Glycerol uptake is by passive diffusion in the heart but by facilitated transport in RBCs at high glycerol levels in cold acclimated rainbow smelt (Osmerus mordax)

Kathy A. Clow and William R. Driedzic
Ocean Sciences Centre, Memorial University of Newfoundland, St. John’s, Newfoundland, Canada
Submitted 1 December 2011; accepted in final form 7 February 2012

Clow KA, Driedzic WR. Glycerol uptake is by passive diffusion in the heart but by facilitated transport in RBCs at high glycerol levels in cold acclimated rainbow smelt (Osmerus mordax). Am J Physiol Regul Integr Comp Physiol 302: R1012–R1021, 2012. First published February 8, 2012; doi:10.1152/ajpregu.00645.2011.—Rainbow smelt (Osmerus mordax) is a small fish that accumulates glycerol at low winter seawater temperatures. In laboratory-held fish, glycerol concentration typically reaches 225 mM in plasma and in all cells. Glycerol uptake by the heart and red blood cells (RBCs) was assessed by tracking [14C(U)]glycerol into the acid-soluble pool. In fish acclimated to 9–10°C a decrease in perfusion/incubation temperature from 8 to 1°C resulted in a decrease in glycerol uptake with a Q10 of 3.2 in heart and 2.4 in RBCs. Acclimation to ~1.5°C did not result in an adaptive enhancement of glycerol uptake as rates were unchanged in heart and RBCs. Glycerol uptake at 1°C was by passive diffusion in heart as evidenced by a linear relationship between glycerol uptake and extracellular glycerol concentration and a lack of inhibition by phloretin. In contrast, in RBCs, glycerol uptake with respect to glycerol concentration showed two linear relationships with a transition point around 50 mM extracellular glycerol. The slope of the second phase was much steeper and eliminated with the inclusion of phloretin. In RBCs from Atlantic salmon (Salmo salar), a related species that does not accumulate glycerol, glycerol uptake showed only a single linear curve and was not inhibited by phloretin. The data imply a strong facilitated component to glycerol uptake in rainbow smelt RBCs at high glycerol concentrations. We propose this is related to cyclic changes in RBC glycerol content involving a loss of glycerol at the gill and a reaccumulation during passage through the liver.

RHYTHMIC TRANSPORT: Glycerol transport; glycerol permeability; heart; red blood cell

RAINBOW SMELT (Osmerus mordax) is a small anadromous teleost that over winters in seawater under the inshore ice where water temperatures reach −1.8°C. Under these conditions both an antifreeze protein and glycerol accumulation as part of the cold defense mechanism (8) Glycerol levels in plasma may reach 400 mM resulting in plasma osmotic pressures similar to seawater (7, 23, 24). In aquaria-held animals, tracking a natural decrease in water temperature, levels of 225 mM at 0°C are typical (6, 17). The liver is the primary site of glycerol production (3, 25), and glycerol equilibrates or reaches close to equilibrium levels with the intracellular space in all tissues via delivery through plasma (9). This study addresses the mechanisms by which glycerol enters the heart and red blood cells (RBCs). These tissues were selected as models to study the uptake process since they are readily amenable to control of the extracellular environment in isolated preparations.

There are many reports dealing with glycerol uptake in mammalian RBCs. These studies utilize RBCs as a tool to assess membrane function and/or address other issues such as the cryopreservation of cells (19). Considerable species variability exists in rates of glycerol permeation into mammalian RBCs. For instance, when assessed by 50% hemolysis time in 300 mM glycerol, RBCs from rats, mice, hamsters, and humans have half-times of <50 s; rabbit, guinea pig, and horse RBCs of 1–3 min; sheep, oxen, and camel RBCs of 5–50 min (18). Glycerol uptake by cells with low rates of entry is primarily by passive diffusion. Although it is likely that some passive diffusion occurs in all cell types, high rates of glycerol uptake are associated with the presence of facilitated transport mechanisms that are inhibited by phloretin and other agents (2, 32). More specifically, human and rat RBCs have aquaglyceroporin (AQP) 3 (27), whereas, mice have AQP 9 (20) that serves as glycerol conduits. For most species a decrease in incubation temperature results in a decrease in rate of uptake of glycerol, although human and rat RBCs with particularly high uptake rates may be exceptions to this rule (5, 21, 32, 34).

