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Parallelism in the oxygen transport system of the lake whitefish: the role of physiological divergence in ecological speciation

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Abstract

In North America, populations of lake whitefish (*Coregonus clupeaformis*) have evolved sympatric 'dwarf' and 'normal' ecotypes that are associated with distinct trophic niches within lakes. Trophic specialization should place diverging physiological demands on individuals, and thus, genes and phenotypes associated with energy production represent ideal candidates for studies of adaptation. Here, we test for the parallel divergence of traits involved in oxygen transport in dwarf and normal lake whitefish from Québec, Canada and Maine, USA. We observed significant differences in red blood cell morphology between the ecotypes. Specifically, dwarfs exhibited larger nuclei and a higher nucleus area/total cell area than normal whitefish in all of the lakes examined. In addition, isoelectric focusing gels revealed variation in the haemoglobin protein components found in whitefish. Dwarf and normal whitefish exhibited a similar number of protein components, but the composition of these components differed, with dwarf whitefish bearing a greater proportion of cathodic components compared to the normals. Furthermore, dwarf whitefish showed significant haemoglobin gene upregulation in the brain compared with the levels shown in normals. Together, our results indicate that metabolic traits involved in oxygen transport differ between the whitefish ecotypes and the strong parallel patterns of divergence observed across lakes implicates ecologically driven selection pressures. We discuss the function of these traits in relation to the differing trophic niches occupied by the whitefish and the potential contributions of trait plasticity and genetic divergence to energetic adaptation.

Keywords: ecological speciation, gene expression, Haemoglobin, physiological divergence, red blood cells, single-nucleotide polymorphism, whitefish

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Introduction

The parallel evolution of traits across replicated populations provides an important framework for investigations of the nature and form of selection operating in the wild (Elmer & Meyer 2011). Indeed, the repeated evolution of the same phenotype across independent lineages is best explained through the operation of similar natural selection pressures (Endler 1986; Rundle *et al.* 2000; Elmer & Meyer 2011). In this regard, studies of fish species that have colonized lakes since the recession of the last major glaciation event, and evolved sympatric populations associated with trophic polymorphism, have become important systems for investigating the role of ecologically driven adaptive divergence (e.g. Bernatchez & Dodson 1990; McPhail 1993; Schluter 2000; MacQueen *et al.* 2011). In eastern North America and Europe, multiple lakes containing whitefish (*Coregonus* spp.) have evolved such trophic polymorphisms (Bernatchez *et al.* 2010; Siwertsson *et al.* 2010). In the

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North American lake whitefish (*Coregonus clupeaformis*), a 'dwarf' ecotype occupies the limnetic zone and has evolved from the ancestral benthic 'normal' ecotype in response to competitive interactions and ecological opportunity (Bernatchez *et al.* 2010). Natural selection has been implicated as a driver of character displacement between the ecotypes as parallel patterns of morphological, behavioural and life history divergence occur across independently colonized lakes (Schluter & McPhail 1993; Bernatchez 2004; Bernatchez *et al.* 2010).

In addition to the above-mentioned traits, recent work has pointed to physiological divergence as a potentially critical facilitator of whitefish adaptive radiation. For instance, comparisons of transcriptomic variation have shown differences between dwarf and normal whitefish at the coding and expression levels for genes associated with energy production (St-Cyr *et al.* 2008; Renaut *et al.* 2010, 2011). Furthermore, studies have shown that dwarf whitefish allocate greater energetic investment into metabolism and less to growth and reproduction, compared with normals (Trudel *et al.* 2001; Rogers & Bernatchez 2005). Ultimately, these physiological differences may be driven by diverging biotic (differing prey sources and predation pressures) or abiotic conditions encountered across the benthic–limnetic resource axis (Landry *et al.* 2007; Landry & Bernatchez 2010; also see Kahilainen & Lehtonen 2003). Nevertheless, considerable gaps remain in our understanding of the specific physiological processes and traits underlying the adaptive divergence of dwarf and normal lake whitefish, particularly as they relate to ecological variation within and among lakes. Here, we investigate the potential for physiological trait divergence associated with molecular oxygen transport in whitefish.

Haemoglobin proteins transport oxygen molecules from respiratory surfaces to tissues for use in cellular respiration. Given their continuous contact with external and internal environments, haemoglobin proteins make ideal candidates for studies of physiological divergence and adaptation (Weber 1990; Nikinmaa 1997; Storz *et al.* 2009). Indeed, many fish species bear diverse haemoglobin protein types that are adapted to oxygen loading and transport under variable temperatures (Sick 1961; Verde *et al.* 2006; Andersen *et al.* 2009; Wetten *et al.* 2010; Star *et al.* 2011). For instance, the predominant haemoglobin proteins found in northern populations of Atlantic cod (*Gadus morhua*) exhibit relatively high oxygen affinities at cooler temperatures, whereas the proteins found in southern populations exhibit relatively high oxygen affinities at warmer temperatures (Brix *et al.* 2004; Andersen *et al.* 2009). The differences in oxygen binding affinities among the haemoglobin protein types appear to be genetically determined which further implicates adaptive processes in the evolution of protein variation (Andersen *et al.* 2009; Star *et al.* 2011). It was also recently shown *in vitro* that northern populations of cod may be able to compensate for the reduced oxygen affinities of their haemoglobin proteins in warmer waters by upregulating the expression of haemoglobin genes (Star *et al.* 2011; also see Brix *et al.* 2004). Thus, haemoglobin protein variation and enhanced haemoglobin protein production via gene upregulation are probably important physiological responses to the variable energetic and ecological constraints faced by fishes.

As in all vertebrates, the red blood cells (RBCs) of fishes are the primary carriers of haemoglobin (Jensen 2009). However, in contrast to mammals, mature fish RBCs are nucleated, contain mitochondria and are metabolically active at levels comparable with ordinary muscle tissues (Itazawa & Oikawa 1983). Given that haemoglobin is carried in the cytoplasm, the nucleus to total cell area ratio in fish RBCs could impact oxygen delivery capacity (Lay & Baldwin 1999). Furthermore, it has been proposed that smaller RBC size should facilitate the delivery of oxygen across the cell membrane (Lay & Baldwin 1999; Snyder & Sheafor 1999). In a comparative examination of RBCs across 52 teleost species, cell size was negatively correlated with activity levels, supporting the hypothesis that oxygen delivery is enhanced in smaller RBCs (Lay & Baldwin 1999). Thus, examinations of RBC morphology may provide important insight into the energetic requirements of populations inhabiting different environments.

