Glycerol-3-phosphatase and not lipid recycling is the primary pathway in the accumulation of high concentrations of glycerol in rainbow smelt (Osmerus mordax)

Delphine Ditlecadet and William R. Driedzic

Department of Ocean Sciences, Ocean Sciences Centre, Memorial University of Newfoundland, St. John’s, Newfoundland and Labrador, Canada

Submitted 12 October 2012; accepted in final form 19 December 2012

Ditlecadet D, Driedzic WR. Glycerol-3-phosphatase and not lipid recycling is the primary pathway in the accumulation of high concentrations of glycerol in rainbow smelt (Osmerus mordax). Am J Physiol Regul Integr Comp Physiol 304: R304–R312, 2013. First published December 26, 2012; doi:10.1152/ajpregu.00468.2012.—Rainbow smelt is a small fish that accumulates glycerol in winter as a cryoprotectant when the animal is in seawater. Glycerol is synthesized in liver from different substrates that all lead to the formation of glycerol-3-phosphate (G3P). This study assesses whether glycerol is produced by a direct dephosphorylation of G3P by a phosphatase (G3Pase) or by a cycling through the glycerolipid pool followed by lipolysis. Foremost, concentrations of on-board glycerolipids and activity of G3Pase and of enzymes involved in lipid metabolism were measured in smelt liver over the glycerol cycle. Concentrations of on-board glycerolipids did not change over the cycle and were too low to significantly contribute directly to glycerol production but activities of enzymes involved in both potential pathways were up-regulated at the onset of glycerol accumulation. A second experiment conducted with isolated hepatic cells producing glycerol showed 1) that on-board glycerolipids were not sufficient to produce the glycerol released even though phospholipids could account for up to 17% of it, 2) that carbon cycling through the glycerolipid pool was not involved as glycerol was produced at similar rates following inhibition of this pathway, and 3) that G3Pase activity measured was sufficient to allow the synthesis of glycerol at the rate observed. These results are the first to clearly support G3Pase as the metabolic step leading to glycerol production in rainbow smelt and the first to provide strong support for a G3Pase in any animal species.

glycerol synthesis; glycerol-3-phosphate; glycerol-3-phosphatase; glycerolipids; lipid metabolism

NUMEROUS TERRESTRIAL ANIMAL species accumulate unusually high amounts of glycerol in winter as a cryoprotectant (31). In fish that inhabit freezing environments, the synthesis of antifreeze proteins is the common strategy used, whereas accumulation of glycerol is not anticipated because of the chemical properties of glycerol. This small sugar alcohol (C₃H₈O₃) is highly soluble in both water and lipid and may be transported across cell membranes through aquaglyceroporins (10), making direct loss to the environment most likely to occur in species that are in constant contact with the surrounding water. Four fish species were, however, shown to accumulate significant amounts of glycerol during winter at subzero temperatures, including two species of smelt (Osmerus mordax and Hypomesus pretiosus) and two species of greenling (Hexagrammos stelleri and Hexagrammos octogrammus) (23).

In a small population of rainbow smelt, indigenous to Newfoundland, Canada, glycerol concentrations follow a predictable cycle that starts with an accumulation period from late fall (<5 mM) to the middle of February (>200 mM), in parallel with the decrease of water temperature. Thereafter, there is a period of decrease that is most likely induced by a change in the photoperiod and extends until the beginning of May (<5 mM) (3, 14). About 10% of the total glycerol content of fish is lost daily to the surrounding water, with no apparent mechanisms for the retention of glycerol during the critical accumulation period (3, 24). Therefore, glycerol concentration is primarily dictated by its rate of synthesis, which needs to be up-regulated during the accumulation period. This characteristic makes smelt an ideal model to study metabolic pathways involved in glycerol synthesis in vertebrates.

Glycerol is well known as a by-product of lipolysis being the backbone of triglycerides (TG) and of most phospholipids (PL). Lipid reserves, such as TG, would be the most direct source of glycerol; however, on-board TG is unlikely to be sufficient to produce the high concentrations accumulated in winter (25). In smelt, carbon sources necessary for the synthesis of glycerol are glucose, glycerogen, and amino acids (26, 27, 34), with dietary glucose and amino acids being essential to maintain rates of glycerol synthesis (1). Synthesis is localized in liver and involves a transition through the C₃ intermediate dihydroxyacetone phosphate (DHAP). From there, DHAP is converted to glycerol-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase (GPDH), a key enzyme in glycerol synthesis (14, 15). The remaining question concerns the last step of the pathway leading to the formation of glycerol from G3P and is at the center of this work.

