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Glycerol production in rainbow smelt (*Osmerus mordax*) may be triggered by low temperature alone and is associated with the activation of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase

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Summary

Rainbow smelt (*Osmerus mordax*) accumulate high levels of glycerol in winter that serves as an antifreeze. Fish were subjected to controlled decreases in water temperature and levels of plasma glycerol, liver metabolites and liver enzymes were determined in order to identify control mechanisms for the initiation of glycerol synthesis. In two separate experiments, decreases in temperature from 8°C to 0°C over a period of 10–11 days resulted in increases in plasma glycerol from levels of less than 4 mmol l^{-1} to approximate mean levels of 40 (first experiment) and 150 mmol l^{-1} (second experiment). In a third experiment, decreases in temperature to –1°C resulted in plasma glycerol levels approaching 500 mmol l^{-1}. The accumulation of glycerol could be driven in either December or March, thus eliminating decreasing photoperiod as a necessary cue for glycerol accumulation. Glycerol accumulation in plasma was associated with changes in metabolites in liver leading to increases in the mass action ratio across the reactions catalyzed by glycerol-3-phosphate dehydrogenase (GPDH) and glycerol-3-phosphatase (G3Pase). The maximal, *in vitro* activity of GPDH, increased twofold in association with a sharp increase in plasma glycerol level. The metabolite levels and enzyme activities provide complementary evidence that GPDH is a regulatory site in the low temperature triggered synthesis of glycerol. Indirect evidence, based on calculated rates of *in vivo* glycerol production by liver, suggests that G3Pase is a potential rate-limiting step. As well, transient increases in glyceraldehyde-3-phosphate dehydrogenase and alanine aminotransferase suggest that these sites are components of a suite of responses, in rainbow smelt liver, induced by low temperature.

Key words: rainbow smelt, *osmerus mordax*, glycerol, glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, low temperature, freeze resistance.

Introduction

Rainbow smelt (*Osmerus mordax*) is a small fish (up to 20 cm in length) that remains active and forages for food in icy seawater during winter. Rainbow smelt avoid freezing by producing an antifreeze protein (Ewart and Fletcher, 1990) and by accumulating high concentrations of organic solutes, especially glycerol (up to 400 mmol l^{-1}), that lower the freeze point colligatively (Raymond, 1992; Raymond, 1993). Plasma glycerol levels in rainbow smelt are sub-millimolar from spring to late summer. Under conditions of natural water temperature and seasonal photoperiod, glycerol begins to accumulate in the plasma when temperature reaches approximately 5°C in November. As the water temperature decreases further, over the next 4–6 weeks, to about 2°C, there is a sharp increase in plasma glycerol level to about 100 mmol l^{-1}. As winter progresses, glycerol increases to values in excess of 250 mmol l^{-1} (Treberg et al., 2002b; Lewis et al., 2004). Temperature has a regulatory role in glycerol accumulation, as maintenance of fish at warm (8°C) temperatures over the winter suppress glycerol accumulation (Lewis et al., 2004) and premature exposure of fish to cold temperatures for 3 weeks in the fall results in glycerol accumulation (Raymond et al., 1996). In this study, we assess if the onset of glycerol accumulation is due to a temperature trigger alone or whether a shortening photoperiod is required. Photoperiod was considered to be a potential requirement since in another freeze resistant species, winter flounder, light and not temperature, is the main environmental factor regulating antifreeze production (Fletcher et al., 1998). All studies, to date, that show glycerol accumulation in rainbow smelt have involved decreases in temperature in association with shorter day lengths. Assessing the direct impact of temperature on the metabolic response leading to freeze resistance was also considered to be important since in rainbow smelt the decrease in glycerol level in spring

is not coupled to an increase in temperature *per se* (Treberg et al., 2002b; Lewis et al., 2004).

In the synthetic pathway leading to glycerol production, dihydroxyacetone phosphate (DHAP) is converted to glycerol 3-phosphate (G3P) and subsequently to glycerol *via* the reactions catalyzed by glycerol-3-phosphate dehydrogenase (GPDH) and glycerol-3-phosphatase (G3Pase), respectively. This contention is based on higher maximal *in vitro* activities of GPDH and G3Pase in rainbow smelt liver compared to other species that do not produce glycerol (Driedzic et al., 1998; Treberg et al., 2002a), a correlation between liver GPDH activity and plasma glycerol level over a seasonal time frame (Lewis et al., 2004), decreases in liver GPDH mRNA associated with decreases in plasma glycerol level when cold-acclimated fish are transferred to warm water (Ewart et al., 2001), and increases in liver GPDH mRNA in association with the initial seasonal increases in plasma glycerol level (Liebscher et al., in press). We report here the relationship between acute decrease in temperature and indices of metabolic activation at GPDH and G3Pase.

