The Journal of Experimental Biology 210, 2866-2872
Published by The Company of Biologists 2007
doi:10.1242/jeb.003749

Relationship between food availability, glycerol and glycogen levels in low-temperature challenged rainbow smelt *Osmerus mordax*

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Accepted 11 June 2007

Summary

Rainbow smelt *Osmerus modax* accumulate glycerol in winter that serves as an antifreeze. Fish were held at 8°C, or subjected to a decrease in water temperature to –1°C over a 19 day period, and subsequently maintained at –1°C from 15 January to 11 May 2004. Starved fish did not survive the challenge of temperature decrease, with death ensuing above the typical freeze point for marine teleosts (–0.8°C). A decrease in temperature activates the glycerol accumulation mechanism at about 5°C with peak plasma levels exceeding 300 μmol ml^{-1}. Glycerol levels begin to decrease in late February even at water temperatures below –1°C, suggesting either an inherent circannual or photoperiod trigger, possibly in association with sufficiently high levels of antifreeze protein. Glycogen levels in liver did not change significantly in starved fish maintained at 8°C. However, liver glycogen was depleted in fish subjected to the low-temperature challenge and at a faster rate in starved than in fed fish. Stored glycogen in liver and other tissues can account for only a small amount of the total glycerol production, suggesting a strong requirement for food during accelerated glycerol production. Liver glycogen levels increased in April and May in association with the decrease in glycerol. Levels of glycerol in liver, kidney, spleen, gill, intestine, heart, muscle and brain follow the same pattern as that in plasma. During the early part of the glycerol accumulation phase, all tissues except for liver have lower levels of glycerol in the intracellular space than the levels in plasma. In liver, glycerol is in equilibrium between the two compartments.

Key words: rainbow smelt, *Osmerus mordax*, glycerol, low temperature, freeze resistance.

Introduction

Rainbow smelt *Osmerus mordax* depress the freezing point of their body fluids through a combination of antifreeze protein (Ewart and Fletcher, 1990) and elevated glycerol levels (Raymond, 1992). These fish remain active and continue to feed in icy seawater, but face the challenge of continual loss of glycerol across the epithelia (Raymond, 1993), resulting in the necessity for an ongoing synthesis of glycerol. Studies involving injection of radioisotopes and heavy isotopes reveal that glycerol is produced from glucose and amino acids, which would be obtained from the diet and/or on-board reserves (Raymond, 1995; Raymond and Driedzic, 1997; Walter et al., 2006). Liver is a primary site of glycerol production, as indicated by enzyme complement, gene expression and synthesis by isolated liver preparations (Driedzic and Ewart, 2004; Driedzic et al., 2006; Liebscher et al., 2006). Glycogen in liver is important as a metabolic source of glycerol. Glycogen in liver decreased by 75 h post capture in fish held without feeding at –1°C and muscle glycogen content was only a small percentage of the level in liver (Raymond, 1995). Liver glycogen declined significantly more at –1°C than +1°C following 14 or 20 days without food (Raymond et al., 1996) but even in fed fish, under laboratory conditions, liver glycogen decreased as winter progressed from December to April (Treberg et al., 2002). The picture that emerges is that dietary glucose, amino acids and glycogen reserves support glycerol synthesis for antifreeze protection. In the present study, details of the interplay between temperature, food availability, on-board fuel reserves and tissue glycerol content are addressed.

Glycerol content in plasma of rainbow smelt begins to increase when the temperature decreases to about 4°C, and may reach levels approaching 500 mmol l^{-1} (Lewis et al., 2004; Driedzic et al., 2006). Glycerol levels in tissues other than plasma are not well understood. In rainbow smelt sampled at one time point in winter at –2°C, the glycerol content in a number of different tissues was similar to that in plasma (Raymond, 1992). In a partial seasonal study, glycerol levels in muscle followed a similar profile as in plasma; however, at the peak of plasma glycerol levels the content of glycerol in liver appeared to be lower than in plasma (Treberg et al., 2002). A similar relationship was noted in fish that were force-chilled (Driedzic et al., 2006).

Plasma glycerol content peaks in February and thereafter decreases, even though water temperature is still below 0°C. Plasma glycerol content reaches 5 mmol l^{-1} in May, similar to levels in the fall. As plasma glycerol decreases, antifreeze protein becomes the dominant freeze protection mechanism (Lewis et al., 2004). Although decreases in plasma glycerol

content while water temperatures are still very low are documented, it is not clear if other tissues follow this pattern and if the decrease in plasma glycerol is triggered by small upswings in temperature towards 0°C or some other factor such as photoperiod. Also, it is not known if one of the fates of plasma glycerol is reincorporation into the glycogen pool.