Two pathways can result in the synthesis of glycerol from G3P: one that involves a direct dephosphorylation by a phosphatase, the glycerol-3-phosphatase (G3Pase), and one that involves the transition of G3P through the glycerolipid pool (TG + PL) prior to the release of glycerol following lipolysis. In yeast (Saccharomyces cerevisiae) that produces glycerol to avoid dehydration in response to osmotic stress, two isoforms of G3Pase were identified and are essential to glycerol synthesis (19, 20). In smelt, existence of G3Pase is supported by its identification in liver and involvement in the transition through the C₃ intermediate dihydroxyacetone phosphate (DHAP). From there, DHAP is converted to glycerol-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase (GPDH), a key enzyme in glycerol synthesis (14, 15). The remaining question concerns the last step of the pathway leading to the formation of glycerol from G3P and is at the center of this work.

Two pathways can result in the synthesis of glycerol from G3P: one that involves a direct dephosphorylation by a phosphatase, the glycerol-3-phosphatase (G3Pase), and one that involves the transition of G3P through the glycerolipid pool (TG + PL) prior to the release of glycerol following lipolysis. In yeast (Saccharomyces cerevisiae) that produces glycerol to avoid dehydration in response to osmotic stress, two isoforms of G3Pase were identified and are essential to glycerol synthesis (19, 20). In smelt, existence of G3Pase is supported by its activity being higher than in a coexisting species not producing glycerol (6), as well as by the mass action ratio across G3Pase (i.e., glycerol][P][G3P] that increased in association with an increase in glycerol production (4). However, as phosphatases can dephosphorylate different substrates in vitro, the report of G3Pase-like activity is not sufficient to validate a pathway that
is still hypothetical in animals. Even though cycling through glycerolipids would be more demanding energetically, as it requires different enzymes but also free fatty acids (FFA) and cofactors, it would be premature to rule it out since all of the enzymes necessary for this pathway should be in place. Furthermore, in mammals, only a very small fraction of the FFA released following lipolysis under starvation is actually oxidized to fuel the organism with energy; most of the released FFA being recycled back to TG, with G3P synthesized through glyceroneogenesis (i.e., synthesis from amino acids) as new backbone (9, 28). This recycling could take place in smelt liver to produce glycerol constantly, as glyceroneogenesis is activated in winter in this tissue (14). In isolated hepatic cells incubated at cold temperature and producing glycerol, there is an up-regulation of mRNA levels of different genes involved in lipid metabolism and of an uncharacterized phosphatase, which may encode for G3Pase (8). As such, the final steps to glycerol production remain unresolved.

To provide a better understanding of the respective roles of glycerolipids and G3Pase in the synthesis of glycerol, we used two approaches. The first one investigated the profile of G3Pase activity and of different components of lipid metabolism in smelt liver over the complete glycerol cycle. This approach offered a representation of how activities of potentially key enzymes are modulated in winter in parallel to changes in glycerol concentrations. The second approach took advantage of the capacity of isolated smelt hepatic cells to produce glycerol when incubated at low temperature. This allowed us to compare more accurately the relation between the rate of glycerol synthesis and the two potential metabolic routes in a closed system, without any confounding extracellular effects. Findings highlight the seasonal regulation of both G3Pase activity and lipid metabolism in liver of smelt but also the impossibility for lipid recycling to be at the origin of all the glycerol produced, pointing out for the first time the direct dephosphorylation of G3P by G3Pase as the most likely pathway to synthesize glycerol in an animal species.

MATERIALS AND METHODS

Animals

Rainbow smelt (Osmerus mordax), hereafter referred to as smelt, were collected by seine netting from Mount Arlington Heights, Placentia Bay, Newfoundland, in October 2008, transported to the Ocean Sciences Centre, Memorial University of Newfoundland (St. John’s, Canada), and transferred to two 3,000-liter tanks with flow-through seawater. One group was maintained at warm temperature fluctuating around 11°C, hereafter referred to as warm smelt. A second group tracked ambient temperature, hereafter referred to as ambient smelt. Fish maintained at ambient temperature that reaches 0°C accumulate glycerol, whereas fish maintained at warm temperature do not (14). Fish were kept on a natural photoperiod using chopped herring twice a week to satiation. Experiments were carried out in accordance with an Animal Utilization Protocol issued by Memorial University of Newfoundland’s Animal Care Committee.

Hepatocyte Preparation

Hepatocytes were isolated from smelt maintained at warm temperature, as these cells produce more glycerol when incubated at low temperature than cells isolated from smelt acclimated to cold winter temperatures (1). Animals were chosen randomly with no consideration of the sex and killed by a sharp blow to the head. Liver was immediately exposed, and hepatocyte suspensions were prepared by perfusing the liver with collagenase (type IV from Clostridium histolyticum, Sigma C5138), as described by Mommsen et al. (16) and adapted for smelt by Clow et al. (1).

