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SPECIAL ISSUE: THE MOLECULAR MECHANISMS OF ADAPTATION AND SPECIATION: INTEGRATING GENOMIC AND MOLECULAR APPROACHES

Convergence in organ size but not energy metabolism enzyme activities among wild Lake Whitefish (*Coregonus clupeaformis*) species pairs

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Abstract

The repeated evolution of similar phenotypes by similar mechanisms can be indicative of local adaptation, constraints or biases in the evolutionary process. Little is known about the incidence of physiological convergence in natural populations, so here we test whether energy metabolism in 'dwarf' and 'normal' Lake Whitefish evolves by similar mechanisms. Prior genomic and transcriptomic studies have found that divergence in energy metabolism is key to local adaptation in whitefish species pairs, but that distinct genetic and transcriptomic changes often underlie phenotypic evolution among lakes. Here, we predicted that traits at higher levels of biological organization, including the activities of energy metabolism enzymes (the product of enzyme concentration and turnover rate) and the relative proportions of metabolically active tissues (heart, liver, skeletal muscle), would show greater convergence than genetic and transcriptomic variation. We compared four whitefish species pairs and found convergence in organ size whereby all dwarf whitefish populations have a higher proportion of red skeletal muscle, three have relatively larger livers and two have relatively larger ventricles than normal fish. On the other hand, hepatic and muscle enzyme activities showed little convergence and were largely dependent on lake of origin. Only the most genetically divergent species pair (Cliff Lake) displayed white muscle enzyme activities matching results from laboratory-reared normal and dwarf whitefish. Overall, these data show convergence in the evolution of organ size, but not in the activities of candidate enzymes of energy metabolism, which may have evolved mainly as a consequence of demographic or ecological differences among lakes.

Keywords: adaptation, aerobic energy metabolism, fish, glycolysis, heart, red muscle

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Introduction

A major goal in evolutionary biology is to discover whether similar underlying genetic, biochemical and physiological mechanisms contribute to the evolution of similar phenotypes (Losos 2011; Elmer & Meyer 2011; Wake *et al.* 2011; Conte *et al.* 2012; Rosenblum *et al.* 2014). Determining when similar phenotypes do, or do not, evolve by the same mechanisms will help identify the demographic, developmental, physiological and genetic factors that constrain or bias evolutionary pathways and allow biologists to better predict evolutionary change (Schwenk & Wagner 2004; Brakefield 2006; Losos 2011; Wake *et al.* 2011; Stern 2013; Rosenblum *et al.* 2014). The incidence of convergence during the evolution of complex phenotypic traits is of particular interest because these traits arise from the integration of multiple physiological processes, biochemical pathways

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and genes, so can theoretically evolve in many different ways (Conte *et al.* 2012). To date, the most informative studies of the mechanisms leading to the repeated evolution of complex traits in natural populations have focused on morphology, including changes in coloration and form (Gompel & Prud'homme 2009; Elmer & Meyer 2011; Conte *et al.* 2012; Rosenblum *et al.* 2014). Less attention has been paid to the incidence and mechanisms of physiological and behavioural convergence in the wild (but see Conte *et al.* 2012; Lenser & Theissen 2013; Foll *et al.* 2014; Dennis *et al.* 2015; Pfenninger *et al.* 2015; Qi *et al.* 2015). Note that we follow Rosenblum *et al.*'s (2014) terminology and use the term 'convergence' to describe the incidence of similar phenotypic patterns and 'parallelism' to describe similar molecular mechanisms underlying phenotypic convergence.

The incidence of parallelism may vary during the evolution of 'morphological', 'behavioural' and 'physiological' traits as a result of differences in the genetic bases for trait variation (Brakefield 2011). For example, morphological evolution may arise as a result of constitutively expressed genetic changes or changes in developmental plasticity, but many 'morphological traits' are irreversible in adulthood (Brakefield 2011; Wake *et al.* 2011; Stern 2013; Martin & Orgogozo 2013; but see examples in Moczek *et al.* 2011). On the other hand, most physiological traits can evolve via constitutively expressed genetic changes, changes in genes influencing developmental plasticity and also the evolution of genes underlying reversible phenotypic plasticity (acclimation/acclimatization; Piersma & Drent 2003). While developmental plasticity (normally irreversible), and acclimation (normally reversible) often interact, their genetic bases may differ (Beaman *et al.* 2016). Thus, at least theoretically, there may be more evolutionary 'routes' available for reversible physiological evolution than irreversible morphological evolution, possibly leading to a decreased incidence of parallelism in the former when all else is equal.

Here, we study multiple species pairs of 'dwarf' and 'normal' Lake Whitefish (*Coregonus clupeaformis*) that have evolved independently in four different lakes as a model system in which to test whether physiological adaptation occurs by similar mechanisms (Bernatchez *et al.* 2010). Lake Whitefish live in freshwaters across North America (Scott & Crossmann 1998). During the Pleistocene glaciation (~60 000 years ago), populations were divided into different glacial refugia (e.g. Acadian, Atlantic–Mississippian; Bernatchez & Dodson 1990, 1991; Pigeon *et al.* 1997; Jacobsen *et al.* 2012). Approximately 10–12 000 years ago, these glacial lineages came back into secondary contact in the four lakes investigated in this study (Rougeux *et al.* 2016). Since this time, some Acadian lineages have undergone continued genetic and phenotypic divergence leading to the evolution of a derived, limnetic 'dwarf' species from an ancestral, 'normal' benthic species (reviewed by Bernatchez *et al.* 2010).

The smaller 'dwarf' Lake Whitefish is a more active swimmer with slower growth rates than the larger, ancestral 'normal' form (Trudel *et al.* 2001; Rogers *et al.* 2002; Bernatchez *et al.* 2010). These repeatedly evolved species pairs show extensive genetic (Bernatchez & Dodson 1990, 1991; Pigeon *et al.* 1997; Campbell & Bernatchez 2004; Gagnaire *et al.* 2013a; Dion-Côté *et al.* 2016), physiological (transcriptional divergence in the liver and white skeletal muscle, organ size; Trudel *et al.* 2001; Derome *et al.* 2006, 2008; St-Cyr *et al.* 2008; Nolte *et al.* 2009; Bernatchez *et al.* 2010; Evans *et al.* 2012, 2013), ecological (Landry *et al.* 2007; Landry & Bernatchez 2010), morphological (trophic morphology, body shape; Lu & Bernatchez 1999; Laporte *et al.* 2015) and microbiont (Sevellec *et al.* 2014) divergence. A suite of genetic and transcriptomic studies have highlighted central energy metabolism as a key biochemical network underlying local adaptation and speciation in this system (including mitochondrial oxidative phosphorylation, the citric acid cycle, glycolysis, glycogen metabolism and creatine phosphokinase; reviewed by Bernatchez *et al.* 2010; summarized in Table 1, Tables S6–S8, Supporting information). Furthermore, laboratory-reared dwarf fish have an increased capacity for oxygen transport, increased activities of glycolytic and mitochondrial enzymes in muscle, and decreased activities of mitochondrial enzymes in liver (Derome *et al.* 2008; Jeukens *et al.* 2009; Dalziel *et al.* 2015; Laporte *et al.* 2016; A.C. Dalziel, M. Laporte, H. Guderley & L. Bernatchez, in preparation).

Although many genes involved in central energy metabolism vary in allele frequencies and gene expression among dwarf–normal species pairs, studies to date have found limited parallelism among repeatedly evolved dwarf species from different lakes (Derome *et al.* 2006; Rogers & Bernatchez 2007; St-Cyr *et al.* 2008; Renaut *et al.* 2011; Evans *et al.* 2012; Gagnaire *et al.* 2013a; but see Jeukens *et al.* 2009). As with many 'physiological traits', the activities and expression of enzymes involved in energy metabolism can vary constitutively or inducibly as a result of genetic differentiation (e.g. Dayan *et al.* 2015; Scott *et al.* 2015), show developmental plasticity (e.g. Schnurr *et al.* 2014) and show reversible phenotypic plasticity (e.g. Davison 1997; Baris *et al.* 2016). Therefore, differences in energy metabolism could evolve in many different ways (Eanes 2011; Zera 2011; but see Lavington *et al.* 2014 for potential constraints). However, the incidence of pathway or network-wide parallelism during the evolution of central energy metabolism is largely unknown.

In this study, we test whether convergence is found in the activities of candidate enzymes and the relative

Table 1 Summary of genomic and transcriptomic surveys of the loci examined in this study in the same wild dwarf and normal whitefish populations. See Tables S6–S8 (Supporting information) for more information on these and additional studies of genomic and transcriptomic divergence among whitefish species pairs. Blank spaces indicate that no information is known, and 'ns' indicates that a particular gene was studied but no significant differences were found among species pairs