We examined variation in traits critical to the metabolic uptake and delivery of oxygen in dwarf and normal lake whitefish from lakes in Maine, USA and Québec, Canada. We examined both ecotypes for RBC morphology, including cell and nucleus size, haemoglobin protein variation and haemoglobin gene expression. The potential for genetically determined variation in haemoglobin proteins or gene expression was also investigated using Sanger sequencing and SNP genotyping of portions of the alpha and beta haemoglobin genes. Because dwarfs allocate a greater proportion of consumed energy to metabolism compared with normals (Trudel *et al.* 2001), we predicted that dwarfs would require an enhanced ability to take up and transport oxygen for use in cellular respiration and that diverging energetic requirements between dwarfs and normals should be reflected in patterns of RBC morphology, haemoglobin protein variation and haemoglobin gene expression.

Methods

Sampling

Lake whitefish were sampled with gill nets from Cliff Lake and Indian Pond in Maine, USA, in June 2010,

and from East and Témiscouata Lakes in Québec, Canada in July 2010. In all lakes but East, Acadian and Atlantic lineages of whitefish came into secondary contact following the last glacial retreat, whereas East Lake was most likely colonized by the Acadian lineage only. Previous phylogeographic work has shown that all lakes were colonized independently and that different levels of admixture between founding lineages occurred in the different lakes (Bernatchez & Dodson 1990; Pigeon *et al.* 1997; Lu *et al.* 2001). Dissections of freshly killed fish were conducted in the field. We collected *c.* 300 mg of tissue from the kidney and gills and whole brain from each individual and these tissues were flash-frozen in liquid nitrogen and later stored at −80 °C. For this study, we collected tissues from a total of 12 dwarf and 12 normal whitefish from each of the lakes. We also collected blood samples from 10 dwarf and 11 normal whitefish from Cliff Lake, eight dwarf and 10 normal whitefish from East Lake, and nine dwarf and three normal whitefish from Témiscouata Lake. All fish examined were sexually mature. Between 100 and 500 μL of blood was collected by puncturing the caudal vein with either a 21- or a 23-gauge syringe. Blood samples were immediately transferred to a 1.5-mL eppendorf tube containing 25 μL of 5000 IU/mL heparin and stored at 4 °C for subsequent analysis.

RBC morphology

Blood smears were made from each of the freshly collected samples (Houwen 2000). The smears were air-dried and fixed with 95% methanol in the field and later stained with Giemsa stain in the laboratory. We examined the smears under a light microscope at 200× magnification and a digital photograph was taken and imported into IMAGEJ (http://rsbweb.nih.gov/ij/index.html). Fifty cells per individual were measured for nucleus and total cell area (dry elliptical area in μm^2) allowing for the calculation of the nucleus area to total cell area. We were unable to analyse all of the blood smears for RBC morphology as a consequence of blood coagulation in the field for some of the samples. Final sample sizes are given in Table 1.

Haemoglobin isolation and characterization

Haemolysate was isolated from RBCs using a standard fractionation technique. Briefly, RBCs were pelleted by centrifugation at 750 ***g*** and the plasma/buffy layer was removed and discarded. The RBCs were washed three times in phosphate-buffered saline (PBS pH 7.4, 316 mOsm) and then lysed using a 3:1 ratio of 4 °C distilled water to RBC volume for 60 min. A final 30 min centrifugation step at 13 000 ***g*** was used to remove cell organelles and membranes from the haemolysate. Following centrifugation, the supernatant containing the haemolysate was collected and stored at −20 °C.

The number of haemoglobin protein components in each sample was determined using isoelectric focus (IEF) gel electrophoresis. This method separates haemoglobin components in a pH gradient across the gel, based on electric charge. A haemoglobin protein component's isoelectric point (pI), is defined as the position in the gel, corresponding to pH, where the protein becomes neutrally charged (Sick 1961; Husebø *et al.* 2004). No previous studies have examined haemoglobin protein component diversity in *Coregonine* fishes. However, other members of the Salmonidae exhibit diverse haemoglobin protein components that correspond to genetic differences (e.g. Fyhn & Withler 1991; Quinn

Table 1. Red blood cell morphological and haemoglobin (Hb) protein variation found in dwarf and normal lake whitefish (*Coregonus clupeaformis*) in three lakes in eastern North America

Lake	Form	N RBC, N Hb	Nuc area (μm^2)	Total cell area (μm^2)	Nuc/total cell area	Anod Lake	Cath Lake	Anod Ind.	Cath Ind.	Total Hb Ind.	Prop Cath Ind.
Témiscouata	Dwarf	3, 9	25.3 ± 2.3	126.1 ± 9.3	0.20 ± 0.03	3	3	2.3 ± 0.9	2.3 ± 0.5	4.7 ± 1.0	0.51 ± 0.12
	Normal	2, 3	25.5 ± 0.1	128.7 ± 2.8	0.20 ± 0.01	3	2	3.0	2.0	5.0	0.40
East	Dwarf	7, 8	26.2 ± 2.5	119.9 ± 9.9	0.22 ± 0.01	4	3	3.6 ± 0.7	2.6 ± 0.5	6.3 ± 0.7	0.42 ± 0.09
	Normal	8, 10	22.3 ± 1.7	116.5 ± 5.0	0.20 ± 0.02	4	2	4.0	2.0	6.0	0.33
Cliff	Dwarf	10, 10	25.8 ± 2.6	127.2 ± 8.3	0.21 ± 0.03	4	3	2.6 ± 0.5	2.2 ± 0.4	4.8 ± 0.8	0.46 ± 0.05
	Normal	7, 11	22.4 ± 3.3	128.1 ± 8.3	0.17 ± 0.02	4	2	3.2 ± 0.8	2.0	5.2 ± 0.8	0.39 ± 0.06

