

## RESEARCH ARTICLE

# Extracellular glucose supports lactate production but not aerobic metabolism in cardiomyocytes from both normoglycemic Atlantic cod and low glycemic short-horned sculpin

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## ABSTRACT

Fish exhibit a wide range of species-specific blood glucose levels. How this relates to glucose utilization is yet to be fully realized. Here, we assessed glucose transport and metabolism in myocytes isolated from Atlantic cod (*Gadus morhua*) and short-horned sculpin (*Myoxocephalus scorpius*), species with blood glucose levels of 3.7 and 0.57 mmol l<sup>-1</sup>, respectively. Glucose metabolism was assessed by the production of <sup>3</sup>H<sub>2</sub>O from [2-<sup>3</sup>H]glucose. Glucose metabolism was 3.5- to 6-fold higher by myocytes from Atlantic cod than by those from short-horned sculpin at the same level of extracellular glucose. In Atlantic cod myocytes, glucose metabolism displayed what appears to be a saturable component with respect to extracellular glucose, and cytochalasin B inhibited glucose metabolism. These features revealed a facilitated glucose diffusion mechanism that accounts for between 30% and 55% of glucose entry at physiological levels of extracellular glucose. Facilitated glucose diffusion appears to be minimal in myocytes for short-horned sculpin. Glucose entry by simple diffusion occurs in both cell types with the same linear relationship between glucose metabolism and extracellular glucose concentration, presumably due to similarities in membrane composition. Oxygen consumption by myocytes incubated in medium containing physiological levels of extracellular glucose (Atlantic cod 5 mmol l<sup>-1</sup>, short-horned sculpin 0.5 mmol l<sup>-1</sup>) was similar in the two species and was not decreased by cytochalasin B, suggesting that these cells have the capability of oxidizing alternative on-board metabolic fuels. Cells produced lactate at low rates but glycogen levels did not change during the incubation period. In cells from both species, glucose utilization assessed by both simple chemical analysis of glucose disappearance from the medium and <sup>3</sup>H<sub>2</sub>O production was half the rate of lactate production and as such extracellular glucose was not available for oxidative metabolism. Overall, extracellular glucose makes only a minor contribution to ATP production but a sustained glycolysis may be necessary to support Ca<sup>2+</sup> transport mechanisms at either the sarcoplasmic reticulum or the sarcolemmal membrane.

**KEY WORDS:** *Gadus morhua*, *Myoxocephalus scorpius*, ATP production, Cytochalasin B, Facilitated glucose diffusion, Glucose metabolism, Heart cells, Lactate production

## INTRODUCTION

Fish exhibit large species-specific variability in plasma glucose content, with most species having levels between 2 and 8 mmol l<sup>-1</sup>

but with a few species having exceptionally low levels by vertebrate standards (<1 mmol l<sup>-1</sup>) (Chavin and Young, 1970; Polakof et al., 2012). How extracellular glucose availability relates to glucose transport mechanisms and influences the use of glucose as a fuel for energy metabolism, especially in species with very low levels of blood glucose, is yet to be fully resolved. Glucose enters fish red blood cells (RBCs) by both facilitated transport and simple diffusive mechanisms (Driedzic et al., 2013). The steady-state rate of metabolism by RBCs from different species is proportional to extracellular physiological levels of glucose (Driedzic et al., 2013). For instance, glucose metabolism by Atlantic cod (*Gadus morhua* Linnaeus 1758) and short-horned sculpin [*Myoxocephalus scorpius* (Linnaeus 1758)] RBCs was 4.2 and 0.7 nmol g<sup>-1</sup> min<sup>-1</sup> at plasma glucose concentrations of 3.1 and 0.26 mmol l<sup>-1</sup>, respectively (Driedzic et al., 2014). Under conditions of adequate oxygenation, extracellular glucose utilization was sufficient to provide the sum of glycosyl equivalents to support aerobic metabolism and lactate production by Atlantic cod RBCs. In contrast, in RBCs from short-horned sculpin, extracellular glucose could account for lactate production but only 10% of the aerobic metabolic rate (Driedzic et al., 2014). Here, we extended the analysis to isolated heart cells from Atlantic cod and short-horned sculpin. It was anticipated that energy demand would be much higher in myocytes than in RBCs, leading to the following questions: what is the contribution of facilitated glucose transport to glucose entry?; how important is extracellular glucose to ATP production?; and are there differences between species at physiological levels of plasma glucose?

Glucose is considered to cross cell membranes by either simple diffusion down a concentration gradient or facilitated diffusion dependent upon transport proteins. In the latter case, a concentration gradient is still required for net glucose movement. In RBCs from Atlantic cod, Atlantic salmon (*Salmo salar*), cunner (*Tautoglabrus adspersus*) and short-horned sculpin, about 80% of glucose enters via facilitated diffusion. Facilitated glucose diffusion is important in fish hearts as well, where the demand for ATP is much higher. Under aerobic conditions, the uptake of 2-deoxyglucose is inhibited 50–80% by cytochalasin B or phloridzin in ventricle strips from American eel (*Anguilla rostrata*) (Rodnick et al., 1997), Atlantic cod (Clow et al., 2004) and rainbow trout (*Oncorhynchus mykiss*) (Becker et al., 2013). None of these species have exceptionally low levels of blood glucose. The relative importance of facilitated glucose transport in hearts from Atlantic cod, which has high levels of blood glucose, and short-horned sculpin, which has routinely low glucose levels, is one subject of this investigation. Our initial thinking was that facilitated glucose diffusion may be more important in short-horned sculpin cells where the concentration difference between the extracellular and intracellular space (i.e. the diffusion gradient) is lower.

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A second aspect of the current study relates to the relationship between the level of plasma glucose and rates of glucose metabolism. Studies to date lend only partial insight into the matter. Isolated heart preparations from brook trout (*Salvelinus fontinalis*), sea raven (*Hemiripertus americanus*) and rainbow trout can oxidize glucose to  $\text{CO}_2$  (Lanctin et al., 1980; Sephton et al., 1990; Milligan, 1991) and under aerobic conditions there is a low rate of lactate production in fish hearts, typically contributing approximately 5% of the total ATP production (Driedzic et al., 1983; Arthur et al., 1992; West et al., 1993; Overgaard et al., 2007; Lague et al., 2012; Speers-Roesch et al., 2013). But these studies do not reveal what proportion of the aerobic ATP production is supported by extracellular glucose. In isolated ventricle strips from rainbow trout, the rate of uptake of the glucose analog 2-deoxyglucose was similar to the rate of lactate efflux (Becker et al., 2013). As such, metabolism of extracellular glucose could account for all of the lactate produced with one-half of the available glucose still free to support an unknown percentage of aerobic metabolism. Sea raven have a plasma glucose level of approximately  $1 \text{ mmol l}^{-1}$  (Walsh et al., 1985). In isolated sea raven hearts, perfused with medium containing  $1 \text{ mmol l}^{-1}$  glucose, the utilization of extracellular glucose was  $1.6 \text{ nmol g}^{-1} \text{ min}^{-1}$ , based on the production of  $^{14}\text{CO}_2$  from  $[^{14}\text{C}]$ glucose. This rate of glucose utilization could support only 1.5% of the oxygen consumption ( $650 \text{ nmol g}^{-1} \text{ min}^{-1}$ ) (Sephton et al., 1990). However, the rate of glucose metabolism, and rates presented in other studies cited above, based on  $^{14}\text{CO}_2$  production, may be underestimates as the rate of oxidation was calculated from the specific activity of extracellular glucose and not the metabolites that actually yield  $\text{CO}_2$ . For instance, the specific activity of intracellular pyruvate, which is converted to acetyl coenzyme A plus  $\text{CO}_2$  via pyruvate dehydrogenase, is required to calculate the rate of  $[6\text{-}^{14}\text{C}]$  glucose oxidation; however, the specific activity of pyruvate may be considerably lower than that of extracellular glucose as a result of dilution of metabolites during passage through the glycolytic pathway. An alternative approach was applied with perfused, isolated rainbow trout hearts, in which glucose metabolism was assessed based on the release of  $^3\text{H}_2\text{O}$  from  $[5\text{-}^3\text{H}]$ glucose (West et al., 1993). The perfusion medium contained a physiological level of glucose at  $5 \text{ mmol l}^{-1}$ . In this procedure, at least in rat heart perfusions, the specific activity of the substrates that lead to  $^3\text{H}_2\text{O}$  release is about 80% that of extracellular glucose (Neely et al., 1972). In the case of rainbow trout, extracellular glucose utilization at  $121 \text{ nmol g}^{-1} \text{ min}^{-1}$  was the same as the rate of release of lactate, leaving  $60 \text{ nmol g}^{-1} \text{ min}^{-1}$  available to support 25% of the oxygen consumed ( $1468 \text{ nmol g}^{-1} \text{ min}^{-1}$ ). It is not known whether the difference between sea raven and rainbow trout hearts in the proportion of extracellular glucose available to support aerobic metabolism (1.5% versus 25%) is due to inherent properties of myocyte metabolism, different levels of plasma glucose, or different methodologies. The current experiments addressed these issues with the simultaneous measurement of oxygen consumption, lactate production, glycogen utilization and glucose metabolism in myocytes isolated from Atlantic cod and short-horned sculpin (i.e. representative species that have a large difference in plasma glucose level).

