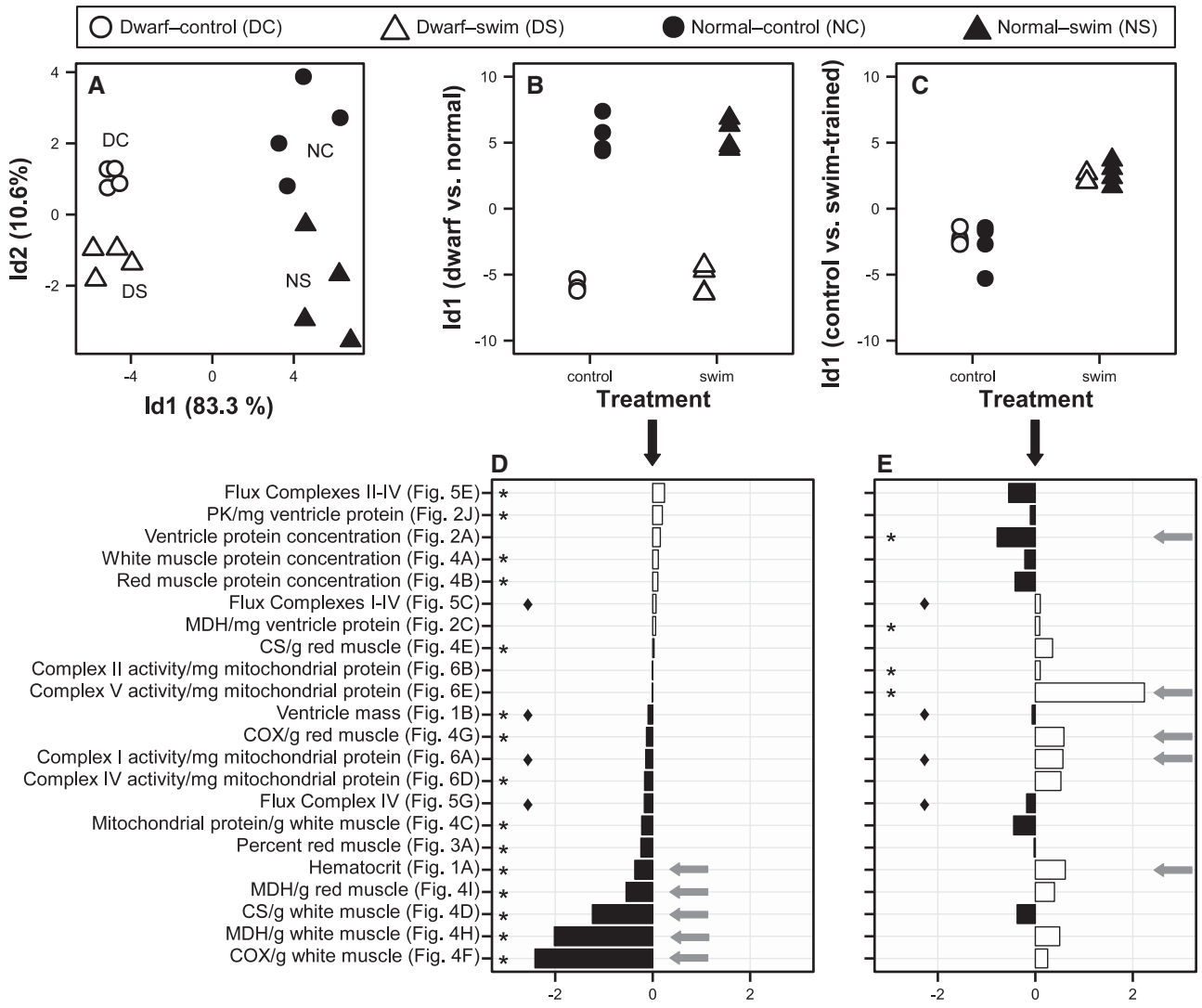


**Figure 7. Multivariate analyses of differences in aerobic exercise physiology due to phenotypic plasticity and evolutionary variation between ecotypes. (A) Mean tank values for the first two linear discriminants (ld) from a DFA discriminating fish by group (dwarf-control, dwarf-swim trained, normal-control, normal-swim trained) with the percentage of variation explained by each listed on the axes. ld values from two additional DFAs that discriminated fish by either ecotype (B) or treatment (C), and the factor loadings contributing to each, respectively (D, E). (D, E) Asterisks are placed next to traits that show a significant effect of ecotype (D) or treatment (E) and diamonds are placed next to traits that have a significant interaction among treatment and ecotype in univariate analyses. Gray arrows point to the five traits with the highest factor loadings for the DFAs discriminating fish by ecotype (D) or treatment (E).**

