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RESEARCH ARTICLE

Extracellular glucose can fuel metabolism in red blood cells from high glycemic Atlantic cod (*Gadus morhua*) but not low glycemic short-horned sculpin (*Myoxocephalus scorpius*)

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ABSTRACT

Energy metabolism was assessed in red blood cells (RBCs) from Atlantic cod and short-horned sculpin, two species that have markedly different levels of blood glucose. The objective was to determine whether the level of extracellular glucose has an impact on rates of glucose metabolism. The blood glucose level was 2.5 mmol l^{-1} in Atlantic cod and 0.2 mmol l^{-1} in short-horned sculpin, respectively. Oxygen consumption, lactate production and glucose utilization were measured in whole blood and related to grams of RBCs. Glucose utilization was assessed by measuring both glucose disappearance and the production of ^3H_2O from [2-^{3}H]-glucose. RBCs from both species have an aerobic-based metabolism. In Atlantic cod, extracellular glucose is sufficient to provide the sum of glucosyl equivalents to support both oxidative metabolism and lactate production. In contrast, extracellular glucose can account for only 10% of the metabolic rate in short-horned sculpin RBCs. In both species, about 70% of glucose enters the RBCs via facilitated transport. The difference in rates of extracellular glucose utilization is related to the extremely low levels of blood glucose in short-horned sculpin. In this species energy metabolism by RBCs must be supported by alternative fuels.

KEY WORDS: Glucose metabolism, [2-^{3}H]-glucose, Cytochalasin B, Red blood cell

INTRODUCTION

Red blood cells (RBCs) of fish have an aerobic-based metabolism, as evident from measurements of oxygen uptake rate ($\dot{M}_{O_2}$) and lactate production in three species of salmonids [rainbow trout, *Onchorhynchus mykiss* (Ferguson et al., 1989; Sephton and Driedzic, 1994; Phillips et al., 2000); brown trout, *Salmo trutta* (Pesquero et al., 1992); Atlantic salmon, *Salmo solar* (Ferguson and Boutilier, 1988)] and the scorpioforme sea raven (*Hemitripterus americanus*) (Sephton et al., 1991; Sephton and Driedzic, 1994). What metabolic fuels support oxygen consumption remains a controversial issue and may be species specific dependent upon levels of blood-borne substrates.

The $\dot{M}_{O_2}$ of isolated RBCs from brown trout is similar without substrate or with glucose or pyruvate availability (Pesquero et al., 1992; Pesquero et al., 1994). It is possible that these cells utilize glucose and/or pyruvate when available but call upon endogenous fuels when deprived of extracellular substrate. A few studies have attempted to address the issue of metabolic fuel use by assessing rates of oxygen consumption and $^{14}CO_2$ production from ^{14}C-labelled substrates. Rainbow trout RBCs produced $^{14}CO_2$ from exogenous glucose, lactate, alanine and oleate (Walsh et al., 1990); sea raven and rainbow trout RBCs oxidized glucose to CO_2 (Sephton et al., 1991; Sephton and Driedzic, 1994); and brown trout RBCs produced $^{14}CO_2$ from labelled glucose and pyruvate (Pesquero et al., 1994). In each of these studies the calculated rate of metabolism of the exogenous fuel, based on the specific activity of the fuel, was well below that required to support oxygen consumption. These studies are compromised because to quantitate actual rates of oxidation of extracellular substrate the specific activity of the intracellular compound that releases $^{14}CO_2$ is required. For instance, the specific activity of intracellular pyruvate that is converted to acetyl coenzyme A plus CO_2 via pyruvate dehydrogenase is required to calculate the rate of [6-^{14}C]-glucose oxidation; however, the specific activity of pyruvate will be considerably lower than the added extracellular glucose due to dilution of metabolites in passage through the glycolytic pathway. In other studies it has been shown that RBCs from American eel (*Anguilla rostrata*) can oxidize glucose, lactate and alanine (Soengas and Moon, 1995), while RBCs from carp (*Cyprinus carpio*), in addition to the above-mentioned fuels, can utilize adenosine and a number of amino acids (Tiihonen and Nikinmaa, 1991); however, it is not possible to calculate the actual rates of metabolism from extracellular specific activities. An alternative approach is required to resolve the question of what metabolic fuels support aerobic metabolism. In this study, we focus on glucose as a likely candidate to be an important fuel source.

Hutton (Hutton, 1972) developed an approach with human RBCs to assess rates of glucose metabolism. Cells are offered [2-^{3}H]-glucose and rates of ^3H_2O produced from the conversion of [2-^{3}H]-glucose 6-phosphate to fructose 6-phosphate catalysed by phosphohexose isomerase is used to measure rates of glycolysis. The specific activity of [2-^{3}H]-glucose 6-phosphate is approximately 80% of that of extracellular glucose (Neely et al., 1972). The technique was applied to isolated RBCs from four species of fish and found to be extremely robust, with linearity of ^3H_2O production for at least 6 h (Driedzic et al., 2013). Here we apply this approach and that of simply measuring glucose disappearance and lactate production of incubated blood to determine the contribution of extracellular glucose in supporting $\dot{M}_{O_2}$ of fish RBCs.