The overall objectives of the study were to assess the importance of facilitated glucose transport and the contribution of extracellular glucose to supporting aerobic metabolism and lactate production in fish hearts. A further novel aspect to the experiment was a comparison of short-horned sculpin, a species with extremely low plasma glucose levels by vertebrate standards, with Atlantic cod, a

species that has typical plasma glucose levels relative to other fish. The most salient findings are that for Atlantic cod, 25–50% of glucose entry is via facilitated glucose transport but in short-horned sculpin, glucose entry is primarily via simple diffusion, and essentially all of the extracellular glucose consumed by the heart in both species is directed to lactate production.

## MATERIALS AND METHODS

### Animal collection and husbandry

Hatchery-reared Atlantic cod (*G. morhua*) were obtained from the Joe Brown Aquatic Resource Building (Logy Bay, Newfoundland) and received commercial pellets daily as is the practice under aquacultured conditions. Short-horned sculpin (*M. scorpius*) were collected locally as required by the field services unit (Ocean Sciences Centre, Logy Bay) and were fed to satiation weekly with chopped herring. All animals appeared to be healthy and had blood glucose levels consistent with previous experiments. 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. Body mass was  $667 \pm 61$  and  $976 \pm 80 \text{ g}$  for Atlantic cod and short-horned sculpin, respectively. Atlantic cod were sampled the day after feeding and short-horned sculpin between 1 and 6 days after feeding. Food deprivation for up to 10 days does not alter blood glucose levels in Atlantic cod (Hall et al., 2009); similar data are not available for short-horned sculpin. On the day of the experiment, approximately 1 ml of blood was collected from the caudal vessel of the fish with a heparinized syringe and kept on ice until cell preparations were completed. Both Atlantic cod and short-horned sculpin were then killed with a sharp blow to the head and the hearts were quickly excised for the isolation of myocytes. Blood was then centrifuged at  $1500 \text{ g}$  for 5 min. Plasma was collected and frozen for future glucose analysis. Animal protocols were approved by the Animal Care Committee at the Memorial University of Newfoundland.

### Preparation of myocytes

Atlantic cod and short-horned sculpin myocytes were isolated by a method previously described for Atlantic cod (Hall et al., 2014) and rainbow trout (Shiels et al., 2000) with minor modifications. Hearts were perfused at 10°C with an isolating solution containing (in  $\text{mmol l}^{-1}$ ): 100 NaCl, 10 KCl, 1.2  $\text{KH}_2\text{PO}_4$ , 4  $\text{MgSO}_4$ , 30 taurine, 20 glucose and 10 HEPES, adjusted to pH 6.9. After 10–15 min, an enzyme solution containing  $0.75 \text{ mg ml}^{-1}$  collagenase (type 1A),  $0.5 \text{ mg ml}^{-1}$  trypsin (type IX.S) and  $0.75 \text{ mg ml}^{-1}$  BSA in the isolation solution was perfused through Atlantic cod hearts for 15–20 min and short-horned sculpin hearts for 45–55 min. Following enzymatic digestion, the atrium and bulbus arteriosus were removed from the ventricle; the ventricle was cut into small pieces and then gently agitated using the opening of a plastic Pasteur pipette. The isolated cells were passed through a  $150 \mu\text{m}$  filter and centrifuged at  $200 \text{ g}$  for 5 min in pre-weighed 2 ml microcentrifuge tubes. Cells were subsequently resuspended at 50 mg cell wet mass  $\text{ml}^{-1}$  in incubation medium containing ( $\text{mmol l}^{-1}$ ): 155 NaCl, 5 KCl, 1  $\text{NaH}_2\text{PO}_4$ , 2  $\text{MgSO}_4$ , 2  $\text{CaCl}_2$ , 10 HEPES and 5 (Atlantic cod) or 0.5 (short-horned sculpin) glucose, adjusted to pH 7.6. Initial experiments confirmed spindle-shaped myocytes and viability by dye exclusion. Linearity between various metabolic parameters and time provided evidence that preparations were viable for the course of the experiments. All discrete experiments utilized cells from the same individual or, in some experiments, cells pooled from two hearts.

## Experimental protocols

### Experiment 1: linearity of glucose metabolism and lactate production

The procedure for determining glucose metabolism in myocytes was similar to the protocol used to measure glucose metabolism in isolated RBCs (Driedzic et al., 2013): 200  $\mu\text{l}$  of a 50 mg cell wet mass  $\text{ml}^{-1}$  medium was incubated in 16 $\times$ 100 mm glass tubes containing [2- $^3\text{H}$ ]glucose (7.4 kBq; American Radiolabeled Chemicals, Burnaby, Canada). Atlantic cod cell suspensions containing 5  $\text{mmol l}^{-1}$  glucose (7.4 kBq  $\mu\text{mol}^{-1}$  glucose) and sculpin cell suspensions containing 1  $\text{mmol l}^{-1}$  glucose (37 kBq  $\mu\text{mol}^{-1}$  glucose) were incubated for 1, 2 and 3 h at 8°C. After each time point, the sample was spun at 10,000  $g$  for 30 s; the supernatant was collected and frozen. The  $^3\text{H}_2\text{O}$  produced from [2- $^3\text{H}$ ]glucose during the incubation was separated from the [2- $^3\text{H}$ ]glucose in the supernatant using chromatography as previously described (Driedzic et al., 2013). Fractions containing  $^3\text{H}_2\text{O}$  were counted using a Packard 2500TR liquid scintillation counter (PerkinElmer, Woodbridge, ON, Canada). Background counts were obtained by adding the label to a 200  $\mu\text{l}$  aliquot of cell suspension, spinning immediately and flash freezing the supernatant. These counts were subtracted from all time points.

Lactate was measured from the supernatant collected after each time point. The supernatant was deproteinized 1:1 with 6% perchloric acid and 25  $\mu\text{l}$  of this extract was added to an assay medium containing glycine buffer (Sigma, G5418) and 2.5  $\text{mmol l}^{-1}$   $\text{NAD}^+$ , pH 9.0. Absorbance was determined at 340 nm using a DTX 880 microplate reader (Beckman Coulter, Mississauga, ON, Canada) before the addition of 40 IU  $\text{ml}^{-1}$  lactate dehydrogenase. Absorbance was then read for another 30 min or until stable.