The current experiment addresses a number of questions relevant to glycogen and glycerol management in freeze-resistant rainbow smelt. Foremost, we tested the hypothesis that continual feeding is a requirement to survive low temperatures. The initial glycogen content in a number of tissues is reported. Thereafter, glycogen levels in liver and glycerol level in numerous tissues were determined in fish maintained at low temperature in winter through to the spring period. The quantitative importance of exchange between these two metabolic pools and the relationship between plasma glycerol and tissue glycerol levels as the former changes was assessed.

Materials and methods

Animals and experimental protocols

Rainbow smelt *Osmerus mordax* Mitchill 1814 were collected by seine netting from Long Harbour, Placentia Bay, Newfoundland, Canada in late October 2003 and 2005, transported to the Ocean Sciences Centre, Memorial University of Newfoundland, and transferred to 3000 l (2003) or 1800 l tanks (2005) with flow-through seawater. Fish were kept on a natural photoperiod with fluorescent lights set on an outdoor photocell and fed a diet of chopped herring unless otherwise indicated. During the 2003/2004 period, fish were fed twice a week; during the 2005/2006 season, they were fed daily.

Some fish were held at 8±0.5°C for the duration of the experiments in both the 2003/2004 and the 2005/2006 studies. Most of the experiments were conducted over the 2003/2004 season with fish subjected to a controlled decrease in water temperature. In these studies, rainbow smelt were transferred to a 500 l tank set at 8°C, 2 weeks prior to a reduction in water temperature. On 15 January 2004, temperature was decreased over a 19-day period to –1°C and subsequently maintained at approximately –1°C until the final sampling point on 11 May 2004. In this experiment, fish either continued to receive food or were starved from the initiation of the water temperature decrease. A population of fish was also allowed to track ambient water temperature during the 2003/2004 season. A group of fish from the October 2005 collection was fed and maintained at 8°C until sampled on 26 January 2006. Thereafter, this population was starved for 26 days and sampled again. All experiments involved both male and female fish.

At sampling times, fish were randomly selected, weighed, measured for length, and blood drawn *via* a caudal vessel. Fish were then killed with a blow to the head, tissues removed and stored at temperatures below –65°C for later glycerol and/or glycogen analysis. Blood was centrifuged at 9300 ***g*** immediately after sampling, plasma was collected and frozen in liquid nitrogen.

Biochemical assays and wet weight determinations

Glycerol level in the plasma was determined directly using a colorimetric detection kit (F6428, Sigma-Aldrich, St Louis, MO, USA). Samples were read at 540 nm after a 15 min incubation at room temperature. Tissues were homogenized in 9 vol. 10% perchloric acid, the homogenate centrifuged at 1500 ***g*** and the supernatant assayed for glycerol. Glycogen was measured by the