Metabolic pathway or cellular function	Tissue	Gene (gene symbol*, isoform)	mRNA content & populations studied	Pathway mRNA content	Genetic variation detected?	Enzyme data
Glycolysis	WM	Phosphoglucose isomerase (GPI)	ns in Indian & Cliff[1]	Significant differences among species pairs, but variation among lakes and probes/isoforms[1,3]		Fig. 4
	WM	Phosphofructokinase (PFKM, muscle)	ns in Indian & Cliff[1]			
	WM	Aldolase (ALDOA, muscle)	↑ in Indian dwarf, ↓ in Cliff dwarf[1]			
	WM	Triosephosphate isomerase (TPI1)	↓ in Cliff & Indian dwarf[1]		Yes[6] – in Cliff & associated with condition factor in Témiscouata dwarf x Aylmer normal backcross (noncoding SNP)	
	WM	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Variation among probes in Indian, ↓ in Cliff dwarf[1]		Yes[5,9], outlier in Cliff (noncoding SNP)	
	WM	Pyruvate kinase (PKM, muscle)	ns in Indian & Cliff[1]		Yes[9], outlier in Cliff (noncoding SNP)	
	WM	Lactate dehydrogenase (LDHA, muscle)	↑ in Cliff & Indian dwarf[3]		Yes[9], outlier in Cliff (noncoding SNP)	
	Liver	GPI	↑ in Cliff[4] dwarf & ns in Indian[2]	↑ in dwarf fish in Cliff (all genes measured) and Indian lakes (ALDO, GAPDH)[2,4]		Fig. 4
	Liver	PFKL, liver	ns in Indian & Cliff[2,4]			
	Liver	ALDOB, liver	↑ in Indian & Cliff dwarf[2,4]			
	Liver	TPI1	↑ in Cliff[4] dwarf & ns Indian[2]		Yes (see above)	
	Liver	GAPDH	↑ in Indian & Cliff dwarf[2,4]		Yes (see above)	
	Liver	PKLR, liver & red blood cell	ns in Indian & Cliff[2,4]		Yes (see above)	
	Liver	LDHB, liver	↑ in Cliff[4] dwarf & ns Indian[2]		Yes (see above)	
Glycogenesis	WM	Glycogen synthase (GYS1, muscle)				Fig. 5
	Liver	GYS2, liver	ns in Cliff[4]	ns in Cliff Lake[4]		
Glycogenolysis	WM	Glycogen Phosphorylase (PYGM, muscle)		ns in Cliff & Indian lakes[1]		Fig. 5
	Liver	PYGL, liver	One contig ↑ in Cliff dwarf, ns for other[4]	↑ in Cliff dwarf[4]		

Table 1 *Continued*

Metabolic pathway or cellular function	Tissue	Gene (gene symbol*, isoform)	mRNA content & populations studied	Pathway mRNA content	Genetic variation detected?	Enzyme data
Glycerol phosphate shuttle, links carbohydrate and lipid metabolism	WM	Glycerol-3-phosphate dehydrogenase (GPD1, cytoplasmic; GPD2, mitochondrial)	GPD1 - ↑ in Cliff dwarf, ↓ in Indian dwarf[1]	Variation among lakes[1]		Fig. 5
	Liver	GPD1; GPD2	GPD1 - ↑ in Cliff dwarf[4]	↑ in Cliff dwarf[3]		
Substrate-level phosphorylation	WM	Creatine phosphokinase (CKM, cytosolic muscle; CKMT1, mitochondrial; CKMT2, mitochondrial)	CKM - ↑ in Indian dwarf, ↓ in Cliff dwarf[1]	Variation among lakes[1]	Yes[5,6,9]. CKM many nonsynonymous substitutions, CKM & CKMT2 are outliers in Cliff (noncoding SNPs)	Fig. 5
	Liver	CKB, cytosolic brain and liver; CKMT1, mitochondrial; CKMT2, mitochondrial	CKB & CKMT1 - ns in Indian & Cliff[2,4]	ns in Cliff & Indian lakes[2,3]	Yes (see above)	
Citric acid cycle (mainly mitochondrial)	WM	Citrate synthase (CS)		ns in Cliff & Indian lakes[1]		Fig. 3
	WM	Malate dehydrogenase (MDH1, cytosolic; MDH2, mitochondrial)	ns in Indian & Cliff[1]		Yes[5,7], MDH1 outlier in Cliff & Indian lakes (coding and noncoding SNPs)	
	Liver	CS	ns in Cliff[3]	Variation among lakes and probes/isoforms, generally ↑ in Cliff dwarf compared to normal[2,4]		
	Liver	MDH1; MDH2	MDH1 – Microarray finds two contigs ↑ in Cliff & Indian dwarf, one contig ↓ in Cliff & Indian dwarf[2], RNA-seq finds ↑ in Cliff dwarf[4] MDH2 - ↓ in Cliff dwarf[4]		Yes (see above)	
Electron transport chain, oxidative phosphorylation (mitochondrial)	WM	Cytochrome *c* oxidase (composed of three mitochondrial and 10 nuclear genes)	MT-COX3 ↑ in Cliff and ↓ in Indian dwarf[1,8] COX6A2 ↑ in Cliff lake dwarf[8]	Variation among lakes[1,8]	Yes[5,6], MT-COX1, COX2, COX3 outliers in Cliff (all on mitochondrial genome, coding SNPs)	Fig. 3
	Liver	Cytochrome *c* oxidase (composed of three mitochondrial and 10 nuclear genes)	MT-CO1, MT-CO2, COX5B, COX6C2, COX8A: one contig ↑ in Cliff & Indian dwarf, one contig ↓[4] MT-CO3: one contig ↑ in Cliff dwarf, one contig	Variation among contigs and lakes, generally ↑ in Cliff dwarf compared to normal[2,4]	Yes (see above)	

Table 1 *Continued*

Metabolic pathway or cellular function	Tissue	Gene (gene symbol*, isoform)	mRNA content & populations studied	Pathway mRNA content	Genetic variation detected?	Enzyme data
			↓[4]; ↓ in Indian dwarf[2] COX4I1, COX5A, COX 7A2: ↑ in Cliff dwarf[4] COX6A: ↑ in Cliff & Indian dwarf[2,4] COX4 I2, COX6B: one contig ↑ in Cliff dwarf, one contig ↓[4]			
Fatty acid metabolism (mitochondrial)	WM	β-hydroxyacyl CoA dehydrogenase (HADH)				Fig. 3
	Liver	HADH	ns in Indian & Cliff[2,4]			

*Gene symbols follow the HUGO gene nomenclature committee.
(1) Derome *et al.* (2006), (2) St-Cyr *et al.* (2008), (3) Jeukens *et al.* (2009) (4) Jeukens *et al.* (2010), (5) Renaut *et al.* (2010), (6) Renaut *et al.* (2011), (7) Jeukens & Bernatchez 2012; (8) Evans & Bernatchez (2012), (9) Hébert *et al.* (2013).

proportions of metabolically active tissues [ventricle mass (Evans *et al.* 2013), liver mass, percentage of red skeletal muscle]. In particular, we measured the activities of seven enzymes in glycolysis, two enzymes in glycogen metabolism, an enzyme involved in ATP production via phosphoryl transfer, an enzyme involved in cellular energy shuttling and four enzymes involved in mitochondrial energy metabolism (Table 1). All of these genes have been previously found to differ in allele frequencies, expression or activity between at least one whitefish species pair (Table 1, Tables S6–S8, Supporting information; Derome *et al.* 2008; St-Cyr *et al.* 2008; Jeukens *et al.* 2009; Renaut *et al.* 2011; Jeukens & Bernatchez 2012; Dalziel *et al.* 2015; Laporte *et al.* 2016). In contrast to the lack of parallelism among species pairs at the transcriptomic and genomic levels, we predict increased convergence in enzyme activities. This is because the incidence of parallelism is predicted to increase with the level of biological organization due to the hierarchical nature of biological traits (Losos 2011; Tenaillon *et al.* 2012; Bailey *et al.* 2015). For example, many mutations can affect a gene, many genes can influence a biochemical pathway, and many biochemical pathways can influence a metabolic network. Therefore, when studying phenotypic adaptation, all else being equal, it is more likely the same metabolic network will contribute to phenotypic variation than the same gene (reviewed by Rosenblum *et al.* 2014). We predict increased convergence in the evolution of enzyme activity compared with gene expression or gene sequence. This is because the maximal rate of enzyme catalysis per gram of tissue (maximal enzyme velocity, V_{max}) is the product of enzyme concentration ([E]) and turnover number (catalytic events per active site per unit time, k_{cat}). Therefore, both coding and regulatory region divergence can influence maximal activity and different mechanisms of underlying variation have the potential to lead to similar enzyme activities per gram tissue. Furthermore, increases in enzyme activity per gram of tissue or in tissue proportions may lead to functionally equivalent changes in an organ's energetic capacity.

To test for convergence in energy metabolism, we collected dwarf and normal Lake Whitefish from four lakes (Cliff, Indian, Témiscouata and East) and measured the relative sizes of metabolically active tissues [liver, ventricle (also previously studied by Evans *et al.* 2013), skeletal muscle fibre types] and activities of candidate enzymes in liver and white muscle. These lakes were specifically chosen because they fall along a 'speciation continuum' such that the Cliff Lake species pair is the most genetically divergent, followed by Indian Pond, East Lake and finally Témiscouata Lake (Fig. 1; Campbell & Bernatchez 2004; Renaut *et al.* 2011;

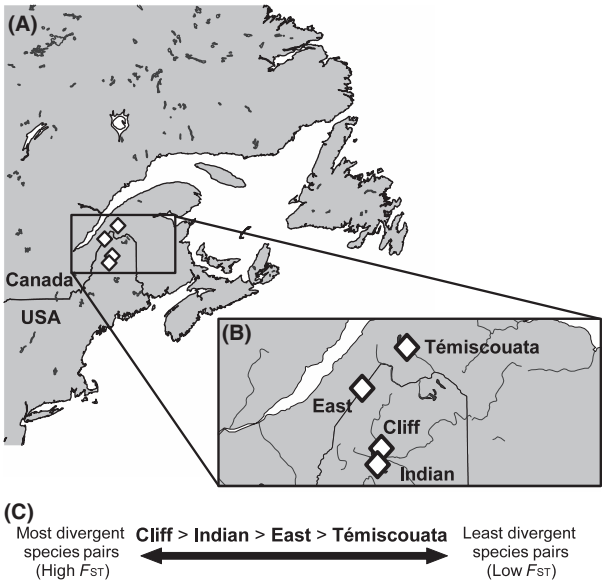

Fig. 1 Location of sampling sites. (A) A map of northeastern North America with the four study lakes indicated with diamonds, and (B) a close-up of the St. John River Basin. (C) Levels of genetic divergence among dwarf–normal species pairs from the four lakes, listed from the most to least genetically divergent (Campbell & Bernatchez 2004; Renaut *et al.* 2011; Gagnaire *et al.* 2013a).