Radioisotope and stable isotope studies reveal that the carbon sources of glycerol are glycogen/glucose and free amino acids (Raymond, 1995; Raymond and Driedzic, 1997; Walter et al., 2006). It is likely that liver is the exclusive or at least the major site of glycerol synthesis. Isolated liver sections produce glycerol (Driedzic et al., 1998), glycogen levels are much higher in liver than other tissues of rainbow smelt (Short and Driedzic, unpublished), and liver glycogen decreases in association with the accumulation of glycerol in plasma, liver, and muscle during the fall-winter transition (Treberg et al., 2002b). The importance of amino acids as a fuel for glycerol production in liver *via* a truncated gluconeogenesis, referred to as glyceroneogenesis (Hanson and Reshef, 2003), is consistent with higher activities of enzymes associated with amino acid metabolism in rainbow smelt captured in winter than other non-glycerol producing species captured at the same time (Driedzic et al., 1998). In addition, on a seasonal basis, there is a correlation between *in vitro* alanine aminotransferase (AlaT) and phospho-enolpyruvate carboxykinase (PEPCK) activities in liver with plasma glycerol levels (Lewis et al., 2004) and a similar pattern of PEPCK mRNA levels in liver as plasma glycerol (Liebscher et al., in press). Another enzyme that is potentially critical to increases in glycerol production is glyceraldehyde phosphate dehydrogenase (GAPDH) as this enzyme is a necessary component of gluconeogenesis.

Although there is now an understanding of the metabolic pathways leading to glycerol production and evidence that the activities of some enzymes are altered in concert with this production, details of the control mechanisms are yet to be fully defined. In the current experiment, the impact of a controlled and acute decrease in water temperature on glycerol production is determined at different times of the year. Levels of key metabolites and activities of enzymes were measured to assess control points of glycerol production during the transition state. The most important new findings are that a decrease in temperature alone is sufficient to activate glycerol production and that an increase in GPDH activity plays a critical role in the early stages of the process.

Materials and methods

Animals

Rainbow smelt (*Osmerus mordax* Mitchill) were collected by seine netting from Long Harbour, Placentia Bay, Newfoundland in late October 2001 and 2002, transported to the Ocean Sciences Centre, Memorial University of Newfoundland, and transferred to 3000 l (2001) or 1800 l tanks (2002) with flow-through seawater. Fish were kept on a natural photoperiod with fluorescent lights set on an outdoor photocell, and fed a diet of chopped herring twice a week. Stock fish were held at approximately 8°C from the time of capture to the initiation of experiments. Whole animal temperature transition or 'step down' experiments were performed in March 2002 (collected in October. 2001), December 2002 (collected in October 2002) and March 2003 (collected in October 2002). For these experiments, smelt were transferred to a 500 l tank set at 8°C, two weeks prior to a controlled decrease in temperature. Mass of smelt in March 2002, December 2002 and March 2003 studies was 45.3±1.4 g (*N*=42), 51.6±1.5 g (*N*=42), and 39.7±1.5 g (*N*=63), respectively. There was no significant difference in initial condition factor amongst the three groups which overall was 0.76±0.02 and decreased between 8 and 18% during the course of the experiments, dependent upon the length of time held at decreased temperature. Initial hepatosomatic index (HSI) was 1.63±0.22 (*N*=6) and 1.17±0.1 (*N*=12) for the December 2002 and March 2003 experiments, respectively. This significant difference between groups may be due to the holding of the same population of fish for a longer period at elevated temperatures and laboratory feeding protocols. HSI did not change over the course of the temperature decrease challenges in either experiment. HSI was not recorded for the March 2002 study. All fish remained in apparent good health as evidenced by colour and activity patterns.

Experimental protocol

March 2002

The purpose of this initial experiment was to determine if a rapid temperature decrease, is sufficient to activate glycerol accumulation. Furthermore, this study was conducted when day length was increasing, such that photoperiod should not be a trigger. Water temperature on day 0 was 8°C and was then decreased to 5, 3, 1, 0 and –1°C on days 3, 6, 8, 10 and 13, respectively. On day 0 of the experiment, fish were randomly selected, weighed, measured for length, and blood was drawn *via* the caudal vessel. Fish were then killed with a blow to the head, and livers were removed, freeze clamped and subsequently stored at temperatures below –65°C. Blood was centrifuged at 9300 ***g*** immediately after sampling, plasma was collected and frozen in liquid nitrogen. Blood and liver samples were taken at each time point similar to day 0.

December 2002

The goals of this experiment were to repeat the March 2002 study at a time when day length was decreasing and to determine changes in levels of metabolites associated with glycerol production. The experimental protocol was similar to that of March 2002, except sampling occurred on days 0, 2, 4, 8, 11, 15 and 18 at water temperatures of 8, 5, 3, 1, 0, –0.5 and –0.5°C, respectively. Glycerol assays were performed on both the liver and plasma. Liver was also analyzed for the following: pyruvate, dihydroxyacetone phosphate (DHAP), lactate, glycerol 3-phosphate (G3P), glucose and inorganic phosphate (P_i).