In the current study the importance of glucose as an aerobic metabolic fuel for RBCs from Atlantic cod (*Gadus morhua* Linnaeus 1758) and short-horned sculpin [*Myoxocephalus scorpius* (Linnaeus 1758)] is assessed. These two species were selected because they show significant differences in blood glucose levels and thus potential differences in the contribution of glucose to metabolism. In Atlantic cod, basal blood glucose ranges from approximately 3 to 7 mmol l^{-1} (Fletcher et al., 1982), values that are typical for most teleost fish (Polakof et al., 2012). In contrast, blood

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glucose in short-horned sculpin is 0.5 mmol l^{-1} (MacCormack and Driedzic, 2007; Driedzic et al., 2013), a basal level that is low by teleost standards (Polakof et al., 2012). A further aspect to the current work is an assessment of what percentage of glucose enters via facilitated glucose transport. In many but not all fish RBCs, glucose entry is via a combination of simple diffusion and facilitated glucose transport. This viewpoint is based upon studies that show saturation of uptake of non-metabolizable radiolabelled glucose analogs and glucose metabolism, as well as inhibition of these processes by cytochalasin B and/or phloretin (Tse and Young, 1990; Soengas and Moon, 1995; Driedzic et al., 2013). Furthermore, GLUT1 mRNA is expressed in RBCs of Atlantic cod (Hall et al., 2014). GLUT1 is a member of the class 1 sodium-independent transmembrane proteins responsible for facilitated glucose diffusion. Here we also address the contribution of facilitated glucose diffusion by RBCs that differ in blood glucose levels.

RESULTS

Basic blood parameters

Haematocrit was significantly higher in blood of Atlantic cod than in short-horned sculpin (Fig. 1). Blood and plasma glucose levels were 12-fold higher in Atlantic cod than in short-horned sculpin. Intracellular glucose calculated from these values was 0.54±0.35 mmol l^{-1} in Atlantic cod and essentially zero in short-horned sculpin. Therefore the glucose gradient from the extracellular space to the RBC intracellular space was approximately 2 mol l^{-1} for Atlantic cod and 0.2 mmol l^{-1} for short-horned sculpin; that is, 10-fold higher in Atlantic cod.

Oxygen consumption

The disappearance of oxygen in sealed vessels was linear for at least 300 min for blood from Atlantic cod and short-horned sculpin under both control conditions and with the addition of cytochalasin B (Fig. 2A,B). Oxygen consumption was expressed as nmol g^{-1} RBC min^{-1} based on the linear regressions for each experiment. The rate of oxygen consumption was the same for RBCs from both species and cytochalasin B had no effect. Mean oxygen consumption rates were typically 25 nmol g^{-1} RBC min^{-1} (Fig. 2C).

Lactate production

The appearance of lactate was linear for at least 300 min in blood from Atlantic cod and short-horned sculpin when incubated under aerated conditions with or without cytochalasin B treatment (Fig. 3A,B). The rate of lactate production was expressed as nmol g RBC^{-1} min^{-1} based on the linear regressions (Fig. 3C). There was no difference between Atlantic cod and short-horned sculpin in the rate of lactate production in untreated preparations. Cytochalasin B treatment resulted in a significant decrease in the rate of lactate production by Atlantic cod RBCs but had no effect on short-horned sculpin RBCs.

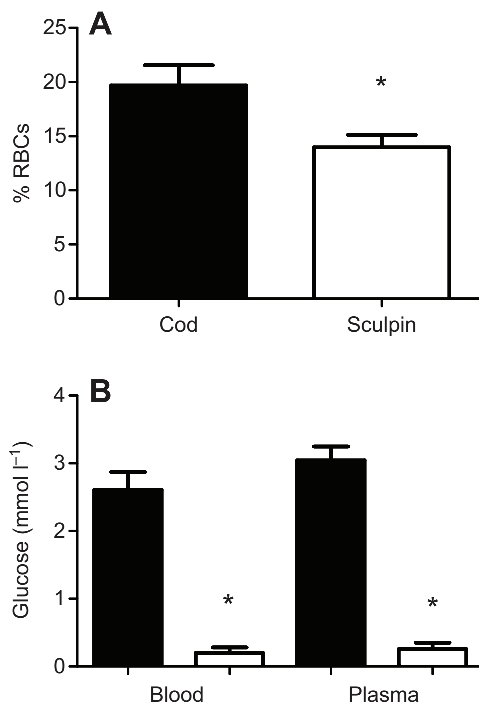

Fig. 1. Blood parameters for Atlantic cod (filled bars) and short-horned sculpin (open bars). (A) Haematocrit. (B) Glucose levels in whole blood and plasma. *N*=5 in all cases with the exception of Atlantic cod blood glucose, in which case *N*=4. *Significant difference between Atlantic cod and short-horned sculpin.

