The Journal of Experimental Biology **198**, 2569–2573 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
JEB0009

GLYCEROL SYNTHESIS IN THE RAINBOW SMELT *OSMERUS MORDAX*

JAMES A. RAYMOND

Department of Biological Sciences, University of Nevada, Las Vegas, Las Vegas, NV 89154, USA

Accepted 6 September 1995

Summary

Rainbow smelt, *Osmerus mordax*, maintain high glycerol levels in winter to avoid freezing. After intramuscular injection of ^{14}C-labeled glucose, [^{14}C]glycerol was found in the blood, liver and muscle, indicating that glycogen is a source of glycerol. Levels of both the active and inactive forms of glycogen phosphorylase were higher in muscle in winter than in autumn, although the fraction in the active form did not change significantly. More of the phosphorylase was in the active form in the liver than in the muscle. Short-term starvation resulted in a significant decrease in the level of glycogen soon after the stomachs were emptied, presumably to replace glycerol lost to the water. However, tissue glycerol levels remained relatively high, despite a near depletion of glycogen reserves. Triglyceride levels increased slightly during starvation, indicating that triglycerides were not involved in glycerol synthesis. After intramuscular injection of ^{14}C-labeled pyruvate, [^{14}C]glycerol was found in the blood, liver and muscle, indicating a second route, presumably from muscle protein, to glycerol synthesis. Liver phosphoenolpyruvate carboxykinase activity was slightly higher in winter, possibly to assist in the conversion of pyruvate to glycerol.

Key words: glycerol synthesis, *Osmerus mordax*, glucose, pyruvate, glycogen, glycogen phosphorylase, phosphoenolpyruvate carboxykinase.

Introduction

The rainbow smelt, *Osmerus mordax*, is unusual among freeze-resistant fishes in that it produces a large amount of glycerol in winter that acts as a colligative antifreeze (Raymond, 1992). Unlike some frogs and insects that also produce glycerol in winter (Storey and Storey, 1988), the smelt live in an aqueous environment and are thus subject to glycerol losses through their epithelia. These losses can exceed 10 % of body glycerol stores per day (Raymond, 1993) and appear to double the daily energy requirement (Raymond, 1995). In insects and frogs, glycerol is synthesized from glycogen. Glycogen also appears to be a source of glycerol in the smelt, as acclimation and acclimatization studies of rainbow smelt and the related surf smelt *Hypomesus pretiosus* in Japan (J. A. Raymond, H. Hattori and K. Tsumura, in preparation) have shown an inverse relationship between glycogen levels and glycerol production. However, these studies also revealed some puzzling aspects of glycerol production in the smelt. Glycogen levels appeared to be unusually depressed after short-term starvation and glycerol levels remained relatively high, despite the low glycogen levels. The present study was undertaken to investigate these phenomena as well as to obtain more direct evidence that glycogen was a source of glycerol.

Materials and methods

Winter-acclimatized rainbow smelt *Osmerus mordax* Mitchell were caught by fishermen with under-ice traps in the Kouchibouguac River and Miramichi River estuaries in New Brunswick, Canada, between late January and late February of 1993 and 1994. Surface water temperature during these periods was approximately −0.4 to −0.7 ˚C. Additional fish were caught in late November 1993, before freeze-up, from a similar trap (water temperature approximately 3 ˚C).

For a glycogen depletion study, approximately 40 fish (average body mass 40±21 g, mean ± S.D.) were retrieved from a trap on 19 February, approximately 16 h after it was set, and maintained in two filtered, aerated 47 l ice chests without feeding for several days at a nearby motel. The water temperature was kept near −1 ˚C by moving the ice chests in and out of doors. The water was replaced on the second day. The fish were calm and appeared to be in good health. Samples of fish (N=5) were taken from the ice chests for tissue samples and stomach analyses at intervals of 12–24 h. Stomach fullness was estimated visually, with 100 % corresponding to a full stomach. Serum samples were taken as described previously (Raymond, 1992). The livers and a sample of muscle from the rear ventral flank were taken, wrapped in aluminum foil and quickly frozen between blocks of dry ice. In the laboratory, glycerol levels were measured using a diagnostic kit (Sigma no. 337) (Raymond, 1992). Glycogen levels were measured according to the method of Keppler and Decker (1974) except that glucose was measured using a Sigma glucose H-K kit. This had the effect of treating glucose 6-phosphate as glycogen, but