regions of many metabolic enzymes (Renaut et al. 2010). The adaptive significance of these differences in energetic enzymes are currently being tested by determining if this trait has evolved repeatedly in multiple, independently evolved Lake Whitefish species pairs (A. C. Dalziel et al. unpubl. ms.).

In addition to having more mitochondria per gram muscle, dwarf whitefish may have higher quality mitochondria, as they have higher Complex IV activity per milligram mitochondria. Even more pronounced were the ecotype-specific effects of swim training (i.e., evolutionary variation in plasticity); after training, dwarf whitefish had higher Complex I activity per milligram protein and increased flux through Complexes I–IV and IV. These same traits either stayed the same (Complex I) or decreased (flux through Complexes I–IV and IV) after swim training in normal whitefish. Although the effects of exercise training on mitochondrial quality and exercise capacity have not been studied in fishes, mammalian studies suggest that increases in mitochondrial respiration (Daussin et al. 2008; Jacobs and Lundby 2013) and electron transport chain protein levels (Egan et al. 2011) per unit mitochondria are associated with increased exercise capacity (Boushel et al. 2014). Therefore, we hypothesize that these increases in mitochondrial function in dwarf fish after training represent beneficial

**Table 2. Patterns of trait variation between dwarf and normal whitefish in high-flow (swim training) and low-flow (control) environments.**

| Trait response: | No variation | Evolution of constitutive values | Evolution of plasticity—I | Evolution of plasticity—II | Evolutionarily conserved plasticity | Genetic accommodation |
|---|---|---|---|---|---|---|
| Effect of swim training? (treatment effect) | No | No | No | No | Yes | No |
| Interaction? | No | No | Yes | Yes | No | Yes |
| Normal plastic? (ancestral) | No | No | No | Yes | Yes | Yes |
| Dwarf plastic? (derived) | No | No | Yes | Yes | Yes | No |
| Evolutionary change? (ecotype effect) | No | Yes | Yes, increased plasticity in dwarf fish. | Yes, direction of plasticity varies among ecotypes. | No | Yes, loss of plasticity in dwarf |
| Direction and extent of plasticity in dwarf and normal fish? | – | – | – | Direction reversed, extent similar | Similar direction and extent | – |
| Control trait values? | Similar | Different | Similar | Similar | Similar | Different |
| Does trait evolution match direction of plastic change? | – | – | – | No | – | Yes |
| Pattern consistent with genetic accommodation (sensu Schitling and Wund 2014)? | No | No | No (no ancestral plasticity) | No (ancestral plasticity not predicted to lead to beneficial acclimation) | No | Yes (genetic assimilation) |
| Traits matching this pattern: | → Ventricle enzymes (Fig. 2B, D–I, K)<br>→ Capillary density (Fig. 3C, E)<br>→ States 2, 3, 4 (Fig. 5B, D, F)<br>→ Complex III (Fig. 6C) | → Hematocrit (Fig. 1A)<br>→ PK/mg ventricle protein (Fig. 2J)<br>→ Percent red muscle (Fig. 3A)<br>→ Total protein content in red and white muscle (Fig. 4A, B)<br>→ Mitochondrial content in white muscle (Fig. 4C)<br>→ Mitochondrial enzyme activities in red and white muscle (Fig. 4D–I)<br>→ Flux Complexes II–IV display trend (Fig. 5E, ecotype, $P = 0.06$)<br>→ Complex IV activity (Fig. 6D) | → Complex I (Fig. 6A) | → Flux Complexes I–IV and IV (Fig. 5C, G) | → Ventricle protein content (Fig. 2A)<br>→ Ventricle MDH activity (Fig. 2C)<br>→ Complex II and V activity (Fig. 6B, E) | → Ventricle mass display trend (interaction, $P = 0.06$, Fig. 1B) |

**Traits are grouped following the general patterns of variation described in Figure 2 of Renn and Schumer (2013). In our study, the normal whitefish ecotype is considered to be very similar to the ancestral form and the dwarf ecotype is the derived form. We use these data to determine if individual traits match the predictions for genetic accommodation outlined by Schlichting and Wund (2014). Note that not all possible patterns for trait evolution and plasticity are included, only those detected in this study.**

acclimation or "adaptive phenotypic plasticity" (sensu Ghalambor et al. 2007). Conversely, we predict that the lack of response, or decreases, in mitochondrial respiration after training in normal fish is a form of "nonadaptive plasticity" (sensu Ghalambor et al. 2007), possibly associated with a stress-response to exercise.

