The Journal of Experimental Biology 216, 3422-3432

doi:10.1242/jeb.085688

RESEARCH ARTICLE

Limited effects of exogenous glucose during severe hypoxia and a lack of hypoxia-stimulated glucose uptake in isolated rainbow trout cardiac muscle

Tracy A. Becker[1], Brian DellaValle[2], Hans Gesser[2] and Kenneth J. Rodnick[1,*]

[1]Department of Biological Sciences, Idaho State University, Pocatello, ID 83209-8007, USA and [2]Institute of Biological Sciences, University of Aarhus, Building 1131, DK-8000 Aarhus C, Denmark

*Author for correspondence (rodnkenn@isu.edu)

SUMMARY

We examined whether exogenous glucose affects contractile performance of electrically paced ventricle strips from rainbow trout under conditions known to alter cardiomyocyte performance, ion regulation and energy demands. Physiological levels of D-glucose did not influence twitch force development for aerobic preparations (1) paced at 0.5 or 1.1 Hz, (2) at 15 or 23°C, (3) receiving adrenergic stimulation or (4) during reoxygenation with or without adrenaline after severe hypoxia. Contractile responses to ryanodine, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum, were also not affected by exogenous glucose. However, glucose did attenuate the fall in twitch force during severe hypoxia. Glucose uptake was assayed in non-contracting ventricle strips using 2-[^{3}H] deoxy-D-glucose (2-DG) under aerobic and hypoxic conditions, at different incubation temperatures and with different inhibitors. Based upon a lack of saturation of 2-DG uptake and incomplete inhibition of uptake by cytochalasin B and D-glucose, 2-DG uptake was mediated by a combination of facilitated transport and simple diffusion. Hypoxia stimulated lactate efflux sixfold to sevenfold with glucose present, but did not increase 2-DG uptake or reduce lactate efflux in the presence of cytochalasin B. Increasing temperature (14 to 24°C) also did not increase 2-DG uptake, but decreasing temperature (14 to 4°C) reduced 2-DG uptake by 45%. In conclusion, exogenous glucose improves mechanical performance under hypoxia but not under any of the aerobic conditions applied. The extracellular concentration of glucose and cold temperature appear to determine and limit cardiomyocyte glucose uptake, respectively, and together may help define a metabolic strategy that relies predominantly on intracellular energy stores.

Key words: rainbow trout, heart, glucose, 2-deoxyglucose, contractility, hypoxia, reoxygenation, adrenaline, lactate.

Received 20 January 2013; Accepted 12 May 2013

INTRODUCTION

The vertebrate heart must remain active to support whole-animal homeostasis and normal function. As a result, cardiomyocytes depend on an uninterrupted provision of energy substrates – either exogenous or endogenous – for production of ATP to sustain contractile function and the Ca^{2+} regulation governing it. The heart of aquatic ectotherms faces unique metabolic challenges because of the possibility of environmental and myocardial hypoxia. In particular, fishes possess a wide range of tolerances to reduced oxygen levels in water or by cardiac tissue. Similar to the mammalian heart, which fails within minutes during oxygen limitation (Allen et al., 1985; Matthews et al., 1986), athletic fishes, such as the rainbow trout (*Oncorhynchus mykiss* Walbaum), possess hearts that cannot maintain force development and cellular energy state during hypoxia (Gesser, 1977; Arthur et al., 1992; Driedzic and Gesser, 1994; Hartmund and Gesser, 1996). However, the heart of rainbow trout is capable of surviving severe hypoxia, without irreversible damage, and sustains contractile function at a reduced work rate (Arthur et al., 1992; Faust et al., 2004). At the same time, energetic needs of hypoxic cardiomyocytes are met by increasing anaerobic glycolysis (10-fold) and lactate output (>35-fold) for ATP production (Arthur et al., 1992).

Although the link between cardiac energy metabolism and function is of fundamental significance, the question of what metabolic fuel source is utilized or preferred under different conditions has not been defined in non-mammalian species, and it appears to be species and possibly sex specific. Similar to mammalian cardiac preparations, the use of exogenous glucose as a 'potential' energy substrate is a time-honored practice for perfused fish hearts and isolated cardiac tissue. The underlying assumption has been that glucose is beneficial for the support of myocardial ATP production and performance. When perfused hearts from sea raven (*Hemitripterus americanus*) are aerated (21% O_2) and deprived of exogenous fuel, they exhibit reduced contractile performance within 30 min (Driedzic and Hart, 1984). Inclusion of 10 mmol l^{-1} glucose in the perfusion medium resulted in improved performance (15%) over preparations without exogenous substrate. In contrast, hearts from dogfish (*Mustelus canis*), little skate (*Raja erinecea*), American eel (*Anguilla rostrata*), Atlantic cod (*Gadus morhua*) and eelpout (*Zoarces viviparus*) do not require extracellular glucose to preserve contractile performance under aerobic conditions (Driedzic, 1978; Driedzic and Hart, 1984; Driedzic et al., 1984; Bailey et al., 2000a; Clow et al., 2004). Under aerobic conditions, exogenous glucose accounts for a very minor component (<10%) of energy metabolism of intact (West et al., 1993; Blasco et al., 1996) or isolated hearts of trout (Bailey and Driedzic, 1993). Conversely, physiological levels of exogenous glucose (5 mmol l^{-1}) maintained resting tension and increased developed tension in isolated ventricle strips from immature female and maturing males, respectively (Farrar et al., 2006).

Possibly related to this beneficial effect on myofilament function, glucose may influence the participation of the sarcoplasmic reticulum (SR) in cardiac excitation–contraction (EC) coupling. In American eel ventricle strips, extracellular glucose counteracts increases in resting tension in the face of elevated extracellular Ca^{2+} under fully oxygenated conditions. This effect is eliminated by blocking the release of Ca^{2+} from the SR with ryanodine (Bailey et al., 2000b). Experiments with cat heart muscle also suggest that the efficiency of EC coupling is locally controlled at the SR Ca^{2+} release sites by mechanisms utilizing ATP, produced by glycolytic enzymes closely associated with the release channel (Hüser et al., 2000). Thus, evidence from fishes and mammals demonstrates that an active glycolytic pathway is required to maintain cardiac EC coupling for intracellular Ca^{2+} homeostasis and mechanical performance under aerobic and oxygen-limiting conditions (Driedzic and Gesser, 1994; Gesser, 2002). Furthermore, under aerobic conditions, exogenous glucose appears to enhance SR Ca^{2+} uptake, storage and release in ventricle strips from sexually immature rainbow trout (males and females), but only helps to maintain resting tension in females (Farrar et al., 2006). To what extent exogenous glucose or tissue glycogen fuels glycolytic activity and ATP production in teleost hearts is unknown, although there is evidence for functional compartmentation of glycolysis and glycogenolysis in the isolated working rat heart (Anousis et al., 2004).

In the complete absence of oxygen, maintenance of cardiomyocyte ATP levels (and therefore cardiac performance) must rely solely on anaerobic glycolysis and either exogenous glucose or stored glycogen for metabolic fuel. Whether the ability to use exogenous glucose for anaerobic glycolysis and energy production defines hypoxia tolerance of the teleost myocardium remains unanswered and controversial. In the American eel (a hypoxia-tolerant species), incubation of ventricle strips under severe hypoxia activates glucose uptake (Rodnick et al., 1997) and the presence of exogenous glucose allows ventricle strips to maintain force production during hypoxia (Bailey et al., 2000a). Conversely, exposure of hypoxia-tolerant short-horned sculpin (*Myoxocephalus scorpius*) to 2.0 mg O_2 l^{-1} (62.5 μmol l^{-1}) for 240 min did not increase cardiac glucose uptake, and this may be related to the corresponding bradycardia and reduced energy demands of the myocardium *in vivo* (MacCormack and Driedzic, 2007). It is also unclear whether substrate preference and utilization changes with metabolic state, such as reoxygenation immediately following a bout of severe hypoxia.

The primary objective of the present study was to test the hypothesis that exogenous glucose increases contractile performance of isolated cardiac tissue from rainbow trout subjected to conditions that challenge energy homeostasis and EC coupling. Based on limited evidence for a mechanical benefit of exogenous glucose, a secondary objective was to determine mechanisms and kinetics of glucose uptake in cardiac tissue. Contrary to the proposed hypothesis, our data demonstrate a lack of dependence of contractile performance on exogenous glucose in this species and, for the first time, an ability to modulate cardiac glucose uptake *via* substrate concentration and reduction in tissue temperature.

MATERIALS AND METHODS

Animals and rearing conditions

Aarhus

Female rainbow trout (~250–550 g) were obtained from a local breeder (Funder-Holme, Jutland, Denmark), transported to the University of Aarhus, Department of Zoophysiology, and maintained in aerated, flow-through freshwater tanks (900 l) at 15±2°C and under a controlled photoperiod (12 h:12 h light:dark cycle). Fish were fed an extruded pellet diet consisting of 44% protein, 29% fat, 12% carbohydrate and 7% ash (available energy 22.1 MJ kg^{-1}, Efico Enviro 920, BioMar A/S, Brande, Denmark) *ad libitum*. All animals were sexually immature at the time of sampling. Fish care and the sampling protocol were approved by the Institutional Animal Care and Use Committee at the University of Aarhus.

Pocatello

Sexually immature male and female rainbow trout (~300–500 g) were obtained from Clear Springs Foods (Buhl, ID, USA) and transported to the Aquatic Research Facility at Idaho State University. Fish were maintained in 1000 l circular tanks receiving recirculating, filtered, UV-sterilized freshwater at 14±1°C. These fish were also exposed to a 12 h:12 h light:dark cycle and fed extruded pellets (1% of body mass, 46% protein, 21% fat, 18% carbohydrate and 8% ash, available energy 22.5 MJ kg^{-1}, produced by Clear Springs Foods) every other day. All experiments performed at Idaho State University were approved by the university's Institutional Animal Care and Use Committee.