as glucose 6-phosphate levels appear to be small compared with glucose levels (Storey and Storey, 1984), the error was assumed to be small. Triglycerides were measured with a diagnostic kit (Sigma no. 337) (based on measurement of glycerol before and after lipase digestion) using the same tissue homogenate (in 0.5 mol l^{-1} $HClO_4$) used for the glycogen analysis, diluted by a factor of 20 to reduce glycerol levels. Triglyceride concentrations were based on an estimated molecular mass of 880 Da.

For the radioisotope studies, fish were transported in an aerated ice chest to Mount Allison University, Sackville, New Brunswick. Water temperature was maintained in the range 0 to −1 °C by occasionally moving the ice chests outdoors. Water was replaced approximately daily by mixing equal parts of fresh sea water and well water and allowing it to cool to about −1 °C outdoors. In each experiment ([^{14}C]glucose and [^{14}C]pyruvate), two fish were injected intramuscularly with approximately 0.55 MBq of substrate and kept in a 10 l gently aerated bucket that was placed in one of the ice chests for temperature control. The fish were killed 24 h later and tissue samples were taken as described above. Liquid extracts of the tissues were filtered with ultrafiltration tubes (Millipore, 10 000 M_r cut-off pore size) and the filtrate was applied to a carbohydrate HPLC column (Phenomenex, RCM), as described previously (Raymond, 1992). The eluate was monitored with a refractive index detector. Fractions were collected so that, as far as possible, they corresponded to the refractive index peaks. Samples of the HPLC fractions (200 μl) were then counted in a liquid scintillation counter.

Glycogen phosphorylase activity was measured according to the method of Storey and Storey (1984) with slight modifications. Tissue samples were homogenized in homogenizing buffer (50 mmol l^{-1} imidazole, 100 mmol l^{-1} NaF, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA, 15 mmol l^{-1} β-mercaptoethanol, adjusted to pH 7.5) at a concentration of 5 μl mg^{-1} tissue. The homogenate was quickly centrifuged at 16 000 ***g*** for 30 s. Phosphorylase *a* activity was measured by monitoring the increase in absorbance at 340 nm of a mixture containing 950 μl of reaction mixture (50 mmol l^{-1} phosphate buffer, pH 7.3, 2 mg ml^{-1} glycogen, 15 mmol l^{-1} $MgSO_4$, 0.25 mmol l^{-1} EDTA, 0.5 mmol l^{-1} NAD, 0.01 mmol l^{-1} glucose 1,6-diphosphate, 0.5 mmol l^{-1} dithiothreitol and 1 unit each of phosphoglucomutase and glucose-6-phosphate dehydrogenase), 50 μl of phosphate buffer and 10 μl of supernatant. (Unit enzyme activities are a defined by Sigma.) The phosphate buffer was replaced with 50 μl of 20 mmol l^{-1} AMP in order to measure total phosphorylase activity.

Phosphoenolpyruvate carboxykinase (PEPCK) activity was measured using the method of Seubert and Huth (1965), in which oxaloacetate was used as the substrate, with a few modifications. Liver tissues that had been stored at −70 °C for 12–24 months were homogenized in 5 volumes of ice-cold 50 mmol l^{-1} imidazole–Tris, pH 7.5, with 1 mmol l^{-1} EDTA and 15 mmol l^{-1} 2-mercaptoethanol. Approximately 15 μl of homogenate was incubated for approximately 20 min with gentle shaking in a water bath at 15 °C. The amount of phosphoenolpyruvate (PEP) generated was converted to lactate in a reaction mixture containing 3 mmol l^{-1} NADH, 3 mmol l^{-1} ADP, 150 mmol l^{-1} $MgSO_4$ and 50 mmol l^{-1} Tris, pH 8.0. The reaction was started by adding 2 μl of a solution containing approximately 3 units each of pyruvate kinase and lactate dehydrogenase. The resulting oxidation of NADH was monitored at 340 nm. Initial experiments confirmed that the amount of PEP produced was proportional to both the incubation time and the incubation temperature.

