Low temperature directly activates the initial glycerol antifreeze response in isolated rainbow smelt (*Osmerus mordax*) liver cells

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Short title: Low temperature glycerol production in smelt hepatocytes
ABSTRACT

Rainbow smelt (Osmerus mordax) accumulate high levels of glycerol in winter that serves as an antifreeze. Liver glycogen is a source of glycerol during the early stages of glycerol accumulation while dietary glucose and amino acids are essential to maintain rates of glycerol synthesis. Here we report rates of glycerol and glucose production by isolated hepatocytes. Cells from fish held at 0.4°C to -1.5°C and incubated at 0.4°C were metabolically quiescent with negligible rates of glycerol or glucose production. Hepatocytes isolated from fish maintained at 8°C and incubated at 8°C produced glucose but not glycerol. Glycerol production was activated in cells isolated from 8°C fish and incubated at 0.4°C without substrate or when glucose, aspartate or pyruvate was available in the medium. Incubation at 0.4°C without substrate resulted in similar molar rates of glucose and glycerol production in concert with glycogen mobilization. Glycogenolysis and glycerol production were associated with increases in total in vitro activities of glycogen phosphorylase and glycerol 3-phosphate dehydrogenase. Maximal in vitro activities of hexokinase (HK) and glucokinase were not influenced by temperature but high activities of a low-\(K_m\) HK may serve to redirect glycogen derived glucose to glycolysis as opposed to releasing it from the cells. Rates of glycerol production were not enhanced in cells from fish held at 8°C and incubated at 0.4°C with adrenergic or glucocorticoid stimulation. As such, low temperature alone is sufficient to activate the glycerol production mechanism and results in a shift from glucose to a mix of glucose and glycerol production.
INTRODUCTION

Rainbow smelt (*Osmerus mordax*) accumulate high levels of glycerol in winter that serves as an antifreeze in plasma and all other tissues. Glycerol level typically increases from less than 5 mM to in excess of 300 mM during the fall-winter transition. Glycerol levels reach a plateau and then in late winter/early spring decrease even at cold temperatures as antifreeze protein assumes more importance in freeze resistance (5, 6, 12). Glycogen in liver is an important source of glycerol during the early stages of glycerol accumulation, while continued feeding is required for survival and continued glycerol production at low temperature (6). A low temperature signal is sufficient to trigger glycerol accumulation in rainbow smelt at the whole animal level (6); however, the sequence of events at the organ-cellular level is not fully understood.

Glycogen level in liver is substantially higher than in all other tissues and a temperature decrease from 8°C to -1.2°C over a period of 12 days essentially depletes glycogen stores in liver of fed fish. The rate of depletion is even faster in food-deprived animals (5). In this study, we assess the impact of temperature on the ability of isolated liver cells to mobilize glycogen with the associated production of glycerol. The control of glycogen utilization and how it relates to glucose release in fish liver cells has been well studied. The first step in glycogen breakdown is catalyzed by glycogen phosphorylase (GPase) which is to a large extent under catecholamine control. Adrenergic stimulation results in increased % GPase a activity in association with elevated glucose release (16). Potentially relevant to understanding the processes in liver of rainbow smelt, as relates to freeze tolerance, are studies with amphibians. For instance, the wood frog (*Rana sylvatica*) upon the initiation of freezing produces glucose from large glycogen reserves in liver, a process that is under adrenergic control (27, 28). The situation is probably similar in the gray tree frog (*Hyla versicolor*), a species that produces glycerol as an antifreeze agent, as cold exposure results in increases in % GPase a activity (26). Here we examine the impact of temperature and adrenergic stimulation on GPase activity in isolated
hepatocytes of rainbow smelt. Although there is compelling evidence that liver of rainbow smelt produces glycerol, the possibility that glucose produced by liver is also cycled to other tissues to be converted to glycerol extrahepatically cannot be ruled out. In experiments executed here it was noted that rates of glycogen depletion exceeded rates of glycerol production. We therefore assessed the rates of glucose and glycerol production by isolated liver cells again as influenced by temperature and adrenergic stimulation.

Further aspects of this study relate to control of glycerol production at the level of glycerol-3-phosphate dehydrogenase (GPDH) and the determination if other potential hormonal signals can enhance glycerol production. Glycerol is formed from glycogen, glucose and amino acids via the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (catalyzed by GPDH) and subsequently to glycerol (4). Cytosolic GPDH activity and gene expression are increased during the active phase of glycerol accumulation in rainbow smelt following the natural thermal cycle (12, 13), GPDH activity increases in liver of fish subjected to acute decreases in water temperature (6), and GPDH mRNA decreases in fish subjected to acute temperature increase (8). GPDH activity is stimulated in rat hepatoma cell lines and transformed rat hepatocytes following three days of in vitro culture with glucocorticoids (14) while GPDH activity and mRNA level are stimulated by glucocorticoids in the rat brain (2). As well, in mouse liver the GPDH gene has a functional peroxisome proliferator-activated receptor (PPAR) response element in its promoter that is a direct target of PPARα (21). Here we determine the activity of GPDH in isolated cells subjected to acute temperature decrease and the impact of glucocorticoid and PPARα stimulation on glycerol production.

In addition to glycogen, glucose is an important substrate for glycerol production by liver of rainbow smelt as evidence by injected radioisotopes and stable isotopes of glucose appearing in labelled glycerol (22, 32) and the necessity for continuous food intake for survival and glycerol production at low temperature (5). In this study, we assessed the enzymatic capacity for glucose
phosphorylation by measuring low \( K_m \) hexokinase (HK) and high-\( K_m \) glucokinase (GK) activities. GK activity, GK gene expression, and HK activity are induced by dietary carbohydrate in liver of rainbow trout (\textit{Onchorynchus mykiss}) (25) presumably in association with hormonal signalling. Here we assess if a low temperature induced change in glucose trafficking may directly result in changes in enzyme activity patterns.

The most important findings are that low temperature challenge alone is sufficient to activate the glycerol production mechanism (i.e. hormonal signals to liver are not a requirement) in isolated cells. Low temperature activation of glycogenolysis is associated with an increase in total GPase activity and there is a switch from glucose release at high temperature to a mix of glucose plus glycerol release at low temperature.