March 2003

The objectives of this study were to increase the time at –1°C to assess if the phase of glycerol accumulation could be extended and to determine activity levels of enzymes associated with glycerol production. Sampling occurred on days 0, 2, 4, 10, 18, 25 and 29, at water temperatures of 8, 5, 3, 1.5, 0, –1 and –1°C, respectively. Glycerol was measured in the plasma as described above. Liver was assayed for the following enzymes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) and alanine aminotransferase (AlaAT, E.C. 2.6.1.2).

Biochemical assays

Glycerol level in the plasma was determined directly using a colorimetric detection kit (F6428, Sigma-Aldrich, MO, USA). Samples were read at 540 nm after a 15-min incubation at room temperature. Liver was homogenized in nine volumes of 10% perchloric acid, the homogenate was centrifuged at 1500 ***g*** and the supernatant was assayed for glycerol as described above.

For metabolite analysis, pieces of frozen liver were weighed and homogenized in nine volumes of ice-cold 6% perchloric acid. Following homogenization, samples were centrifuged at 10 000 ***g*** in an Eppendorf centrifuge for 10 min at 4°C and neutralized with 5 mol l^{-1} KOH. Pyruvate and DHAP assays were performed immediately; lactate, G3P, glucose and P_i assays were performed on the frozen extracts 1–2 weeks later. All assays were performed on a Beckman DU640 spectrophotometer at 340 nm with the exception of P_i measurements. Assay conditions were as follows.

Pyruvate and DHAP: 100–200 μl of liver extract were added to 50 mmol l^{-1} triethanolamine buffer (pH 7.5 at room temperature) and 0.6 mmol l^{-1} reduced nicotinamide-adenine dinucleotide (NADH). Samples were read after 10 min before adding either 45 IU ml^{-1} lactate dehydrogenase (LDH) for the pyruvate assay or 1 IU ml^{-1} of GPDH for the DHAP assay. Absorbances were read for another 20 min or until stable.

Lactate and G3P: 30-100 μl of liver extract were added to an assay medium containing glycine buffer (Sigma, 8263) and 2.5 mmol l^{-1} oxidised nicotinamide-adenine dinucleotide (NAD^+), pH 9.0 at room temperature. Samples were read after 10 min before adding 30 IU ml^{-1} LDH for the lactate assay or 3.5 IU ml^{-1} GPDH for the G3P assay. Absorbances were read for another 30 min or until stable.

Glucose: assay conditions were based on a procedure modified from the method of Bergmeyer (Bergmeyer, 1974). Briefly, 100 μl of liver extract were diluted 1:10 with the assay medium (250 mmol l^{-1} imidazole, 5 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} ATP and 0.8 mmol l^{-1} $NADP^+$). 10 μl of glucose-6-phosphate dehydrogenase were added to remove any endogenous glucose 6-phosphate. Absorbance was read after 10 min, and hexokinase was then added and the absorbance read after 25–30 min.

P_i: assay conditions were based on those of Rockstein and Herron (Rockstein and Herron, 1951). A 200 μl sample of liver extract was added to 200 μl 3.3% ammonium molybdate in 5 N sulphuric acid and 400 μl distilled water. Colour was initiated by adding 200 μl of 0.26 mol l^{-1} ferrous sulfate and the absorbance was read at 700 nm after 12–14 min.

For enzyme activity analysis, pieces of frozen liver were weighed and homogenized in nine volumes of ice-cold extraction buffer (20 mmol l^{-1} imidazole, 5.0 mmol l^{-1} EGTA, 5.0 mmol l^{-1} EDTA, 10 mmol l^{-1} mercaptoethanol, 50 mmol l^{-1} sodium fluoride and 0.1 mmol l^{-1} phenylmethylsulfonyl fluoride, pH 7.4 at 4°C) and centrifuged at 10 000 ***g*** for 10 min at 4°C. All three enzyme assays were performed immediately following homogenization and centrifugation. Control reaction rates were determined prior to the addition of the substrate. Assay conditions for GAPDH and AlaAT are described by Treberg et al. (Treberg et al., 2002a). GPDH assays conditions included 20 mmol l^{-1} imidazole, pH 7.2 at 20°C, 0.15 mmol l^{-1} NADH and 2 mmol l^{-1} DHAP that initiated the reaction. Enzyme activities determined at 340 nm were calculated based on the millimolar extinction coefficient of 6.22. All enzyme activities were determined at 20°C to facilitate analysis at higher activity levels since we were primarily interested in relative not absolute activities during exposure of fish to decreased temperature. This was considered acceptable with the assumption that enzyme activity is proportional to enzyme content.

Data analysis

Mass action ratio across GPDH was calculated as: [G3P][pyruvate]/[DHAP][lactate]. This is based on the premise that the LDH reaction is at equilibrium and, as such, the [pyruvate]/[lactate] ratio reflects the [NADH]/[NAD^+] ratio. The mass action ratio across G3Pase was calculated as: [glycerol][P_i]/[G3P]. Means were compared with a one-way analysis of variance (ANOVA) for all measurements followed by Tukey's *post-hoc* test. A P value of <0.05 was considered to be statistically significant for all studies.