Results

[^{14}C]glucose metabolism

Twenty-four hours after the intramuscular injection of two fish with [^{14}C]glucose, the ^{14}C label appeared in glycerol in the blood, liver and muscle of both fish. Example

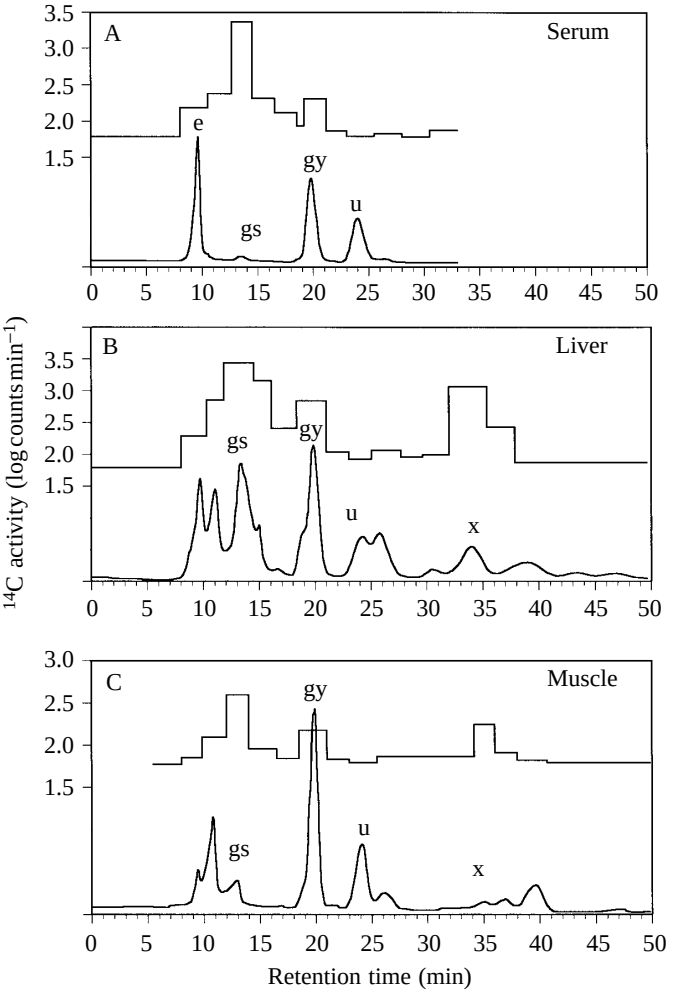

Fig. 1. Metabolism of intramuscularly injected [^{14}C]glucose in winter-acclimatized rainbow smelt. Lower curves, HPLC chromatograms of serum and supernatant from homogenized tissues using a carbohydrate column with refractive index detection. Upper curves, counts per minute of HPLC fractions. (A) Serum; (B) liver; (C) muscle. e, electrolytes; gs, glucose; gy, glycerol; u, urea; x, unknown.

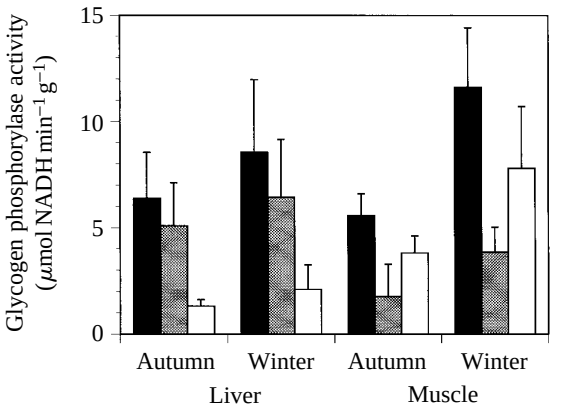

Fig. 2. Glycogen phosphorylase activities in liver and muscle of autumn- and winter-acclimatized rainbow smelt. Filled bars, total phosphorylase; stippled bars, phosphorylase *a*; open bars, phosphorylase *b*. Sample size for each bar is five fish. Error bars show 1 S.D.