Liver washing. The hepatic vein of the exposed liver was cannulated (retrocannulation), and the venous input was nicked for further drainage. Blood was washed out using a perfusion medium (PM) adjusted to pH 7.6 that contained (in mM) 176 NaCl, 5.4 KCl, 0.81 MgSO4, 0.44 KH2PO4, 0.35 Na2HPO4, 5 NaHCO3, 10 HEPES, and 1 EGTA.

Hepatocyte isolation. Liver was perfused with a collagenase solution (PM without EGTA + 0.3 mg/ml collagenase) until the tissue was soft enough to be gently teased apart (25–30 min). Up to this point, each step was performed at room temperature. Cell suspensions were then kept on ice when possible.

Cells were filtered twice (250 and 50 µm), washed using a BSA solution (PM supplemented with 2% BSA and 1.5 mM CaCl2) and counted using a Neubauer hemocytometer. Viability was assessed by Trypan blue exclusion, and any preparations with viability lower than 90% were rejected from further experiments. Concentration of the cell suspensions was adjusted to 40 × 106 cells/ml with the BSA solution. One hundred and fifty microliters of each suspension (25–40 mg of cells) were aliquoted into separate 20-ml glass scintillation vials containing 2 ml of incubation medium (BSA solution + 5 mM glucose) and incubated in the conditions described below for up to 72 h. Viabilities at any sampling points were usually above 90%, and any viability below this was rejected.

Experimental Protocols

G3Pase activity and lipid metabolism over the glycerol cycle. Blood and liver of six ambient and six warm individuals were sampled monthly from November 2008 to May 2009 with the exception that in April and May 2009, only ambient smelt were sampled, as warm smelt started to die from the end of March. The occurrence of high mortalities from March to April is a recurrent phenomenon for smelt maintained at warm temperature. For each individual, one piece of liver was immediately frozen in liquid nitrogen and kept at −80°C until use. Enzyme activities were measured. A second fraction was sampled from five individuals per group and was placed in a lipid-free glass tube with 2 ml of chloroform under nitrogen. Teflon-sealed tubes were held at −80°C until total lipid extraction and analysis. Blood samples were collected with syringes rinsed with 2% EDTA and centrifuged at 10,000 g for 10 min at 4°C. Plasma collected was processed in the same manner as liver samples for glycerol concentrations and lipid extraction.

G3Pase activity was measured in all livers sampled. Lipid metabolism was investigated through the measurement of activities of key enzymes in liver and through the quantification of TG, PL, and FFA in both liver and plasma. Activity of lipase, hydroxyacyl CoA dehydrogenase (HOAD), carnitine palmitoyltransferase (CPT), malic enzyme (ME), and glucose-6-phosphate dehydrogenase (G6PDH) were measured in all livers sampled. Lipase, HOAD, and CPT are all directly involved in lipid degradation. Both ME and G6PDH are indirectly involved in lipid synthesis, as they provide major sources of NADPH necessary during fatty acid synthesis.

Respective roles of G3Pase and glycerolipids in glycerol synthesis in isolated hepatocytes. The first experiment showed that activity of G3Pase and of a number of enzymes of lipid metabolism was increased in ambient smelt producing glycerol. As such, the metabolic route to glycerol required further clarity.

Hepatocytes isolated from warm smelt produce glycerol linearly for up to 72 h when placed at 0.4°C in presence of glucose (1). On this basis, we investigated the effect of the inhibition of lipid synthesis on glycerol production over this period. Inhibition was induced using triacsin C, a fungal metabolite and potent inhibitor of
long-chain fatty acyl-CoA synthases (ACS) and, in consequence, of de novo lipid synthesis (2,33).

The effect of triacsin C was first evaluated on the activity of palmitoyl-CoA synthase (pACS) in smelt hepatocytes. Hepatocytes of warm fish (n = 3) were incubated at 0.4°C in the incubation medium described above with 10 μM triacsin C in DMSO (0.1% vol/vol) (T−) or with DMSO only (T−) for 0, 6, 12, and 72 h. At each time point, an aliquot of the hepatocyte suspension was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was discarded, and the cell pellet was kept at −80°C until pACS activity was measured. The percentage of inhibition (% inhib.) was calculated for each time point and for each suspension considered using the following formula: % inhib. = 100 × [pACS− − pACS+, pAPACS]−.[1]

After demonstrating that triacsin C significantly decreased pACS activity in smelt hepatocytes, an experiment was designed to estimate its effect on glycerol synthesis. The effect of triacsin C was determined on six fish with duplicate assays. Cell suspensions were prepared from individual animals, aliquoted into 16 different vials and incubated at 0.4°C in either incubation medium with 10 μM triacsin C (T−) or in control medium (T−). Four vials per treatment were immediately sampled and considered as preincubation samples, while the remaining vials were sampled after 72 h incubation (T−). For each sampling, the contents of two vials were transferred to Eppendorf tubes and centrifuged at 1,000 g for 10 min at 4°C. Supernatants were transferred to new tubes, and both fractions were kept frozen at −80°C until glycerol and G3Pase analyses. The content of the two other vials was centrifuged in lipid-free glass tubes at 1,000 g for 10 min at 4°C, and the supernatant was transferred to new lipid-free glass tubes. Chloroform was added to both fractions, and all tubes were placed under nitrogen and sealed with Teflon before being placed at −80°C until further analysis of lipids.