Experimental preparations for cardiac muscle performance studies

Individual animals in Aarhus were netted, euthanized by a blow to the head, decapitated, and the heart was quickly excised and transferred to ice-cold oxygenated physiological saline in a Petri dish, in which one to four longitudinal myocardial strips were prepared from each ventricle. Each strip represented a complete cross-section of the ventricle and consisted of both compact (epicardial) and spongy (endocardial) tissue. The physiological saline consisted of (mmol l^{-1}): 115 NaCl, 2.5 KCl, 1 $MgSO_4$, 1 NaH_2PO_4, 1.5 $CaCl_2$ and 15 $NaHCO_3$. The saline was equilibrated with 0.5% CO_2 and 99.5% O_2, and pH was 7.6 at 15°C. Ventricular strips [N=56, thickness: 1.4±0.03 mm, assuming a square cross-section, mass: 11.9±0.6 mg (means ± s.e.m.)] were mounted vertically using 3-0 surgical silk; one end was attached to a thin glass rod and the other end to one of the two platinum stimulation electrodes. The second stimulation electrode was positioned close to the upper end of the preparation. Both stimulation electrodes were connected to a Grass SD 9 stimulator (Quincy, MA, USA). The glass rod was connected to a force transducer (Fort 10, World Precision Instruments, Sarasota, FL, USA). Glass, water-jacketed muscle baths contained 50 ml of physiological saline and were maintained at 15 or 23°C with a refrigerated recirculating water bath (Lauda K2 RD, Königshofen, Germany). Unless stated otherwise, ventricle strips were electrically paced at 0.5 Hz, with pulses of 5 ms and a voltage 1.5 times that eliciting maximal response.

Each preparation was allowed to stabilize for 20 min before tissue length was adjusted with a micrometer screw to the peak of the contractile force–length relationship. Preparations were then left to stabilize for another 30 min before experimentation. Signals from the force transducers were recorded by an MP100 data-acquisition system (Biopac, Santa Barbara, CA, USA) at 200 Hz and analyzed by AcqKnowledge software (Version 3.7.1, Biopac). At the end of each experiment, length and wet mass of each cardiac strip were measured. Force (mN) relative to cross-sectional area (mm^2) was estimated assuming a tissue density of 1.06 mg mm^{-3} (Layland et al., 1995) and uniform thickness of the strips.

Protocol 1: Effects of exogenous glucose on contractility and contributions of the SR, adrenergic stimulation and temperature

After stabilization of twitch force in the absence of exogenous substrate, two of four ventricle strips from each of six hearts (run

in parallel) received 5 mmol l^{-1} glucose (D-glucose, unless stated otherwise) and the other two received 5 mmol l^{-1} mannitol (membrane impermeable control). To determine SR participation in EC coupling, one of each of the two ventricle strips in glucose and mannitol was exposed to 10 μmol l^{-1} ryanodine. Ryanodine at this concentration provides maximal effect on twitch force development of trout heart preparations (Hove-Madsen, 1992), probably by locking the SR Ca^{2+} release channel in a closed state and rendering the SR ineffective in Ca^{2+} cycling (Rousseau et al., 1987). After 40 min, electrical stimulation was turned off and was then resumed after 5 min to assess post-rest potentiation (Bers, 1985). When force was stable, stimulation rate was elevated from 0.5 to 1.1 Hz for 15 min and then returned to 0.5 Hz. These rates are comparable to heart rates for quiescent and active rainbow trout held at similar temperatures (Wood et al., 1979; Gamperl et al., 2011), and we noted in preliminary trials that some preparations become arrhythmic at frequencies >1.5 Hz (data not shown). After an additional 15 min at 0.5 Hz, a maximally effective dose of adrenaline (10 μmol l^{-1}) was added to all four preparations. Ten minutes later, stimulation rate was elevated again to 1.1 Hz for 15 min and finally returned to 0.5 Hz for 15 min at 0.5 Hz.

To address potential effects of elevated temperature on myocardial substrate preference and cardiac performance, experiments were repeated with ventricle strips from six hearts, except that 30 min after the addition of glucose or mannitol temperature was elevated from 15 to 23°C over 10–15 min and maintained at 23°C for 30 min before the rest of the protocol was applied.

Protocol 2: Effects of exogenous glucose and adrenaline during severe hypoxia

Four strips from each of 10 ventricles were run in parallel. After stabilization of twitch force at a stimulation rate of 0.5 Hz, two ventricle strips received 5 mmol l^{-1} glucose and the other two 5 mmol l^{-1} mannitol (controls). Fifteen minutes thereafter, severe hypoxia was imposed for 100 min by replacing O_2 with N_2 in the gas mixture. After 10 min of severe hypoxia, one of the two ventricle strips in glucose and mannitol was exposed to 10 μmol l^{-1} adrenaline. To assess temporal changes in ventricle strip performance under aerobic conditions, additional preparations were oxygenated continuously and subjected to the same time schedule and treatments as the above experiments (N=4).

Protocol 3: Effect of exogenous glucose on force recovery and its adrenaline dependence following severe hypoxia

To assess the potential importance of glucose and increasing concentrations of adrenaline to tissue mechanical performance during aerobic recovery, severe hypoxia was imposed on paced (0.5 Hz) ventricle strips not receiving exogenous substrate. Five minutes before reoxygenation, three of the four ventricle strips were exposed to 5 mmol l^{-1} glucose, 10 mmol l^{-1} glucose, or 5 mmol l^{-1} mannitol. The fourth ventricle strip received 5 mmol l^{-1} L-glucose (N=4), 5 mmol l^{-1} sucrose (N=1) or nothing (N=1) for a total of 6 experiments. After 60 min of reoxygenation, adrenaline was added stepwise to attain final concentrations of 0.1, 0.5, 2 and 20 μmol l^{-1} with stabilization of force at each concentration. To control for oxygenation state, additional experiments were performed with identical treatments but under aerobic conditions throughout (N=10).

Protocol 4: Recovery after severe hypoxia in the presence of non-carbohydrate substrates or glucose

Four ventricle strips from each of six hearts received 2 mmol l^{-1} of Na-butyrate, Na-acetate or Na-octanoate, or 5 mmol l^{-1} of glucose after 55 min of severe hypoxia. Five minutes later, N_2 was replaced by O_2 for aerobic recovery and ventricle strips remained oxygenated for 60 min.

Protocol 5: 2-DG uptake in ventricle strips

Similar to mechanical performance studies performed in Aarhus, ventricle strips were prepared from rainbow trout in Pocatello. Glucose uptake was measured for individual ventricle strips using the glucose analog 2-deoxy D-glucose (2-DG) as described previously for American eel (Rodnick et al., 1997) and Atlantic cod (Clow et al., 2004). 2-DG is transported into cardiomyocytes, phosphorylated by hexokinase and trapped inside the myocyte as 2-deoxyglucose 6-phosphate (2-DG-6-P), allowing for the determination of rates of sugar uptake over extended periods (minutes *versus* seconds). Uniform ventricle strips (1.6–2.0 mm thick, weighing 14–24 mg) were rinsed in an ice-cold, filtered (0.22 μm) teleost Ringer's solution consisting of (in mmol l^{-1}) 111 NaCl, 5.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 0.5 NaH_2PO_4, 10 $NaHCO_3$ and 5 glucose, pH 7.6 at 14°C. The solution was equilibrated with 0.5% CO_2 and 99.5% O_2 and osmolality was kept constant (~300 mOsmol kg^{-1} water) by varying the concentration of mannitol, such that the sum of glucose, pyruvate, mannitol and 2-DG was 40 mmol l^{-1}. Select measurements of 2-DG uptake were conducted on individual strips from the same animal at multiple temperatures (4, 14 and 24°C, N=6 for each temperature) and over a range of 2-DG concentrations (1–40 mmol l^{-1}, N=2–5 per concentration) to define thermal sensitivity and kinetics. All solutions were equilibrated in stoppered, 25 ml Erlenmeyer flasks at the appropriate temperature before experiments and serial incubations occurred in a reciprocating water bath (Haake-Fisons SWB20, Karlsuhe, Germany, set at 60 cycles min^{-1}) and two custom flask incubators connected to refrigerated recirculating baths (Neslab RTE 7, Portsmouth, NH, USA).

Ventricle strips were (1) preincubated for 1 h in 2 ml of Ringer's solution containing 0.1% (w/v) bovine serum albumin (BSA; fraction V, fatty acid free) pregassed with either 0.5% CO_2:99.5% O_2 (aerobic, ~640 mmHg P_{O_2}) or 0.5% CO_2:99.5% N_2 (severe hypoxia, 6–10 mmHg P_{O_2}), (2) rinsed for 10 min in Ringer's solution containing 40 mmol l^{-1} mannitol plus 0.1% BSA, minus glucose to remove glucose from the extracellular space, and (3) incubated for 20 min in 1.5 ml of glucose free Ringer's solution containing 0.1% BSA, 2 mmol l^{-1} sodium pyruvate (extracellular substrate to support oxidative metabolism in the absence of glucose), 1–40 mmol l^{-1} 2-DG including 1,2-[^{3}H(N)] deoxy D-glucose (40 Ci mmol $^{-1}$, specific activity 1.0 μCi ml^{-1}) and D-mannitol-1 [^{14}C] (53 mCi mmol $^{-1}$, specific activity 0.1 μCi ml^{-1}). Incubations were terminated and extracellular space and intracellular 2-DG and 2-DG-6-P were determined as described previously (Rodnick et al., 1997; Clow et al., 2004). Preliminary studies (N=4) demonstrated that 2-DG uptake (at 5 mmol l^{-1} and 14°C) remains linear for at least 60 min, which is consistent with measurements on ventricle strips from American eel (Rodnick et al., 1997). For comparative purposes, data are reported as μmol 2-DG ml^{-1} intracellular water 20 min^{-1} or nmol 2-DG g^{-1} tissue min^{-1}. Cytochalasin B (25 μmol l^{-1}, final concentration), a nonselective inhibitor of facilitative glucose transport proteins (GLUTs) in rainbow trout (Teerijoki et al., 2001), or excess D-glucose (25–100 mmol l^{-1}, final concentrations) were included in some incubations to determine the relative contribution of GLUTs *versus* other transmembrane mechanisms (e.g. simple diffusion). Cytochalasin B was dissolved in absolute ethanol [final concentration 0.025% (v/v)] and added to incubation flasks just prior to experiments. Separate controls demonstrated that the presence

of ethanol did not affect 2-DG uptake measurements, and incubations with increased cytochalasin B (100 μmol l^{-1}) did not significantly reduce 2-DG uptake compared with 25 μmol l^{-1} cytochalasin B (data not shown).