Results

Plasma glycerol

Rainbow smelt maintained at 8°C and sampled in March 2002, December 2002 and March 2003, had very low plasma glycerol levels of 0.8, 3.7 and 1.1 mmol l^{-1}, respectively (Fig. 1). When fish were challenged with a drop in water temperature from 8 to 0°C over 10–11 days (March 2002 and

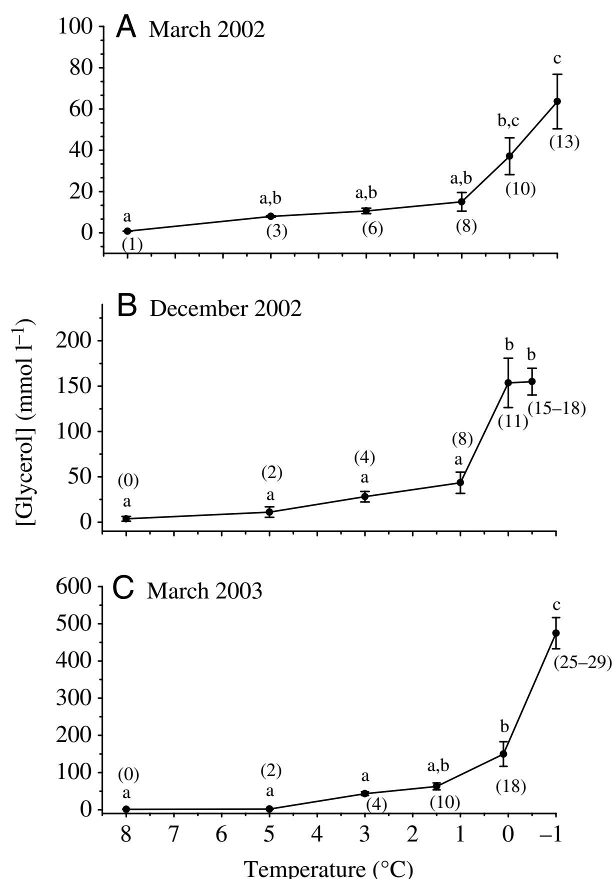

Fig. 1. Plasma glycerol levels in rainbow smelt subjected to a controlled decrease in temperature during three separate experiments: (A) March 2002; (B) December 2002; (C) March 2003. Days following the temperature transition are shown in parentheses. Values are means ± s.e.m. In March 2002 and December 2002 experiments *N*=6 at each temperature with the exception of December 2002, –0.5°C where *N*=12. In the March 2003 experiment *N*=12 for all points except temperatures of 1.5°C where *N*=10 and 0°C where *N*=11. Different letters indicate significant differences between two temperatures.

Dec. 2002; Fig. 1A,B), a significant increase in plasma glycerol occurred ($P<0.001$). A further drop from 0°C to –1°C in the Mar 2002 study, resulted in a plasma glycerol level of 64 mmol l^{-1}. These experiments show that a temperature decrease alone is sufficient to drive an increase in glycerol production and that the glycerol increase is not photoperiod dependent. Fish challenged with a decrease in temperature in the March 2003 experiment (Fig. 1C) showed a similar pattern of glycerol increase ($P<0.001$). Rainbow smelt kept at –1°C, to day 29, accumulated extremely high levels of plasma glycerol (484 mmol l^{-1}).

Metabolite analysis (December 2002 study)

A significant increase in liver glycerol paralleled that measured in the plasma (compare Fig. 2 and Fig. 1B). Liver glycerol levels increased significantly ($P<0.01$) as compared to 8°C values once temperatures reached 0°C on day 11. Liver glycerol level was substantially lower than plasma glycerol at this same time point, 57 μmol g^{-1} in liver, compared to 154 μmol ml^{-1} in plasma. Lower levels of glycerol also occurred in liver than in plasma at –0.5°C on days 15–18 following the transition. Glycerol synthesis requires the conversion of either glucose/glycogen or amino acids by DHAP and G3P. Significant (P=0.011) changes occurred in DHAP levels as a function of time. There was a tendency for DHAP to increase during the initial decrease in water temperature with maximum values occurring at 3°C; this was followed by a significant decrease to the lowest value at –0.5°C. Neither G3P nor P_i, one of the breakdown products of G3P, showed significant change as temperature decreased. Pyruvate, a potential substrate for glyceroneogenesis showed a tendency to change over temperature (P=0.068) with the mean level being lower at –0.5°C (0.14±0.02 μmol g^{-1}) than at 8°C (0.25±0.03 μmol g^{-1}). Lactate did not change significantly over the course of the experiment. The level of glucose in liver changed with temperature (P=0.003) with the value at –0.5°C being lower than the highest amount noted at 0°C.