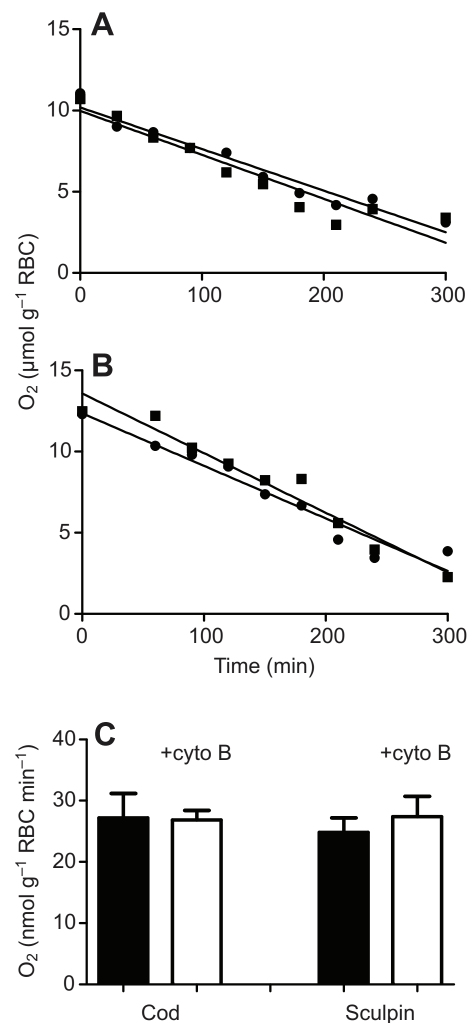

Fig. 2. Oxygen consumption of red blood cells (RBCs) of Atlantic cod and short-horned sculpin. Examples of the relationship between oxygen content and time for blood incubated in a sealed syringe at 8°C for (A) Atlantic cod and (B) short-horned sculpin. Data are normalized to grams of RBCs. Circles, control; squares, plus cytochalasin B (cyto B). (C) Oxygen consumption expressed as nmol g^{-1} RBC min^{-1}. Filled bars, control; open bars, plus cyto B. *N*=5 for both species.

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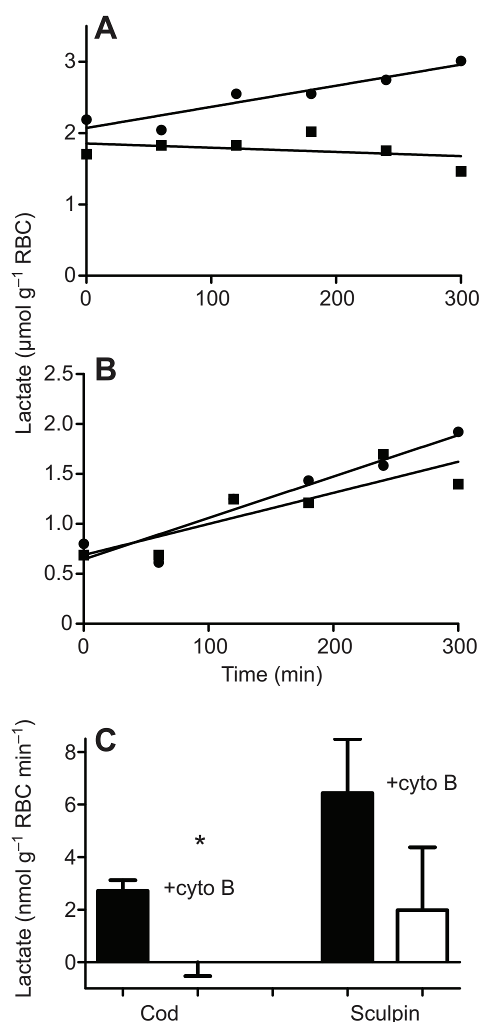

Fig. 3. Lactate production by RBCs of Atlantic cod and short-horned sculpin. Examples of the relationship between lactate level and time in whole blood incubated at 8°C for (A) Atlantic cod and (B) short-horned sculpin. Data are normalized to grams of RBCs. Circles, control; squares, plus cytochalasin B (cyto B). (C) Lactate production expressed as nmol g^{-1} RBC min^{-1}. Filled bars, control; open bars, plus cyto B. *N*=5 for both species. *Significant difference between control and cyto B in Atlantic cod.

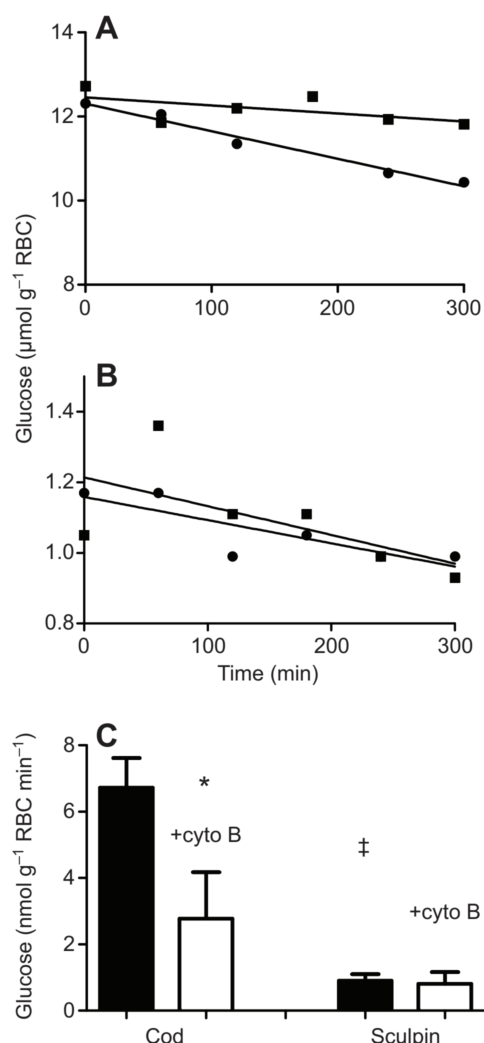

Fig. 4. Glucose consumption by RBCs of Atlantic cod and short-horned sculpin. Examples of the relationship between glucose level and time in whole blood incubated at 8°C for (A) Atlantic cod and (B) short-horned sculpin. Data are normalized to grams of RBCs. Circles, control; squares, plus cytochalasin B (cyto B). (C) Glucose consumption expressed as nmol g^{-1} RBC min^{-1}. Filled bars, control; open bars, plus cyto B. *N*=4 for Atlantic cod; *N*=5 for short-horned sculpin. *Significant difference between control and cyto B in Atlantic cod; ‡significant difference between control Atlantic cod and short-horned sculpin.