Protocol 6: Tissue lactate efflux under aerobic conditions and severe hypoxia

To confirm that incubation of ventricle strips under severe hypoxia stimulated anaerobic glycolysis and examine the importance of exogenous glucose uptake for glycolytic metabolism, we included 5 mmol l^{-1} glucose in the aerobic and hypoxic Ringer's solution instead of 2-DG, and the final incubation was extended from 20 to 60 min. Cytochalasin B (25 μmol l^{-1}) was added to some preparations to inhibit facilitated glucose uptake. Net lactate efflux from ventricle strips (N=9–10 per treatment) was determined by sampling the incubation solution and measuring this metabolite according to the method of Fleischer (Fleischer, 1970) as modified by Battiprolu et al. (Battiprolu et al., 2007).

Chemicals, calculations and data analysis

Unless specified otherwise, chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). We used several indices of mechanical performance to assess cardiac contractility and relaxation (Hartmund and Gesser, 1996; Gesser, 2002). Twitch force development was normalized (%) to values measured after the initial stabilization period. Although twitch force was calculated in absolute terms and standardized for ventricle strip cross-sectional area (values for 56 Aarhus preparations were 11.1±0.5 mN mm^{-2}), relative values were used in assessing the effects of experimental interventions. Rates of rise of contraction and 50% relaxation were also measured from waveforms.

The adrenaline-dependent change in twitch force (ΔF) was calculated as the force just before adrenaline additions subtracted from the twitch force in the presence of adrenaline. The adrenaline concentration (ED_{50}) eliciting half of the maximal adrenaline effect on force was assessed with the Michaelis–Menten equation written as $\Delta F=\Delta F_m-ED_{50}(\Delta F/[Adr])$, whereby ED_{50} is provided by the slope of ΔF plotted against $\Delta F/[Adr]$. The change in 2-DG uptake with incubation temperature was quantified using Q_{10} and the formula $Q_{10}=(R_2/R_1)^{[10/(T_2-T_1)]}$, where R_1=2-DG uptake at the lower temperature (T_1), and R_2=2-DG uptake at the higher temperature (T_2).

Prior to statistical analysis, a variety of tests were conducted to determine whether the data were normally distributed and justify the use of parametric tests. For normalized measurements of contractile performance (values ranging from above 100% to well below 100%), graphical assessments were performed according to Zar (Zar, 1999). Additional tests for normality included Kolmogorov–Smirnov and Shapiro–Wilk tests (2-DG uptake and dose response data), and Mauchly's test of sphericity (for repeated measurements of lactate efflux and temperature effects on 2-DG uptake). In each case the data were normally distributed. However, it is also worth pointing out that the parametric tests used in the present study are robust enough to be applied to data not severely deviating from normality (Zar, 1999). Comparisons of ventricle strip performance were assessed by either one-way ANOVA with Tukey's *post hoc* test or Student's *t*-test. Analyses of 2-DG uptake measurements and net lactate efflux from ventricle strips were initially completed using a mixed model protocol (SPSS software, Version 16.0, Chicago, IL, USA) or repeated measures two-way ANOVA. If no differences were found between male and female fish in Pocatello, the data for both sexes were pooled and analyzed by repeated-measures, one-way ANOVA. Data are expressed as means ± s.e.m. and statistical significance was established at $P<0.05$.

RESULTS

Effects of exogenous glucose on cardiac performance in the presence of ryanodine and adrenaline, and at an elevated temperature

Preliminary experiments demonstrated that twitch force in the presence of 5 mmol l^{-1} glucose or 2 mmol l^{-1} lactate or pyruvate (N=5) did not differ significantly under aerobic conditions at 0.5 and 1.1 Hz stimulation rates (data not shown). Therefore, subsequent aerobic studies focused on possible effects of exogenous glucose on cardiac performance. In the presence of ryanodine and adrenaline, twitch force was similar in ventricle strips receiving glucose and isomolar mannitol, at both 0.5 and 1.1 Hz (Fig. 1A). With exogenous glucose or mannitol, ryanodine significantly depressed twitch force, not only after 5 min of rest, but also in the presence of adrenaline and at the higher stimulation frequency (Fig. 1A,B). The addition of adrenaline resulted in a twofold increase in force development (positive inotropy) at 15°C.

Consistent with studies performed at 15°C, twitch force for ventricle strips at 23°C was comparable with glucose and mannitol under all conditions applied (Fig. 1C). At the warmer temperature, negative effects of ryanodine on twitch force also occurred (Fig. 1C), although ryanodine did not diminish force at the elevated stimulation rate (Fig. 1D). The results suggest that exogenous glucose did not affect contractile performance under aerobic conditions with elevated metabolic demands, in the presence of intact or reduced SR function.

Mixed effects of glucose on contractile performance during severe hypoxia

Ventricle strips did not maintain contractile performance during oxygen limitation (Fig. 2A,B). Force development decreased rapidly or gradually in the presence of exogenous mannitol and glucose, respectively. In the absence of added adrenaline, there was a biphasic response in ventricle strips receiving glucose, whereby twitch force was greater compared with controls for approximately 30 min after the onset of hypoxia, but not during the remainder of the hypoxic period. In fact, twitch force was significantly lower with exogenous glucose at the end of the hypoxic exposure (Fig. 2A). Ten minutes after the onset of hypoxia, the addition of adrenaline increased twitch force to that of baseline values under aerobic conditions after 20 min (Fig. 2B). However, independent of the presence of exogenous glucose, and similar to studies without added adrenaline, twitch force decreased progressively during severe hypoxia. Ventricle strips receiving glucose did maintain twitch force at a higher value compared with mannitol-treated ventricle strips at the end of the exposure to N_2. The possibility that degradation of adrenaline limited twitch force production was discounted by the finding that supplemental adrenaline added after 45 min of hypoxia was without effects on force development (data not shown). Furthermore, the adrenaline response did not diminish in similar experiments run with continuous oxygenation instead of hypoxia. In contrast to hypoxic experiments, aerobic controls with added adrenaline displayed limited force decay during the experiment (Fig. 2C).

Glucose does not affect adrenaline-induced contractility and kinetics during reoxygenation

Following severe hypoxia, the recovery of twitch force during 60 min of reoxygenation was not affected by the addition of exogenous

