Glycerol loss to water exceeds glycerol catabolism via glycerol kinase in freeze-resistant rainbow smelt (Osmerus mordax)

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Ditlecadet D, Short CE, Driedzic WR. Glycerol loss to water exceeds glycerol catabolism via glycerol kinase in freeze-resistant rainbow smelt (Osmerus mordax). Am J Physiol Regul Integr Comp Physiol 300: R674–R684, 2011. First published December 22, 2010; doi:10.1152/ajpregu.00700.2010.—Rainbow smelt accumulate high amounts of glycerol in winter. In smelt, there is a predictable profile of plasma glycerol levels that starts to increase in November (<5 μmol/ml), peaks in mid-February (>200 μmol/ml), and thereafter decreases to reach the initial levels in the beginning of May. The aim of this study was to investigate the respective role of the two main mechanisms that might be involved in glycerol clearance from mid-February: 1) breakdown of glycerol to glycerol-3-phosphate through the action of the glycerol kinase (GK) and 2) direct loss toward the environment. Over the entire glycerol cycle, loss to water represents a daily loss of ~10% of the total glycerol content of fish. GK activities were very low in all tissues investigated and likely have a minor quantitative role in the glycerol cycle. These results suggest that glycerol levels are dictated by the rate of glycerol synthesis (accelerated and deactivated during the accumulation and decrease stages, respectively). Although not important in glycerol clearance, GK in liver might have an important metabolic function for other purposes, such as gluconeogenesis, as evidenced by the significant increase of activity at the end of the cycle.

cold adaptation; glycerol kinase; diffusion to water

RAINBOW SMELT (Osmerus mordax) is an unusual fish species with regard to the strategies it uses in winter to remain fully active at temperatures lower than the usual freezing point of teleost blood. As with many other species inhabiting the same environment, rainbow smelt produce antifreeze proteins (10) but also accumulate high amounts of glycerol (17, 25, 35). Accumulation of glycerol, in addition to other solutes like urea and TMAO, may participate in the decrease of the freezing point by colligative mechanisms (25), but it may also serve as a chemical chaperon to promote or preserve protein folding (11), or, as it is the case in some insects, contribute to promote supercooling (14).

It was first reported that plasma glycerol levels of rainbow smelt caught in late fall were very low compared with the levels measured in winter when water temperature was colder (25, 27). Tracking of glycerol levels from an indigenous Canadian population during the cold period, from December (5°C, decreasing water temperature) to the beginning of April (1°C, increasing water temperature) showed that glycerol levels peaked between late January and late February and decreased from there even if water temperature remained low (35). These data were confirmed by Lewis et al. (17), who reported the complete cycle of glycerol. This cycle started in late fall with an increase of glycerol levels in parallel with the decrease in water temperature. Levels increased until late February when they peaked at concentrations > 200 μmol/ml and then decreased until the beginning of May when values were back to levels measured in November (<5 μmol/ml).

A drop of temperature was determined as the trigger of glycerol accumulation, while a change in the photoperiod is more likely the trigger for the decrease in plasma glycerol reported from late January/late February (3, 7). Liver is the major site of production for the entire animal (reviewed in Ref. 6) with glucose, glycogen, and amino acids serving as fuels (28, 29, 36) and the cytosolic form of glycerol-phosphate dehydrogenase (cGPDH) functioning as a key enzyme in the control of glycerol synthesis (5, 17) (Fig. 1). Furthermore, glycerol accumulation requires a very active synthesis, as it is constantly challenged by direct losses to the surrounding water (26). Physiological mechanisms involved in the glycerol decrease phase are not as well documented. The role of the mitochondrial form of glycerol-phosphate dehydrogenase (mGPDH) that catalyses an irreversible dehydrogenation of glycerol-3-phosphate (G3P) to dihydroxyacetone (DHAP) (as opposed to cGPDH involved in reversible reactions) has been assessed (Robinson JL, Hall JR, Charman M, Ewart KV, Driedzic WR, unpublished observations) and thus is only related to glycerol breakdown (Fig. 1) (14, 18). Those results clearly show an increase of the mGPDH activity in April and May with average activities higher during the decrease period of glycerol, suggesting a channeling of glycerol toward the gluconeogenic pathway as plasma glycerol returned to low levels.