Biochemical Analysis

Enzyme assays. Liver samples or cell pellets were thawed on ice and homogenized using a polytron in four volumes of ice-cold triethanolamine buffer 0.1 M at pH 7.4. All assays were run in duplicate at 15°C with the exception of lipase that was run at 25°C. In all cases, concentration of the homogenate was chosen to provide a linear response over the period specified below. The activity of all enzymes was shown to be freeze stable. Except for the cell experiments in which enzyme activities were expressed per gram of cells, all activities were assessed per gram of liver and thereafter normalized to grams of fish taken into consideration the liver mass for each individual animal. This method of analysis was selected to gain insight into the capacity of the whole liver to produce glycerol for the whole animal and to take into consideration any change of relative liver mass over the glycerol cycle. The relative mass of liver, represented by the hepatosomatic index—HSI (%); [mass of liver/mass of fish] × 100—was significantly higher in ambient smelt in December than for any other month over the glycerol cycle except January (1.59 ± 0.18, 2.60 ± 0.15, 1.89 ± 0.17, 1.73 ± 0.21, 1.45 ± 0.21, 1.64 ± 0.13, and 1.79 ± 0.10% for November, December, January, February, March, April, and May, respectively; ANOVA; P < 0.05). In warm smelt, HSI was significantly lower in March than in December, January, or February, with average values of 2.01 ± 0.25, 2.67 ± 0.24, 2.83 ± 0.17, 2.45 ± 0.15 and 1.58 ± 0.21% for November, December, January, February and March, respectively (ANOVA; P < 0.05). HSI was higher in warm and in ambient smelt in January and February (t-test; P < 0.05).

Lipase activity was measured using 4-methylumbelliferyl as substrate (4-MUH) by a method modified by Lemieux et al. (13) from Roberts (29). A liposomal dispersion was prepared by dissolving 2 mM 4-MUH and 2 mM soybean lecithin in chloroform methanol (2:1). This mix was evaporated under a nitrogen stream and dissolved back in 150 mM NaCl by sonicating. The reaction assay consisted of 3 ml of 1 mM Tris-HCl (pH 7.5), 20 μl of liposomal dispersion, and 20 μl of homogenate diluted to give a linear response for up to 10 min. The change in fluorescence was recorded every 20 s for 10 min using excitation/emission of 365/450 nm. A standard curve of 4-MU was prepared, and lipase activity was assessed from it as millimoles of 4-MU produced per minute per gram of liver and thereafter converted to mmol4-MU/min g−1 liver.

HOAD assay was run in a triethanolamine buffer (100 mM triethanolamine; pH 7.0) containing 5 mM EDTA, 1 mM KCN, 0.115 mM NADH, and 0.05 mM acetoacetoyl CoA. Disappearance of NADH was monitored at 340 nm for 5 min and HOAD activity assessed as units per gram of fish (U/gfish), 1 unit corresponding to the disappearance of 1 μmol of NADH/min (NADH extinction coefficient = 6.22 mM/cm). Calculations were the same for other enzymes described below.

CPT assay was run in a Tris-HCl buffer (75 mM; pH 8.0) containing 1.5 mM EDTA, 0.25 mM DTNB, 0.035 mM palmitoyl-CoA, and 2 mM l-carnitine (omitted for controls). Formation of a yellow DTNB-CoA complex (extinction coefficient = 13.6 mM/cm) was monitored at 412 nm for 5 min.

ME assay was run in a triethanolamine buffer (65 mM; pH 7.4) containing 5 mM MnCl2, 3.5 mM l-malic acid and 0.35 mM NADP. Appearance of NADP+ (NADP+ extinction coefficient = 6.22 M/cm) was monitored at 340 nm for 5 min.

G6PDH assay was run in a Tris-HCl buffer (55 mM, pH 8.0) containing 3.3 mM MgCl2, 6 mM NADP, and 100 mM glucose-6-phosphate. The appearance of NADPH was monitored at 340 nm for 5 min, and G6PDH activity was assessed as units per gram of fish (U/gfish), 1 unit corresponding to the appearance of 1 μmol of NADPH/min.