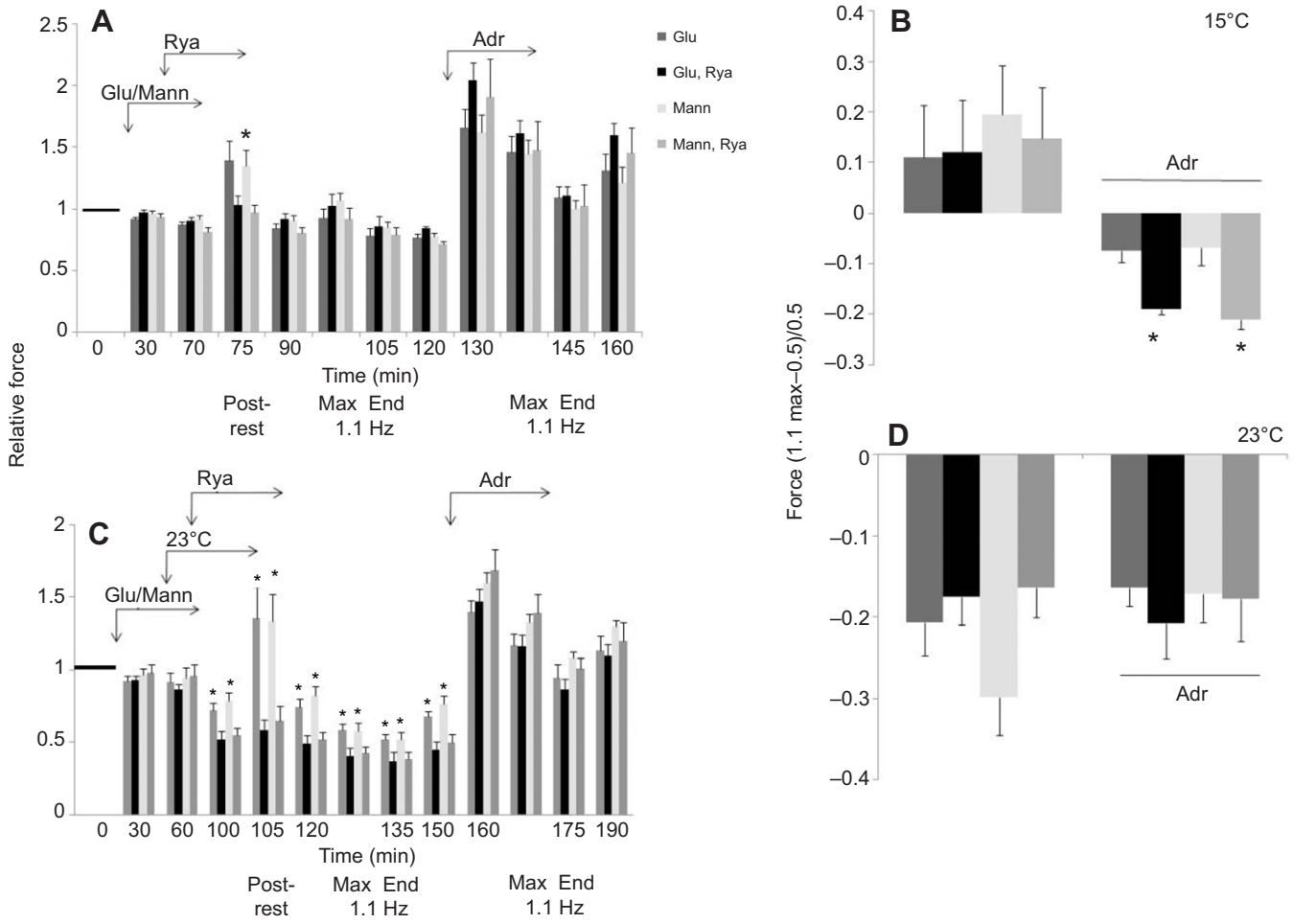

Fig. 1. Normalized twitch force development of cardiac muscle from rainbow trout exposed to different treatments sequentially. In each experiment (N=6) four strips from each ventricle were run in parallel. After 30 min without exogenous substrate, two strips electrically stimulated at 0.5 Hz received 5 mmol l^{-1} glucose (Glu) and the other two 5 mmol l^{-1} mannitol (Mann). One strip receiving Glu and one in Mann were exposed to 10 µmol l^{-1} ryanodine (Rya). After 40 min, stimulation was turned off for 5 min and resumed to assess post-rest potentiation (post-rest). Stimulation rate was then increased from 0.5 Hz to 1.1 Hz and switched back to 0.5 Hz after 15 min. After 15 min, 10 µmol l^{-1} adrenaline (Adr) was added to all preparations. Ten minutes later the stimulation rate was increased to 1.1 Hz for 15 min and then reduced back to 0.5 Hz for 15 min. Horizontal bars at time zero set the references for relative force development. Vertical arrows pointing down indicate the time of addition of different chemicals or the change in temperature. Horizontal arrows indicate the presence of compounds or a particular temperature during the rest of the experiment. During stimulation at 1.1 Hz, maximal force and the force at the end of exposure to 1.1 Hz are depicted as 'Max' and 'End', respectively. The time at which maximal force occurred varied between experiments and is therefore not indicated on the x-axis. Results are means ± s.e.m. (A) Twitch force development at 15°C (acclimation temperature). (B) Effects of the elevation of stimulation rate on twitch force calculated as the difference between the maximal force at 1.1 Hz and the preceding force at 0.5 Hz divided by the preceding force at 0.5 Hz at 15°C in the absence and presence of Adr. *Significant effect of ryanodine *versus* baseline (P<0.01, t-test). (C) Twitch force development after acute elevation of temperature to 23°C. Six new experiments were run as above except that temperature was increased from 15 to 23°C 30 min after the addition of Glu or Mann. *Significant effect of ryanodine in the absence of Adr (P<0.05, one-way ANOVA with Tukey's test). (D) Effects of increased stimulation rate on twitch force calculated as in B at 23°C, in the absence and presence of Adr. In contrast to results at 15°C, there was no effect of Rya in the presence of Adr.

glucose (5 or 10 mmol l^{-1}) compared with controls (Fig. 3A). Increasing concentrations of adrenaline promoted contractile performance in a staircase manner; however, the presence of glucose did not alter the maximal or submaximal responses. Similarly, when ventricle strips were oxygenated continuously, twitch force was identical in the presence or absence of glucose, and in response to incremental additions of adrenaline (Fig. 3B). The presence of glucose did not alter the results under any conditions. In addition, the concentration of adrenaline eliciting half of the maximal effect on twitch force (ED_{50}) ranged from 0.3 to 0.5 µmol l^{-1}, and was not influenced by variations in substrate availability (Table 1). Results obtained with the ventricle preparations exposed to either 5 mmol l^{-1} L-glucose (N=4), 5 mmol l^{-1} sucrose (N=1) or no addition (N=1) were similar to the results for the other three groups (data not shown) and support the lack of dependence of adrenaline-induced intropism on exogenous glucose.

Various exogenous substrates do not improve recovery of twitch force after severe hypoxia

When recovering after oxygen deprivation, force development by cardiac muscle from rainbow trout did not increase when exposed to non-carbohydrate compared with carbohydrate substrates. During severe hypoxia, force development by ventricle strips decreased by 80–90% over 60 min (Fig. 4). The recovery of twitch force during reoxygenation was not different between strips receiving glucose, butyrate and acetate, and was decreased with octanoate relative to

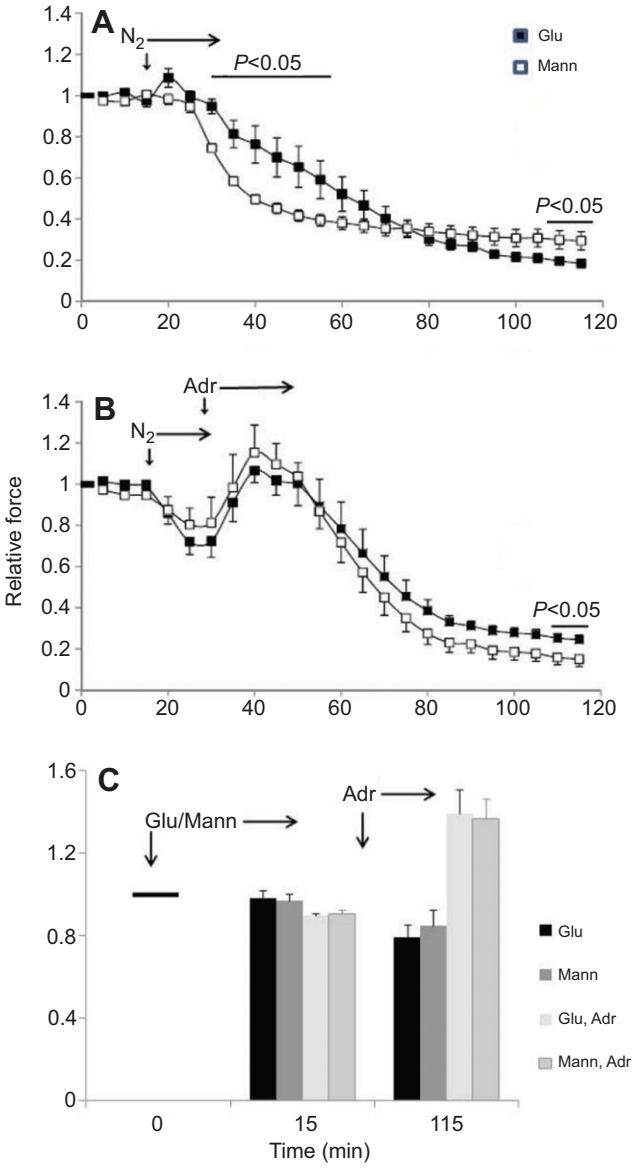

Fig. 2. Effects of 100 min of severe hypoxia on normalized twitch force development in rainbow trout ventricle strips with 5 mmol l^{-1} exogenous glucose (Glu) or mannitol (Mann), in the absence (A) and presence (B) of 10 μmol l^{-1} adrenaline (Adr). After stabilization for 60 min at 0.5 Hz and 15°C, two strips of four from each heart (*N*=10) received Glu and two Mann. Horizontal bars at time zero set the references for relative force development. The horizontal lines above the bars indicate either the period of severe hypoxia (N_2) or the presence of different compounds. After 15 min, severe hypoxia was imposed by replacing O_2 with N_2. After 10 min of hypoxia, one of the two strips with Glu and Mann was exposed to Adr. Results are means ± s.e.m. Significant differences (*P*<0.05, one-way ANOVA with Tukey's test) occurred between ventricle strips receiving Glu and Mann and varied with time. (C) Normalized twitch force of oxygenated ventricle strips serving as controls (*N*=4).

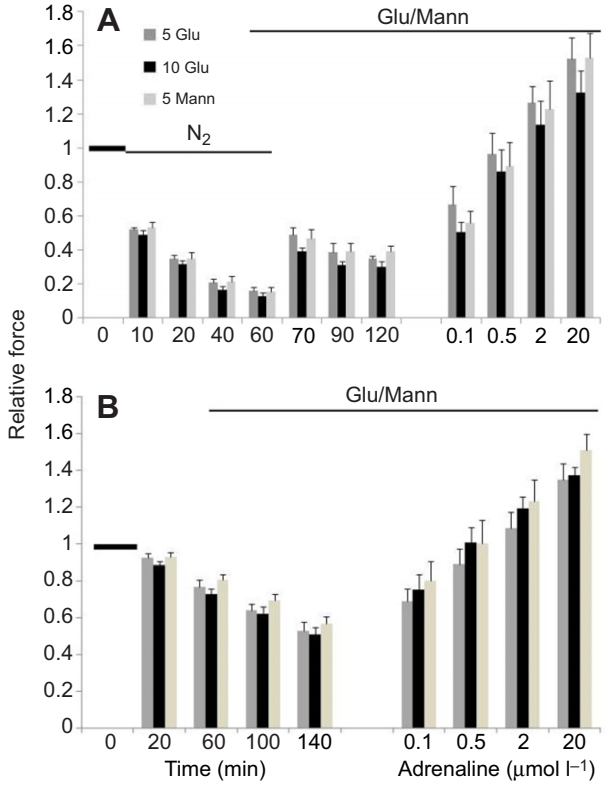

Fig. 3. Normalized twitch force in response to increasing adrenaline (Adr) concentrations in the presence of various compounds during reoxygenation after severe hypoxia. Horizontal bars at time zero set the references for relative force development. The horizontal lines above the bars indicate either the period of severe hypoxia (N_2) or the presence of different compounds. Four strips from each ventricle of rainbow trout (*N*=6) were stimulated at 0.5 Hz and subjected to 60 min of severe hypoxia (N_2) at 15°C. Five minutes before reoxygenation, preparations received exogenous substrate [i.e. 5 or 10 mmol l^{-1} glucose (Glu)], or 5 mmol l^{-1} mannitol (Mann). The fourth strip (not shown) received 5 mmol l^{-1} L-glucose (*N*=4), 5 mmol l^{-1} sucrose (*N*=1) or nothing (*N*=1). After 60 min of reoxygenation, Adr was added stepwise with stabilization of force at each concentration. (A) Effects of exogenous Glu (5 or 10 mmol l^{-1}) or Mann. (B) Twitch force response to Adr in the presence of Glu (5 or 10 mmol l^{-1}), or no substrate (Mann) and continuous oxygenation, but otherwise as in A (*N*=10). Results are means ± s.e.m. and demonstrate that exogenous Glu had no effect on contractile performance.

the recovery with added glucose and acetate after 40 to 60 min of incubation.

