Freeze Resistance in Rainbow Smelt (*Osmerus mordax*): Seasonal Pattern of Glycerol and Antifreeze Protein Levels and Liver Enzyme Activity Associated with Glycerol Production

Johanne M. Lewis[1]
K. Vanya Ewart[2]
William R. Driedzic[1,*]
[1]Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland A1C 5S7, Canada; [2]National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada

Accepted 9/29/03

ABSTRACT

Rainbow smelt (*Osmerus mordax*) inhabit inshore waters along the North American Atlantic coast. During the winter, these waters are frequently ice covered and can reach temperatures as low as −1.9°C. To prevent freezing, smelt accumulate high levels of glycerol, which lower the freezing point via colligative means, and antifreeze proteins (AFP). The up-regulation of the antifreeze response (both glycerol and AFP) occurs in early fall, when water temperatures are 5°–6°C. The accumulation of glycerol appears to be the main mechanism of freeze resistance in smelt because it contributes more to the lowering of the body's freezing point than the activity of the AFP (0.5°C vs. 0.25°C for glycerol and AFP, respectively) at a water temperature of −1.5°C. Moreover, AFP in smelt appears to be a safeguard mechanism to prevent freezing when glycerol levels are low. Significant increases in activities of the liver enzymes glycerol 3-phosphate dehydrogenase (GPDH), alanine aminotransferase (AlaAT), and phosphoenolpyruvate carboxykinase (PEPCK) during the initiation of glycerol production and significant correlations between enzyme activities and plasma glycerol levels suggest that these enzymes are closely associated with the synthesis and maintenance of elevated glycerol levels for use as an antifreeze. These findings add further support to the concept that carbon for glycerol is derived from amino acids.

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

Physiological and Biochemical Zoology 77(3):415–422. 2004. 1522-2152/2004/7703-3014$15.00

Introduction

Ectothermic animals that live in the northern temperate regions are exposed seasonally to subzero temperatures. To survive such harsh environments, these organisms have developed various strategies to prevent death from freezing (Storey and Storey 1988). One such species is the rainbow smelt (*Osmerus mordax*), which is an anadromous teleost that overwinters in estuaries and inshore waters along the North American Atlantic coastline (Scott and Scott 1988). These waters are frequently ice covered and can reach temperatures as low as −1.9°C in the winter. To survive in this environment, smelt use biochemical means of freeze resistance. As seen in other teleosts that inhabit these inshore waters, smelt produce antifreeze proteins (AFP) to help protect themselves from freezing. However, at water temperatures of −1.5°C, the level of AFP activity (difference between melting and freezing point) in smelt is 0.3°C, which is insufficient to prevent freezing at water temperatures that low (Ewart and Fletcher 1990). It was later found that in smelt, the production of AFP is not the main method of lowering the blood freezing point. Smelt rely mainly on the accumulation of high levels of organic solutes, especially glycerol (200–400 mM), which lower the blood freezing point through colligative properties (Raymond 1992).

A comparison of the liver enzyme profile of winter-caught smelt with two species from the same environment (*Microgadus tomcod* and *Liopsetta putmani*) with low glycerol levels suggested that metabolism in the liver of smelt is specifically designed for increased glycerol production (Driedzic et al. 1998). This finding was further supported by Treberg et al. (2002*a*) in work that compared the liver enzyme profile of smelt to another osmerid, capelin (*Mallotus villosus*), which does not produce high levels of glycerol or AFP but survives the winter by existing in a supercooled state (Raymond and Hassel 2000). In both studies, key enzymes of amino acid breakdown, gluconeogenesis, glycolysis, and glycerol production are all significantly higher in smelt than in the other species (Driedzic et al. 1998; Treberg et al. 2002*a*). Additionally, high levels of the liver enzyme glycerol 3-phosphate dehydrogenase (GPDH) suggest that glycerol production in smelt may occur through the reduction of dihydroxyacetone phosphate (DHAP) to produce glycerol 3-phosphate (G3P; Driedzic et al. 1998; Treberg et al. 2002*a*) as opposed to the pathway favored in insects where glycerol is produced via the dephosphorylation of glyceralde-

hyde phosphate (GAP) to glyceraldehyde (Storey and Storey 1988).

Treberg et al. (2002*b*) examined the antifreeze response in smelt held in a laboratory setting. This work was limited to five time points from December to May and, as such, failed to describe the up-regulation of glycerol production in the fall of the year and the suppression of the freeze response in the spring. It was demonstrated that constant low temperatures are necessary for the maintenance of high levels of osmolytes acting as antifreeze and that the production and loss of glycerol and AFP are asynchronous (Treberg et al. 2002*b*). Glycerol levels and AFP activity at the initial sampling date in December were in the range of 80 mM and 0.2°C, respectively, indicating that the antifreeze response is initiated well before the threat of freezing. The decrease in glycerol levels in smelt exposed to warm temperatures was not associated with decreased activity of liver enzymes required for glycerol production (GPDH and amino acid transferases). Also, like the production of glycerol, any up-regulation of these particular enzymes would have occurred well before the initial sampling date in December (5°C; Treberg et al. 2002*b*).