G3Pase assay was run in an acetate buffer (100 mM; pH 5.5) containing 10 mM G3P. Released inorganic phosphate (P1) was determined at three time points over 30 min with a colorimetric assay based on Rockstein and Herron (30). Briefly, 40 μl of the reaction mix was added to 80 μl of 3.3% ammonium molybdate in 5 N sulfuric acid: distilled water (2:1; vol/vol) to stop the reaction. Color was initiated by adding 40 μl of 260 mM FeSO4, and absorbance was measured after 5 min on a plate reader at 620 nm. A standard curve of P1 was run from different concentrations of Na2HPO4.

pACS assay was run in a Tris-HCl buffer (100 mM; pH 8.0) containing 5 mM DTT, 150 mM KCl, 15 mM MgCl2, 10 mM ATP, 1 mM coenzyme A and 0.25 mM palmitic acid. After 5 and 20 min of incubation, 100 μl of the reaction mix was stopped by adding 360 μl acetonitrile and 40 μl of 1 M phosphoric acid. The mixture was filtered using a syringe filter (50 μm), and 100 μl of the filtrate was used to determine the amount of palmitoyl-CoA formed using HPLC, according to Tomoda et al. (33). Measurements were made on a C18 WATERS column using WATERS HPLC system. The conditions of the HPLC were as follows: 10-min linear gradient from 40–60% acetonitrile in 25 mM KH2PO4 followed by 10-min isocratic run with 60% acetonitrile in 25 mM KH2PO4, with a flow rate of 1 ml/min and detection UV at 254 nm. The retention time of palmitoyl-CoA was 15 min. A standard curve of palmitoyl-CoA was run, and pACS activity was assessed from it.

Lipid analysis. Lipids were extracted using chloroform/methanol, according to Parrish (22). This method was used principally to measure TG concentrations, avoiding using the usual assay that involves a measure of glycerol, as the high concentrations of glycerol accumulated in winter may affect the accuracy of the results. Concentrations of PL and FFA were determined at the same time. Lipid classes were determined using thin-layer chromatography with flame ionization detection using a MARK V Intracan (Intron Laboratories, Tokyo, Japan) as described by Parrish (21). Extracts were spotted on silica-gel-coated rods, and a three-step development system was used to differentiate lipid classes. A first separation consisted of a 20-min development in hexane:diethyl ether:formic acid (99:1:0.05), the second separation consisted of a 40-min development in hexane:diethyl ether:formic acid (80:20:1), and the last development consisted of 2 × 15 min in 100% ace tone followed by 2 × 10 min in chloroform:
methanol:water (5:4:1). After each development system, the rods were scanned and the chromatograms were analyzed using PEAKSIMPLE 2.83 software system (Shell USA, Fredericksburg, VA). Concentrations of TG, FFA, and PL were quantified using the following lipid standards as reference: tripalmitin (TG), palmitic acid (FFA), and phosphatidylcholine dipalmitoyl (PL). For each class, concentrations measured in liver were determined per gram of tissue and thereafter normalized to gram of fish, taking into consideration the liver mass for each individual animal (μg/gfish). Concentrations measured in plasma were expressed as milligrams per milliliter of plasma (mg/ml/plasma).

Glycerol and protein measurements. Glycerol concentrations were determined by using the free glycerol kit from Sigma (Oakville, ON, Canada) following the manufacturer’s instructions. In experiment 1, glycerol concentrations measured in plasma were expressed as micromoles per milliliter. In experiment 2, glycerol content measured in the medium and pellet was summed and expressed per gram of cells (μmol/gcell).

Statistical Analysis

Values are presented as means ± SE. For all specific parameters followed over the glycerol cycle, significant differences between treatments at a particular sampling point were assessed using Student’s t-tests. One-way ANOVAs with Tukey post hoc test were used to determine differences between sampling points within a treatment. Data were log-transformed when homoscedasticity was not met. In isolated cell experiments, significant differences in pACS activity over time were assessed for each treatment using repeated-measures one-way ANOVAs. Student’s t-tests with repeated measures were used to assess difference in activity between treatments for a particular sampling point. Significant differences between Preincubation, T−, and T+ were assessed using repeated-measures one-way ANOVAs.

RESULTS

G3Pase and Lipid Metabolism Over the Glycerol Cycle

Water temperature and glycerol concentrations. The profiles of water temperature and of glycerol concentrations were presented in a previous paper (3). Briefly, in the ambient tank, temperature started to decrease in November to reach a minimum at 0°C by mid-March and increased from there to reach 2.1°C at the beginning of May 2009. Water temperature in the warm tank fluctuated around 11°C during the entire period. In fish held at ambient temperature, plasma glycerol concentrations increased from 0.71 ± 0.47 to 221.4 ± 23.4 mM between November 2008 and February 2009. After this point, glycerol concentrations decreased until the end of the experiment to 0.02 mM at the beginning of May. Glycerol concentrations in plasma were expressed as micrograms per milliliter of plasma. In ambient smelt, activity was lower in November than in December and January (ANOVA; P < 0.05). In warm smelt, lipase activity was higher in December and January than in March with average values of 99.9 ± 22.0, 90.9 ± 5.0, and 42.3 ± 7.5 mmol4-MU/min gfish, respectively (ANOVA; P < 0.05). Activities were not different between warm and ambient smelt for any sampling points.