### Experiment 2: relationship between extracellular glucose and glucose metabolism and the effect of cytochalasin B

Glucose metabolism by Atlantic cod and short-horned sculpin myocytes was assessed at extracellular glucose concentrations of 0.3, 0.625, 1.25, 2.5, 5, 10, 15, 20  $\text{mmol l}^{-1}$  in the incubation medium after 2 h at 8°C. The specific activity ranged from 123 to 1.85 kBq  $\mu\text{mol}^{-1}$  glucose. After 2 h, the incubation was terminated and glucose metabolism was measured as described in experiment 1. Background counts were obtained as described above except the cell suspension contained 20  $\text{mmol l}^{-1}$  of extracellular glucose.

The effect of 25  $\mu\text{mol l}^{-1}$  cytochalasin B, a glucose transporter inhibitor, was tested. Cytochalasin B was added 30 min prior to the addition of [2- $^3\text{H}$ ]glucose, to either Atlantic cod or short-horned sculpin myocyte suspension containing 0.5, 2.5, 5 or 10  $\text{mmol l}^{-1}$  extracellular glucose. As cytochalasin B was dissolved in 0.33% DMSO, cells were also incubated with DMSO in incubation medium containing 0.5 and 10  $\text{mmol l}^{-1}$  glucose to assess whether DMSO impaired metabolism.

### Experiment 3: oxygen consumption, lactate production, glucose disappearance, glucose metabolism and glycogen utilization

All metabolic measurements in this experiment were performed with myocytes incubated either with or without 25  $\mu\text{mol l}^{-1}$  cytochalasin B. All Atlantic cod cell suspensions contained 5  $\text{mmol l}^{-1}$  glucose and all short-horned sculpin cell suspensions contained 0.5  $\text{mmol l}^{-1}$  glucose.

A sample of the myocytes was made up to a concentration of 50 mg cell wet mass  $\text{ml}^{-1}$  and allowed to rest on ice for approximately 2 h. After 2 h, 25  $\mu\text{mol l}^{-1}$  cytochalasin B was added to one aliquot of cells and incubated in an 8°C water bath with another aliquot containing no cytochalasin B (control). Thirty minutes later, approximately 2 ml of each cell suspension was put

into two separate temperature-controlled (8°C) closed glass chambers housing E101 oxygen electrodes (Analytical Sensors Inc., Sugar Land, TX, USA) connected to an OM-200 oxygen meter (Cameron Instrument Company, Port Aransas, TX, USA). The change in partial pressure ( $P_{\text{O}_2}$ ) was acquired with a MP100A-CE data acquisition system (BIOPAC Systems) and analyzed using AcqKnowledge software (v3.7.2, BIOPAC Systems). The rate of oxygen consumption ( $\dot{M}_{\text{O}_2}$ ) was expressed as  $\text{nmol O}_2 \text{ g}^{-1} \text{ min}^{-1}$  based on the oxygen solubility coefficient in the reagent  $\alpha=0.05234$  (Graham, 1987). Preliminary results showed that using a cell concentration of 50 mg cell wet mass  $\text{ml}^{-1}$  was ideal for both species as oxygen decline was neither too fast nor too slow. Resting periods up to 1–1.5 h produced steep and inconsistent slopes but cells that were rested between 2 and 6 h had similar  $\dot{M}_{\text{O}_2}$ .

Glucose disappearance and lactate production were determined by incubating approximately 700  $\mu\text{l}$  of 200 mg cell wet mass  $\text{ml}^{-1}$  cell suspension with or without 25  $\mu\text{mol l}^{-1}$  cytochalasin B in an 8°C shaking water bath for 240 min. At time 0, 30, 60, 120, 180 and 240 min, a 100  $\mu\text{l}$  aliquot was taken and frozen in liquid nitrogen for later analysis of glucose and lactate. Glucose was measured as previously described by Clow et al. (2004) and lactate as described above. Glucose disappearance and lactate appearance were determined from the linear regression of concentration versus time.

Glucose metabolism was measured by the same method described in experiment 1 except only background (i.e. time=0) and 2 h time points were performed. Glycogen was measured in myocytes obtained from the pellet collected in the metabolism protocol. The method was based upon a procedure modified from Keppler and Decker (1974) using amyloglucosidase (EC 3.2.1.3) to hydrolyse glycogen. Briefly, myocyte pellets were deproteinized in 12% perchloric acid, centrifuged and neutralized with 2  $\text{mol l}^{-1}$  potassium bicarbonate. The neutralized homogenate was added to 56 IU  $\text{ml}^{-1}$  amyloglucosidase in 0.4  $\text{mol l}^{-1}$  acetate buffer, pH 4.8, and heated at 40°C. Hydrolysis was stopped after 2 h by adding 12% perchloric acid. Glucose from these samples was measured as described by Clow et al. (2004). Free glucose from these samples without hydrolysis was also measured and subtracted from the glucose produced from glycogen.

### Experiment 4: effect of etomoxir on $\dot{M}_{\text{O}_2}$ and glucose metabolism

After resting for 2 h, 50 mg cell wet mass  $\text{ml}^{-1}$  cod myocytes was added to closed  $\text{O}_2$  chambers as described above and oxygen consumption was recorded. Once the decrease in  $\text{O}_2$  was steady, 1  $\text{mmol l}^{-1}$  etomoxir, an inhibitor of fatty acid metabolism, was added to one chamber and water (vehicle) was added to the other. To determine the effect of this inhibitor on glucose metabolism, 1  $\text{mmol l}^{-1}$  etomoxir was added to myocyte incubations as in experiment 1 but without a pre-incubation period. Only background and 2 h time points were taken.

### Data analysis

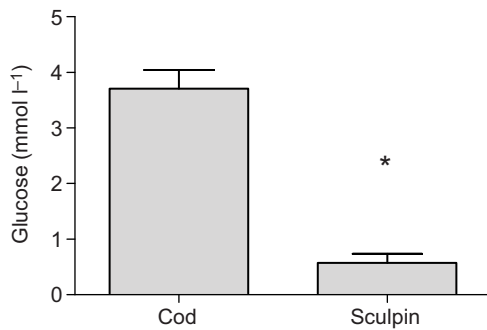
Values are expressed as means $\pm$ s.e.m. Statistical analyses applied are stated in the legends to the figures. In all cases  $P<0.05$  was considered to be significant.

### RESULTS

Plasma glucose levels were significantly higher (6.5-fold) in Atlantic cod ( $3.7\pm 0.34 \text{ mmol l}^{-1}$ ) than in short-horned sculpin ( $0.57\pm 0.16 \text{ mmol l}^{-1}$ ) (Fig. 1).