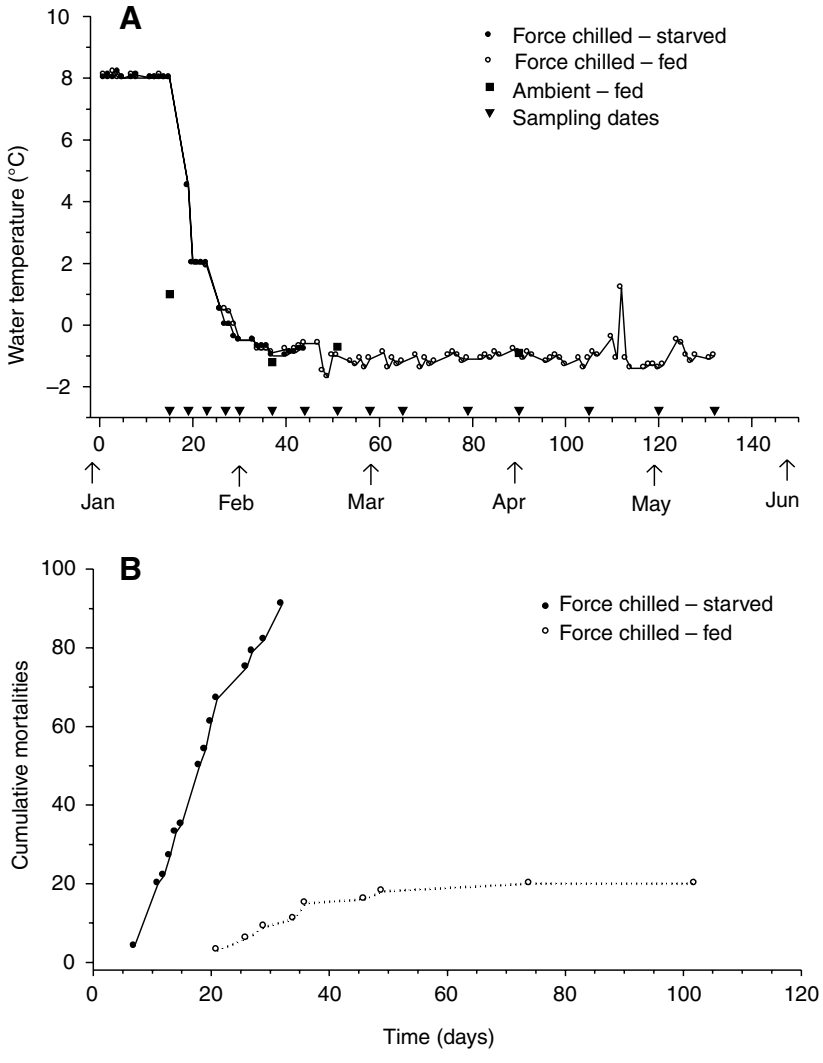

Fig. 1. (A) Temperature profiles. Water temperature was decreased by cooling, beginning on 15 January 2004, and fish were either starved (filled circles) or fed (open circles). Control fish (squares) were maintained under ambient water temperature conditions (2004) and fed. Filled inverted triangles indicate sampling dates. (B) Cumulative mortalities for fed and starved rainbow smelt *Osmerus mordax* subjected to a controlled decrease in water temperature. The date at which the temperature decrease was initiated (i.e. 15 January 2004) is used as day 0. Initial population size was 130 for both groups.

method of Walaas and Walaas (Walaas and Walaas, 1950) as described by Driedzic et al. (Driedzic et al., 1998).

Percentage water content was calculated from the difference in mass between fresh tissue and after drying to a constant mass at 90°C.

Data analysis

Unless otherwise indicated, values (means ± s.e.m.) were compared with a one-way analysis of variance (ANOVA) for all measurements followed by Duncan's *post-hoc* test. In some situations involving comparisons between two values, a *t*-test was applied; $P<0.05$ considered as statistically significant.

Results

Relationship between temperature and viability in fed and starved fish

Temperature profiles are presented in Fig. 1A. Most of the experiments were conducted with fish maintained at 8°C from the time of capture on 26 October 2003 and subsequently subjected to a controlled decrease in temperature on 15 January 2004. Water temperature was decreased over 19 days to –1°C and, with the exception of a 1-day spike to 1.2°C on 9 March 2004, maintained under 0°C until 11 May 2004. A control population of fish was maintained at normal seasonal temperatures. On 15 January 2004 ambient water temperature was 1°C and decreased to –1.2°C by 6 February 2004. Additional experiments were conducted on a small number of animals that were maintained at 8°C in January/February 2004 and 2006.

The cumulative mortalities associated with the controlled decrease in temperature are shown in Fig. 1B. The figure is set with day 0 as the start of temperature decrease. Each group started with 130 fish, of which six healthy fish were removed on each of the dates shown in Fig. 1A. There is a clear difference in the spontaneous death rate between fed and starved rainbow smelt. Fish that were fed survived well. By day 30 at –1.2°C, there were nine recorded deaths with 11 more between day 30 and day 115 at temperatures of –0°C or lower. In contrast, in the group of starved fish, all individuals had succumbed by 30 days (14 February 2004), when the temperature had reached –1.2°C. Many of the fish died at temperatures well above the anticipated freezing point of body fluids. For instance, 50 fish died by day 20 (20 February 2004), when the water temperature was still above –0.8°C. A typical teleost should be able to survive these temperatures even in the absence of antifreeze mechanisms.

In the 2003/2004 experiment, there were no inexplicable mortalities in fish that were fed and maintained at either ambient or elevated temperatures over the time window of the above described experiment. In the 2005/2006 study, eight apparently healthy fish that had been maintained on heated water, were food deprived beginning 16 January 2006. One fish died over the next 26 days, when all had been sampled.

Glycogen levels

Tissue-specific glycogen levels were determined in rainbow smelt held at 8°C (Fig. 2A). Fish were sampled on 15 January 2004. Glycogen content was highest in liver at 172±64 μmol glucosyl unit g^{-1}, followed by heart at 50±12 μmol glucosyl unit g^{-1}. Muscle and gut levels were approximately 17 μmol glucosyl unit g^{-1}, with lower levels being noted in the other tissues.

In fish that were fed and subjected to a decrease in temperature, there was a significant decrease in liver glycogen by day 15 (Fig. 2B). The glycogen level remained low until day 104 (29 April 2004; –1.4°C temperature) and day 116 (11 May 2004; –1.2°C temperature), at which time glycogen levels increased to amounts not significantly different from the initial value. Liver glycogen in starved fish decreased to a minimum level of 7.7±5.2 μmol glucosyl unit g^{-1} by day 12 and remained low until the last sample was taken on day 22. The glycogen level on day 12 was significantly lower in starved than in fed fish (*t*-test; $P=0.032$).

