Molecular Analysis, Tissue Profiles, and Seasonal Patterns of Cytosolic and Mitochondrial GPDH in Freeze-Resistant Rainbow Smelt (*Osmerus mordax*)

Jason L. Robinson[1]
Jennifer R. Hall[1]
Mark Charman[2]
K. Vanya Ewart[2]
William R. Driedzic[1,*]
[1]Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland A1C 5S7, Canada; [2]National Research Council Institute for Marine Biosciences, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada

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ABSTRACT

Rainbow smelt (*Osmerus mordax*) is an anadromous teleost that, beginning in late fall, accumulates plasma glycerol in excess of 200 mM, which subsequently decreases in the spring. The activity of cytosolic glycerol-3-phosphate dehydrogenase (cGPDH) is higher (*i*) in liver of smelt than in that of Atlantic salmon and capelin (nonglycerol accumulators), (*ii*) in liver of smelt maintained at 1°C than in that of smelt held at 8°–10°C, and (*iii*) in smelt liver than in smelt muscle, heart, brain, or kidney. In addition, transcript levels of cGPDH in liver peak in December during the onset of glycerol production and then decline over the remainder of the season. There are four cGPDH protein isoforms in smelt liver that are present regardless of glycerol production status. A minimum of four cGPDH gene copies identified by Southern blotting provide adequate genetic potential to yield multiple protein isoforms. A full-length cDNA for smelt mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) was cloned and characterized. The 2,790-bp cDNA contains a 109-bp 5′UTR, a 2,193-bp open reading frame, and a 488-bp 3′UTR; transcripts are ubiquitously expressed in both warm- and cold-acclimated smelt tissues. Smelt mGPDH encodes a 730-aa protein that clusters with that of zebrafish and frog and contains several common structural motifs. mGPDH transcript levels generally increase late in the seasonal glycerol cycle, and mGPDH enzyme activity increases significantly during the glycerol decrease phase. Taken together, these findings suggest that liver cGPDH and mGPDH play a key role in the glycerol accumulation and decrease phases, respectively.

* Corresponding author; e-mail: wdriedzic@mun.ca.

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Introduction

Rainbow smelt (*Osmerus mordax*), hereafter referred to as smelt, is an anadromous teleost that accumulates plasma glycerol to levels typically in excess of 200 mM in response to cold temperatures (Raymond 1992; Treberg et al. 2002*b*; Lewis et al. 2004). Glycerol acts colligatively to lower the point of freezing and allows smelt to remain active when water temperatures are well below the typical freezing point of fish plasma. For smelt, being active at subzero temperatures is advantageous because it allows access to an invertebrate food source without competition from non-antifreeze-producing fish species. The trade-off is that smelt must continue to fuel glycerol production through prey capture at low temperatures (Driedzic and Ewart 2004).

Glycerol production begins in the fall as temperatures drop to 3°–5°C (Lewis et al. 2004). Initial glycerol accumulation is fueled through the mobilization of glycogen (Driedzic and Short 2007); however, in order to sustain the carbon demand for glycerol production, smelt must feed constantly. Tracer studies show that glycerol is predominantly derived from exogenous amino acids and glucose (Raymond 1995; Raymond and Driedzic 1997; Walter et al. 2006). Amino acids are metabolized to glycerol by glyceroneogenesis, which is a truncated gluconeogenic pathway resulting in the accumulation of glycerol rather than glucose. Essential to glycerol synthesis is the flux of dihydroxyacetone phosphate (DHAP) into glycerol-3-phosphate (G3P) via the cytosolic form of glycerol-3-phosphate dehydrogenase (cGPDH).

The activity of cGPDH plays an important role in glycerol accumulation. cGPDH activity in liver from smelt living at cold temperature is higher than that in non-glycerol-producing species (Driedzic et al. 1998; Treberg et al. 2002*a*). In smelt, liver cGPDH activity increases in association with decreases in water temperature and elevated glycerol accumulation (Lewis et al. 2004; Driedzic et al. 2006). To date, all studies of cGPDH enzyme activity in smelt have focused on liver, but glycerol accumulates in all tissues at low temperature (Driedzic and Short 2007). Therefore, the possibility remains that other tissues have the capacity for glycerol synthesis. Here, we report cGPDH

activity in various tissues in addition to liver from smelt and two non-glycerol-producing species that are closely related to smelt—capelin (*Mallotus villosus*) and Atlantic salmon (*Salmo salar*)—to assess the potential to produce high levels of glycerol. Statements regarding enzyme activity above are based on total maximal cGPDH activity measured in tissue homogenates; however, in numerous fish, electrophoretic activity patterns reveal two prominent isoforms in muscle and liver (Phillipp et al. 1979; Van der Bank et al. 1989). In black bullhead (*Ictalurus melas*), there are four isoforms in liver and two in muscle (Basaglia and Cucchi 1995). In this study, the number of and the impact of temperature on cGPDH isoforms in smelt liver and muscle were measured to determine whether smelt as a glycerol producer is unique at this locus. To further our understanding of the basis of multiple cGPDH isoforms, we assessed gene copy number.

Consistent with the pattern for cGPDH enzyme activity is the observation that liver cGPDH transcript levels increase several days before the onset of seasonal glycerol accumulation in plasma (Liebscher et al. 2006). It is unclear whether this is a response to declining water temperature per se or to some other factor such as photoperiod. To address this question, we measured cGPDH transcript levels in smelt tracking ambient water temperature, as well as in smelt maintained at an elevated temperature.

Smelt maintain glycerol at a high level until early spring, when levels begin to fall. This sharp and predictable glycerol decrease occurs while water temperature is still low, and thus, it is not a function of warming water. Whole-body glycerol is constantly lost through the skin and gills of smelt at a rate of about 10% glycerol per day (Raymond 1993; Ditlecadet et al. 2011). In concert with the decrease in plasma glycerol that occurs in spring is an increase in liver glycogen content (Driedzic and Short 2007). This presents the possibility that a portion of the glycerol is retained and directed toward gluconeogenesis. Glycerol catabolism must first occur through glycerol kinase, which phosphorylates glycerol to G3P. The activity of glycerol kinase increases in smelt liver late in the glycerol cycle, when plasma glycerol is returning to low levels (Ditlecadet et al. 2011). The G3P produced by the activity of this enzyme can then be channeled into lipid biosynthesis or flux back to DHAP through cGPDH or mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH). mGPDH is an irreversible bisubstrate enzyme located on the outer face of the inner mitochondrial membrane (Klingenberg 1970). mGPDH reduces FAD to $FADH_2$ within the matrix, thus yielding reducing equivalents for ATP production during the metabolism of glycerol. In rat liver, increased capacity for conversion of extracellular glycerol into glycogen (induced by dietary manipulation) is associated with increases in mGPDH maximal activity (Piquet et al. 1996). There are currently no data concerning mGPDH in smelt, at either the transcript level or the enzyme level.

This study better describes smelt cGPDH biochemistry through tissue and species distribution assessment of enzyme activity, electrophoretic analysis of cGPDH protein isozymes, and quantification of liver gene copy number. Furthermore, a full-length cDNA for smelt mGPDH was cloned and sequenced, its transcript tissue distribution pattern determined, and its deduced amino acid sequence characterized by phylogenetic and functional domain analyses. Finally, a seasonal study was performed (encompassing the glycerol production and termination phases) in which smelt cGPDH and mGPDH transcript levels and mGPDH activity were measured in liver.

Material and Methods

Study Overview

A series of experiments related to cGPDH and mGPDH were conducted. In series 1, the maximal in vitro activity of cGPDH in liver, skeletal muscle, heart, brain, and kidney in smelt, Atlantic salmon, and capelin was determined. Smelt and Atlantic salmon were maintained at warm and cold temperatures; capelin were sampled from the field. In series 2, the pattern of cGPDH isoforms was determined in smelt liver and muscle in fish held at warm and cold temperature. Given that there were multiple isoforms, this was complemented with a determination of gene copy number in series 3 studies. In series 4, liver cGPDH transcript levels were determined in smelt maintained at warm temperature and in fish tracking ambient temperatures during the fall to spring period. The second major part of the study relates to mGPDH. In series 5, mGPDH was cloned and characterized. Finally in series 6, liver mGPDH transcript and activity levels were assessed as above for cGPDH transcript levels.