### Validation of methodology to determine glucose metabolism

The objective of experiment 1 was to assess whether isolated myocytes incubated with [2- $^3\text{H}$ ]glucose in the bathing medium



**Fig. 1. Plasma glucose levels in Atlantic cod and short-horned sculpin.** Means and s.e.m.,  $N=29$  for Atlantic cod and  $N=27$  for short-horned sculpin. \*Significant difference following a  $t$ -test between Atlantic cod and short-horned sculpin.

produced  $^3\text{H}_2\text{O}$  in a linear fashion with respect to time. Glucose metabolism was linear for at least 3 h, as was the production of lactate (Fig. 2). On the basis of this information, all further experiments involving  $[2\text{-}^3\text{H}]\text{glucose}$  utilized an incubation time of 2 h. In these experiments, the rate of glucose metabolism based on the slope of the curves was significantly higher (5.5-fold) in myocytes from Atlantic cod ( $21 \pm 2.8 \text{ nmol g}^{-1} \text{ min}^{-1}$ ;  $y=1.28x-0.13$ ,  $r^2=0.78$ ) than from short-horned sculpin ( $3.8 \pm 0.7 \text{ nmol g}^{-1} \text{ min}^{-1}$ ;  $y=0.23x+0.2$ ,  $r^2=0.63$ ). The rate of lactate production by myocytes from Atlantic cod was  $33 \pm 3.8 \text{ nmol g}^{-1} \text{ min}^{-1}$  ( $y=2.0x+0.78$ ,  $r^2=0.71$ ) and that by short-horned sculpin myocytes was  $23 \pm 11.6 \text{ nmol g}^{-1} \text{ min}^{-1}$  ( $y=1.39x+4.38$ ,  $r^2=0.18$ ). The slopes of the curves were not significantly different. For myocytes from Atlantic cod, the rate of lactate production was 1.6 times the rate of glucose metabolism but for short-horned sculpin myocytes, the rate of lactate production was 6 times the rate of extracellular glucose metabolism.

#### Assessment of the importance of facilitated glucose transport

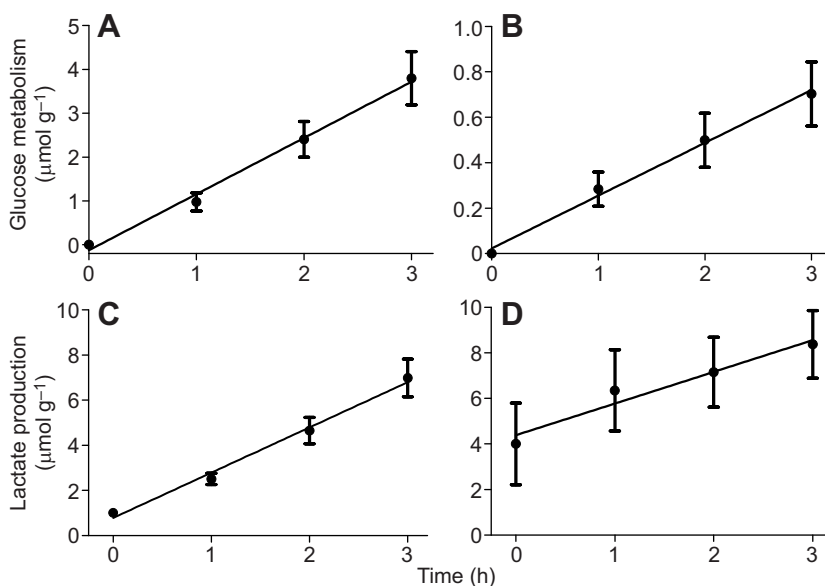
The objective of experiment 2 was to gain insight into the importance of facilitated glucose diffusion. This was first assessed by determining the relationship between glucose metabolism and extracellular glucose based on the rationale that a leveling out of the

rate of glucose metabolism would suggest saturation of a transport mechanism. There appeared to be a biphasic relationship for myocytes from Atlantic cod, with a hyperbolic component up to approximately  $5 \text{ mmol l}^{-1}$  glucose and a linear relationship thereafter (Fig. 3A). The relationship for short-horned sculpin is more difficult to interpret as a linear curve could fit the data points from the lowest to the highest level of extracellular glucose (Fig. 3B). Fig. 3C shows the linear relationship between glucose metabolism and extracellular glucose over an arbitrarily selected concentration range from  $2.5$  to  $20 \text{ mmol l}^{-1}$  (i.e. at the higher end of the concentration range). The slopes of the linear regressions were not significantly different between Atlantic cod ( $y=0.84x+24.8$ ,  $r^2=0.29$ ) and short-horned sculpin ( $y=0.73x+2.9$ ,  $r^2=0.51$ ).

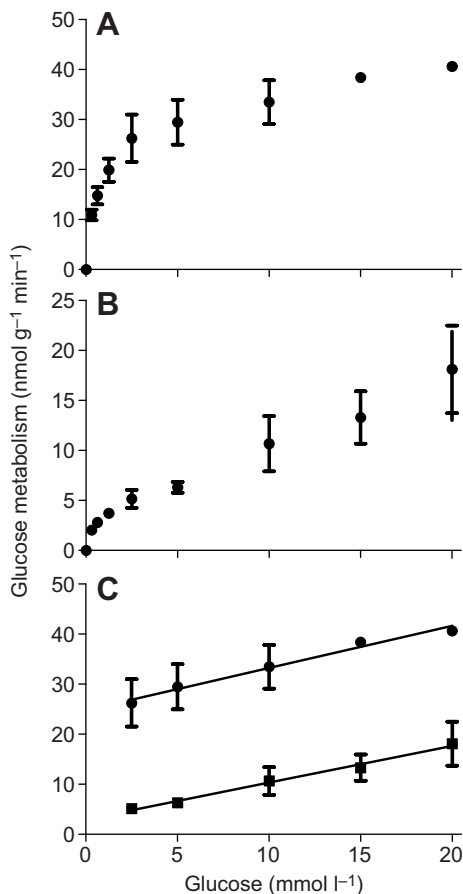
To further assess the importance of facilitated glucose diffusion, the impact of cytochalasin B, a well-recognized inhibitor of glucose transport, was determined. The addition of DMSO alone to the incubation medium had no effect on glucose metabolism (Fig. 4). Cytochalasin B significantly inhibited glucose metabolism by myocytes from Atlantic cod at extracellular glucose levels of  $2.5 \text{ mmol l}^{-1}$  and above (Fig. 4A). At  $2.5$  and  $5 \text{ mmol l}^{-1}$  glucose (i.e. close to physiological plasma level), glucose metabolism was inhibited by 43% and 30%, respectively. Glucose metabolism by myocytes from short-horned sculpin was significantly inhibited only at  $10 \text{ mmol l}^{-1}$  glucose, a level well above physiological concentrations.

#### Metabolic and glucose budget

Oxygen consumption, lactate production, glucose disappearance from the incubation medium, glucose metabolism (assessed from  $^3\text{H}_2\text{O}$  production) and glycogen levels were subsequently determined in the same cell preparations at physiological levels of extracellular glucose (experiment 3). The intent was to establish a metabolic budget that would allow determination of the importance of extracellular glucose as a metabolic fuel. In addition, preparations were treated with cytochalasin B to further address the issue of facilitated glucose transport. Oxygen consumption was linear with respect to time, with oxygen typically being decreased to close to zero in closed systems in approximately 80 min. Fig. 5A,B shows representative traces for myocytes from both species. There was no significant difference in  $\dot{M}_{\text{O}_2}$  by myocytes from the two species



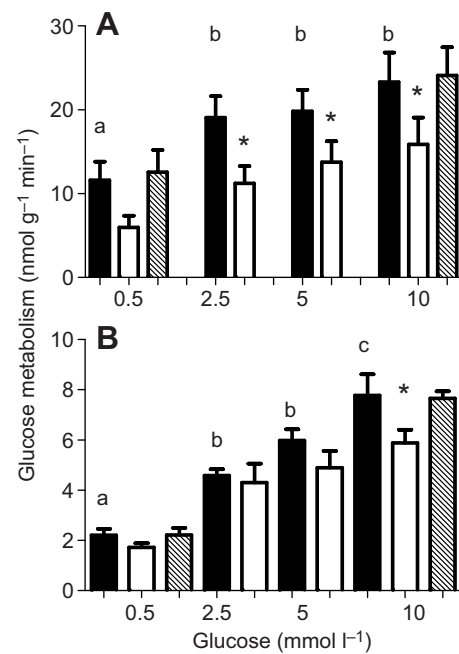
**Fig. 2. Glucose metabolism and lactate production by isolated myocytes.** Myocytes were incubated in  $5 \text{ mmol l}^{-1}$  glucose (Atlantic cod; A,C) or  $1 \text{ mmol l}^{-1}$  glucose (short-horned sculpin; B,D) for 3 h at  $8^\circ\text{C}$ . (A,B) Glucose metabolism, measured by  $^3\text{H}_2\text{O}$  production from  $[2\text{-}^3\text{H}]\text{glucose}$ . (C,D) Lactate production.  $N=8$  for Atlantic cod;  $N=4$  for short-horned sculpin.