**MATERIALS AND METHODS**

*Animals*

Rainbow smelt, \textit{Osmerus mordax}, Mitchell, were collected by seine netting from Long Harbour, Newfoundland in October between the years 2002 and 2007, transported to the Ocean Sciences Centre, Memorial University of Newfoundland, and held in 3000 l or 1800 l tanks with flow-through seawater. Fish were kept on a natural photoperiod with fluorescent lights set by an outdoor photocell and fed a diet of chopped herring twice a week. After 4 weeks while water temperature was at 8-10\(^\circ\)C, some of the fish were separated into two (500 l) experimental tanks, one maintained at 8\(^\circ\)C ("warm smelt") and the other tracked ambient temperature ("cold smelt"). Ambient water temperature at the time of experimentation ranged between 0.4 and \(-1.5\(^\circ\)C (see reference 12 for a typical water temperature profile). Cold smelt were at these low temperatures for at least two weeks before isolation of hepatocytes. All experiments were conducted between December and April the period over which rainbow smelt normally have high levels of glycerol (12). Fish were not held past April in any given
year, as such, a new group of animals was collected every October. Experiments were conducted over six separate years. Both male and female fish were utilized and fish ranged in mass from 24 to 132 g. Animal protocols were approved by the Institutional Animal Care Committee, Memorial University of Newfoundland.

_Hepatocyte isolation_

Rainbow smelt were randomly removed from both the warm and cold water tanks, killed by a quick blow to the head, and the liver was immediately exposed. Hepatocytes were isolated by perfusing the liver with collagenase as described by Moon et al. (17) with minor modifications. Briefly, a retrograde perfusion was performed by cannulating the hepatic vein and nicking the venous input for drainage. Perfusion medium containing (in mM) 176 NaCl, 5.4 KCl, 0.81 MgSO₄, 0.44 KH₂PO₄, 0.35 Na₂HPO₄, 5 NaHCO₃, 10 Hepes, and 1 EGTA, adjusted to pH 7.63 was used to clear blood. This was the basic medium for all isolation and cell suspension protocols. The perfusion was carried out at room temperature at a flow rate of 1-1.5 ml min⁻¹. After 10 min, the liver was transferred to a watch glass and perfused with the above medium containing 0.3 mg ml⁻¹ collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and no EGTA. The liver was perfused with this recirculated collagenase medium for 25 min or until the tissue was soft. The liver was gently teased apart and the isolated cells were filtered, washed 3 times in medium containing 2% BSA (fraction V, fatty acid free, ICN Biochemicals) and 1.5 mM CaCl₂, and counted using a Neubauer haemocytometer. Cell viability was assessed at this time by trypan blue exclusion and was usually greater than 95%. Any preparations with viabilities below 90% were rejected. Cells were resuspended at a concentration of 40 x 10⁶ cells ml⁻¹. In some cases, hepatocytes from two individuals were pooled in order to obtain enough cells to complete one experiment. Initial (“pre-incubation” or “0 h”) hepatocyte samples were collected by adding 150 µl of the cell suspension or 6 x 10⁶ cells (corresponding to 10 –30 mg cells) to 2 or 3
microcentrifuge tubes. These tubes were centrifuged at 1000 g for 10 min, the supernatant was transferred to a cryovial, and both the cell pellet and supernatant were snap frozen in liquid nitrogen. Samples were stored at -80°C until glycerol, glucose, glycogen content and/or enzyme activities were measured. The remaining cell suspension was divided into 20 ml glass scintillation vials (10-30 mg cells per vial) containing 2 ml of medium with 2% BSA and 1.5 mM CaCl₂ and incubated at either 0.4°C (cold incubation) or 8°C (warm incubation). Preliminary experiments comparing cells plated in six-well Primaria plates (Falcon) to cells plated in 20 ml scintillation vials indicated no difference in viability and glycerol production after 24, 48 and 72 h. By 72 h, hepatocytes established cell-to-cell connections and formed monolayers. Viabilities at 72 h were usually greater than 90% and any viabilities below this were rejected.

**Experimental Protocols**

1. **Linearity of glycerol production.** Initial experiments were performed to determine if glycerol was produced at a linear rate. Hepatocytes from warm or cold smelt were incubated at 0.4°C with 5 mM glucose or without substrate in the medium for 24, 48, and 72 hr. At each time point, the hepatocyte suspension was transferred to separate centrifuge tubes, spun at 1000 g for 10 min, the supernatant collected, and both portions frozen in liquid nitrogen for glycerol analysis.

2. **Impact of acclimation/incubation temperature and substrate availability on glycerol production.** After confirming that glycerol was produced in a linear fashion by isolated cells, we next determined if supplementing the medium with potential substrates could enhance glycerol production and assessed the impact of acclimation/incubation temperatures on rates of glycerol production. Hepatocytes from warm fish were incubated at either 0.4°C or 8°C in medium without substrate or containing one of the following (in mM): 5 glucose, 5 L-alanine, 5 L-aspartate, 1 pyruvate.
Hepatocytes from cold fish were incubated at 0.4°C. Glycerol production and cell viability were determined after 72 h.

3. Glycerol production, GPase (EC 2.4.1.1) and potential adrenergic activation of glycogenolysis. Experiment 2 showed that even in the absence of substrate, hepatocytes from warm acclimated fish produce glycerol when incubated at cold temperature. The next series of experiments determined if glycogen was a potential source of glycerol and the state of GPase. As well, it was assessed if glycerol production and GPase activation state could be enhanced by adrenergic stimulation.

Samples were collected at pre-incubation and following 72 h incubation with no exogenous substrate in the medium. Glycogen level was determined in cells isolated from warm-acclimated rainbow smelt and incubated at 0.4°C or in two cases also at 8°C. Glycogen was not measured in fish held at cold temperature since it is known to be minimal (5). Thereafter, experiments consisted of three acclimation/incubation groups: warm fish/8°C incubation, warm fish/0.4°C incubation, and cold fish/0.4°C incubation. Cells were incubated without additional components or with 1 or 10 µM isoproterenol (a non-specific β-agonist) (Sigma-Aldrich, U.S.A.) in the medium. Hepatocytes were assayed for glycerol production and GPase activity (total and α). The level of isoproterenol used was based on the review of Fabbri et al. (9) who concluded, on the basis of many studies, that the general concentration of catecholamine required to stimulate maximal glucose production by isolated fish hepatocytes is in the 10 µM range although much lower concentrations may be effective.

4. Glycerol and glucose production. Experiment 3 revealed that decreases in glycogen could not be accounted for by increases in glycerol alone. This led to studies that included glucose measurements. Samples were collected at pre-incubation and following 72 h incubation with no exogenous substrate in the medium. Experiments consisted of three acclimation/incubation groups: warm fish/8°C incubation, warm fish/0.4°C incubation, and cold fish/0.4°C incubation. Cells were
incubated without additional components or with 1 or 10 µM isoproterenol in the medium. Rates of glucose and glycerol production were determined.