The mass action ratio across GPDH and GPase were calculated from the various metabolites measured above. The overall GPDH mass action ratio (Fig. 3A) changed significantly (P=0.033) as temperature decreased. Although the analysis did not reveal any significant difference between specific points, the mean value at –0.5°C (14.3±2.5) was three times as high as the ratio at 1°C (4.7±1.2). The mass action ratio across G3Pase (Fig. 3B) increased significantly (P=0.023) as the temperature decreased, peaking at 0°C.

Enzyme activity levels (March 2003 study)

Activities of key enzymes involved in gluconeogenesis (GAPDH), the terminal steps to glycerol synthesis (GPDH) and amino acid breakdown (AlaAT), either changed significantly or showed a strong tendency to change as temperature was decreased (P=0.056, P=0.034 and P=0.052, respectively) (Fig. 4). Mean activity levels of all three enzymes were higher at 0°C, compared to warmer temperatures in association with an increase in plasma glycerol (compare Fig. 4 and Fig. 1C). The elevation in plasma glycerol was associated with a 2.1-fold change in mean liver GPDH from 136±25 to 292±51 μmol g^{-1} min^{-1} between 1.5°C and 0°C. Average GAPDH and AlaAT activities were 1.5- and 1.4-fold higher, respectively during this transition.

Discussion

Plasma and liver glycerol levels

Plasma glycerol begins to increase as temperature falls below 3–5°C. This contention is based on the gradual elevation in mean plasma level shown in each of three independent experiments. This is followed by sharp increases in glycerol levels at temperatures of 1°C to 0°C. These findings are very similar to changes observed under natural decreases in water temperature and photoperiod (Lewis et al., 2004). The level of

glycerol in liver followed a similar pattern to plasma glycerol, with significant elevation relative to the initial level, in association with temperatures of 0°C and below. A decrease in temperature alone is sufficient to result in an accumulation of glycerol. A decrease in photoperiod is not a requirement for glycerol production as the low temperature response may be induced in either December (shortening day length) or in March (increasing day length). It was previously shown that rainbow smelt maintained at high temperature do not accumulate glycerol when subjected to seasonal decreases in photoperiod (Lewis et al., 2004). Taken together these studies reveal that a lowering of temperature is the key environmental cue to glycerol accumulation, whereas photoperiod is not a factor.