This study expands on previous work to describe the antifreeze response of rainbow smelt, from initiation of the response in October, through high levels of antifreeze components in winter, to suppression of the response in June. In addition, one group of fish was held at a higher temperature (8°C) than previously (5°C) because glycerol production was shown to occur around 5°C (Treberg et al. 2002*b*); moreover, the full extent of the antifreeze response was assessed by challenging smelt in water that was chilled below ambient water temperatures. Particular emphasis is placed on the biochemical mechanisms of glycerol production by assessment of activities of key enzymes. The activities of liver enzymes involved in the breakdown of amino acids, gluconeogenesis, and glycerol production were explored to determine potential regulatory loci of glycerol synthesis. The extension of the experimental design compared with the Treberg et al. (2002*b*) article expands our understanding of the low temperature response in smelt by revealing that glycerol is important in antifreeze defense early in the season, whereas AFP is more critical in late winter. Additionally, it shows that plasma glycerol levels in animals tracking ambient temperature correlate with maximal liver enzyme activities of GPDH, alanine aminotransferase (AlaAT), and phosphoenolpyruvate carboxykinase (PEPCK).

Material and Methods

Animal Collection

Smelt were obtained from freshwater streams near estuaries in mid-October 2000. Water temperature was approximately 11°C. Fifty smelt were caught by dip net in the Gambo River, and 400 smelt were caught by seine netting in Long Harbour, Placentia Bay, Newfoundland. All fish were transported in freshwater to the Ocean Sciences Centre, Memorial University of Newfoundland, where they were immediately transferred to a 4.0-m^3 tank with flow-through ambient seawater (11°C) with minimal mortality (<1%). For the first 2 wk, fish were fed three times a week with frozen brine shrimp, after which they were switched to a diet of chopped frozen herring for the duration of the study. Fish were exposed to a natural photoperiod with fluorescent lights set on an outdoor photocell. On November 26, 2000 (ambient temperature 6°C), 230 fish were transferred to a second 4.0-m^3 tank with flow-through heated seawater maintained at 7°–12°C throughout the study. On March 14, 2001, 40 fish from the ambient tank were moved to an insulated 1.0-m^3 tank with flow-through chilled seawater to study the effect of extreme cold temperatures on glycerol production. These three tanks comprised the ambient, heated, and chilled seawater treatments, respectively.

Sample Collection

Blood was drawn from five fish from the Gambo River while in the field to obtain an initial blood glycerol level reading. After placement in the ambient tank, five fish were randomly sampled from each treatment at approximately every 1°C drop in ambient seawater temperature. Fish were killed with a blow to the head, and blood was drawn via the caudal vein with a heparinized 25-gauge needle. Livers were removed, cut into sections, and frozen in liquid nitrogen. The treatment of fish and sampling procedures were in accordance with Canadian Council for Animal Care guidelines. Blood samples were stored on ice for approximately 20 min before centrifuging for 5 min. Plasma was then removed, divided into two parts for glycerol and AFP analysis, and frozen in liquid nitrogen. All samples were stored at −80°C until analysis. Each fish sampled was individually numbered, and gonadosomatic index (GSI = gonad weight/somatic weight × 100), sex, and presence of food in the stomach were recorded.

Water temperature and general fish health were observed and recorded for each tank on a daily basis throughout the study. All animals exhibited good health under laboratory conditions, and death rates were low with one exception. The fish held in heated seawater were in good health and had low mortality rates until February–March when they underwent early spawning, presumably due to the elevated seawater temperatures. After spawning, these fish experienced high mortality rates because the experimental population was composed mainly of males (80%), which are one-time spawners (Buckley 1989).

Plasma Glycerol Levels

Plasma samples were analyzed using Sigma diagnostic kit 337-40A. Plasma samples were diluted to remain in the linear portion of the assay. Tubes were incubated at room temperature for 15 min, after which absorbance at 540 nm was measured.