5. **GPDH (EC 1.1.1.8), HK (EC 2.7.1.1) and GK (EC 2.7.1.2) activity.** The aim of this experiment was to assess if enhanced glycerol production was associated with increases in the activity levels of key enzymes. Cells were collected at pre-incubation and following 72 h incubation with 5 mM glucose in the medium. Experiments involved fish acclimated to warm temperature with hepatocytes from the same population of fish incubated at 8ºC and 0.4ºC. Glycerol production was assessed along with GPDH, HK, and GK activities. Six whole livers from other fish were also collected and frozen at this time.

6. **Glucocorticoid and PPAR activation.** We assessed if the presence of glucocorticoids or a putative PPAR activator could enhance glycerol production. Cells were collected at pre-incubation and following 72 h incubation with 5 mM glucose in the medium. Experiments consisted of cells prepared from warm fish and incubated at 0.4ºC. Glycerol production was assessed without any further additions or with 100 or 1000 ng ml⁻¹ hydrocortisone (Sigma-Aldrich, St. Louis MO, USA), 100 or 1000 ng ml⁻¹ dexamethasone (Sigma-Aldrich), or 50 µM Wy 14643 (Sigma-Aldrich) in the medium. Three preparations were utilized with isolated cells in every experiment being subject to all of the above conditions. The level of glucocorticoid activators was based on amounts shown to increase medium glucose and increase glycogen breakdown in tilapia and rainbow trout hepatocytes (15). Wy 14643 is a PPAR ligand and in mouse fibroblast cells a concentration of 10 µM was sufficient to activate a GPDH reporter construct (21).

**Analytical**

**Glycerol, glycogen, and glucose.** Both media and the cell pellet were analyzed for glycerol in duplicate. Glycerol was directly determined from the media samples using a free glycerol kit (F6428;
Sigma-Aldrich). Cell pellets were dissolved in nine volumes of 6% perchloric acid, the homogenate was neutralized with 20% KOH, spun at 10,000 g for 10 min, and the supernatant was assayed for glycerol. In experiments in which glucose was also measured, cell pellets were treated as above. Glycerol or glucose content in the medium and pellet were summed and expressed per g cell. The rate of glycerol or glucose production was calculated by subtracting post-incubation from pre-incubation levels and dividing by time.

For glycogen analysis nine volumes of 30% KOH were added to each cell pellet and boiled for 10 min. Glycogen was precipitated by adding 0.15 ml of 2% aqueous Na₂SO₄ and 1.0 ml absolute ethanol. After the mixture was centrifuged for 10 min at 1500 g, the supernatant was decanted. The pellet was washed with 1 ml 66% ethanol and dissolved in 0.5 ml warm distilled water. An equal volume of 1.2 M HCl was then added, and the sample heated in a boiling water bath for 2 h (31). Hydrolysates were frozen in liquid nitrogen and stored at -80°C until analyzed for glucose content.

Assay conditions for glucose released during glycogen measurements and free glucose produced during hepatocyte incubation were based on a procedure modified from Bergmeyer et al. (1). Briefly, an assay media was prepared containing (in mM) 250 imidazole, 5 MgSO₄, 10 ATP, and 0.8 NADP⁺. A 25 or 50 µl aliquot of the sample was added to a 96 well plate and diluted 1:10 or 1:5 with the assay medium. Twenty five µl of 10 U ml⁻¹ glucose-6-phosphate dehydrogenase (G6PDH) was added to remove any endogenous glucose-6-phosphate (G-6-P). Absorbance was read at 340 nm on a DTX 880 plate reader (Beckman Coulter) after 10 min. Twenty five µl of 10 U ml⁻¹ HK was then added and the absorbance read after 45 min.

**Enzyme assays.** For GPase cell pellets were homogenized in nineteen volumes of ice-cold extraction buffer (in mM) 50 imidazole, 5.0 EGTA, 2.0 EDTA, 30 mercaptoethanol, 100 sodium fluoride, and 0.1 phenylmethylsulfonyl fluoride, pH=7.4 at 4°C. Samples were incubated on ice for 30 min and the upper phase was assayed for GPase a and total (a + b) activity. Assay conditions were
based on a procedure modified from Joanisse and Storey (11). Total GPase was measured in (in mM) 50 potassium phosphate buffer (pH 7.0) containing 0.4 NAD⁺, 0.010 glucose-1,6-bisphosphate, 0.25 EDTA, 15 MgCl₂, 1 U ml⁻¹ G6PDH, 1 U ml⁻¹ phosphoglucomutase, and 1.6 AMP. The reaction was initiated by adding 2 mg ml⁻¹ glycogen (oyster muscle, dialyzed; Sigma- Aldrich). Control and reaction rates were determined separately. GPase a activity was measured in the absence of AMP. The % GPase a activity was calculated by dividing GPase a by total GPase (a + b).

For GPDH, HK and GK activities, frozen cell pellets or pieces of liver were homogenized in nine volumes of ice-cold extraction buffer containing (in mM) 50 triethanolamine, 1.0 DTT, and 5.0 EDTA, pH 7.6 at 4°C. The homogenate was spun at 1000 g for 5 min, an aliquot of the supernatant was diluted 1:20 for GPDH determination and the rest was used for HK/GK determination. GPDH assay conditions included (in mM) 20 imidazole, pH 7.2 at 20°C, 0.15 NADH and 2.0 DHAP which was used to initiate the reaction. HK/GK assay conditions included (in mM) 50 triethanolamine, pH 7.6 at 20°C, 0.2 NADP⁺, 8.0 MgCl₂, 7.0 ATP, and 0.4 U ml⁻¹ G6PDH. The reaction was initiated by 1 mM glucose for HK (low 𝐾ₘ) determination and 100 mM glucose for total glucose phosphorylation activity. Control rates were determined prior to the addition of the substrate. In addition, glucose dehydrogenase (G1DH; EC 1.1.1.47), a microsomal enzyme in fish liver, was measured in order to correct GK activities in these frozen samples. G1DH can introduce significant bias into GK measurements in frozen tissues thus GK activity was calculated as the total glucose phosphorylation activity minus low 𝐾ₘ HK activity minus 1/3 of G1DH activity (20). G1DH assay conditions were similar to total glucose phosphorylation activity conditions except ATP and G6PDH were omitted. The above homogenates were also assayed for protein using a Coomassie (Bradford) protein assay kit (23200; Pierce, Rockford IL).