chromatograms are shown in Fig. 1A–C. An unknown ^{14}C species appeared in the liver and muscle samples with an elution time of approximately 35 min. This peak, which was outside the region of mono- and disaccharides, could not be attributed to phosphoenolpyruvate, oxaloacetic acid, citrate, acetyl coenzyme A, acetate or bicarbonate.

Glycogen phosphorylase activity

Total phosphorylase activity in muscle tissue was significantly higher in mid winter than before freeze-up in the autumn (*t*-test, $P<0.01$) (Fig. 2). The activities of phosphorylase *a* (the active form) and phosphorylase *b* were also higher in winter ($P<0.05$ for each). Sample sizes were not large enough to determine whether similar differences were present in the liver. Total phosphorylase activities were not significantly different in liver and muscle tissues. However, a greater percentage of the phosphorylase was in the active form in the liver than in the muscle both in the autumn (77 *versus* 28 %) and in the winter (75 *versus* 35 %) ($P<0.001$ for each).

Short-term starvation

The stomachs of fresh, winter-caught rainbow smelt were relatively full, indicating that they were feeding actively in winter. Mysid shrimp, amphipods and polychaetes were the main food items. Stomachs were almost empty within about 2 days after capture (Fig. 3A). Liver glycogen (Fig. 3B), liver glucose (Fig. 3C) and serum glycerol (Fig. 3E) levels all decreased and serum triglyceride (Fig. 3D) level increased in the period after the stomachs emptied. In the final three sampling periods, glycogen, glucose and glycerol levels were significantly lower ($P<0.001$) and triglyceride levels were higher ($P<0.05$) than in the first four sampling periods. Females in the sample (11 of 35), which were preparing for spawning, tended to have emptier stomachs and lower levels of liver glycogen and serum triglyceride than the males and, thus, were largely responsible for the early variability in these parameters. The lower levels of liver glycogen in the females were partially compensated by the fact that the livers were significantly larger in the females than in the males (3.2±0.4 *versus* 1.8±0.4 % of body mass, $P<0.001$). This is a common phenomenon among spawning fishes (e.g. Henderson *et al.* 1984). Serum glycerol levels were correlated with liver glycogen levels ($r=0.39$, $N=35$, $P<0.02$), although when the latter dropped sharply, serum glycerol levels decreased by a relatively small amount. No correlation was found between serum glycerol and liver triglyceride levels. Liver glycerol and triglyceride levels (data not shown) closely resembled those in the serum, although the absolute glycerol values were slightly lower in the liver because of its lower water content.