Animals

Smelt were captured by seine netting from Mount Arlington Heights, Placentia Bay, Newfoundland, Canada, in late October to mid-November in each of 2006, 2007, and 2008 and transported to the Ocean Sciences Centre, Memorial University of Newfoundland. The one exception to the above was a specimen used for genomic DNA analysis that was captured in the fall of 2002 and assayed in the winter of 2003. Fish were maintained in seawater, on a natural photoperiod with fluorescent lights set on an outdoor photocell, and were fed chopped herring twice a week to satiation. On arrival at the laboratory, smelt were randomly sorted into tanks that received either seawater heated to 8°–10°C for the duration of the experiments or water that tracked ambient temperature, reaching approximately −1°C in February. Fish held at 8°–10°C are hereafter referred to as "warm-acclimated fish," while fish that were held in ambient water are referred to as "cold-acclimated fish." A typical temperature profile is presented by Lewis et al. (2004). Fish used in experiments to determine maximal cGPDH activity were collected in November 2006 and sampled on March 29 and April 13, 2007. Because there was no statistically significant difference between enzyme activities determined at the two sample dates, values were pooled for further analysis. In experiments involving isozyme number, the fish were collected in November 2007 and sampled on January 14, February 18, and March 27, 2008. Fish used in the tissue distribution of

mGPDH transcripts were collected in 2009 and sampled on March 5, 2010. One fish was held at warm temperature. A second fish was sampled from a population that was subjected to a controlled temperature decrease from 8° to 5 °C on February 12, to 3°C on February 15, to 1°C on February 22 and held at 1°C until sampled. Fish used in the measurement of seasonal cGPDH and mGPDH transcript levels and mGPDH enzyme activity were collected in November 2008 and sampled approximately every 30 d thereafter. Fish held at cold temperature were available until May 2009. Fish held at elevated temperature, in common with earlier studies, become gravid and typically could not be maintained past mid-April. Average body mass of smelt was 55.3 ± 1.1 g.

Atlantic salmon (*Salmo salar*), hereafter referred to as salmon, were purchased from Cooke Aquaculture, Daniel's Harbour, Newfoundland, Canada; maintained at the Ocean Sciences Centre; and fed commercially available 3.5-mm pellets (EWOS, Surrey, British Columbia). On January 7, 2008, fish were placed in either seawater set to ~10°C (i.e., warm tank) or in seawater set to 5°C (cold tank). Water temperature in the cold tank was further decreased to 3°C on January 16, 2008, and finally to 1°C on January 18, 2008. Fish were sampled on January 28 and February 29, 2008, for assessment of maximal cGPDH activity. Because there was no statistically significant difference between enzyme activities determined at the two sample dates, values were pooled for further analysis. Average body mass was 170 ± 13 and 223 ± 12 g for salmon held at warm and cold temperature, respectively.

Capelin (*Mallotus villosus*) were captured from Middle Cove, Newfoundland, by seine netting on July 5, 2007, and placed in a holding tank at ambient temperature (8°C). Capelin were sampled on July 6, 2007, for assessment of maximal cGPDH activity. Average body mass was 37 ± 1.4 g.

In all cases, blood was extracted via caudal puncture, using heparanized syringes. Fish were killed with a sharp blow to the head, and tissues were harvested immediately, frozen in liquid N_2, and stored at −80°C for future analysis. The treatment of fish and sampling protocols were according to guidelines of the Canadian Council on Animal Care.

Glycerol and Protein Concentration

Blood was centrifuged for 5 min at 5000 *g*, and plasma was removed and stored at −80°C. Plasma glycerol concentrations were determined using a diagnostic kit (33740A, Sigma-Aldrich, Oakville, Ontario), following the manufacturer's instructions. Protein concentrations in mitochondrial suspensions were measured using a commercially available version of the Bradford method (Coomassie Plus Bradford Protein Assay, Thermo Scientific, Rockford, IL).

cGPDH Activity Tissue Distribution

The maximal activity of cGPDH was measured using a method adapted from Joanisse and Storey (1994). Samples of heart, brain, kidney, liver, and white muscle from smelt, salmon, and capelin were analyzed. Tissues were weighed and homogenized in nine volumes of ice-cold extraction buffer (20 mM imidazole, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 0.1 mM PMSF, 5 mM DTT, pH 7.4). Tissue homogenates were assayed in reaction medium containing 20 mM imidazole, 1.5 mM NADH, pH 7.2. The assay was initiated with 1.87 mM DHAP, and the change in absorbance at 340 nm was measured at 20°C. Data are expressed as micromoles per minute gram tissue, based on a millimolar extinction coefficient of 6.22. This assay will not detect mGPDH, which requires FAD^+ as a cofactor.

cGPDH Isozyme Separation

cGPDH was partially purified from smelt liver and muscle. Tissues were homogenized in nine volumes of extraction buffer and centrifuged for 2 min at 2,000 *g* to remove cellular debris. Fifty-millimolar ammonium sulfate was added to the supernatant, samples were centrifuged at 4°C for 10 min at 10,000 *g*, and the supernatant was desalted in a 10-DG desalting column (Bio-Gel P-6 Desalting Gel, Bio-Rad, Mississauga, Ontario). Bound proteins in the 10-DG column were eluted, and the buffer was exchanged with 50 mM K_2PO_4. Affi-gel blue gel (Bio-Rad) columns were manually packed with ~5 mL of blue gel per column. Approximately 20 mg of desalted protein was added to blue gel columns and washed with two bed volumes of 50 mM K_2PO_4. Bound fractions were competitively eluted using 5 mL buffer (10 mM NADH, 1 M NH_4Cl , pH 7.4, 4°C). Protein concentration was measured, and cGPDH activity was verified.

Enzyme activity of cGPDH isozymes was visualized using cellulose-acetate gel electrophoresis, using the method of Rothe (1994). Samples were added to Titan III cellulose acetate gels and placed in a Titan Gel horizontal chamber (Helena Laboratories, Beaumont, TX) for 45 min at 0.98 mA, 116 V. Gels were immersed in reaction medium (100 mg G3P, 200 mg pyruvic acid, 20 mg NAD^+, 1 mL 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, 5 mg/mL phenazine methosulfate, 1% agarose, TRIS-HCl, 45°C, pH 7.4) and incubated in the dark at 37°C for 45 min. The reaction was stopped with 10% acetic acid, and gels were briefly washed in water and imaged using a Syngene G:Box imager (Syngene, Frederick, MD).

cGPDH Gene Copy Number

Genomic DNA was obtained from smelt liver. The DNA was isolated using a kit based on proteinase K treatment (Gentra, QIAGEN, Mississauga, Ontario). Restriction digests were performed to completion at 37°C on genomic DNA, using enzymes *Eco*RI, *Bam*HI, *Xba*I, and *Pst*I (Fermentas, Burlington, Ontario). Digestion products (20 μg per lane) were electrophoretically separated on a 0.7% agarose gel in 1 × TBE buffer. The DNA was transferred to a positively charged nylon membrane (Roche Applied Science, Laval, Quebec) by downward capillary transfer under alkaline conditions.

The probe sequence was selected to amplify a stretch of genomic DNA encoding a single exon, as predicted by align-

ment of the smelt cGPDH cDNA (GenBank accession no. AY024368) with the Gpd1 genes of fugu (*Fugu rubripes*) and zebrafish (*Danio rerio*) and with no restriction sites for the above enzymes. The probe was a 169-bp polymerase chain reaction (PCR)–amplified fragment obtained using primers presented in Table 1 and the full-length cGPDH cDNA cloned previously (Ewart et al. 2001) as template. The PCR product was gel purified using the QIAquick Gel Extraction Kit (QIAGEN) with the manufacturer's instructions. It was then subcloned into a TOPO TA vector (Invitrogen, Burlington, Ontario) with the manufacturer's instructions, and individual clones were sequenced. Plasmid DNA containing a correct clone was digested with *Eco*RI to excise the fragment, which was then gel purified using the QIAquick Gel Extraction Kit with the manufacturer's instructions. The isolated fragment (400 ng) was labeled using the AlkPhos Direct labeling kit (GE Healthcare Life Sciences, Baie d'Urfe, Quebec), and CDP-Star chemiluminescent detection was used as described by the manufacturer.