**Fig. 3. Relationship between rate of glucose metabolism by myocytes and extracellular glucose concentration.** Myocytes were incubated for 2 h at 8°C in extracellular glucose, and glucose metabolism was measured by <sup>3</sup>H<sub>2</sub>O production from [2-<sup>3</sup>H]glucose. (A,B) Glucose metabolism for Atlantic cod (A) and short-horned sculpin (B) up to 20 mmol l<sup>-1</sup> extracellular glucose. (C) Glucose metabolism between 2.5 and 20 mmol l<sup>-1</sup> extracellular glucose for Atlantic cod (circles) and short-horned sculpin (squares). *N*=4 for all time points except Atlantic cod at 15 and 20 mmol l<sup>-1</sup>, where *N*=2.

either with or without cytochalasin B (Fig. 5C). Cytochalasin B had no effect upon  $\dot{M}_{O_2}$  in cells within species. Lactate production was calculated from individual preparations based on regression equations including at least 5 time points. Lactate production was significantly higher in myocytes from Atlantic cod than from short-horned sculpin in both control and cytochalasin B-treated preparations. Cytochalasin B had no effect upon lactate production in cells within species.

The rate of glucose disappearance from the incubation medium was typically linear with respect to time. Representative traces are shown for Atlantic cod (Fig. 6A) and short-horned sculpin myocytes (Fig. 6B). Glucose disappearance was calculated from individual preparations based on regression equations including at least 5 time points. The rate of glucose disappearance for myocytes from control preparations of Atlantic cod was significantly (2.9-fold) greater than that for cells from short-horned sculpin (Fig. 6C). There was no significant impact of cytochalasin B on the rate of glucose disappearance. Glucose metabolism determined from the production of <sup>3</sup>H<sub>2</sub>O was significantly (3.8-fold) higher by myocytes from control preparations of Atlantic cod than of short-horned sculpin (Fig. 6D). Cytochalasin B treatment resulted in a significant decrease in glucose metabolism by myocytes from Atlantic cod (55%) but not for short-horned sculpin.



**Fig. 4. Effect of cytochalasin B on glucose metabolism by myocytes at different extracellular glucose concentrations.** (A) Atlantic cod; (B) short-horned sculpin. Filled bars, control; open bars, 25 μmol l<sup>-1</sup> cytochalasin B in DMSO; hatched bars, DMSO. *N*=4 for both species. Data were analyzed by one-way repeated measures ANOVA followed by Tukey's multiple comparison test. Lowercase letters refer to the effect of different levels of extracellular glucose in control preparations; values having different letters are significantly different. \*Significant difference between control and cytochalasin B treatments at the same concentration of glucose.

Initial glycogen levels were significantly (13-fold) higher in myocytes from short-horned sculpin than from Atlantic cod (Fig. 7). There was no apparent decrease in glycogen level following 2 h of incubation for myocytes from either species. Furthermore, there was no significant change in final glycogen level when cells were incubated in the presence of cytochalasin B.

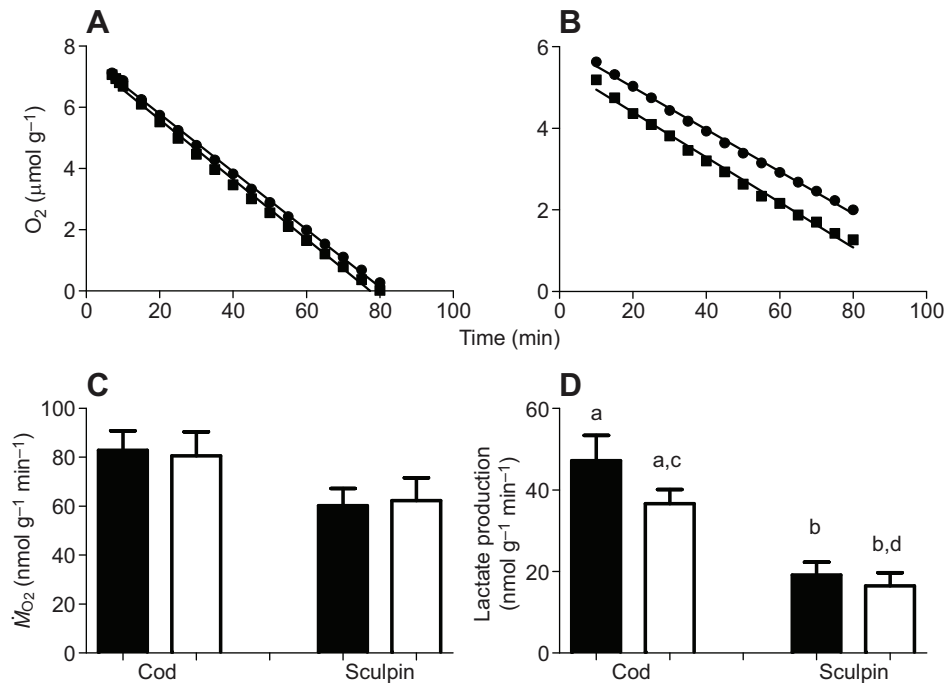
#### Inhibition of lipid metabolism

Experiment 3 showed that although cytochalasin B inhibited glucose metabolism in myocytes from Atlantic cod, there was no impact upon oxygen consumption or glycogen utilization. This suggests that either glucose is not used as an aerobic metabolic fuel or that in the presence of cytochalasin B an on-board metabolic fuel other than glycogen is called upon to make up the shortfall in providing NADH to the electron transport chain. The objective of experiment 4 was to indirectly assess whether stored lipids were being utilized by pharmacologically inhibiting fatty acid breakdown. Treatment of myocytes from Atlantic cod with etomoxir did not result in a change in oxygen consumption (Fig. 8A); however, there was a small but significant increase in glucose metabolism (Fig. 8B).

#### DISCUSSION

##### Glucose levels

As anticipated, plasma glucose levels in Atlantic cod (3.7 mmol l<sup>-1</sup>) were higher than in short-horned sculpin (0.57 mmol l<sup>-1</sup>). These values are based on blood sampled from caudal venous vessels and in the absence of further information are assumed to be the same as levels that would ultimately nourish the compact layer of the heart. This contention, though, remains to be tested.