Enzyme activities were determined at 340 nm on a Beckman DU640 spectrophotometer and activity calculated on the basis of a millimolar extinction coefficient of 6.22. All enzyme activities
were determined at 20ºC to facilitate analysis at higher activity levels since we were primarily interested in relative not absolute activities during exposure of fish to decreased temperature. This was considered acceptable with the assumption that enzyme activity is proportional to enzyme content. Enzymes activities were expressed as either µmol min⁻¹ g⁻¹ cells or µmol min⁻¹ mg⁻¹ protein.

Data analysis

Pre-incubation versus 72 h incubation values or treatment effects after 72 h incubation were compared with the Wilcoxon signed-rank tests when paired samples from the same cell preparation were available. The Mann-Whitney U test was utilized in situations where some but not all of the values were obtained from the same population of cells. The Kruskal-Wallis test was used to determine the statistical differences when three groups were compared. This was followed by the Mann-Whitney U test to identify between treatment differences. Student’s t-test was used to compare GPDH, HK, and GK activity levels in whole liver versus activity levels in freshly isolated hepatocytes from different fish. P ≤ 0.05 was considered to be statistically significant for all studies. A statistical software package (SPSS) was used for all statistical analysis.

RESULTS

Initial glycerol levels and linearity of glycerol production. Total glycerol measured in pre-incubation cells isolated from warm smelt was 11.41 ± 1.28 µmol g⁻¹ (N=17; data from experiment 2 only). This level is similar to that found in whole livers from smelt prior to the antifreeze response (12) and in smelt maintained at 8ºC (6). Glycerol level in pre-incubation hepatocytes from cold fish was 8.63 ± 0.97 µmol g⁻¹ (N=8), a value lower than expected when compared to levels found in vivo at that acclimation temperature. Analysis of the post-wash solution indicated that most of the glycerol in the hepatocytes was lost during washing and/or liver perfusion. Attempts to increase the osmolality of the
perfusion and washing solutions (to equal the osmolality of the in vivo liver) resulted in unacceptable viability.

The production of glycerol, assessed as the sum of glycerol in the cells plus glycerol released into the incubation medium was linear for at least 72 h (Fig. 1). In groups that showed elevated levels of glycerol, approximately 90% of the total glycerol was found in the incubation medium. Subsequent experiments used 72 h incubations.

*Hepatocytes from warm fish produce glycerol at low temperature.* Total glycerol concentrations at pre-incubation and following 72 h incubation, either without substrate or with (in mM) 5 glucose, 5 alanine, 5 aspartate or 1 pyruvate in the incubation media are compared in Fig. 2. Fish held at warm temperatures, and not yet engaged in enhanced glycerol production in vivo, did not show an increase in glycerol levels when hepatocytes were isolated and incubated at 8ºC (Fig. 2A). Incubation without substrate or with alanine in the medium resulted in a small but significant decrease in total glycerol levels. In contrast, when hepatocytes from warm fish were incubated at 0.4ºC for 72 h, glycerol synthesis was activated (Fig. 2B). Significant increases in glycerol occurred when either glucose or pyruvate were added to the medium (104.8 µmol g⁻¹ and 76.8 µmol g⁻¹, respectively versus a pre-incubation level of 11.2 µmol g⁻¹). When incubated without substrate or with aspartate in the medium, there was a tendency for an increase in glycerol production with respect to pre-incubation levels as P values were 0.059 and 0.052, respectively. No significant changes in glycerol were seen when hepatocytes from cold fish were incubated at 0.4ºC with the exception of when glucose was added to the medium (Fig. 2C). Under these conditions glycerol levels increased from the pre-incubation values of 8.63 µmol g⁻¹ to 17.64 µmol g⁻¹.

Rates of glycerol production were calculated in order to compare glycerol production at different acclimation/incubation temperatures (Table 1). The rate of glycerol accumulation at 0.4ºC
incubation temperature with either glucose or aspartate in the medium was significantly higher in warm- than cold-acclimated fish. In the absence of exogenous substrate, the average glycerol production was higher in cells from warm-acclimated than cold acclimated fish when tested at 0.4°C; however, in this experiment, this difference was not statistically significant. Glycerol production by cells from warm fish incubated at 0.4°C was significantly higher than cells from warm fish incubated at 8°C with glucose, aspartate, or no substrate in the media. There were no significant differences between groups, in rates of glycerol production, when the media contained either alanine or pyruvate (data not shown).

*Glycerol production is associated with an increase in total GPase.* Initial glycogen levels were measured from hepatocytes prepared from warm acclimated smelt. When these cells were incubated in the absence of substrate at 0.4°C for 72 h, there was a significant decrease in glycogen content (214.9 ± 79.8 to 17.4 ± 12.4 µmoles glucosyl units g⁻¹ : N=7). Cells from two of these warm fish were also incubated at 8°C and showed a similar decrease in glycogen levels (21 and 42 µmoles glucosyl units g⁻¹).

Hepatocytes from warm fish incubated at 0.4°C produced significant amounts of glycerol after 72 h compared to the pre-incubation level (180.3 ± 31.64 µmol g⁻¹ compared to 11.37 ± 1.69 µmol g⁻¹) (Fig. 3). The direction of change in glycerol level in this study was consistent with the previous experiment although the magnitude of change was greater. Preparations of cold fish/0.4°C cells or warm fish/8°C cells did not produce glycerol in the absence of substrate. Instead, cells from warm fish incubated at 8°C showed significant glycerol decrease. When values were expressed as rates, glycerol production in warm fish/0.4°C cells was significantly higher than glycerol production in warm fish/8°C cells or in cold fish/0.4°C cells (Table 2). If all of the glycogen in the cells from warm fish incubated at 0.4°C were converted to glycerol this would result in a rate of (215 – 17 /72)(2) = 5.5 µmol g⁻¹ h⁻¹ a value about twice as high as the observed rate of glycerol production.
Total GPase and GPase \(a\) activities were determined in the same population of cells as shown above for glycerol production. There was a significant increase in total GPase and GPase \(a\) activities when hepatocytes from warm fish were incubated at 0.4°C, compared to pre-incubation activities (Fig. 4B), whereas, an 8°C incubation showed significant decrease in total GPase and GPase \(a\) activities (Fig. 4A). The directions of change match the patterns seen in glycerol production in hepatocytes isolated from warm fish at 0.4°C and 8°C. There was no change in % GPase \(a\) for either of these two groups. Individual variability in enzyme activity was much greater in the cold fish/0.4°C cells group and as a result, although average total GPase (7.02 µmol min\(^{-1}\) g\(^{-1}\) vs. 11.75 µmol \(\cdot\) min\(^{-1}\) g\(^{-1}\)) and GPase \(a\) (5.73 µmol min\(^{-1}\) g\(^{-1}\) vs. 11.58 µmol min\(^{-1}\) g\(^{-1}\)) activities were higher after incubation, they were not significantly different from pre-incubation values.