Gagnaire *et al.* 2013a). Thus, we predict that, similar to genetic divergence, divergence in energy metabolism will be greatest between Cliff Lake species pairs and least in Témiscouata species pairs. We also predict that enzyme activities and overall tissue proportions will show more convergence among species pairs than has been found for allele frequencies and gene expression evolution.

Methods

Samples, study system and measurement of tissue proportions

We collected dwarf and normal Lake Whitefish using gill nets from four lakes in the St. John River drainage in June and July of 2013: Témiscouata Lake (47°40′22″N, 68°49′41″W) and East Lake (47°11′6″N, 69°33′35″W) in Québec, Canada and Cliff Lake (46°23′51″N, 69°15′05″W) and Indian Pond (46°15′25″N, 69°18′05″W) in Maine, USA (Fig. 1). We checked gillnets every hour and stored fish in a live well during transport to ensure that fish were alive prior to sampling. We euthanized the fish with a stunning blow to the head followed by cervical dislocation and weighed and measured fish prior to cutting a one-inch-thick muscle steak just posterior to the cloaca. We photographed the muscle steak to determine the percentage of muscle mass taken up by red muscle and then separated red muscle fibres (along lateral line) from white muscle (remaining muscle tissue) prior to flash-freezing in liquid nitrogen. We found no evidence for the presence of pink muscle fibres in Lake Whitefish muscles in prior histological studies (Dalziel *et al.* 2015). We removed the heart (ventricle only, extra blood blotted away) and liver, weighed organs and then flash-froze these samples in liquid nitrogen. Some fish collected from Indian Pond and Témiscouata Lake (both dwarf and normal) died ~ 10–20 min prior to sampling, but as no major differences were observed among these fish and other fish from the same lake, we retained these individuals in our analyses (noted in raw data file, Dryad: http://datadryad.org/resource/doi:10.5061/dryad.686sq). We measured the total red and white muscle cross-sectional area in photographs of muscle steaks with ImageJ (Rasband 2014).

Enzyme assays

We followed the methods of Milligan (2003), Martinez *et al.* (2006), Driedzic *et al.* (2006) and Dalziel *et al.* (2015) to measure enzyme activities in white muscle and liver. We weighed and immediately homogenized frozen tissues in either 20 volumes of chilled homogenization buffer (50 mM Hepes, 1 mM EDTA and 0.1% Triton X-100, pH 7.4; creatine phosphokinase, glycolytic and mitochondrial enzymes) or 10 volumes of buffer with DTT, phosphatase inhibitors and protease inhibitors added just prior to use [50 $mmol^{-1}$ imidazole, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, 1 mM Roche Pefabloc SC, 1× Roche PhosSTOP, pH 7.5; glycogen metabolism enzymes and glycerol-3-phosphate dehydrogenase (GPD)], in 4 mL Wheaton glass homogenizers. We assayed enzyme activities from homogenates at 26 °C with a 96-well plate spectrophotometer (SPECTRAMAX 190, Molecular Devices). We ran all samples in triplicate and optimized all assays to ensure substrates, cofactors and linking enzymes were not limiting and that we obtained maximal rates of enzymatic catalysis.

We used the following final reaction conditions for mitochondrial enzymes in liver and muscle: (i) cytochrome *c* oxidase (COX; EC 1.9.3.1), 50 μM reduced cytochrome *c*, 0.5% Tween 20, in 50 mM Tris, pH 8, (ii) 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, (iii) 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, (iv) 3-hydroxyacyl CoA dehydrogenase (HADH; EC 1.1.1.35), 0.15 mM Aceto-acetyl CoA, 0.5 mM NADH, 0.2 mM DTT, 0.1% TritonX-100 in 50 mM Tris, pH 8.

For glycolytic enzymes, we used the following conditions in liver and muscle: (i) phosphoglucose isomerase (GPI; 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, (ii) phosphofructokinase (PFK; 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, (iii) Aldolase (ALDO; EC 4.1.2.13) 10 mM KCl, 0.2 mM NADH, 5 U/mL GPD, 14.5 U/mL TPI and 0.75 mM fructose 1,6-bisphosphate in 100 mM Hepes, pH 7.4, (iv) triosephosphate isomerase (TPI; EC 5.3.1.1), 10 mM KCl, 0.2 mM NADH, 10 U/mL GPD, 2.9 mmol glyceraldehyde 3-phosphate in 100 mM Hepes, pH 7.4, (v) glyceraldehyde 3 phosphate dehydrogenase (GAPDH, EC 1.2.1.12), 10 mM KCl, 2 mM $MgCl_2$, 3.1 mM ATP, 0.2 mM NADH, 8 U/mL PGK and 2.8 mM phosphoglycerate in 100 mM Hepes, pH 7.4, (vi) pyruvate kinase (PK; EC2.7.1.40), 5 mM phosphoenol pyruvate, 0.15 mM NADH, 5 mM ADP, 10 mM KCl, 10 mM $MgCl_2$, 10 μM fructose 1,6-bisphosphate and 1 U/mL LDH in 100 mM Hepes, pH 7.4, (vii) lactate dehydrogenase (LDH; EC 1.1.1.27), 25 mM pyruvate and 0.5 mM NADH in 50 mM Tris, pH 7.4.

The activity of creatine phosphokinase (CK; EC 2.7.3.2), an enzyme involved in cellular energy transfer, was measured in muscle only using the following reaction conditions: 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. Glycerol 3-phosphate dehydrogenase (GPD; EC 1.1.1.8), an enzyme involved in cellular energy shuttling and linking carbohydrate to lipid metabolism, was also measured only in muscle under the following conditions: 0.15 mM NADH and 2 mM DHAP in 50 mM imidazole, pH 7.2).

The activities of two enzymes involved in glycogen metabolism were measured using the following conditions in muscle only: (i) glycogen phosphorylase (PYG; EC 2.4.1.1), 15 mM $MgSO_4$, 0.5 mM DTT, 0.5 mM NADP, 0.25 mM EDTA, 1 U/mL glucose-6-phosphate dehydrogenase, 1.5 U/mL phosphoglucomutase, 0.01 mM glucose 1,6-bisphosphate, 2 mg/mL glycogen, in 50 mM potassium phosphate, pH 7.3, (ii) glycogen synthase (GYS; EC 2.4.1.11), 70 mM KCl, 4 mM $MgCl_2$, 0.5 mM PEP, 0.2 mM NADH, 5 U/mL LDH, 5 U/mL PK, 2 mg/mL glycogen in 50 mM Tris, pH 7.8.

We calculated background rates in wells lacking specific substrates, with the exception of COX. 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) and 29.5 mM^{-1} cm^{-1} for cytochrome *c* (550 nm) to calculate enzyme activities and measured tissue protein content in quadruplicate using the Bradford Reagent.

Statistical analyses

We conducted all statistical analyses with R version 3.2.3 (R Development Core Team, 2014). To control for differences in size between dwarf and normal whitefish, and among individuals within each population, we calculated the residuals from a best-fit least-squared linear regression against mass for all response variables that were significantly correlated with body mass (using all individuals from all lakes). We tested that all 26 response variables met the assumptions of ANOVA by viewing diagnostic plots in R and directly testing for homogeneity of variances using the Fligner–Killeen Test. When heteroscedasticity was detected, we log-transformed response variables (muscle COX and CS) or calculated the residuals from log-transformed data and log mass (ventricle and liver mass) (Quinn & Keough 2002). This transformation did not lead to homogeneity of variances for ventricle mass residuals, so we also Box–Cox-transformed ventricle residuals to obtain homoscedasticity. We then conducted univariate three-way ANOVAs on all 26 response variables to examine the influence of lake (fixed effect: Cliff, Indian, East or Témiscouata), species (fixed effect: dwarf or normal), sex (fixed effect: male or female) and interactions among factors. If the effect of sex was not significant, we cut this variable and conducted two-way ANOVAs testing the effects of lake, species and interactions on pooled sexes. In cases where sex was not significant, we present data from two-way ANOVAs pooling sexes, as differences among species and lakes, and not sexes was the focus of this study [see Casselman & Schulte-Hostedde (2004) for a summary of sex differences in organ mass in Lake Whitefish]. We tested for correlations among response variables (Pearson's correlation coefficient), and to account for multiple testing, we adjusted *P*-value significance cut-off from 0.05 with the 'Brainwaver' package in R using the function 'compute.FDR' to determine the false discovery rate (Achard 2010). Plots were created in R with ggplot2 (Wickham 2009) and corrplot (Wei & Simko 2016).

We also conducted a multivariate analysis of variance (MANOVA) on response variables with 'species' and 'lake' as fixed effects with the MASS package in R (Venables & Ripley 2002). Because some enzyme activity values were significantly correlated, we selected a subset of enzyme variables to reduce multicollinearity among response variables. In particular, we retained only one enzymatic variable from each group of correlated response variables that provided biologically (from the same major biochemical network in a tissue – e.g.

carbohydrate metabolism or mitochondrial oxidative phosphorylation) and statistically redundant data (significantly correlated). We preferentially retained the enzyme activity response variables with the largest sample size (see Figure S1, Supporting information for information about missing data). We standardized all variables to have a mean of zero and variance of one prior to analyses. To visualize how fish from different species and lakes clustered in terms of metabolic phenotype, we conducted a discriminant function analysis (DFA) with species and lake as predefined groups. We also conducted a principal components analysis (PCA) to determine whether the associations found in the DFA would remain without predefined groupings, and tested the effect of varying the 12 response variables used in our PCA and DFA.

Results

Organ size

There was a significant effect of 'species' on the percentage of red muscle ($P < 0.001$, $F_{1,116} = 20.816$), relative liver mass ($P = 0.019$; $F_{1,145} = 5.610$) and relative ventricular mass ($P = 0.019$, $F_{1,134} = 5.672$), with dwarf whitefish having more red muscle and proportionally larger livers and ventricles (Fig. 2; Table S1, Supporting information). In particular, the percentage of red muscle was higher in dwarf compared with normal fish in all four lakes, relative liver mass was larger in dwarf fish from Cliff, Indian and East lakes, but not Témiscouata Lake, and relative ventricular mass was larger in dwarf fish from Cliff and Indian lakes (the two lakes with the most genetically divergent species pairs) and similar to normal fish in East and Témiscouata lakes (Fig. 2).

