Seasonal Freeze Resistance of Rainbow Smelt (*Osmerus mordax*) Is Generated by Differential Expression of Glycerol-3-Phosphate Dehydrogenase, Phosphoenolpyruvate Carboxykinase, and Antifreeze Protein Genes

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ABSTRACT

In winter, rainbow smelt (*Osmerus mordax*) accumulate glycerol and produce an antifreeze protein (AFP), which both contribute to freeze resistance. The role of differential gene expression in the seasonal pattern of these adaptations was investigated. First, cDNAs encoding smelt and Atlantic salmon (*Salmo salar*) phosphoenolpyruvate carboxykinase (PEPCK) and smelt glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were cloned so that all sequences required for expression analysis would be available. Using quantitative PCR, expression of beta actin in rainbow smelt liver was compared with that of GAPDH in order to determine its validity as a reference gene. Then, levels of glycerol-3-phosphate dehydrogenase (GPDH), PEPCK, and AFP relative to beta actin were measured in smelt liver over a fall-winter-spring interval. Levels of GPDH mRNA increased in the fall just before plasma glycerol accumulation, implying a driving role in glycerol synthesis. GPDH mRNA levels then declined during winter, well in advance of serum glycerol, suggesting the possibility of GPDH enzyme or glycerol conservation in smelt during the winter months. PEPCK mRNA levels rose in parallel with serum glycerol in the fall, consistent with an increasing requirement for amino acids as metabolic precursors, remained elevated for much of the winter, and then declined in advance of the decline in plasma glycerol. AFP mRNA was elevated at the onset of fall sampling in October and remained elevated until April, implying separate regulation from GPDH and PEPCK. Thus, winter freezing point depression in smelt appears to result from a seasonal cycle of GPDH gene expression, with an ensuing increase in the expression of PEPCK, and a similar but independent cycle of AFP gene expression.

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Introduction

Temperate fish species inhabiting cold oceans have various adaptations to avoid freezing. During winter, a number of fish species migrate to deep or relatively warm water, where freezing will not occur, whereas others have biochemical adaptations that lower their freezing points to that of the surrounding seawater and prevent them from freezing (Goddard and Fletcher 2002; Driedzic and Ewart 2004). Many fish species produce antifreeze proteins (AFPs), which depress the freezing point of fluids noncolligatively by binding to ice crystals and inhibiting their growth (Ewart et al. 1999; Fletcher et al. 2001). These proteins can produce a substantial freezing point depression far beyond their colligative effects. In addition, a few species, including rainbow smelt, surf smelt (*Hypomesus pretiosus japonica*), and two greenlings (*Hexagrammos octogrammus* and *Hexagrammos stelleri*), accumulate glycerol to very high levels, which contributes to the lowering of the freezing point to safe levels by colligative means (Raymond 1992). In rainbow smelt, glycerol provides most of the freezing point depression during winter (Driedzic and Ewart 2004). Rainbow smelt also produce an AFP, although levels are modest and lower the freezing point by only about 0.3°C (Ewart and Fletcher 1990). Nonetheless, the AFP appears to be essential to reduce the freezing point to safe levels in winter. It also appears

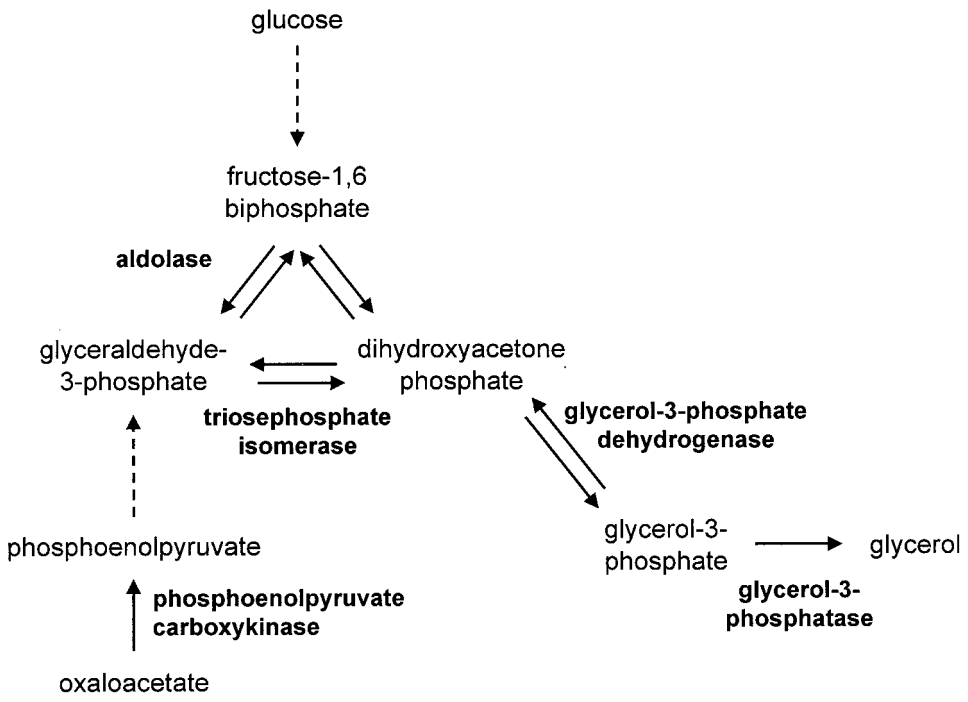

Figure 1. Major metabolic pathway for the production of glycerol in smelt liver. Dashed lines indicate multiple steps. The names of metabolites are in regular type, and enzyme names are in bold.

necessary in spring, when glycerol levels decrease and water temperatures are still very low (Driedzic and Ewart 2004). Trimethylamine oxide and urea make colligative contributions to freezing point depression in rainbow smelt plasma, but only in a minor fashion (Raymond 1994). Full freeze resistance in rainbow smelt appears to be provided by AFP and glycerol.

Initial studies of glycerol synthesis in rainbow smelt (hereafter referred to as smelt) suggested that low water temperatures were required for glycerol accumulation (Raymond et al. 1996), with carbohydrates and amino acids serving as metabolic precursors for glycerol synthesis (Raymond 1995; Raymond and Driedzic 1997). Triglyceride was ruled out as a source of glycerol because levels were not depleted in starved smelt producing glycerol (Raymond et al. 1996). Further work implicated glycerol-3-phosphate dehydrogenase (GPDH; enzyme commission no. [EC] 1.1.1.8) in liver as a key enzyme in glycerol synthesis. Polyol dehydrogenase activity in smelt liver was found to be similar to those in livers of two sympatric species with no glycerol accumulation during winter, whereas GPDH activities were highly elevated and glycerol-3-phosphatase activities modestly elevated in smelt compared with the other species (Driedzic et al. 1998). Smelt also had liver GPDH activities far higher than those of the capelin (*Mallotus villosus*), a closely related osmerid that occupies deeper water in winter and does not accumulate glycerol (Treberg et al. 2002*a*). A partial seasonal cycle of glycerol accumulation in smelt was described by Treberg et al. (2002*b*), and this was followed by a full seasonal study (Lewis et al. 2004). Liver glycogen decreased steadily over the winter months in smelt (Treberg et al. 2002*b*), suggesting that gluconeogenic precursors would be increasingly important in glycerol synthesis as the season progressed. Taking into account data from all individuals over a full fall-spring season, significant correlations were found between plasma glycerol levels and activities of liver GPDH, phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), and alanine aminotransferase (AlaAT; EC 2.6.1.2), suggesting metabolic upregulation at these loci in smelt producing glycerol (Lewis et al. 2004). The pathways that appear central to glycerol synthesis in smelt are presented in Figure 1. The smelt showed a clear seasonal variation in plasma antifreeze activity, although activities were elevated at initial sampling, well in advance of plasma glycerol accumulation (Lewis et al. 2004).