Glucose utilization

Rates of glucose utilization were assessed with two different approaches: direct analysis of glucose disappearance from plasma, and with the use of glucose labelled with a radioisotope.

Glucose disappearance from plasma

Glucose decreased in plasma in a linear fashion in both Atlantic cod and short-horned sculpin (Fig. 4A,B). The rate of glucose disappearance was expressed as nmol g^{-1} RBC min^{-1} based on the linear regressions (Fig. 4C). Glucose utilization was 8-fold faster in Atlantic cod than short-horned sculpin under control conditions. Cytochalasin B significantly decreased glucose utilization (59%) by Atlantic cod RBCs but had no impact on the already low rates of glucose utilization by short-horned sculpin RBCs.

Production of ^3H_2O from [2-^{3}H]-glucose

It was previously reported that the production of ^3H_2O from [2-^{3}H]-glucose by isolated RBCs from Atlantic cod and short-horned sculpin is linear for at least 6 h (Driedzic et al., 2013). Preliminary experiments with whole blood confirmed this finding. As such, in the current experiments, samples were taken at only one time point following 3 h of incubation. The rate of glucose metabolism was significantly higher (6.2-fold) in Atlantic cod than short-horned sculpin under control conditions (Fig. 5). Cytochalasin B inclusion led to a significant decrease in glucose metabolism for both Atlantic cod (81%) and short-horned sculpin (85%) RBCs.

Glycogen levels

Glycogen levels decreased during the course of a 3 h incubation of RBCs from Atlantic cod in both control preparations and in the presence of cytochalasin B (Fig. 6A). The rate of decrease was similar in both control and treated preparations, i.e. impairing glucose uptake did not increase the rate of glycogenolysis (Fig. 6C). The was no change in glycogen level in short-horned sculpin RBCs following 3 h of incubation in either control or cytochalasin B-enriched preparations (Fig. 6B). Initial glycogen levels were

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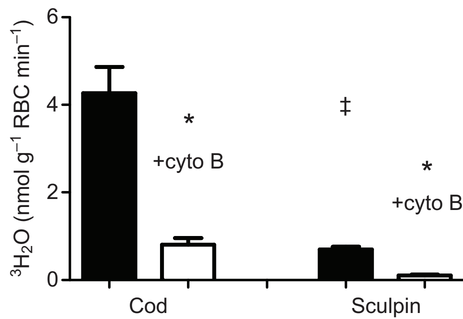

Fig. 5. Glucose metabolism by RBCs of Atlantic cod and short-horned sculpin calculated from the production of ^3H_2O released from [2-^{3}H]-glucose following incubation of whole blood for 3 h at 8°C. Data are normalized to grams of RBCs. Filled bars, control; open bars, plus cytochalasin B (cyto B). *N*=5 in all cases. *Significant difference between control and cyto B in both species; ‡significant difference between control Atlantic cod and short-horned sculpin.

significantly higher (6-fold) in short-horned sculpin than in Atlantic cod RBCs (cf. first bar of Fig. 6B with Fig. 6A).

DISCUSSION

Methodology

The objective of the study was to assess rates of glucose utilization by RBCs under physiological conditions. As such, experiments were conducted with whole blood. All measurements were normalized to grams of RBCs on the premise that these cells are the major contributor to metabolic changes. This was considered appropriate on the following grounds. Centrifuged blood from healthy Atlantic cod and short-horned sculpin does not yield a low density 'fluffy layer' usually associated with white blood cells. Preliminary experiments showed that the oxygen consumption of blood normalized to grams of RBCs is the same as that of isolated RBCs (data not shown). RBCs were exposed to normal plasma levels of glucose, as well as other potential extracellular metabolic fuels. Furthermore, because RBCs were not subjected to isolation and washing, the cells will have physiological levels of potential on-board metabolic fuels.

Glucose utilization was measured by two methods. The disappearance of glucose in the blood was determined with enzymatic analysis. This measurement does not take into account other fates of glucose, for example possible incorporation into the glycogen pool, conversion to other compounds such as ribose for nucleotide synthesis, and binding of glucose to proteins. In the current experiments glycogen did not serve as a net sink for extracellular glucose as this compound either did not change or decreased during the course of incubation; however, the possibility that some of the extracellular glucose is being incorporated into other intracellular compounds cannot be ruled out. Therefore, the direct disappearance of glucose may be an overestimate of rates of glycolysis. Rates of glycolysis were determined by adding trace amounts of [2-^{3}H]-glucose to whole blood. Glucose is taken up by cells and converted via hexokinase to [2-^{3}H]-glucose-6-phosphate (glucose-6P), which in turn is converted via phosphohexose isomerase to fructose 6-phosphate with the release of ^3H_2O. Glycolytic rate is subsequently calculated from the released ^3H_2O radioactivity based on the specific activity of extracellular glucose that will be higher than [2-^{3}H]-glucose-6P due to dilution of the intracellular pool. We have been unable to measure the specific activity of [2-^{3}H]-glucose-6P given its very low level but in perfused rat heart the specific activity of [2-^{3}H]-glucose-6P is 80% of