Despite elevated glycolytic activity, 2-DG uptake is not stimulated by severe hypoxia in ventricle strips

Under aerobic conditions, uptake rates for 5 mmol l^{-1} 2-DG at 14°C were similar for male (1.16±0.08 μmol ml^{-1} intracellular water 20 min^{-1}, *N*=6) and female (1.09±0.03 μmol ml^{-1} intracellular water 20 min^{-1}, *N*=6; Table 2) ventricle strips from Pocatello rainbow trout, so data for both sexes were combined. Exposure of non-contracting ventricle strips to severe hypoxia for 60 min did not stimulate 2-DG uptake; however, hypoxic exposure increased net lactate efflux by nearly sixfold above aerobic values for male and females (sexes combined, *N*=9–10; Table 2).

2-DG uptake is mediated by facilitated and simple diffusion

Cytochalasin B inhibited uptake of 2-DG (5 mmol l^{-1}) by 59±3 and 59±2% under aerobic and hypoxic conditions, respectively (Table 2), suggesting that GLUTs are responsible for a significant portion of extracellular glucose uptake by quiescent cardiomyocytes, independent of oxygenation state. It also appears that another uptake mechanism (i.e. passive or simple diffusion) is responsible for the balance (~41%). Similar to cytochalasin B, when 50 or 100 mmol l^{-1} glucose were added to the Ringer's

Table 1. Effective dose of adrenaline producing 50% of the maximal effect (ED_{50}; mean ± s.e.m.) on increasing twitch force in D-glucose (5 or 10 mmol l^{-1}), mannitol (5 mmol l^{-1}), or additional control conditions [5 mmol l^{-1} L-glucose (*N*=4), 5 mmol l^{-1} sucrose (*N*=1) or no additions (*N*=1)]

	ED_{50} (μmol l^{-1})	*N*
Glucose (5 mmol l^{-1})	0.37±0.08	8
Glucose (10 mmol l^{-1})	0.36±0.07	9
Mannitol (5 mmol l^{-1})	0.33±0.08	9
Controls (mixed)	0.55±0.09	6

For calculations of the adrenaline-dependent change in twitch force, see Materials and methods.

solution (*N*=8), uptake of 2-DG was inhibited by 25±8 and 43±6%, respectively (data not shown). These data provide additional evidence for a common, competitive uptake mechanism for both sugars.

Consistent with an incomplete inhibition of 2-DG uptake with cytochalasin B and D-glucose, we observed a linear increase for 2-DG uptake and lack of saturation between 1 and 40 mmol l^{-1} (Fig. 5). In addition, cytochalasin B inhibited proportionately less of the total 2-DG uptake as 2-DG concentration increased. At 40 mmol l^{-1} 2-DG, cytochalasin B inhibited just 25% of total 2-DG uptake, suggesting a greater enhancement of simple *versus* facilitated diffusion at increasing sugar concentrations. At each concentration of 2-DG, 92–95% of the 2-DG taken up by cardiac tissue was phosphorylated to 2-DG-6-P and this was unaffected by the presence of cytochalasin B (*N*=4, data not shown). Together, these results provide evidence that both facilitated and passive transport mechanisms exist for glucose uptake and cardiac tissue has a high capacity to phosphorylate intracellular 2-DG.

As shown in Fig. 6, when ventricle strips were warmed acutely from the acclimation temperature (14°C) to 24°C, there were no significant changes in total 2-DG uptake (Q_{10}=1.24±0.21). In contrast, reducing temperature from 14 to 4°C decreased overall 2-DG uptake by 45±5% (P<0.01, Q_{10}=1.91±0.18). There were no significant differences between ventricle strips from male and female rainbow trout and data for the sexes were combined. The addition of cytochalasin B decreased 2-DG uptake *via* facilitated glucose transporters to a similar extent at every temperature tested (51±6% at 24°C, 68±6% at 14°C, and 61±9% at 4°C), and yet the absolute contribution of non-transporter mediated 2-DG uptake was decreased at 4°C compared with 14 and 24°C (Fig. 6).

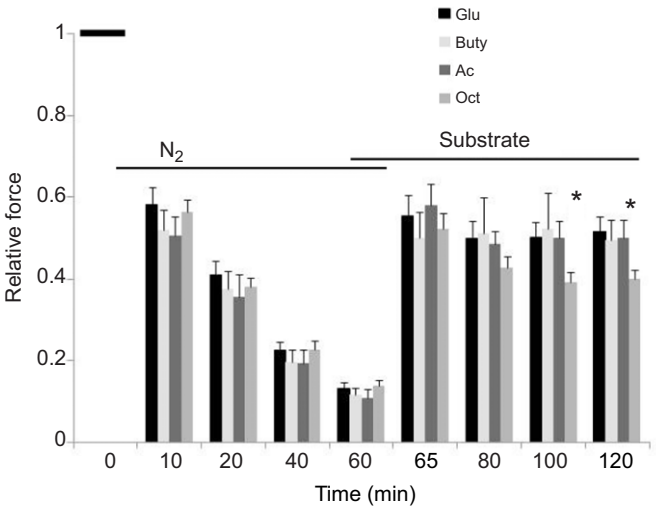

Fig. 4. Post-hypoxia twitch force recovery with 5 mmol l^{-1} glucose (Glu), or 2 mmol l^{-1} butyrate (Buty), acetate (Ac) or octanoate (Oct). Four ventricle strips were prepared from each of six hearts and stimulated at 0.5 Hz. Horizontal bars at time zero set the references for relative force development. The period of severe hypoxia (N_2) or the presence of different substrates is indicated by horizontal lines above the bars. After stabilization for 45–60 min, O_2 was replaced by N_2. After 55 min with N_2, the four exogenous substrates were given one to each strip (*N*=6). Five minutes later, N_2 was replaced by O_2 and strips remained under aerobic conditions for 60 min. Results are means ± s.e.m. *Significantly different from Glu and Ac (P<0.05, one-way ANOVA with Tukey's test).

DISCUSSION

The initial objective of this study was to examine potential effects of exogenous glucose on contractile performance of the rainbow trout heart under conditions known to alter performance, challenge ion regulation and increase energy demands. The addition of glucose has been a mainstay for heart preparations from fish because of (1) historical use of glucose in buffers for similar mammalian preparations, (2) the presence of glucose in extracellular fluid of fishes, and (3) the assumption that exogenous glucose is required to support energy production, especially during hypoxia. However, the use and benefits of exogenous *versus* endogenous energy substrates in the teleost heart appear to be species and sex specific with diverse links to contractility and tolerance to varied extracellular conditions (Driedzic, 1992; Battiprolu et al., 2007).

For rainbow trout in Aarhus, glucose did not alter contractile performance when energy demands of oxygenated cardiac muscle were increased due to chronotropic (0.5 to 1.1 Hz), inotropic (adrenaline) or temperature (15 to 23°C) stimuli. As a result, the current findings extend previous results that question the value and use of exogenous substrates as effective fuel for aerobic energy metabolism in the fish heart (Driedzic and Hart, 1984). It appears that isolated cardiac tissue from rainbow trout can be supported principally – if not completely – by endogenous energy stores (i.e. glycogen and or triglyceride) under most conditions. Conversely, in the presence of substrate-free buffer, endogenous substrates are not able to support the higher energy needs of the mammalian heart, even under aerobic conditions (Neely et al., 1970) and increased heart work stimulates the uptake of exogenous glucose (Depre et al., 1999). While it appears that hearts from American eel (Rodnick et al., 1997), Atlantic cod (Clow et al., 2004) and rainbow trout at Aarhus (present study) do not require extracellular glucose to maintain mechanical function *in vitro*, over extended periods (several hours), low levels of exogenous glucose uptake may be important in rainbow trout to replenish and maintain glycogen reserves and support the oxidative pentose phosphate pathway, and could help explain intraspecific differences in cardiac energy metabolism (Battiprolu et al., 2007) and hypoxia tolerance (Faust et al., 2004).

The EC coupling of the vertebrate heart depends upon trans-sarcolemmal movement of extracellular Ca^{2+} and to a degree that varies among species on an intracellular Ca^{2+} regulation by SR (Tibbits et al., 1990). The majority (60–70%) of energy needs of the mammalian myocardium fuels the myosin ATPase for cross-bridge cycling and the creation of muscle tension. However, ~15% of metabolic energy needs of contracting cardiomyocytes is devoted to pumping Ca^{2+} (two Ca^{2+} ions per ATP consumed) from the cytoplasm into the SR by its Ca^{2+}ATPase for mechanical relaxation (Schramm et al., 1994). Several lines of evidence support an active role of the SR in cycling Ca^{2+} during EC coupling in the rainbow trout (reviewed in Shiels and White, 2005). In the present study we

Table 2. Effects of severe hypoxia on 2-deoxyglucose (2-DG) uptake and net lactate efflux from rainbow trout ventricle strips

Variable	Cytochalasin B	Aerobic	Severe hypoxia
2-DG uptake (nmol ml^{-1} intracellular water 20 min^{-1})	0	1123±43	1132±100
	+	456±46†	456±48†
2-DG uptake (nmol g^{-1} tissue min^{-1})	0	29.7±1.1	29.4±2.3
	+	12.1±1.1†	11.4±0.9†
Lactate efflux (nmol g^{-1} tissue min^{-1})	0	37.7±5.1	228.1±17.1*
	+	39.7±5.2	212.9±10.3

Non-contracting ventricles strips were exposed to either aerobic conditions (99.5% O_2/0.5% CO_2) or severe hypoxia (99.5% N_2/0.5% CO_2) at 14°C. 2-DG uptake was determined over 20 min with 5 mmol l^{-1} 2-DG, 1,2-[^{3}H(N)] deoxy D-glucose and D-mannitol-1 [^{14}C] (see Materials and methods for details), whereas net lactate efflux was measured over 60 min in the presence of exogenous (5 mmol l^{-1}) glucose. Additional strips were exposed to a saturating concentration of cytochalasin B (25 µmol l^{-1}) to block facilitated glucose transport. There were no differences between female and male fish; therefore, data for both sexes were combined (N=9–12 per group) and analyzed using a one-way ANOVA with Tukey's test. Results are means ± s.e.m. *P<0.001 for severe hypoxia *versus* aerobic conditions; $^{\dagger}P$<0.001 for the presence (+) *versus* absence (0) of cytochalasin B.

used ryanodine to confirm SR participation in cardiomyocyte EC coupling. Ryanodine reduced twitch force development at the first stimulus after a prolonged rest period [post-rest potentiation (Bers, 1985)] and the force developed at 1.1 Hz in the presence of adrenaline at 15°C and in the absence of adrenaline at 23°C – a temperature close to the upper thermal limits of this species (Rodnick et al., 2004). Overall, the current results for Aarhus rainbow trout confirm the importance of the SR as a source of activator Ca^{2+} and, in contrast to a previous study (Farrar et al., 2006), do not support an essential role of exogenous glucose for Ca^{2+} loading/release in the SR of female rainbow trout.