Jacobs et al. (16) assessed glycerol permeability in fish RBCs via the time to 75% hemolysis in solution containing 500 mM glycerol. As with mammals there were extreme differences even between closely related species such as cunner and tautog. In the only other fish report that we are aware of RBC swelling and shrinking studies revealed that glycerol permeability in four species of salmonids is by passive diffusion alone (15). More recently, Goldstein et al. (13) examined the uptake of glycerol by RBCs from warm- and cold-acclimated gray treefrog, a species that in the winter also accumulates glycerol up to 100 mM. Glycerol permeation as measured by cell lysis was substantially slower at a test temperature of 5°C than at 20°C, for both cold- and warm-acclimated gray treefrogs, and was abolished by 0.3 mM HgCl2, implying a facilitated transport process. The response was similar in animals acclimated to either 5°C or 21°C. The uptake of radiolabeled glycerol, at an extracellular concentration of 1 mM glycerol, was decreased by 67% in the presence of 0.3 mM HgCl2 in RBCs from treefrogs acclimated to warm temperature and tested at warm temperature. An AQP-like transcript was evident in RBCs from both warm- and cold-acclimated treefrogs, whereas protein expression was two- to threefold higher in cold- than warm-acclimated animals. These data are consistent with an AQP-mediated entry of glycerol in treefrog RBCs, but this process is not enhanced in cells from cold-acclimated animals. Two AQP3-like transcripts have been detected in the nucleated RBCs from rainbow smelt, albeit at extremely low levels.
relative to other tissues, such as posterior kidney and brain. An AQP9-like transcript has also been identified in rainbow smelt RBCs but again at low levels relative to liver and kidney (Hall JR, unpublished observations).

Rat heart displays glycerol kinase activity and can utilize glycerol as a metabolic fuel and for incorporation into the lipid pool. Increases in extracellular glycerol up to 0.7 mM with neonatal cardiac cells and up to 3.5 mM with perfused isolated hearts led to increases in glycerol uptake. With isolated cardiac cells the inclusion of phloretin in the medium decreased glycerol uptake by 60% at 0.7 mM glycerol (10, 11). Knockdown of AQP7 in rat cardiomyocytes resulted in lower rates of glycerol uptake and in perfused isolated hearts lower rates of glycerol extraction from the medium (14). It appears that at least in the rat heart, glycerol permeation is via a facilitated transport mechanism involving AQP7. The heart of rainbow smelt displays glycerokinase activity (6) and expresses two AQP3-like transcripts at levels comparable to RBCs and an AQP9-like transcript but at relatively low levels compared with the liver, kidney, and spleen (Hall JR, unpublished observations).

In the current study, we assess whether glycerol uptake by the heart and RBCs of rainbow smelt is enhanced in fish acclimated to low temperature and whether glycerol uptake is via passive and/or facilitated transport. A fewer number of experiments were also conducted with Atlantic salmon (Salmo salar), a species that is closely related to rainbow smelt (31) but does not accumulate glycerol (25). Uptake was assessed by following the incorporation of radiolabeled glycerol into the acid-soluble pool, similar to other protocols with RBCs and hearts (10, 11, 13, 20). Our most important findings are that in rainbow smelt acute temperature, but not thermal history, has a substantial impact on glycerol uptake, and in RBCs there is a facilitated transport component at high extracellular glycerol level.

MATERIALS AND METHODS

Animals. Rainbow smelt (Osmerus mordax) were collected in October 2008 and 2009 by seine netting from Long Harbour, Placentia Bay, Newfoundland and transported to the Ocean Sciences Centre. Atlantic salmon (Salmo salar) were hatchery reared by Cold Ocean Salmon (Daniel’s Harbour, Newfoundland) and transferred to the Ocean Sciences Centre in September 2008. All fish were held in tanks with either flow-through seawater maintained at 8–10°C or seawater that tracked ambient temperature reaching ~1°C in February. A typical seasonal water temperature profile is presented in Lewis et al. (17). All fish were kept on a natural photoperiod with fluorescent lights set by an outdoor photocell. Rainbow smelt were fed chopped herring twice a week; Atlantic salmon received commercial pellets.