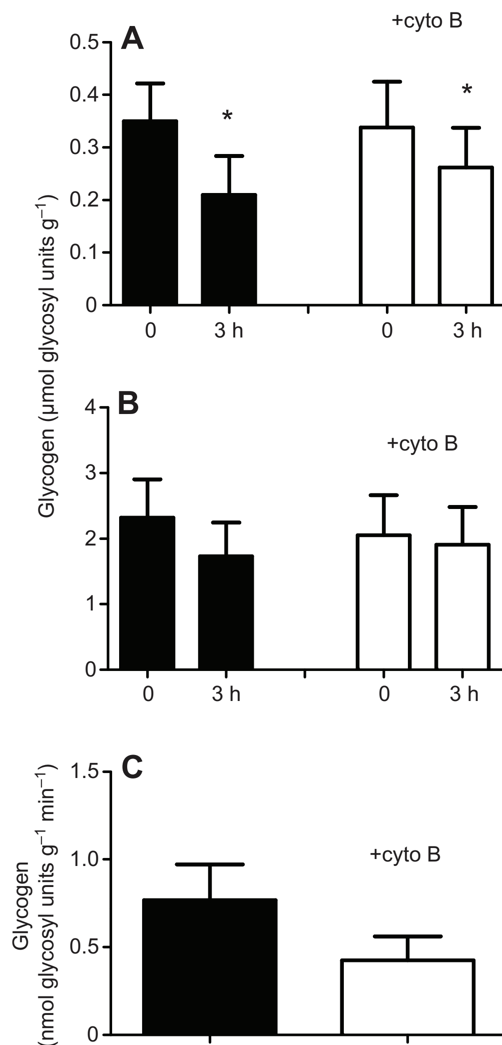

Fig. 6. Glycogen levels in RBCs at time 0 and following 3 h incubation. (A) Atlantic cod. (B) Short-horned sculpin. *Significant difference between time 0 and 3 h. (C) Glycogen consumption by Atlantic cod RBCs expressed as nmol g^{-1} RBC min^{-1}. Filled bars, control; open bars, plus cytochalasin B (cyto B). *N*=5 in all cases.

extracellular glucose (Neely et al., 1972). Thus the calculated rate of glycolysis for the RBCs will be somewhat of an underestimate.

There is a linear correlation between the two measurements of glucose utilization (Fig. 7), with glucose disappearance being higher than glucose metabolism. This would be consistent with the specific activity of glucose-6P being lower than that of extracellular glucose. The true rate of glucose metabolism is somewhere between the values of the two approaches in this study. In our hands, the production of ^3H_2O is a much more robust method with less noise within an experiment than the enzymatic measurement of small changes in glucose levels. The power of the isotope approach is that it is extremely sensitive, only a small volume of cells is required, and it avoids the difficulty of measuring small changes in glucose level against a high background.

Blood parameters

Metabolite levels were determined in blood collected from quiescent animals that were fed on a regular basis. Blood and plasma glucose were significantly higher in Atlantic cod than short-horned sculpin, as expected from previous studies (Driedzic et al., 2013). The blood

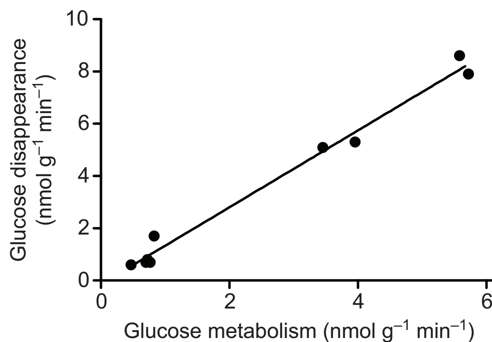

Fig. 7. Linear relationship (y=1.47x–0.13; P<0.0001) between glucose consumption determined by direct glucose disappearance and glucose metabolism determined from ^3H_2O released from [2-^{3}H]-glucose. Data are extracted from Figs 4 and 5.

glucose level in short-horned sculpin of 0.2 mmol l^{-1} is at the low extreme of levels reported for in excess of 1000 species (Polakof et al., 2011; Polakof et al., 2012). The calculated glucose gradient from the extracellular to the intracellular space was 10-fold higher in Atlantic cod than short-horned sculpin. This is an important consideration in the current experiment as it establishes that high plasma glucose is associated with a high gradient and not simply high intracellular glucose levels.

Aerobic-based metabolism in fish red blood cells

The $\dot{M}_{O_2}$ of Atlantic cod and short-horned sculpin RBCs was the same at 27 and 25 nmol O_2 g^{-1} RBC min^{-1} at 8°C. This is within the range reported for salmonids, American eel and sea raven RBCs (5–90 nmol O_2 g^{-1} RBC min^{-1} at various temperatures) (Ferguson and Boutilier, 1988; Ferguson et al., 1989; Walsh et al., 1990; Sephton et al., 1991; Pesquero et al., 1992; Sephton and Driedzic, 1994; Soengas and Moon, 1995; Phillips et al., 2000). Similarly, rates of lactate production by Atlantic cod and short-horned sculpin RBCs at 2.7 and 6.4 nmol lactate g^{-1} RBC min^{-1} were within the same range reported for RBCs from other species (0–10 nmol lactate g^{-1} RBC min^{-1}) under aerobic conditions (Ferguson and Boutilier, 1988; Ferguson et al., 1989; Walsh et al., 1990; Sephton et al., 1991; Pesquero et al., 1992; Sephton and Driedzic, 1994; Soengas and Moon, 1995). Simultaneous measurements of oxygen consumption and lactate production allow a calculation of what percentage of ATP generation is via aerobic and anaerobic respiration. Based on a P:O ratio of 2.5, and 1 for ATP:lactate, more than 95% of energy production is provided by aerobic metabolism in Atlantic cod and short-horned sculpin RBCs. This extends the number of species in which a predominantly based aerobic metabolism has been observed in fish RBCs.