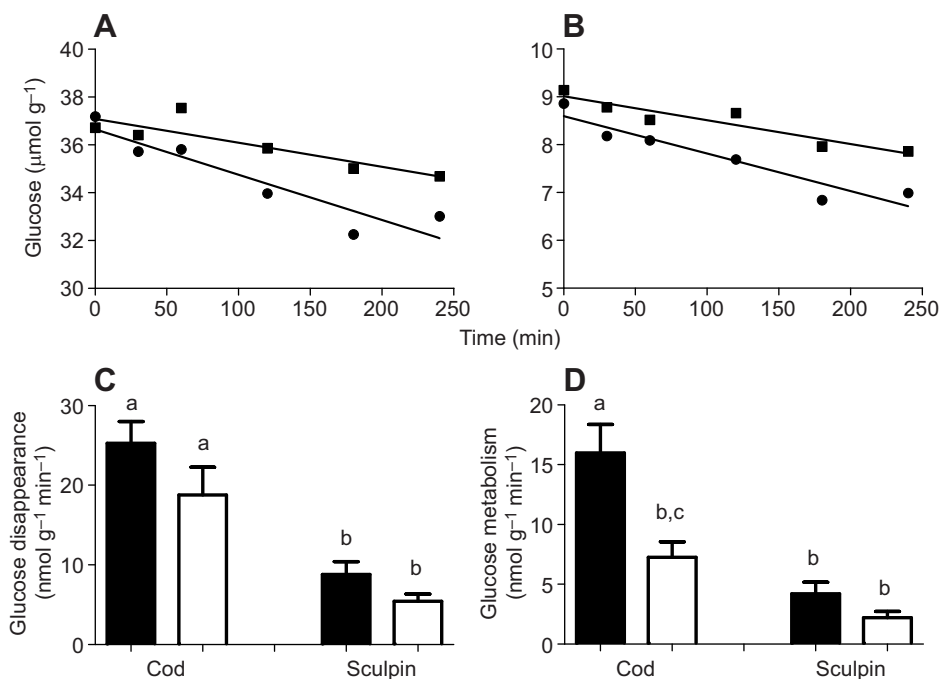
**Fig. 5. Oxygen consumption and lactate production by isolated myocytes.** Atlantic cod myocytes were incubated in  $5 \text{ mmol l}^{-1}$  glucose and short-horned sculpin in  $0.5 \text{ mmol l}^{-1}$  glucose in medium. Experiments were conducted at  $8^\circ\text{C}$ . (A,B) Examples of the relationship between oxygen content and time for Atlantic cod (A) and short-horned sculpin (B) myocytes incubated in closed chambers. Oxygen level in the medium was normalized to grams wet mass of cells. Circles, control; squares, cytochalasin B. (C) Oxygen consumption ( $\dot{M}_{\text{O}_2}$ ;  $N=6$  for both species). (D) Lactate production ( $N=7$  for both species). For C and D, filled bars, control; open bars, cytochalasin B. Statistically significant differences between species were assessed with a two-way ANOVA and the impact of cytochalasin B treatment with repeated measures two-way ANOVA with the Bonferroni post-test applied in both cases. Values sharing a letter are not significantly different.

In experiments designed to assess the linearity of glucose metabolism and lactate production (i.e. experiment 1) and for the determination of multiple parameters (i.e. experiments 3 and 4), the level of extracellular glucose for Atlantic cod myocytes was set to  $5 \text{ mmol l}^{-1}$  and for short-horned sculpin myocytes to either  $1 \text{ mmol l}^{-1}$  (experiment 1) or  $0.5 \text{ mmol l}^{-1}$  (experiments 3 and 4). The concentrations used in these studies were based on anticipated levels from earlier experiments involving fish from the same populations (MacCormack and Driedzic, 2007; Hall et al., 2009; Driedzic et al., 2013, 2014). Although the mean glucose level of plasma for the Atlantic cod sampled here was lower than  $5 \text{ mmol l}^{-1}$ , four of the 29 specimens had plasma glucose levels of  $5 \text{ mmol l}^{-1}$  or higher; as such, the experimentally controlled

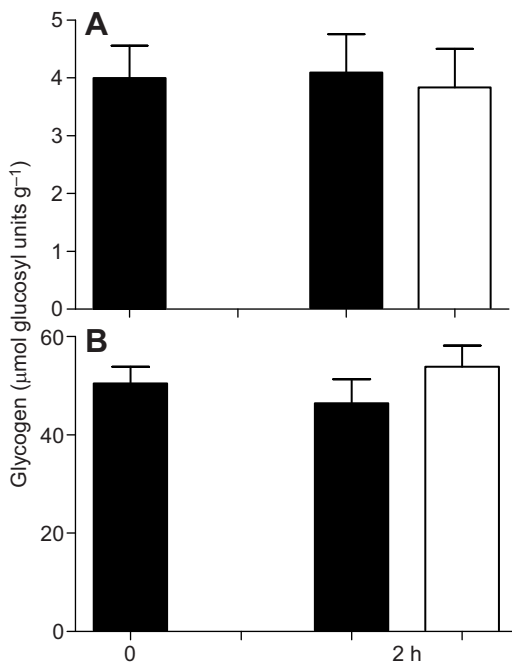
concentration was within the physiological range. The concentration of  $0.5$  or  $1 \text{ mmol l}^{-1}$  used for the incubation of myocytes from short-horned sculpin was also within the physiological window. The significant difference between plasma glucose levels in Atlantic cod and short-horned sculpin sets the stage for analysis of whether the species-specific concentration of extracellular glucose is an important determinant of cardiac energy metabolism.

#### Methodology

The experiments utilized isolated cell preparations as this approach allows time course measurements and multiple trial conditions with the same population of cells. In some experiments, glucose



**Fig. 6. Glucose disappearance from medium and glucose metabolism.** Atlantic cod myocytes were incubated with  $5 \text{ mmol l}^{-1}$  glucose and short-horned sculpin with  $0.5 \text{ mmol l}^{-1}$  glucose in medium. Experiments were conducted at  $8^\circ\text{C}$ . (A,B) Examples of the relationship between glucose disappearance and time for Atlantic cod (A) and short-horned sculpin (B). Circles, control; squares, cytochalasin B. (C) Glucose disappearance ( $N=7$  for both species). (D) Glucose metabolism, measured by  $^3\text{H}_2\text{O}$  production from  $[2\text{-}^3\text{H}]\text{glucose}$  ( $N=7$  for Atlantic cod;  $N=5$  for short-horned sculpin). For C and D, filled bars, control; open bars, cytochalasin B. Statistical significance was assessed as described for Fig. 5. Values sharing a letter are not significantly different.

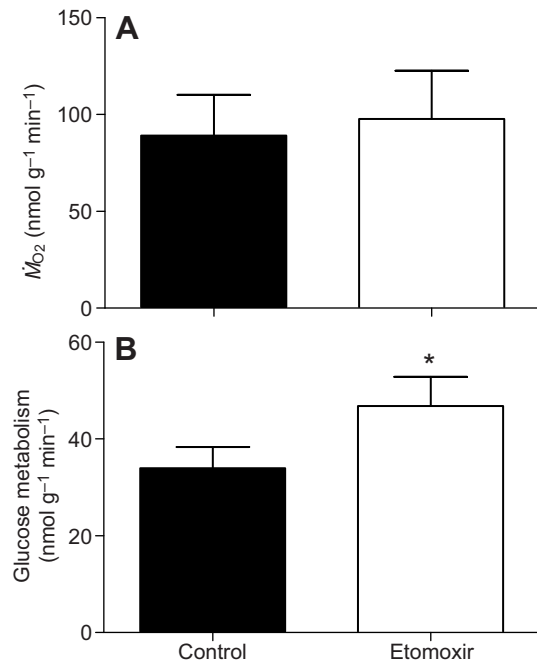


**Fig. 7. Glycogen levels in myocytes at time zero and following 2 h incubation at 8°C.** (A) Atlantic cod; (B) short-horned sculpin. Filled bars, control; open bars, cytochalasin B.  $N=6$  (with one preparation consisting of myocytes from two animals) for Atlantic cod;  $N=4$  (with one preparation consisting of myocytes from two animals) for short-horned sculpin. Statistical significance was assessed with a one-way repeated measures ANOVA.