Muscle glycogen levels (0.6±0.4 mg g^{-1} at 2 h,

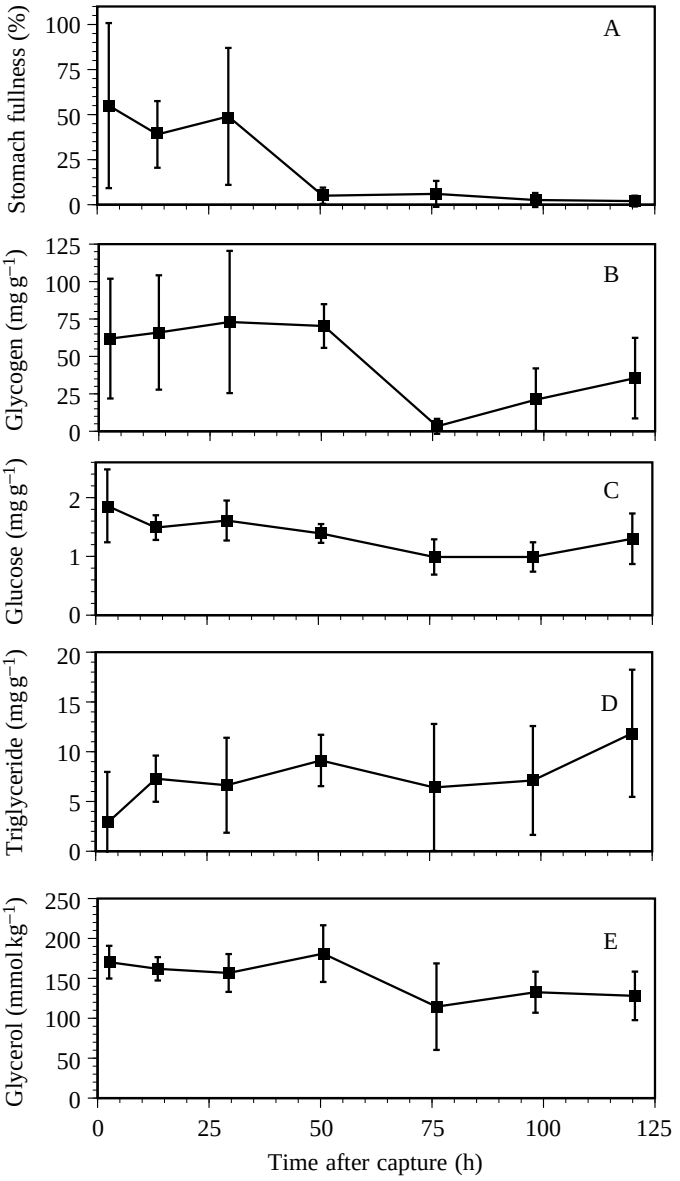

Fig. 3. Changes in stomach fullness and metabolite levels in winter-acclimatized rainbow smelt during short-term starvation. (A) Stomach fullness; (B) liver glycogen; (C) liver glucose; (D) serum triglyceride; (E) serum glycerol. Sample size for each point is five fish. Error bars show 1 S.D.

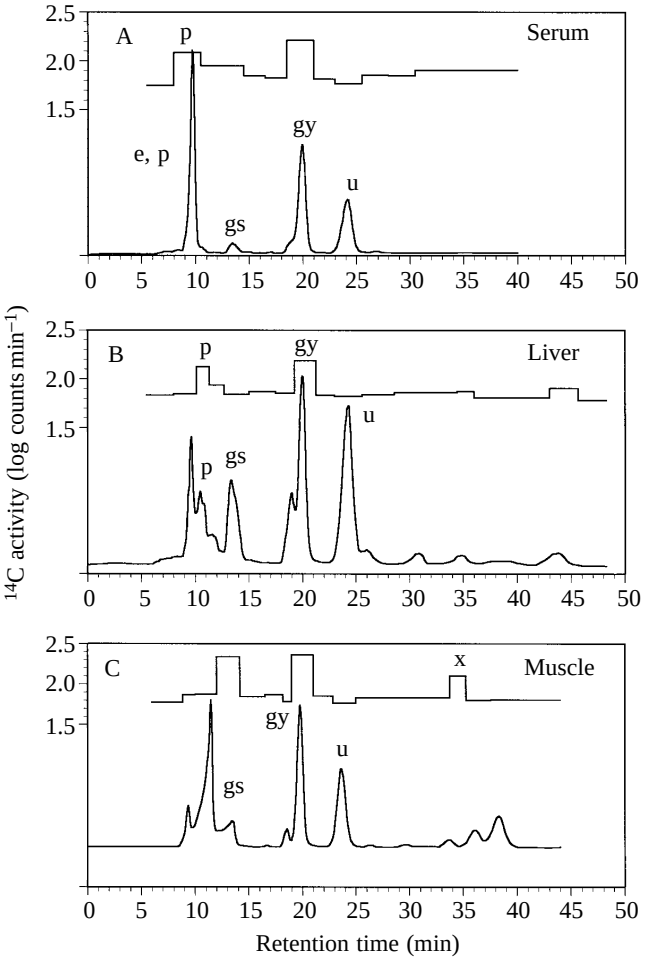

Fig. 4. Metabolism of intramuscularly injected [^{14}C]pyruvate in winter-acclimatized rainbow smelt. Data and symbols are as described in Fig. 1. p, pyruvate.