The regulation of seasonal variations in activities of GPDH and PEPCK and in levels of AFP remains to be determined. In mammals and yeast, GPDH is transcriptionally regulated in response to specific environmental perturbations (Burg et al. 1996; Cheng et al. 1997). In smelt, an interruption of cold exposure by transfer to warmer water midwinter brought about concomitant decreases in plasma glycerol and liver GPDH mRNA relative to controls, suggesting that glycerol accumulation in smelt in response to low temperature might also be regulated at the level of GPDH gene expression (Ewart et al. 2001). PEPCK is regarded as a classic example among transcriptionally regulated metabolic genes in mammals (Hanson

and Reshef 1997), and it appears similarly regulated in carp (*Cyprinus carpio*; Panserat et al. 2002). The seasonal regulation of the C-type lectin-like smelt AFP gene expression has not been investigated, although plasma levels of this AFP and others are known to vary seasonally; in the case of winter flounder (*Pleuronectes americanus*), this is mainly directed through gene expression (Fletcher et al. 2001). Therefore, our hypothesis is that all three genes are regulated seasonally in smelt.

In this study, GPDH, PEPCK, and AFP gene expression were quantified over a seasonal cycle. Because controls for gene regulation in fish in response to temperature are not well established, seasonal expression of our beta actin standard was compared with a common reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), before its use as a standard in this study. The cDNA sequences encoding smelt GPDH and AFP have previously been reported (Ewart et al. 1992, 2001). For polymerase chain reaction (PCR) primer design, partial PEPCK cDNAs were cloned from smelt and Atlantic salmon (*Salmo salar*), and the GAPDH cDNA was cloned from smelt. Then, seasonal samples were analyzed for GPDH, AFP, and PEPCK gene expression by real-time quantitative PCR (qPCR).

Material and Methods

Animals and Collection of Samples

Smelt were obtained from freshwater streams near estuaries in eastern Newfoundland in mid-October 2000, transported to the Ocean Sciences Centre (Memorial University of Newfoundland), held in tanks, and sampled (Lewis et al. 2004). Briefly, smelt were housed in a 4-m^3 tank with flow-through seawater at ambient temperature and a natural photoperiod using fluorescent lights set with an outdoor photocell. Five smelt were sampled after every approximately 1°C drop in ambient water temperature, and sampling was in accordance with the Canadian Council on Animal Care guidelines. Blood plasmas were obtained, and liver sections were quickly frozen in liquid nitrogen and then stored at −80°C until use. Only male fish were used in this gene expression study because transcript levels in livers of female fish were found to be highly variable in preliminary analyses. Variability may have resulted from the considerable transcriptional activity devoted to vitellogenin synthesis in maturing female fish.

Liver samples were obtained from Atlantic salmon (hereafter referred to as salmon) held in saltwater at the National Research Council of Canada Institute for Marine Biosciences Research Station and placed in RNALater solution (Ambion) overnight. The solution was decanted, and the samples were then stored at −80°C until use.

Cloning and Sequence Analysis

Degenerate oligonucleotide primers based on an alignment of the cDNA sequences of trout, chicken, and human PEPCK (accession no. AF246149, AY275429, NM002591; Cook et al. 1986; Panserat et al. 2001; Strausberg et al. 2002) were designed using Primer3 (Rozen and Skaletsky 2000), available through the Canadian Bioinformatics Resource (CBR) at http://cbr-rbc.nrc-cnrc.gc.ca/cgi-bin/primer3_www.cgi, and used to amplify a fragment of the salmon and smelt PEPCK cDNAs from salmon and smelt liver cDNA libraries. For all cloning, messenger RNA was prepared from salmon and smelt liver using the FastTrack 2.0 kit (Invitrogen Canada, Burlington, Ontario) and treated with DNase to minimize contaminating genomic DNA (RNase-free DNase kit, Qiagen, Mississauga, Ontario). The ProSTAR Ultra HF RT-PCR system (Stratagene, La Jolla, CA) was used to synthesize the first strand of cDNA in a 50-μL reaction per the manufacturer's directions, using $p(dT)_{12–18}$ as primer. The cDNA template was amplified using PfuTurbo DNA polymerase using additional oligonucleotide primers that were designed to perform 5′- and 3′-RACE to complete the cDNA sequences or to produce a full-length cDNA clone for the salmon PEPCK (Table 1). However, this approach was not successful with the smelt PEPCK. Additional 5′ and 3′ sequence fragments were obtained using the GeneRacer kit (Invitrogen) following the manufacturer's instructions, starting with DNase-treated total RNA and treating with calf intestinal phosphatase, tobacco acid pyrophosphatase, and T4 RNA ligase to produce a cDNA product after reverse transcription using Thermoscript RT (Invitrogen) at 65°C. Amplification of the cDNA template was performed using oligonucleotides specifically designed for high melting temperatures (Primer3; Table 1) on the basis of the known smelt DNA sequence as well as the included GeneRacer 5′, 3′, and nested 5′ or 3′ oligonucleotides. All oligonucleotides were either synthesized in house (Expedite 8909, PerSeptive Biosystems, Framingham, MA) or purchased from Integrated DNA Technologies (Coralville, IA). Amplification was done according to the manufacturer's instructions using either Platinum Pfx DNA polymerase (Invitrogen) or recombinant Taq DNA polymerase (GE Healthcare, Amersham Biosciences, Baie d'Urfé, Quebec), and fragments were inserted into either the pCR 4Blunt-TOPO or the pCR 2.1-TOPO vector for transformation of TOP10 competent cells (Invitrogen).

DNA sequencing was performed using the ABI Prism 377 DNA sequencer with the ABI sequencing analysis software (PerkinElmer, Boston). The ABI sequencing protocol was used for the majority of the sequencing reactions, with minor modifications. Briefly, 300 ng of cloned vector template, 50 ng of primer, 2 μL of BigDye terminator, and 6 μL of BigDye terminator buffer (166 mM Tris-HCl pH 9.0, 4.2 mM $MgCl_2$) were combined in a total volume of 20 μL and subjected to amplification on a PerkinElmer Cycler 9700 with the following thermal cycling parameters: 96°C for 5 min followed by 25 cycles at 96°C for 20 s, 55°C for 5 s, and 60°C for 4 min followed by a 4°C hold. Additional sequencing reactions were performed by the Atlantic Genome Centre using the MegaBACE capillary sequencer (GE Healthcare, Amersham Biosciences). Templates

Table 1: Synthetic oligonucleotides used in molecular cloning of GAPDH and PEPCK cDNAs and in analysis of expression for these and other transcripts