We next assessed if adrenergic activation could work synergistically with low temperature to further increase glycerol production by isolated rainbow smelt hepatocytes (Table 2). The inclusion of 1 or 10 µM isoproterenol during the incubation period did not result in statistically significant changes in the production of glycerol relative to preparations without adrenergic stimulation. However, in warm cells incubated at 0.4°C the average value of glycerol production decreased from 2.35 to 1.52 µmol g\(^{-1}\) h\(^{-1}\). In five experiments in which paired samples were available the rate of glycerol production was decreased by 27 ± 8 % with the addition of 1 µM isoproterenol. Isoproterenol did not change the activity patterns of total GPase, GPase \(a\) (data not shown), or %GPase \(a\) (data not shown) relative to incubation without adrenergic stimulation. Total GPase activity in cells from both warm and cold acclimated fish was significantly higher in cells incubated at 0.4°C than in cells incubated at 8°C.

*Low temperature incubation redirects glycogen derived glucose to glycerol.* In this series of experiments the hypothesis was tested that isolated hepatocytes produce glucose as well as glycerol. Figure 5 compares glucose and glycerol levels after 72 h incubation with and without 1 µM
isoproterenol in the incubation medium. In all three groups, in the absence of isoproterenol, glucose levels increased significantly after 72 h compared to pre-incubation levels. This increase was highest in the warm fish/8°C cells (pre-incubation, 26.7 ± 7.8 µmol g\(^{-1}\) vs. 72 h incubation, 203.5 ± 71.6 µmol g\(^{-1}\) ) and lowest in the cold fish/0.4°C cells (pre-incubation, 1.06 ± 0.13 µmol g\(^{-1}\) vs. 72 h incubation, 8.81 ± 3.02 µmol g\(^{-1}\) ). Glycerol concentration in the warm fish/8°C cells was significantly decreased after 72 h. As anticipated, cells collected from warm fish and incubated at 0.4°C produced glycerol. Levels of glycerol in cells from warm fish following incubation were higher compared to levels in cells collected from cold fish and incubated at the same temperature.

The inclusion of 1 or 10 µM isoproterenol (10 µM data not shown) in the incubation medium had no impact on the final level of glucose accumulation relative to incubation without isoproterenol in any of the conditions and no impact on final glycerol level in cells from warm fish incubated at 8°C or cells from cold fish incubated at 0.4°C. However, 1 and 10 µM isoproterenol resulted in a significant decrease in the level of glycerol accumulation in cells from warm fish incubated at 0.4°C (79.0 ± 12.83 to 42.6 ± 6.35 µmol g\(^{-1}\) and to 40.1 ± 6.28 µmol g\(^{-1}\), with 1 and 10 µM isoproterenol, respectively). The only other impact of 10 µmol l\(^{-1}\) isoproterenol was a significant increase in glucose production by cells from cold fish incubated at 0.4°C (1.06 ± 0.13, pre-incubation to 9.22 ± 2.97 µmol g\(^{-1}\)).

Rates of glucose and glycerol production/consumption are presented in Fig. 6 to allow comparisons across experimental preparations. Although not statistically significant the average rate of glucose production in cells from warm acclimated fish was lower at 0.4°C than in cells incubated at 8°C with or without isoproterenol in the medium. Hepatocytes isolated from cold fish and incubated at 0.4°C had the lowest rates of glucose production. When isoproterenol was included in the medium a slight increase in rate of glucose production in the warm acclimation/0.4°C incubation condition was enough to make this group significantly different than the cold acclimation/0.4°C incubation group. As well, the inclusion of isoproterenol resulted in a significant increase in the rate of glucose
production from $0.74 \pm 0.36$ to $0.95 \pm 0.36 \mu\text{mol g}^{-1} \text{h}^{-1}$ in cells from warm fish incubated at $0.4^\circ\text{C}$. As in all previous experiments, cells from warm fish consumed glycerol when incubated at $8^\circ\text{C}$ but produced glycerol when incubated at $0.4^\circ\text{C}$. In cells from warm fish, incubated at $0.4^\circ\text{C}$, the inclusion of $1 \mu\text{M}$ isoproterenol resulted in a significant decrease in the rate of glycerol production ($0.77 \pm 0.17$ to $0.27 \pm 0.09 \mu\text{mol g}^{-1} \text{h}^{-1}$). These data show that there is a redirection from glucose to glycerol production in hepatocytes isolated from warm rainbow smelt and incubated at low temperature. Furthermore isoproterenol decreases the rate of glycerol production and increases the rate of glucose production.

*Activity levels of GPDH, hexokinase, and glucokinase in isolated hepatocytes.* This experiment involved only fish acclimated to warm temperature. The rate of glycerol production was four-fold higher in cells incubated at $0.4^\circ\text{C}$ than at $8^\circ\text{C}$ (Table 3).

GPDH activity in whole liver was $142.6 \pm 19.5 \mu\text{mol min}^{-1} \text{g}^{-1}$ ($N = 6$). The activity in pre-incubation cells ($226.2 \pm 65 \mu\text{mol min}^{-1} \text{g}^{-1}$), from other fish, was not significantly different than the level found in the whole liver. Cells that were sampled prior to incubation had significantly higher activity levels of GPDH than cells harvested after 72 h, regardless of incubation temperature. Cells which were subjected to $0.4^\circ\text{C}$ incubation had a significantly higher level of GPDH activity than cells which were incubated at $8^\circ\text{C}$. When data were expressed on a protein basis, GPDH activity in cells incubated at $0.4^\circ\text{C}$ ($1.45 \pm 0.40 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$) was significantly higher than in cells incubated at $8^\circ\text{C}$ ($1.21 \pm 0.32 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$) thus eliminating the possibility that the difference in activity level based on wet weight was related to water content.

HK and GK activities in whole liver were $0.13 \pm 0.02$ and $0.199 \pm 0.04 \mu\text{mol min}^{-1} \text{g}^{-1}$, respectively. There was no significant difference between HK or GK between activities in fresh liver and values in cells isolated from different fish at the pre-incubation stage. Incubation of cells at either $0.4^\circ\text{C}$ or $8^\circ\text{C}$ for 72 h did not alter HK of GK activities with respect to pre-incubation levels, nor was
there a difference in enzyme activities between cells incubated at 0.4°C and cells incubated at 8°C. The same conclusions were reached when activities were expressed on a mg protein basis (data not shown).