disappearance from the incubation medium was measured; in all experiments, steady-state glucose metabolism was assessed by tracking  $^3\text{H}_2\text{O}$  production from  $[\text{^3H}]\text{glucose}$ . The latter approach was used by West et al. (1993) with rainbow trout heart preparations and more recently was shown to be a robust technique with RBCs from fish (Driedzic et al., 2013, 2014). The production of  $^3\text{H}_2\text{O}$  from  $[\text{2-}^3\text{H}]\text{glucose}$  is here shown to be linear for at least 3 h with isolated heart cells. The merits of this procedure are (i) it minimizes the problem of dilution of the radioisotope in commonly used  $^{14}\text{CO}_2$  studies in that the specific activity of glucose 6-phosphate that releases the labeled water is probably close to that of extracellular glucose (Neely et al., 1972), (ii) it provides a measure of the steady-state rate of glycolysis as opposed to 2-deoxyglucose, which gives the initial rate of uptake of a glucose analog that is not metabolized past glucose 6-phosphate and eventually equilibrates with the extracellular isotope, and (iii) it is more accurate than measurements of disappearance of glucose especially when small changes are measured against a large background. Furthermore, changes in extracellular glucose do not give a rate of glycolysis, per se, as glucose may be incorporated into other cellular constituents. As such, we have confidence that the methodology of tracking  $^3\text{H}_2\text{O}$  production provides a sound reflection of the steady-state rate of glucose metabolism leading to either  $\text{CO}_2$  or lactate production.

#### How important is facilitated glucose diffusion?

The importance of facilitated glucose transport was first probed by determining the relationship between glucose metabolism and extracellular glucose concentration. The rationale for this approach is that facilitated transport should saturate at high levels of extracellular glucose. For myocytes from Atlantic cod, at concentrations of glucose below approximately  $5 \text{ mmol l}^{-1}$  there is a hyperbolic relationship between the rate of steady-state metabolism of glucose and



**Fig. 8. Oxygen consumption and glucose metabolism by isolated Atlantic myocytes incubated with or without  $1 \text{ mmol l}^{-1}$  etomoxir.** (A) Oxygen consumption ( $N=7$ ). (B) Glucose metabolism ( $N=8$ ). Cells were incubated in medium containing  $5 \text{ mmol l}^{-1}$  glucose. \*Significant difference following a  $t$ -test between control and etomoxir treatment.

extracellular glucose concentration, suggesting a facilitated transport component, although the relationship could also be explained by the availability of substrate to enzyme-catalyzed reactions in glycolysis. The curve for myocytes from short-horned sculpin is less clear as the relationship between glucose metabolism and extracellular glucose level could be defined by a straight-line relationship. Myocytes from both Atlantic cod and short-horned sculpin showed a linear relationship between steady-state metabolism of glucose and extracellular glucose concentrations above  $2.5 \text{ mmol l}^{-1}$  with the same slope implying a simple diffusive process that is fundamental to both preparations and presumably related to similar membrane compositions.

Further insight into the contribution of facilitated glucose diffusion was obtained with cytochalasin B, a cell-permeable fungal metabolite that inhibits glucose transport by binding to glucose transporters on the inside and inhibiting glucose exit (Basketter and Widdas, 1978). Cytochalasin B has been shown to inhibit glucose transport in a variety of mammalian tissues (Silverman, 1991), including mammalian heart (Ramasamy et al., 2001) and heart from other species of fish (Rodnick et al., 1997; Becker et al., 2013). Regardless, we cannot rule out secondary target effects of the pharmacological treatment as cytochalasin B binds to actin filaments (MacLean-Fletcher and Pollard, 1980). In perfused rat heart, cytochalasin B does not influence systolic or diastolic function, suggesting that an indirect effect on contractility of the myocytes in the current experiment may not be a problem. Nevertheless, interpretation of cytochalasin B effects in the current study must be viewed with this caveat. Cytochalasin B significantly inhibited glucose metabolism in Atlantic cod myocytes at physiological levels of glucose concentration for this species. This finding is similar to that obtained with the inhibition of 2-deoxyglucose uptake in non-contracting ventricle strips from eel, Atlantic cod and rainbow trout under conditions of aerobic metabolism (Rodnick et al.,

1997; Clow et al., 2004; Becker et al., 2013). Inhibition of glucose metabolism by cytochalasin B in short-horned sculpin myocytes was observed only under conditions of 10 mmol l<sup>-1</sup> extracellular glucose, a level 20-fold higher than physiological concentrations.

Together, the glucose concentration and the cytochalasin B experiments provide evidence that a portion of glucose entry into Atlantic cod myocytes is via facilitated diffusion. At physiological levels of glucose, facilitated diffusion may account for 30–55% of the glucose entry. This interpretation is consistent with the presence of glucose transporter transcripts in Atlantic cod heart where GLUT1 is highly expressed relative to levels in many other tissues and accounts for 96% of the class 1 GLUTs (Hall et al., 2014). Although the levels were not quantified, Teerijoki et al. (2000) also report abundant GLUT1 transcript in rainbow trout heart relative to other tissues. In contrast, GLUT4 is absent or poorly expressed in the heart of Atlantic cod (Hall et al., 2014) and brown trout (*Salmo trutta*) (Planas et al., 2000). It appears that, at least in the heart of Atlantic cod and rainbow trout, facilitated glucose diffusion via GLUT1 is important in glucose trafficking.

The situation in short-horned sculpin heart is not as clear. We found no convincing evidence of a facilitative diffusive component to glucose metabolism at the low physiological levels of glucose characteristic of this species. Myocytes from both Atlantic cod and short-horned sculpin exhibited a simple diffusive component in glucose trafficking. The rate of glucose metabolism at the same extracellular glucose concentration was higher for Atlantic cod than for short-horned sculpin myocytes. Given that the simple diffusive component is similar, it appears that the difference may be due to a more active facilitated diffusion process in Atlantic cod than in short-horned sculpin heart cells. This interpretation is contrary to an anticipated higher affinity for glucose by short-horned sculpin than for Atlantic cod myocytes in concert with lower levels of glucose in the former species. To our knowledge, information at the gene and transcript level of GLUTs is not available for short-horned sculpin, a fish that has exceptionally low levels of blood glucose. It would be of great interest to determine whether GLUTs are present in heart and other tissues.

### Contribution of extracellular glucose to aerobic metabolism and lactate production

Oxygen consumption, lactate production, glycogen levels and glucose disappearance/metabolism were all determined in the same population of cells at physiological levels of extracellular glucose. Rates of oxygen consumption were similar in Atlantic cod and short-horned sculpin myocytes. This is not surprising given the cells are non-contracting and require only low level basal metabolic rates. Lactate production, though, was significantly higher in Atlantic cod heart cells, consistent with higher rates of glucose metabolism. Glycogen levels in short-horned sculpin myocytes were approximately 10-fold higher than in Atlantic cod heart cells. A similar relationship is found in RBCs from these species, although the absolute levels of glycogen are about 10-fold lower (Driedzic et al., 2014). The source of glycogen, potentially plasma glucose or amino acids, is not known. Glycogen levels in myocytes did not change during the incubation period; as such, all of the lactate produced must have been derived from exogenous glucose. Based on a P:O ratio of 2.5 and a yield of 1 ATP per lactate, aerobic respiration would account for 90% of ATP production for Atlantic cod and 94% for short-horned sculpin myocytes. This is similar to the relationship in perfused sea raven, rainbow trout and tilapia hearts (Driedzic et al., 1983; Arthur et al., 1992; West et al., 1993; Speers-Roesch et al., 2013). Not surprisingly, glycolysis leading to

lactate production makes only a minimal contribution to ATP production under normoxic conditions.