In heart experiments, fish were sampled as the water temperature of perfusion medium consisted of (in mM): 182 NaCl, 5 KCl, 1.99 MgSO4, 2.3 CaCl2, 3.73 N-Tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) base, 2.58 TES acid, and 5 glucose (pH 7.8 at 10°C). In heart glucose uptake studies 10 mM glucose was included. Various glycerol concentrations were added to the medium and in some studies, phloretin, an inhibitor of facilitative transport was included. The glucose uptake studies served as a positive control to assess the efficacy of phloretin inhibition. A stock solution of 25 mg phloretin/ml DMSO was prepared, and an aliquot was added to the medium to make a final concentration of 0.3 mM phloretin with 0.33% DMSO. Attempts at making more concentrated solution were aborted due to the low solubility of phloretin at 1°C. A DMSO vehicle group was added to experiments using phloretin.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Isolated hearts. The heart perfusion apparatus was similar to the system described in Clow et al. (4). The system was designed to recirculate 30 ml of perfusion medium at a temperature of 1°C or 8°C. These temperatures were selected as they represent physiological conditions associated with high extracellular and intracellular glycerol accumulation (1°C) versus low extracellular glycerol (8°C). Temperature control of the reservoir and input chamber was maintained by using a recirculating water bath. A metal cannula attached to the input chamber was placed just above the heart allowing the perfusate to fill the heart by gravity. A stainless steel screen was placed under the heart to support it. The input cannula was inserted into the atrium, and the heart was perfused without recirculation for 30 s to allow washout of blood. The aorta was then cannulated with PE-60 tubing to effectively collect the fluid into a graduated cylinder for recirculation. The fluid in the cylinder was then pumped back to the input chamber. Hearts were electrically paced using a Grass model SD9 square-wave generator set at 5 V and 200 ms duration. One electrode was fused to the input cannula and a second electrode was attached to the metal screen support. Contraction rate was set to 20–25 beats/min, typical for marine teleosts at the temperatures of this study, and only those hearts that showed visible contractions were used in analysis. Hearts were perfused for 5 to 10 min with basic medium to allow contraction to stabilize and for endogenous glycerol to be washed out. Thereafter, hearts were perfused with recirculating medium containing [14C(U)]-glycerol (5.55 kBq/ml) (Perkin Elmer, Canada), an extracellular marker [1-13C(N)]mannitol (11.1 kBq/ml), and various levels of glycerol. Details of experiments involving radiolabeled glycerol are presented below.

Time-series experiments were performed to determine the range of linear uptake of glycerol. Hearts were perfused with media at either 8°C or 1°C. All time-series experiments included 50 mM glycerol in the perfusate and were terminated after 10, 20, 30, 40, 50, or 60 min of perfusion with radioisotope. The atrium and bulbous were cut away from the ventricle, and the ventricle was cut into four pieces, rinsed in...
ice-cold glycerol-free medium, and weighed. Pieces of ventricle weighing ~60–70 mg were homogenized in 9 volumes of 6% perchloric acid. Two hundred microliters of this homogenate were added to 10 ml of Ecolume (MP Biomedicals, Canada) and counted for both $^3$H and $^{14}$C on a Packard 2500TR liquid scintillation counter. Extracellular space (ml/g ventricle) and the intracellular concentration of glycerol ($\mu$mol/g ventricle) were calculated as previously described for glucose uptake (26).

Substrate saturation experiments were performed to assess whether glycerol uptake in hearts removed from rainbow smelt held at 1.7°C and Atlantic salmon held at 2.5°C could be saturated. Perfusion temperature was set to 1°C, and either 25, 50, 100, 150, 200, or 300 mM of unlabeled glycerol was added to the radioactive perfusate. Rainbow smelt and Atlantic salmon hearts were perfused with radiolabeled blood at the final hematocrits of 20% and 25%, respectively. These times were considered to be on the linear portion of the glycerol uptake curves. The second part of this experiment determined whether glycerol uptake in the rainbow smelt heart was decreased by phloretin, a well-recognized inhibitor of facilitated diffusion. Phloretin was added to the perfusate during both washout and perfusion with medium containing radiolabeled glycerol at a glycerol concentration of 200 mM. Additional experiments were performed to assess the efficacy of phloretin action during both washout and perfusion with medium containing radiolabeled glycerol. Phloretin was added to the perfusate to be on the linear portion of the glycerol uptake curves. The second time point was chosen to perform concentration curves. Incubations were performed similar to the time-course experiments except glycerol uptake values were subtracted by the 20-s time point to account for any nonspecific binding of glycerol in rainbow smelt hearts was calculated as described above. The y-intercept of 0.92 $\mu$mol/g was calculated from 12 data points obtained after 10, 20, and 30 min of perfusion, and this value subtracted from each of the smelt data points.

Values are expressed as means ± SE. Significant differences involving two conditions were assessed with Student's $t$-test and in the case of multiple conditions a one-way ANOVA with Tukey's post hoc test. In all cases $P < 0.05$ was considered to be significant.