There was a significant effect of 'lake' (Cliff, Indian, Témiscouata or East) on relative ventricular mass ($P < 0.001$, $F_{3,134} = 14.320$) and liver mass ($P < 0.001$, $F_{3,145} = 10.511$), but not on the percentage of red muscle ($P = 0.294$, $F_{3,116} = 1.253$). Post hoc tests found that the effect of lake on liver mass arose due to Indian Pond fish having larger livers than fish from all other lakes (Table S1, Supporting information). East Lake relative heart mass was significantly smaller than all other lakes, and Indian Lake fish had significantly larger ventricles than Témiscouata fish as well (Table S1, Supporting information).

The interaction between 'lake' and 'species' was significant for ventricle mass ($P = 0.013$, $F_{3,134} = 3.752$), but not relative liver mass ($P = 0.137$, $F_{3,145} = 1.872$) or the percentage of red muscle ($P = 0.989$, $F_{3,116} = 0.045$). Post hoc tests on this interaction showed that dwarf fish from Cliff Lake had significantly larger relative ventricle sizes than normal fish, but the Indian Lake dwarf and normal fish were not significantly different (Fig. 2, Table S1, Supporting information). Sex had a significant effect on the percentage of red muscle ($P = 0.023$,

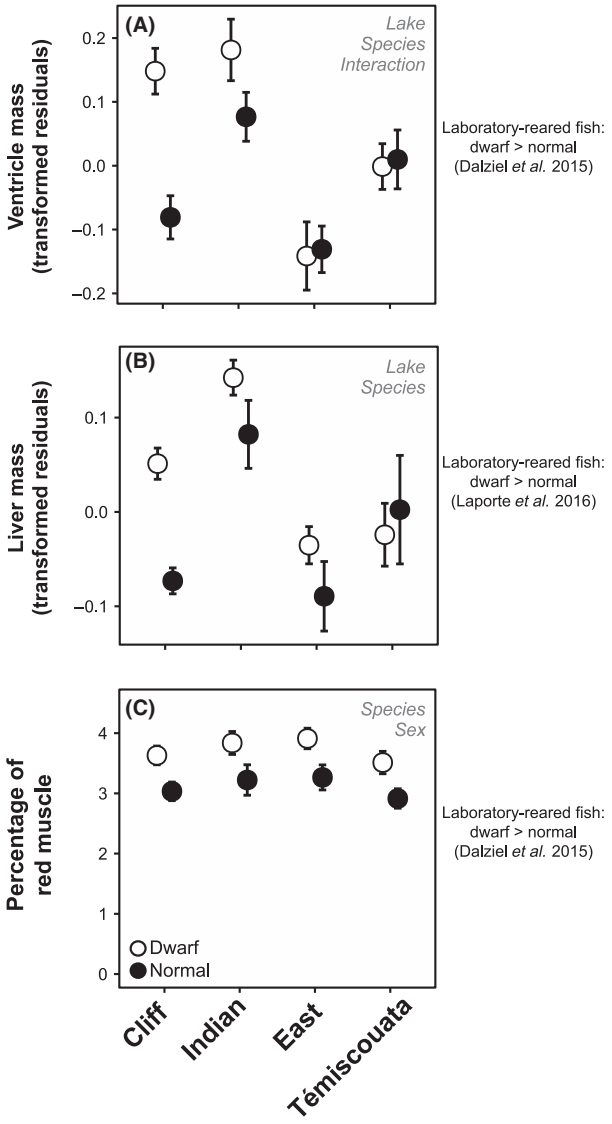

Fig. 2 Relative organ size and tissue proportions among species pairs. Residual ventricle mass (A, $n = 142$), liver mass (B, $n = 153$) and the proportion of red skeletal muscle (C, $n = 134$) in dwarf (white circles) and normal (black circles) whitefish from four lakes. These lakes are listed from those containing the most (Cliff) to least (Témiscouata) genetically divergent species pairs. Data are presented as the mean for each species pair in each lake ± SEM with males and females combined for clarity. Data for laboratory-reared crosses from Dalziel *et al.* (2015) and Laporte *et al.* (2016) are noted next to each panel (Témiscouata Lake dwarf and Aylmer Lake normal whitefish) for comparison. Statistical results are from three-way ANOVAs testing the effects of species, lake and sex (C) and two-way ANOVAs testing the effect of species and lake (A and B). Significant effects of these factors ($P < 0.05$) are noted in the figure, with full statistical results and sample sizes for each group included in Table S1 (Supporting information).

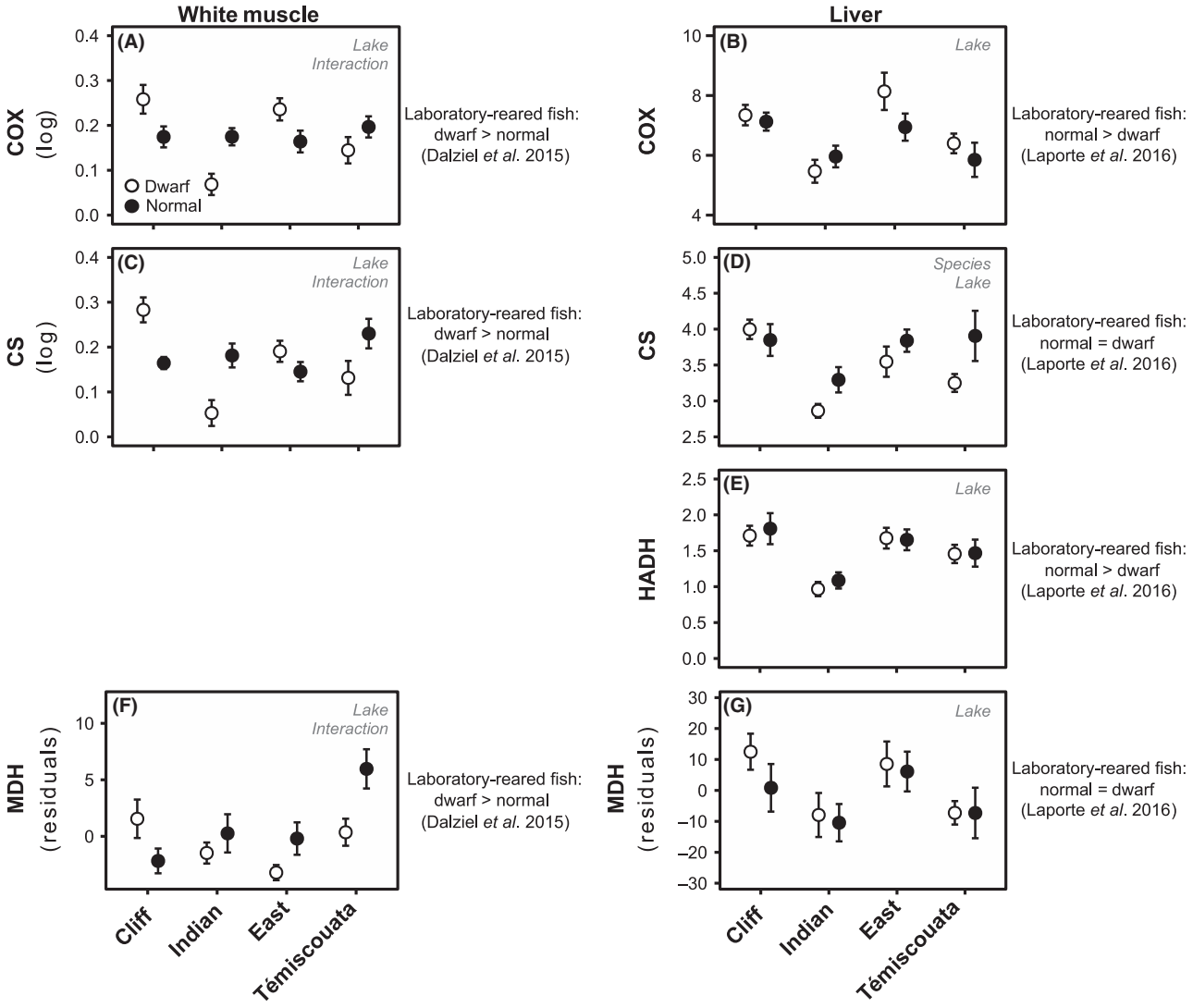

Fig. 3 Muscle and hepatic mitochondrial enzyme activities among species pairs. Activities of mitochondrial enzymes in dwarf (white circles) and normal (black circles) whitefish. (A, B) White muscle (n = 122) and liver (n = 116) cytochrome c oxidase (COX), (C, D) white muscle (n = 122) and liver (n = 116) citrate synthase (CS), (E) liver B-hydroxyacyl CoA dehydrogenase (β-HADH; n = 107), and (D) white muscle (n = 121) and liver (n = 114) malate dehydrogenase (MDH; combination of cytoplasmic and mitochondrial isoforms). Activities are expressed as μM/min/g tissue. Data are presented as in Fig. 2, with Tables S2 & S3 (Supporting information) containing full statistical results and activities per mg of total tissue protein^{-1} for comparison.

$F_{1,116}$ = 5.278), but not relative ventricle mass or relative liver mass (data not shown). Female fish generally had a higher percentage of red muscle than male fish. See Table S1 (Supporting information) for full statistical results.