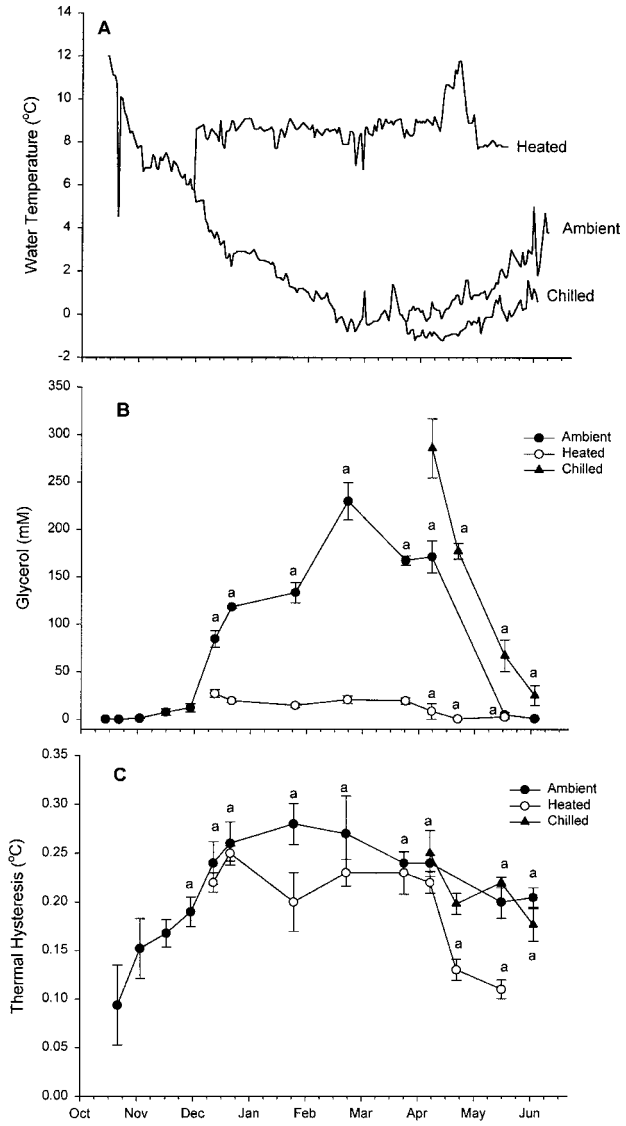

Figure 1. Temperature profile for smelt held in a long-term acclimation study from October 2000 to June 2001 and seasonal pattern of plasma glycerol levels (mM) and antifreeze protein activity (thermal hysteresis, degrees Celsius; *filled circles* = ambient, *open circles* = heated, *triangles* = chilled). Symbols represent mean ± SEM ($n = 5$); "a" indicates significance (ANOVA: $P < 0.05$) from initial sampling point within a treatment.

Antifreeze Protein Activity

Antifreeze protein activity analysis followed the methods used in Ewart et al. (2000). Values for AFP activity were based on the difference between the melting point and freezing point of the sample (thermal hysteresis, °C). These values were obtained by recording the melting and freezing temperatures of a single ice crystal on a cooling stage using a Clifton nanoliter osmometer. These temperatures were obtained in triplicate and averaged to obtain the AFP activity for an individual plasma sample.

In Vitro Liver Enzyme Activity

Liver samples were weighed and homogenized in 9 volumes of extraction buffer for three 10-s bursts with a Polytron tissue homogenizer. Extraction buffer (pH 7.4 at 5°C) contained 20 mM imidazole, 5 mM EGTA, 5 mM EDTA, 10 mM mercaptoethanol, and 50 mM sodium fluoride. Maximum enzyme activities in crude homogenate were determined spectrophotometrically under optimal pH. Enzyme activities are expressed as micromoles substrate converted to product min^{-1} g^{-1} tissue. All activities were assayed using a Beckman 640 spectrophotometer with a circulating water-jacketed cell holder maintained at 15°C. Procedures and conditions for each enzyme assay were taken from Driedzic et al. (1998).

Glycerol-3-Phosphate Dehydrogenase (*EC 1.1.1.8*). Assay medium contained 20 mM imidazole and 0.15 mM NADH at pH 7.6 at 20°C. Reaction was initiated with 2 mM DHAP.

Phosphoenolpyruvate Carboxykinase (*EC 4.1.1.32*). Assay medium contained 80 mM Tris-HCl 7.4, 1 mM KCN, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1.5 mM IDP, 1.1 mM PEP, 0.17 NADH, 19 IU mL^{-1} of MDH in glycerol at pH 7.0 at 20°C. Reaction was initiated with 20 mM $NaHCO_3$.

Alanine Aminotransferase (*EC 2.6.1.2*). Assay medium contained 50 mM imidazole, 1 mM KCN, 200 mM alanine, 0.05 mM pyridoxal-5-phosphate, 0.2 mM NADH, and 1 IU mL^{-1} of LDH at pH 7.4 at 20°C. Reaction was initiated with 10 mM α-ketoglutarate.

Aspartate Aminotransferase (*EC 2.6.1.1*). Assay medium contained 50 mM imidazole, 1 mM KCN, 30 mM aspartate, 0.05 mM pyridoxal-5-phosphate, 0.2 mM NADH, and 7 IU mL^{-1} of MDH at pH 7.4 at 20°C. Reaction was initiated with 7 mM α-ketoglutarate.

Data Analysis

For each treatment, values for the fish killed per sampling time are expressed as mean ± SEM ($n = 5$). To assess significant differences between treatments at a particular sampling time, values of specific parameters were compared using a *t*-test ($P < 0.05$ significant). A one-way ANOVA with Tukey's post-test was used to determine significant differences within a treatment throughout the study. The initial sampling point in each treatment was used as a reference point with which all remaining sampling points in that particular treatment were compared. Regression analysis was conducted to determine whether there is a correlation between enzyme activity levels and plasma glycerol content for fish in the ambient and chilled treatments. Fish held at high temperatures were excluded from this analysis because glycerol levels did not increase and sexual maturation added a variable on metabolic organization. Results are ex-