RESULTS

Plasma glycerol levels. Rainbow smelt maintained at temperatures above 8°C always had plasma glycerol levels below 10 $\mu$mol/ml (Table 1). Levels of plasma glycerol increased as water temperature decreased either due to a natural seasonal decline or, as was the case in some RBC studies, a controlled decrease over a period of 2 to 3 wk. Plasma glycerol levels in Atlantic salmon were always <0.1 $\mu$mol/ml, regardless of temperature.

Glycerol uptake by heart. The time necessary to achieve equilibration of the radiolabeled perfusate in the extracellular space (ECS) of the heart was determined with $[^3]$Hmannitol (Figs. 1A and 2A). In both rainbow smelt and Atlantic salmon stability of marker in the ECS was achieved within 10 min of perfusion (i.e., the initial time point in the study), regardless of the initial temperature of the fish or perfusion temperature of the isolated hearts. The occasional aberration to this interpretation, and in the glycerol uptake data discussed below, is most likely due to the fact that each point on the figures represents one heart only.

In all of the time-course studies the perfusion medium contained 50 mM glycerol. Glycerol uptake by hearts from rainbow smelt initially increased in a linear fashion (Fig. 1B). More specifically, under conditions of acclimation 10.8°C/8.8°C 8.2

<table>
<thead>
<tr>
<th>Acclimation Temperature</th>
<th>Rainbow Smelt</th>
<th>Atlantic Salmon</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC experiments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.8°C</td>
<td>8.2 ± 0.28, n = 5</td>
<td>0.10 ± 0.02, n = 6</td>
</tr>
<tr>
<td>1.2°C</td>
<td>116.6 ± 9.2*, n = 10</td>
<td>0.07 ± 0.01, n = 5</td>
</tr>
<tr>
<td>Heart experiments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.8°C</td>
<td>8.8 ± 0.78, n = 4</td>
<td></td>
</tr>
<tr>
<td>4.6°C</td>
<td>52.3 ± 4.95*, n = 4</td>
<td></td>
</tr>
<tr>
<td>2.8°C</td>
<td>99.0 ± 7.63*, n = 9</td>
<td></td>
</tr>
<tr>
<td>1.7°C</td>
<td>162.9 ± 6.43*, n = 33</td>
<td></td>
</tr>
</tbody>
</table>

Plasma glycerol ($\mu$mol/ml) was not measured in salmon during the heart perfusion experiments. Values are means ± SE; $n$ = number of experiments. *Significant difference in value between fish acclimated to 9–11°C and fish sampled at the lower temperatures.

Table 1. Glycerol concentrations in plasma of rainbow smelt (Osmerus mordax) and Atlantic salmon (Salmo salar)
perfusion 8°C, acclimation 4.6°C/perfusion 1°C, acclimation 2.8°C/perfusion 1°C for the periods of 60, 40, and 60 min, respectively, glycerol uptake followed a statistically significant linear regression (\(P < 0.005\)) in all cases. Under conditions of acclimation 10.8°C/perfusion 1°C glycerol uptake tended to be linear for the first 30 min (\(r^2 = 0.97; P = 0.12\)). The raw data reveal that glycerol uptake by hearts perfused at 8°C is qualitatively higher than uptake by hearts perfused at 1°C, and that hearts perfused at 1°C all have similar rates of glycerol accumulation. Rates of uptake calculated from the initial linear portions of the curves in Fig. 1B are presented in Fig. 1C. Hearts perfused at 1°C all showed significantly lower rates of glycerol uptake than the hearts perfused at 8°C. For hearts from fish acclimated to 10.8°C glycerol uptake at 8°C was 0.65 \(\mu\text{mol·g}^{-1}·\text{min}^{-1}\) compared with 0.29 \(\mu\text{mol·g}^{-1}·\text{min}^{-1}\) at 1°C yielding a \(Q_{10}\) of 3.2. Thermal history of the fish had no impact on the rate of glycerol uptake by the heart, at least over the temperature range of 10.8–1.7°C.

Glycerol uptake by hearts from Atlantic salmon also initially increased in a linear fashion regardless of thermal history or perfusion temperature with 50 mM glycerol in the medium (Fig. 2B). More specifically, under conditions of acclimation 9.8°C/perfusion 1°C and acclimation 2.4°C/perfusion 1°C for

---

**Fig. 1.** Extracellular space (ECS) and glycerol uptake by hearts from rainbow smelt that were perfused for various times with 50 mM glycerol in the medium. ECS was determined with \(^{3}H\)mannitol and glycerol uptake with \(^{14}C\)glycerol. A: ECS. B: glycerol uptake. Each point represents ECS or uptake by one individual heart. Different symbols represent different rainbow smelt acclimation/heart perfusion temperatures. C: rate of glycerol uptake calculated from data shown in B for 10-, 20-, and 30-min time points. The value for fish acclimated to 1.7°C is taken from the glycerol concentration series presented in Fig. 3. These rates were also adjusted to account for nonspecific binding as described in MATERIALS AND METHODS. Values are means ± SE; \(n = 3\) with the exception of fish acclimated to 1.7°C where \(n = 4\). Values not sharing a common letter are significantly different.