The hybridization employed solutions and protocols supplied by the label manufacturer (AlkPhos Direct). Posthybridization stringency washes were performed as recommended by the label manufacturer, except that the temperature was elevated to 60°C. The membrane was exposed to autoradiographic film (Hyperfilm ECL, GE Healthcare Life Sciences), which was scanned, and the images were captured and analyzed using a Gel Doc system (Bio-Rad).

mGPDH Cloning and Gene expression

RNA preparation. Total RNA was extracted from smelt liver, using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. RNA integrity was verified by 1% agarose gel electrophoresis, and purity was assessed by A260/280 and A260/230 UV spectrophotometry. For quantitative reverse transcription PCR (QPCR), total RNA was treated with TURBO DNA-free (Ambion, Austin, TX). Briefly, 10 μg of total RNA was incubated in a 50-μL final volume with two units of Turbo DNase for 30 min at 37°C; an additional two units were added, and the incubation continued for an additional 30 min. DNase Inactivation Reagent (10 μL) was added to the reaction, incubated at room temperature for 5 min, and centrifuged at 10,000 *g* for 1.5 min. The supernatant containing the RNA was transferred to a fresh tube.

mGPDH cDNA Cloning

The full-length cDNA for smelt mGPDH was cloned using reverse transcription (RT-PCR) and RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (RLM-RACE). The sequences of all primers used in cDNA cloning and their application are presented in Table 1.

A partial cDNA for mGPDH was amplified using RT-PCR. Degenerate primers were designed on the basis of consensus sequences from conserved areas of aligned vertebrate cDNAs. DNase I–treated liver total RNA (1 μg) was reverse transcribed in a 20-μL reaction, using random primers (250 ng; Invitrogen) and M-MLV reverse transcriptase (200 units; Invitrogen) with the manufacturer's first strand buffer (1 × final concentration) and DTT (10 mM final concentration) at 37°C for 50 min. PCR amplification was performed using DyNAzyme EXT (MJ Research, Waltham, MA). Briefly, 50-μL reactions were prepared containing 2 μL of cDNA (corresponding to 100 ng of input total RNA), DyNAzyme EXT DNA polymerase (one unit), the manufacturer's Optimized DyNAzyme EXT Buffer (1 × final concentration), 0.2 mM dNTPs, and 0.2 μM each

Table 1: Sequences of oligonucleotides used in cGPDH gene copy number probe PCR, mGPDH cDNA cloning, and QPCR

cDNA	Nucleotide Sequence (5′–3′)[a]	Direction[b]	Application	Position of 5′ End in cDNA
cGPDH	GGTTTCTGTGACGGTCTTGG	F	Gene copy no. probe	677
cGPDH	CGTAGCAGGTTGTGATGAGG	R	Gene copy no. probe	845
mGPDH	CYTGCCCTGGGAGAAGATGAC	F	Degenerate	1,148
mGPDH	CCGCTGTC**G**CTGACGTTGAC	R	Degenerate	1,401
mGPDH	GGTTCCTGCAGATGGACTGCGT	R	5′ RACE	1,376
mGPDH	CGTATTCCACTCCACGCTGCCA	R	5′ RACE (nested)	1,323
mGPDH	GACATCAACTTCATCCTGACGGA	F	3′ RACE	1,229
mGPDH	GGCAGCGTGGAGTGGAATACG	F	3′ RACE (nested)	1,303
mGPDH	TGGCCAAGATGGCACATGT	F	QPCR	1,641
mGPDH	CATGGCATACAACACCTCGCT	R	QPCR	1,741
cGPDH	GAGGCGTTCGCTAAAACAGGA	F	QPCR	869
cGPDH	ATGGTGGACTTCTGATGCCGT	R	QPCR	970

Note. cGPDH, cytosolic glycerol-3-phosphate dehydrogenase; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase; PCR, polymerase chain reaction; QPCR, quantitative reverse transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends.

[a]Nucleotide highlighted in bold differs from the actual cDNA sequence.

[b]F, forward; R, reverse.

of forward and reverse primers. Touchdown PCR was used with 40 cycles of 94°C for 30 s, 65°C decreasing by 0.5°C per cycle (to 45.5°C at cycle 40) for 30 s, and 72°C for 30 s. The PCR product was electrophoresed on a 1% agarose gel, excised, and purified using the QIAquick Gel Extraction Kit with the manufacturer's instructions. It was then subcloned into pGEM-T Easy (Promega, Madison, WI) with the manufacturer's instructions and triplicate clones sequenced on both strands at the McMaster Institute for Molecular Biology and Biotechnology (McMaster University, Ontario).

The 5′ and 3′ ends of the partial mGPDH cDNA were cloned using a commercial kit for RLM-RACE (GeneRacer Kit, Invitrogen), using total RNA from liver. PCR amplification was performed at 94°C for 30 s, 70°C decreasing by 0.3°C per cycle (to 58.3°C at cycle 40) for 30 s, and 72°C for 2 min for 40 cycles, using DyNAzyme EXT (MJ Research), with core reaction component concentrations as described for RT-PCR.

Sequence Analysis of mGPDH

Sequence data were compiled and analyzed using Vector NTI (Vector NTI Advance 10, Invitrogen). Alignments were performed using AlignX (Vector NTI Advance 10), which uses the CLUSTAL W algorithm (Thompson et al. 1994). For phylogenetic and molecular evolutionary analyses, alignments were imported in MSF format into MEGA, version 4.0.2 (Tamura et al. 2007). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) with Poisson correction. Bootstrap analysis was performed with 1,000 replicates.

QPCR Analysis of c- and mGPDH Transcripts

mRNA levels of the target genes (cGPDH, mGPDH) were quantified by QPCR, using SYBR Green I dye chemistry. Levels were normalized to 18S ribosomal RNA, quantified using a commercially available TaqMan assay. QPCR assays were performed using the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). The sequences of the QPCR primer pairs are presented in Table 1. The cDNA for smelt cGPDH had been previously cloned (GenBank accession no. AY024368), and primers were derived from this sequence. These primers were quality tested to ensure that a single product was amplified (dissociation curve analysis) and that there was no primer dimer present in the no-template control. Amplicons were electrophoresed on 2% agarose gels and compared with a 1-kb-plus ladder (Invitrogen) to ensure that the correct size fragment was being amplified. Finally, amplification efficiencies (Pfaffl 2001) were calculated and were required to be between 90% and 110%. Briefly, amplification efficiencies were calculated for both warm-acclimated fish and cold-acclimated fish. A five-point 1 : 5 dilution series starting with 10 ng of cDNA was analyzed for both, and the reported efficiencies are an average of the two values. cGPDH primers generated a 102-bp amplicon with an efficiency of 104%, and the mGPDH primers generated a 101-bp amplicon with an efficiency of 97%. This dilution series was also performed for the 18S ribosomal RNA TaqMan assay to ensure that 18S levels were consistent between the cold-acclimated fish and the warm-acclimated fish and hence that it was an acceptable normalizer.

First-strand cDNA was synthesized from 1 μg of DNaseI-treated total RNA, using random primers and M-MLV reverse transcriptase (Invitrogen) as described for mGPDH cloning. PCR amplification of the target genes was performed in a 25-μL reaction, using either 10 ng (cGPDH) of cDNA (corresponding to 10 ng of input total RNA) or 50 ng (mGPDH) of cDNA (corresponding to 50 ng of input total RNA), 50 nM of each forward and reverse primer, and 1 × Power SYBR Green PCR Master Mix (Applied Biosystems). Expression levels of the target genes were normalized to 18S ribosomal RNA, using the Eukaryotic 18S rRNA Endogenous Control (VIC/MGB probe, Primer Limited, Applied Biosystems). PCR amplification of 18S was performed in a separate 25-μL reaction, using 0.4 ng of cDNA (corresponding to 0.4 ng of input total RNA), 1 × probe/primer mix, and 1 × TaqMan Universal PCR Master Mix, with AmpErase UNG (Applied Biosystems). The real-time analysis program consisted of one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. On each plate, for every sample, the target gene and endogenous control were tested in duplicate (Nolan et al. 2006). The fluorescence threshold cycle (C_T) was determined using the 7300 PCR Detection System SDS Relative Quantification Study Application software (ver. 1.2.3; Applied Biosystems). The relative starting quantity (RQ) of each transcript was determined with this software, using the $2^{-\Delta\Delta CT}$ relative quantification method and assuming 100% amplification efficiencies (Livak and Schmittgen 2001). For each target gene, the individual or tissue with the lowest normalized expression (mRNA) level was set as the calibrator sample (assigned a value = 1). Gene expression data are presented as mean ($\pm$SE) RQ relative to the calibrator.