Even under aerobic conditions, glycolysis appears to be crucial for normal EC coupling in the heart of rainbow trout (Gesser, 2002) and mammals (Kockskämper et al., 2005). For example, there is close association between glycolytic enzymes and the SR, and ATP generated from these enzymes may selectively support SR Ca^{2+} uptake in the mammalian heart (Xu and Becker, 1998; Zima et al., 2006). Intermediates and products of glycolysis also modulate the open probability of the RyR and SR Ca^{2+} release (Kockskämper et al., 2005; Zima et al., 2006). However, there is also evidence for glycogenolytic enzymes associated with the cardiac SR (Entman et al., 1977), suggesting that endogenous glycogen might serve as a preferential substrate over exogenous glucose for glycolysis and Ca^{2+} homeostasis.

It is well established that adrenergic stimulation increases contractile performance and energy demands of the vertebrate heart. Conversely, very little is known about how adrenaline might affect cardiac fuel use and energy metabolism in the teleost heart. In the rainbow trout, inotropic effects of adrenaline are due to activation of the β-adrenergic signal-transduction pathway, leading to greater trans-sarcolemmal Ca^{2+} influx (Tibbits et al., 1992; Shiels and Farrell, 1997) and possibly increased Ca^{2+}-induced release of Ca^{2+} from the SR. β-Adrenergic stimulation is also thought to accelerate the Ca^{2+} pumping rate of the SR to shorten diastole and accelerate relaxation (Tada et al., 1975). Consistent with previous studies (Gesser et al., 1982; Gesser, 1996), adrenaline resulted in a rapid (<5 min) doubling of twitch contractile force of ventricle strips from Aarhus rainbow trout under aerobic conditions. The expectation is that increased energy consumption with adrenaline will help support the myosin ATPase and Ca^{2+} transport mechanisms to enhance

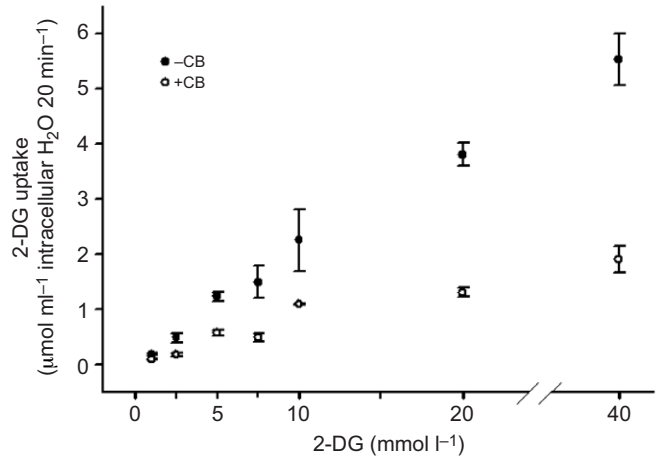

Fig. 5. Effects of increasing concentrations of 2-deoxyglucose (2-DG) on 2-DG uptake in isolated, non-contracting ventricle strips from rainbow trout. After a 60 min recovery period, excised cardiac tissue was rinsed for 10 min to remove extracellular glucose and then assayed for 2-DG uptake at 14°C for 20 min in medium containing 2 mmol l^{-1} sodium pyruvate and the presence or absence of cytochalasin B (CB, 25 µmol l^{-1}, final concentration). Values are means ± s.e.m. for two to five ventricle strips per point. The absence of error bars indicates that s.e.m. fell with the symbol area.

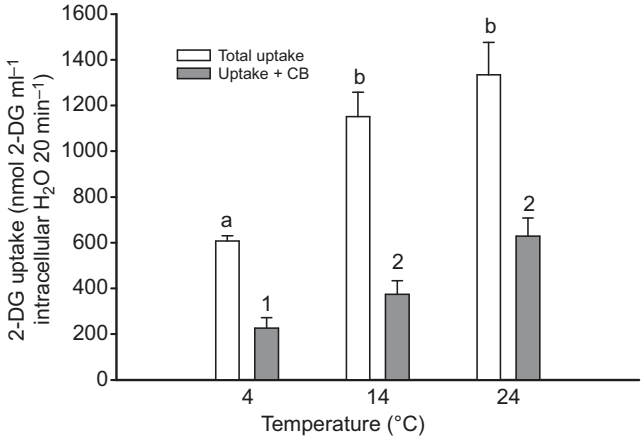

Fig. 6. Acute effects of temperature and cytochalasin B (CB) on 2-deoxyglucose (2-DG) uptake in isolated, non-contracting ventricle strips from rainbow trout. After a 60 min recovery period at 4, 14 or 24°C, excised cardiac tissue was rinsed for 10 min at the same temperature to remove extracellular glucose and then assayed simultaneously for 2-DG uptake at 4, 14 or 24°C for 20 min in medium containing 2 mmol l^{-1} sodium pyruvate, in the presence or absence of CB (25 µmol l^{-1}, final concentration). Results are means ± s.e.m., N=6 for each group. Dissimilar letters and numbers denote significant differences in 2-DG uptake for ventricle strips in the absence or presence of CB, respectively (P<0.05, one-way ANOVA with Tukey's test).

inotropic and lusitropic responses, respectively. However, previous (Gesser et al., 1982; Farrar et al., 2006; Battiprolu et al., 2007) and current data demonstrate that positive inotropic effects of a maximally effective dose of adrenaline occur during oxygen deprivation and recovery after oxygen deprivation, and these effects are independent of the presence of exogenous glucose. An additional study using iodoacetate to block glycolysis showed that inotropic responses to adrenaline under aerobic conditions do not require an active glycolytic pathway (Farrar et al., 2006). Together, these results suggest that aerobic and anaerobic metabolic pathways are capable of supporting adrenaline-induced changes in mechanical performance of the rainbow trout heart without exogenous glucose.

We also considered the possibility that exogenous energy sources may be important for adrenergic sensitivity of the increased contractile response. The concentration of adrenaline eliciting half of the maximal inotropic effect on force production in ventricle strips from Aarhus rainbow trout (ED_{50}, 0.33–0.55 $\mu mol\,l^{-1}$) is similar to values reported for (1) adrenaline (0.28 $\mu mol\,l^{-1}$) stimulation of CO_2 production from glucose in isolated cardiomyocytes from rainbow trout (Milligan, 1991) and (2) adrenaline (0.46 $\mu mol\,l^{-1}$)-induced glucose release from hepatocytes of fed rainbow trout at similar acclimation temperatures (McKinley and Hazel, 1993), and is within the range of resting and stress levels of adrenaline (1 $nmol\,l^{-1}$ to 1 $\mu mol\,l^{-1}$) for this species (Tetens et al., 1988). Our results showing that exogenous glucose did not alter sensitivity of ventricle strips to adrenaline do not discount the possibility that overall cardiac substrate availability (exogenous and endogenous) modulates adrenergic control of inotropy and lusitropy, and non-mechanical functions *in vivo*.

Assuming that oxygen is not limiting, the majority of ATP production to support heart performance in fish comes from aerobic metabolism (Driedzic et al., 1983). Salmonid fishes have been labelled as 'hypoxia sensitive' and respond to environmental hypoxia with a rapid reflex bradycardia and yet maintain cardiac output because of an increase in stroke volume (Wood and Shelton, 1980). The heart must rely on anaerobic glycolysis for energy production as oxygen supply becomes limiting. Importantly, environmental hypoxia increases plasma levels of glucose (Van Raaij et al., 1996) and adrenaline (Tetens and Christensen, 1987) in rainbow trout. This combination could potentially increase cardiac glucose uptake and utilization *via* concentration- or hormonal-dependent mechanisms. In the present study, the transition from aerobic to severe hypoxic conditions resulted in a gradual decline of twitch force development to just 10–20% of original values, which agrees with previous studies on this species (Gesser et al., 1982; Hartmund and Gesser, 1992; Overgaard and Gesser, 2004; Battiprolu et al., 2007). Also consistent with previous findings for various teleost species [American eel (Bailey et al., 2000a) and Atlantic cod (Clow et al., 2004)] was the observation that exogenous glucose reduced the decline in twitch force production in ventricle strips during exposure to severe hypoxia (Fig. 2). However, in the absence of added adrenaline we observed a biphasic response, with early contractile performance in the presence of glucose above that of the controls, whereas it fell below controls by the end of experiments. This response may reflect enhanced ATP production from extracellular glucose and glycolysis (after 15–45 min) followed by inhibition of glycolytic activity and/or reduced glycogenolysis (later, after 100 min).

In the presence of adrenaline, twitch force development was enhanced, but this gain was not significantly influenced by extracellular glucose. A likely explanation for the reduced influence of extracellular glucose on contractile function during adrenergic stimulation involves the increased provision of glycolytic substrate (glucose and G6P) *via* intracellular glycogenolysis. Furthermore, regardless of oxygenation state, lactate efflux from non-contracting ventricle strips did not change when cytochalasin B was present (Table 2), indicating that facilitative uptake of exogenous glucose is not vital for increasing glycolytic flux under these conditions.