---

**Fig. 2.** ECS and glycerol uptake (B) by hearts from Atlantic salmon perfused for various lengths of time with 50 mM glycerol in the medium. ECS was determined with \(^{3}H\)mannitol and glycerol uptake with \(^{14}C\)glycerol. A: ECS. B: glycerol uptake. Each point represents ECS or uptake by one individual heart. Different symbols represent different Atlantic salmon acclimation/heart perfusion temperatures. C: rate of glycerol uptake calculated from data shown in B for 10-, 20-, and 30-min time points. Values are means ± SE; \(n = 3–5\). Values not sharing a common letter are significantly different.
the periods of 50 and 30 min, respectively, glycerol uptake followed a statistically significant linear regression \((P < 0.05)\). Under conditions of acclimation 9.8°C/perfusion 8°C, glycerol uptake tended to be linear for the first 30 min \((r^2 = 0.96; P = 0.13)\). The curves do not go through the zero time point. We cannot distinguish whether this is due to an extremely high rate of glycerol uptake between 0 and 10 min or to a nonspecific and rapid binding. Our best estimates of the rate of uptake based on assuming nonspecific binding are presented in Fig. 2C. The rate of glycerol uptake by hearts from fish held at 9.8°C and perfused at 8°C was significantly higher than the rate of uptake by hearts from fish acclimated to 2.4°C and perfused at 1°C. Although the mean rate of glycerol uptake by hearts from fish held at 9.8°C and perfused at 8°C was higher than the mean rate of uptake by hearts from fish acclimated to the same temperature and perfused at 1°C, the values only tended to be different \((P = 0.14)\). The rate of glycerol uptake was similar in the two groups perfused at 1°C, implying that thermal history does not influence this process at least over the range of 9.8 to 2.4°C.

There were no significant differences in the rates of uptake between rainbow smelt and Atlantic salmon hearts compared at the same perfusion temperature and similar acclimation temperatures (Figs. 1C and 2C). We consider that this supports the position of a nonspecific glycerol binding in hearts from Atlantic salmon.

The relationship between the rate of glycerol uptake and extracellular glycerol concentration in hearts from rainbow smelt is presented in Fig. 3. In this experiment perfusion temperature was 1°C and acclimation temperature of fish was 1.7°C. Glycerol uptake increased in a linear fashion by hearts from Atlantic salmon (data not shown).

In the next series of experiments, hearts from rainbow smelt were perfused with medium containing phloretin, a general inhibitor of transport proteins. Phloretin had no impact on glycerol uptake by hearts from rainbow smelt acclimated to 1.7°C and perfused at 1°C with 200 mM glycerol in the medium. To assess the efficacy of phloretin at 1°C, the rate of uptake of glucose was determined (Fig. 4). In this case, phloretin resulted in a significant 41% decrease in glucose uptake by hearts from fish acclimated to 0.7°C and perfused at 1°C.

**Glycerol uptake by RBCs.** The initial uptake of glycerol by rainbow smelt and Atlantic salmon RBCs at 25 mM glycerol was linear, regardless of acclimation temperature of the fish or incubation temperature of the cells (Figs. 5, A and B). More specifically, for rainbow smelt RBC under conditions of acclimation 8.8°C/incubation 8°C, acclimation 8.8°C/incubation 1°C, and acclimation 1.2°C/incubation 1°C for the periods of 120, 60, and 120 s, respectively, glycerol uptake followed a statistically significant linear regression \((P < 0.05)\). For At-

![Fig. 3. Uptake of glycerol by hearts from rainbow smelt perfused for 20 min with medium containing various glycerol concentrations at a temperature of 1°C. Rainbow smelt were acclimated to 1.7°C. Values are means \(\pm SE\); \(n = 3–4\).](http://ajpregu.physiology.org/)