Cytochalasin B did not affect oxygen consumption by RBCs from either species. This treatment decreases glucose metabolism by ~75% in isolated RBCs from Atlantic cod and short-horned sculpin (Driedzic et al., 2013). This suggests that RBCs in whole blood are either not utilizing glucose as a metabolic fuel under control conditions or alternatively they switch to other metabolic fuels when glucose uptake is compromised. RBCs from a variety of species have been shown to oxidize lactate, pyruvate, oleate, adenosine and a number of amino acids (Walsh et al., 1990; Sephton et al., 1991; Tiihonen and Nikinmaa, 1991; Pesquero et al., 1994; Sephton and Driedzic, 1994; Soengas and Moon, 1995). In this experiment RBCs are not utilizing exogenous lactate as an energy source as this metabolite increases during incubation. It is not known whether blood levels of pyruvate, oleate or adenosine are sufficiently high to support metabolism; however, there should be a large reserve of amino acids either in plasma or from the breakdown of on-board protein.

Glucose utilization

Under control conditions, glucose utilization by Atlantic cod RBCs was significantly higher than short-horned sculpin RBCs when assessed by either tracking glucose disappearance from blood (8-fold higher) or the production of ^3H_2O from labelled glucose (6-fold higher). Both approaches are consistent in showing that glucose utilization is higher in Atlantic cod than short-horned sculpin RBCs, despite the similarity in oxygen consumption. For Atlantic cod RBCs the rate of glucose metabolism assessed by ^3H_2O is the same in whole blood and isolated RBCs (Driedzic et al., 2013). For short-horned sculpin RBCs the rate of metabolism in whole blood is only about a third of that observed for isolated cells offered only glucose as a metabolic fuel. The difference may be due to RBCs in whole blood oxidizing additional/alternative fuels available in plasma.

Cytochalasin B treatment resulted in a decrease in glucose utilization by Atlantic cod RBCs when assessed by either glucose disappearance or ^3H_2O production. These experiments suggest that 60–80% of the glucose enters by facilitated glucose transport. A similar decrease in glucose utilization was noted for short-horned sculpin RBCs with the radioisotope approach, but not the direct glucose measurement procedure. We caution that the latter method is far less sensitive and are of the opinion that facilitated glucose transport is important glucose trafficking in short-horned sculpin RBCs. Overall, the general finding of impairment of glucose utilization with the same blood samples as used for oxygen consumption leads to the conclusion that RBCs from both species have the capacity to use metabolic fuels in addition to glucose.

What proportion of oxygen uptake rate can be supported by extracellular glucose?

The data set as a whole allows us to quantitate the importance of extracellular glucose in supporting metabolism in RBCs from Atlantic cod and short-horned sculpin. The latter species has blood glucose levels that are extremely low by vertebrate standards.

The situation with Atlantic cod seems relatively straightforward. Based on the standard equation of glucose+6O_2=6CO_2+12H_2O, there would be a glucose requirement of 4.53 nmol g^{-1} RBC min^{-1} to fully meet the aerobic metabolism requirements and an additional demand of 1.35 nmol g^{-1} RBC min^{-1} to meet the rate of lactate production for a total of 5.88 nmol glycosyl equivalents g^{-1} RBC min^{-1}. The rate of glucose disappearance is 14% higher than this value [(6.72–5.88)/5.88=0.14] and the rate of metabolism determined by the radioisotope approach is 28% lower [(5.88–4.26)/5.88=0.28] than the calculated glycosyl demand. Given the limitation of the two approaches there is a good match-up between glucose requirement calculated from oxygen consumption/lactate production and glucose utilization determined by the two methodologies. As such, extracellular glucose is quite sufficient to fully satisfy the metabolic requirements of Atlantic cod RBCs. Under cytochalasin B inhibition of glucose uptake, the rates of glucose utilization and glycogenolysis fall short of matching energetic demand. Under these conditions alternative metabolic fuels must be called upon, as discussed above. Preliminary studies reveal that RBCs from Atlantic cod produce NH_4^+, implying that these cells may utilize amino acids if deprived of glucose. RBCs from rainbow trout and American eel are able to oxidize alanine (Walsh et al., 1990; Soengas and Moon, 1995), while carp RBCs

produce $^{14}CO_2$ from labelled glutamine, glutamate, aspartate and isoleucine (Tiihonen and Nikinmaa, 1991).

Energy metabolism in short-horned sculpin RBCs that function in plasma with low extracellular glucose is quite different. Under control conditions, there is a demand of 7.3 nmol glucosyl equivalents g^{-1} RBC min^{-1} to support the sum of oxidative metabolism (4.13 nmol g^{-1} RBC min^{-1}) and lactate (3.2 nmol g^{-1} RBC min^{-1}) production. Measurements of glucose utilization yield only 0.9 nmol g^{-1} RBC min^{-1} (glucose disappearance) and 0.7 nmol g^{-1} RBC min^{-1} (^3H_2O production), revealing that in contrast to Atlantic cod RBCs, extracellular glucose is not a major contributor to energy metabolism in short-horned sculpin. In the presence of cytochalasin B the rate of glucose utilization may be even much lower. In neither situation is the glycogen pool called upon. As such, aerobic metabolism must be supported by an alternative fuel(s). Similar to Atlantic cod RBCs, preliminary evidence shows that short-horned sculpin RBCs produce NH_4^+, implying the oxidation of amino acids during *in vitro* incubation. Under *in vivo* conditions there may be a variety of metabolic fuels that could be utilized by RBCs and replenished from other tissues. These fuels could include pyruvate, adenosine and selected amino acids, as has been shown for RBCs from other species (Walsh et al., 1990; Tiihonen and Nikinmaa, 1991; Pesquero et al., 1994; Soengas and Moon, 1995).