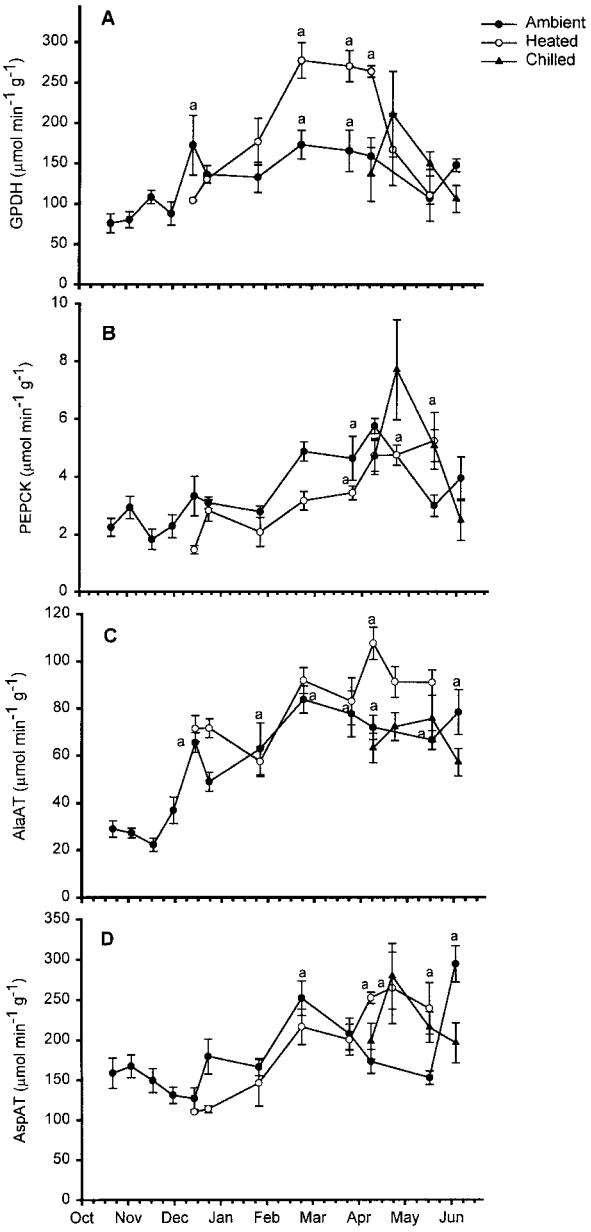

Figure 2. Seasonal pattern of in vitro enzyme activity (μmol g^{-1} min^{-1}) of glycerol 3-phosphate dehydrogenase (GPDH), phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAT) in the livers of rainbow smelt held in a long-term acclimation study in ambient (*filled circles*), heated (*open circles*), and chilled (*triangles*) seawater from October 2000 to June 2001. Values represented as mean ± SEM ($n = 5$); "a" indicates significance (ANOVA: $P < 0.05$) from initial sampling point within a treatment.

pressed as individual sample points ($n = 76$; $P < 0.05$ is significant).

Results

Plasma Glycerol and AFP Activity

The temperature profile is presented in Figure 1*A*. Ambient water temperature decreased to −1°C by February; further chilling lowered the temperature to −1.5°C. Heated seawater ranged from 7° to 12°C throughout the study.

Initial glycerol levels, from fish sampled in the field, were 0.3 mM (Fig. 1*B*). Fish that tracked ambient seawater temperature showed a significant increase to 84 mM in December when water temperatures reached 5°C. A glycerol concentration of 230 mM was reached in these fish in February (−1°C), after which levels began to decrease. A significant reduction to 5 mM was observed in May, bringing the glycerol levels close to those obtained in early fall. The glycerol level in fish, 10 d after being transferred to the heated seawater, was 27 mM in December. Smelt maintained glycerol concentrations at this slightly elevated level until April, when there was a significant decrease to 0.7 mM. By exposing fish to chilled seawater temperatures as low as −1.5°C, glycerol levels were elevated to 286 mM, after which smelt began to steadily lose glycerol at a substantial rate, regardless of the extreme cold temperatures, to a final concentration of 25 mM in May.

Initial thermal hysteresis values obtained in October were 0.09°C (Fig. 1*C*). A significant increase to 0.18°C was observed in November when water temperature was approximately 6°C. A maximum value of 0.28°C was reached in January (water temperature of 1°C). There was no significant difference in AFP activity between the three treatments until April, when fish in the heated treatment experienced a significant decrease in thermal hysteresis to 0.10°C, whereas AFP activity remained elevated in fish in the ambient and chilled treatments for the duration of the study.

Liver Enzyme Activities

Initial activity of GPDH in smelt sampled in October was 76 μmol g^{-1} min^{-1} (Fig. 2*A*). In smelt from the ambient treatment, activity significantly increased in December to 173 μmol g^{-1} min^{-1}, after which it decreased to the initial range of activity. Another significant increase was observed in February when activity returned to 173 μmol g^{-1} min^{-1}, after which activity once again decreased to initial levels. Activity for GPDH in fish sampled from the chilled seawater was not significantly different from activity levels in fish in ambient seawater. However, activity levels from smelt sampled from the heated seawater treatment gradually increased from initial values (104 μmol g^{-1} min^{-1}) to a significant peak in February, when activity levels reached 278 μmol g^{-1} min^{-1}. Enzyme activity remained at this elevated level until April, when it began to decrease, approaching initial levels for activity for smelt in the heated treatment.