Hepatic Seasonal mGPDH Activity

Mitochondria were extracted from smelt liver, using a method adapted from Ballantyne (1994). Briefly, whole livers were ground with a pestle in nine times the volume of ice-cold incubation media (500 mM sucrose, 30 mM Hepes, 5 mM EDTA, 5 mM EGTA, pH 7.4). The homogenate was passed through cheesecloth and centrifuged for 10 min at 800 *g*. The supernatant was again passed through cheesecloth and centrifuged at 9,000 *g* for 10 min to sediment the mitochondria. The supernatant was discarded, and the mitochondrial pellets were resuspended and centrifuged at 9,500 *g* for 10 min in resuspension media (300 mM KCl, 30 mM Hepes, 10 mM K_2HPO_4, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4). The mitochondrial pellet was taken up once again in resuspension media. Measurement of maximal mGPDH activity (i.e., FAD-linked activity) was adapted from Gardner (1974). Briefly, 250 μL mitochondrial suspension was added to 250 μL iodoformazan buffer (4 mM iodoformazan, 1 mM KCN, 400 mM K_2PO_4, 0.1 mM FAD^+, 1 μM antimycin A, pH 7.4) in a 2-mL reaction

tube. The reaction was initiated with 50 μL of 40 mM G3P and took place in the dark for 30 min at room temperature. The reaction was terminated by adding 100 μL acetic acid and 1 mL ethyl acetate. Reaction tubes were vortexed for 15 s to separate the organic and aqueous phases. Absorbance of the upper organic phase was measured at 490 nm. Data are expressed as micromoles per minute milligram mitochondrial protein, on the basis of a millimolar extinction coefficient of 18.5 (Gardner 1974). This assay will not detect cGPDH, which requires NAD^+ as a cofactor.

Data Analysis

Values are presented as mean ± SEM. Within groups of samples, the values obtained at the various sampling dates were compared by one-way ANOVA, followed by Tukey's HSD post hoc tests. Independent-sample *t*-tests were performed to compare warm- and cold-acclimated fish at each sampling date. In all cases, $P < 0.05$ was considered statistically significant.

Results

cGPDH Enzyme Activity

Plasma glycerol concentrations for fish used in the cGPDH maximal enzyme activity study are shown in Figure 1*A*. Glycerol concentrations were more than 100-fold greater in cold-acclimated smelt than in warm-acclimated smelt, capelin, or salmon (at either temperature).

Maximal in vitro cGPDH activity was determined in multiple species, tissues, and temperature conditions. The activity of cGPDH in liver from cold-acclimated smelt approached 200 μmol/min g tissue and was significantly higher than the level measured in liver from warm-acclimated smelt (107 μmol/min g tissue; Fig. 1*B*). The cGPDH activities in liver of smelt were significantly higher than levels in capelin and salmon liver, which approached only 25 and 10 μmol/min g tissue, respectively.

cGPDH activity in white muscle from cold-acclimated smelt was significantly higher than that in white muscle from warm-acclimated smelt (11.99 ± 1.00 vs. 6.75 ± 0.95 μmol/min g tissue) but significantly lower than that found in white muscle from warm- or cold-acclimated salmon (19.83 ± 1.26 and 21.12 ± 1.77 μmol/min g tissue, respectively; Fig. 1*C*). Enzyme activity in salmon muscle was significantly higher than that in salmon liver. Capelin muscle cGPDH activity was the lowest of all species and conditions, at 3.42 ± 0.41 μmol/min g tissue.

Heart, brain, and kidney cGPDH activities are shown in Table 2. Activities in these tissues were substantially lower than those observed in liver within the same species. cGPDH activity in heart and brain were generally similar across the species sampled or across the acclimation temperature within species. Exceptions to this were a significantly higher activity in brain from warm-acclimated salmon versus cold-acclimated salmon and higher activity in heart from capelin than in that from cold-acclimated salmon. The cGPDH activity in the kidney of salmon was significantly lower than that of the other species,

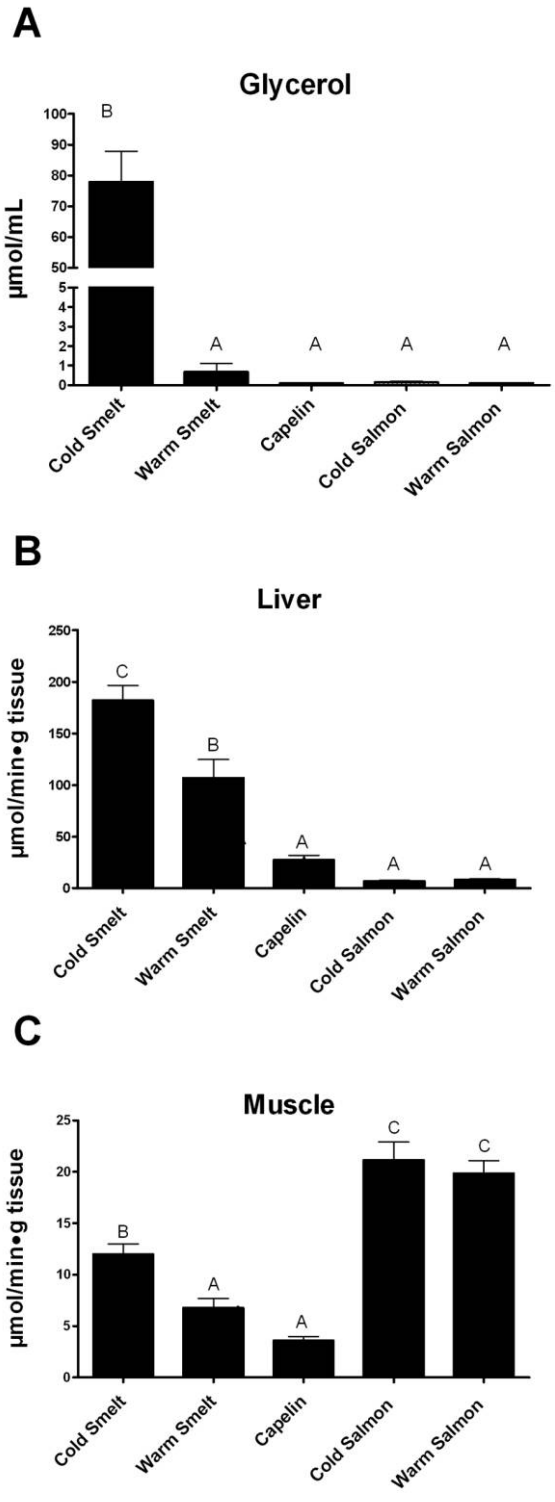

Figure 1. Plasma glycerol levels (*A*) and tissue cytosolic glycerol-3-phosphate dehydrogenase (cGPDH) activity (*B*, *C*) of cold-acclimated (1°C) and warm-acclimated (8°–10°C) smelt, cold-acclimated (1°C) and warm-acclimated (10°C) salmon, and capelin (8°C). Different letters above bars indicate significant differences between groups. Values are mean ± SEM. For smelt, $N = 14$; for capelin, $N = 8$; and for salmon, $N = 12$.