Glycerol production is not additionally activated by glucocorticoid or PPARα agonists. In the next series of experiments we assessed if additional potential candidates of GPDH stimulation could result in further increases in glycerol production than that due to low temperature alone. Cells isolated from warm acclimated fish were incubated at 0.4°C for 72 h with 5 mM glucose in the incubation medium. As predicted glycerol level was produced by cells with no additional agents (Fig. 7). Neither hydrocortisone (100 or 1000 ng ml⁻¹), dexamethasone (100 or 1000 ng ml⁻¹), or Wy 14643 (50 µM) resulted in a further increase in glycerol accumulation.

DISCUSSION

The most important finding of this study is that in vitro low temperature (0.4°C) challenge is sufficient to directly activate the glycerol production mechanism by liver cells isolated from rainbow smelt held at high temperature. Isolated hepatocytes incubated at 8°C did not produce glycerol. This was expected given that in vivo studies show that temperatures lower than 5°C are needed in order to activate glycerol synthesis (6, 12). Hepatocytes from cold-acclimated fish incubated at 0.4°C produced small amounts of glycerol when glucose was available but not under any other condition. This is consistent with low rates of glycerol production by fish in the plateau or glycerol decrease phase of the annual cycle. We emphasize that the current work focuses on the use of glycogen/glucose during a shift from warm to cold temperature. This study does not fully probe mechanisms for the maintenance
of glycerol production at low temperature which may involve a complex relationship between substrate availability and hormonal signalling.

The activation of glycerol production by cells prepared from warm fish and incubated at 0.4°C was observed in five separate experiments. The rate of glycerol production showed considerable range between studies but was typically about 1 µmol g⁻¹ h⁻¹. There may be a number of reasons for variability amongst preparations. Foremost, experiments were performed over six separate years and with six different populations of fish. Secondly, the time of year that fish were sampled, associated with the rapid increase, plateau or decrease phase of glycerol production may account for some variability in the glycerogenic potential of isolated liver cells. Further, since sexually mature males and females were used in this study, variation in the glycerol production rates may be related to the reproductive cycle and lipid synthesis for gonad development. Regardless, there is absolute consistency within experiments in the activation of glycerol production by cells isolated from rainbow smelt at 8°C and incubated at 0.4°C. Additional extracellular signals related to adrenergic, glucocorticoid, or PPAR activation did not enhance glycerol production over that of low temperature challenge alone. Thus the activation mechanisms for glycerol production are inherent to the hepatocytes.

In one experiment it was noted that when various substrates such as glucose, aspartate, and pyruvate were added to the hepatocyte medium, rates of glycerol production were greater than the no substrate group. The enhanced production of glycerol when pyruvate or aspartate was available is in keeping with the concept that glycerol can be produced via the gluco- or glyceroneogenic pathway. Evidence for this includes i) elevated levels of amino transferases (7), PEPCK (phosphoenolpyruvate carboxykinase) (12, 22), and PEPCK mRNA (13) in rainbow smelt liver either in comparison to other species or during the phase of rapid glycerol accumulation, and ii) the incorporation of injected radiolabelled and stable isotopes of pyruvate and amino acids into labelled glycerol (22, 23, 32).
Glycerol was produced by hepatocytes isolated from warm fish and incubated at 0.4°C even in the absence of exogenous substrate. Under these conditions endogenous glycogen was utilized with most of the reserve being depleted after 72 h. This finding is consistent with whole animal studies in which glycogen levels in liver of rainbow smelt were decreased after several weeks of low temperature exposure under a natural seasonal regime (29) and in days following an imposed decrease in temperature from 8°C to -1.2°C (6). The glycogenolysis was associated with an increase in the total amount of measurable GPase activity and more specifically GPase $a$ activity. The low temperature challenge may have resulted in an increase in GPase gene expression; however, an alternative possibility exists. The activation of glycogen breakdown has been well studied in the wood frog, a species that produces glucose from massive glycogen stores as a primary cryoprotectant when exposed to ice crystallization. Cold exposure results in the conversion of an inactive, unmeasurable (by standard activity assays) $b$ form into an active $a$ form in the liver of this species (27). We cannot rule out the possibility that this occurs in liver of rainbow smelt as well. Glycogenolysis in the wood frog is under strong adrenergic control (27). This does not appear to be the case in rainbow smelt, at least with isolated liver cells. Exposure to isoproterenol (1 or 10µM) in these experiments did not result in changes in GPase activity, led to only small increases in glucose production, and most unexpectedly decreases in glycerol production.

Glycogen utilization at 8°C did not result in glycerol production and moreover glycogen utilization in cells from warm fish incubated at 0.4°C exceeded that of glycerol accumulation. These findings motivated us to assess if isolated liver cells were producing glucose, as occurs in many other species of fish (16). Hepatocytes from warm acclimated fish produced glucose at 8°C incubation temperature but not glycerol; however, incubation at 0.4°C resulted not only in glycerol production but equimolar release of glucose. An increase in GPase $a$ activity, following incubation at 0.4°C, was sufficient to allow similar molar rates of glucose (at 8°C) versus glucose plus glycerol release (at
0.4°C) despite the thermal difference. The switch from glucose production to a glucose plus glycerol production mode must involve control site(s) in addition to GPase. The finding that hepatocytes release glucose at low temperature also raises the possibility that glucose is being exported to other tissues for extrahepatic glycerol production as opposed to the liver being the sole site of glycerol production for the animal.

A likely contributor to enhanced glycerol production at low temperature in the isolated cells is the enzyme GPDH. Whole animal studies involving enzyme activity, metabolite levels, and gene expression levels reveal that enhanced glycerol production is associated with increased flux through GPDH (6, 12, 13). In the current study a four-fold increase in rate of glycerol production was associated with maximal in vitro GPDH activities that were about 40% higher after 72 h incubation at 0.4°C than at 8°C. GPDH activity in freshly isolated cells was higher than levels following incubation. We cannot account for this finding but suggest that there may be a transient increase in GPDH during the preparation of hepatocytes perhaps associated with a stress response. Regardless various experiments have consistently shown that high activities of GPDH are associated with glycerol production. Given that the in vitro activity of GPDH is thousands of times higher than rates of glycerol production it is unlikely that this is the only rate controlling step in the metabolic pathway. As presented earlier it may be that glycerol-3-phosphatase which catalyzes the conversion of glycerol 3-phosphate to glycerol plus Pᵢ, is a primary regulatory site (6). The maximal in vitro activity of glycerol-3-phosphatase at 20°C is 117 µmol h⁻¹ g⁻¹ (7). Assuming a Q₁₀ of 2 this would equate to an activity of approximately 30 µmol h⁻¹ g⁻¹, a value much closer to the highest rates of glycerol production observed here of 2.35 µmol h⁻¹ g⁻¹ than the activities of GPDH.