Oligonucleotide Name[a]	Nucleotide Sequence (5′–3′)	Application
GAPDH:		
Omogla-1	CCC TCA AGG TTG TCA GCA AT	Initial sequencing
Omogla-2	GAT GAA GGG GTC ATT GAT GG	5′-RACE
Omogla-3	TAG TCA GCA CCA GCC TCT CC	5′-RACE
Omogla-4	TAA GAC CCT CGA TGA TGT GG	5′-RACE
Omogla-5	AGT GGT CGA CCT GAC TGT CC	3′-RACE
Omogla-6	GCA TTC TGG GAT ACA CAG AGC	3′-RACE
Omogla-7	CAC TCC TCC ATC TTT GAT GC	3′-RACE
Omogla-8	TAA GAC CCT CGA TGA TGT GG	Sequencing
Omogla-9	GAG GCA GGA ATT ATG TTC TGG	Sequencing
Omogla-10	GCC ACA GCT TAC CAG AAG G	Sequencing
Omogla-11	ACA GAG GAC TAG ACC AAC TCA GG	Full-length cloning
Omogla-12	TGG GTC ATG TGA CGT AGT GC	Full-length cloning
Omogla-13	AGG AAG CAT TGC TGA CAA CC	Quantitative PCR
Omogla-14	AGA GGC TGG TGC TGA CTA CG	Quantitative PCR
PEPCK:		
Ssaph-1	AGT ATG AG/CA ACT GCT GGC/T TGG C	Initial sequencing
Ssaph-2	GTG/C ATC CCC/A TTC AGC ATG GG	Initial sequencing
Ssaph-3	GCT GTT/G GCT/C TCT/A GAT/C CTC AT	Initial sequencing/5′-RACE
Ssaph-4	GTT GGG ATG AGC ACA CGG GC	5′-RACE
Ssaph-5	GCC AGG TTA GTT TTC CCA CA	5′-RACE
Ssaph-6	GGC ACA CGA CGC TCT TAT TC	Full-length clone
Ssaph-7	TTC CTC ATC ACG CTC CAA G	Full-length cloning
Omoph-1	GAT CCT GTC GTT CGG CAG TGG CTA CG	3′ GeneRacer
Omoph-2	CCC TGA CCG ACT GGC ACG GTA AAT CC	3′ GeneRacer
Omoph-3	CTG GGT TAC ACG GCC ACG AGT TGA CC	5′ GeneRacer
Omoph-4	CCC CAG CAG TGA GTT GCC TCC GTA GC	5′ GeneRacer
Omoph-5	CAA CTC ACT GCT GGG GAA GAA GT	Quantitative PCR
Omoph-6	ATG CCC AGG ATC AGC ATG TGT T	Quantitative PCR
AFP:		
Omoaf-1	GCT CTA CTT GTT TGT GCC ATG GTG	Quantitative PCR
Omoaf-2	GCC TGT GTA TGG ATG CAA GGT TTG	Quantitative PCR
GPDH:		
Omogl-1	TTC GTA CAG AAT GGC AGC AC	Quantitative PCR
Omogl-2	ACA TTC CCA CTG CAT CCT TC	Quantitative PCR
Beta actin:		
Ssaac-1	CTA CGA GGG TTA CGC TCT GC	Quantitative PCR
Ssaac-2	AGC ACT GTG TTG GCG TAC AG	Quantitative PCR

[a] Names are derived from the first letter of the genus, the first two letters of the species, and early letters of the enzyme.

for MegaBACE sequencing were generated directly from glycerol stock cells using Templiphi technology (Amersham Biosciences). Sequencing reactions were performed using ET-terminator chemistry (Amersham Biosciences) and M13 forward or M13 reverse primers. All DNA sequences were analyzed using Sequencher version 4.0.5 analysis software (Gene Codes, Ann Arbor, MI). Predicted amino acid sequences obtained from the cDNAs were aligned with the five known proteins with the lowest E scores in search of the Swiss-Prot database using BLAST (Altschul et al. 1990), through CBR (http://blast.cbr.nrc.ca/). Related sequences for each protein were aligned using ClustalW (Thompson et al. 1994) at CBR (http://cbr-rbc.nrc-cnrc.gc.ca/services/clustalw_form.html). For percentage identity determination between sequences, single alignments were performed using SIM (Huang and Miller 1991), also at CBR (http://ca.expasy.org/).

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Smelt          --MVKVGINGFGRIGRLVTRAGFSSKKVEIVAINDPFIDLEYMVYMFKYDSTHGRYKGEV
Eel            --MVKVGVNGFGRIGRLVTRAAFHSGKVQVVAINDPFIDLDYMVYMFKYDSTHGRFHGEV
Trout          MSDLCVGINGFGRIGRLVLRACLQKG-IKVVAINDPFIDLQYMVYMFKYDSTHGRYKGEV
Cichlid        MSDLCVGINGFGRIGRLVLRACLQKG-IKVVAINDPFIDLQYMVYMFKYDSTHGRYRGEV
                    ** ********** **          ********** **************  ***

Smelt          KADNGKLVIDGHAITVFHERDPTAIKWGEAGADYVVESTGVFTTIEKASAHLKGGAKRVI
Eel            KAEGGKLHIAGHVITVFHERDPTNIKWGEAGADYVVESTGVFTTIEKASTHLKGGAKRVI
Trout          SMEDGKLIVDDHSISVFQCMKPHEIPWGKAGADYVVESTGVFLSIDKASSHIQGGAKRVV
Cichlid        SADNGKLVVDGQAISVFQCMKPAEIPWGSSGAKYVVESTGVFLSVEKASSHIQGGAKRVV
                   ***      * **    *  * **  ** *********    *** *  ******

Smelt          ISAPSADAPMFVMGVNHEKYENS-LKVVSNASCTTNCLAPLAKVINDNFHIIEGLMSTVH
Eel            ISAPSADAPMFVMGVNHEKYDKS-LKVVSNASCTTNCLAPLAKVVNDKFVIIEGLMSTVH
Trout          VSAPSPDAPMFVMGVNEDKFDPSSMTIVSNASCTTNCLAPLAKVIHDNFGIEEALMTTVH
Cichlid        VSAPSPDAPMFVMGVNEDKYDPSSMTIVSNASCTTNCLAPLAKVIHDSFGIEEALMTTVH
                **** **********  *   *    ****************  * * * * ** ***

Smelt          AVTATQKTVDGPSGKLWRDGRGASQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPN
Eel            AITATQKTVDGPSGKLWRDGRGASQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPN
Trout          AYTATQKTVDGPSAKAWRDGRGAHQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPVAD
Cichlid        AYTATQKTVDGPSAKAWRDGRGAHANIIPASTGAAKAVGKVIPELNGKLTGMAFRVPVAD
               * ********** * *******  ******************************

Smelt          VSVVDLTVRLEKPASYDAIKKVVKAASEGPMKGILGYTEHHVVSSDFNGDTHSSIFDAGA
Eel            VSVVDLTVRLEKPAKYDDIKKVIKEAAEGPMKGILGYTEHQVVSTDFNGDTHSSIFDAGA
Trout          VSVVELTCRLSRPGSYAEIKGAVKKAAEGPMKGYVGYTEYSVVSSDFIGDTHSSMFDAGA
Cichlid        VSVVDLTCRLSKPASYAEIKEAVKKAAEGPLKGVLGYTEDQVVSSDFIGDTHSSIFDAGA
               **** ** **  *  *  **   * * *** **  ****  *** ** ****** *****

Smelt          GIALNDHFVKLVSWYDNEFAYSQRVCDLMAHMASKE
Eel            GIALNDHFVKLVSWYDNEFGYSNRVVDLMVHMASKE
Trout          GISFNDNFVKLISWYDNEFGYSHRVADLLLYMHFKE
Cichlid        GISLNDNFVKLISWYDNEYGYSNRVADLLLYMHSKE
               **  ** **** ******  ** ** **   *  **
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Figure 2. Alignment of rainbow smelt GAPDH protein sequence with those of other fish species. Other sequences are from rainbow trout (accession no. O42259), Japanese eel (*Anguilla japonica*, accession no. BAC06416; Okubo et al. 2002), and a cichlid (*Haplochromis burtoni*, accession no. AAD23573). Alignment was performed as described in "Material and Methods." Residues conserved among all species are indicated with an asterisk.