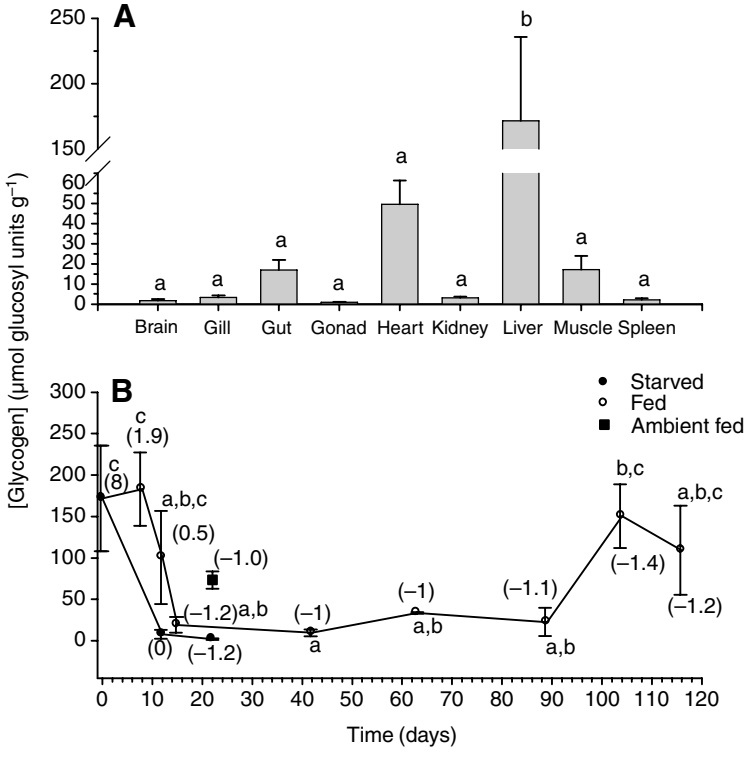

Fig. 2. Glycogen levels in rainbow smelt *Osmerus mordax*. (A) Glycogen levels in various tissues of fish maintained at 8°C and sampled on 15 January 2004, immediately prior to the temperature decrease. Note the break in axis that separates liver from other tissues. Values are means ± s.e.m., $N=4$. Different letters over bars indicate a statistically significant difference between tissues. (B) Glycogen levels in liver of fish subjected to a controlled decrease in water temperature with either feeding (open circles) or food deprivation (filled circles). The date at which the temperature decrease was initiated (i.e. 15 January 2004) is used as day 0. Filled square, liver glycogen content in fish maintained at ambient temperature. Values are means ± s.e.m., $N=4$. Different letters indicate a statistically significant difference between time points for the fed group. Water temperature (°C) is shown in parentheses.

Table 1. *Liver glycogen, and plasma and liver glycerol levels in rainbow smelt Osmerus mordax maintained at 8°C and either fed or food deprived*

	Fed	Starved
[Glycogen] (μmol glucosyl unit g^{-1})	153±23.1	119±16
[Glycerol]		
Plasma (μmol ml^{-1})	13.4±2.6	3.61±1.39*
Liver (μmol ml^{-1})	39.8±5.0	16.5±5.23*

Fed fish were sampled on 26 January 2006 (*N*=11). Starved fish were sampled on 20 February 2006 (i.e. following 26 days of without food) (*N*=6).

Asterisks indicate values that are significantly different between fed and starved fish.

Table 2. *Percentage water content in tissues of rainbow smelt Osmerus mordax*

	% Water
Liver	72.1±1.1
Kidney	80.1±0.5
Spleen	72.5±1.8
Gill	84.3±0.6
Intestine	52.1±3.4
Heart	78.9±0.5
Muscle	77.6±0.3

N=6 in each case.

Fish that were fed and held under ambient temperature conditions had a liver glycogen level of 73.3±10.5 μmol glucosyl unit g^{-1} (sampled 6 February 2004; –1°C) (Fig. 2B; square symbol). This value tended to be lower than the levels of 172±64 and 153±23 μmol glucosyl unit g^{-1} noted in liver of fish held at 8°C and sampled on 15 January 2004 (Fig. 2) or 26 January 2006 (Table 1), respectively but significantly (ANOVA; *P*=0.001) higher than the 2.0±0.9 μmol glucosyl unit g^{-1} in fish subjected to the forced decreased in temperature (Fig. 2B; day 22). In the 2006 experiment, the liver glycogen content in rainbow smelt held at 8°C and fed was no different than in fish subjected to 26 days of starvation at this temperature (Table 1).