For both groups, HOAD and CPT activities were linearly correlated (linear regression P < 0.05; data not shown). No changes of activities were detected for either HOAD or CPT in warm smelt (Fig. 1C and D). In ambient smelt, activity increased early in the cycle, reaching its highest point in March with the average activity of 115.8 ± 13.5 mU/gfish and 12.7 ± 2.2 mU/gfish for HOAD and CPT, respectively. After this point, activity decreased to reach values not different from those measured in November (ANOVA, P < 0.05). For both enzymes, no difference was noted between warm and ambient smelt for any sampling point considered.

ME activity was higher in February than in November in ambient smelt with average activities of 49.8 ± 4.3 mU/gfish and 31.9 ± 4.2 mU/gfish, respectively (Fig. 1E). Activities measured in warm smelt were more variable than in ambient smelt; no significant differences were detected over the period considered. ME activities were higher overall in warm smelt than in ambient smelt with significantly higher activities measured in December, January, and February (Fig. 1E).

G6PDH activity did not change over the glycerol cycle in ambient smelt (Fig. 1F). In warm smelt, activity was higher in January than in November, December, or March (ANOVA; P < 0.05). Activity measured in warm smelt were overall higher than activities measured in ambient smelt and were significantly higher in January (t-test; P < 0.05).

Lipid content in liver and in plasma. Because of technical issues, a number of liver samples were lost in the process of lipid extraction, reducing the number of samples to two or three in some groups. The low number of samples tested decreased the power of statistical analysis and, as such, may not be sufficient to detect subtle differences between groups. The available data, however, provide a general picture of the lipid concentrations present in liver. TG and PL were detected in liver samples; FFA was below levels of detection. Concentrations of TG and PL did not change over the glycerol cycle in warm or ambient smelt. Average concentrations were thus pooled within a group and are presented in Table 1. No differences were detected between warm and ambient smelt.

TG and PL were also detected in plasma samples but not FFA. There was no significant difference in average concentrations in any of the measured lipids between ambient and warm smelt (Table 1). In some cases, there were changes within a lipid class over the time course, but this information is peripheral to the overall scope of this study (PL for ambient smelt and TG for warm smelt; data not shown).

Respective Roles of G3Pase and Glycerolipids in Glycerol Synthesis in Isolated Hepatocytes

Effect of triacsin C on pACS activity. Triacsin C significantly inhibited pACS activity in isolated hepatocytes incubated at cold temperature. Over 72 h, pACS activity increased almost
two times in the absence of triacsin C, while it decreased by 3.5 times in its presence, being already significantly reduced after 6 h incubation (Fig. 2). pACS activity was inhibited by 52.8/1100616.9, 61.5/1100613.6, and 85/110061.36% after 6, 12, and 72 h of incubation, respectively.

Effect of triacsin C on glycerol production, G3Pase activity, and lipid content. Glycerol accumulated significantly in all preparations incubated for 72 h at 0.4°C, with no difference between treatments (Table 2). On average, 67.7 ± 17.0 and 78.7 ± 20.6 μmol/g cell of glycerol were produced by T– and
Table 1. Triglycerides and phospholipids concentrations in rainbow smelt (Osmerus mordax)

<table>
<thead>
<tr>
<th></th>
<th>Ambient Smelt</th>
<th>Warm Smelt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver, µg/gfish</td>
<td>Plasma, µg/mlplasma</td>
</tr>
<tr>
<td>TG</td>
<td>156.9 ± 37.5</td>
<td>5.30 ± 0.38</td>
</tr>
<tr>
<td>equivalent glycerol</td>
<td>0.20 µmol/gfish</td>
<td>18.8 ± 1.0*</td>
</tr>
<tr>
<td>PL</td>
<td>532.0 ± 75.0</td>
<td>18.8 ± 1.0*</td>
</tr>
<tr>
<td>equivalent glycerol</td>
<td>0.71 µmol/gfish</td>
<td>18.8 ± 1.0*</td>
</tr>
</tbody>
</table>

Average concentrations ± SE measured in liver and plasma of ambient and warm smelt from November 2008 to May 2009, and November 2008 to March 2009, respectively. Data collected monthly were pooled within a group (n = 28 and n = 22 for ambient and warm smelt, respectively). *Significant differences were detected over the period considered. Glycerol-equivalent concentrations of triglycerides (TG) and phospholipids (PL) were estimated in liver based on a molecular weight of 800 and 750 g/mol, respectively.