The first enzyme directly involved in glycerol breakdown is glycerol kinase (GK). This enzyme phosphorylates glycerol to G3P that can then enter glycerolipid synthesis (e.g., triglycerides and phospholipids) or be converted back to DHAP by cGPDH or mGPDH and eventually flux back to glucose (Fig. 1). In mammals, normal plasma glycerol levels range between 0.04 and 0.4 mM (18) with higher levels reported in cases subject to GK deficiency (32). Glycerol levels generally reflect the state of fat mobilization. It is released following lipid degradation and is phosphorylated back to G3P by GK mainly in the liver, which has the highest levels of GK (18). In mammals, GK activity can be regulated dependent on the physiological status of the animal. Insulin injections resulted in a decrease of glycerol levels in plasma in vivo in rabbits (12), while it was shown to induce GK activity in liver tissue and adipose cells of rats (16). High-fat diet also resulted in an increase of GK activity in adipocytes of obese Zucker rats, maybe as a consequence of the well-known increase of glycerol levels in plasma following this kind of diet (33). These indirect links between glycerol and GK levels were observed in species where glycerol is a product of lipid degradation. They show that GK activity can be regulated depending on the physiological needs of the animal considered. Smelt survival in...
cold freezing waters relies on the accumulation of glycerol to levels more than five hundred times what is considered normal in mammals. It would thus seem probable that GK activity, in this particular species, would be regulated. Activity might thus be downregulated during the accumulation period, while it might be upregulated during the decrease period of glycerol.

A balance between glycerol inputs and outputs sets glycerol levels. For glycerol levels to decrease, outputs must exceed inputs. In addition to biochemical pathways involving GK, direct loss in water might be an important part of the glycerol decrease phase. Raymond (26) estimated these daily losses might be an important part of the glycerol loss in smelt. In 2008, fish were immediately transferred to two tanks. For both years and for the rest of the experiment, one group was maintained at warm temperature fluctuating around 9°C and 11°C for the 2007–2008 and 2008–2009 seasons, respectively, (warm smelt); and one group tracked ambient temperature (ambient smelt). Fish were kept on a natural photoperiod with fluorescent lights set by an outdoor photocell and were fed a diet of chopped herring twice a week to satiety.

**Materials and Methods**

**Animal Collection and Sampling**

Rainbow smelt (*O. mordax* Mitchill) were collected by seine netting from Mount Arlington Heights, Placentia Bay, Newfoundland, in October 2007 and 2008, transported to the Ocean Sciences Centre, Memorial University of Newfoundland (St. John’s, Canada), and transferred to 3,000-liter tanks with flow-through seawater. In 2007, all fish were first held in one tank tracking ambient temperature (9.3°C the day of transfer), and thereafter one-half of those fish were transferred to a second tank maintained at 8–10°C immediately after the first sampling in November. In 2008, fish were immediately transferred to two tanks. For both years and for the rest of the experiment, one group was maintained at warm temperature fluctuating around 9°C and 11°C for the 2007–2008 and 2008–2009 seasons, respectively, (warm smelt); and one group tracked ambient temperature (ambient smelt). Fish were kept on a natural photoperiod with fluorescent lights set by an outdoor photocell and were fed a diet of chopped herring twice a week to satiety.

**Experimental Protocols**

**Experiment 1: GK activity and expression.** From November 2007 to May 2008, blood and liver were sampled monthly from six fish per tank (except for March and April where samples were taken bimonthly). In November 2007, only ambient smelt were sampled, as the second tank was yet to be set up. In May 2008, only ambient smelt were sampled, since warm smelt started to die from the end of April. Occurrence of high mortalities in March-April is a recurrent phenomenon for smelt maintained at warm temperature, and this is thought to be the result of early spawning, males being predominant and one-time spawners in this population. Blood was used to measured glycerol levels in plasma, and liver was used to clone GK transcript (GK mRNA) and to measure seasonal expression and activity of GK.

From November 2008 to May 2009, blood, liver, white muscle, brain, gill, and heart were sampled monthly from six fish per tank. Only ambient smelt were sampled in April and May. Blood was used to measured seasonal glycerol levels in plasma, and white muscle, brain, gill, and heart were used to measure seasonal activity of GK as a complement to the previous year. A few samples of liver (*n* = 3–5) were also run for GK activity on November, and from February to May in both groups to confirm the pattern observed the season before.

In all cases, fish were killed with a sharp blow to the head, and tissues immediately harvested. Samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Blood was extracted via caudal puncture by using heparinized syringes and centrifuged at 10,000 g for 10 min. Plasma was then separated from the blood cell pellet and stored at −80°C until analysis.

**Experiment 2: seasonal rate of glycerol loss in water.** The rate of glycerol release in water was estimated at different points of the glycerol cycle during the 2007–2008 and 2008–2009 seasons. Five points between February 2008 and April 2008 (Feb 4, Feb 20, Feb 27,
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March 25, April 9) and six points between December 2008 and February 2009 (Dec 11, Dec 23, Jan 27, Feb 10, Feb 12, Feb 24) were selected. The first group captured the glycerol decline period, whereas the second group tracked the glycerol increase period. As such, two different populations of fish were used in these studies.