Plasma glycerol levels

Plasma glycerol in fish that were force chilled and fed increased from 5 μmol ml^{-1} to levels in excess of 300 μmol ml^{-1} at day 42 (27 February 2004; –1°C) (Fig. 3A). Thereafter, plasma glycerol concentration decreased to 37.4±9.7 μmol ml^{-1} on the final sample date (11 May 2004; –1.2°C).

Rainbow smelt subjected to the challenge of a decrease in water temperature, without food, showed an increase in plasma glycerol content similar to fed fish for the first 22 days of the experiment (Fig. 3B). Glycerol levels reached 272±17.4 μmol ml^{-1} by the final sampling point (6 February 2004; –1.2°C). These data are based only on those fish that survived the temperature decrease challenge.

Rainbow smelt maintained at elevated temperatures had plasma glycerol levels of less than 10 μmol ml^{-1} (Fig. 3A). Food deprivation for 26 days at 8°C resulted in a significant decrease in plasma glycerol level (Table 1).

Tissue glycerol levels

The content of glycerol in various tissues in fish that were subjected to a forced decrease in water temperature and fed is presented in Fig. 4. These values follow the general pattern of that in plasma with an increase to day 42 (17 February 2004; –1°C), followed by a continual decrease. This pattern occurred in all tissues sampled. The level of tissue glycerol at the apex of the curves was highest in kidney at 263±37 μmol g^{-1} and lowest in gill at 125±13 μmol g^{-1}. Glycerol levels in fish that were deprived of food, under the same thermal conditions, followed the same pattern up to day 22, at which time the study ended (data not shown).

The level of glycerol in plasma, expressed as μmol ml^{-1}, was generally higher than in tissue, expressed as μmol g^{-1}, over the first 42 days of the temperature decrease. As plasma glycerol levels decreased, tissue levels did as well. This prompted a first approximation analysis of the intra- to extracellular glycerol gradient. The percentage water

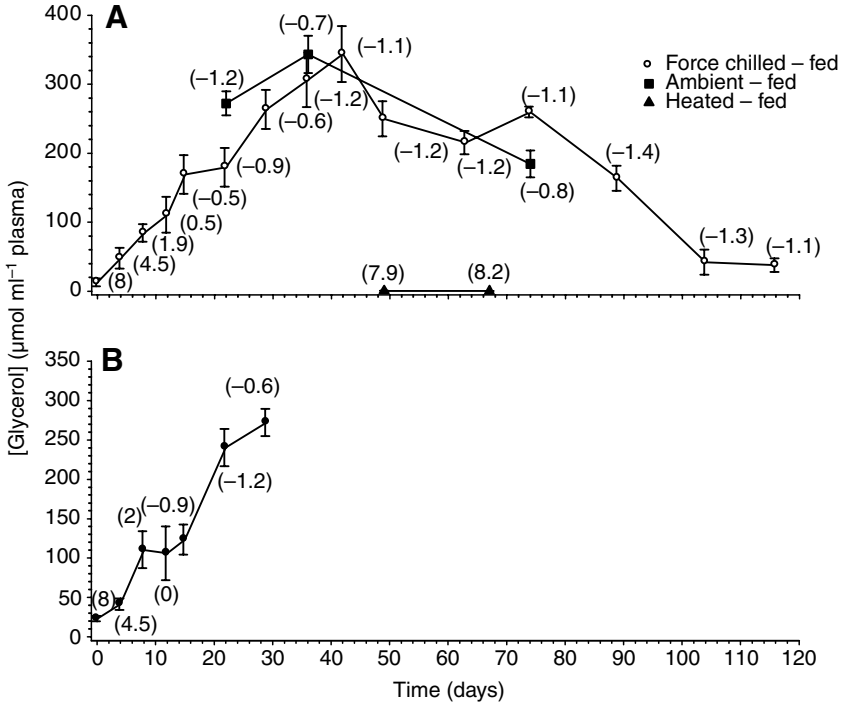

Fig. 3. Plasma glycerol levels in rainbow smelt *Osmerus mordax*. The date at which a controlled temperature decrease was initiated (i.e. 15 January 2004) is used as day 0. Water temperature (°C) is shown in parentheses. Values are means ± s.e.m., *N*=5 or 6 for all points. (A) Open circles, fish subjected to a controlled decrease in water temperature; filled squares, fish maintained at natural ambient temperature; filled triangles, fish maintained at elevated temperature. All fish were fed. (B) Fish subjected to a controlled decrease in water temperature without feeding.