White muscle and liver enzyme activities

The lake of origin had a stronger effect on hepatic and white muscle enzyme activities than species (Figs 3–5). In particular, all mitochondrial enzymes in liver and white muscle (Fig. 3), all but one glycolytic enzyme in liver and white muscle (Fig. 4), and all enzymes involved in glycogen and glycerol metabolism in white muscle (Fig. 5) differed significantly among lakes (Tables S2 & S3, Supporting information). Of the 15 enzymes measured in muscle and liver, only hepatic CS (Fig. 3D; P = 0.032, $F_{1,108}$ = 4.711) was significantly affected by species (Figs 3–5; Tables S2 & S3, Supporting information). In addition, hepatic enzymes showed no significant interaction effects. There was a significant interaction between species and lake for many muscle enzymes, including all three mitochondrial enzymes (Fig. 3), the glycolytic enzymes PFK, TPI, PK, LDH

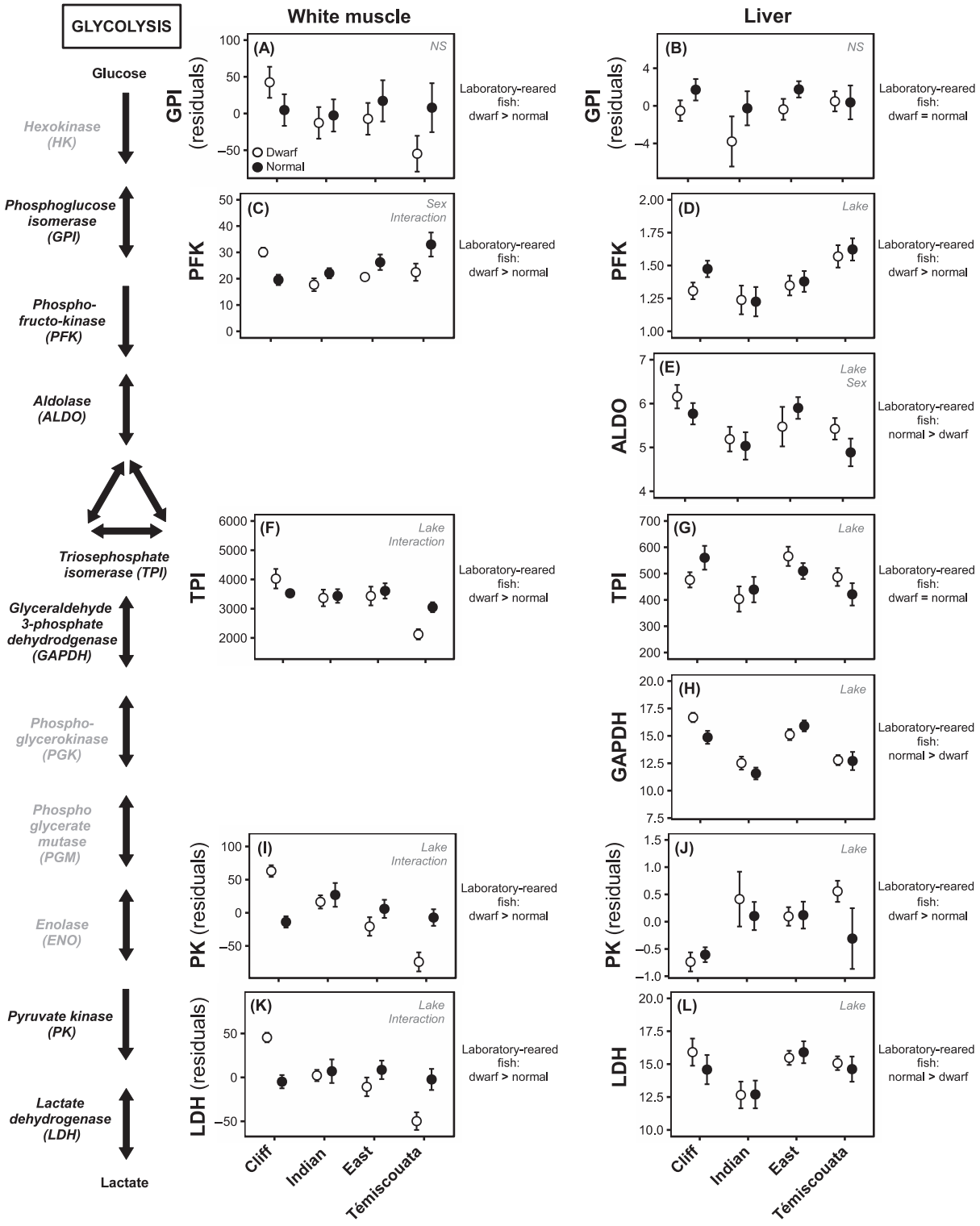

Fig. 4 Muscle and hepatic glycolytic enzyme activities among species pairs. Activities of glycolytic enzymes in dwarf (white circles) and normal (black circles) whitefish. (A, B) White muscle (n = 121) and liver (n = 114) phosphoglucose isomerase (GPI), (C, D) white muscle (n = 122) and liver (n = 115) phosphofructokinase (PFK), (E) Liver (n = 116) aldolase (ALDO), (F, G) white muscle (n = 121) and liver (n = 100) triose phosphate isomerase (TPI), (H) liver (n = 116) glyceraldehyde phosphate dehydrogenase (GAPDH), (I, J) white muscle (n = 121) and liver (n = 90) pyruvate kinase (PK), (K, L) white muscle (n = 121) and liver (n = 116) lactate dehydrogenase (LDH). Activities are expressed as μM/min/g tissue. Data are presented as in Fig. 3. Data from laboratory-reared fish were collected by A.C. Dalziel, M. Laporte, H. Guderley & L. Bernatchez (in preparation).

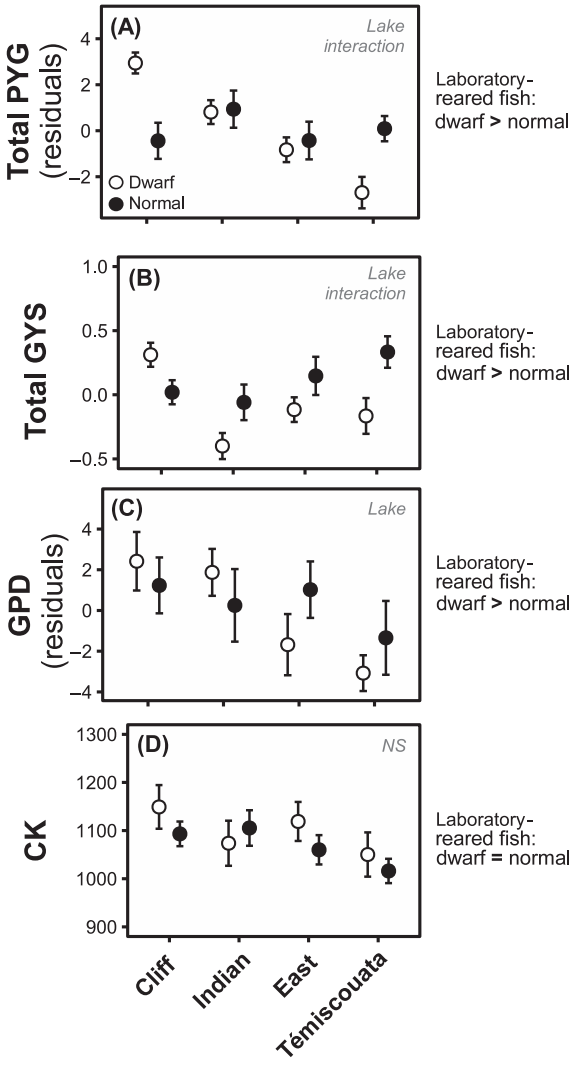

Fig. 5 Activities of enzymes involved in glycogen, glycerol and creatine metabolism in white skeletal muscle. Activities of enzymes in dwarf (white circles) and normal (black circles) whitefish. (A) Total glycogen phosphorylase (PYG, $n = 95$), (B) total glycogen synthase (GYS, $n = 95$), (C) glycerol 3-phosphate dehydrogenase (GPD, $n = 95$) and (D) creatine phosphokinase (CK, $n = 122$). Activities are expressed as μM/min/g tissue. Data are presented as in Fig. 3. Data from laboratory-reared fish were collected by A.C. Dalziel, M. Laporte, H. Guderley & L. Bernatchez (in preparation).

(Fig. 4), and glycogen metabolism enzymes PYG, and GYS (Fig. 5). In some cases, this interaction indicated a difference in sign (i.e. dwarf > normal in some lakes but normal > dwarf in others) among lakes. For example, Cliff lake dwarf fish had higher activities than Cliff normal fish for muscle PK, LDH and PYG, but Témiscouata Lake normal fish had higher activities than Témiscouata dwarf fish (Figs 3–5, Table S2, Supporting information). See Tables S2 and S3 (Supporting information) for full statistical results for enzyme activities expressed per gram tissue and per mg protein for comparison.

In white muscle, the activities of enzymes in similar biochemical pathways and networks generally followed similar trends among species pairs, suggesting pathways are primarily regulated as a whole and not on an enzyme by enzyme basis. For example, white muscle mitochondrial, glycolytic and glycogen metabolism enzyme activities were all higher in Cliff lake dwarf fish than Cliff normal fish, and all lower in Témiscouata dwarf fish than Témiscouata normal fish (although not all were significantly so; see Figs 3, 4, 5, Table S2, Supporting information). Most muscle enzymes within similar biochemical networks were also correlated across all samples (Fig. 6), including most white muscle mitochondrial enzymes (e.g. COX and CS, CS and MDH, but not COX and MDH), glycogen metabolism (e.g. PYG and GYS) and glycolytic enzymes (e.g. GPI is significantly correlated with TPI, PK, LDH), with the exception of PFK, which was only correlated with one other glycolytic enzyme, LDH (Fig. 6).