The number of individuals examined from each lake and for each ecotype for red blood cell morphology (N RBC) and haemoglobin protein variation (N Hb) are indicated. For RBCs, the mean dry nucleus (Nuc) area, the dry total cell area, and the nucleus area/total cell area (Nuc/total cell area) is shown ±1SD. For the haemoglobin proteins, the mean total number of anodic (Anod) and cathodic (Cath) protein components found in each lake is shown ±1SD, as is the mean number of each protein component type, the mean number of total haemoglobin protein components and the proportion of cathodic protein components found in individual (Ind.) whitefish ±1SD.

et al. 2010). Studies of haemoglobin protein components in cod have shown that 'anodic' components (i.e. that run towards the anode in IEF gels) exhibit relatively high oxygen affinities at low temperatures, whereas 'cathodic' components (i.e. that run towards the cathode) show relatively high oxygen affinities at warmer temperatures (Weber 1990; e.g. Brix *et al.* 2004). Also, it has been suggested that cathodic haemoglobins may serve as an emergency source of oxygen that can be utilized under hyperactive metabolic conditions (Weber 1990).

Isoelectric focus gels were run following manufacturer's instructions and details on the use of this method in the context of fish haemoglobin component partitioning can be found in Husebø *et al.* (2004). Briefly, we determined haemoglobin concentration in the haemolysate spectrophotometrically at 540 nm using Drabkin's reagent (D 5941; Sigma, Saint Louis, MO, USA) and a bovine haemoglobin standard (H2500-1G; Sigma) according to the manufacturer's instructions (Sigma). Novex® IEF gels (pH = 3–10; Invitrogen Life Technologies Co., USA) were loaded with *c.* 50 μg of haemoglobin per sample, along with a protein marker (Novex® IEF-Marker; Invitrogen), run for 2.5 h at 4 °C and then fixed and stained in accordance with the manufacturer's instructions (Invitrogen). We determined the pI of each haemoglobin component with reference to this marker. To facilitate scoring, each gel was photographed, using transmitted light, with the Eagle-eye imaging system (Stratagene, La Jolla, CA, USA), and the photographs were processed in IMAGEJ. Only gels with regression coefficients (R^2) > 0.97, when plotting the pI of the standard proteins against the corresponding migration distance, were included in the study. All gels were scored in duplicate.

RNA extraction and cDNA synthesis

Total RNA was extracted from kidney, gill and whole-brain tissue using the Ambion PureLink RNA Mini Kit according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). We were interested in examining the expression of haemoglobin in the kidney because it is a primary organ involved in the production and storage of RBCs in fishes (Fänge 1994; Abdel-Aziz *et al.* 2010). Gills are the site of oxygen uptake from the environment, and thus, we were interested in potential differences in the quantity of haemoglobin produced in these tissues. Finally, the brain was examined for haemoglobin expression levels because previous microarray-based studies have shown that dwarf and normal whitefish differentially express an EST associated with haemoglobin proteins (Whiteley *et al.* 2008; J. St-Cyr, unpublished data), suggesting that enhanced production of haemoglobin in the brain may be involved in supporting the higher metabolic rate of the dwarf whitefish.

RNA extracts were treated with Ambion Superase-In, an RNAase inhibitor (Applied Biosystems). RNA purity and concentration was evaluated by measuring the 260/230 and 260/280 nm absorbance ratios using the Nanodrop 2000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was confirmed using the Experion Automated Electrophoresis system (Bio-Rad, Mississauga, ON, Canada). We synthesized cDNA from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription reactions were performed in 20 μL volumes containing 500 ng RNA and using random primers.

Haemoglobin expression assays

Vertebrate haemoglobin proteins are composed of two alpha-type and two beta-type polypeptide chains that are joined noncovalently to form a tetramer (Weber 1990). We designed TaqMan (Applied Biosystems) qPCR assays to examine the expression of the alpha and beta chains of haemoglobin. TaqMan Minor Groove Binder (MGB) probes and primers were designed in Primer Express v. 3.0 (Applied Biosystems) using target sequences specific to the lake whitefish haemoglobin. The template sequences for the haemoglobin assays were taken from 454 sequencing data derived from the lake whitefish liver transcriptome (Renaut *et al.* 2010) and subsequently validated using cloning and Sanger sequencing of between four and eight clones containing inserts (also see Jeukens *et al.* 2009). Both the alpha and beta haemoglobin ESTs were associated with Bohr effect haemoglobin molecules, based on alignment with the annotated Atlantic salmon haemoglobin genome (GenBank Accession X97285.1; McMorrow *et al.* 1996). For the alpha haemoglobin assay, the combination of forward primer 5′-TGGACCCACCAACTTCAA-3′, reverse primer 5′-GCGGCAACGACCACAATC-3′, and probe 5′-ATCCTGGCTCACAACC-3′ was used. For the beta haemoglobin assay, the combination of forward primer 5′-GTGCAGTTTCTCCGAGTGCAT-3′, reverse primer 5′-AGAACCTGGATGACATCAAAAACA-3′ and probe 5′-ACACTCAGTGCAGTATAG-3′ was used. The efficiency of each assay was evaluated using a validation experiment (Applied Biosystems 2008) and was found to be 100% and 99%, for the alpha and beta assays, respectively. Because the common ancestor of all salmonid fishes underwent a whole-genome duplication event, it is probable that our assays are amplifying two or more copies of the same gene (see Quinn *et al.* 2010).

qPCR

We utilized the comparative C_T method ($\Delta\Delta C_T$ method; Applied Biosystems) to quantify interindividual variation in the relative amounts of target haemoglobin cDNAs in the kidney, gill and brain. This method calculates the relative quantity (RQ) of cDNA compared with an internal reference sample (calibrator) and RQ values represent fold changes in expression relative to the calibrator, which has a value of one (Applied Biosystems 2008). Due to the potential for large differences in haemoglobin expression levels among the three tissue types examined in this study, we elected to use a calibrator sample derived from the same tissue type in each expression assay. Thus, we generally did not compare variation in expression levels among tissue types. However, for a subset of individuals (six Témiscouata dwarf whitefish, six Témiscouata normal whitefish, six Cliff normal whitefish and three Cliff dwarf whitefish), we compared RQ levels for the alpha haemoglobin EST among the kidney, gill and brain tissues utilizing a calibrator derived from kidney tissue.