While several studies demonstrate that exogenous glucose improves contractile function in the teleost heart during hypoxia (Driedzic and Gesser, 1994; Bailey et al., 2000a; Gesser, 2002; Clow et al., 2004), it was not known whether various exogenous substrates could help support recovery of mechanical function during reoxygenation following severe hypoxia. A previous study demonstrated that glucose was beneficial for rainbow trout ventricle strips during reoxygenation following an extended period (120 min) of severe hypoxia and adrenaline exposure in the absence of exogenous glucose in order to deplete glycogen (Gesser, 2002). Early studies on hearts from frogs (Clark et al., 1938) and turtles (Reeves, 1963) indicate that the use of exogenous glucose may require a high rate of glycogenolysis and/or significant glycogen depletion. Results from the present study indicate that ventricle strips deprived of exogenous substrate recover contractile function following 60 min of severe hypoxia, and the addition of various substrates during reoxygenation had no (acetate, butyrate or glucose) or even negative (octanoate) inotropic effects. Whether more prolonged periods of hypoxia – and significant glycogen depletion – could shift fuel selection in favor of exogenous substrates during reoxygenation will require further investigation. In contrast to the present findings, exogenous glucose helps the recovery of hypoxia-induced contractile depression and sarcolemma integrity in isolated feline cardiac muscle (Burton et al., 1980).

Given the limited effects of exogenous glucose on mechanical performance or post-hypoxic recovery of rainbow trout cardiac tissue, a second objective of this study was to define the mechanism(s) and kinetics of glucose uptake in non-contracting ventricle strips. Under aerobic conditions, the uptake of 2-DG (~30 $nmol\,g^{-1}\,min^{-1}$; Table 2) in quiescent ventricle strips from Pocatello rainbow trout was comparable to values reported for American eel (Rodnick et al., 1997), Atlantic cod (Clow et al., 2004) and rates of glucose utilization in perfused, normoxic rainbow trout hearts (West et al., 1993), but greater than *in vivo* cardiac glucose utilization [5 $nmol\,g^{-1}\,min^{-1}$ (West et al., 1993)]. In the present study, the majority of intracellular 2-DG was phosphorylated to 2-DG-6-P, suggesting that hexokinase activity was greater than transmembrane transport and glucose transport was a rate-limiting step for glucose utilization. These data agree with previous measurements of maximal hexokinase activity (12 $\mu mol\,g^{-1}\,min^{-1}$) in ventricles from Pocatello rainbow trout (Battiprolu et al., 2007), which exceed 2-DG uptake rates by 400-fold. Conversely, *in vivo* studies of brown trout, *Salmo trutta* (Blasco et al., 1996), and short-horned sculpin, *Myoxocephalus scorpius* (MacCormack and Driedzic, 2007), report the accumulation of much more cardiac 2-DG than 2-DG-6-P and a greater importance of hexokinase over glucose transport in controlling glucose uptake. Similar to in mammals, the control of cardiac glucose utilization in rainbow trout and other teleosts may vary between enzymatic steps depending on experimental conditions (Kashiwaya et al., 1994).

The importance of facilitated glucose transport across the sarcolemma for rainbow trout cardiomyocytes was suggested by a major cytochalasin B-sensitive component of 2-DG uptake (Fig. 5) and is consistent with similar studies of American eel (Rodnick et al., 1997) and Atlantic cod (Clow et al., 2004). The current finding of non-carrier-mediated glucose uptake in rainbow trout ventricle strips also agrees with studies of American eel and Atlantic cod

hearts, and other tissues of rainbow trout [erythrocytes (Tse and Young, 1990) and hepatocytes (Mannerström et al., 2003)]. The existence of multiple mechanisms for trans-sarcolemmal permeability of glucose raises important new questions about the regulation of cardiac glucose utilization and the possibility that glucose uptake is determined primarily by extracellular conditions and not cardiac performance in rainbow trout.

In contrast to previous studies on hearts from American eel (Rodnick et al., 1997) and Atlantic cod (Clow et al., 2004), our results are the first to demonstrate an inability of severe hypoxia and elevated glycolytic activity to stimulate 2-DG uptake in rainbow trout cardiac muscle *in vitro*. It has been known for over 50 years that oxygen limitation stimulates membrane transport and intracellular phosphorylation of glucose in isolated rat hearts (Morgan et al., 1959). Constraints involving the stimulation of trans-sarcolemmal transport of extracellular glucose could define a fundamental difference between the rainbow trout, some hypoxia-tolerant teleosts, and mammals. Although GLUTs have been identified in striated muscle from trout (Planas et al., 2000; Teerijoki et al., 2000), altered intracellular trafficking may limit sarcolemmal permeability of glucose in rainbow trout compared with mammals (Díaz et al., 2007). It is noteworthy that limiting the increase of glucose transport and glycolytic energy production by cardiomyocytes during hypoxia could be advantageous by preventing the accumulation of metabolic end products (lactate and protons) in the cytosol, which is consistent with reduced cardiac function in rainbow trout. It is also possible that our *in vitro* preparations lack essential extracellular factors to stimulate or inhibit the utilization of exogenous substrates for energy production and improved contractile performance of rainbow trout cardiac muscle.

The present study provides new and compelling information about the concentration and temperature dependence of glucose uptake in the rainbow trout ventricle. The importance of sugar concentration is not surprising given the presence of a cytochalasin B-insensitive component to glucose uptake and may highlight an opportunistic strategy to increase the utilization of extracellular glucose. While severe hypoxia, *per se*, did not stimulate 2-DG uptake in rainbow trout ventricle strips, hypoxic hearts will be exposed to elevated extracellular glucose levels *in vivo* (Van Raaij et al., 1996), and a more favorable concentration gradient should increase membrane transfer of glucose proportionately. In contrast to varying exogenous substrate levels, an acute increase in temperature (14 to 24°C) did not affect overall 2-DG uptake (at 5 mmol l^{-1}) in ventricle strips. This indicates a lack of thermal sensitivity as fish approach their physiological limits and may necessitate the use of other energy substrates (e.g. fatty acids or glycogen) at proportionately higher rates to increase cardiac ATP production. However, our data show temperature dependence of 2-DG uptake between 14 and 4°C, which may parallel tissue oxygen consumption requirements over this range and reflect a phase transition of membrane lipids, reduced membrane fluidity and/or reduced intrinsic activity of membrane-bound glucose transporters (Whitesell et al., 1989).

In the absence of increasing rates of extracellular glucose uptake, glycogen is the primary substrate for elevating glycolytic flux (Newsholme and Leech, 1983), especially during severe hypoxia (Reeves, 1963). A possible explanation for differences in cardiac use of exogenous glucose in rainbow trout (Battiprolu et al., 2007) and a lack of stimulation of 2-DG uptake in the present study may be that high glycogen levels in ventricle strips promote glycogenolysis, which in turn elevates intracellular glucose-6-phosphate, inhibits hexokinase and limits glucose uptake. The importance of glycogen in regulating glucose uptake in mammalian skeletal muscle stimulated by contractions and hypoxia has been documented (Hespel and Richter, 1990; Reynolds et al., 1998) and there may be a threshold for glycogen below which cardiomyocyte uptake and utilization of exogenous glucose is increased in rainbow trout. During hypoxia, the preferential use of glycogen could also be related to a sizable energetic advantage (50%, three *versus* two ATP per glucose unit) over exogenous glucose for anaerobic glycolysis (Newsholme and Leech, 1983).

In summary, this study demonstrates that despite greater energetic needs (increased frequency of contraction, temperature and inotropism) and glycolytic requirements (severe hypoxia), isolated cardiac tissue from rainbow trout can rely predominantly on intracellular energy stores and maintain mechanical performance independent of exogenous energy substrates. Depending on the timing of measurements, exogenous glucose can have beneficial and negative effects on contractility during severe hypoxia, and the addition of various exogenous substrates did not impact mechanical recovery following oxygen deprivation or the sensitivity to adrenaline. Rainbow trout use a combination of facilitated glucose transport and simple diffusion for 2-DG uptake in cardiac tissue, and yet severe hypoxia or elevated temperature does not increase 2-DG uptake under basal conditions. Ultimately, the extracellular concentration of glucose may be a key determinant of myocardial glucose metabolism in this species, and glucose uptake is compromised at cold temperatures.

ACKNOWLEDGEMENTS

The authors extend a special thanks to Dr William Driedzic at Memorial University for valuable comments and suggestions, and to technician Dorte Olsson at the University of Aarhus for excellent assistance.

AUTHOR CONTRIBUTIONS

K.J.R., H.G. and T.A.B. designed the experiments, which were carried out and interpreted by the four authors. The article was written by K.J.R. in collaboration with the other three authors.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This research was supported by the Danish Research Council, the National Institutes of Health of the United States [grant number P20RR16454], the INBRE Program of the National Center for Research Resources and the National Science Foundation of the United States [award number IOB-0517669]. Deposited in PMC for release after 12 months.

REFERENCES

Allen, D. G., Morris, P. G., Orchard, C. H. and Pirolo, J. S. (1985). A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolysis. *J. Physiol.* **361**, 185-204.

Anousis, N., Carvalho, R. A., Zhao, P., Malloy, C. R. and Sherry, A. D. (2004). Compartmentation of glycolysis and glycogenolysis in the perfused rat heart. *NMR Biomed.* **17**, 51-59.

Arthur, P. G., Keen, J. E., Hochachka, P. W. and Farrell, A. P. (1992). Metabolic state of the *in situ* perfused trout heart during severe hypoxia. *Am. J. Physiol.* **263**, R798-R804.

Bailey, J. R. and Driedzic, W. R. (1993). Influence of low-temperature acclimation on fate of metabolic fuels in rainbow trout (*Oncorhynchus mykiss*) hearts. *Can. J. Zool.* **71**, 2167-2173.