HK and GK levels were similar in isolated cells and in directly homogenized liver preparations lending credibility to the concept that there was a transient stress induced elevation in GPDH. The low-\(K_m\) HK and high-\(K_m\) GK activities are similar in rainbow smelt liver cells. This is different to the
situation in rainbow trout where GK is typically 10 to 15-fold higher than HK activity. In the common carp (*Cyprinus carpio*) which has a high capacity to utilize dietary carbohydrate HK activity is usually higher than GK activity (20). The data suggest that rainbow smelt, despite having a natural high protein diet and being more closely related to rainbow trout than common carp, has a liver metabolism designed for the glucose utilization perhaps to fuel essential glycerol production at low temperatures. The activities of HK and GK were not altered by incubation at either 0.4°C or 8 °C. HK kinetics have been studied in the glycerol producing larvae of the goldenrod gall moth (*Epiblema scudderiana*). At low temperature the enzyme has a higher affinity for glucose and ATP and reduced product inhibition by glucose-6-phosphate (G6P) and ADP than at high temperature (18). If this were to occur in rainbow smelt liver as well it could contribute to the utilization of extracellular glucose for glycerol production and might be one of the factors involved in higher rates of glycerol formation in cells incubated at 0.4°C than at 8 °C where glucose was available in the medium (e.g. Tables 1 and 3). The kinetic properties of HK may also be critical in the switch from glucose release at high temperature to a mixed glucose and glycerol release at low temperature if the enzyme served to salvage glucose released from glycogenolysis and redirect glucose into the glycolytic pathway. Any downstream regulation between fructose-6-phosphate and glycerol could decrease the level of G6P and thus relieve any potential product inhibition on HK. This scenario remains speculative until detailed analysis of metabolite levels and the kinetic properties of HK are achieved.

One feature of the study that remains unresolved is an apparent inhibitory effect of isoproterenol on glycerol production as suggested in the experiment reported in Table 2 and reported as a significant reduction of 65% in Fig. 6. Isoproterenol inhibits pyruvate kinase (PK) in rainbow trout liver (33). It may be that a requirement for glycerol production is an on going glycolysis to provide cytosolic NADH for GPDH activity. An inhibition of glycolysis through PK activity would reduce flux though glyceraldehyde 3-phosphate dehydrogenase and in turn could impede glycerol production. In
the whole animal context it may be that adrenergic control is involved in the decrease in glycerol production as season progresses and not the increase in glycerol accumulation in the fall.

**Perspectives and Significance.** Rainbow smelt accumulate high levels of glycerol throughout the body as an antifreeze mechanism. At the whole animal level glycerol production is triggered by a decrease in water temperature to 3-5°C (6). Here it is shown that a low temperature signal alone is sufficient to activate the glycerol production mechanism in liver cells as additional putative signals have no further stimulatory impact. Glycerol production in isolated hepatocytes may be fuelled by a variety of substrates such as glucose and amino acids; however, cells make glycerol in the absence of substrate from glycogen stores. A decrease in temperature results in a shift from glucose release to a mix of glucose and glycerol release. Glycogenolysis is activated in part through an increase in the total activity of GPase and high levels of a low-$K_m$ HK may serve to redirect glycogen derived glucose to glycolysis as opposed to release from the cells. Increases in GPDH activity are associated with elevated levels of glycerol production but strong metabolic control most likely exists at other sites as well. An understanding of glycerol production mechanisms has relevance beyond the current model system of rainbow smelt per se. The process of glyceroneogenesis leading to glycerol 3-phosphate in mammalian liver is an active area of research as it is essential to the triglyceride/fatty acid cycle and is a target for treatment of type 2 diabetes (10, 24, 30). As well, activity of a putative glycerol 3-phosphatase has been suggested in oxygen limited rat heart and brain although neither the protein nor the transcript have been identified (3, 19). The current work with rainbow smelt presents an ideal model system to study the control mechanisms of glyceroneogenesis and the final step in glycerol production. As well, along with earlier studies, it is shown that GPDH is an unanticipated modulated step in the process.
ACKNOWLEDGEMENTS

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GRANTS

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Table 1. *Rates of glycerol production by hepatocytes isolated from rainbow smelt (Osmerus mordax)*.

<table>
<thead>
<tr>
<th></th>
<th>Warm fish 8°C incubation</th>
<th>Warm fish 0.4°C incubation</th>
<th>Cold fish 0.4°C incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>-0.047 ± 0.037&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.294 ± 0.544&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.125 ± 0.051&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspartate</td>
<td>-0.056 ± 0.060&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.409 ± 0.188&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.025 ± 0.011&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>No substrate</td>
<td>-0.067 ± 0.021&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.227 ± 0.094&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007 ± 0.006&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Fish were acclimated to either warm (8°C) or cold temperature (0.4 to -1.5°C) and cells were incubated for 72 h at either 8°C or 0.4°C. Media contained either 5 mM glucose, 5 mM aspartate, or no substrate. Rates were calculated by subtracting pre-incubation levels from the post incubation levels and dividing by 72 h. Glycerol production is in µmol g<sup>-1</sup> hr<sup>-1</sup>. Values are expressed as means ± s.e.m. N= 8 for glucose (all conditions) and no substrate (0.4°C incubation). N = 3-5 for all other conditions. Different letters indicate significant differences between different acclimation/incubation groups with glucose, aspartate, or no substrate in the medium.
Hepatocytes were isolated from rainbow smelt (*Osmerus mordax*) acclimated to either warm (8°C) or cold temperature (0.4 to -1.5°C) and cells were incubated for 72 h at either 8°C or 0.4°C. Hepatocytes were incubated for 72 h with no substrate in the medium and with/without addition of isoproterenol. Glycerol production is in µmol g⁻¹ hr⁻¹. GPase activity is in µmol min⁻¹ g⁻¹ cells. Values are expressed as means ± s.e.m. N = 6-7 for all conditions except for glycerol production: 1 µM isoproterenol:warm fish/0.4°C incubation where N=5 and glycerol production: 10 µM isoproterenol:warm fish/0.4°C incubation where N=4. Different letters indicate significant differences between different acclimation/incubation groups with either no additions or isoproterenol in the medium.
Table 3. Rates of glycerol production and glycerol-3-phosphate dehydrogenase (GPDH), hexokinase (HK; low $K_m$), and glucokinase (GK; high $K_m$) activities in rainbow smelt (Osmerus mordax) hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>Glycerol</th>
<th>GPDH</th>
<th>HK</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>226 ± 64.7$^a$</td>
<td>0.20 ± 0.04</td>
<td>0.25 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>0.4 °C incubation</td>
<td>1.41 ± 0.55$^a$</td>
<td>141 ± 41.8$^b$</td>
<td>0.28 ± 0.05</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>8 °C incubation</td>
<td>0.37 ± 0.32$^b$</td>
<td>102 ± 28.4$^c$</td>
<td>0.26 ± 0.05</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