The glycogen perplexities

Short-horned sculpin RBCs had considerably higher levels of glycogen than Atlantic cod RBCs (2.3 and 0.35 μmol glycosyl units g^{-1} RBC, respectively). The glycogen level in short-horned sculpin RBCs is similar to that in sea raven (3.9 μmol glycosyl units g^{-1} RBC), a closely related scorpioforme (Sephton and Driedzic, 1994), whereas glycogen level in Atlantic cod RBCs is similar to rainbow trout (Walsh et al., 1990; Sephton and Driedzic, 1994). During incubation, glycogen was mobilized in Atlantic cod RBCs but only at approximately a tenth of the rate of use of extracellular glucose. Glycogen was apparently not utilized by short-horned sculpin RBCs; however, if there was only a low rate of utilization this could have been missed against the high levels of glycogen. The high amount of glycogen in scorpioforme RBCs raises the question of when it is mobilized. It is unlikely that glycogen in RBCs is there to support anaerobic metabolism as oxygen levels in a RBC would rarely reach the point of compromising mitochondrial respiration. Glycogen may serve as a fuel reserve when the RBCs are deprived of whatever metabolite typically supports energy metabolism. Also perplexing is what fuels the synthesis of glycogen when rates of glucose disappearance from plasma are so low. It may be that there is a slow and continuous rate of glycogen synthesis from any glucose that is taken up and not catabolized; however, we cannot rule out the possibility that alternative fuels such as amino acids via gluconeogenesis are involved.

Conclusions

RBCs from both Atlantic cod and short-horned sculpin have an aerobic-based metabolism that is not impaired by decreasing the capacity for facilitated glucose transport by cytochalasin B. Thus these cells have the capacity to utilize other endogenous or plasma-borne fuels. Plasma glucose content in Atlantic cod was 3 mmol l^{-1}. In this species extracellular glucose can fully support aerobic metabolism and lactate production by RBCs, and about 70% of glucose enters by facilitated transport. Short-horned sculpin with a plasma glucose of 0.26 mmol l^{-1} presents a very different picture. Extracellular glucose makes a minor contribution to metabolism in satisfying only about 10% of energy demand and again, the sensitive radioisotope approach shows that a large percentage of glucose entry is via facilitated transport. We suggest that *in vivo* a variety of plasma-borne fuels, such as pyruvate, adenosine and amino acids could be called upon for oxidative metabolism.

The difference in metabolism between Atlantic cod and short-horned sculpin RBCs is most likely to be due to the plasma level of glucose. Low glucose levels have been noted previously for short-horned sculpin and do not increase above 1 mmol l^{-1} even under hypoxia (MacCormack and Driedzic, 2007; Driedzic et al., 2013). Glucose metabolism, by isolated RBCS from Atlantic cod and short-horned sculpin, determined by the radiometric method used here, rises sharply up to 1 mmol l^{-1} glucose and essentially achieves maximal rates at this concentration (Driedzic et al., 2013). Plasma glucose levels in Atlantic cod are sufficient to support the energetic demand of RBCs but not so in short-horned sculpin. Quite simply the use of glucose by short-horned sculpin RBCs is limited by the plasma concentration of 0.26 mmol l^{-1}. For unknown reasons, it is unlikely that blood glucose ever increases to a level required to support energy metabolism. Although by far in the minority a few other species of teleosts, such as Atlantic wolffish (*Anarhichas lupus*), ocean pout (*Macrozoarces americanus*) and monkfish (*Lophius americanus*) have blood glucose levels under 1 mmol l^{-1} (Chavin and Young, 1970). It would be interesting to assess the range of blood glucose levels in these species and whether RBC glucose metabolism is also limited.

The low level of blood glucose forces short-horned sculpin RBCs into an alternative metabolic strategy. Unlike other tissues, RBCs traverse the circulatory system and are exposed to an ever changing and complex milieu. For instance, it is possible that RBCs are fuelled by lactate during passage through white muscle and by amino acids during passage through the intestine. What fuels are used remains to be resolved and challenges us to design experiments to assess the dynamics of energy metabolism of RBCs.

MATERIALS AND METHODS

Animal collection and husbandry

Atlantic cod (*Gadus morhua*) were reared in a hatchery at the Ocean Sciences Centre, Memorial University of Newfoundland and received commercial pellets every day. Short-horned sculpin (*Myoxocephalus scorpius*) were collected locally as required by the field services unit (Ocean Sciences Centre) and were fed to satiation weekly with chopped herring. Body masses ranged from 1000 to 1500 g for Atlantic cod and from 500 to 1000 g for short-horned sculpin. Both species were maintained in running seawater at a temperature of 8–10°C and kept on a natural photoperiod with fluorescent lights set by an outdoor photocell. Animal protocols were approved by the Animal Care Committee at the Memorial University of Newfoundland.

Blood preparation

Approximately 1.25 ml of blood was removed from the caudal vessel of each fish with a heparinized syringe and transferred to a glass tube. For each experiment, blood from multiple fish was pooled. An aliquot was taken for haematocrit. A second aliquot was centrifuged at 1500 ***g*** for 5 min and the plasma assayed for glucose and lactate. The remainder of the whole blood was used for the measurements of O_2 consumption, lactate production, glucose utilization and glycogen levels. Experiments were conducted without (control) or with the addition of 25 μmol l^{-1} cytochalasin B. The blood was pre-incubated for 30 min with either cytochalasin B in dimethylsulphoxide (DMSO), or DMSO alone. All incubations started within 1–2 h from the time of blood collection and were at a temperature of 8°C. An incubation temperature of 8°C was selected as it falls within the range of the holding temperature of the fish.