Glycerol is accumulated at equivalent concentrations in all tissues (5, 23). Therefore, it is possible to estimate the rate of glycerol synthesis for the whole animal. Over the accumulation period, from November to February, the rate of glycerol accumulation is estimated to be close to 1.2 nmol/min gfish; 215 µmol/gfish/(120 days × 24 h × 60 min). The actual rate of synthesis should be higher considering the daily 10% loss to the water (3). In November, G3Pase activity was 10 mU/gfish the lowest activity measured over the accumulation period. This activity would allow the production of 10 nmol/min gfish glycerol under optimal conditions at 15°C. Over this period, average water temperature was about 4.5°C. Assuming a Q10 of 2, actual average activity of G3Pase could result in a rate of glycerol production closer to 5 nmol/min gfish. As such, this activity may be sufficient to produce glycerol at the rate observed over the accumulation period without any need of up-regulation, but it may be limiting, considering it is very close to the rate of synthesis needed and that the actual physiological activity could be lower. The sharp increase of G3Pase activity observed at the onset of glycerol accumulation in December and January could thus be a requirement to support glycerol synthesis over this critical period.

**DISCUSSION**

G3Pase and Lipid Metabolism Over the Glycerol Cycle


In ambient smelt, G3Pase activity increased early in the cycle, with the highest values recorded in December and January at the onset of glycerol accumulation. Activities started to decrease after these points to reach the initial values by the end of the cycle. The increase of activity observed in December and January may be a way to compensate for the decrease of activity induced by the decreasing water temperature, suggesting the activity of this enzyme is important enough to be up-regulated at the onset of the glycerol cycle in smelt. This pattern of activity is consistent with that reported by Lewis et al. (14) for GPDH, supporting a channeling of DHAP toward glycerol production through a direct dephosphorylation of G3P. How this increase of activity is achieved, either by increasing the amount of enzymes or by modification of the protein is still to be resolved.
Concentrations of none of the lipid classes changed in smelt liver over the experiment and were very similar between warm and cold smelt. Concentrations of TG measured in liver over the experiment and were very similar between warm and cold smelt. As such, TG reserves per se cannot be the source of glycerol, confirming a report by Raymond (25) and do not seem to have a significant role at the onset of glycerol synthesis. Similar to what was seen for G3Pase, activities of enzymes involved in lipid degradation (lipase, HOAD, and CPT) increased at the onset of glycerol accumulation. The assay used to measure lipase activity was directed to triglyceride lipases (EC 3.1.1.3) and, thus, should reflect the potential of smelt liver to lyse TG, even though its actual contribution cannot be estimated due to the synthetic nature of the substrate used (29). Both CPT and HOAD are key to the oxidation of FFA, supporting the high correlation observed between their activities. Up-regulation of these three enzymes early in the cycle supports the importance of lipids as a metabolic fuel when water temperature decreases.

Taken together, these results suggest that G3Pase activity is important for glycerol synthesis and that TG reserves do not have an important role at the onset of glycerol accumulation in December. The unchanged activities of enzymes involved providing reducing equivalents for lipid synthesis (ME and G6PDH) do not support an active synthesis of FFA for the formation of new glycerolipids following an active lipolysis. However, the data do not rule out any potential recycling of released FFA into new glycerolipids, a pathway that may provide glycerol in addition to that synthesized through G3Pase. The second experiment using isolated smelt hepatic cells as a model was conducted to answer this question.

**Glycerol Synthesis in Isolated Hepatic Cells**

Hepatic cells isolated from smelt maintained at warm temperature and incubated at cold temperature (0.4°C) produce glycerol in a linear manner for up to 72 h (1). In this experiment, isolated cells received only glucose as external fuel. Under these conditions, the potential sources of glycerol over a short-term period may be 1) the direct dephosphorylation of G3P by G3Pase, 2) the lipolysis of on-board glycerolipids, and 3) a continuous cycle involving de novo synthesis of glycerolipids from newly synthesized G3P and recycled FFA, followed by lipolysis.

As it was reported in all previous animal cell models assayed under similar conditions [Raji cells, (32); human fibroblasts (11, 12); and rat hepatic cells (17)], triacsin C strongly affected ACS activity in smelt-isolated hepatic cells. A strongly impaired de novo synthesis of glycerolipids did not, however, affect glycerol production, ruling out de novo synthesis and subsequent degradation of glycerolipids as a possible source of glycerol in isolated hepatic cells.