We amplified the haemoglobin targets from cDNA in triplicate using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Universal PCR Master Mix. Quantitative PCRs were conducted in 10 μL reactions and using the default thermocycler setting for Fast qPCR TaqMan assays, which consist of an initial denaturation step at 95 °C for 20 s, and then 40 cycles of 95 °C for 3 s and 60 °C for 30 s of annealing/extension. We used ABI's Human Euk 18S rRNA gene assay as an endogenous control to normalize the total quantity of cDNA loaded into the PCR wells. The Human Euk 18S rRNA assay has previously been shown to be an appropriate target for the normalization of expression assays in whitefish (Jeukens *et al.* 2009) and other salmonids (e.g. Olsvik *et al.* 2005).

Repeatability of the expression assays was confirmed by conducting two independent qPCR runs on a subset of the cDNA samples for each tissue (kidney: $N = 9$; brain: $N = 8$, gill: $N = 8$). Moreover, we found that the RQ for the alpha and beta haemoglobin assays were highly correlated within individuals and in each of the tissue types (Spearman's rank correlation: kidney: $\rho = 0.92$; brain: $\rho = 0.83$; gill: $\rho = 0.96$), suggesting that the reproducibility of our expression assays was high. This also confirmed that we were probably amplifying alpha and beta coding regions from the same type of haemoglobin molecule. We ensured that genomic DNA was not contaminating our results by conducting an RT reaction without reverse transcriptase and running these samples in the qPCRs (Bustin *et al.* 2009). Additionally, the alpha haemoglobin probe spans exons two and three of the alpha haemoglobin chain, which will preclude amplification of genomic DNA (containing introns) when using this assay.

SNP identification, validation and KASPAR SNP genotyping

We used Sanger sequencing to investigate the occurrence of single-nucleotide polymorphisms (SNPs) in a subset of haemoglobin genes. Species-specific primers for portions of haemoglobin alpha and beta genes were designed based on 454 sequencing data of the whitefish transcriptome (Table S1, Supporting information; see Renaut *et al.* 2010). In total, we examined 1376 bp of sequence distributed across alpha and beta introns and exons and identified 12 putative SNPs (Table S1, Supporting information). For broad scale SNP genotyping across whitefish populations, we developed KASPar SNP genotyping assays (KBiosciences, Hoddesdon, UK) for each of the 12 SNPs (Table S1, Supporting information). KASPar assays are composed of fluorescently labelled SNP-specific primers that enable the determination of homozygous or heterozygous genotypes based on the differential amplification of alleles associated with each of the uniquely tagged primers.

KASPar assays were run on the Applied Biosystems 7500 Real-time PCR system under the genotyping application. KASPar genotyping reactions were run in 8 μL volumes consisting of 4 μL DNA, 4 μL 2 × reaction mix, 0.11 μL Assay Mix (see Table S1, Supporting information) and 0.064 μL 50 mM $MgCl_2$ (KBiosciences). We genotyped all individuals examined for haemoglobin gene expression levels in this study at the SNP markers and conducted a population genetic evaluation of SNP allele frequencies in a total of 323 whitefish (Table S2, Supporting information). The allele frequencies for all of the SNP markers, excluding 1544-242, did not conform to Hardy–Weinberg equilibrium (HWE) expectations, indicating that these putative SNPs are probably polymorphisms in haemoglobin gene paralogs. Thus, we excluded all but SNP 1544-242, an A/G polymorphism located in intron 1 of the alpha (Bohr effect) haemoglobin gene, from further analysis. For this marker, observed and expected heterozygosities for dwarf and normal individuals in each population were calculated in Genepop v. 4.0.10 (Raymond & Rousset 1995) and are reported in Table S2 (Supporting information).

Statistical analyses

The measurements of the RBCs and haemoglobin protein components were approximately normally distributed (Shapiro–Wilk test: $P > 0.47$). In contrast, the alpha and beta gene expression levels were not normally distributed, but did conform to a Log Normal distribution in all

tissue types (Kolmogorov–Smirnov tests: $P > 0.15$); thus, we used Log+1 transformed expression data as dependent variables in all statistical analyses of expression. Dwarfs and normals showed similar variance in the RBC and haemoglobin measurements (analysis of means for variance: $P > 0.05$).

Two-way ANOVA was used to partition variation in RBC nucleus area, total cell area, nucleus area/total cell area, number of haemoglobin protein components, and the proportion of cathodic haemoglobin protein components to lake, ecotype and lake×ecotype effects.

We used the first factor of a principal components analysis (PCA, on covariances) of the alpha and beta expression values (Log+1 transformed) to describe variation in expression at the two genes. Factor 1 of the PCA captured 91%, 90% and 95% of the variation in expression in the kidney, brain and gill tissues, respectively.

Previous studies in lake whitefish have shown sex-specific differences in the expression of some genes (Jeukens *et al.* 2009). We did not have information on the sex of the fish examined for haemoglobin expression in either Cliff Lake or Indian Pond. However, we were able to examine sex effects on haemoglobin expression levels in both East and Témiscouata lakes. We did not detect a difference in expression between males and females in any of the tissues examined (Independent samples *t*-test: kidney: $t_{49} = 0.32$, $P = 0.75$; brain: $t_{49} = -0.52$, $P = 0.61$; gill: $t_{53} = -0.125$, $P = 0.21$). Thus, we used two-way ANOVA to examine the contribution of lake, ecotype and lake×ecotype effects to variation in PCA Factor 1 haemoglobin expression levels in each of the tissues. We used ANOVA, with lake and ecotype included as fixed factors in the model, to examine variation in alpha haemoglobin expression levels among the kidney, brain and gill tissues. Nested ANOVA was used to examine the relationship between SNP 1544-242 genotype (AA, AG and GG) and alpha haemoglobin gene expression in each of the tissues. In these models, ecotype was nested within SNP genotype, as not all of the SNP genotypes were found in each ecotype in each lake. Lake was also included in the model to control for lake-specific effects, independent of SNP genotype, on gene expression.

All statistical analyses were run in JMP v.9 (SAS Institute Inc., Cary, NC, USA). Throughout, means are reported ±1 standard deviation (SD), except where indicated, and we used a threshold significance level of $\alpha = 0.05$ for all statistical tests.