Table 2: cGPDH activity for heart, brain, and kidney of cold- and warm-acclimated smelt, cold- and warm-acclimated salmon, and capelin

Fish	Heart	Brain	Kidney
Cold-acclimated rainbow smelt	2.97 ± .37 (14)^{AB}	.94 ± .14 (14)^{AB}	7.09 ± .75 (14)^{B}
Warm-acclimated rainbow smelt	2.19 ± .41 (14)^{AB}	.78 ± .09 (14)^{AB}	6.14 ± .99 (14)^{B}
Capelin	2.03 ± .22 (8)^{B}	1.31 ± .10 (8)^{AB}	6.66 ± .62 (8)^{B}
Cold-acclimated Atlantic salmon	1.07 ± .19 (12)^{A}	.51 ± .07 (12)^{A}	.77 ± .11 (11)^{A}
Warm-acclimated Atlantic salmon	2.21 ± .52 (12)^{AB}	1.50 ± .35 (12)^{B}	1.89 ± .58 (12)^{A}

Note. Activity is expressed as micromoles per minute gram tissue. Values are means ± SEM, with the *N* value in parentheses. Values not sharing a common letter indicate a significant difference ($P < 0.05$) within a tissue. cGPDH, cytosolic glycerol-3-phosphate dehydrogenase.

regardless of acclimation temperature. Within species, there was no difference in enzyme activity in kidney as a function of temperature.

cGPDH Isozymes

Staining patterns following electrophoresis showing cGPDH activity (Fig. A1 in the online edition of *Physiological and Biochemical Zoology*) were similar to those presented for black bullhead (Basaglia and Cucchi 1993, 1995). In both liver and muscle, there was one major band that had high mobility in the electric field and a qualitatively weaker but still quite prominent band that had little mobility. Liver showed two additional relatively faint bands of intermediate mobility. These bands were never apparent in muscle samples. The number and presence of smelt cGPDH isozymes were independent of acclimation temperature at this level of qualitative resolution.

Gene Copy Number

Southern blotting analysis suggests the presence of at least four copies of the cGPDH gene in the smelt genome, as shown in the *Xba*I- and *Pst*I-digested DNA (Fig. 2). Only two bands were detected in the *Eco*RI and *Bam*HI digests, and three bands were visible in the *Xho*I digest, suggesting that gene copies are closely linked. Although no genomic sequence is available for the cGPDH gene of smelt, the 169-bp probe sequence used corresponds to a single exon in the cGPDH genes of two fish species and should therefore also fall within a single exon in smelt. Furthermore, none of the restriction enzymes used in this analysis cleaves within this sequence. Therefore, a single gene copy should not give rise to more than one band. Conversely, one band can contain two tandem gene copies (or more) if there is no restriction site for the enzyme used to digest the DNA between the copies. Consequently, each band on the blot should represent a minimum of one and possibly more complete copies of the cGPDH gene.

Cloning and Characterization of Smelt mGPDH

A full-length cDNA for smelt mGPDH was cloned using a combination of RT-PCR and RLM-RACE, and the sequence was deposited in GenBank (accession no. FJ797643). mGPDH is a 2,790-bp cDNA that contains a 109-bp 5′UTR, a 2,193-bp open reading frame, and a 488-bp 3′UTR. It encodes a 730-aa protein that has a predicted molecular mass of 81.1 kDa and an isoelectric point of 6.24.

A BLASTx 2.2.20 search of the nonredundant protein sequences database that used a translated mGPDH cDNA sequence was performed to compare the relatedness of smelt

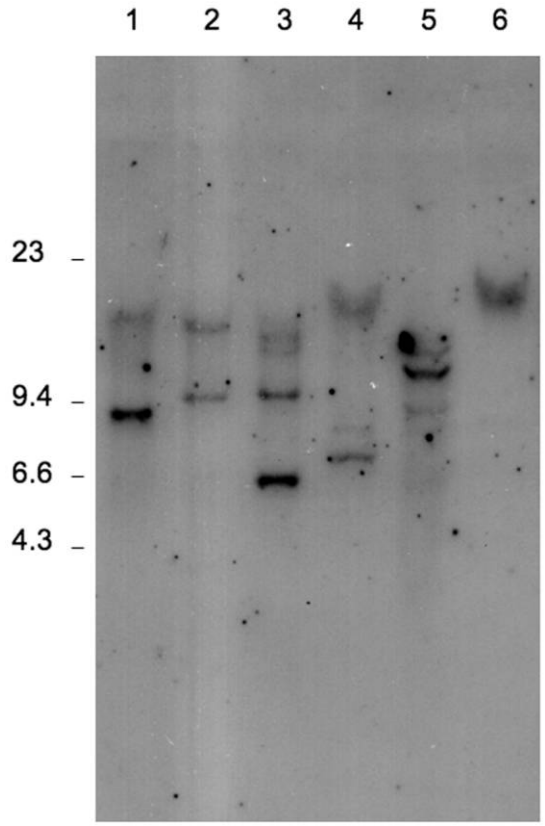

Figure 2. Southern blot analysis of smelt cytosolic glycerol-3-phosphate dehydrogenase (cGPDH). Genomic DNA was separated on a 0.7% agarose gel after digestion with *Eco*RI (lane 1), *Bam*H1 (lane 2), *Xba*I (lane 3), *Xho*I (lane 4), and *Pst*I (lane 5) or without digestion (lane 6) and then blotted and probed as described in "Material and Methods." Molecular size markers (kb) are indicated on the left of the blot.

mGPDH to protein sequences from other species. At the amino acid level, smelt mGPDH had the highest sequence identity with that of zebrafish. The zebrafish protein was truncated (536 aa); however, there was 86% identity over that alignment length. Amino acid identities ranged from 72% (dog) to 79% (frog) for other vertebrates and from 64% to 57% for the vase tunicate and mosquito, respectively. A multiple alignment of 18 mGPDH protein sequences (16 vertebrates, two invertebrates), identified by BLASTx analysis, was performed, and a phylogenetic tree was constructed (Fig. 3). Smelt mGPDH clusters with that of zebrafish and frog and concurs with percentage amino acid identities.

mGPDH proteins are characterized by the presence of several structural motifs. They contain a mitochondrial leader peptide, an FAD-binding domain, a G3P-binding domain, and a region near the carboxyl terminus that contains a pair of sequences homologous to EF-hand Ca^{2+}-binding domains, the second of which has features consistent with high Ca^{2+} affinity. EF-hands are around 29 amino acids in length and form a helix-loop-helix structure in which the Ca^{2+} ion is coordinated by oxygen atoms from six amino acids in the loop. They typically occur in groups of two or four, with pairings of high- and low-affinity Ca^{2+}-binding domains being commonplace (Brown et al. 1994; Koza et al. 1996; Matsutani et al. 1996). An alignment of the amino acid sequence of mGPDH from smelt, human, rat, and frog showing these structural features is presented in Figure 4. Amino acid sequences pertaining to the mitochondrial leader peptide, the FAD-binding domain, and the G3P-binding domain were derived from Brown et al. (1994), Koza et al. (1996), and Matsutani et al. (1996). The two EF-hand Ca^{2+}-binding domains, with their eight Ca^{2+}-binding residues, were identified using the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The mGPDH protein from zebrafish was not included in this alignment because it is truncated and therefore does not contain the EF-hand calcium-binding domains.