For all experiments, four plastic containers [21 cm (L) × 16.5 cm (W) × 13 cm (H)] were placed on a platform in a one cubic meter tank with flow-through seawater that tracked ambient temperature. This allowed maintaining the containers at the same temperature as the holding tank. Each container had its own air and water lines. On the day of the experiment, two ambient smelt were placed in each container, the water line was removed, and a lid with holes to allow air circulation and water sampling was secured. The level of water was set to fill the container to 6.5 cm in height. One milliliter of water was sampled from each container at this time and after 1, 3, 6, 12, and 24 h. Water samples were frozen and kept at −80°C for further glycerol analysis. After 24 h, plasma was sampled from each fish and processed as previously described, and total volume of water was determined. For each container, the rate of accumulation was expressed in micromoles per day and in micromoles per gram of fish per day. For the latter, the rate of glycerol accumulation in micromoles per day was divided by the combined mass of the two fish present in the container. The daily percentage of glycerol loss (%loss/day) was calculated based on the premise that the plasma glycerol level is similar to the glycerol content in all tissues in the fish (7). The following formula was applied: %loss/day = total amount of glycerol accumulated in the container (µmol) × 100/[plasma glycerol mass1 (µmol/ml) × massfish1 (g)] + [plasma glycerol mass2 (µmol/ml) × massfish2 (g)].

Length (cm), mass (g), and sex were recorded for all fish in all experiments. Animal protocols were approved by the Institutional Animal Care Committee, Memorial University of Newfoundland, St. John’s, NL, Canada.

Seasonal Expression and Activity of GK

Preparation of RNA from liver. Total RNA was extracted from liver using Trizol reagent (Invitrogen, Burlington, ON, Canada). Extracted RNA was quantitated by UV absorption at 260 nm, and its quality (integrity and purity) verified prior to cDNA synthesis. Integrity and purity were verified by agarose gel electrophoresis and by calculating the OD260/OD280 ratio, respectively. Ten micrograms of RNA were treated with DNAse using the TURBO DNA-free kit (Ambion, Austin, TX), and treated RNA was quantitated a second time prior to cDNA synthesis. One microgram of DNAse-treated RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Burlington, ON, Canada). cDNA so generated was used for cloning and real-time quantitative PCR (qPCR). DNAse treatment and reverse transcription were performed following manufacturers’ instructions.

GK and 18S cloning. A partial sequence of smelt 18S rRNA was obtained using PCR from freshly synthesized cDNA, while the complete sequence of the GK transcript was obtained following a combination of PCR and RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). Touchdown PCR was used to obtain the 18S rRNA amplicon and the central fragment of GK used for RLM-RACE. For GK, a second PCR was necessary to obtain enough product for the subsequent steps. This second PCR was the same as the first one except for the use of nested primers and of 5′ of the first reaction as a template. All PCRs were performed in a total volume of 50 µl containing 1× dyNaZyme buffer, 0.2 µM of each forward and reverse primer, 0.2 mM of dNTPs mix, and 2 units of dyNaZyme II DNA polymerase. Twenty nanograms of cDNA were used as template for the 18S rRNA and the first GK reaction, while 5 µl of the first reaction were used as template in the second GK reaction. Conditions of the PCR were as follows: 94°C for 2 min of initial denaturation; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s; 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s; and a final extension of 10 min at 72°C. All primer sequences used to obtain the 18S rRNA and GK sequences are listed in Table 1. 18S rRNA primers were designed from Salmo salar 18rRNA sequence (AJ427629), while GK primers were designed from Danio rerio (NP_001108056.1) and conserved region of vertebrate’s consensus sequences.

Amplics of 1,298 bp and 826 bp were amplified for 18S rRNA and GK, respectively. Those products were electrophoresed on a 1.5% agarose gel, excised, and purified using a QIAquick gel extraction kit (QIAGEN, Mississauga, ON, Canada). They were then subcloned into pGEM-T Easy vectors (Promega, Madison, WI) following the manufacturer’s instructions, and triplicate clones were sequenced on both strands at the Genomics and Proteomics Facility, Core Research Equipment and Instrument Training (CREAIT) Network, Memorial University, St. John’s, NL, Canada.

The 5′- and 3′-ends of the GK transcript were cloned using the GeneRacer kit for RLM-RACE (Invitrogen, Burlington, ON, Canada) following the manufacturer’s instructions. For both ends, two consecutive reactions were necessary to obtain enough products (the second reaction used nested primers and 5′ of the previous reaction as template). Primers used are listed in Table 1. For each reaction, one primer was furnished with the kit; the second was designed from the central fragment of GK previously sequenced. Resulting products were then processed in the same way as the central GK product previously sequenced (gel extracted, subcloned, and sequenced).