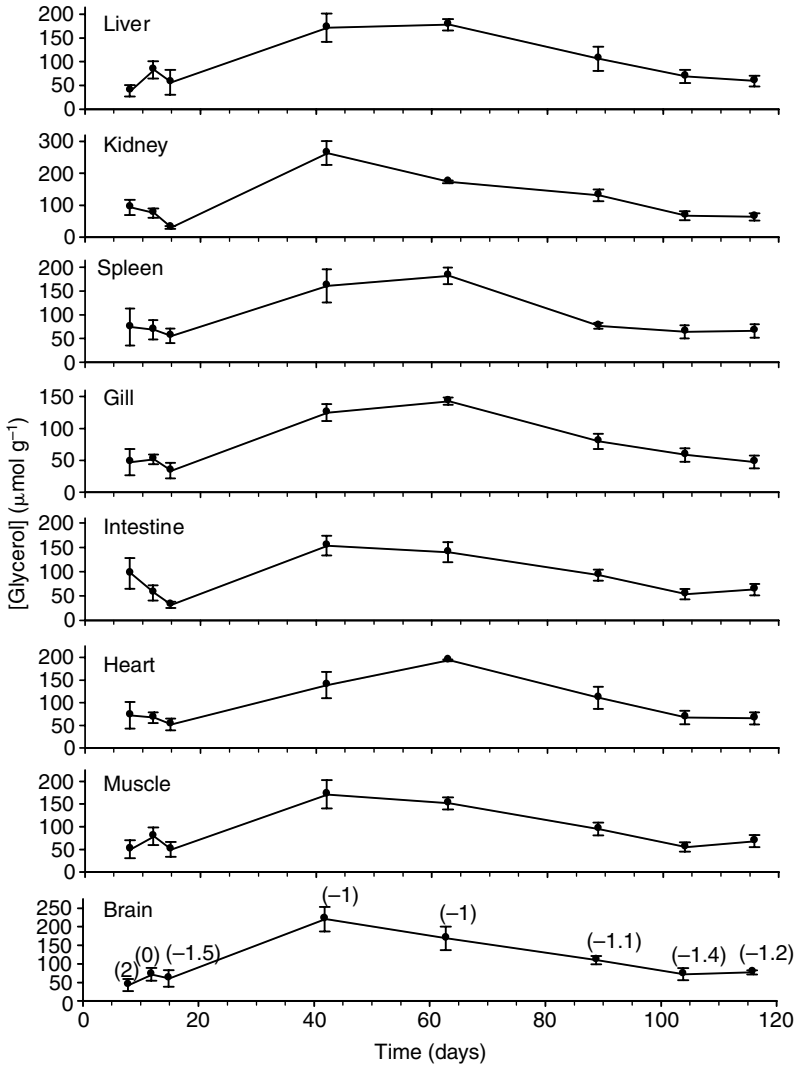

Fig. 4. Glycerol levels in tissues of rainbow smelt *Osmerus mordax* subjected to a controlled decrease in water temperature. The date at which a controlled temperature decrease was initiated (i.e. 15 January 2004) was used as day 0. Water temperature (°C) on each sampling day is shown on bottom panel in parentheses. Values are means ± s.e.m., *N*=4 for all time points.

content was calculated for each of the tissues (Table 2) and ranged from 52% water for gut to 84% water for gill. Water content did not change with temperature (data not shown). Tissue levels were converted from μmol g^{-1} to μmol ml^{-1} tissue water and divided by μmol ml^{-1} plasma to obtain the ratio of glycerol from total tissue water to plasma (Fig. 5). A ratio of 1.0 indicates equilibration of glycerol between the intra- and extracellular compartments. Values less than 1.0 indicate a diffusion gradient from plasma to tissue, whereas values greater than 1.0 indicate a diffusion gradient from tissue to plasma. Precise intra- to extracellular gradients cannot be calculated without knowledge of extracellular tissue water.

As plasma glycerol increased to the apex on day 42 the level of glycerol was either close to equilibrium between the two compartments or there was a gradient from the plasma space to the tissue space. Late in the experiment (i.e. days 104 and 116), as plasma glycerol levels decreased, the average ratio for all tissues was in excess of 1.0. Although the value for any specific tissue is not significantly different from 1.0, the overall picture shows them to be consistently positive, suggesting that further study of this may be required in order to determine whether glycerol content in tissue water might exceed that in plasma.

Discussion

Rainbow smelt subjected to low temperature with access to food or maintained at high temperature without food survive quite well in captivity. However, the combination of food deprivation and transition to low temperature is lethal. This is most unusual as it is expected that low temperature should prolong survival in the absence of food by reducing energy demand. Moreover, death ensued at temperatures well above the freezing point of body fluids. This could have important ecological consequences as it implies that under field conditions, an absence of usual food sources as the temperature decreases in the fall, could result in massive mortality. We do not know the cause for the premature death with respect to temperature and food deprivation; however, it is clearly not a matter of simple energy balance, as rainbow smelt maintained at 8°C tolerate longer periods of starvation than fish challenged with lower temperatures. An understanding of this phenomenon will be dependent upon detailed analysis of both surviving and dying individuals.