Results

RBC morphology

Across all whitefish examined, dry RBC nucleus area averaged 24.4 ± 2.9μm^2 (range = 19.3–29.9μm^2) and the nucleus area/total cell area averaged 0.20 ± 0.02 (range = 0.15–0.25). Dwarf whitefish in all three populations examined exhibited significantly larger nuclei and nucleus area/total cell areas than normal whitefish (Tables 1–2). We also detected a significant difference among lakes in RBC morphology, independent of ecotype, with East Lake whitefish showing the smallest RBCs compared with whitefish in Cliff and Témiscouata lakes (Tables 1–2).

Haemoglobin proteins

We identified four major anodic (pI = 6.1–6.7) and four major cathodic (pI = 7.5–8.3) haemoglobin protein components in whitefish via IEF (e.g. Fig S1, Supporting information). In total, these components produced 12 different IEF gel banding patterns (Table S3, Supporting information). The frequencies of each haemoglobin protein component are shown in Table S4 (Supporting information) for each ecotype in each of the three lakes

Table 2 Results of two-way ANOVA examining the influence of ecotype and lake on red blood cell morphology and the proportion of cathodic haemoglobin (Hb) protein components in lake whitefish (*Coregonus clupeaformis*)

	Nucleus area			Total cell area			Nucleus/total cell area			Total Hb individuals			Proportion Cath Hb individuals		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Model	3.60	5, 31	**0.011**	2.63	5, 31	**0.043**	3.16	5, 31	**0.020**	7.36	5, 45	**<0.001**	6.76	5, 45	**<0.001**
Lake	0.50	2	0.609	6.05	2	**0.006**	1.90	2	0.166	15.56	2	**<0.001**	4.10	2	**0.023**
Ecotype	6.09	1	**0.019**	<0.01	1	0.997	5.70	1	**0.023**	0.49	1	0.484	15.84	1	**<0.001**
Lake × ecotype	1.30	2	0.286	0.41	2	0.669	0.88	2	0.424	1.06	2	0.356	0.34	2	0.715

The ANOVA models examined the contribution of lake, ecotype and lake×ecotype to variation in nucleus area, total cell area, nucleus/total cell area, the total number of haemoglobin protein components per individual and the proportion of cathodic (Cath) haemoglobin protein components found per individual. *F*-statistics, numerator and denominator degrees of freedom (d.f.) for the model, d.f. for each fixed effect, and *P*-values are indicated. Significant *P*-values are bolded.

examined. On average, individual whitefish exhibited 5.3 ± 0.9 (range = 3–7) haemoglobin protein components composed of an average of 2.2 ± 0.4 (range = 2–4) cathodic components and 3.1 ± 0.9 (range = 1–4) anodic components. The total number of haemoglobin components exhibited by individual whitefish did not differ significantly between the dwarf and normal ecotypes, albeit we did detect a lake effect on haemoglobin component number, with individuals in East Lake exhibiting a significantly greater number of components than individuals in both Cliff and Témiscouata lakes (Tables 1–2; Tukey's *post hoc* test: $P < 0.05$). However, we did observe parallel patterns of divergence in the proportion of cathodic haemoglobin components found in the whitefish ecotypes in each of the three lakes; specifically, dwarf whitefish exhibited a significantly greater proportion of cathodic components compared with normal whitefish (*Post hoc* Student's *t*-test: $P < 0.05$; Tables 1–2). Furthermore, East Lake whitefish showed a significantly smaller proportion of cathodic protein components than whitefish from Témiscouata Lake (Tukey's *post hoc* test: $P < 0.05$).

Haemoglobin gene expression

Alpha haemoglobin expression in the kidney was significantly greater than expression levels in either the brain or gill tissues (ANOVA: $F_{4, 40} = 23.2$, $P < 0.001$; Tukey's *post hoc* test: $P < 0.05$). Indeed, levels of alpha haemoglobin expression in the kidney tissue (RQ = 1.08 ± 0.11) were 14 times greater than what was observed in the brain (RQ = 0.08 ± 0.12) and 27 times greater than what was observed in the gill tissues (RQ = 0.04 ± 0.07), albeit haemoglobin expression levels did not differ significantly between the brain and gill tissues (Tukey's *post hoc* test: $P > 0.05$).

Haemoglobin expression (PCA Factor 1) in the kidney tissues did not vary significantly between dwarf and normal lake whitefish, although we did detect some differences in expression among lakes (Table 3). Specifically, East and Témiscouata lake whitefish exhibited greater levels of haemoglobin expression in the kidneys than did Indian Pond whitefish (Fig. 1a, Tukey's *post hoc* test: $P < 0.05$). In the brain tissue, dwarf whitefish showed greater expression levels than normal whitefish in each of the four lakes examined (Fig. 1b, Tukey's *post hoc* test: $P < 0.05$). We also observed significant variation in haemoglobin expression in the brain among lakes, with greater expression found in Indian Pond whitefish compared with whitefish from Cliff Lake (Tukey's *post hoc* test: $P < 0.05$). For the gill tissue, variation in haemoglobin expression was not explained by ecotype (Fig. 1c, Table 3). We did observe a significant lake effect on gill haemoglobin expression (Table 3); however, the difference among lakes was not significant when examined using a *post hoc* test (Tukey's *post hoc* test: $P > 0.05$).

Haemoglobin SNP genotype and expression

Of the 323 whitefish genotyped at SNP 1544-242, only two normal individuals from Témiscouata Lake were homozygous for the AA genotype. The GG genotype was the most common in each of the lakes and ecotypes (Table S2, Supporting information). We did not observe a significant relationship between alpha haemoglobin expression and SNP 1544-242 genotype in any of the tissues examined (Table 4). However, in the kidney tissue, there was a trend for alpha haemoglobin expression to increase as a function of number of G nucleotide copies at SNP 1544-242 (Fig. 2). We also observed a significant effect of form nested within SNP in the brain and gill tissues; dwarf whitefish bearing the GG genotype exhibited significantly greater expression than normals bearing the same genotype (Tukey's posthoc test: $P < 0.05$).