Table 1. Sequences of primers used for glycerol kinase (GK) and 18S rRNA cloning (PCR and RLM-PCR) and quantitative (qPCR)

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Direction_Sequence (5′-3′)</th>
<th>Application</th>
<th>Position from 5′-End</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>F_AAGACCGCTACAGATCCAGAG</td>
<td>PCR1</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>R_CGAAAGACCTCTCAAAACCA</td>
<td>PCR1</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F_GAGGCAAAAGGATGTCGCA</td>
<td>qPCR</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>R_GGATATGTTTATGGTCGGAAC</td>
<td>qPCR</td>
<td></td>
</tr>
<tr>
<td>GK</td>
<td>F_AGAGTTGGTGRARSARG</td>
<td>PCR1 (central)</td>
<td></td>
</tr>
<tr>
<td>GK</td>
<td>R_GGTCGAAACGATCAGCTTCA</td>
<td>PCR1 (central)</td>
<td></td>
</tr>
<tr>
<td>GK</td>
<td>F_AAGATGTTGAGAAATGAGTC</td>
<td>PCR2 (central)</td>
<td>265</td>
</tr>
<tr>
<td>GK</td>
<td>R_GGTCGAAACGATCAGCTTCA</td>
<td>PCR2 (central)</td>
<td>1074</td>
</tr>
<tr>
<td>GK</td>
<td>F_CTGGCTTCTGTTGATTTCC AAC</td>
<td>RLM-RACE1 (3′-end)</td>
<td>857</td>
</tr>
<tr>
<td>GK</td>
<td>R_CTGGTCGAAACGATCAGCTTCA</td>
<td>RLM-RACE2 (3′-end)</td>
<td>950</td>
</tr>
<tr>
<td>GK</td>
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<td>RLM-RACE1 (5′-end)</td>
<td>452</td>
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<tr>
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<td>R_GGTGCACAAGGCTAGCTGCA</td>
<td>RLM-RACE2 (5′-end)</td>
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<tr>
<td>GK</td>
<td>R_GGAAGACGACACATTTCTGCA</td>
<td>qPCR</td>
<td>1153</td>
</tr>
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F, Forward; R, Reverse.

AJP-Regul Integr Comp Physiol • VOL 300 • MARCH 2011 • ajpregu.org
Sequence analysis. Sequences were analyzed using Vector NTI Advance 10 (Invitrogen, Burlington, ON, Canada). Percentages of identity with other available sequences were obtained at the nucleotide and at the amino acid levels using the Basic Local Alignment Tool (BLAST) 2.2.23 (1). A multiple alignment of 21 GK protein sequences (16 vertebrates and 5 invertebrates) was performed using MUSCLE version 3.8.31 (9) and used to construct a phylogenetic tree. The tree was constructed using MEGA version 4.0.2 (34) following the Neighbor-Joining method (31) with Poisson correction. Bootstrap analysis was performed with 1,000 replicates.

Real-time qPCR. GK expression was quantified as levels of GK mRNA. Total RNA was extracted and treated as previously described, and the cDNA synthesized was used for quantification by qPCR with normalization to 18S rRNA using SYBR Green I dye chemistry and the 7,300 real-time PCR system (Applied Biosystems, Foster City, CA). Primers used are listed in Table 1 for both 18S rRNA and GK and were designed from the sequences previously cloned. For both transcripts, efficiency and specificity of the reactions were confirmed before running the samples. Efficiencies (% of the reactions were estimated using a five-points serial dilution and were 97.9% (r², 0.997) and 99.9% (r², 0.999) for 18S rRNA and GK, respectively. Specificity of each reaction was verified by running a melt curve analysis and by running the reaction products on a 2% agarose gel. Both methods showed a single specific product of the expected size for both genes. Samples were then run in duplicates in separate reactions. qPCR reactions were performed in a total volume of 25 µl containing 1× power SYBR Green PCR Master Mix (Applied Biosystems); 50 nm and 250 nm of each forward and reverse primers for GK and 18S rRNA, respectively; and 10 ng of cDNA. The qPCR program consisted of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold was determined using the 7300 PCR Detection System SDS software version 1.2.3 from Applied Biosystems, and the relative quantity of GK mRNA was determined following the Pfaffl method (24). This mathematical model takes into account the respective efficiencies of the gene considered. The individual with the lowest normalized expression level was set as the calibrator sample (assigned value = 1). Data are presented as mean relative quantity (RQ) ± SE relative to the calibrator.

GK activity. GK is known to catalyse the following reaction: Glycerol + ATP → glyceraldehyde 3-phosphate + ADP. Commonly used assays to measure GK activity based on NAD/NADH-coupled reactions were found unsuitable for smelt liver due to high background activities that could not be dampened. The assay used was based on a colorimetric procedure that results in the production of quinoneimine dye that absorbs light at 540 nm (20). Briefly, tissues were thawed on ice and homogenized using a polytron in 4 volumes of ice-cold triethanolamine buffer 0.1 M at pH 7.4. Assays were run in duplicates in a Tris·HCl buffer (50 mM, pH 7.5) containing 1 mM ATP, 5 mM MgCl2, 1 mM 4-aminoantipyrine, 2 mM N-ethyl-N-((3-sulfopropyl)-m-anisidine, 5 U/ml glycerol phosphate oxidase, and 2 U/ml peroxidase. Concentration of the homogenate was chosen to provide a linear response over 15 min. Reactions were run at 30°C, and GK activity was monitored after a 5-min incubation period, for 15 min at 540 nm on a Beckman DU640 spectrophotometer to detect the liberated dye (extinction coefficient = 14.1 µM/cm). The assay temperature was selected to allow for the detection of GK activity in a reasonable time and is thought to be acceptable as we were interested in relative, rather than absolute, activities. GK activities are expressed as unit per gram of protein, one unit being the formation of one micromole of dye per minute at 30°C. Samples for which any activity was not detected were attributed the minimal activity detectable with this method (Δabsorbance of 0.00012 per min).