An imposed temperature decrease leads to increases in glycerol levels in plasma and other tissues, as has been observed in earlier studies (Driedzic et al., 2006). Even though the temperature was held below –1°C, glycerol in plasma and other tissues began to decrease in samples taken after 27 February 2004 (i.e. day 42), with tissue levels of glycerol generally following the same pattern as plasma glycerol. This pattern of glycerol accumulation and subsequent decrease is strikingly similar to that observed in two other experiments in which rainbow smelt were maintained under natural thermal and light conditions (Treberg et al., 2002; Lewis et al., 2004). A decrease in temperature alone is sufficient to activate the glycerol accumulation mechanism and occurs at about 5°C, but the signal for reducing the level of glycerol resides elsewhere. Potential candiates are photoperiod or an inherent circannual rhythm, possibly in association with sufficient levels of antifreeze protein.

The highest level of glycogen occurs in liver, followed by heart, with lower levels in a number of other tissues. At 8°C, starvation alone for a 26-day period did not result in a significant decrease in liver glycogen, although the mean value decreased by 22%. This is common for fish, for example Atlantic cod held at 8°C showed about a 60% decrease in liver glycogen following 39 days of food deprivation (Hall et al., 2006). In rainbow smelt,

however, a decrease in temperature from 8° to –1.2°C over only 12 days resulted in liver glycogen levels close to zero. The decrease in liver glycogen occurs even in fed fish, but not to as great an extent. As such, either starvation or low temperature results in glycogen mobilization, with the combination of both having the most impact.

Liver glycogen reserves can only account for a small proportion of the glycerol that accumulates. For instance, liver glycogen with an initial level of 175 μmol glucosyl units g^{-1} could produce 350 μmol glycerol g^{-1}. Of this about 150 μmol g^{-1} would be retained in the liver with the remainder (i.e. 350–150=200 μmol g^{-1}) available for export to other tissues. A 50 g fish with a liver mass of 0.75 g could release 150 μmol of glycerol, which if distributed evenly across all tissues, would result in a glycerol increment of only 3 μmol g^{-1}. With the possible exception of heart, none of the other tissues have adequate glycogen reserves to account for the increase in glycerol content. There is a clear need for dietary intake of fuels or additional on-board reserves such as amino acids from protein to support glycerol production. In this context it is important to note that glycerol from triglycerides is not considered to be a contributor to glycerol production (Raymond, 1995).

The decrease in glycerol content in all tissues observed late in the study is associated with an increase in glycogen content in liver, and this occurs while the temperature is below –1°C. It is likely that one fate of glycerol is incorporation into the glycogen pools. Between days 89 and 116 the content of glycogen in liver increased on average by about 125 μmol glucosyl units g^{-1}, or the equivalent of 250 μmol glycerol g^{-1}. This could account for the decrease in liver glycerol level of about 50 μmol g^{-1} and still provide room to accommodate some of the decrease of glycerol in the plasma and other tissues. Glycogen levels may cycle in other tissues as well. The important point is that glycogen synthesis can occur at low temperatures and could serve as a sink for glycerol, suggesting that glycerol need not be lost to the environment nor metabolized to CO_2 during this period.

Glycerol level in tissues follows the same general profile as that in plasma, with an increase as temperature is reduced followed by a decrease in level. As plasma glycerol increased there was either a gradient from the plasma space to the tissue space or the level of glycerol was close to equilibrium between the two compartments. In liver, on day 15, there is no significant difference between glycerol in tissue water and in plasma, but for all other tissues tested glycerol is lower in the tissue water than in plasma. This would be consistent with a movement of glycerol from liver into plasma and subsequently down a concentration gradient from plasma into other tissues. This contention is supported by the release of glycerol by isolated preparations (Driedzic et al., 1998; Driedzic and Ewart, 2004). However, on day 42, when plasma glycerol is at its highest level, glycerol in liver tissue water is less than in plasma. This situation, of lower than expected concentration of glycerol in liver at times of high plasma glycerol, has been observed in two previous experiments (Treberg et al., 2002; Driedzic et al., 2006). There are a number of possible explanations to account for this. As previously suggested, based on findings with yeast, there may be active pumping of glycerol out of liver into plasma (Driedzic et al., 2006). An alternative explanation is that the process of glycerol sequestering into glycogen may be starting with the intracellular metabolic steps proceeding faster than diffusion of glycerol into the liver. A third possibility is that other tissues, such as kidney (a known gluconeogenic tissue),