PEPCK activity levels in October were 2.9 μmol g^{-1} min^{-1} (Fig. 2*B*). During the study there were no significant differences

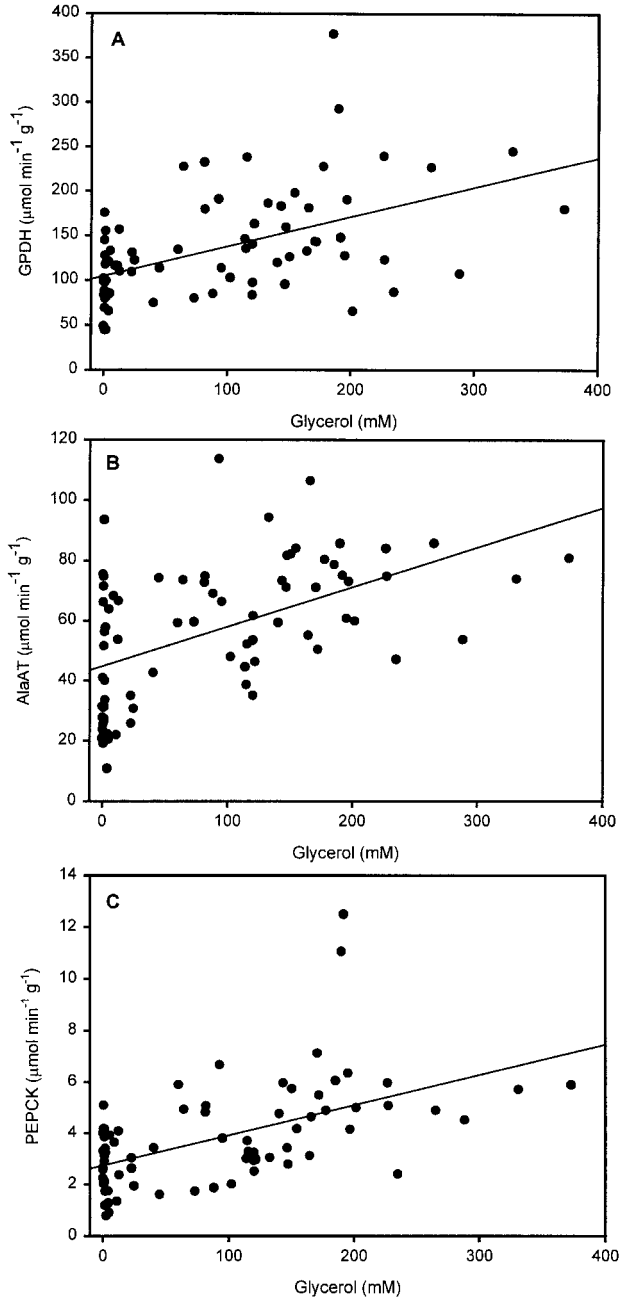

Figure 3. Regression analysis comparing plasma glycerol level (mM) and in vitro liver enzyme activity for glycerol 3-phosphate dehydrogenase (GPDH), alanine aminotransferase (AlaAT), and phosphoenolpyruvate carboxykinase (PEPCK; μmol g^{-1} min^{-1}) in fish held in ambient and chilled treatments. Circles represent individual fish (n = 76); $P < 0.001$ in all cases.

in activity levels in smelt between the ambient, heated, or chilled seawater treatments. There was a gradual increase in activity throughout the study, which reached a significant difference from initial levels in fish sampled in April, at which time the ambient and heated treatment had activity levels of 5.8 and 4.7 μmol g^{-1} min^{-1}, respectively.

Activity levels for AlaAT in early fall were 30 μmol g^{-1} min^{-1} (Fig. 2*C*). There was no significant difference in enzyme activity in smelt sampled from the ambient, chilled, or heated seawater treatments. Activity levels gradually increased throughout the study, with significant increases in the fish from the ambient seawater in December, when levels reached 65 μmol g^{-1} min^{-1}. Another significant increase in enzyme activity was observed in February for fish in the ambient treatment when levels reached 84 μmol g^{-1} min^{-1}. AlaAT activity was maintained at this elevated level for the duration of the study.

Initial aspartate aminotransferase (AspAT) activity levels for smelt in October were 159 μmol g^{-1} min^{-1} (Fig. 2*D*). Enzyme activity remained at this level for fish in both the heated and ambient seawater treatments until February, when a significant increase in activity (252 μmol g^{-1} min^{-1}) was observed in fish from the ambient seawater, which increased again in late May to 294 μmol g^{-1} min^{-1}. Fish exposed to heated seawater throughout the study showed an increase in enzyme activity from initial values to levels of 265 μmol g^{-1} min^{-1} in fish sampled in April. These activities were maintained at high levels for the duration of the study.