In liver, enzymes within the same biochemical pathway often had opposite trends in activity between dwarf and normal whitefish; in Cliff Lake, the glycolytic enzymes ALDO, GAPDH and LDH were higher in dwarf fish, but GPI, PFK, TPI, and PK were higher in normal fish (Fig. 4; although not all significantly so based upon post hoc tests, Table S3, Supporting information). When all samples were combined (Fig. 6), hepatic mitochondrial enzymes were often significantly correlated (e.g. hepatic CS with MDH, COX and HADH), but there were few significant correlations among glycolytic enzymes (e.g. GPI was not significantly correlated with any other glycolytic enzyme and TPI was only significantly correlated with LDH). Because some glycolytic enzymes (e.g. GPI, ALDO, TPI, GAPDH, LDH) also participate in gluconeogenesis, this reduced covariation could stem from a differential regulation of gluconeogenesis and glycolysis in the liver.

As many enzyme activities were correlated (Fig. 6), we selected a subset for further multivariate analyses to reduce collinearity and increase our statistical power. We chose to exclude enzymatic variables that were significantly correlated with another enzymatic variable in the same biochemical network (e.g. mitochondrial energy metabolism: citric acid cycle, electron transport chain or fatty acid B oxidation) or carbohydrate metabolism (glycolysis, glycogenolysis, glycogenesis) in the same tissue. The 14 enzymes excluded (and nine retained) were as follows: muscle COX and MDH (correlated with CS), muscle GPI, TPI, LDH, GYS, PYG (correlated with PFK and PK), muscle GPD (correlated

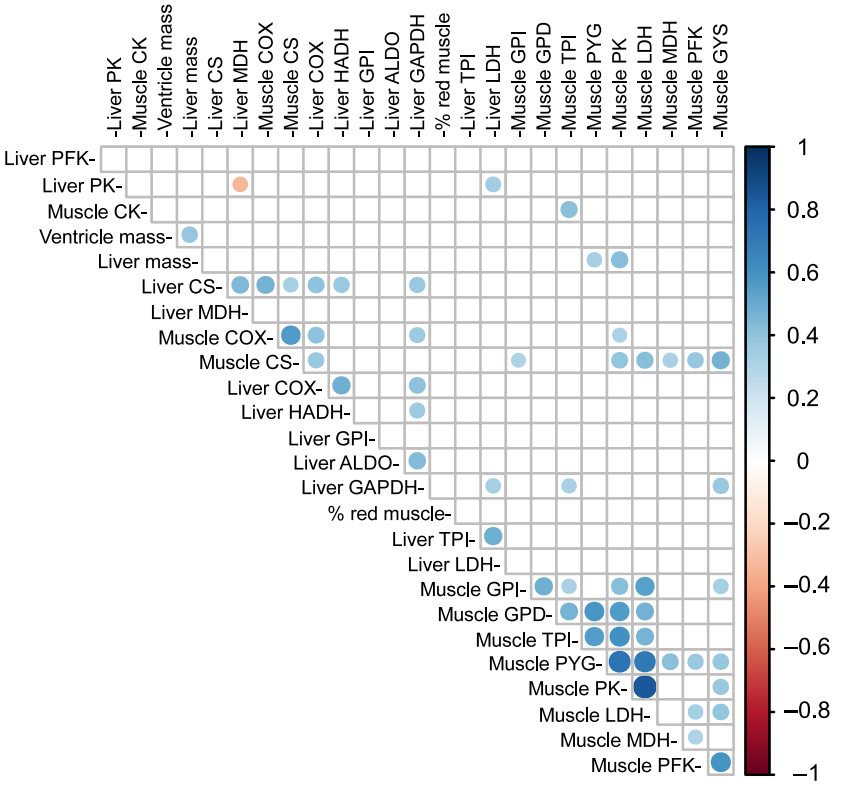

Fig. 6 Correlation matrix of energy metabolism response variables. A correlation matrix for the 26 response variables presented in Figs 2–5. Only Pearson's correlation coefficients with *P*-values surpassing the false discovery rate-adjusted *P*-value cut-off of 0.0015 are shown. The strength and direction of the Pearson correlation coefficients are indicated by colour, with self-correlations excluded.

with PK), hepatic COX, MDH, HADH (correlated with CS), hepatic PK, TPI (correlated with LDH), hepatic ALDO (correlated with GAPDH) (Fig. 6). The 12 response variables used in multivariate analyses were as follows: relative ventricle mass (Fig. 2A), relative liver mass (Fig. 2B), percentage of red muscle (Fig. 2C), muscle CS (Fig. 3C), PFK (Fig. 4C), PK (Fig. 4I), CK (Fig. 5D) and hepatic CS (Fig. 3D), GPI (Fig. 4B), PFK (Fig. 4D), GAPDH (Fig. 4H) and LDH (Fig. 4L).

Multivariate analyses

A MANOVA of the 12 selected explanatory variables found a significant effect of 'lake', 'species' and their interaction (Table 2). Subsequent two-way ANOVAs on the 85 individuals with no missing data that could be included in the MANOVA detected a significant effect of 'species' on relative ventricle mass, percentage of red muscle and liver CS. The 'lake' of origin had a significant effect on relative ventricle mass, relative liver mass, muscle CS, PK and liver CS, GAPDH, PFK and LDH. We also found a significant interaction among 'species' and 'lake' on relative ventricle mass, white muscle CS, PFK and PK (Table 2). Note that ANOVAs using data from this subset of 85 individuals gave slightly different statistical results than the univariate analysis incorporating all individuals for which we measured each response variable (n = 91–153), in Figs 2–5 (Tables S1–S3, Supporting information).

Table 2 Results of a MANOVA testing the influence of 'lake' and 'species' on a subset of 12 energetic traits (relative liver mass, relative ventricle mass, percentage of red muscle, white muscle CS, CK, PFK, PK and hepatic CS, GPI, GAPDH, PFK and LDH). Traits were selected based upon tissue-specific biological networks to reduce collinearity and maximize sample size (see Methods)

Independent variables [response variables which are significantly different in subsequent Two-way ANOVAs]	Pillai–Bartlett Trace	Approximate F	Numerator (hypothesis) d.f., denominator (error) d.f.	*P*-value
Species (1, 77) [relative ventricle mass, percentage of red muscle, liver CS]	0.326	2.656	12, 66	0.006
Lake (3, 77) [relative ventricle mass, relative liver mass, muscle CS, PK, liver CS, GAPDH, PFK, LDH]	1.436	5.202	36, 204	<0.001
Lake x species (3, 77) [relative ventricle mass, muscle CS, PFK, PK]	0.776	1.978	36, 204	0.002

Discriminant function analyses (DFAs, grouped by lake of origin and species) and principal component analyses (PCAs) of the 12 variables used in the MANOVA showed that lake of origin had a major influence; the values for the first two linear discriminants (LD1 and LD2) and principal components (PC1 and PC2) all found a significant effect of lake (Table S4, Supporting information). In particular, Indian Lake Whitefish consistently grouped separately from the other three lakes (Fig. 7). The DFA and PCA also revealed that the transition from normal to dwarf phenotypes did not follow a similar trajectory in all species pairs, as illustrated by the direction of arrows linking normal to dwarf species within a lake (Fig. 7). Indeed, while the effect of species was statistically significant for PC2 and East, Indian and Témiscouata lakes show a similar trajectory, Cliff lake dwarf and normal fish did not vary along this axis (Fig. 7, Table S4, Supporting information). Furthermore, Cliff and Témiscouata species pairs were significantly different in LD2, but in the opposite direction. Cliff Lake dwarf and normal fish showed significant differentiation along PC1 as well, while species pairs from East and Indian lakes did not significantly differ in any composite measures (Fig. 7; Table S4, Supporting information). Thus, the species pair from Cliff Lake showed the greatest divergence among the four lakes studied (Fig. 7).

We also tested the effect of substituting the subset of enzymes included in our analyses (Fig. S2, Supporting information). There was an effect of variable selection (Fig. S2, Supporting information), but the major findings were similar: (i) there is a strong effect of lake with Indian Lake diverging the most from other lakes, (ii) the transition from the normal to dwarf phenotype does not follow the same trajectory in all four species pairs, and (iii) Cliff Lake dwarf and normal fish are the most divergent species pairs (Fig. 7 and Fig. S2, Supporting information).

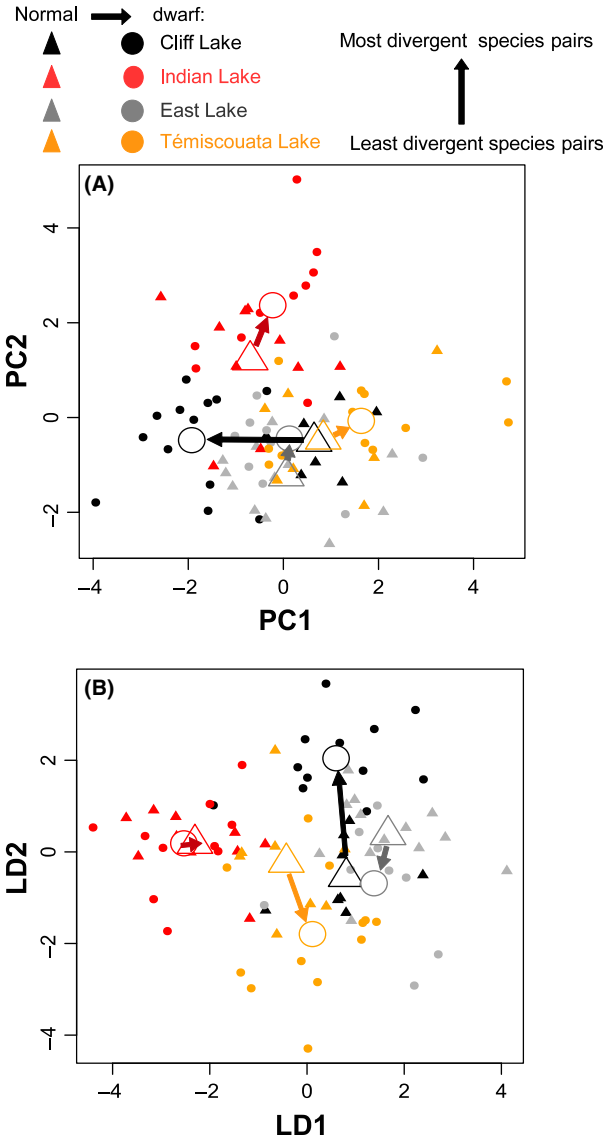

Fig. 7 Multivariate analysis of energy metabolism enzyme activities and tissue masses. Values for dwarf (circles) and normal (triangles) species pairs from four lakes (colour coded) for the first two principal components (A) or linear discriminants (B) from respective multivariate analyses on a subset of 12 'energy metabolism' response variables (those used in the MANOVA, Table 1; $n = 85$ individuals). Each point represents an individual fish, and larger points represent group means. Arrows are included to display the trajectory of variation from 'normal' (the hypothesized ancestral phenotype) to 'dwarf' (the derived phenotype) metabolic phenotypes within each lake. Significant differences among groups are listed in Table S4 (Supporting information).