After 72 h of incubation, average TG concentration did not change significantly in the control group, while it decreased by 25% when cells were exposed to triacsin C. Assuming TG degradation may have occurred in both groups of cells, broken-down TG may have been replaced by de novo synthesis in the control group, while the inhibition of ACS would block this replacement in cells exposed to triacsin C, supporting the decrease of TG in this group. Regardless, initial concentrations of TG would be sufficient to provide only 2.5% of the glycerol produced. This rough estimation is based on an average molecular weight of 800 and 750 g/mol, respectively.

**Table 2. Glycerol concentrations, G3Pase activity, and lipid concentrations in hepatocytes from rainbow smelt (Osmerus mordax)**

<table>
<thead>
<tr>
<th>Glycerol, μmol/gcells</th>
<th>Preincubation</th>
<th>T72/T-</th>
<th>T72/T+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol, μmol/gcells</td>
<td>10.06 ± 0.37a</td>
<td>77.5 ± 17.1b</td>
<td>88.8 ± 20.7b</td>
</tr>
<tr>
<td>G3Pase, U/gcells</td>
<td>1.18 ± 0.26</td>
<td>1.00 ± 0.14</td>
<td>1.21 ± 0.19</td>
</tr>
<tr>
<td>TG, mg/gcells</td>
<td>1.38 ± 0.19a</td>
<td>1.31 ± 0.26a,b</td>
<td>1.03 ± 0.19b</td>
</tr>
<tr>
<td>equivalent glycerol, μmol/gcells</td>
<td>1.72</td>
<td>1.64</td>
<td>1.29</td>
</tr>
<tr>
<td>PL, mg/gcells</td>
<td>20.9 ± 1.4a</td>
<td>12.7 ± 2.3b</td>
<td>11.5 ± 1.8b</td>
</tr>
<tr>
<td>equivalent glycerol, μmol/gcells</td>
<td>27.9</td>
<td>16.9</td>
<td>15.1</td>
</tr>
</tbody>
</table>

Average concentrations ± SE measured in isolated hepatic cells before (Preincubation) and after 72 h (T72) incubation at 0.4°C in presence (T+) or absence (T−) of 10 μM triacsin C (n = 6). a,bSignificantly different values. Glycerol-equivalent concentrations of TG and PL were estimated on the basis of a molecular weight of 800 and 750 g/mol, respectively.

---

Fig. 3. Linear correlation between rates of glycerol production by hepatic cells isolated from smelt and incubated at 0.4°C with (T+) or without (T−) 10 μM triacsin C. Each dot represents data obtained from a different hepatic cell preparation (n = 6).
TG cannot be an important source of glycerol in the isolated cells, as it was concluded from the whole animal experiments. PL concentrations decreased in both groups of cells (T− and T+) by 40–45% over the incubation period, with initial concentrations comparable to concentrations observed in whole livers (20.9 ± 1.4 mg/gcells vs. 27.5 ± 2.9 mg/gliver, respectively). Applying the same approach that we used to estimate the amount of glycerol that could be generated by TG, the amount of PL used could generate about 12 μmol glycerol or about 17% of the total glycerol produced, assuming all PL were glycerophospholipids ([9 mgPL/gcells ± 0.75 mg/μmol] × 100 ÷ 70 μmol glycerol produced). The main function of PL is to form cellular membranes. Such a decrease in the amount of PL would most likely not occur in vivo and, therefore, should not be a source of glycerol of equivalent importance at the whole animal level. Rather, it may be a response to the shock the cells received following the rapid transfer from acclimation temperature of the fish used (8°C) to the low temperature at which cells were incubated (0.4°C).

FFA concentrations measured in the supernatant were not sufficient to make up for the large decrease measured in pellets (about 35% of it), suggesting most FFA released by lipolysis were oxidized by the cell.

G3Pase activity did not increase over the experiment in any groups of cells and was not affected by triacsin C. Activity would, however, be sufficient to produce glycerol at the rate observed. Indeed, 1 μmol/min gcells glycerol could be produced at 15°C. Assuming a Q10 of 2, actual G3Pase activity would be closer to 0.35 μmol/min gcells at 0°C, still more than sufficient to produce the average 0.02 μmol glycerol/min gcells measured.

Perspectives and Significance

This study is the first to clearly support that the metabolic pathway through G3Pase is the main source of glycerol in smelt during the winter season. Adjustments of lipid metabolism occur in winter, as suggested by regulation of different enzymes, but this is more likely associated with adaptations to cold in general than to the specific production of glycerol. On-board TG is not a direct source of glycerol nor is cycling cold in general than to the specific production of glycerol.

ACKNOWLEDGMENTS

We thank the Field Services Unit of the Ocean Sciences Centre for the collection of specimens. Kathy Clow for the technical assistance she offered in isolating smelt hepatic cells, and Connie Short for ongoing support.

REFERENCES