Regression Analysis

The purpose of performing regression analysis was to determine whether there was a significant relationship between various enzyme activities and the increased level of glycerol in response to low temperatures. To achieve this, only fish held under "natural" temperature conditions, which included the ambient and chilled treatments, were used for the regression analysis. Fish from the heated treatment were not analyzed because there may have been additional demands on the glycerol production pathway that were not associated with temperature, such as the production of triglycerides during the period when fish were becoming gravid. A relationship to glycerol production in the liver becomes apparent when enzyme activities for fish in the ambient and chilled water treatments are plotted against plasma glycerol levels for the same fish. A significant positive correlation was found between increasing enzyme activity and plasma glycerol content for GPDH, AlaAT, and PEPCK (P values of 2.77×10^{-6}, 3.97×10^{-7}, and 1.12×10^{-6}), respectively (Fig. 3). There was no significant correlation found between increasing enzyme activity for AspAT and plasma glycerol content.

Discussion

Plasma Glycerol and AFP Activity

Results from this study confirm the concept proposed by Treberg et al. (2002*b*) that antifreeze response in smelt begins well before there is any threat of freezing. Both AFP activity and

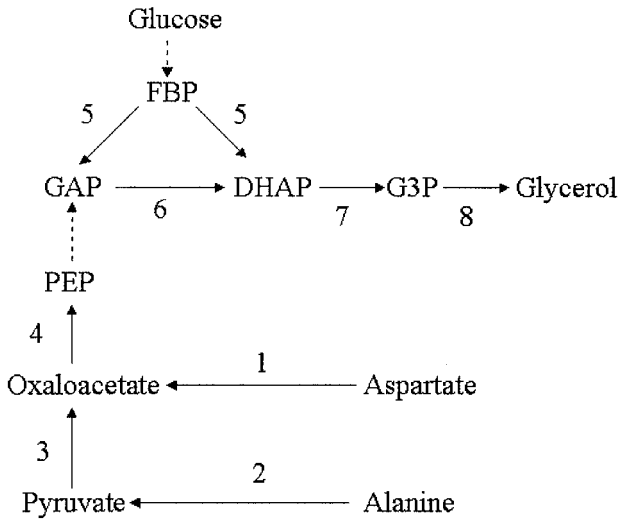

Figure 4. Proposed pathway for carbon flow from amino acids into glycerol in smelt liver. FBP = fructose-1,6-bisphosphate; GAP = glyceraldehyde-3-phosphate; DHAP = dihydroxyacetone phosphate; G3P = glycerol-3-phosphate; PEP = phosphoenolpyruvate. *1* = aspartate aminotransferase; *2* = alanine aminotransferase; *3* = pyruvate carboxylase; *4* = phosphoenolpyruvate carboxykinase; *5* = aldolase; *6* = triose isomerase; *7* = glycerol-3-phosphate dehydrogenase; *8* = glycerol-3-phosphatase. Dotted arrows indicate multiple conversion steps.

plasma glycerol levels showed a significant increase in early December, when water temperatures were 5°–6°C. Glycerol levels increased to 85 mM, and the increase in AFP activity resulted in a 0.2°C decrease in the freezing point of the plasma, which matches the initial values for December measured in smelt from the Treberg et al. (2002*b*) study.

The seasonal pattern of glycerol accumulation in smelt appears to be similar to that of an insect, the gall fly, with production beginning in early fall, reaching a peak mid-winter (after which levels begin to decrease), and complete loss occurring in the spring (Storey and Storey 1988; Joanisse and Storey 1994*a*, 1994*b*). It is not known if the production of glycerol in the fall is triggered by exposure to temperature, a photoperiod cue, or some other factor. The loss of glycerol from the blood in the spring is not regulated by temperature because there was a significant decrease in blood glycerol levels in all three treatments at the same point of time, regardless of the water temperature.

The seasonal pattern of AFP in smelt follows the same general pattern as that of glycerol accumulation. However, there are some differences between the two methods of freeze resistance that warrant mentioning. Synthesis of AFP is up-regulated a few weeks earlier, and the increase in the fall of the year is more gradual than that of glycerol. During the fall and winter, AFP activity levels were not significantly different between the various treatments, indicating that the initiation of AFP production is not temperature dependent in smelt. Studies on winter flounder (*Pleuronectes americanus*) have suggested the production and loss of AFP may be controlled by a predetermined endogenous cycle, which is regulated by the central nervous system (Fletcher et al. 1998). This may also be the case with AFP production in smelt; however, the disappearance of AFP from smelt plasma appears to be influenced by temperature. After a certain point, AFP activity declined in the fish held in heated seawater in April, whereas activity levels were maintained in fish in the ambient and chilled treatments (water temperatures less than 2°C). These results are consistent with those obtained by Duman and DeVries (1974) in which smelt transferred to warmer temperatures and a lengthened photoperiod demonstrated a decrease in AFP activity.