Tissue Distribution of Smelt mGPDH

mGPDH transcript levels from tissues of one warm-acclimated smelt and one cold-acclimated smelt were analyzed by QPCR (Fig. A2 in the online edition of *Physiological and Biochemical Zoology*). The calibrator sample (the sample with the lowest normalized expression level) was the warm-acclimated smelt

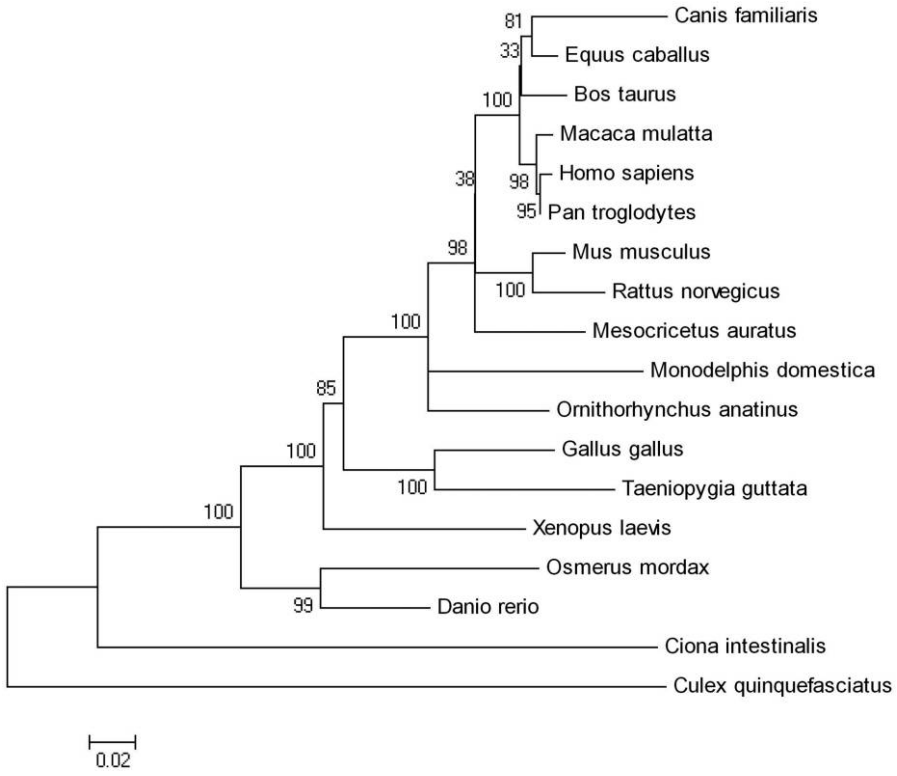

Figure 3. Phylogenetic analysis of smelt mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH). A phylogenetic tree (bootstrap) of protein sequences for mGPDH from a variety of species. Protein accession numbers are as follows: *Osmerus mordax* (ACO34845), *Danio rerio* (AAI65374), *Homo sapiens* (AAB60403), *Mus musculus* (AAH21359), *Rattus norvegicus* (AAB60443), *Mesocricetus auratus* (CAO79918), *Bos taurus* (AAI48086), *Xenopus laevis* (AAH73694), *Gallus gallus* (XP_422168), *Macaca mulatta* (XP_001086947), *Ornithorhynchus anatinus* (XP_001509091), *Equus caballus* (XP_001490923), *Monodelphis domestica* (XP_001365971), *Taeniopygia guttata* (XP_002187076), *Pan troglodytes* (XP_001142893), *Canis familiaris* (XP_848389), *Ciona intestinalis* (XP_002123583), and *Culex quinquefasciatus* (XP_001845276). The tree was constructed as described in the text. The scale bar represents the number of substitutions per amino acid site.

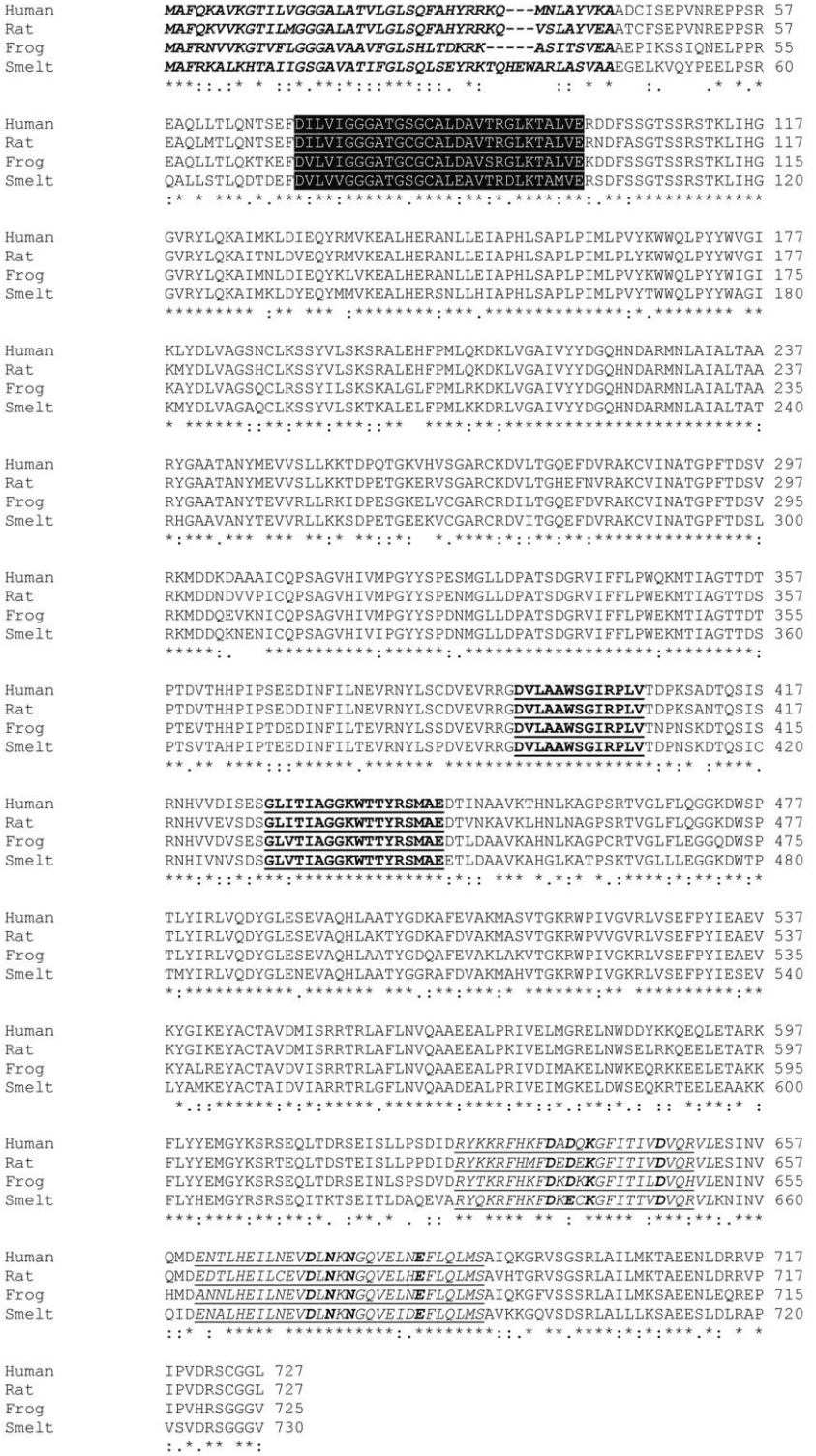

Figure 4. Amino acid alignment of mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) from human (AAB60403), rat (AAB60443), frog (AAH73694), and smelt (ACO34845). Amino acids are represented by the single-letter code. Dashes are inserted where gaps are present in the amino acid sequences. Asterisks indicate residues that are identical among all four sequences shown, whereas colons indicate residues that are similar. Residues highlighted with bold italic characters form the mitochondrial leader peptide; those highlighted with a black background form the FAD-binding domain; those highlighted with bold underlined characters form the glycerol-3-phosphate binding domain; and those highlighted with underlined italic characters form the two EF-hands, with their eight calcium-binding residues highlighted in bold underlined italic characters.

liver. mGPDH is ubiquitously expressed in both warm- and cold-acclimated smelt. Transcript levels were highest in red blood cells, gill, spleen, and kidney in the warm-acclimated smelt and in gill, kidney, liver, and spleen in the cold-acclimated smelt. With the exception of the red blood cells and gonad, mGPDH transcript levels were higher in the cold-acclimated smelt than in the warm-acclimated smelt. However, these results are based on the comparison of two fish and should be viewed with caution.