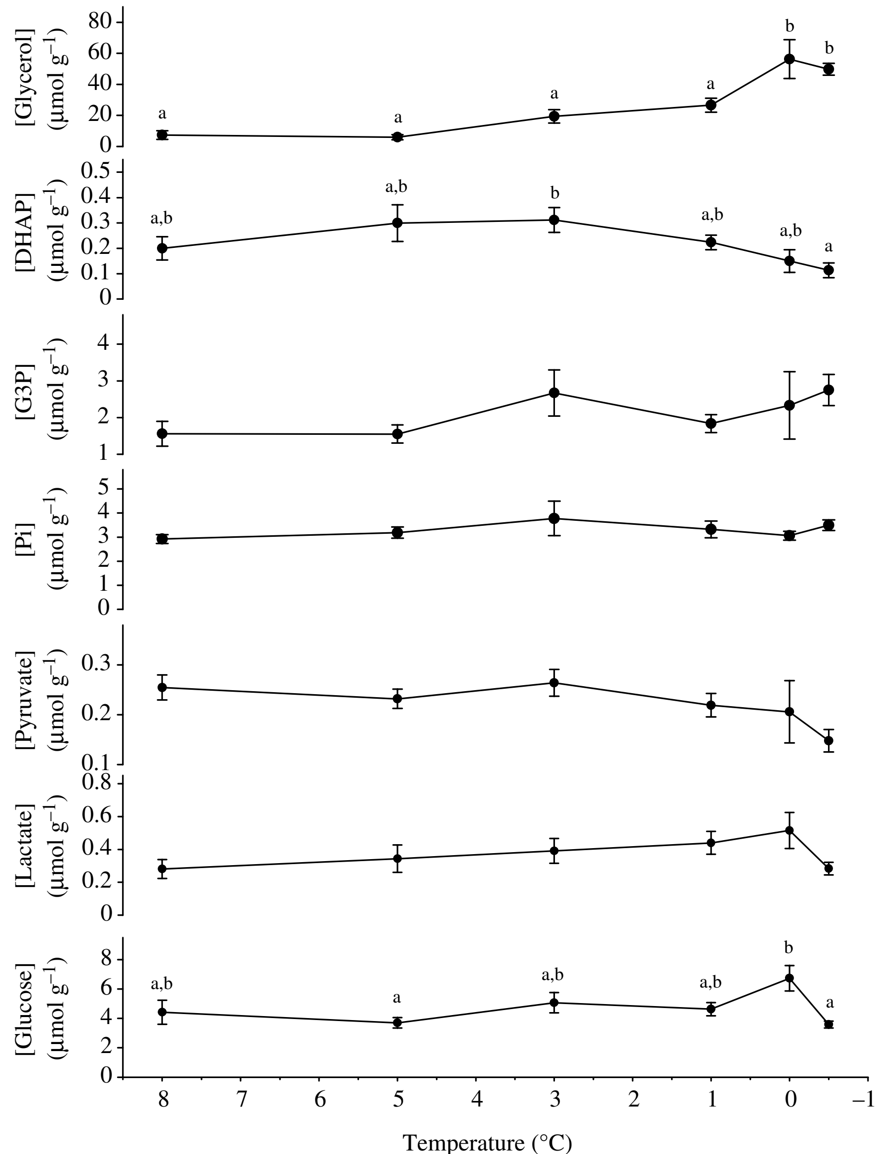

Fig. 2. Glycerol, dihydroxyacetone phosphate (DHAP), glycerol 3-phosphate (G3P), inorganic phosphate (P_i), pyruvate, lactate and glucose levels in liver of rainbow smelt subjected to a controlled decrease in temperature. Values are means ± s.e.m., N=6 at each point with the exception of –0.5°C where N=12. Different letters indicate significant differences between values. Transition time and plasma glycerol levels for this experiment are presented in Fig. 1B.

Once elevated levels in plasma are achieved, the concentration of glycerol in intracellular water must be substantially lower than that in the extracellular space given levels of 50 μmol g^{-1} liver *vs* 150 μmol ml^{-1} in plasma (December 2002 study). This finding is consistent with the ratio of plasma to liver glycerol in rainbow smelt following seasonal changes in temperature (Treberg et al., 2002b). The true difference in glycerol between the two compartments awaits the accurate determination of intracellular water. Regardless, the qualitative observation implies an active export of glycerol from liver cells to plasma against a concentration gradient. The yeast, *Saccharomyces cerevisiae*, actively transports glycerol into the cell (Holst et al., 2000), whether similar active transport mechanisms exist in rainbow smelt to move glycerol out of liver into the plasma is not known. An aquaglycerporin cDNA from sea bream, a marine teleost, has been sequenced and when expressed in *Xenopus* oocytes results in an increase in glycerol permeability (Santos et al., 2004). A similar mRNA transcript has been detected in rainbow smelt liver (J. R. Hall and W.R.D., unpublished) and may play a role in glycerol permeability; however, an aquaglyceroporin, which could serve as a glycerol conduit, in itself cannot account for the large extra- to intracellular differences in glycerol levels.

The maximal rate of glycerol synthesis in liver may be estimated from the increase in plasma glycerol. A consideration of the Dec. 2002 study between days 8 and 11 serves as an example. Over this time period, plasma glycerol increased from 44 to 153 mmol l^{-1}. A typical body mass for rainbow smelt is 50 g, of which about 5 g are bone. Although glycerol content may be lower in liver than in plasma, the content in most other soft tissues is similar to that of plasma (Raymond, 1992). Thus, the increase in glycerol in the total fish between days 8 and 11 would approximate 153 μmol g^{-1} (i.e. level at day 11)–44 μmol g^{-1} (i.e. level at day 8)×45 g, which equals 4905 μmol. A typical liver mass, in a 50 g rainbow smelt is 0.75 g. Accepting that liver is the primary site of glycerol formation, the rate of glycerol production over the 3 days would be 4905 μmol glycerol×0.75 g^{-1}×4320 min^{-1} which equals 1.5 μmol glycerol g^{-1} min^{-1}. Given that the loss of glycerol to water is about 10% per day (Raymond, 1993),

our best estimate of the rate of glycerol production by liver is 1.65 μmol glycerol g^{-1} min^{-1} at 0°C. Similar calculations for the March 2003 experiment between days 18 and 25 and for March 2002 between days 8 and 13 yield rates of 1.5 and 0.5 μmol g^{-1} min^{-1}, respectively.

Sites of metabolic regulation

GPDH catalyzes the conversion of DHAP plus NADH to G3P plus NAD^+. Independent assessment of metabolite levels and enzyme activities suggests that this site is critical to activation of glycerol production during acute low temperature challenge. The initial stages of glycerol production are associated with a transient increase in DHAP followed by a decrease in this metabolite. The mass action ratio of this reaction increased at 0.5°C, relative to higher temperatures. The mass action ratio is calculated on the premise that the reaction catalyzed by LDH remains in equilibrium and thus the pyruvate/lactate ratio reflects the NAD^+/NADH ratio. This assumption may not be correct during the transition period to high rates of glycerol production and may account for the calculated change in mass action ratio occurring only after steady state levels of liver glycerol are achieved. As such, although fine details of the temporal analysis may be in question, activation of GPDH based on metabolite analysis, is revealed at high levels of glycerol accumulation. In a separate experiment, there was a 2-fold change in, *in vitro* GPDH activity at 0°C relative to 1.5°C, in association with increases in plasma glycerol level. Again this implies that this site is critical in the process of high rates of glycerol production at low temperature. The maximal *in vitro* activity of GPDH is ~275 μmol g^{-1} min^{-1} at 20°C. Assuming a Q_{10} of 2 this would equate to 68 μmol g^{-1} min^{-1} at 0°C, a value that is well in excess of the calculated rate of glycerol production by liver. Even if the Q_{10} was higher, for instance, if the Q_{10} equalled 4,