The accumulation of glycerol appears to be the main mechanism of freeze resistance in smelt because during mid-winter it contributes a greater degree of the lowering of the body's freezing point than the activity of the AFP (0.5°C vs. 0.25°C for glycerol and AFP, respectively). The earlier up-regulation of AFP activity in the fall and the maintenance of activity in the spring of the year, well after glycerol levels had returned to low values, suggest that the production of AFP may serve as a safeguard against unexpected freezing events that may occur when glycerol levels are insufficient to prevent freezing.

It has been suggested that activity of AFP in insects may be enhanced by the presence of high levels of low molecular weight solutes such as glycerol (Li et al. 1998). Preliminary studies in this area with respect to smelt AFP have shown that at concentrations of 250 and 500 mM, glycerol does increase the activity of AFP in smelt. However, the effect is most evident at very low antifreeze concentrations and activities (K. V. Ewart, unpublished data). The increase in antifreeze activity generated by glycerol is not sufficient to account for the seasonal cycle of antifreeze activity seen in this study due to the higher levels of antifreeze activity seen in smelt. Moreover, the antifreeze activity remained high in smelt that were in heated water, whereas glycerol levels were low (0–20 mM). This supports the contention that the seasonal variation in antifreeze activity is not solely attributed to enhancement by glycerol.

The freezing point of plasma will be set by colligative and noncolligative agents. In the most extreme conditions of this study, at a water temperature of −1.5°C, glycerol level was approximately 300 mM. This would contribute to a freeze point depression of approximately 0.54°C, and freeze point depression through AFP was approximately 0.24°C. It was previously reported that plasma osmolality in smelt sampled in February was 840 mOsm kg^{-1}, with glycerol levels of 250 mM (Treberg et al. 2002*b*). Organic solutes, most notably NaCl (Raymond 1994) and to a lesser extent trimethylamine oxide (TMAO) and urea (Raymond 1994; Treberg et al. 2002*b*), would contribute to the remaining 590 mOsm kg^{-1}, resulting in a contribution of 1.06°C to freeze point depression. If the osmolality of fish in the current study is similar to that reported by Treberg et

al. (2002*b*), it is apparent that the glycerol component is absolutely essential to avoid freezing at −1.5°C because freeze point depression by the sum of AFP and glycerol free plasma would only amount to 1.30°C.

In Vitro Liver Enzyme Activity

In vitro enzyme activity profiles for key enzymes in the amino acid breakdown and gluconeogenic and glycerol production pathways in smelt were obtained from the initial up-regulation of glycerol production in the fall to the return to low levels of glycerol in the spring. A simple schematic of the metabolic pathway is presented in Figure 4. Except for AspAT, which is approximately threefold higher, the enzyme activity levels obtained in this study were in the same range as described by Treberg et al. (2002*b*). The initial increases in the enzyme activities of GPDH and AlaAT correspond with the initial up-regulation of glycerol that occurs in the fall of the year at approximately 5°–6°C. Also, the second increase in GPDH levels in February coincides with the significant increase in glycerol levels in the ambient treatment, bringing the glycerol levels to the highest measurement obtained for the ambient treatment. These results suggest that these enzymes play a key role in the rapid synthesis of glycerol in the fall of the year, and increases in GPDH may be linked to increases in blood glycerol levels throughout the season. The findings also support the conjecture of Treberg et al. (2002*b*) that the regulatory events of glycerol production occur well before the fish are exposed to subzero temperatures. Even though levels of GPDH decrease slightly after both significant increases, enzyme activity is maintained at an elevated level throughout the study, compared with initial values. AlaAT continues to increase to the time of the highest glycerol levels and then remains relatively constant. PEPCK has been designated as one of the key regulatory enzymes that allows the flow of carbon intermediates into the gluconeogenic pathway (Suarez and Mommsen 1987). Levels of this enzyme are closely associated with glycerol levels during winter. In addition, PEPCK undergoes a gradual increase in enzyme activity from winter to spring that is probably related to its key function in energy metabolism, which is required for various other processes during the warmer seasons.

The decrease in plasma glycerol levels clearly is not strongly associated with decreases in enzyme activity levels but rather may be due to increased rates of loss via skin, gills, or the kidneys to the surrounding environment or through the catabolism of glycerol within the body. Despite the general trend of a seasonal increase among the enzymes studied, there was only a single significant difference in enzyme activity between fish that had high glycerol levels (ambient and chilled treatments) and those that did not (heated treatment). This was for GPDH, in which the levels in fish from the heated treatment underwent a significant increase between February and April. There was a steady increase in GPDH activity levels in smelt from the heated treatment to levels that were two to threefold higher than levels in glycerol-producing fish. The elevated activity of GPDH when glycerol levels are low suggests this enzyme is functioning for other purposes besides glycerol accumulation at this time. One possibility is that increased GPDH could be serving to produce glycerol for triacylglycerol (lipid) synthesis, associated with gonad production, as smelt in the heated treatment underwent spawning earlier than smelt exposed to the natural water temperatures. This spawning event occurred from late February to March, which coincided with the period of time when the extremely high levels of GPDH activity are found in these fish.