Quantitative PCR

Total RNA was isolated from smelt liver samples using the RNeasy Mini kit (Qiagen) and following the manufacturer's recommendations. The liver samples were homogenized using a 7-mm generator on a Polytron standard rotor-stator homogenizer (Kinematica, Brinkmann Instruments, Mississauga, Ontario). Before elution of RNA with RNase-free water from the columns provided, each sample was treated for 15 min with 27 Kunitz units of RNase-free DNase 1 (Qiagen) to eliminate any contaminating genomic DNA. Absorbance (260 nm) of diluted RNA samples was measured and used to calculate equal quantities of RNA for gel electrophoresis. For each RNA sample, a 3-μg aliquot was resolved on a 1% agarose formaldehyde/MOPS gel in loading buffer that contained ethidium bromide. The gel was checked for high RNA quality by observing the integrity of the 18S and 28S ribosomal bands before proceeding to cDNA synthesis.

For each RNA sample, cDNA was prepared using the EndoFree RT kit (Ambion, Austin, TX) according to manufacturer's directions, with minor changes. Briefly, 10 μg of RNA from each smelt liver sample was heat denatured to 80°C with 100 pmol of oligo (dT) primer for 5 min before transfer to a 49°C water bath and the addition of a warmed master mix that contained the appropriate buffer, 5 nmol of dNTPs, and 20 units of RNase inhibitor. One microliter of reverse transcriptase solution was added, and then the samples were gently mixed,

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Salmon          ----------MSCLLLGLIRRRSGVGTSVGVRSLASIPSLPPAVADFVKRAVDECKPAN
Trout-m         ----------MSCLLLGLIRRRGGVGTSVGVRSLASIPSLPPAVADFVKRAVDECKPAN
Smelt           ----------MSCLLLGLIRRRSGVGTSVGVRSLASIPSLPPAVADFVKRAVDECKPAN
Zebrafish-c     ----------MPPQLQSQDR-------SCPRVLQGDLASLSASVREFIDSSVSLCQPDA
Chicken-C       ----------MAPELKTEVN-------IISKVIQGDLESLPPQVREFIESNAKLCQPES
Chicken-M       MFWLRGGAQSCRGGETEDRMQRGMWGVGLARRRLSTSLSALPAAARDFVEEAVRLCRPRE
                                                        *      *       * *

Salmon          VHVVTGSSEESAQILAGLEKDGMVKRLPKYENCWLARTDPKDVARVESKTVIVTKNQRDT
Trout-m         VHVVTGSAEESAHILAGLEKDGMVKRLPKYENCWLARTDPKDVARVESKTVIVTKNQRDT
Smelt           VHVVTGSSEESAQILAGLEKDGMVKRLPKYENCWLARTDPKDVARVESKTVIVTKNQRDT
Zebrafish-c     LHICDGSEQENSTILSLLEEQGAIKRLRKYSNCWLARTDPRDVARVESKTVIVTAEQRDT
Chicken-C       IHICDGSEEENKKILDIMVEQGMIKKLSKYENCWLALTNPRDVARIESKTVIITQEQRDT
Chicken-M       VLLCDGSEEEGKELLRGLQDDGVLHPLPKYDNCWLARTDPRDVARVQSKTVLVTPEQSDA
                     **  *    *      *    * ** ***** * * ****  ****  *  * *

Salmon          IPIPDGGAKSQLGSWMSEGDFHKARQDRFPGCMAGRTMYVIPFSMGPVGSPLSKFGVQVT
Trout-m         IPIPDGGAKSQLGSWMSEGDFQKARQDRFPGCMSGRTMYVIPFSMGPVGSPLSKFGVQVT
Smelt           IPIPDGGAKSQLGSWMSEGDFQKARQDRFPGCMAGRTMYVIPFSMGPVGSPLSKFGVQVT
Zebrafish-c     VPTPTGGGVSQLGRWMCPEEWDKAMNLRFPGCMKGRVMYVIPFSMGPVGSPLSKIGVELT
Chicken-C       IPIPKTG-SSQLGRWMSEEDFEKAFNTRFPGCMQGRTMYVIPFSMGPIGSPLAKIGIELT
Chicken-M       VPPPPPSGSPQLGNWMSPNAFQAAVQERFPGCMAGRPLYVIPFSMGPPTSPLAKLGVQVT
                 * *      *** **       *   ****** **  *********  *** * *   *

Salmon          DSPYVVASMGIMTRMGTPVMDKLAQGAEFVRCQHSLGRPLPLKAPLVNSWPCNPEKVLIS
Trout-m         DSPYVVASMGIMTRMGTPVMDKLAQGAEFVRCQHSLGRPLPLKAPLVNSWPCNPEKVLIS
Smelt           DSPYVVASMGIMTRMGTPVMDKLAQGAEFVRCQHSLGRPLPLKAPLVNSWPCNPEKVLIS
Zebrafish-c     DSPYVVASMRIMTRMGKTVLSALGNG-EFVRCLHSVGCPLPLKKPLVNNWPCNPELTLVA
Chicken-C       DSPYVVASMRMMTRMGTAALKALGNG-EFVKCLHSVGCPLPLKEPLINNWPCNPELTLIA
Chicken-M       DSPYVVLSMRIMTRVGPAVLQRLDDD--FVRCLHSVGRPLPLTEPLVSSWPCDRSRVLVA
                ****** **  *** *      *     ** * ** * ****  **   ***     *

Salmon          HLPDTRQILSFGSGYGGNSLLGKKCFALRIASRIAKDEGWLAEHMLILGITNPQGVKRYV
Trout-m         HLPDTRQILSFGSGYGGNSLLGKKCFALRIASRIAKDEGWLAEHMLILGITNPQGVKRYV
Smelt           HLPDTRQILSFGSGYGGNSLLGKKCFALRIASRIAKDEGWLAEHMLILGITNPQGVKRYV
Zebrafish-c     HIPDQRKIVSFGSGYGGNSLLGKKCFALRIASRIAKEEGWLAEHMLILGITNPAGQKKYF
Chicken-C       HLPDRREIISFGSGYGGNSLLGKKCFALRIASRIAKEEGWLAEHMLILGITNPEGEKKYF
Chicken-M       HIPSERRIVSFGSGYGGNSLLGKKCFALAIASRMAQQQGWLAEHMLILGVTSPSGEKRYM
                * *  * * ******************** **** *   *********** * * * * *