Hepatocytes were isolated from rainbow smelt (Osmerus mordax) acclimated to warm temperature (8°C) and incubated either at 0.4°C or 8°C for 72 h. Glycerol production is in µmol g$^{-1}$ h$^{-1}$. Enzyme activities are in µmol min$^{-1}$ g$^{-1}$. Values are expressed as means ± s.e.m. N=6. Different letters indicate significant differences between pre-incubation, 0.4 °C and 8 °C incubation groups.
Fig. 1. Linear production of glycerol by hepatocytes isolated from rainbow smelt (*Osmerus mordax*) and incubated at 0.4°C. Each line represents an example of production from one individual. Open circles; fish acclimated to warm temperature (8°C) with 5 mM glucose in the medium, left y-axis applies. Closed circles; fish acclimated to cold temperature (0.4-1.5°C) with 5 mM glucose in the medium; right y-axis applies. Triangles; fish acclimated to 8°C with no additional substrate in medium, right y-axis applies.

Fig. 2. Initial and final total (i.e. cellular plus extracellular) concentration of glycerol in hepatocytes isolated from rainbow smelt (*Osmerus mordax*) and incubated for 72 h in medium without substrate or containing (in mM) 5 glucose, 5 alanine, 5 aspartate, or 1 pyruvate. A) Hepatocytes isolated from rainbow smelt held at warm temperature (8°C) and incubated at 8°C. B) Hepatocytes isolated from rainbow smelt held warm temperature and incubated at 0.4°C. C) Hepatocytes isolated from rainbow smelt held low temperature (0.4-1.5°C) and incubated at 0.4°C. All values are means ± s.e.m., N = 8-9 for all pre-incubation values and N = 3 to 8 for all incubation conditions. *Significantly different from pre-incubation within the same acclimation/incubation temperature group.

Fig. 3. Total glycerol concentration in hepatocytes isolated from rainbow smelt (*Osmerus mordax*) prior to incubation (■) and 72 h after incubation (■). Fish were either acclimated to warm temperature (8°C) or cold temperature (0.4-1.5°C) and cells incubated at 8°C or 0.4°C without additional substrate in the medium. All values are means ± s.e.m. and N = 6-7. *Significantly different from pre-incubation within the same acclimation/incubation temperature group.

Fig. 4. Glycogen phosphorylase activities (total GPase and GPase a, left y-axis applies; % GPase a, right y-axis applies) in hepatocytes isolated from rainbow smelt (*Osmerus mordax*) prior to incubation (■) and after a 72 h incubation (■). A) Hepatocytes isolated from fish held at 8°C and incubated at 8°C. B) Hepatocytes isolated from fish held at 8°C and incubated at 0.4°C. C) Hepatocytes isolated
from fish held at 0.4-1.5°C and incubated at 0.4°C. All activities are means ± s.e.m. and N = 6-7.

*Significantly different from pre-incubation within the same acclimation/incubation temperature group.

Fig. 5. Glucose (■) and glycerol (■) content in pre-incubation and following 72 h incubation with or without 1 µM isoproterenol (+iso.) in the medium by hepatocytes isolated from rainbow smelt (Osmerus mordax). Each panel is a different acclimation/incubation group. All concentrations are means ± s.e.m. and N = 5-6. *Glucose or glycerol 72 h incubation content (with or without isoproterenol) are significantly different from the pre-incubation within the same acclimation/incubation temperature group. + Indicates significant differences in glycerol level between incubation with or without 1 µM isoproterenol within the warm temperature/0.4°C cell groups.

Fig. 6. Rates of glucose (A) and glycerol (B) production by hepatocytes isolated from rainbow smelt (Osmerus mordax) incubated with or without 1 µM isoproterenol in the medium. Data are derived from Fig. 5 and include three acclimation/incubation groups; warm fish/8°C incubation, warm fish/0.4°C incubation, and cold fish/0.4°C incubation. All rates are means ± s.e.m. and N = 5-6. Different letters indicate significant differences in either glucose or glycerol rates across acclimation/incubation temperature groups with or without 1 µM isoproterenol. * indicates significant differences in rates of glucose and glycerol production between incubations with or without 1 µM isoproterenol within the warm temperature/0.4°C cell groups.

Fig. 7. Total glycerol production in rainbow smelt hepatocytes isolated from warm acclimated fish and incubated at 0.4°C for 72 h. All groups were incubated with 5 mM glucose in the medim. In addition, medim also included 100 or 1000 ng ml⁻¹ HC (hydrocortisone), 100 or 1000 ng ml⁻¹ Dex (dexamethasone), or 50 µM l⁻¹ Wy 14643 (PPARα agonist). Glycerol concentrations are means ± s.e.m. and N = 3.
Total Glycerol (μmol g⁻¹)

Warm fish/8°C cells

Warm fish/0.4°C cells

Cold fish/0.4°C cells

*
A

Rate (μmol g\(^{-1}\) hr\(^{-1}\))

- isoproterenol

+ isoproterenol

B

Rate (μmol g\(^{-1}\) hr\(^{-1}\))

- isoproterenol

+ isoproterenol
Total Glycerol (μmol g⁻¹)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glucose</th>
<th>HC</th>
<th>Dex</th>
<th>Wy 14643</th>
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<tbody>
<tr>
<td>5 mmol⁻¹</td>
<td>80</td>
<td>90</td>
<td>85</td>
<td>85</td>
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<tr>
<td>10 ng ml⁻¹</td>
<td>90</td>
<td>95</td>
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</tr>
<tr>
<td>1000 ng ml⁻¹</td>
<td>100</td>
<td>105</td>
<td>100</td>
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Pre-Inc