Seasonal Glycerol, cGPDH, and mGPDH Transcript Levels and mGPDH Activity

The seasonal plasma glycerol concentration was monitored for smelt used in cGPDH and mGPDH transcript and mGPDH enzyme activity seasonal measurements. In cold-acclimated smelt, glycerol increased to maximal levels by March 12, 2009, with plasma concentrations reaching 128 ± 9.4 μmol/mL (water temperature, 0.4°C; Fig. 5*A*). Glycerol concentrations dropped significantly after this date, and plasma glycerol reached initial levels (0.36 ± 0.1 μmol/mL) by the final sampling point (May 4, 2009). The decrease in glycerol level occurred before the increase in water temperature, which rose from 0.5°C on April 6, 2009, to 2.1°C on May 2, 2009. The plasma glycerol level in warm-acclimated smelt bordered the levels of detection (<1 μmol/mL) throughout the study.

The seasonal levels of hepatic smelt GPDH transcripts were measured by QPCR. The seasonal profile of cGPDH transcripts was marked by differences between cold- and warm-acclimated smelt during the initiation of glycerol production (Fig. 5*B*). Transcript levels of cGPDH in cold-acclimated smelt increased to maximal values in December (December 9, 2009) and slowly declined to baseline values by February 9, 2009, which were maintained up to May 4, 2009. In contrast, the warm-acclimated smelt showed no variation throughout the season.

Transcript levels of mGPDH did not show significant variation over the sampling period in either cold-acclimated smelt or warm-acclimated smelt (Fig. 5*C*). mGPDH was significantly higher in the cold-acclimated group than in the warm-acclimated group on December 9 and February 9 and tended to be significantly higher on April 6, 2009 ($P = 0.06$). The average mGPDH transcript levels in cold-acclimated smelt on April 6, 2009, when plasma glycerol was clearly declining, was fourfold higher than November levels, when glycerol was not accumulating. The average transcript levels between March 12 and May 4 were all higher than the November levels. Collectively, it appears that transcript levels of mGPDH are higher at the end of the winter season than during the preglycerol accumulation stage. The combined value during the glycerol decrease phase from April 6 to May 4 is 16.83 ± 3.06 ($N = 21$) and is significantly higher than the glycerol accumulation stage from November 13 to February 9, which has a value of 8.27 ± 1.25 ($N = 34$). Data from March 12, 2009, were not included in this analysis because it cannot be stated with certainty that maximal glycerol levels were attained by that date.

The seasonal hepatic mGPDH enzyme activity in cold-acclimated smelt was characterized by significant increases at the end of the season as compared to November 2008 samples (Fig. 5*D*). The highest mGPDH activity was observed in the final sample (May 4, 2009), with a maximal activity of 4.71 ± 0.45 μmol/min mg mitochondrial protein. This activity is 2.4-fold higher than the initial November 13, 2008, level. The mGPDH activity in the warm-acclimated smelt did not change between the November 13, 2008, and March 12, 2009, sampling dates. Unfortunately, because of a technical error, values are not available for April 6, 2009.

Discussion

This work confirms previous reports of elevated plasma glycerol levels in smelt (Raymond 1992; Treberg et al. 2002*b*; Lewis et al. 2004) and lack of accumulation in capelin (Raymond and Hassel 2000). Atlantic salmon are not freeze resistant (Fletcher et al. 1988), and, as such, low levels of plasma glycerol were anticipated. The cold-acclimated smelt had higher concentrations of plasma glycerol than did salmon, capelin, and warm-acclimated smelt. There was a difference in plasma glycerol between cold-acclimated smelt and warm-acclimated smelt even though animals were sampled during the time window when plasma glycerol levels would be on the decline in fish held at ambient temperature. The seasonal profile of glycerol levels in smelt tracking ambient water temperatures confirmed that previously reported (Lewis et al. 2004; Ditlecadet et al. 2011). Glycerol accumulation commences at around 3°–5°C in the fall; levels peak in March and sharply decline thereafter. Glycerol levels decline while water temperature is still low; thus, the decline phase does not seem to be dependent on temperature per se. Increasing day length is hypothesized to trigger the decrease in glycerol level (Driedzic and Short 2007). The differences noted here in plasma glycerol levels between smelt and other species and within smelt in association with the seasonal cycle are important in setting the context for the cGPDH and mGPDH studies because we can be confident with respect to statements made about glycerol-producing and non-glycerol-producing individuals.

Earlier studies provide evidence for the production of glycerol by liver and the importance of cGPDH in response to a decrease in temperature. For instance, hepatocytes isolated from smelt held at warm temperature and incubated at warm temperature release glucose, but when these cells are incubated at low temperature, glycerol is also produced (Clow et al. 2008). When smelt are subjected to a rapid temperature decrease from 8° to 1°C over a few days, plasma glycerol rapidly increases in association with a change in the liver mass action ratio at the cGPDH locus and an increase in maximal in vitro enzyme activity (Driedzic et al. 2006). In smelt tracking seasonal changes in water temperature, the onset of the increase in plasma glycerol is closely preceded by an increase in cGPDH transcript level and coincides with an increase in cGPDH enzyme activity (Liebscher et al. 2006). Thereafter, transcript levels decrease, but high enzyme activity levels may persist (Lewis et al. 2004; Liebscher et al. 2006). It is clear that smelt liver is a