Although dwarf whitefish generally had higher values for traits in the oxygen transport cascade, this trend did not hold for all traits. In particular, two traits related to oxygen uptake have increased values in normal fish: hematocrit (this study, may also influence oxygen transport) and gill surface area (Evans et al. 2013). Additional parameters, including gill diffusion distance, hemoglobin content and hemoglobin-oxygen binding affinity must be measured to determine how these changes affect oxygen uptake, but these data do suggest that normal fish may have an increased capacity for oxygen uptake. At first glance, this seems counterintuitive with respect to the higher activity levels of dwarf fish (Rogers et al. 2002), and may simply be the result of stochastic evolutionary processes. However, we hypothesize that these data could also reflect the influence of additional selective pressures, such as hypoxia. Although the hypoxia tolerances of these ecotypes are not known, normal whitefish may experience environmental hypoxia in the benthic zone during the summer (Landry et al. 2007), so increased oxygen extraction capacity may be beneficial. Alternatively, Evans et al. (2012) found that dwarf fish have more cathodic hemoglobins, which aid in oxygen uptake during hypoxia, at high temperatures (which can induce

hypoxia), and with metabolic acidosis (Mairbãurl and Weber 2012). These are conditions that dwarf fish may experience while foraging in the warmer limnetic zone in the summer (Gorsky et al. 2012; Evans et al. 2014). Together, these data hint that differences in oxygen transport cascade-related traits in Lake Whitefish ecotypes may be the product of multiple interacting selective pressures, including activity levels, environmental oxygen content, and temperature. We note that while increases in hypoxia tolerance and sustained exercise performance are both predicted to occur via increases in the capacity of the oxygen transport cascade, empirical studies often find trade-offs among these performance traits in fish (reviewed by Farrell and Richards 2009). Therefore, these factors (environmental and exercise-induced hypoxia) may lead to selection for similar values in some traits and divergent values in others (Farrell and Richards 2009). Future studies examining maximal aerobic capacity, hypoxia tolerance, and temperature tolerance, in combination with studies of independently evolved species pairs, should shed more light on the demographic and ecological factors influencing the evolution of the oxygen transport cascade in Lake Whitefish.

Together, our data argue that dwarf whitefish have evolved an increased constitutive capacity for aerobic energy metabolism and "adaptive" plasticity of mitochondrial quality in the skeletal muscle. These evolved differences, which span the "transport and use" steps of the oxygen cascade, are predicted to increase the aerobic capacity of dwarf compared to normal whitefish and as such, corroborate studies of swimming behavior, metabolic energy expenditure (Trudel et al. 2001; Rogers et al. 2002), and gene expression (Derome et al. 2006; Evans et al. 2012). Aerobic capacity is hypothesized to be a common target of natural selection in vertebrates (Hayes and O'Connor 1999; Irschick et al. 2008), so an understanding the mechanisms by which it evolves can help to reveal the mechanistic bases of local adaptation in a number of species. Data from natural (e.g., Eliason et al. 2011; Cheviron et al. 2012; Dalziel et al. 2012) and experimentally evolved populations (e.g., Swallow et al. 2009; Gębczyński and Konarzewski 2011) have found that multiple steps in the oxygen transport cascade can evolve relatively rapidly and that the particular steps that evolve are a combination population-specific and common mechanisms (e.g., Garland et al. 2011). For example, increases in ventricle size are associated with the evolution of aerobic capacity in Lake Whitefish, Sockeye Salmon (Eliason et al. 2011), and Threespine Stickleback (Dalziel et al. 2012), suggesting that this could be a common mechanism underlying increases in prolonged swimming capacity in fish.