Glycerol and Protein Assays

Glycerol concentrations were determined by using the free glycerol determination kit from Sigma (Oakville, ON, Canada) following the manufacturer’s instructions. Protein concentrations in tissues were measured from homogenates used for GK assays with the Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada).

Statistical Analysis

For all specific parameters followed, values are presented as means ± SE, and significant differences between treatments at a particular sampling point were assessed using Student’s t-tests. One-way ANOVA and Tukey’s post hoc test were used to determine differences between sampling points within a treatment.

RESULTS

Water Temperature and Glycerol Level Profiles

Profiles of water temperature are presented in Fig. 2A for both the 2007–2008 and 2008–2009 periods. Temperature of ambient water was higher between November and mid-January in 2008–2009 than in 2007–2008. For both periods, tempera-
ture decreased until March to reach minima at −0.4°C and 0°C for 2007–2008 and 2008–2009, respectively, and increased from there to reach 1.1°C and 2.1°C at the end of the experiment in the beginning of May 2008 and 2009, respectively. For technical reasons temperature of the warm tank was higher in 2008–2009 with values fluctuating around 11°C in contrast to 9°C in 2007–2008.

In both cases, glycerol levels accumulated in plasma of ambient rainbow smelt to levels > 200 μmol/ml in mid-February, while they remained < 10 μmol/ml in warm smelt (Fig. 2B). Plasma glycerol profiles in fish held in ambient water were very similar in both experiments with the highest levels recorded in the middle of February (216.6 ± 15.4 and 221.4 ± 23.4 μmol/ml in 2008 and 2009, respectively). The only significant differences between studies were observed in January and April when levels were significantly lower in the 2008–2009 study (t-test, P < 0.05). For rainbow smelt held at warm temperature, glycerol levels were higher in 2008–2009 with average values reaching levels > 7 μmol/ml in January and February, while they never exceeded 4 μmol/ml in 2007–2008 (ANOVA, P < 0.05) (Fig. 2B).

**Seasonal Activity of GK**

The complete profile of GK activity in the liver is presented for 2007–2008 in Fig. 3A. In warm smelt, GK activity did not change over the whole period with an average activity of 79.41 ± 9.54 mU/g protein. In ambient smelt, activity remained constant until April when it started to increase significantly to reach a maximum in May at levels almost 25 times higher than those recorded in November (18.37 ± 2.06 and 452.91 ± 74.73 mU/g protein for November and May, respectively). When

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**Fig. 3.** Seasonal activity (A) and expression (B) of GK in smelt liver (2007–2008 experiment). A also includes a partial temperature profile. GK activity is presented as means ± SE (n = 6 per point). GK expression is measured as transcript levels. Those levels are presented as mean relative quantity (RQ) ± SE for GK normalized to 18S rRNA and calibrated to the individual with the lowest normalized expression (n = 6 per point). Different letters indicate GK activities significantly different within ambient smelt. *Significantly different (P < 0.05) and **close to different (P < 0.06), respectively, between warm and ambient smelt for the sampling date considered.
comparison was possible, GK activity of ambient smelt was not different than activity of warm smelt except at the beginning of April where activity was higher in ambient smelt. The increase of GK activity detected from the beginning of April corresponded to the period where water temperature started to increase again (Fig. 3A). Average activity during the accumulation period of glycerol (from November to February) was 43.04 ± 6.39 mU/g protein, while it was 197.69 ± 29.34 mU/g protein during the decrease period (from February to May).

A few liver samples were processed for the 2008–2009 experiment for GK activity to confirm the pattern observed during the decrease period of the previous 2007–2008 experiment. Values were in the same range as the ones reported in 2007–2008 and again a clear significant increase was observed from April with the maximum GK activity measured in May 380.79 ± 46.77 mU/g protein. GK activities measured in November (43.78 ± 13.61), February (142.16 ± 46.39), March (108.61 ± 62.00), and April (204.43 ± 30.20) were not different from one another, but activities measured in May 2009 were significantly different from November, February, and March but were not different from April, resulting in exactly the same pattern as the one reported on Fig. 3A for 2007–2008. When considering each month separately, GK activities were not different between the two studies. As in 2007–2008, GK activity of warm smelt was not different from activity of ambient smelt for the months considered [November (71.61 ± 36.58), February (40.27 ± 17.48), and March (52.56 ± 37.34)] and did not change with respect to time.