Salmon          AAAFPSACGKTNLAMMKPALPGWTVECVGDDIAWMKFDSQGKLRAINPENGFFGVAPGTS
Trout-m         AAAFPSACGKTNLAMMKPALPGWTVECVGDDIAWMKFDSQGKLRAINPENGFFGVAPGTS
Smelt           AAAFPSACGKTNLAMMKPALPGWTVECVGDDIAWMKFDSQGKLRAINPENGFFGVAPGTS
Zebrafish-c     AAAFPSACGKTNLAMLKPSLPGWKVECVGDDIAWMKFDKEGNLRAINPENGFFGVAPGTS
Chicken-C       AAAFPSACGKTNLAMMNPSRPGWKIECVGDDIAWMKFDELGNLRAINPENGFFGVAPGTS
Chicken-M       AAAFPSACGKTNLAMMTPSLPGWRIHCVGDDIAWMKFDDRGRLRAINPERGFFGVAPGTS
                ***************  *  ***   ************  * ******* **********

Salmon          LKTNPHAMATIAKNTVFTNVGETSDGGVWWEGLDPP-AAGVSLTDWHGKSWKAGDSGPCA
Trout-m         LKTNPHAMATIAKNTVFTNVGETSDGGVWWEGLDPP-AAGVSLTDWHGKSWKAGDSGPCA
Smelt           LKTNPHAMATIAKNTVFTNVGETSDGGVWWEGLDPP-AAGVSLTDWH-------------
Zebrafish-c     SKTNPNAMSTISCNTLFTNVAESSDGGVFWEGMDEDLPEGVTLTSWKNQPWTPEDGEPCA
Chicken-C       IKTNPNAIKTIFKNTIFTNVAETSDGGVYWEGIDEPLPPGVTLTSWKNKDWTPDNGEPCA
Chicken-M       SRTNPNAMATIARNTIFTNVGLRSDGGVYWDGLDEPTEPGVTYTSWLGKPWKHGDPEPCA
                  *** *  **  ** ****   ***** * * *     **  * *    .      ...
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Salmon          HPNSRFCTPAAQCPIIDPQWESDEGVPIDAIIFGGRRPEGVPLVYESFNWRHGVFVGASM
Trout-m         HPNSRFCTPAAQCPIIDPQWESDEGVPIDAIIFGGRRPEGVPLVYESFNWRHGVFVGASM
Smelt           ------------------------------------------------------------
Zebrafish-c     HPNSRFCTPAAQCPIIDPQWESPEGVPIEAIIFGGRRPQGVPLVYEAFDWAHGVFVGASM
Chicken-C       HPNSRFCTPASQCPIMDPAWESPEGVPIEGIIFGGRRPAGVPLVYEAFNWQHGVFIGAAM
Chicken-M       HPNSRFCAPADQCPIMDPRWDDPEGVPIDAIIFGGRRPRGVPLVVEAFGWRHGVFMGSAM
                ....... .  .... .. .   .....  .......  ..... . . . .... .  .

Salmon          RSEATAAAEYKGKVIMHDPFAMRPFFGYNFGDYLAHWLSMETRKGATHLPKIFHVNWFRK
Trout-m         RSEATAAAEYKGKVIMHDPFAMRPFFGYNFGDYLAHWLSMETRKGATHLPKIFHVNWFRK
Smelt           ------------------------------------------------------------
Zebrafish-c     RSEATAAAEHKGKVIMHDPFAMRPFFGYNFGQYLSHWLSMEQRP-GAKLPKIFHVNWFGR
Chicken-C       RSEATAAAEHKGKIIMHDPFAMRPFFGYNFGKYLAHWLSMAHRP-AAKLPRIFHVNWFRK
Chicken-M       RSEATAAAEHKGGRLMHDPFAMSPFFGYNAGRYLEHWLSTGLRS-NARLPRLFHVNWFLR
                ......... ..   ....... ...... . .. ....         ..  ......

Salmon          DPTSGSFLWPGFGDNARVLEWIFKRCSREREDEAAKKSMVGWVPLEGAINLQGLGSKVDM
Trout-m         DPTSGSFLWPGFGDNARVLEWIFKRCSREREDEAAKKSMVGWVPLEGAINLQGLGSKVDM
Smelt           ------------------------------------------------------------
Zebrafish-c     S-SSGRFLWPGFGENIRVLEWMFGRLSG---GAEARTTAVGLVPADGALNLHGL-PDVEP
Chicken-C       D-SQGKFLWPGYGENSRVLEWMFNRIQG---KASAKSTAIGYIPADTALNLKGL-EDINL
Chicken-M       D-NEGRFVWPGFGHNARVLAWIFGRIQG---RDTARPTPIGWVPKEGDLDLGGL-PGVDY
                    . . ... . . ... .                   .  .      . ..