O_2 consumption

Blood was drawn into a 1 cm^3 BD Glaspak tuberculin glass syringe and incubated in a shaking water bath at 8°C. Every 30 min the syringe was taken out of the water bath and gently agitated in order to redistribute the red blood cells. A Hamilton syringe was inserted into the end and 50 µl of blood was carefully withdrawn. The air at the end of the tuberculin syringe was pushed out, the syringe recapped and put back into the water bath. The blood in the Hamilton syringe was injected into a temperature-controlled (37°C) glass chamber housing an E101 oxygen electrode (Analytical Sensors Inc., Sugar Land, TX, USA) connected to an OM-200 oxygen meter (Cameron Instrument Company, Port Aransas, TX, USA). The chamber contained a degassed mixture of 6 g l^{-1} ferricyanide and 3 g l^{-1} saponin, which caused the release of hemoglobin-bound oxygen from the blood sample (Tucker, 1967). The change in partial pressure (P_{O_2}) was acquired with a MP100A-CE data acquisition system (BIOPAC Systems) and analysed using AcqKnowledge software (version 3.7.2, BIOPAC Systems). Oxygen concentration was calculated based on the oxygen solubility coefficient in the ferricyanide reagent α=0.02386 (Tucker, 1967). Oxygen consumption was determined from the linear regression of oxygen concentration versus time and expressed as nmol O_2 g^{-1} RBC min^{-1}. The mass of RBCs was calculated from the initial haematocrit value based on 1 ml RBC being equivalent to 1 g RBC.

Glucose metabolism

The metabolism of glucose was determined by measuring the rate of ^3H_2O production formed from [^{2}H-3]-glucose via water exchange in the reaction catalysed by phosphoglucose isomerase (Hutton, 1972). The protocol used here with whole blood was similar to that used to measure glucose metabolism in isolated RBCs (Driedzic et al., 2013). Whole blood (200 µl) was incubated in 16×100 mm glass tubes containing [2-^{3}H]-glucose (7.4 kBq; American Radiolabeled Chemicals, Burnaby, Canada) for 3 h at 8°C. Preliminary time course experiments showed a linear increase in glucose metabolism over 5 h. Radiolabelled glucose was added after the pre-incubation period; no additional glucose was added to the whole blood. After 3 h the sample was centrifuged at 12,000 ***g*** for 30 s, and the plasma was collected and frozen. RBC pellets were weighed and frozen for later determination of glycogen. The ^3H_2O produced during the incubation was separated from the [2-^{3}H]-glucose in the plasma by using chromatography as previously described (Driedzic et al., 2013). Background counts (time 0) were obtained by adding the label to a 200 µl aliquot of blood, spinning immediately and flash freezing the plasma. These counts were subtracted from the 3 h incubation.

Measurement of changes in blood glucose and lactate

Following pre-incubation, ~500–800 µl of blood was put into an 8°C shaking water bath for 300 min. At time 0, 60, 120, 180, 240 and 300 min, a 50 µl aliquot was taken and frozen in liquid nitrogen for glucose and lactate measurements. Glucose disappearance and lactate appearance were determined from the linear regression of concentrations versus time and expressed as nmol O_2 g^{-1} RBC min^{-1}, as described for oxygen consumption.

Glucose, lactate and glycogen analysis

Plasma glucose was assayed as described by Clow et al. (Clow et al., 2004). For the analysis of glucose and lactate in whole blood, samples were thawed and 200 µl of 6% perchloric acid (PCA) was added directly to the tube. Samples were spun at 12,000 ***g*** for 5 min and the supernatant was collected. Glucose was measured as described above for plasma. For lactate, 25–50 µl of extract was added to an assay medium containing glycine buffer (Sigma-Aldrich, G5418) and 2.5 mmol l^{-1} NAD$^+$ at pH 9.0. Samples were read at 340 nm before adding 40 IU ml^{-1} lactate dehydrogenase. Absorbances were then read for another 30 min or until stable. All absorbances were determined with a DTX 880 microplate reader (Beckman Coulter, Mississauga, ON, Canada).

Glycogen was measured in the RBCs obtained from the metabolism experiment. The method was based upon a procedure modified from Keppler and Decker (Keppler and Decker, 1974) using amyloglucosidase (EC 3.21.3) to hydrolyse glycogen. Briefly, RBCs were deproteinized in 12% PCA, centrifuged and neutralized with 2 mol l^{-1} potassium bicarbonate. The neutralized homogenate was added to 56 IU ml^{-1} amyloglucosidase in 0.4 mol l^{-1} acetate buffer at pH 4.8 and heated to 40°C. Hydrolysis was stopped after 2 h by adding 12% PCA. Glucose from these samples was measured as described above for plasma glucose. In addition, free glucose from these samples without hydrolysis was measured and subtracted from the glucose produced from glycogen.

Data analysis

Values obtained following incubation of blood were normalized to grams of RBCs. Intracellular glucose was calculated from the formula [whole blood glucose–plasma glucose (1–haematocrit)]/haematocrit (Pesquero et al., 1994). Values are expressed as means ± s.e.m. Comparisons between Atlantic cod and short-horned sculpin were made with an unpaired *t*-test. Comparisons between preparations within species were made with a paired *t*-test. In all cases, $P<0.05$ was considered to be significant.

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Competing interests
The authors declare no competing financial interests.

Author contributions
K.A.C. and C.E.S. were responsible for the execution of the studies and reviewing drafts of the manuscript. W.R.D. was responsible for experimental design, interpretation of the findings and drafting of the article.

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