## PLASTICITY OF EXERCISE PHYSIOLOGY

The effects of training were not as pronounced as the effects of evolutionary divergence among ecotypes and many of the traits predicted to increase with training did not (Table 1). Indeed, only MDH activity per milligram ventricle protein, Complex V activity per milligram mitochondrial protein, ventricle size in normal fish, and flux through mitochondrial OXPHOS Complexes II–IV and IV in dwarf fish increased as predicted. Two traits, Complex II activity and ventricle protein content, responded in a manner contrary to our predictions in both ecotypes. The limited response to swim training could be due to a reduced response to exercise training in Lake Whitefish compared to other fishes or because our training regime was not rigorous enough to induce significant trait variation. The first possibility is supported by data showing that cold acclimation, a treatment that often increases mitochondrial enzyme activity and the percentage of red muscle in salmonids, did not induce changes in juvenile Lake Whitefish (Blier and Guderley 1988). Such species-specific responses to training are commonly found in fishes (see Table 1). We do note that in European Whitefish (*Coregonus lavaretus*), muscle calcium handling and the activity of a mitochondrial enzyme are plastic (Anttila et al. 2008), as are feeding behaviors related to local adaptation (Lundsgaard-Hansen et al. 2013). On the other hand, the effects of swim training also depend on training intensity (e.g., Anttila et al. 2008; Castro et al. 2013; but see Farrell et al. 1991), and our swim-training protocol was less rigorous than prior studies in European Whitefish (Anttila et al. 2008). This is because of the allometric relationship between fish body size and swimming capacity (Kolok 1999), so a training regime of ~1.5 BL/s for juvenile whitefish was less rigorous than for large adult European Whitefish (Anttila et al. 2008). Based upon results for slightly larger fish (Bernatchez and Dodson 1985), our training speeds were at approximately 33% of the critical swimming speed ($U_{crit}$) whereas Anttila et al. (2008) trained European Whitefish at 50–80% of $U_{crit}$. Whatever the basis, it seems that evolutionary divergence, rather than moderate differences in routine activity, best explain differentiation in exercise physiology between dwarf and normal whitefish.

We predicted that exercise training would increase cardiac citric acid cycle enzyme activity (Farrell et al. 1990; but see Farrell et al. 1991), and found an increase in the activity of MDH, but not CS per milligram protein. This may be because we combined spongy and compact myocardium tissues or because MDH also functions in the malate-aspartate shuttle, the dominant NAD/NADH shuttle in heart (e.g., Scholz et al. 2000). Indeed, the proteins in this shuttle increase in rat heart after exercise training (Ferreira et al. 2015). In Lake Whitefish, the cytoplasmic isoform of MDH (cMDH) is upregulated in the liver of dwarf populations and linked *cis*-regulatory sites display evidence of selection (Jeukens and Bernatchez 2012). Hepatic upregulation is accompanied by increases in many mitochondrial proteins (St-Cyr et al. 2008), similar to our findings in dwarf skeletal muscles. Although our enzyme assay cannot distinguish between MDH isoforms, it suggests that constitutive increases in enzyme activities evolved

in dwarf fish liver and skeletal muscle while maintaining plasticity in cardiac muscle.

Exercise training also led to increases in OXPHOS Complex V activity per milligram mitochondrial protein, decreases in Complex II activity, and no changes in the activities of other OXPHOS complexes in both ecotypes. This lack of coordination in OXPHOS complex regulation after exercise training is also found in human skeletal muscle mitochondria (Egan et al. 2011) and increased respiration can occur via increased flux through a single OXPHOS complex (Daussin et al. 2008; Jacobs and Lundby 2013). In dwarf Lake Whitefish, increases in Complex V coupled with training-induced increases in Complex I are associated with significant increases in mitochondrial respiration.

Finally, we found that training decreased total ventricle protein per gram tissue in both ecotypes, which could reflect differences in tissue hydration or rates of protein synthesis/breakdown. This was not a trait that we originally predicted to influence cardiac output independently of changes in ventricle size and metabolic capacity and our data show a trend opposite to the increases heart protein concentration after swim training in Rainbow Trout (Houlihan and Laurent 1987). However, exercise-induced changes in protein content are age, training regime, and population specific in mammalian skeletal muscle (e.g., Harber et al. 2009, 2012), so further studies are needed to determine the mechanisms leading to, and any functional implications of, changes in ventricle protein content in Lake Whitefish.