Salmon          GALFDLPKAFWEKETQELRAYFTQQVGADLPHQVEGELKALEDRIRN--------
Trout-m         GALFDLPKAFWEKETQELRAYFTQQVGADLPQQVEGELKALEDRIRN--------
Smelt           ------------------------------------------------------------
Zebrafish-c     LELFRVSQEFWMQELQEIREYFSRELNRDLPQEMQRQLELLEHRLTHTHVSSKHG
Chicken-C       TELFNISKEFWEKEVEEIKQYFEGQVNADLPYEIERELLALEMRIKQL-------
Chicken-M       SQLFPMEKGFWEEECRQLREYYGENFGADLPRDVMAELEGLEERVRKM-------
                  ..     ..  .      .       ...      .  .. .
```

Figure 3. Alignment of rainbow smelt and salmon PEPCK protein sequences with those of other vertebrate species. Other sequences include one from zebrafish that is predicted to encode a cytosolic form (zebrafish-c, *Danio rerio*, accession no. NP_999916; Strausberg et al. 2002), a cytosolic chicken sequence (chicken-C, accession no. VP05153; Cook et al. 1986), a predicted mitochondrial sequence from rainbow trout (trout-m, accession no. AAK28384; Panserat et al. 2001), and a mitochondrial sequence from chicken (chicken-M, accession no. J05419.1; Weldon et al. 1990). Alignment was performed as described in "Material and Methods." Residues conserved among all species are indicated with an asterisk, and those conserved among all but smelt outside of the sequenced smelt region are indicated by a dot.

briefly centrifuged, and incubated at 49°C for 2 h. To control for DNA contamination, replicate cDNA reactions without added reverse transcriptase were performed for ~10% (randomly picked) of the RNA samples.

The reverse-transcribed cDNAs encoding beta actin, GPDH, and PEPCK were each amplified in triplicate from the smelt liver cDNA preparations using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, Oakville, Ontario) in a 25-μL reaction. Briefly, 12.5 μL of the ReadyMix, 1 μL of FAM (1 : 2,000) internal fluorescence reference (Sigma-Aldrich), and 20 ng each of gene-specific primers (Table 1) were added as a master mix to either 4 μL of a 1 : 10 v/v dilution in water (Sigma-Aldrich) of the cDNA template (beta actin, GPDH) or 2 μL of 1 : 10 diluted cDNA template (PEPCK). Additionally, 2 μL of either 1 : 10 or 1 : 100 v/v dilutions of the cDNA preparations were analyzed for expression of GAPDH and AFP, respectively, in a similar manner using the iQ SYBR Green Supermix (Bio-Rad Laboratories Canada, Mississauga, Ontario) except replacing the added FAM internal standard with additional water, since the Supermix already contained an internal fluorescein reference. Reaction mixtures were cycled in a Bio-Rad iCycler iQ Real-Time PCR Thermal Cycler as follows: 10 min at 95°C followed by 38 cycles (beta actin, GPDH) or 36 cycles (PEPCK) or 45 cycles (GAPDH) or 40 cycles (AFP) of 30 s at 95°C, 60 s at 54°C (beta actin, GPDH) or 58°C (PEPCK) or 56.5°C (GAPDH) or 58°C (AFP) and 30 s at 72°C, followed by a hold at 16°C. Reactions were monitored by melt curve analysis after cycling was complete to ensure that the observed fluorescent signal was due solely to the specific amplicon. To set up a standard curve to quantify cDNA in the samples, plasmids containing each gene noted above were serially diluted and amplified as described above (2-μL template per reaction).

These standards were amplified along with the experimental samples during every reaction to ensure result consistency, because operator and reagent variability is of notable concern with quantitative real-time PCR (Bustin 2002). The standards from each experiment were averaged to obtain a final standard curve to be used in cDNA quantification. In addition, aliquots of randomly selected reactions (approximately every tenth one) were resolved on a 2% agarose gel to confirm the presence of a single PCR product and the absence of artifacts such as primer dimers, because either of these would confound the quantified data.

Data Analysis

Data were analyzed using the iCycler iQ Real-Time Detection System Software (Bio-Rad). Beta actin was amplified as a control (housekeeping) gene, and to support those data, GAPDH was also amplified and compared with beta actin. The triplicate averages for GPDH, PEPCK, AFP, and GAPDH were divided by the control (beta actin) values for each fish to normalize the data. In addition, for each sampling date, relative expression values calculated for individual fish were averaged and standard errors determined. This provided relative quantification of expression changes that occurred in male smelt liver over the fall-spring seasonal cycle. For statistical analysis, the data sets were $\log_{10}$ transformed, and then means were compared by ANOVA with a Tukey post hoc test considering $P < 0.05$ to be significant.

Results

cDNA Sequences

The smelt GAPDH cDNA was 1,075 bp in length and contained a complete open reading frame predicting a 333-residue GAPDH protein (GenBank accession no. DQ138967). No polyadenylation signal was evident in the 3′ untranslated region, but the sequence was short and probably contained only the proximal portion of the 3′ untranslated region. The smelt GAPDH protein sequence was aligned with those from other fish species, showing extensive sequence identity (Fig. 2).

A 1,185-bp partial, internal sequence for smelt PEPCK, encoding 395 residues from the center of the protein sequence, was also obtained (GenBank accession no. DQ230919). Because further PCR cloning of PEPCK cDNA from smelt was unsuccessful, a longer ortholog was sought from salmon in order to provide sequence information on a full coding sequence for alignment and comparison with other PEPCK sequences. The PEPCK cDNA sequence from salmon was 1,908 bp in length and encoded a complete open reading frame that predicted a 635-residue PEPCK protein (GenBank accession no. DQ144903). The 5′ and 3′ untranslated regions were incomplete and extended just past the protein-coding region. Over its length, the smelt PEPCK cDNA sequence is highly identical to that of salmon, and the encoded protein fragment is an exact match to its salmon counterpart. The salmon and partial smelt PEPCK protein sequences were aligned with full-length sequences from other fish species, and the mitochondrial and cytosolic PEPCK isoforms from chicken (Fig. 3). An earlier report suggested that the rainbow trout (*Oncorhynchus mykiss*) PEPCK sequence might be mitochondrial, on the basis of a putative mitochondrial import signal (Panserat et al. 2001). Nonetheless, pairwise alignments of salmon PEPCK with mitochondrial and cytosolic PEPCK sequences from chicken (Weldon et al. 1990) revealed that the salmon sequence shares 416 identical residues with the cytosolic form and 395 with the mitochondrial form of chicken PEPCK over the range of common sequence overlap when aligned using SIM (results not shown). Therefore, the smelt and salmon clones can be considered to encode cytosolic PEPCK.

Seasonal Variation in the Expression of Genes

In order to select a suitable reference gene for seasonal expression studies, the expression of two commonly used reference genes, beta actin and GAPDH, was determined at each sampling date over the course of the fall-winter-spring interval. The expression of GAPDH relative to actin was unchanged (no significant difference) over the season (results not shown), suggesting that both genes had very stable expression. Therefore, actin was considered to be an appropriate reference gene for this seasonal study.