Four other tissues were investigated in 2008–2009. GK activity was not detected in gills, and brain homogenates yielded too high of a nonspecific background for the accurate quantitation of enzyme activity. GK activity was, however, easily detectable in heart and white muscle over the entire 2008–2009 study (Fig. 4). In heart of ambient smelt producing glycerol, activity did not change over the cycle with an average activity of 89.61 ± 5.64 mU/g protein (Fig. 4A). In warm fish, GK activity was higher in March than in all other months. At this point, activity was also higher in the warm than in the ambient group. GK activity in white muscle did not change over time in either warm or ambient fish but was overall higher in warm fish with average activities of 144.65 ± 10.49 and 182.57 ± 19.35 mU/g protein for ambient and warm smelt, respectively (Fig. 4B).

**GK Expression in Liver**

18S and GK cloning. A partial 18S rRNA sequence of 1,298 bp was cloned (HM856872). This sequence is very well conserved among vertebrates with sequence identities of 99.8% and 95% compared with *Plecopterus altivelis* and *Homo sapiens*, respectively.

By combining RT-PCR and RLM-RACE PCR, a full-length cDNA was cloned for GK (HM856871). It is a 1,869 bp cDNA that contains a 101 bp 5’-UTR, a 1,599 bp open-reading frame and a 169 bp 3’-UTR. At the nucleotide level, this sequence had the highest identity to *G. salar* (84.2%), followed by *D. rerio* (79.3%) and *Xenopus tropicalis* (73.2%). The 1,599 bp open-reading frame encodes for a 533 aa protein that clusters with other GK fish sequences within the vertebrate groups (Fig. 5). At the amino acid level, smelt GK had the highest identity to *S. salar*, *D. rerio*, and *X. laevis* (87, 85, and 78% respectively). The 533 aa protein features the two expected conserved domains for glycerol kinase: FGGY-N and FGGY-C superfamily domains. These domains adopt a ribonuclease H-like fold and are structurally related to the NH2- and COOH-terminal domains of GK, respectively (data not shown).

**Seasonal expression of GK in liver.** 18S expression was stable over the season and was thus determined as a suitable housekeeping gene for our experiment. Expression of GK did not change over the tested time period for either ambient or warm smelt and was not different between both groups (Fig. 3B). GK levels of transcripts are very low as shown by the need of two PCRs in a row to be able to amplify sufficient products (for size, >400 bp) and the high cycle threshold values measured during the qPCR experiment.

**Glycerol Loss in Water**

Plasma glycerol levels confirm that the periods considered are related to a decrease period from February 2008 to April 2008 and to an increase period from December 2007 to February 2009 (Fig. 6A). Water temperature ranged between 1.2°C and 0.3°C during the decrease period and between 6.5°C and 0.3°C during the increase period (Fig. 6A). The highest temperature was recorded at the beginning of the increase period and slowly decreased from there to reach 0.3°C at the end of February. Glycerol accumulated in the water in a linear fashion over the 24-h incubation period (data not shown). Over both periods considered, glycerol loss is positively linearly correlated to the glycerol concentration in plasma (Fig. 6B). The relationship between glycerol loss and plasma glycerol was not significantly different between the increase or decrease in glycerol time periods even though the two groups of fish differed in mass with the February 2008 to April 2008 group being 75.22 ± 5.29 g and the December 2008 to February 2009 group being 49.81 ± 1.27 g. As the two rates were similar, the data sets have been combined to yield the following relationship: glycerol loss (μmol/g protein/day) = 0.047 × plasma glycerol (μmol/ml) + 1.290.

When expressed as a daily percentage of glycerol loss, most values were below 10%. The one exception was at the beginning of the increase period where the percentage was the most variable. In early December, the average percentage loss was higher, close to 15% (Fig. 3C).

**DISCUSSION**

The first notable result of this study is the similarity between glycerol cycles measured in 2007–2008 and 2008–2009 with the same peak detected in mid-February at levels > 200 μmol/ml. The cycles differ slightly with January and April concentrations higher in 2008 than in 2009. Those differences, however, did not have any effect on the overall rate of glycerol accumulation and decrease between both seasons. These patterns are also very similar to the one reported by Lewis et al. (17), all of them closely fitting one on each other. The predictable decrease in glycerol levels observed in February supports the hypothesis that the decrease of glycerol levels from mid-February is not triggered by temperature but by some other factor, such as photoperiod (7).

Glycerol loss to water was determined during both the glycerol accumulation and glycerol decrease periods. Fish in
experimental chambers did not show any signs of agitation, and the rate of glycerol accumulation was linear to 24 h. As such, we consider the measurements of rate of glycerol loss to the environment to be a normal physiological process and not one induced by experimental stress. Dates chosen for those experiments covered an important part of both periods with plasma glycerol values ranging from 17 to 211 μmol/ml and from 212 to 50 μmol/ml for the accumulation and decrease periods, respectively. A positive linear correlation between plasma glycerol levels and the rate of glycerol loss is observed for both periods with no significant difference between both increase and decrease periods. As such, no obvious mechanism seems to be in place to retain glycerol during the accumulation period.