It is possible to assess how much of the extracellular glucose is required to support lactate production. In the case of Atlantic cod myocytes, lactate was produced at a rate of 47 nmol g<sup>-1</sup> min<sup>-1</sup>. Glucose disappeared from the bathing medium at a rate of 25 nmol g<sup>-1</sup> min<sup>-1</sup>. This value is probably somewhat of an overestimate of the rate of glycolysis as a portion of the glucose may be incorporated into other cellular constituents or simply bound to protein. Glucose metabolism assessed from <sup>3</sup>H<sub>2</sub>O production yielded a rate of 16 nmol g<sup>-1</sup> min<sup>-1</sup>. This value will be somewhat of an underestimate because of dilution of the glucose 6-phosphate pool. Accepting the rat heart value of glucose 6-phosphate having a specific activity of 80% of extracellular glucose (Neely et al., 1972), the true rate of glucose metabolism would be closer to 20 nmol g<sup>-1</sup> min<sup>-1</sup>. The two approaches lead to a similar conclusion in that the rate of glucose utilization approximates half the rate of lactate production. That is, all of the glucose is converted to lactate with none remaining for oxidative metabolism. A similar situation occurs for short-horned sculpin myocytes, where the rate of lactate production was 19 nmol g<sup>-1</sup> min<sup>-1</sup>. Glucose disappeared from the medium at a rate of 8.8 nmol g<sup>-1</sup> min<sup>-1</sup>; glucose metabolism assessed from <sup>3</sup>H<sub>2</sub>O production was 4.2 nmol g<sup>-1</sup> min<sup>-1</sup> and again, rounding up because of isotope dilution, the true rate is probably closer to 5.3 nmol g<sup>-1</sup> min<sup>-1</sup>. Once more, the data reveal that all of the extracellular glucose would be required to support lactate production. This finding is consistent with the absolute lack of any impact of cytochalasin B on the oxygen consumption of myocytes from either experimental species. Although lactate production plays only a small role in ATP provision, it places a huge demand on the rate of utilization of the extracellular glucose pool.

A corollary to the above viewpoints is that cytochalasin B should inhibit lactate production by Atlantic cod myocytes. The mean rate of lactate production decreased under cytochalasin B treatment from 47 to 37 nmol g<sup>-1</sup> min<sup>-1</sup> the difference was not significantly different. It is possible that under conditions of cytochalasin B treatment, lactate production is supported in part from the glycogen pool. In the current study, even in Atlantic cod, the size of the glycogen pool in glucosyl units is large relative to the rate of lactate production. Even a small decrease of the glycogen pool, which could be missed with the current protocols, could elevate the rate of lactate production under cytochalasin B treatment, thus masking an anticipated decrease in rate. All of the lactate production need not be accounted for by glycogen mobilization, only a small rate of lactate production that would elevate the rate under cytochalasin B treatment such that it is not significantly different from control preparations.

There is evidence that the glycolytic generation of ATP is required to support heart resting tension under normoxic conditions. This contention is based on findings with isometrically contracting ventricle strips where (i) preparations from American eel showed an increase in resting tension when challenged with increases in extracellular Ca<sup>2+</sup> in the absence of glucose but not when glucose was available in the medium (Bailey et al., 2000), and (ii) ventricle strips from rainbow trout challenged with high frequency showed a greater elevation in resting tension when glycolysis was pharmacologically blocked than control preparations even with abundant alternative metabolic fuels in the medium (Gesser, 2002). The underlying mechanism appears to be a necessity for glycolytically generated ATP to support some aspects of intracellular Ca<sup>2+</sup> regulation, either directly or indirectly, at either the sarcoplasmic reticulum or the sarcolemmal membrane (Bailey et al., 2000; Gesser, 2002). It is



possible that in the current study, glycolytically generated ATP, although only a minor component of overall ATP production, is required to maintain basal  $\text{Ca}^{2+}$  trafficking. There is a functional coupling between glycolysis and  $\text{Ca}^{2+}$  transport in sarcoplasmic reticulum isolated from heart and skeletal muscle of rabbits. Glycolytic enzymes are associated with the sarcoplasmic reticulum and ATP generated from glycolysis is 15-fold more effective in supporting  $\text{Ca}^{2+}$  transport than exogenous ATP (Xu et al., 1995). Furthermore, in perfused rat hearts, glycolysis is required to support  $\text{Na}^+$  balance possibly through preferential fueling of  $\text{Na}^+/\text{K}^+$ -ATPase by glycolytically derived ATP (Dizon et al., 1998).  $\text{Na}^+$  balance in turn impacts upon intracellular  $\text{Ca}^{2+}$  levels by mechanisms such as  $\text{Na}^+/\text{Ca}^{2+}$  exchange. It is possible that these processes relate to the current observations of extracellular glucose being metabolized to lactate in Atlantic cod and short-horned sculpin myocytes even under aerobic conditions.

The exclusive utilization of extracellular glucose to support lactate production and not aerobic metabolism in both Atlantic cod and short-horned sculpin myocytes is consistent with findings for sea raven, a cottid that is closely related to short-horned sculpin. In sea raven hearts perfused with [ $^{14}\text{C}$ ]glucose in the medium, only a small percentage of extracellular glucose appeared as  $^{14}\text{CO}_2$  (Sephton et al., 1990); unfortunately, lactate production rates were not reported. Although the data are not as robust, rates of lactate production and glucose metabolism, assessed by a method similar to that utilized here, were reported for perfused rainbow trout hearts (West et al., 1993). Similar to Atlantic cod and short-horned sculpin, it appears that in rainbow trout heart, the extracellular glucose is directed to lactate production and not aerobic metabolism.

Given that most or all of the external glucose is used to support lactate production, the question remains as to what metabolic fuels support oxygen consumption. The possibility that stored lipids were being utilized was assessed by treating myocytes with etomoxir, a chemical that inhibits fatty acid metabolism at the level of carnitine palmitoyl transferase. It was reasoned that if on-board triglycerides were an important fuel, respiration would be decreased or alternative metabolic fuels called upon in the presence of etomoxir. For Atlantic cod myocytes, there was no decrease in oxygen consumption but rather a small but significant increase in glucose metabolism with etomoxir treatment. This finding suggests that under control conditions a portion of oxygen consumption is fueled by on-board fatty acids and when catabolism of fatty acids is impaired, the use of extracellular glucose is increased.

## Conclusions

Plasma glucose in Atlantic cod is approximately 6.5-fold higher than in short-horned sculpin that have extremely low plasma glucose levels ( $<0.6 \text{ mmol l}^{-1}$ ) by vertebrate standards (Polakof et al., 2011). Myocytes from Atlantic cod and short-horned sculpin exhibit a simple diffusive component to glucose entry into the cell that is similar in nature between species and may be set by common membrane compositions. Atlantic cod myocytes also have a facilitated glucose transport component that at physiological levels of extracellular glucose accounts for approximately 30–55% of the glucose entry. Essentially all of the extracellular glucose metabolized goes to supporting lactate production in both Atlantic cod and short-horned sculpin myocytes with little or no glucose available for oxidative metabolism. Thus, the contribution of extracellular glucose to ATP production is minimal. A sustained glycolysis may be necessary to support specific aspects of  $\text{Ca}^{2+}$  transport. The nature of the preparation should be appreciated on at least two points. The current experiments utilized isolated myocytes; it may be that at

elevated workloads, glucose metabolism is enhanced in concert with energy demand and would support a higher percentage of the ATP production. Furthermore, although under the incubation conditions with isolated cells, on-board metabolic fuels may support oxygen consumption, this could not be sustained *in vivo*. The extension of this is that *in vivo* the heart must be supplied with alternative blood-borne metabolic fuels. Under glycolytic inhibition, the performance of perfused isolated hearts from sea raven can be sustained by extracellular palmitate and to a lesser extent ketone bodies (Driedzic and Hart, 1984). The elucidation of whether these fuels actually support heart metabolism *in vivo* remains a challenge.

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## Competing interests

The authors declare no competing or 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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