Discussion

This study provides strong evidence for the parallel divergence of physiological traits involved in oxygen uptake, transport and availability between dwarf and normal lake whitefish from multiple lakes in eastern North America. Variation in RBC morphology in fishes is expected to reflect differences in environmental

Table 3 Results of two-way ANOVA models examining the influence of ecotype and lake on haemoglobin gene expression (principal components analysis Factor 1) in kidney, brain and gill tissues in lake whitefish (*Coregonus clupeaformis*)

	Kidney			Brain			Gill		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Model	2.85	7, 86	**0.010**	6.81	7, 86	**<0.001**	2.46	7, 88	**0.023**
Lake	4.91	3	**0.003**	5.24	3	**0.002**	3.17	3	**0.029**
Ecotype	1.65	1	0.202	30.79	1	**<0.001**	0.75	1	0.388
Lake × ecotype	1.27	3	0.288	0.28	3	0.835	1.86	3	0.141

F-statistics, numerator and denominator degrees of freedom (d.f.) for the model, d.f. for each fixed effect and *P*-values are indicated. Significant *P*-values are bolded.

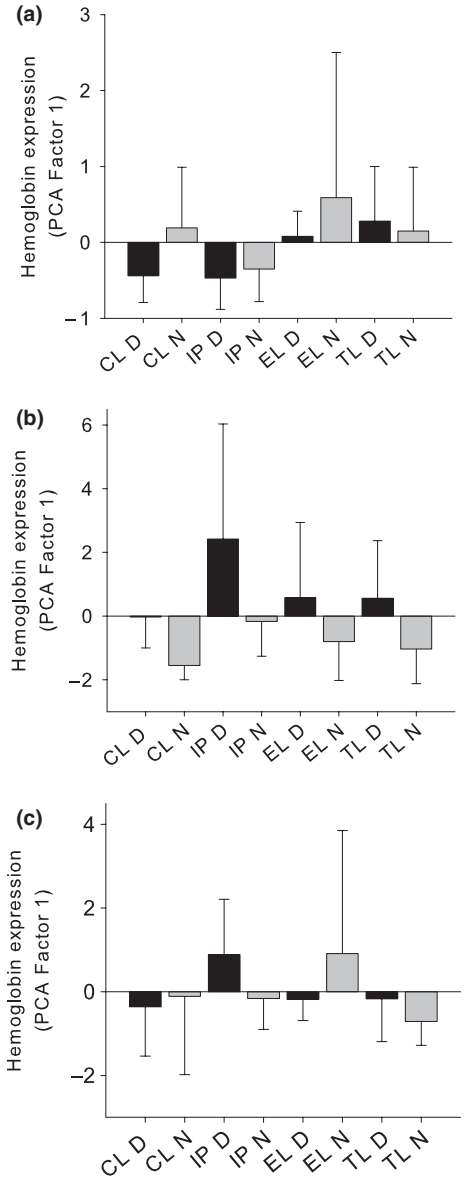

Fig. 1 Variation in haemoglobin gene expression in dwarf and normal lake whitefish (*Coregonus clupeaformis*) from four lakes in eastern North America. Gene expression levels are shown for dwarf (D-black bars) and normal whitefish (N-grey bars) from Cliff Lake (CL) and Indian Pond (IP) in Maine, USA, and East (EL) and Témiscouata (TL) lakes in Québec, Canada in each of the (a) kidney, (b) brain and (c) gill tissues. Variation in the mean expression (±1SD) of the haemoglobin alpha and beta chains is shown using the first factor of a principle components analysis (Factor 1). See Table 3 for two-way ANOVA models examining the contribution of ecotype and lake to variation in haemoglobin expression. Note that the scales on the y-axes differ.

oxygen availability and the energetic requirements of individuals (Wilhem Filho *et al.* 1992; Lay & Baldwin 1999). Dwarf whitefish exhibited significantly larger RBC nuclei, but not overall cell size, compared with normal whitefish across each of the populations examined for this trait. Surprisingly, little is known about the determinants of nucleus size variation in cells (Webster *et al.* 2009), though, it has been suggested that larger nuclei may reflect increased ploidy (Wolters *et al.* 1982; Gregory 2001; Hardie & Hebert 2003) or increased capacity to produce transcription products (Schmidt & Schibler 1995; Nikinmaa 2001; Webster *et al.* 2009). It is also possible that nucleus size in the normal whitefish is constrained by the need for a larger cytoplasmic area. In some lakes, normal whitefish face seasonal hypoxia when foraging in the benthic zone due to lake stratification in the fall (Landry *et al.* 2007; Fig. S2, Supporting information), and RBCs with larger haemoglobin molecule carrying capacities, potentially facilitated through larger cytoplasmic areas, could increase oxygen uptake in an oxygen depleted environment. At present, the potential for differences in RBC DNA and RNA content or haemoglobin protein concentrations remain unstudied for the whitefish ecotypes. However, the parallel divergence observed between dwarf and normal lake whitefish in RBC morphology strongly suggests that the two ecotypes require differing capacities to take up and transport oxygen via RBCs. Further studies are clearly warranted to examine the precise function of these morphological differences.

Haemoglobin protein diversity represents a potentially critical adaptation to the variable environmental conditions found in aquatic environments (Weber 1990). Indeed, we identified a total of eight different haemoglobin components in lake whitefish consisting equally of anodic and cathodic forms, which is similar to what has been found in other salmonid fishes, such as for example, rainbow trout (*Oncorhynchus mykiss*; Fago *et al.* 2001). While the two ecotypes did not differ in total number of haemoglobin protein components, dwarf whitefish exhibited a greater proportion of cathodic components compared with normal whitefish across each of the three lakes examined. At warm temperatures (>15 °C), the cathodic haemoglobin proteins found in Atlantic cod show a higher affinity for oxygen than anodic haemoglobin components (Brix *et al.* 1998); thus, the differences we observed in haemoglobin protein component diversity between dwarf and normal whitefish could be explained by plastic or adaptive responses to temperature (Houston *et al.* 1996; Brix *et al.* 2004; Fig. S2, Supporting information). Indeed, temperature decreases with depth in the whitefish lakes in Québec and Maine during the summer months (Landry *et al.* 2007), so dwarf whitefish inhabiting the shallower limnetic zone probably face environmental conditions favourable for cathodic haemoglobin protein function. It has also been suggested that cathodic haemoglobin proteins provide a reservoir of oxygen available to protect