1.2±0.7 mg g^{-1} at 50 h and 0.4±0.3 mg g^{-1} at 124 h) were very low compared with those in the liver and did not show a significant trend during the 5 day starvation period. Muscle glycerol levels, determined only at 50 and 124 h, were similar to those in the serum. Muscle triglyceride levels were too low to measure accurately.

[^{14}C]pyruvate metabolism

Twenty-four hours after intramuscular injection of two fish with [^{14}C]pyruvate, the ^{14}C label appeared in glycerol in the blood, liver and muscle of both fish. Example chromatograms are shown in Fig. 4A–C. [^{14}C]pyruvate was present in the blood and liver, but not in the muscle, where it appeared to have been converted to glucose. The ^{14}C label was also found in the muscle in an unidentified peak at 35 min.

PEPCK activity

Liver samples were taken from fish collected in early November (before freeze-up, water temperature approximately 3 °C) and late January (water temperature −0.7 °C). PEPCK activity of liver tissue was slightly but significantly higher in the winter (1.64±0.47 μmol PEP g^{-1} min^{-1}, N=7) than in the autumn (1.21±.13 μmol g^{-1} min^{-1}, N=8) (P<0.05).

Discussion

Winter-acclimatized rainbow smelt must maintain high glycerol levels (approximately 200–400 mmol kg^{-1}) for freezing avoidance (Raymond, 1992), despite significant glycerol losses (approximately 3–9 mg h^{-1} 100 g^{-1}) into the water through the gills and skin (and probably feces) (Raymond, 1993). The estimated energy loss associated with the glycerol loss, 2.1 J 100 g^{-1} day^{-1}, is comparable to published values for the metabolic rates of polar fishes (Raymond, 1995) and thus would require an approximate doubling of the minimum food requirement. The observed active feeding of the smelt in winter appears to be a consequence of this. Starvation and cold acclimation studies have previously shown that the glycerol is synthesized rather than obtained through the diet (J. A. Raymond, H. Hattori and K. Tsumura, in preparation). That study, by showing that there was a high demand on glycogen in winter and that glycogen levels were more depressed in fish acclimated to −1 °C than in fish acclimated to +1 °C, strongly pointed to glycogen as a source of glycerol.

In the present study, the conversion of [^{14}C]glucose to glycerol confirms that glycogen is a source of glycerol in rainbow smelt. This is further supported by (1) the increase in glycogen phosphorylase activity in muscle, and perhaps liver, in winter and (2) the rapid depletion of liver glycogen reserves during short-term starvation. The latter result appears to be unusual among fishes, as other studies have shown that liver glycogen levels do not normally change significantly during short-term starvation (Dave *et al.* 1975; Ottolenghi *et al.* 1981; Black and Love, 1986). The rapid consumption of glycogen in the smelt strongly suggests that it was being used to replace glycerol that had been lost to the environment. Glycogen depletion rates are presumably lower in other fishes because they do not experience a significant carbohydrate efflux.

The ability of the smelt to maintain relatively high glycerol levels despite a near depletion of glycogen after both 5 days of starvation (this study) and 2 weeks of starvation (J. A. Raymond, H. Hattori and K. Tsumura, in preparation) suggested that another glycerol synthetic pathway was present. This would be a departure from other glycerol-producing animals in which glycogen appears to be the only source of glycerol (Storey and Storey, 1988). The pattern of relatively constant or possibly increasing triglyceride levels in starved smelt (Fig. 3D) was similar to that observed in a longer acclimation study (J. A. Raymond, H. Hattori and K. Tsumura, in preparation) and indicates that triglycerides are not involved in glycerol synthesis, in agreement with previous studies (Storey and Storey, 1988). This result is not surprising in view of the small contribution of glycerol to the molecular mass of triglycerides and the inability of animals to convert fatty acids into gluconeogenic precursors (Mathews and van Holde, 1990).