![Fig. 4. Glycerol and glucose uptake in hearts from rainbow smelt perfused with or without 0.3 mM phloretin in the medium. All preparations received 0.33% DMSO. A: glycerol uptake with 200 mM glycerol in the medium. Fish were acclimated to 1.7°C. B: glucose uptake with 10 mM glucose in the medium. Fish were acclimated to 0.7°C. All hearts were perfused for 20 min at 1°C. Values are means \(\pm SE\); \(n = 3–5\). *Significant difference in value between the two groups.](http://ajpregu.physiology.org/)
Atlantic salmon RBCs under conditions of acclimation 8.8°C/incubation 8°C, acclimation 8.8°C/incubation 1°C, and acclimation 1.9°C/incubation 1°C for the periods of 120, 240, and 240 s, respectively, glycerol uptake followed a statistically significant linear regression ($P < 0.001$). The rate of uptake was calculated from the initial linear portion of the curves. For rainbow smelt a decrease in incubation temperature resulted in a decrease in rate of uptake (Fig. 5C). For RBCs obtained from fish held at 8.8°C, the rate of uptake between incubation at 8°C (8.06 ± 0.73 μmol·g$^{-1}$·min$^{-1}$) and 1°C (4.41 ± 0.67 μmol·g$^{-1}$·min$^{-1}$) was 45% lower yielding a $Q_{10}$ of 2.4. There was no significant difference in glycerol uptake between RBCs from fish held at 8.8°C and 1.2°C when incubated at 1°C. In RBCs from Atlantic salmon acclimated to 8.8°C, a decrease in incubation temperature resulted in a 66% decrease in glycerol uptake (5.37 ± 0.32 μmol·g$^{-1}$·min$^{-1}$ vs. 1.82 ± 0.05 μmol·g$^{-1}$·min$^{-1}$) (Fig. 5D).
The rate of glycerol uptake by cells from Atlantic salmon acclimated to 1.9°C was no different from that of RBCs from fish held at 8.8°C when incubated at the common temperature of 1°C.

The relationships between the initial rate of glycerol uptake and extracellular glycerol concentration are presented in Fig. 6 for RBCs incubated at 1°C and obtained from rainbow smelt and Atlantic salmon acclimated to 1.2°C or 1.9°C, respectively. For rainbow smelt RBCs there was a biphasic relationship between the rate of glycerol uptake and extracellular glycerol level (Fig. 6A). There was a linear relationship up to 50 mM glycerol ($y = 0.0324x + 0.069, r^2 = 0.98$) and a second significantly ($P = 0.003$) steeper linear relationship from 50 to 150 mM glycerol ($y = 0.0866x - 3.15, r^2 = 0.99$). The addition of phloretin to rainbow smelt RBCs eliminated the biphasic aspect and resulted in a single linear curve up to 100 mM glycerol ($y = 0.0497x - 0.0139, r^2 = 0.995$) (Fig. 6C). Furthermore, with phloretin in the medium the uptake of glycerol became saturated, but this interpretation should be viewed with caution because it is based on only one glycerol concentration above 100 mM. In Atlantic salmon RBCs there was simple linear relationship between glycerol uptake and extracellular glycerol level that was not influenced by phloretin (without phloretin, $y = 0.026x - 0.0461, r^2 = 0.98$; with phloretin, $y = 0.008x + 0.002, r^2 = 0.99$).

---

**Fig. 6.** Uptake of glycerol by RBCs from rainbow smelt and Atlantic salmon versus glycerol concentration. Rainbow smelt were acclimated to 1.2°C; Atlantic salmon were acclimated to 1.9°C. All cells were incubated for 60 s at 1°C. A: glycerol uptake by RBCs from rainbow smelt. B: glycerol uptake by RBCs from Atlantic salmon. C: glycerol uptake by RBCs from rainbow smelt with 0.3 mM phloretin in DMSO (●) or with DMSO alone (▲) included in the medium. D: glycerol uptake by RBCs from Atlantic salmon with 0.3 mM phloretin in DMSO (●) or with DMSO alone (▲) included in the medium. Values are means ± SE; n = 3–6.
The relationship between glycerol uptake and the level of extracellular glycerol concentration among untreated rainbow smelt RBCs (up to 50 mM glycerol), phloretin-treated rainbow smelt RBCs (up to 100 mM glycerol), untreated Atlantic salmon RBCs (up to 150 mM glycerol), and phloretin-treated RBCs (up to 150 mM glycerol) were not significantly different. This conclusion is based on an ANOVA analysis taking into consideration the slope of the regression equation for each individual experiment. The only condition that showed elevated rates of glycerol uptake was in RBCs from rainbow smelt at elevated levels of extracellular glycerol.