Table 4 Results of the nested ANOVA models examining the relationship between haemoglobin SNP 1544-242 genotype and variation in alpha haemoglobin gene expression in lake whitefish (*Coregonus clupeaformis*)

	Kidney			Brain			Gill		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Model	5.56	7, 84	**<0.001**	8.91	7, 85	**<0.001**	4.85	7, 77	**<0.001**
Lake	11.12	3	**<0.001**	9.12	3	**<0.001**	6.16	3	**<0.001**
SNP	1.27	2	0.286	2.19	2	0.118	1.32	2	0.272
SNP [Ecotype]	0.48	2	0.618	15.50	2	**<0.001**	7.22	2	**0.001**

We examined the influence of SNP genotype on Log+1 transformed levels of gene expression in kidney, brain and gill tissues. *F*-statistics and *P*-values are reported for the model and each factor included within the model. Degrees of freedom (d.f.), including model and error d.f. for the model, are also indicated. Significant *P*-values are bolded.

against the effects of hyperactivity acidosis (Weber 1990). Thus, bearing a higher proportion of cathodic haemoglobin components could be an important physiological mechanism used to support the relatively high metabolic rates of dwarfs (Trudel *et al.* 2001; Rogers *et al.* 2002; Derome *et al.* 2006).

We also observed differences among lakes in both haemoglobin protein component diversity and RBC size, with East Lake dwarf and normal whitefish exhibiting the largest number of protein components and the smallest RBCs when compared to whitefish from Cliff and Témiscouata lakes. East Lake is the coldest of the three lakes (Landry *et al.* 2007) which could result in a decreased aerobic scope of activity in whitefish inhabiting this lake compared with whitefish from Cliff and Témiscouata lakes (Bernatchez & Dodson 1990; Pörtner *et al.* 1998; Brix *et al.* 2004). Under colder conditions, a larger haemoglobin repertoire and a greater RBC surface area to volume ratio should facilitate oxygen transport and the diffusion of oxygen across the cell membrane, respectively (Weber 1990; Lay & Baldwin 1999). Concurrent studies of spatial and temporal variation in haemoglobin proteins and ambient temperatures will help to elucidate the potential role of haemoglobin gene–temperature interactions in the regulation of protein variation between the whitefish ecotypes and among lakes.

In addition to exhibiting a diversity of haemoglobin protein components, the increased production of haemoglobin may be an important physiological response to enhanced metabolic oxygen demands or to environmental hypoxia (Star *et al.* 2011). We detected the expression of both the alpha and beta chains of haemoglobin molecules in each of the three tissue types examined in this study. The production of haemoglobin was highest in the kidney tissue when compared to the gill and brain tissues, which, given its primary role in RBC production, was not an unexpected result. More surprising was the observed expression of haemoglobin genes in the brain and gill tissues. The method we used to isolate RNA from the tissues cannot rule out the detection of RBC-associated RNA in these tissues (see Lund *et al.* 2000). However, a recent study in mice showed that neuron cells express haemoglobin genes (Biagioli *et al.* 2009). Furthermore, studies of rats and humans have shown haemoglobin gene expression in individual kidney (mesanglial), lung (epithelial), macrophage and cardiac ventricular cells, suggesting a potentially widespread role for haemoglobin proteins outside of purely RBC-based oxygen transport (see Nishi *et al.* 2008 for a review). Indeed, it has been suggested that haemoglobin protein production in tissues may serve an antioxidant function and play a role in the maintenance of cellular homoeostasis (Nishi *et al.* 2008; Biagioli *et al.* 2009). Studies incorporating the use of cell cultures are needed to confirm the expression of haemoglobin genes in individual kidney, gill and brain cells in whitefish.

Our most salient result relating to gene expression was the strong parallelism in the upregulation of haemoglobin alpha and beta genes in the brains of dwarf whitefish compared with normal whitefish across the four populations examined in this study. In contrast, the expression of haemoglobin genes did not differ significantly between dwarf and normal whitefish in either the kidney or gill tissues. Dwarf whitefish allocate a greater proportion of consumed energy to metabolism compared with normal whitefish (Trudel *et al.* 2001), and so an enhanced availability of oxygen molecules could be necessary for the maintenance of brain cell function in dwarfs (Nishi *et al.* 2008). Differences in prey availability or predation pressures may also alter gene expression directly through gene × environment effects. A recent study in three-spine sticklebacks (*Gasterosteus aculeatus*) showed that experimental predator exposure resulted in the upregulation of genes associated with cellular metabolism in brain tissues (Sanogo *et al.* 2011). Studies in European lakes have shown that dwarf whitefish face higher predation pressures than normals (Kahilainen & Lehtonen 2003), suggesting that