The intimate relationship between liver enzyme activity levels and plasma glycerol is revealed when individuals are teased out of the data array. When animals held under heated conditions, for reasons stated previously, are excluded from the analysis, the activities of liver GPDH, AlaAT, and PEPCK correlate with the plasma glycerol level. Earlier work over a much more limited range of time and glycerol levels failed to reveal these important relationships (Treberg et al. 2002*b*). Highly significant correlations between activity levels of GPDH, AlaAT, and PEPCK and glycerol levels in fish from the ambient and chilled treatments suggest that there is metabolic up-regulation at these specific loci in fish producing glycerol. These results support previous research that implies the liver metabolism of smelt is specially designed to produce increased levels of glycerol during the winter (Driedzic et al. 1998; Treberg et al. 2002*a*), with the main substrate for glycerol production being amino acids (Raymond and Driedzic 1997) as opposed to glucose or glycogen as in insects (Storey and Storey 1988). These results per se do not provide proof of rate-controlling sites for glycerol production. Further research is required to determine the exact regulatory loci for glycerol synthesis in smelt.

Acknowledgments

We would like to thank the Ocean Sciences Centre Field Services Unit for animal collection and Connie Wilson and Jason Treberg for their technical assistance throughout the study. This work was funded by AquaNet, Natural Sciences and Engineering Research Council of Canada, Aqua Bounty Canada, and the National Research Council's Institute for Marine Biosciences. National Research Council publication 42436.

Literature Cited

Buckley J.L. 1989. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (North Atlantic): rainbow smelt. U.S. Fish Wildlife Services Biological Report 82(11.106). U.S. Army Corps Engineers, TR EL-82-4.

Driedzic W.R., J.L. West, D.H. Sephton, and J.A. Raymond.

1998. Enzyme activity levels associated with the production of glycerol as an antifreeze in liver of rainbow smelt (*Osmerus mordax*). Fish Physiol Biochem 18:125–134.

Duman J.G. and A.L. DeVries. 1974. The effects of temperature and photoperiod on antifreeze production in cold water fishes. J Exp Zool 190:89–98.

Ewart K.V., B. Blanchard, S.C. Johnson, W.L. Bailey, D.J. Martin-Robichaud, and M.I. Buzeta. 2000. Freeze susceptibility in haddock (*Melanogrammus aeglefinus*). Aquaculture 188:91–101.

Ewart K.V. and G.L. Fletcher. 1990. Isolation and characterization of antifreeze proteins from smelt (*Osmerus mordax*) and Atlantic herring (*Clupea harengus harengus*). Can J Zool 68:1652–1658.

Fletcher G.L., S.V. Goddard, P.L. Davies, Z. Gong, K.V. Ewart, and C.L. Hew. 1998. New insights into fish antifreeze proteins: physiological significance and molecular regulation. Pp. 239–265 in H.O. Portner and R.C. Playle, eds. Cold Ocean Physiology. Society of Experimental Biology Seminar Series 66. Cambridge University Press, Cambridge.

Joanisse D.R. and K.B. Storey. 1994*a*. Enzyme activity profiles in an overwintering population of freeze-avoiding gall moth larvae, *Epiblema scudderiana*. Can J Zool 72:1079–1086.

———. 1994*b*. Enzyme activity profiles in an overwintering population of freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*. Comp Physiol Biochem 164B:247–255.

Li N., C.A. Andorfer, and J.G. Duman. 1998. Enhancement of insect antifreeze protein activity by solutes of low molecular mass. J Exp Biol 201:2243–2251.

Raymond J.A. 1992. Glycerol is a colligative antifreeze in some northern fishes. J Exp Zool 262:347–352.

———. 1994. Seasonal variation of trimethylamine oxide and urea in the blood of a cold-adapted marine teleost, the rainbow smelt. Fish Physiol Biochem 13:13–22.

Raymond J.A. and W.R. Driedzic. 1997. Amino acids are a source of glycerol in cold-acclimatized rainbow smelt. Comp Biochem Physiol 118B:387–393.

Raymond J.A. and A. Hassel. 2000. Some characteristics of freezing avoidance in two osmerids, rainbow smelt and capelin. J Fish Biol 57:1–7.

Scott W.B. and M.G. Scott. 1988. Atlantic fishes of Canada. Can Bull Fish Aquat Sci 219:761.

Storey K.B. and J.M. Storey. 1988. Freeze tolerance in animals. Physiol Rev 68:27–84.

Suarez R.K. and T.P. Mommsen. 1987. Gluconeogenesis in teleost fishes. Can J Zool 65:1869–1882.

Treberg J.R., J.M. Lewis, and W.R. Driedzic. 2002*a*. Comparison of liver enzymes in osmerid fishes: key differences between a glycerol accumulating species, rainbow smelt (*Osmerus mordax*), and a species which does not accumulate glycerol, capelin (*Mallotus villosus*). Comp Biochem Physiol 132A: 433–438.

Treberg J.R., C.E. Wilson, R.C. Richards, K.V. Ewart, and W.R. Driedzic. 2002*b*. The freeze avoidance response of smelt, *Osmerus mordax*: initiation and subsequent suppression of glycerol, trimethylamine oxide and urea accumulation. J Exp Biol 205:1419–1427.