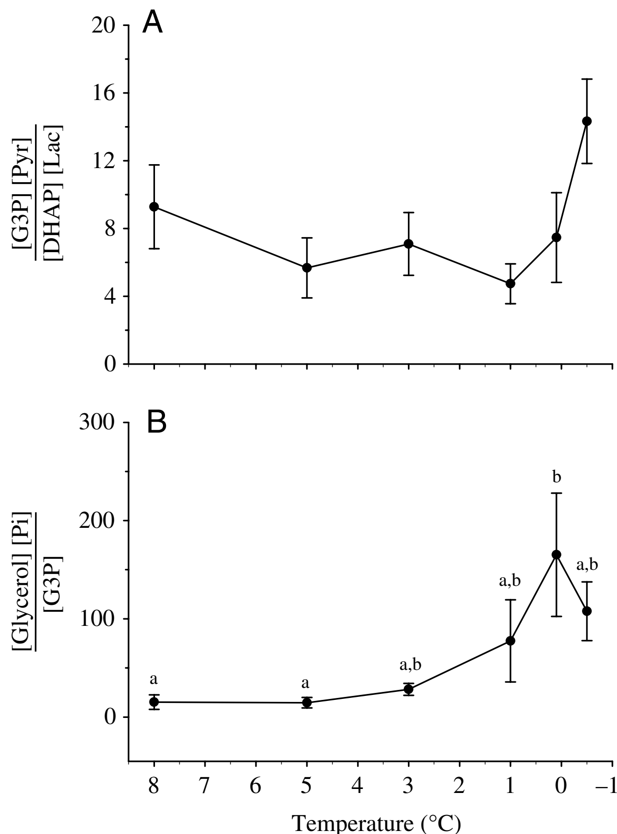

Fig. 3. Mass action ratios in liver of rainbow smelt subjected to a controlled decrease in temperature: (A) across glycerol-3-phosphate dehydrogenase; (B) across glycerol 3-phosphatase. Values are means ± s.e.m., *N*=6 at each point with the exception of –0.5°C, where *N*=12. Different letters indicate significant differences between values. Transition time and plasma glycerol levels for this experiment are presented in Fig. 1B.

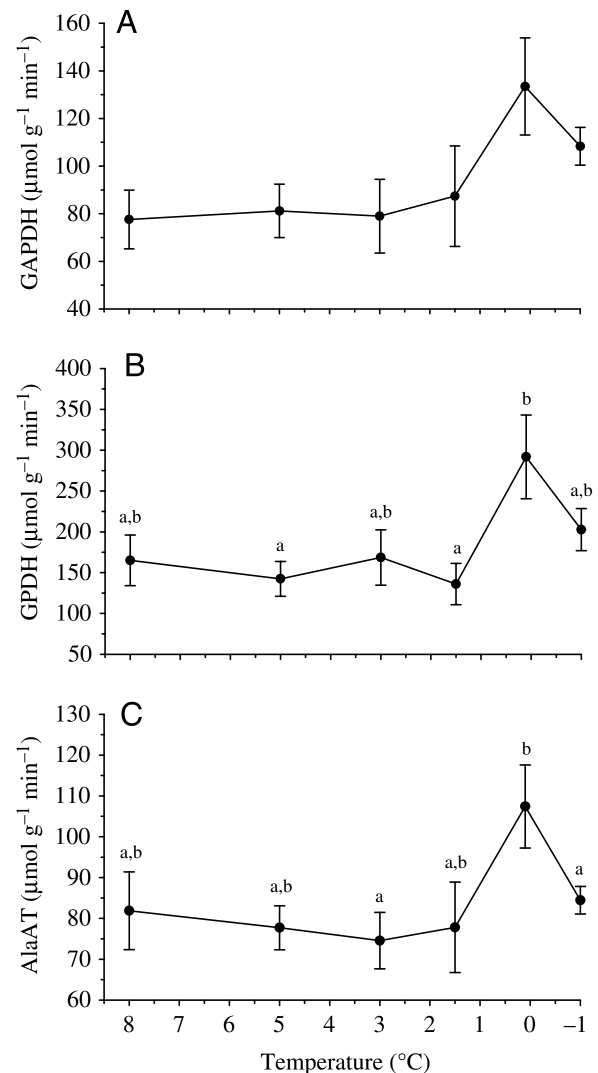

Fig. 4. Activities of enzymes in liver of rainbow smelt subjected to a controlled decrease in temperature: (A) glyceraldehyde-3-phosphate dehydrogenase (GAPDH); (B) glycerol-3-phosphate dehydrogenase (GPDH); (C) alanine aminotransferase (AlaAT). Values are means ± s.e.m., *N*=6 at each point with the exception of 1.5°C, where *N*=5, and –1°C, where *N*=11. Different letters indicate significant differences between values. Transition time and plasma glycerol levels for this experiment are presented in Fig. 1C.

the maximal enzyme activity would still be more than 10 times the estimated rate of *in vivo* glycerol production. The high constitutive activity of total GPDH, measured in homogenates, needs to be reconciled with the far lower estimated rates of flux across this reaction. There are at least 2 genes encoding cytosolic GPDH in rainbow smelt (K.V.E., R. S. Richards and W.R.D., unpublished). Crude enzyme activity measurements report the activities of all encoded GPDH isoforms, whereas it may be that only one form is involved in the cold-induced mechanisms of glycerol production. Although there is now considerable data that points to the importance of GPDH as an important regulatory enzyme in glycerol production, there is mounting evidence that other enzymes contribute to control of the process as well.