The expression of GPDH and PEPCK in smelt liver followed clear seasonal cycles with distinct time courses. The mRNA levels for these enzymes, normalized to actin, are indicated in arbitrary units over the course of the fall-winter-spring seasons (Fig. 4). Overlaid with these data are plasma glycerol values and enzyme activities that were previously obtained from the same fish (Lewis et al. 2004) to allow comparison of seasonal cycles at the gene and product levels. GPDH gene expression in liver appeared to have increased slightly from baseline levels before the first sampling in October, although levels were not significantly higher than those at the end of the seasonal sampling (May). GPDH transcript levels increased rapidly in November, resulting in significant elevations above the first sampling value, with maximal levels occurring in mid-December. The level of liver GPDH mRNA followed the same pattern as that of glycerol in plasma from the onset of sampling to mid-December; however, significant increases in transcript level preceded significant increases in plasma glycerol by approximately 2 wk (Lewis et al. 2004). Levels of GPDH mRNA decreased over the winter months and approached initial values from mid-February onward.

In contrast to the rapid increase in GPDH in early fall, the levels of PEPCK transcript remained low. PEPCK mRNA then increased sharply in November and December to significantly elevated levels concomitantly with rapid plasma glycerol accumulation (Fig. 4*B*). Over the winter months, PEPCK mRNA levels remained elevated above fall starting levels, mirroring the

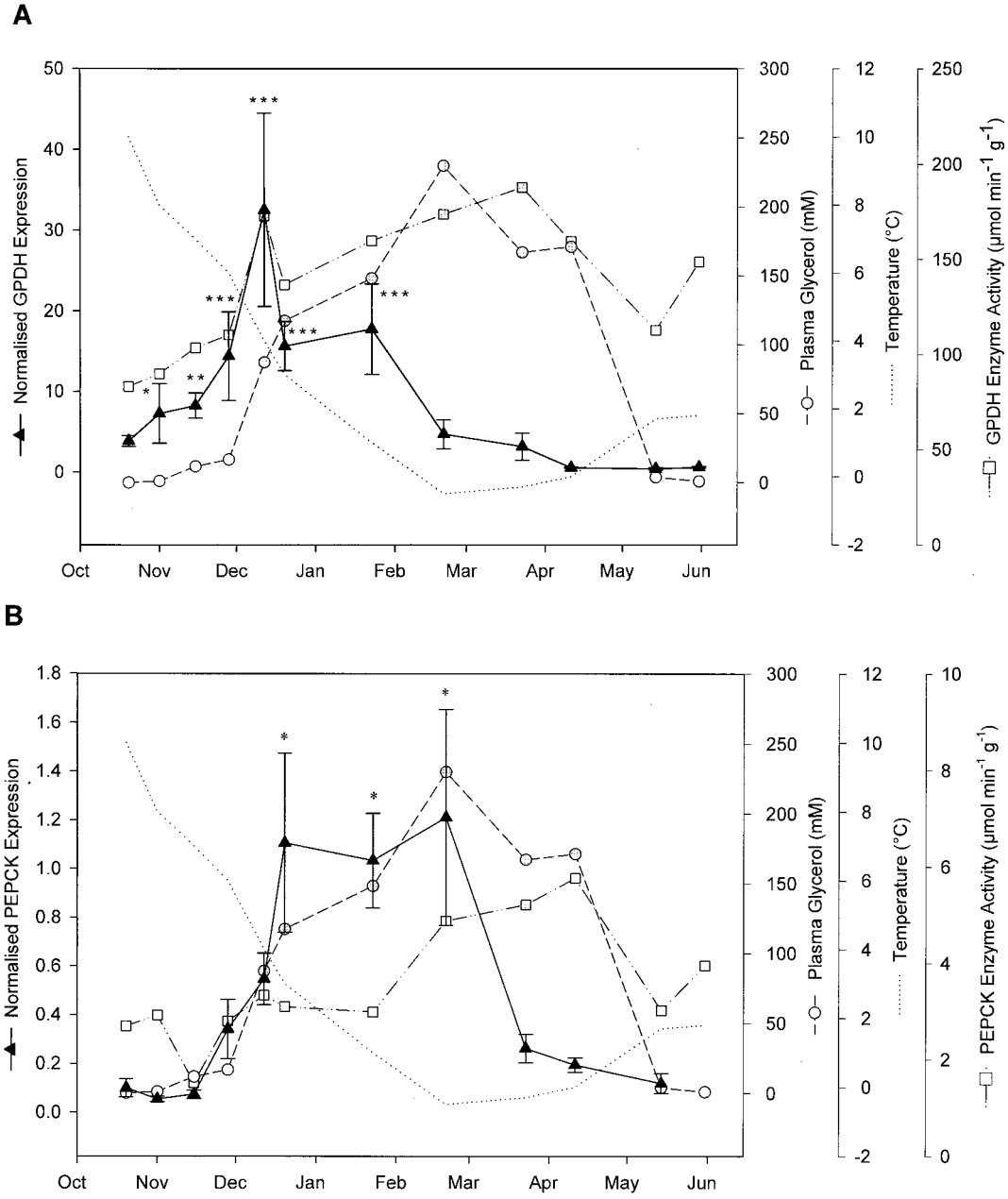

Figure 4. Seasonal variation in levels of GPDH (*A*) and PEPCK (*B*) mRNA in liver of smelt. Transcript levels relative to actin were determined by qPCR, as described in "Material and Methods," and these values are shown as solid triangles. For the November 13 and January 23 sampling dates, $n = 3$; for April 11, $n = 5$; for all other sampling dates, $n = 4$. All data are represented as means ± SEM. Asterisks indicate significant differences compared with initial values. *One asterisk*, $P < 0.05$; *two asterisks*, $P < 0.01$; *three asterisks*, $P < 0.001$. Previously determined water temperatures, smelt plasma glycerol levels, and activity levels for appropriate liver enzymes (Lewis et al. 2004) are shown for comparison. For glycerol and enzyme activities, open symbols represent means that are not significantly different from the initial value, whereas solid symbols show means that are significantly different ($P < 0.05$) from the initial value.

elevated glycerol levels until a decline in mid-March. At the mid-March and mid-April time points, PEPCK mRNA levels decreased while plasma glycerol levels remained elevated.

Smelt liver AFP gene expression also showed seasonal variation (Fig. 5). Plasma antifreeze activity values from these fish (Lewis et al. 2004) are overlaid for comparison. The cycle of AFP synthesis began well in advance of cooling water temperatures in the fall; AFP mRNA levels were significantly elevated

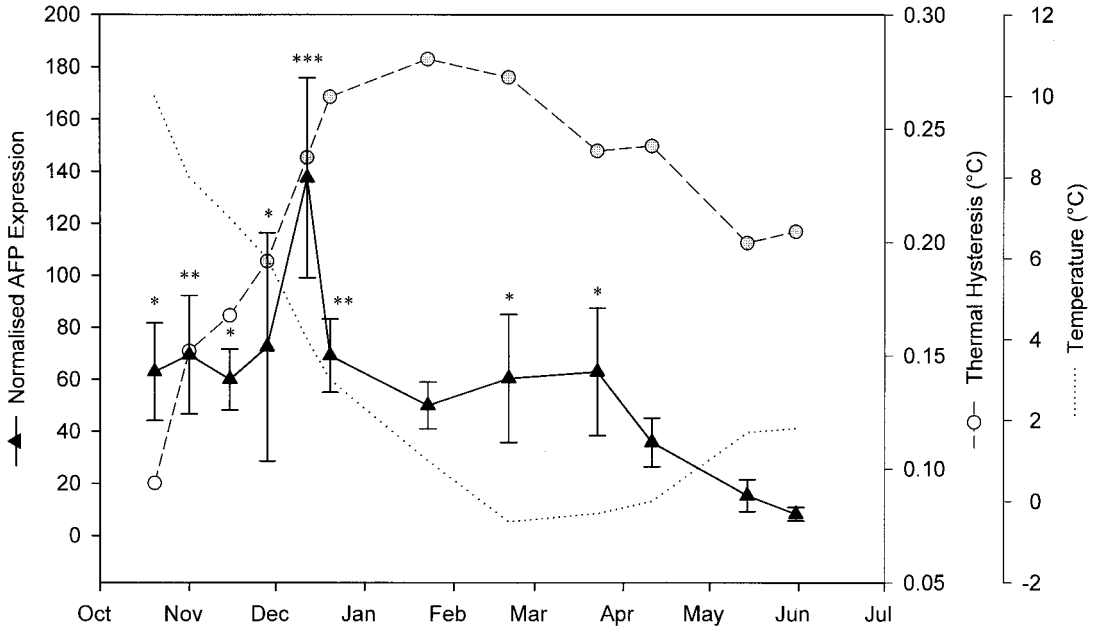

Figure 5. Seasonal variation in levels of AFP in liver of rainbow smelt. Transcript levels relative to actin were determined by qPCR, as described in "Material and Methods," and these values are shown as solid triangles. Sample numbers, representation, and indications of statistical significance are as in Figure 4. Previously determined water temperatures and smelt plasma antifreeze activity levels (Lewis et al. 2004) are shown for comparison. For antifreeze activities, open circles represent means that are not significantly different from the initial value, whereas solid circles show means that are significantly different ($P < 0.05$) from the initial value.

at the first sampling in October compared with the final sampling value in May. For this reason, statistical analysis of significance of elevation in AFP levels over the winter was determined with respect to the final (baseline) sampling values in May rather than the first mean value in October. With the exception of the January time point, levels of AFP mRNA were significantly elevated until April. The pattern of change in AFP mRNA was similar to that of AFP activity in plasma from mid-October to mid-December. Thereafter, transcript levels decreased while AFP activity in plasma remained elevated. The seasonal cycle of AFP gene expression began at least 1 mo earlier than those of GPDH and PEPCK and ended 1 mo later than PEPCK and 2 mo later than GPDH, suggesting a distinct mode of regulation from the other two genes.

Discussion

The increase in GPDH mRNA in the fall closely preceded the acceleration of glycerol accumulation in corresponding blood plasma samples that was previously reported (Lewis et al. 2004). This finding suggests that an autumn increase in GPDH gene expression generates the seasonal accumulation of glycerol in smelt. Similar glycerol accumulation occurs in the baker's yeast *Saccharomyces cerevisiae* (Burg et al. 1996), where a transcriptional increase in yeast GPDH, mediated through the high-osmolarity-glycerol kinase pathway, plays the key role in the metabolic response to osmotic stress (Hohmann 2002). GPDH expression is also upregulated in response to various forms of stress in glial cells of mammalian central nervous system in response to cortisol signaling (Cheng et al. 1997). For smelt, the primary environmental signal triggering the increase in GPDH gene expression is likely to be temperature. Earlier studies have suggested that temperatures in the vicinity of 5°C coincide with natural glycerol accumulation in smelt plasma during fall (Lewis et al. 2004) and that low temperatures (+1 to −1°C) can trigger an early onset of plasma glycerol accumulation (Raymond et al. 1996). Furthermore, seawater temperatures close to 0°C are required for the maintenance of high plasma glycerol levels in winter; transfer of smelt to heated seawater (5°–7°C) leads to a significant decrease in liver GPDH mRNA compared with levels in control smelt held at ambient seawater temperatures (Ewart et al. 2001).

An increase in PEPCK mRNA levels occurred later in the fall than GPDH mRNA, just as plasma glycerol levels increased sharply. This coincident increase suggests that PEPCK gene expression does not regulate initial glycerol synthesis per se but that it may respond to the metabolic demands placed on the smelt liver by rapid glycerol production. Accelerated glycerol synthesis through the GPDH-catalyzed pathway would require a substantial supply of dihydroxyacetone phosphate (DHAP). This can be synthesized from glycogen or glucose, through partial glycolysis, or from amino acids through pyruvate in the early steps of gluconeogenesis. Radiotracer experiments have