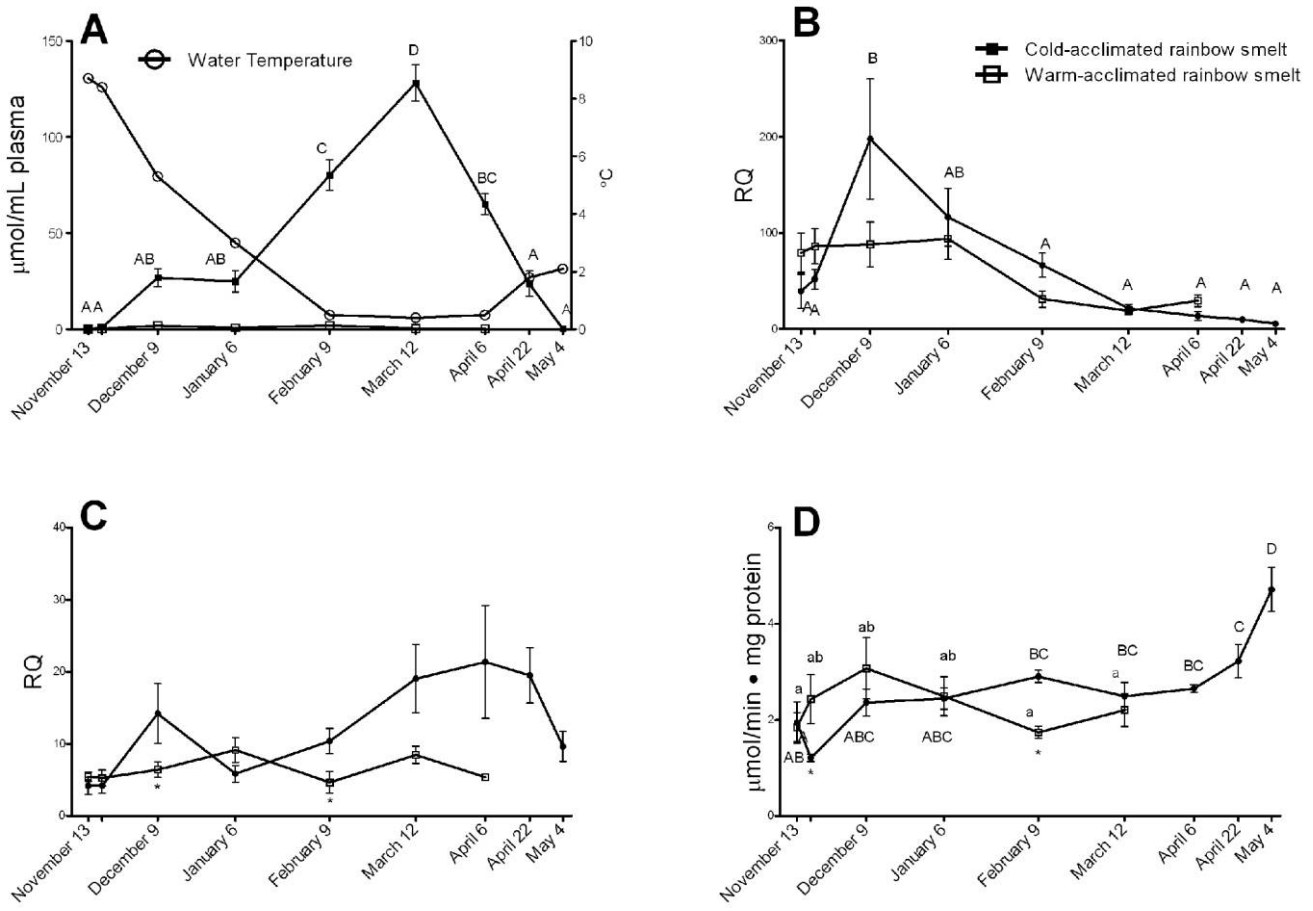

Figure 5. A seasonal (November 2008–May 2009) analysis of plasma glycerol levels (*A*), hepatic cytosolic glycerol-3-phosphate dehydrogenase (cGPDH) transcript levels (*B*), hepatic mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) transcript levels (*C*), and hepatic mGPDH activity (*D*) in warm- and cold-acclimated smelt. Symbols for all panels are shown in *B*. *A*, Plasma glycerol levels (μmol/mL plasma) are presented as mean ± SEM. Ambient water temperature for cold-acclimated smelt is shown. Warm temperature was maintained at ~10°C. cGPDH (*B*) and mGPDH (*C*) transcript levels were measured by quantitative reverse transcription polymerase chain reaction and are presented as mean relative quantity (RQ) ± SEM values for the gene of interest (cGPDH or mGPDH), normalized to 18S ribosomal RNA. Values are calibrated to the individual with the lowest normalized expression of that gene. *D*, mGPDH activities are presented as micromoles per minute milligram protein. In all cases, $N = 4$ for November 13, and $N = 8$ for November 19–February 9; in all other sampling points, $N = 7$. Lowercase letters indicate Tukey's HSD groupings for warm-acclimated smelt, and uppercase letters represent cold-acclimated smelt. Sampling dates without a common letter are significantly different ($P < 0.05$). An asterisk denotes a significant difference between cold-acclimated smelt and warm-acclimated smelt.

source of glycerol and that cGPDH plays an important role in the pathway leading to glycerol synthesis. In this study, evidence is provided that smelt liver is the primary source of glycerol production, on the basis of the catalytic potential of cGPDH. The tissue and species distribution of cGPDH activity reveals that the smelt liver is unique in having very high levels of this enzyme. cGPDH activity in smelt liver is higher than in all other tissues in smelt, salmon, and capelin. This finding confirms earlier reports of higher activities of cGPDH in smelt liver than in capelin, tomcod, and flounder livers (Treberg et al. 2002*a*; Driedzic et al 1998). Of all of the species mentioned, only smelt accumulate glycerol. It is noted for the first time that cGPDH activity in white muscle is higher in cold-acclimated smelt than it is in warm-acclimated smelt. Given the low levels of cGPDH activity in smelt muscle compared to smelt liver and salmon muscle, it is unlikely that the low-temperature-associated increase in cGPDH activity is related to glycerol production in muscle. The finding could be a reflection of membrane lipid reorganization in response to low temperature. The activity of cGPDH in the muscle of salmon (10% of smelt liver) was the next highest of all tissues evaluated but was not matched to a temperature-associated increase in glycerol accumulation. The elevated levels of cGPDH activity in salmon muscle may be related to a high ratio of lipolytic rate to metabolic rate, as exists in the trout (Magnoni et al. 2008), and, as such, might reflect high rates of lipid recycling rather than a specific role in glycerol production. This explanation leads to the concept that glycerol production in smelt liver may in-

volve cycling through the triglyceride pool as opposed to a direct dephosphorylation of G3P, as previously proposed (Driedzic et al. 2006). This issue is beyond this study, and, regardless, both of the aforementioned potential pathways to glycerol require cGPDH.

The seasonal profile of cGPDH transcript levels in smelt tracking ambient water temperature confirms that presented by Liebscher et al. (2006) and extends the findings, with the inclusion of fish held at a non-glycerol-producing temperature. Germane to this profile is the December peak in cGPDH transcript levels that is not observed in fish maintained at 10°C and thus appears to be a response specific to accelerated glycerol production. The important advancement is that the peak in cGPDH transcript levels and concomitant glycerol accumulation are associated with a decrease in water temperature per se.

There appear to be four cGPDH isozymes in smelt liver and at least two in muscle. This is the pattern observed in black bullhead *Ictalurus melas* (Basaglia and Cucchi 1995). Basaglia and Cucchi (1995) suggest that the pattern in liver is due to two codominant alleles of an A locus, resulting in two of the dimerized proteins, a BB isozyme and a heterodimeric AB isozyme. There does not appear to be differential expression in smelt liver in response to temperature because warm- and cold-acclimated individuals display all four isozymes. However, the cellulose-acetate gels are qualitative in nature, and thus, differential regulation may be present but beyond the resolution of the assays employed. There appear to be at least four copies of this gene in smelt. On the basis of the current information, the number of genes appears more than sufficient to encode the detected isozymes. We cannot predict whether all gene copies evident in the Southern blot are functional, although it is reasonable to expect that all the isozymes are encoded by genes among those shown in the Southern blot.

The studies reporting the cloning and sequence analysis of the mGPDH cDNA, tissue distribution, and seasonal pattern of mGPDH transcript levels and enzyme activity of mGPDH are the first involving this locus in glycerol metabolism. Smelt mGPDH contains all the structural motifs characteristic of mGPDH sequences from rat, mouse, and human (Brown et al. 1994; Koza et al. 1996; Matsutani et al. 1996). The ubiquitous tissue distribution is also consistent with that reported in rat (Müller and Seitz 1994) and mouse (Koza et al. 1996). No significant variation was noted in the expression of liver mGPDH transcripts at any specific time point throughout the season; however, there is an indication that transcript levels are higher during glycerol decline than during other phases. This viewpoint is based on the higher average transcript levels at multiple time points late in the season relative to November values and the significantly higher grouped values during the decrease phase than during the accumulation phase. mGPDH enzyme activity was significantly higher in April and May samples than in those from November. The increase in mGPDH activity in the last phases of the glycerol cycle is putatively due to elevated transcript levels and is associated with a sharp increase in water temperature from 0.5°C on April 6 to 2.1°C on May 1. Unfortunately, enzyme activity values for April and May in warm-acclimated fish are not available, so at this point it cannot be stated with certainty that increased mGPDH activities in cold-acclimated fish are due to small water temperature increases. Regardless of the triggering mechanism, high activity levels of liver mGPDH occur when the levels of glycerol in plasma and liver are decreasing as plasma and liver values parallel each other (Driedzic and Short 2007). Elevated activity of mGPDH may be associated with increased rates of oxidative metabolism in spring and an increased need for the transport of reducing equivalents across the mitochondrial membrane via the G3P shuttle. However, in this particular case, mGPDH may be more specifically required for biosynthesis. The increase in activity of mGPDH follows precisely the same pattern as glycerol kinase activity in April and May (Ditlecadet et al. 2011). The parallel increases in mGPDH and glycerol kinase are likely related to the rebuilding of liver glycogen pools that occur in spring (Driedzic and Short 2007). As such, smelt mGPDH may be important in channeling glycerol, via three-carbon intermediates, into the gluconeogenic pathway, as it is in rats (Taleux et al. 2009). Also, in rats, mGPDH is governed by thyroid hormones (Rauchova et al. 2004), and in brook trout, increases in temperature lead to increases in thyroid activity (Smith and Eales 1971). In smelt, the sequelae may be an increase in temperature, leading to increases in thyroid activity and thereafter to elevated mGPDH activity, which ultimately results in glycogen synthesis.

In conclusion, this study confirms the association between cGPDH and the onset of glycerol production in smelt and provides evidence that liver is the dominant production source. The description of cGPDH has been enhanced through the observation of multiple protein isoforms in smelt liver, associated with multiple genes. Smelt mGPDH has been cloned and characterized, shown to have high sequence identity with other ectotherms and the structural motifs common to mGPDHs from other species. Finally, mGPDH has emerged as an important enzyme in the pathway for the clearance of glycerol late in the seasonal cycle.

Acknowledgments

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