Another potential source of glycerol is protein. During

periods of starvation, the catabolism of muscle protein yields several gluconeogenic precursors, including pyruvate (Mathews and van Holde, 1990). This route for glycerol production is suggested (but not proven) by the appearance of [^{14}C]glycerol in all tissues examined shortly after [^{14}C]pyruvate injection and by the increase in the activity of PEPCK in the liver in winter. PEPCK is a key gluconeogenic enzyme that mediates the conversion of pyruvate to phosphoenolpyruvate. These results clearly demonstrate that there is a second route to glycerol production in the smelt. Further studies using labeled amino acids are planned to more clearly define this novel synthetic pathway.

I thank K. Callendar, J. Daigle and K. Sweezey for generously providing the fish used in this study, Dr W. Driedzic, Mount Allison University, for providing facilities for holding the fishes used in the ^{14}C studies, and Drs K. and J. Storey for helpful advice on measuring glycogen phosphorylase activity. This study was supported by NSF grants DPP-9123228 and OPP-9423920.

References

BLACK, D. AND LOVE, R. M. (1986). The sequential mobilisation and restoration of energy reserves in tissues of Atlantic cod during starvation and refeeding. *J. comp. Physiol.* B **156**, 469–479.

DAVE, G., JOHANSSON-SJOBECK, M.-L., LARSSON, A., LEWANDER, K. AND LIDMAN, U. (1975). Metabolic and hematological effects of starvation in the European eel, *Anguilla anguilla* L. I. Carbohydrate, lipid, protein and inorganic ion metabolism. *Comp. Biochem. Physiol.* **52**A, 423–430.

HENDERSON, R. J., SARGENT, J. R. AND HOPKINS, C. C. (1984). Changes in the content and fatty acid composition of lipid in an isolated population of the capelin *Mallotus villosus* during sexual maturation and spawning. *Mar. Biol.* **78**, 255–263.

KEPPLER, D. AND DECKER, K. (1974). Glycogen: Determination with amyloglucosidase. In *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer), pp. 1127–1131. New York: Academic Press.

MATHEWS, C. K. AND VAN HOLDE, K. E. (1990). *Biochemistry*. Redwood City, CA: Benjamin/Cummings. 1129pp.

OTTOLENGHI, C., PUVIANI, A. C. AND BRIGHENTI, L. (1981). Glycogen in liver and other organs of catfish (*Ictalurus melas*): Seasonal changes and fasting effects. *Comp. Biochem. Physiol.* **68**A, 313–321.

RAYMOND, J. A. (1992). Glycerol is a colligative antifreeze in some northern fishes. *J. exp. Zool.* **262**, 347–352.

RAYMOND, J. A. (1993). Glycerol and water balance in a near-isosmotic teleost, winter-acclimatized rainbow smelt. *Can. J. Zool.* **71**, 1849–1854.

RAYMOND, J. A. (1995). Responses of marine fishes to freezing temperatures: A new look at colligative mechanisms. In *Thermobiology* (ed. J. S. Willis), *Advances in Cellular and Molecular Biology* series, Greenwich, CN: JAI Press (in press).

SEUBERT, W. AND HUTH, W. (1965). On the mechanism of gluconeogenesis and its regulation. II. The mechanism of gluconeogenesis from pyruvate to fumarate. *Biochem. Z.* **343**, 176–191.

STOREY, K. B. AND STOREY, J. M. (1984). Biochemical adaption for freezing tolerance in the wood frog, *Rana sylvatica*. *J. comp. Physiol.* B **155**, 29–36.

STOREY, K. B. AND STOREY, J. M. (1988). Freeze tolerance in animals. *Physiol. Rev.* **68**, 27–84.