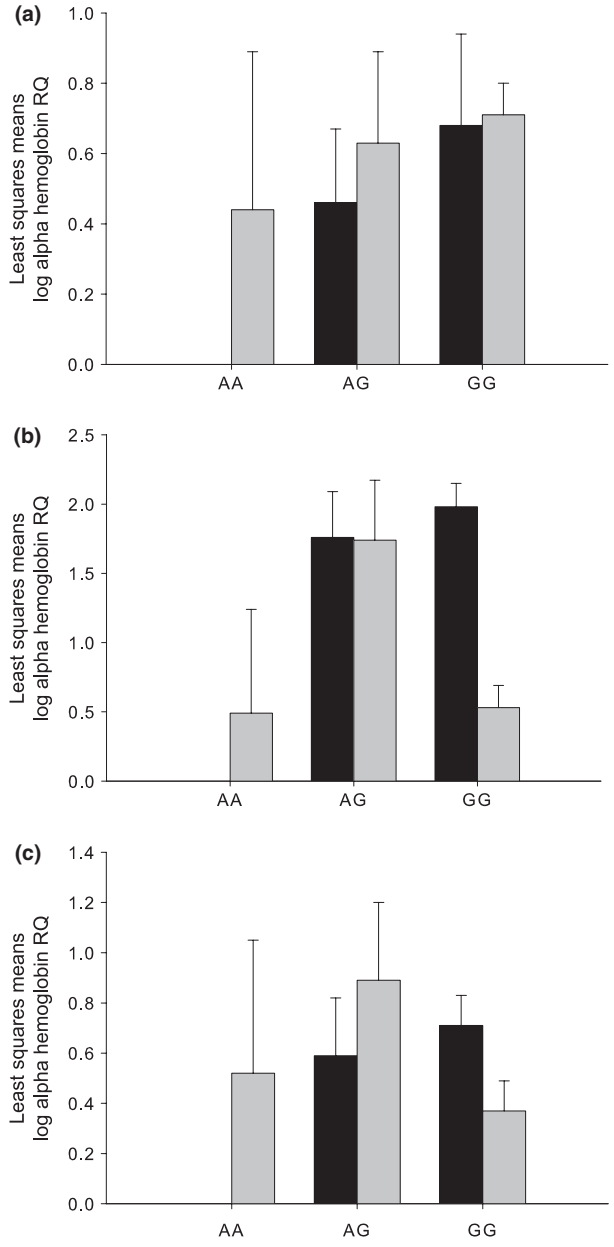

Fig. 2 Relationship between haemoglobin SNP 1544-242 genotype and alpha haemoglobin expression levels in lake whitefish (*Coregonus clupeaformis*) ecotypes. The relationship between the SNP genotype and haemoglobin gene expression in the dwarf (black bars) and normal (grey bars) ecotypes is shown in the (a) kidney, (b) brain and (c) gill tissues. The bars correspond to the least squares means ± 1SE of gene expression (RQ), which corrects levels of gene expression for any lake effects. The statistical analyses corresponding to this figure are shown in Table 4.

this could be an important ecological driver of the observed haemoglobin upregulation in the brains of dwarfs in our lakes. Fish may also upregulate haemoglobin expression in the face of hypoxic conditions (Wawrowski *et al.* 2011; but see Roesner *et al.* 2006) or rising temperatures (Star *et al.* 2011). Common garden experiments will be necessary to further explore the extent to which a plastic response to environmental variables such as predation, temperature or oxygen saturation regulates the differential expression of haemoglobin genes between the dwarf and normal whitefish.

Our targeted Sanger sequencing of haemoglobin genes did not uncover any genetic polymorphisms that significantly explained the observed differences in gene expression or haemoglobin protein variation among individual whitefish. However, we did observe a trend for haemoglobin expression in the kidney tissues to increase with the number of guanine nucleotide copies at SNP 1544-242, suggesting that genetic variation in intron one of the alpha haemoglobin gene could play a role in regulating gene expression. A growing number of studies point to a strong genetic basis underlying the divergence of metabolic traits between dwarf and normal whitefish (e.g. Rogers & Bernatchez 2005; Renaut *et al.* 2010; Jeukens & Bernatchez 2012); thus, it is also possible that haemoglobin expression is being regulated by genes directly involved in cellular respiration via gene–gene interactions or genetic variation found in regulatory regions upstream of the haemoglobin genes. Incorporating the use of BAC library sequencing of the haemoglobin alpha and beta chains (see Jeukens *et al.* 2011), in addition to the sequencing of each of the haemoglobin protein components uncovered via IEF, will provide further detail on the genomic architecture of the whitefish haemoglobin complex and resources for investigations of genetic differences between the dwarf and normal whitefish. This knowledge may also shed light on the potential role of differential haemoglobin paralog regulation in explaining expression differences among tissues.

In summary, we observed strong parallelism in traits involved in the uptake, delivery and availability of oxygen to tissues across each of the lake whitefish populations examined in this study. Dwarf whitefish systematically exhibited larger RBC nuclei, a greater proportion of cathodic haemoglobin protein components and the upregulation of haemoglobin genes in brain tissue, compared with normal whitefish. The strong parallelism observed across multiple independent whitefish populations implies an important role for common ecological factors as drivers of physiological trait variation. The differences we observed between dwarf and normal whitefish could be dependent on both genetically determined and plastic responses to environmental variation, but nonetheless point to a critical role for the integrated coevolution of multiple phenotypic traits in the ability of dwarf and normal whitefish ecotypes to occupy and utilize divergent trophic niches. Thus, this research adds to the growing number of studies that have previously

implicated morphological, life history, physiological, behavioural and gene expression traits in the ecologically driven speciation of dwarf and normal whitefish (Bernatchez 2004; Bernatchez *et al.* 2010). Previous studies have shown that most of these traits are under the control of several genomic regions (Rogers *et al.* 2007; Derome *et al.* 2008; Whiteley *et al.* 2008). Indeed, the process of ecological speciation should be facilitated by an increasing number of traits (and genomic regions) under the effects of divergent selection (Nosil 2008). Altogether this could explain why dwarf and normal lake whitefish species pairs represent one of the most advanced cases of divergence and reproductive isolation along the continuum of ecological speciation in freshwater fishes (Hendry 2009).

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This study is part of M.E.'s postdoctoral research in L.B.'s lab. Both authors are interested in elucidating the traits underlying adaptive divergence. S.P. is interested in fish genetics and aquaculture and K.P.'s research interests cover life history changes and physiological and genetic processes involved in adaptive radiations and speciation.

Data accessibility

Red blood cell measurement, haemoglobin protein component, SNP scoring and haemoglobin gene expression data are uploaded at DRYAD DOI:10.5061/dryad.675tt.

Supporting information

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

Table S1 Putative single nucleotide polymorphisms (SNP) examined in hemoglobin genes of the lake whitefish (*Coregonus clupeaformis*).

Table S2 Genotype frequencies, and observed (H_O) and expected (H_E) heterozygosity found at hemoglobin single nucleotide polymorphism marker 1544-242 in dwarf and normal lake whitefish (*Coregonus clupeaformis*) in four populations in eastern North America.

Table S3 Summary of the 12 different hemoglobin protein banding patterns observed in lake whitefish (*Coregonus clupeaformis*) and their associated cathodic (C1–4) and anodic (A1–4) components.

Table S4 Summary of anodic and cathodic hemoglobin protein component frequencies (proportion of individuals exhibiting a given component) found in dwarf and normal lake whitefish (*Coregonus clupeaformis*) in each of the three lakes examined in eastern North America.

Fig. S1 Examples of banding patterns observed in this study on the IEF gels.

Fig. S2 Oxygen and temperature profiles exhibited by the four lakes examined in this study in June and August 2003.

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