shown that both glucose and amino acids contribute to glycerol synthesis in smelt during winter (Raymond 1995; Raymond and Driedzic 1997). Glycogen is an obvious source of DHAP in fall, but it may not be sufficient to maintain glycerol levels as glycogen pools in liver decrease during the winter (Treberg et al. 2002*b*). Therefore, a gluconeogenic source of DHAP may become progressively more important as glycerol accumulation proceeds over the fall and winter. An abbreviated gluconeogenesis that leads from amino acid precursors to glycerol-3-phosphate accumulation and that is dependent on PEPCK is recognized in mammals and termed glyceroneogenesis (Hanson and Reshef 2003). Recent nuclear magnetic resonance studies have revealed that glyceroneogenesis is a pathway employed in smelt for glycerol synthesis during winter (Walter et al. 2006). In mammals, PEPCK transcription increases in response to carbohydrate depletion and it is upregulated by glucagon, glucocorticoids, prolactin, and thyroid hormone (Hanson and Reshef 1997). The resulting increase in PEPCK enzyme accelerates gluconeogenesis (Hanson and Reshef 1997). The situation is not as clear in fish. In rainbow trout, PEPCK was found to be unaffected by dietary carbohydrate level, whereas in carp (*Cyprinus carpio*), PEPCK gene expression decreased with carbohydrate feeding as in mammals (Panserat et al. 2001, 2002). The substantial increase in PEPCK mRNA in smelt liver during the months of intensive glycerol synthesis, and while glycogen levels are decreasing, suggests an important role for the enzyme in eliciting glyceroneogenesis, which directly supplies glycerol-3-phosphate for dephosphorylation to glycerol.

The seasonal cycle of AFP gene expression is distinct from that of GPDH and the ensuing increase in PEPCK because AFP mRNA in the liver increases much earlier, in advance of the sampling performed in this study, and remains elevated until spring. Levels of AFP mRNA were high in the fall and winter, relative to levels in the spring, and these were reflected by elevated antifreeze activity in the plasma (Lewis et al. 2004). Temperature is an unlikely trigger for the seasonal onset of AFP gene expression in smelt liver, because this cycle was well underway in October when the water temperature was 11°C. In other fish species, the distinct AFPs are regulated primarily through photoperiod and temperature. In winter flounder, AFP mRNA levels have been measured over the course of the season and found to increase from undetectable levels in September to about 20% of the maximal winter level in October and to continue to increase during the fall in response to shortening day length (Idler et al. 1989). Although the smelt AFP, a globular protein belonging to the C-type lectin superfamily, is unrelated to the small alpha-helical AFP of winter flounder (Ewart et al. 1999), the genes encoding these proteins may respond to similar environmental cues that allow a preadaptive response well in advance of seawater freezing. Although low temperature is not a trigger for the increase in smelt AFP transcript level, it appears to be required for the winter maintenance of winter AFP mRNA levels, because smelt transferred to heated water (5°C) in January showed a significant decrease in this transcript compared with control smelt in ambient (0.8°C) seawater (Treberg et al. 2002*b*).

Regression analysis comparing plasma glycerol with all PEPCK activities and with all GPDH activities over the sampling season showed positive correlations for both (Lewis et al. 2004), supporting the contention that these enzymes have key roles in glycerol accumulation. However, maximal activities of GPDH and PEPCK in liver homogenates of these smelt did not follow the pattern of the sharp fall increase and winter-spring decrease in levels evident in their mRNAs. The increase in GPDH activity lagged behind the increase in transcript level in that activity levels were not significantly different from initial values until mid-December. Elevated levels of GPDH then persisted over the winter period while transcript levels decreased. PEPCK activity levels gradually increased from fall through March and, as such, followed the seasonal increase in PEPCK mRNA. Nonetheless, as with GPDH, elevated PEPCK activities were maintained after the decrease in transcript level. The maintenance of enzyme activities following the decrease in transcript level could be explained in part by extended half-lives of the enzymes encoded by these genes in hepatocytes, such that the activities essential for glycerol synthesis are maintained for a long time after decline of their gene expression.

In smelt plasma, glycerol levels remain elevated for several weeks during winter and spring, after GPDH and PEPCK mRNA levels have declined. In addition to the concept of extended half-lives of liver enzymes, there may be mechanisms preserving glycerol in the blood of smelt and thereby reducing the demand for synthesis over the winter. The modest accumulation of urea and TMAO in smelt plasma during winter appears to result from conservation mechanisms occurring at low temperatures rather than increased synthesis (Raymond 1998). Although smelt lose substantial glycerol to the surrounding seawater (Raymond 1993), losses in mid- to late winter may be reduced compared with rates of synthesis by a conservation mechanism in common with that suggested for the other osmolytes. Antifreeze activity in plasma also remains elevated long after liver AFP mRNA levels begin declining, which may reflect a lengthy AFP half-life. The possibility of enhanced retention of small organic molecules, such as glycerol, and assessment of the longevity of AFPs will be intriguing questions in future investigation.

The autumn increase in liver GPDH transcript, preceding a similar increase in plasma glycerol, provides the first indication of a causative role for GPDH in the seasonal onset of glycerol accumulation. Thus, smelt appear to employ unusual regulation of an ordinary gene (GPDH) to generate remarkable freeze resistance. The molecular basis for GPDH upregulation during fall and the decrease in transcription level in winter, even as the temperature reaches lower levels, remains unknown, but it may involve photoperiod or other factors. The increase in PEPCK mRNA corresponding to glycerol accumulation also

varies with season, and this may reflect the metabolic role of the encoded enzyme in providing precursors for glycerol synthesis. It will be interesting to determine whether expression of this gene might be induced in response to hormonal signals, as is the case for its mammalian orthologs (Hanson and Reshef 1997). Freeze resistance conferred by glycerol is further augmented by the synthesis of AFP. It is interesting that the AFP appears to be regulated separately from the other two genes. In essence, the seasonal freeze resistance of smelt appears to result primarily from transcriptional programs that generate seasonal variation in gene expression, with GPDH responding to temperature decline during fall and AFP responding to a cue that precedes that decline, possibly photoperiod. Identification of the precise environmental triggers and hormonal/cellular signals may reveal the general regulatory mechanisms generating winter freeze resistance adaptation in smelt.

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