Glycerol loss to the environment appears to be by diffusion since it is dictated by the concentration difference between plasma and water levels. Raymond (26) previously reported that the major sites of glycerol loss are gill and skin with only small losses in urine. Although the data obtained here are consistent with a passive diffusion, we cannot rule out the
possibility of a more complex scenario, perhaps involving facilitated diffusion via aquaglyceroporins or even different processes in gill, skin, and kidney. For instance, glycerol movement into heart appears to be by simple diffusion alone; however, in red blood cells there is both a simple and a facilitated component to glycerol uptake (K. A. Clow and W. R. Driedzic, unpublished observation).

According to the formula deduced from the linear relationship between glycerol loss (\( \mu \text{mol/gfish/day} \)) and the concentration of plasma in glycerol (\( \mu \text{mol/ml} \)), a fish with a plasma concentration of 100 \( \mu \text{mol/ml} \) would lose around six micro-moles of glycerol per gram of fish per day. Thus, a 60 g fish would lose 360 \( \mu \text{mol/day} \). Given that glycerol levels in all tissues are similar to plasma, a 60 g fish would contain about 6,000 \( \mu \text{mol} \) of glycerol. The rate of glycerol loss to water would be sufficient to clear the animal of glycerol in 16.6 days (6,000/360), assuming glycerol synthesis is highly reduced. It is recognized that this is an over simplification of the rate of glycerol loss, since as plasma glycerol levels decrease so will the rate of loss. Regardless, as discussed below, on the basis of this first approximation analysis, there appears to be no need to involve GK activity to facilitate glycerol removal. This position is further developed below.

When expressed as a percentage of total glycerol in fish, our results fit nicely with the ones reported by Raymond (26), which estimated daily loss between 3 and 13%. Most all percentages estimated in this study were \( \leq 10\% \), the only exceptions being at the beginning of the glycerol cycle where very high daily percentage losses were recorded with values that exceeded 15%. These high values at the beginning of the increase period might be due to the higher water temperatures. This kind of relation between temperature and glycerol loss was previously reported for algae accumulating high amounts of glycerol when subject to osmotic stresses. The percentage of glycerol loss increased drastically with an increase of temperature (37). Higher glycerol loss at higher temperatures might be the result of a higher blood flow through gills generally associated with higher temperature and/or to a different membrane structure at higher temperature making small molecule exchanges easier.

The in vitro activity of GK appears to be a good proxy for in vivo rates of glycerol metabolism. This viewpoint is based on the following findings. Activity of GK was measured in rat liver during optimization of the assay and was slightly higher than 1 U/gliver at 37°C (data not shown). This activity is lower but comparable to the total capacity of glycerol clearance in rat liver set between 2 and 4 U/gliver at 37°C (19). Furthermore, at 30°C, GK activity of the same rate was 0.66 U/gliver and was \( \geq 40 \) times higher than GK activity measured at the same time in liver of two smelts (data not shown). As such, GK activity in liver of smelt is unexpectedly low compared with animals that rarely experienced glycerol plasma concentrations > 0.4 mM (19).

The quantitative contribution of GK to the decrease in glycerol levels at the end of the glycerol cycle is minimal. For instance, GK activity in April is \( \sim 300 \mu \text{U/gprotein} \) when plasma glycerol levels are \( \sim 100 \mu \text{mol/ml} \). This activity overestimates glycerol metabolism in vivo as GK measurements were done at 30°C. Assuming a Q10 of 2, the in vivo activity would be 37.5 \( \mu \text{U/gprotein} \) at 0°C. For a typical 60 g fish with an average weight of liver of 1 g and an average protein content per gram of liver of 0.12 g, the maximal rate of glycerol metabolism in liver would be \( (0.0375 \times 1 \times 0.12 \times 60 \times 24) = 6.48 \mu \text{mol glycerol/day} \). This is in contrast to glycerol loss to the water estimated to be 360 \( \mu \text{mol/day} \) for a 60 g fish.

Regardless of a minimal role in overall glycerol clearance, the seasonal profile of hepatic GK activity reveals changes that
suggest an important metabolic function for this enzyme. GK activity remained constant and at its minimum, while glycerol accumulates from November to mid-February with an average activity of 43 mU/g\text{protein}, while the average activity was 198 mU/g\text{protein} over the decrease period. Activity levels reached a maximum of levels in excess of 400 mU/g\text{protein} at the final sampling point. The seasonal profile of GK activity supports the theory that from February some glycerol is directed toward the synthesis of glucose as suggested by the increase of glycogen in this tissue at the end of the season (7) and by the parallel increase of mGPDH activity that catalyses the formation of DHAP from G3P (Robinson JL, Hall JL, Charman R, Ewart KV, Driedzic WR, unpublished observation). GK activity started to rapidly increase at the same time, as there was a small increase in water temperature above minimal winter temperatures, suggesting that this enzyme is under very fine temperature control.