Discussion

In this study, we examine four independently evolved 'dwarf' and 'normal' Lake Whitefish species pairs to test whether similar biochemical and physiological mechanisms contribute to the evolution of phenotypic convergence. We found convergence in organ size, such that all dwarf populations had proportionally more red muscle, three had relatively larger livers and two had relatively larger ventricles than normal fish from the same lakes. However, muscle and hepatic activities of enzymes of aerobic, glycolytic and glycogen metabolism did not display convergence among lakes and even varied in opposite directions in some cases. Multivariate analyses of combined organ sizes and enzyme activities show that most variation in these metabolic traits is partitioned among lakes and that species pairs from the four lakes do not show a common metabolic trajectory when evolving from normal to dwarf phenotypes. Finally, species pairs from Cliff Lake, the most

genetically divergent species pair, are also the most divergent in terms of organ masses and energy metabolism enzyme activities, supporting findings from genomic and transcriptomic studies.

Convergent evolution of metabolic traits – relative organ sizes

We found strong convergence in relative organ size among species pairs. Most strikingly, the relative percentage of red muscle was higher in dwarf than normal whitefish in all four lakes. Fish skeletal muscle fibre types are divided into three anatomically distinct groups; red fibres that use aerobic energy metabolism to fuel movement, white, glycolytic fibres that rely more on substrate-level phosphorylation (e.g. glycolysis, creatine phosphokinase), and pink fibres that are intermediate in metabolic phenotype to red and white fibres (Johnston *et al.* 2011). Fish with a higher percentage of red skeletal muscle tend to be better endurance swimmers, while those that swim mainly by bursts have a higher proportion of white skeletal muscle (reviewed by Langerhans 2008). Therefore, we predicted that dwarf whitefish, which must swim continuously to capture limnetic prey, and have evolved higher swimming activity (Rogers *et al.* 2002), would have a higher percentage of red muscle than the benthic normal form. We have also recently shown that divergence in this phenotype has a genetic basis as dwarf whitefish raised in the laboratory had a higher percentage of red muscle than normal fish, which remained after swim training (Dalziel *et al.* 2015). Therefore, studies on wild and laboratory-reared whitefish indicate that skeletal muscle fibre types have convergently evolved, supporting the hypothesis that selection has driven the divergence of aerobic swimming capacity among species pairs (Trudel *et al.* 2001; Rogers *et al.* 2002).

We found incomplete convergence in relative liver mass, such that three dwarf populations have relatively larger livers than normal fish from the same lakes. This trait is heritable in whitefish, as laboratory-reared Témiscouata dwarf whitefish have a larger liver than normal Aylmer fish (Laporte *et al.* 2016). Relative liver mass is also plastic in response to swim training in normal fish only, suggesting genetic differentiation has led to both constitutive and inducible variation (Laporte *et al.* 2016). The liver is a multifunctional organ involved in waste management, blood filtration and gluconeogenesis and is a metabolically 'expensive' tissue to maintain (Rolfe & Brown 1997). We do not yet know specifically how variation in liver size influences whitefish whole-animal physiology and performance, but it may facilitate the higher aerobic swimming capacity of dwarf fish as suggested by the 'aerobic capacity model' (Bennett & Ruben 1979).

Finally, relative ventricular size varied significantly among species pairs. Cliff and Indian Lake dwarf fish, the most genetically divergent species pairs, had larger ventricles than normal fish from the same lake. Fish have a single ventricle to pump blood throughout the circulatory system, and the size of this organ is predicted to be positively correlated with oxygen transport capacity and aerobic potential (Farrell & Jones 1992). Indeed, among species, relative ventricular mass is commonly found to be positively correlated with prolonged, endurance swimming capacity in fish (Farrell 1996; Moyes 1996). Intraspecific studies have also found that populations of fish with higher prolonged swimming capacities have evolved larger relative ventricle masses (Eliason *et al.* 2011; Dalziel *et al.* 2012). We have found that relative ventricular size is larger in laboratory-reared dwarf whitefish from Témiscouata Lake than in normal whitefish from Aylmer Lake and that relative ventricular size is plastic in normal whitefish and increases with swim training (see Dalziel *et al.* 2015). Therefore, the finding that dwarf whitefish in Cliff and Indian lakes have larger ventricles than normal fish supports the hypothesis of adaptive evolution of this trait in these lakes.

The relative ventricular mass in the four wild populations examined in this study was also measured by Evans *et al.* (2013) who found that all dwarf fish had larger ventricles than their normal conspecifics in 2010 (although this trend was not significant for all lakes). Thus, our results from East and Témiscouata lakes (no difference observed) differ from those of Evans *et al.* (2013). One possible explanation for this discrepancy is that there has been year-to-year variation in environmental factors that influence phenotypic plasticity in this trait. For example, ventricle mass is plastic in response to swim training in normal fish (Dalziel *et al.* 2015), so annual differences in activity levels could reduce variation among species. We also hypothesize that the reduced divergence in ventricle mass in Lake Témiscouata, the lake with the least divergent species pair, might be due to increasing hybridization (Lu & Bernatchez 1999; Landry *et al.* 2007; L. Bernatchez, unpublished observations).

We note that relative liver size, relative ventricle size and the proportion of red muscle could be classified as either 'morphological' or 'physiological' and that, in general, the distinction between such 'trait categories' is a grey area. We have categorized these traits as physiological mainly because we are studying them in the context of the differences in whole-animal swimming performance, energy metabolism and growth that occur among species pairs (Trudel *et al.* 2001; Rogers *et al.* 2002). Also, both traits are generally plastic during adulthood in response to environmental factors (e.g.

exercise training; Davison 1997) and the relative proportions of these tissues in adults is influenced by developmental variation (Johnston *et al.* 2011; Staudt & Stainier 2012). Thus, there are a wide range of possible genetic changes in either regulatory or effector genes that may underlie these changes in adult tissue proportions. This matches quantitative trait loci (QTL) studies in whitefish that find multiple loci influencing swimming activity and growth, as well as limited evidence for parallelism in QTL among lakes (Rogers & Bernatchez 2005, 2007; Renaut *et al.* 2011; Gagnaire *et al.* 2013a, b).

Nonconvergent evolution of metabolic traits – enzyme activities

We found that lake of origin was the major factor influencing both hepatic and muscle energy metabolism enzyme activities and little evidence for convergence among whitefish species pairs. These findings match Derome *et al.*'s (2006) transcriptomic studies of whitefish muscle and St-Cyr *et al.*'s (2008) study of the liver transcriptome, in which significant expression differences in energy metabolism genes were found in two species pairs from Cliff Lake and Indian Pond, but most changes varied in sign among lakes. Together, with previous quantitative trait mapping, genomic and transcriptomic studies, these data show that there is limited convergence in the evolution of energy metabolism at the genetic, transcriptomic or enzymatic levels of biological organization (Rogers & Bernatchez 2005, 2007; Derome *et al.* 2006; St-Cyr *et al.* 2008; Renaut *et al.* 2011; Gagnaire *et al.* 2013a, b).

Theoretical studies suggest that many factors, including the strength of selection, the distance from fitness optima, genetic and functional constraints and biases, and phylogenetic and demographic characteristics may influence the likelihood of parallelism (Rosenblum *et al.* 2014; Bailey *et al.* 2015; Storz 2016). Specifically, we hypothesize that the following factors act to reduce the likelihood of convergence in Lake Whitefish species pairs and lead to nonconvergence in enzyme activities: (i) differences in population demography among lakes, (ii) differences in selective pressures among lakes, (iii) the polygenic genetic basis of adaptation in Lake Whitefish and (iv) the influence of phenotypic plasticity in wild caught fish.

Rougeux *et al.* (2016) has recently found that all four species pairs examined in this study arose after secondary contact of the Atlantic–Mississippian (normal ancestor) and Acadian (dwarf ancestor) glacial lineages, but have experienced different demographic expansion rates and gene flow since. In particular, East Lake has experienced much stronger historical introgression from the Acadian to the Atlantic–Mississippian lineage than other lakes (Rougeux *et al.* 2016). Furthermore, all populations have small effective population sizes, which is thought to have caused genetic drift, reduced standing genetic variation and decreased the likelihood that the same alleles lead to local adaptation among lakes. This is particularly true for Indian Lake, where dwarf and normal populations have smaller historic and contemporary effective population sizes than the other three lakes (Rougeux *et al.* 2016).

Second, differences in the direction or strength of selection can reduce the incidence of convergence (Rosenblum *et al.* 2014), and lake ecology does vary substantially among species pair locations (Landry *et al.* 2007; Landry & Bernatchez 2010). The evolution of the dwarf form is hypothesized to be driven by a combination of competition for limited resources and ecological opportunity in the limnetic niche, so variation in prey biomass and suitable habitat among lakes should have a strong influence on the strength of selection (Bernatchez 2004; Landry *et al.* 2007; Landry & Bernatchez 2010). Indeed, Cliff and Indian lakes contain the most divergent species pairs and the lowest biomass of zooplankton and a narrower distribution of zooplankton prey sizes, both of which are predicted to increase competition (Landry *et al.* 2007). Cliff and Indian lakes also have greater variation in benthic oxygen content, greater seasonal changes in the benthic invertebrate community and are generally shallower, which may reduce the availability of suitable habitat for whitefish and enhance competition (Landry *et al.* 2007; Landry & Bernatchez 2010). Thus, we predicted that Cliff and Indian lakes would show the most convergence based upon the similar selective pressures in these lakes (Bailey *et al.* 2015). This is what we found for organ proportions, but not for enzyme activities, possibly as a result of the increased genetic drift in Indian Lake populations.