DISCUSSION

All fish in the current study responded as expected with respect to plasma glycerol levels. As water temperature decreased, plasma glycerol levels increased in rainbow smelt but not in Atlantic salmon (8, 25). A parallel increase would occur in rainbow smelt heart and presumably in RBCs as it does in the liver, kidney, muscle, brain, spleen, and ocular fluid (9, 12). In the context of this study, it means that the heart and RBCs from low temperature-acclimated rainbow smelt should be poised to take up glycerol.

Glycerol uptake was assessed by the incorporation of radio-labeled glycerol from the extracellular space into the acid soluble pool. Glycerol kinase is the requisite enzyme for the metabolism of glycerol. Maximal in vitro glycerol kinase activity in the rainbow smelt heart is 100 nmol·min\(^{-1}\)·g protein\(^{-1}\) in animals held between 0°C and 1°C and measured at 30°C (6), and activity level in RBCs is nondetectable (Ditlecaden D, unpublished observations). Based on a protein content of 54 mg/g (Ditlecaden D, unpublished observations) and assuming a Q\(_{10}\) of 2, the maximal glycerol kinase activity at 1°C in the rainbow smelt heart would be in the order of 0.6 nmol·min\(^{-1}\)·g tissue\(^{-1}\) compared with a much higher rate of glycerol uptake, which is about 0.2 µmol·min\(^{-1}\)·g\(^{-1}\) at 50 mM extracellular glycerol (Fig. 2C). Given the low activity of glycerol kinase in both the heart and RBCs, the incorporation of radioactivity assessed here is considered to reside in the free glycerol pool and not other cellular components.

In rainbow smelt hearts, a decrease in perfusion temperature resulted in a decrease in glycerol uptake. The decrease in glycerol uptake due to perfusion temperature alone may be associated with a decrease in membrane fluidity at lower temperature. This is discussed further under RBCs. Thermal history had no impact on the rate of glycerol uptake, suggesting that no specific adaptations are required in the heart of rainbow smelt to allow for the accumulation of high levels of glycerol under winter conditions. The uptake of glycerol, at least between 25 and 300 mM extracellular glycerol, at 1°C appears to be by simple diffusion as evidenced by a linear relationship between the rate of uptake and the lack of inhibition by phloretin a generalized blocker of transport processes. Phloretin is effective as an inhibitor of transport processes at 1°C as shown by a decrease in glucose uptake similar to the impact of cytochalin B on the normoxic eel heart and the hypoxic Atlantic cod heart (4, 26). The smelt heart differs from the mammalian heart where there is a strong facilitated transport component involving AQPs (10, 14). Although expression of two AQP3 transcripts and an AQP9-like transcript has been observed in rainbow smelt heart (Hall JR, personal communication), facilitated glycerol transport does not play a role in the conditions of this study. It may be that similar to the rat heart, AQP is important at very low concentrations of extracellular glycerol resulting in a minimal diffusion gradient as would be experienced by rainbow smelt in summer.

The situation with respect to glycerol uptake by Atlantic salmon heart is for the most part the same as that for rainbow smelt. At an extracellular concentration of 50 mM glycerol, there were no significant differences between the two species in rates of glycerol uptake under similar conditions of thermal history or perfusion temperature. A decrease in perfusion temperature was associated with lower rates of glycerol uptake by Atlantic salmon hearts with the caveat that in one case there was only a tendency in this direction and thermal history had no impact on glycerol uptake. At 1°C there is a linear relationship between uptake and extracellular glycerol at least up to 100 mM glycerol. Atlantic salmon, a species closely related to rainbow smelt, does not accumulate high levels of glycerol. The similarity in glycerol uptake between hearts from Atlantic salmon and rainbow smelt supports the viewpoint that there are no special adaptations in hearts of rainbow smelt associated with the accumulation of glycerol under winter conditions.

Glycerol uptake by smelt RBCs decreased as incubation temperature was lowered from 8 to 1°C when 25 mM glycerol was present in the extracellular medium. A decrease in glycerol uptake at lower temperatures is consistent with findings for many mammalian RBCs (5, 21, 32, 34). Moreover, in RBCs from five species of mammals, in which glycerol uptake is considered to be primarily by passive diffusion, cells with lower levels of unsaturated fatty acids (i.e., lower fluidity) exhibit lower levels of glycerol permeability (31). It is likely that in the current experiments an acute decrease in temperature resulted in immediate decreases in membrane fluidity that could contribute to a decrease in the rate of glycerol entry. There was no significant difference in glycerol uptake between RBCs from fish held at 8.8°C or 1.2°C when measured at 1°C, revealing that thermal history has no impact on this process. In rainbow smelt RBCs, glycerol uptake with respect to glycerol concentration showed two linear relationships with a transition point around 50 mM extracellular glycerol. The slope of the second phase was much steeper and was eliminated with the inclusion of phloretin. The data imply a strong facilitated component to glycerol uptake at high glycerol concentrations. This interpretation is consistent with the observation of AQP-like transcript expression albeit at low levels in rainbow smelt RBCs (Hall JR, unpublished observations).