GK expression in liver did not show any change over the season and was not different between ambient and warm smelt. Only one sequence that encodes for a functional GK was cloned from smelt liver but the possibility of different GK isoforms present in liver cannot be excluded. Primers designed for qPCR might anneal to other isoforms having the same conserved region and would result in a dilution of the results and thus a failure to detect any change in a specific isoform. Alternatively, regulation of GK activity might happen after mRNA synthesis. A change in protein content can occur at a posttranscriptional level without any change in the mRNA levels following different mechanisms that regulate mRNA activity (38). A change in GK activity can also occur with no change in the protein content at a posttranslational level. Regulation of enzyme activity through phosphorylation/dephosphorylation is one of the most common posttranslational modifications that can lead to protein activation or deactivation, similar to an on/off switch (19), and might regulate GK activity in smelt liver as the protein reported in this paper features potential phosphorylation sites (data not shown). Whatever is the way of regulation of the protein activity, GK expression was very low consistent with the low activity of enzyme.

As glycerol accumulates to the same levels in all tissues (7), GK expression in liver did not show any change over the season and was not different between ambient and warm smelt. Only one sequence that encodes for a functional GK was cloned from smelt liver but the possibility of different GK isoforms present in liver cannot be excluded. Primers designed for qPCR might anneal to other isoforms having the same conserved region and would result in a dilution of the results and thus a failure to detect any change in a specific isoform. Alternatively, regulation of GK activity might happen after mRNA synthesis. A change in protein content can occur at a posttranscriptional level without any change in the mRNA levels following different mechanisms that regulate mRNA activity (38). A change in GK activity can also occur with no change in the protein content at a posttranslational level. Regulation of enzyme activity through phosphorylation/dephosphorylation is one of the most common posttranslational modifications that can lead to protein activation or deactivation, similar to an on/off switch (19), and might regulate GK activity in smelt liver as the protein reported in this paper features potential phosphorylation sites (data not shown). Whatever is the way of regulation of the protein activity, GK expression was very low consistent with the low activity of enzyme.

As glycerol accumulates to the same levels in all tissues (7), GK activity was measured elsewhere to assess any significant role of extra hepatic function of this enzyme during the glycerol cycle. GK activity was measured in heart, brain and gills, as they are important tissues for basic function, and in white muscle (WM), as it is a substantial portion of the body (estimated to be 40%). No activity was detected in gills, and the technique used did not allow for accurate measurements in the brain. The latter result is not unexpected, as low activities were reported in brain of mammals (18); however, we expected some in gills, as this tissue is a major site of glycerol loss in smelt (26). GK activities measured in white muscle and heart in smelt are not unexpected, since comparable activities were previously reported in other fish species (21), but activities similar to the ones measured in liver are quite surprising. In mammals, liver is responsible for about three-fourths of the total body capacity of glycerol clearance (18). The similarity of GK activity among the different tissues in smelt might reflect a difference between mammals and fish with respect to the function of glycerol. No change of GK activity in both white muscle and heart over the glycerol cycle support a minor role of this enzyme in the control of glycerol levels and highlight the important metabolic function of GK in liver where activity is upregulated at the end of the glycerol cycle.

![Fig. 6. A: rate of glycerol loss from smelt (average ± SE) and associated water temperature at different points of the glycerol cycle (n = 4 trials per point). B: rate of glycerol loss as a function of glycerol concentration in smelt plasma for the increase and decrease periods of the cycles. C: daily %glycerol loss at different points of the glycerol cycle (n = 4 trials/point).](http://ajpregu.physiology.org/ by 10.220.32.247 on July 4, 2017)
Perspectives and Significance

We have investigated the respective roles of direct loss of glycerol to water and GK in the glycerol cycle repeatedly observed in winter in smelt. The loss of glycerol to water is consistent with a passive diffusion process with no mechanisms to retain glycerol during the accumulation stage. Our study does not rule out the possibility of more complex processes being involved at the level of gill, skin, and/or kidney, as there may be tissue differences in the mechanisms of glycerol movement across cell membranes.

Based on GK activities, no metabolic mechanism is in place to accelerate the decrease of glycerol from mid-February. Glycerol levels seem to be only dictated by the rate of glycerol synthesis that must be accelerated during the accumulation period and deactivated during the decrease period. The direct dephosphorylation of the G3P to glycerol has been proposed to be the pathway used in smelt to directly produce glycerol (5, 8), as it is the case in some algae and yeasts producing high amounts of glycerol in response to environmental stresses (23, 37). Activity of such an enzyme has been suggested in oxygen-limited rat heart and brain, although neither the protein nor the transcript have been identified in a vertebrate (4, 22). Isolation and characterization of such an enzyme would be of interest, as it would open a new avenue of research concerning the role of glycerol in vertebrates.

Although having a minor role in glycerol clearance, GK in liver appears to have an important metabolic function as evidenced by the increase in activity late in the cycle in this tissue only. An elevated GK activity at the end of the cycle might be related to the restoration of glycogen stores in liver suggested previously (7). Regulation of GK activity may be controlled by small increases in water temperature above the winter minima. How this is achieved is yet to be resolved.

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DISCLOSURES

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REFERENCES

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