The final step in glycerol production requires the conversion of G3P to glycerol plus P_i catalyzed by G3Pase. The importance of G3Pase to glycerol production was first implied by maximal *in vitro* enzyme activities in liver, which were significantly higher in rainbow smelt than two other species of non-glycerol producing species captured in winter (Driedzic et al., 1998). In the current experiment, G3P and P_i levels in liver remain constant as glycerol increases in association with a decrease in temperature. As such, the increase in the concentration of products, relative to substrate results in an increase in the mass action ratio across the reaction catalyzed by G3Pase after the initiation of the temperature transition. The maximal *in vitro* activity of G3Pase at 20°C, previously reported (Driedzic et al., 1998), was 1.95 μmol g^{-1} min^{-1}; a similar activity was found by Raymond and Hassel (Raymond and Hassel, 2000). Assuming a Q_{10} of 2, the rate of G3Pase activity would approximate 0.5 μmol g^{-1} min^{-1} at 0°C; a Q_{10} of 4 would result in a rate of 0.12 μmol g^{-1} min^{-1}. Given these assumptions, the calculated rates of glycerol production by liver *in vivo* and the maximal activity of G3Pase *in vitro* are closely matched. Taken together, the change in mass action ratio in association with an increase in glycerol production and the similarity in estimated rates of glycerol production to measurements of *in vitro* activity of G3Pase, suggests that the reaction catalyze by G3Pase is rate limiting and regulatory.

The three enzymes (GPDH, GAPDH, AlaT) measured in this study, all follow the same pattern, with an increase in average activity between days 10 and 18, in association with the upswing in plasma glycerol level. It is unlikely that the increases in mean activity of the three enzymes is spurious but rather reflects change in metabolic organization. AlaAT is required to channel carbon from alanine into pyruvate. Increase in activity of this enzyme is consistent with a correlation between maximal *in vitro* activity in liver and plasma glycerol level during seasonal change in temperature (Lewis et al., 2004) and with nuclear magnetic resonance findings that show neighboring carbons from injected alanine appearing in glycerol (Walter et al., 2006). GAPDH is required for glyceroneogenesis from pyruvate and amino acids. Activities of both AlaT and GAPDH are well in excess of calculated rates of glycerol production and G3Pase. Although it is unlikely these sites are rate limiting, an increase in activity of these reactions is a component of the suite of low temperature responses of rainbow smelt and may contribute to metabolic control.

General conclusions

In rainbow smelt, a decrease in water temperature to less than 5°C, is sufficient to activate the metabolic processes leading to glycerol accumulation. This anticipatory response is similar to activation of glycerol production in a number of insects (Storey and Storey, 1988) and in the gray treefrogs, *Hyla veriscolor and H. chrysoscelis,* where an increase in plasma and organ glycerol occurs in response to extended cold acclimation at temperatures above 0°C (Layne and Jones, 2001; Irwin and Lee, 2003). This situation differs from the accumulation of glucose in these frogs and other frog species that occurs only in response to freezing (Storey and Storey, 1992; Layne and Jones, 2001; Irwin and Lee, 2003).

Glycerol accumulation is associated with activation of GPDH. This contention is based on metabolite levels reported here, enzyme activities reported here and elsewhere (Driedzic et al., 1998; Lewis et al., 2004), and changes in liver GPDH mRNA in the direction of changes in plasma glycerol level (Liebscher et al., in press); however, the high *in vitro* rates of GPDH activity relative to calculated rates of glycerol production remains to be resolved. It may be that in rainbow smelt liver there is a specific GPDH isoform involved in temperature-activated glycerol production. The reaction catalyzed by G3Pase also appears to be important in the metabolic sequence leading to glycerol production. This position is based on mass action ratios of metabolites at this reaction and the similarity between calculated maximal rates of glycerol production *in vivo* compared to the maximal activity of the enzyme *in vitro*. On the basis of levels of three carbon intermediates and glycerol, Churchill and Storey (Churchill and Storey, 1989) proposed that in the larvae of *Epiblema scudderiana*, G3Pase is subject to activation and then inhibition, during the transition to accumulation of glycerol and subsequent maintenance of high glycerol content. How G3Pase activity is regulated in high glycerol producing animals is yet to be addressed. Increases in the activity of other enzymes, including AlaT and GAPDH are also associated with the transition to glycerol accumulation.

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