Third, local adaptation to the limnetic niche by dwarf whitefish is the result of variation in many performance traits (e.g. growth rate, metabolic rate, swimming activity) controlled by multiple genes of moderate to small effect (Rogers & Bernatchez 2007; Bernatchez *et al.* 2010; Gagnaire *et al.* 2013a, b). Such polygenic adaptation decreases the likelihood that local adaptation will occur by parallel mechanisms (Elmer & Meyer 2011; Yeaman 2015; Bernatchez 2016). Indeed, limited evidence of parallelism at the genome level has been found in genome scans (Campbell & Bernatchez 2004; Renaut *et al.* 2011; Gagnaire *et al.* 2013a).

Finally, all fish used in this study were collected from the wild, so trait variation may be the result of genetically based (constitutive or inducible) or plastic divergence. In prior studies, we have found genetically based differences in liver size and muscle and hepatic

enzyme activities that persist after swim training (Dalziel *et al.* 2015; Laporte *et al.* 2016; A.C. Dalziel, M. Laporte, H. Guderley & L. Bernatchez, in preparation), but other environmental factors may influence these traits in the wild (e.g. temperature, Blier & Guderley 1988; Guderley 2004). If the environment among benthic and limnetic niches in all lakes is similar, plasticity could lead to increased phenotypic convergence, but if lakes vary, plasticity could mask genetically based differences or cause extensive, nongenetically based trait divergence (e.g. Oke *et al.* 2016). Given the abiotic and biotic environmental differences among lakes (Landry *et al.* 2007; Landry & Bernatchez 2010), the latter is more likely.

Together, differences in baseline genetic variation, caused by either genetic drift or different demographic histories, differences in the strength of selection among species pairs and the influence of phenotypic plasticity may result in the relatively low genetic, transcriptomic and enzymatic convergence in whitefish species pairs (Derome *et al.* 2006; St-Cyr *et al.* 2008; Renaut *et al.* 2011; Evans & Bernatchez 2012; Gagnaire *et al.* 2013a; present study). However, we do not dismiss the limited, but strong evidence for parallelism at both the genome and transcriptome levels in previous studies (e.g. Jeukens *et al.* 2009). Instead, we want to point out that while parallelism does occur, evidence for parallelism is actually outpaced by that for nonparallelism, as also reported in many other taxa (Bernatchez 2016).

Extent of energetic divergence among species pairs follows a gradient of ecological speciation

Overall, differences in energetic phenotypes generally match predictions from prior genomic studies in whitefish (Renaut *et al.* 2011; Gagnaire *et al.* 2013a; Rougeux *et al.* 2016). In particular, Cliff Lake dwarf and normal whitefish are the most genetically divergent (Lu & Bernatchez 1999; Renaut *et al.* 2011; Gagnaire *et al.* 2013a) and also have the greatest metabolic divergence in this study. Indian Lake species pairs are the next most divergent and show the second highest divergence in organ masses. Finally, East Lake and Témiscouata lakes, which are hypothesized to have much higher levels of historical and current gene flow (Renaut *et al.* 2011; Gagnaire *et al.* 2013a; Rougeux *et al.* 2016), show the least metabolic divergence.

Previous comparisons of the Cliff Lake species pair have found a variety of glycolytic and mitochondrial oxidative phosphorylation genes have experienced selection, including: triose phosphate isomerase (TPI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the muscle isoform of pyruvate kinase (PKM), the muscle isoform of lactate dehydrogenase (LDHA), the cytoplasmic isoform of malate dehydrogenase (MDH1), mitochondrial-encoded subunits of oxidative phosphorylation complexes I, III, IV and V, and creatine phosphokinase (CK) (Jeukens *et al.* 2009; Renaut *et al.* 2010, 2011; Jeukens & Bernatchez 2012; Hébert *et al.* 2013). However, transcriptomic data for these and other energy metabolism genes often varied among studies, lakes and measurement methods. For example, Derome *et al.* (2006) found that dwarf fish downregulated muscle glycolytic gene expression, but Jeukens *et al.* (2009) found an upregulation of a glycolytic gene in Cliff dwarf compared with normal fish. Our data help resolve this transcriptomic variation by measuring enzyme activity; we find that Cliff Lake dwarf whitefish have higher activities of muscle glycolytic, glycogen metabolism and mitochondrial enzymes than normal whitefish. Furthermore, our finding that all enzymes within a metabolic pathway generally covary indicates that differences among species are most likely the result of changes in metabolic regulators (Metallo & Vander Heiden 2013). This information, in combination with previous studies describing enzyme-specific evolutionary variation (e.g. triose phosphate isomerase; Renaut *et al.* 2011; cytoplasmic malate dehydrogenase; Jeukens & Bernatchez 2012), suggests that a combination of trans-regulatory and cis-acting factors has led to the evolution of energy metabolism in the recently diverged Cliff lake species pair.

Conclusions

Differences in energy metabolism are predicted to influence evolutionary fitness and contribute to local adaptation and ecological speciation in many systems (e.g. Eanes 2011; Zera 2011; Marden 2013; Scott *et al.* 2015), including in the dwarf and normal Lake Whitefish species pairs (Bernatchez *et al.* 2010). In whitefish, differences in energy metabolism are predicted to underlie the increased swimming activity and standard metabolic rate that has evolved in the derived, limnetic dwarf whitefish (Trudel *et al.* 2001; Rogers *et al.* 2002), but the specific loci under selection vary among species pairs (Renaut *et al.* 2011; Gagnaire *et al.* 2013a). In this study, we find that independent populations of dwarf whitefish have repeatedly evolved a greater proportion of oxidative, red skeletal muscle and a relatively larger liver and ventricle, suggesting these traits are critical for local adaptation to the limnetic niche and the associated increases in prolonged swimming required for limnetic foraging. This occurs in combination with convergent changes in body shape whereby dwarf whitefish show a more streamlined body shape predicted to facilitate prolonged swimming (Laporte *et al.* 2015). However, we found that the activities of

candidate enzymes involved in central energy metabolism in muscle and liver (glycolysis, glycogen metabolism, mitochondrial oxidative phosphorylation) have not evolved convergently among lakes. The lack of convergence in enzyme activities is not entirely unexpected, given the complex genetic basis for locomotion and energy metabolism in vertebrates (e.g. Kelly & Pomp 2013; Kelly *et al.* 2015). The substantial differences in population demography and selective pressures among lakes may also act to reduce convergence (Rosenblum *et al.* 2014).

Finally, we have found evidence for the evolutionary upregulation of all enzymes involved in glycolysis, glycogen metabolism and mitochondrial oxidative phosphorylation in the white muscle of Cliff Lake dwarf fish compared with normal fish. These data suggest that increases in the size of tissues supporting aerobic energy metabolism (ventricle mass and percentage of red muscle, common to many dwarf populations), in combination with the global upregulation of central energy metabolism in white skeletal muscle and genetic changes in many metabolic genes (Renaut *et al.* 2011; Jeukens & Bernatchez 2012), underlie the evolution of energy metabolism in this highly divergent dwarf and normal species pairs. Together, the genomic, ecological and physiological evidence collected over the years supports the hypothesis that the speciation process is likely complete in Cliff lake but still ongoing in other lakes.

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L.B. leads the long-term, multidisciplinary research programme on speciation and adaptation in whitefish species pairs. L.B., H.G. and A.C.D. designed this experiment. A.C.D. and M.L. collected samples and data. A.C.D. analysed the data, and L.B. H.G., A.C.D., M.L. and C.R. wrote and edited the manuscript.

Data accessibility

All data (mass and lengths of fish, enzyme activities, tissue morphology) and code have been submitted to Dryad (http://datadryad.org/resource/doi:10.5061/dryad.686sq).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Overview of data collection for all 26 metabolic explanatory variables.

Fig. S2 Multivariate analyses using two different sub-sets of correlated enzyme activities.

Table S1 Differences in ventricle mass (residuals of linear regression against mass), liver mass (residuals of linear regression against mass) and percentage red muscle among species and lakes (data presented in Fig. 2).

Table S2 Differences in white skeletal muscle protein content and enzyme activities among species and lakes (data presented in Figs 2-4)

Table S3 Differences in hepatic protein content and enzyme activities among species and lakes (data presented in Figs. 2–4).

Table S4 Differences in discriminant function linear discriminant 1 (ld1) and 2 (ld2) and principal component 1 (PC1) and 2 (PC2) values among species and lakes (data presented in Fig. 7 and S2).

Table S5 Results of the MANOVA testing the influence of 'lake' and 'species' on an alternate subset of 12 energetic traits: relative liver mass, relative ventricle mass, percentage red muscle, white muscle COX (instead of CS), CK, GPI (instead of PFK), LDH (instead of PK) and hepatic COX (instead of CS), GPI, ALDO (instead of GAPDH), TPI (instead of LDH), and PFK.

Table S6 Summary of studies examining genomic, transcriptomic, biochemical, and whole-organismal differentiation of energy metabolism in dwarf and normal Lake Whitefish.

Table S7 Differences in the mRNA content and enzyme activities of genes involved in central energy metabolism in the white muscle of dwarf and normal Lake Whitefish species or whole-juvenile fish.

Table S8 Differences in the mRNA content and enzyme activities of genes involved in central energy metabolism in the liver of dwarf and normal Lake Whitefish species.