RBCs from Atlantic salmon similar to those from rainbow smelt show a decrease in glycerol uptake at lower incubation temperature and no impact of thermal history. More importantly in the context of this study the relationship between glycerol uptake and extracellular glycerol concentration followed a single linear curve and phloretin was without effect. These findings suggest that in marked contrast to rainbow smelt RBCs, glycerol uptake by Atlantic salmon RBCs is by simple diffusion alone.
Perspectives and Significance

Glycerol uptake was reduced by a decrease in temperature in the hearts and RBCs from rainbow smelt and Atlantic salmon. A negative impact of lower temperature on glycerol permeability has been observed repeatedly in numerous cell types such as mammalian RBCs (5, 21, 32, 34), gray treefrog RBCs (13), membrane vesicles of kidney proximal tubule (30), and mitochondria from the brain, liver, and kidney (33). Therefore, our current findings are consistent with a well-established phenomenon in this respect.

Acclimation to low temperature does not result in enhanced glycerol permeability in either the heart or RBCs of rainbow smelt at least at 50 and 25 mM extracellular glycerol, respectively. In this sense, rainbow smelt RBCs are similar to RBCs from gray treefrog, a species that also accumulates glycerol in winter.

Glycerol uptake by rainbow smelt heart between 25 and 300 mM extracellular glycerol is primarily by passive diffusion. Rat heart exhibits a facilitated transport process inhibited by phloretin at 0.7 mM extracellular glycerol (10) and hearts of a number of species express AQPs (1, 14, 28). Rainbow smelt heart expresses low levels of AQP-like transcripts (Hall JR, unpublished observations). It may be that these play a role in facilitated glycerol transport at low extracellular levels that would occur in summer. Regardless, entry of glycerol into the heart by passive diffusion appears to be adequate to support the high levels of glycerol that accumulate in this tissue in winter.

In contrast to the heart, RBCs from rainbow smelt incubated at 1°C exhibit a facilitated transport process inhibited by phloretin at 0.7 mM extracellular glycerol (10) and hearts of a number of species express AQPs (1, 14, 28). Rainbow smelt heart expresses low levels of AQP-like transcripts (Hall JR, unpublished observations). Here again rainbow smelt RBCs are similar to gray treefrog RBCs that also show reduced glycerol permeability when facilitative transport is inhibited (13).

The question becomes as to what value is an enhanced glycerol transport in RBCs at high levels of extracellular glycerol? We suggest that this is related to a cyclic loss and accumulation of glycerol in RBCs. At low temperature and high plasma glycerol, rainbow smelt lose about 10% of glycerol stores per day across the gill and skin (6, 24). This must lead to a decrease in plasma and presumably RBC glycerol as the blood traverses the gill. The major site of glycerol production is the liver that supports glycerol accumulation in all other tissues (4, 9, 25). Glycerol that is lost from RBCs at the gills could then be recharged upon passage through the hepatic circulation. This would occur under conditions of relatively high extracellular glycerol against a lower, but probably still high, intracellular level.

We propose that this process, with a requirement for a rapid loading of cells, may be dependent on a facilitated glycerol transport mechanism.

ACKNOWLEDGMENTS

We thank Connie Short for technical assistance and the Field Services Unit of the Ocean Sciences Centre for the collection of specimens.

GRANTS

This work was supported by the Canadian Institutes of Health Research through the Regional Partnership Program (to W. R. Driedzic and K. Vanya Ewart) matched by the Newfoundland and Labrador Department of Innovation, Trade and Rural Development (to W. R. Driedzic), the Research and Development Corporation of Newfoundland and Labrador, and by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (to W. R. Driedzic). W. R. Driedzic holds the Canada Research Chair in Marine Bioscience.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.A.C. performed experiments; K.A.C. analyzed data; W.R.D. conceived and designed research; W.R.D. interpreted results of experiments; W.R.D. drafted manuscript; W.R.D. approved final version of manuscript.

REFERENCES