### DO SIMILAR MECHANISMS CONTRIBUTE TO PLASTICITY AND THE EVOLUTION OF EXERCISE PHYSIOLOGY?

If common mechanisms contribute to phenotypic plasticity within a population and local adaptation among populations then we would see one of the following patterns in the traits we measured: an effect of both ecotype and treatment, an effect of ecotype and treatment plus an interaction, an effect of ecotype and an interaction, an effect of treatment and an interaction, or an interaction effect. All five patterns suggest that a trait displays some form of plasticity and that there has also been some type of evolutionary divergence among ecotypes, either in constitutive trait values or plasticity. Overall, we found that 15 of the 36 traits we measured evolved in dwarf fish and eight show plasticity in at least one ecotype, but only four of the 36 traits fit the criteria above: ventricle size (significant effect of ecotype and near-significant interaction [$P = 0.06$]), flux through OXPHOS Complexes I–IV and IV (interaction), and Complex I activity (interaction). Our multivariate analyses further argue that the traits that have evolved among ecotypes are generally different than those that vary with swim training as only one of the five traits that best discriminates groups by ecotype or swim training is shared (hematocrit).

Furthermore, if genetic accommodation, "a process (1) initiated by phenotypic responses to an environmental stimuli enabling population persistence, and (2) followed by genetic changes that enhance their adaptive value" (Schlichting and Wund 2014), leads to local adaptation, traits must: (3) display "adaptive" plasticity (sensu Ghalambor et al. 2007) in normal fish (ancestral form), and (4) vary in a similar manner during adaptation in dwarf fish (see Fig. 2 Renn and Schumer 2013 for reaction norms). Flux through Complexes I–IV and IV do not meet the fourth criterion as these traits respond in opposite directions to training in dwarf and normal ecotypes, suggesting these responses are controlled by different mechanisms. Furthermore, these decreases in flux after exercise training in normal fish are not predicted to be beneficial, so do not meet the third criterion of "adaptive" plasticity. Therefore, only ventricle mass follows a pattern consistent with genetic accommodation, although the interaction was not significant ($P = 0.06$).

Recently, studies examining the mechanisms underlying local adaptation to environmental stressors have found that genetic accommodation may be a common route to local adaptation in natural populations of animals (e.g., McCairns and Bernatchez 2010; Scoville and Pfrender 2010; Storz et al. 2010; Lee et al. 2011; Whitehead et al. 2011). However, if ancestral populations display little phenotypic plasticity in ecologically important traits, then local adaptation can only occur via constitutive changes in trait values (e.g., Dhillon and Schulte 2011; Lee et al. 2011) and/or the evolution of increased "adaptive" plasticity (e.g., Dhillon and Schulte 2011; Cheviron et al. 2013, 2014; Morris et al. 2014). Our swim-training experiments, in combination with studies of thermal acclimation by Blier and Guderley (1988), suggest that ancestral populations of Lake Whitefish may have had limited ancestral plasticity in traits related to aerobic capacity, which would preclude evolution by genetic accommodation.

## Conclusions

We found that local adaptation in a more active, derived dwarf whitefish population is associated with the evolution of physiological and biochemical traits related to oxygen transport and use and rule out allometric variation and swim training as major contributors to differences in aerobic physiology among ecotypes. Together, the evolution of increased constitutive trait values, in combination with the evolution of "adaptive" plasticity in mitochondrial function, is predicted to increase the capacity for oxygen transport and use and facilitate active swimming in dwarf fish (Rogers et al. 2002). As well, the mechanistic targets of acclimation largely differed from those affected by local adaptation, supporting the hypothesis that the rapid evolutionary divergence in the oxygen transport cascade between Lake Whitefish ecotypes occurred by mechanisms other than genetic accommodation.

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## *Supporting Information*

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1**. Ventricle mass, protein content, and enzyme activities.
**Table S2**. White skeletal muscle total and mitochondrial protein content and mitochondrial enzyme activities.
**Table S3**. Red skeletal muscle total protein content and mitochondrial enzyme activities.
**Table S4**. Respiration of mitochondria isolated from white skeletal muscle.
**Table S5**. Activities of oxidative phosphorylation (OXPHOS) enzymes expressed per milligram mitochondrial protein.