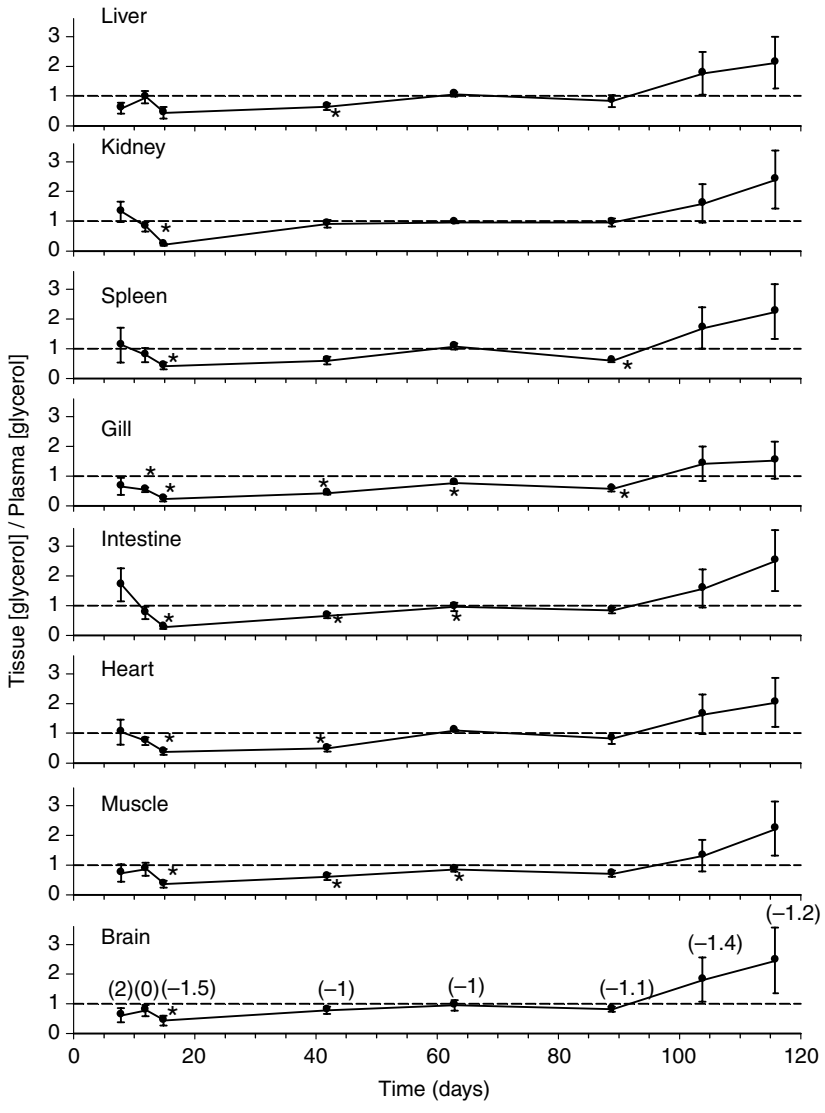

Fig. 5. Ratio of [Glycerol] in tissue (μmol ml^{-1} tissue water)/plasma (μmol ml^{-1} plasma) in rainbow smelt *Osmerus mordax* subjected to a controlled decrease in water temperature. The date at which a controlled temperature decrease was initiated (i.e. 15 January 2004) was used as day 0. The broken line represents equal values for glycerol in tissue water and plasma. Asterisks indicate values significantly different from 1.0. Water temperature (°C) on each sampling day is shown on bottom panel in parentheses. Values are means ± s.e.m., *N*=4 for all points.

may become a site of glycerol release as the season progresses. Finally, when plasma glycerol returns to minimal levels there appear to be higher levels of glycerol in tissues than would be expected if glycerol was in equilibrium between the intra- and extracellular space.

General conclusions

Rainbow smelt must feed to live at low temperatures; in food-deprived fish death ensues at about 5°C. Glycerol accumulation is activated by low temperature alone but the decrease in glycerol that occurs in late winter/early spring is temperature-independent. The early phase of glycerol production is associated with mobilization of liver glycogen; however, glycogen from liver and in all other tissues can account for only a small fraction of the total glycerol that accumulates. This is consistent with the need to continue feeding at low temperature with a constant supply of ingested carbohydrate and amino acids for glycerol synthesis. During late winter/early spring, even while the temperature is still subzero, glycerol levels decrease with a concomitant increase in glycogen.

This work was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (W.R.D.), the Canadian Institutes of Health Research through the Regional Partnership Program, and Newfoundland and Labrador Department of Innovation, Trade and Rural Development. W.R.D. holds the Canada Research Chair in Marine Bioscience. We thank the Field Services Unit of the Ocean Sciences Centre for the collection of specimens and we thank Vanya Ewart (NRC IMB) for helpful discussion and comments on the manuscript.

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