Bailey, J. R., Rodnick, K. J., MacDougall, R., Clowe, S. and Driedzic, W. R. (2000a). Anoxic performance of the American eel (*Anguilla rostrata* L.) heart requires extracellular glucose. *J. Exp. Zool.* **286**, 699-706.

Bailey, J. R., Barter, T. and Driedzic, W. R. (2000b). Maintenance of resting tension in the American eel (*Anguilla rostrata* L.) heart is dependent upon exogenous fuel and the sarcoplasmic reticulum. *J. Exp. Zool.* **286**, 707-717.

Battiprolu, P. K., Harmon, K. J. and Rodnick, K. J. (2007). Sex differences in energy metabolism and performance of teleost cardiac tissue. *Am. J. Physiol.* **292**, R827-R836.

Bers, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *Am. J. Physiol.* **248**, H366-H381.

Blasco, J., Fernàndez-Borràs, J., Marimon, I. and Requena, A. (1996). Plasma glucose kinetics and tissue uptake in brown trout *in vivo*: effect of an intravascular glucose load. *J. Comp. Physiol. B* **165**, 534-541.

Burton, K. P., Templeton, G. H., Hagler, H. K., Willerson, J. T. and Buja, L. M. (1980). Effect of glucose availability on functional membrane integrity, ultrastructure and contractile performance following hypoxia and reoxygenation in isolated feline cardiac muscle. *J. Mol. Cell. Cardiol.* **12**, 109-133.
Clark, A. J., Eggleton, M. G., Eggleton, P., Gaddie, R. and Stewart, C. P. (1938). *The Metabolism of the Frog's Heart*. Edinburgh: Oliver and Boyd.
Clow, K. A., Rodnick, K. J., MacCormack, T. J. and Driedzic, W. R. (2004). The regulation and importance of glucose uptake in the isolated Atlantic cod heart: rate-limiting steps and effects of hypoxia. *J. Exp. Biol.* **207**, 1865-1874.
Depre, C., Vanoverschelde, J.-L. J. and Taegtmeyer, H. (1999). Glucose for the heart. *Circulation* **99**, 578-588.
Díaz, M., Antonescu, C. N., Capilla, E., Klip, A. and Planas, J. V. (2007). Fish glucose transporter (GLUT)-4 differs from rat GLUT4 in its traffic characteristics but can translocate to the cell surface in response to insulin in skeletal muscle cells. *Endocrinology* **148**, 5248-5257.
Driedzic, W. R. (1978). Carbohydrate metabolism in the perfused dogfish heart. *Physiol. Zool.* **51**, 42-50.
Driedzic, W. R. (1992). Cardiac energy metabolism. In *Fish Physiology: The Cardiovascular System*, Vol. XIIA (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 219-266. San Diego, CA: Academic Press.
Driedzic, W. R. and Gesser, H. (1994). Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol. Rev.* **74**, 221-258.
Driedzic, W. R. and Hart, T. (1984). Relationship between exogenous fuel availability and performance by teleost and elasmobranch hearts. *J. Comp. Physiol. B* **154**, 593-599.
Driedzic, W. R., Scott, D. L. and Farrell, A. P. (1983). Aerobic and anaerobic contributions to energy metabolism in perfused isolated sea raven (*Hemitripterus americanus*) hearts. *Can. J. Zool.* **61**, 1880-1883.
Driedzic, W. R., Gesser, H. and Johansen, K. (1984). Effects of hypoxic adaptation on myocardial performance and metabolism of *Zoarces viviparus*. *Can. J. Zool.* **63**, 821-823.
Entman, M. L., Bornet, E. P., Van Winkle, W. B., Goldstein, M. A. and Schwartz, A. (1977). Association of glycogenolysis with cardiac sarcoplasmic reticulum: II. Effect of glycogen depletion, deoxycholate solubilization and cardiac ischemia: evidence for a phorphorylase kinase membrane complex. *J. Mol. Cell. Cardiol.* **9**, 515-528.
Farrar, R. S., Battiprolu, P. K., Pierson, N. S. and Rodnick, K. J. (2006). Steroid-induced cardiac contractility requires exogenous glucose, glycolysis and the sarcoplasmic reticulum in rainbow trout. *J. Exp. Biol.* **209**, 2114-2128.
Faust, H. A., Gamperl, A. K. and Rodnick, K. J. (2004). All rainbow trout (*Oncorhynchus mykiss*) are not created equal: intra-specific variation in cardiac hypoxia tolerance. *J. Exp. Biol.* **207**, 1005-1015.
Fleischer, W. R. (1970). Enzymatic methods for lactic and pyruvic acid. In *Standard Methods in Clinical Chemistry* (ed. R. P. MacDonald), pp. 24-59. New York, NY: Academic Press.
Gamperl, A. K., Swafford, B. and Rodnick, K. J. (2011). Elevated temperature, *per se*, does not limit the ability of rainbow trout to increase stroke volume. *J. Therm. Biol.* **36**, 7-14.
Gesser, H. (1977). The effects of hypoxia and reoxygenation on force development in myocardia of carp and rainbow trout: protective effects of CO_2/HCO_3. *J. Exp. Biol.* **69**, 199-206.
Gesser, H. (1996). Cardiac force-interval relationship, adrenaline and sarcoplasmic reticulum in rainbow trout. *J. Comp. Physiol. B* **166**, 278-285.
Gesser, H. (2002). Mechanical performance and glycolytic requirement in trout ventricular muscle. *J. Exp. Zool.* **293**, 360-367.
Gesser, H., Andresen, P., Brams, P. and Sund-Laursen, J. (1982). Inotropic effects of adrenaline on the anoxic or hypercapnic myocardium of rainbow trout and eel. *J. Comp. Physiol.* **147**, 123-128.
Hartmund, T. and Gesser, H. (1992). Temperature, contractility and high energy phosphates in anoxic fish heart muscle. *J. Comp. Physiol. B* **162**, 714-721.
Hartmund, T. and Gesser, H. (1996). Cardiac force and high-energy phosphates under metabolic inhibition in four ectothermic vertebrates. *Am. J. Physiol.* **271**, R946-R954.
Hespel, P. and Richter, E. A. (1990). Glucose uptake and transport in contracting, perfused rat muscle with different pre-contraction glycogen concentrations. *J. Physiol.* **427**, 347-359.
Hove-Madsen, L. (1992). The influence of temperature on ryanodine sensitivity and the force-frequency relationship in the myocardium of rainbow trout. *J. Exp. Biol.* **167**, 47-60.
Hüser, J., Wang, Y. G., Sheehan, K. A., Cifuentes, F., Lipsius, S. L. and Blatter, L. A. (2000). Functional coupling between glycolysis and excitation-contraction coupling underlies alternans in cat heart cells. *J. Physiol.* **524**, 795-806.
Kashiwaya, Y., Sato, K., Tsuchiya, N., Thomas, S., Fell, D. A., Veech, R. L. and Passonneau, J. V. (1994). Control of glucose utilization in working perfused rat heart. *J. Biol. Chem.* **269**, 25502-25514.
Kockskämper, J., Zima, A. V. and Blatter, L. A. (2005). Modulation of sarcoplasmic reticulum Ca^{2+} release by glycolysis in cat atrial myocytes. *J. Physiol.* **564**, 697-714.
Layland, J., Young, I. S. and Altringham, J. D. (1995). The length dependence of work production in rat papillary muscles *in vitro*. *J. Exp. Biol.* **198**, 2491-2499.
MacCormack, T. J. and Driedzic, W. R. (2007). The impact of hypoxia on *in vivo* glucose uptake in a hypoglycemic fish, *Myoxocephalus scorpius*. *Am. J. Physiol.* **292**, R1033-R1042.
Mannerström, M., Tähti, H., Tiihonen, K. and Salama, A. (2003). D-Glucose uptake in fish hepatocytes: mediated by transporter in rainbow trout (*Oncorhynchus mykiss*), but only by diffusion in prespawning lamprey (*Lampetra fluviatilis*) and in RTH-149 cell line. *Comp. Biochem. Physiol.* **136A**, 779-790.
Matthews, P. M., Taylor, D. J. and Radda, G. K. (1986). Biochemical mechanisms of acute contractile failure in the hypoxic rat heart. *Cardiovasc. Res.* **20**, 13-19.
McKinley, S. J. and Hazel, J. R. (1993). Adrenaline stimulation of glucose release from perfused trout liver: effects of assay and acclimation temperature. *J. Exp. Biol.* **177**, 51-62.
Milligan, C. L. (1991). Adrenergic stimulation of substrate utilization by cardiac myocytes isolated from rainbow trout. *J. Exp. Biol.* **159**, 185-202.
Morgan, H. E., Randle, P. J. and Regen, D. M. (1959). Regulation of glucose uptake by muscle. 3. The effects of insulin, anoxia, salicylate and 2:4-dinitrophenol on membrane transport and intracellular phosphorylation of glucose in the isolated rat heart. *Biochem. J.* **73**, 573-579.
Neely, J. R., Whitfield, C. F. and Morgan, H. E. (1970). Regulation of glycogenolysis in hearts: effects of pressure development, glucose, and FFA. *Am. J. Physiol.* **219**, 1083-1088.
Newsholme, E. A. and Leech, A. R. (1983). *Biochemistry for the Medical Sciences*. Chichester, UK: John Wiley and Sons.
Overgaard, J. and Gesser, H. (2004). Force development, energy state and ATP production of cardiac muscle from turtles and trout during normoxia and severe hypoxia. *J. Exp. Biol.* **207**, 1915-1924.
Planas, J. V., Capilla, E. and Gutiérrez, J. (2000). Molecular identification of a glucose transporter from fish muscle. *FEBS Lett.* **481**, 266-270.
Reeves, R. B. (1963). Control of glycogen utilization and glucose uptake in the anaerobic turtle heart. *Am. J. Physiol.* **205**, 23-29.
Reynolds, T. H., 4th, Brozinick, J. T., Jr, Rogers, M. A. and Cushman, S. W. (1998). Mechanism of hypoxia-stimulated glucose transport in rat skeletal muscle: potential role of glycogen. *Am. J. Physiol.* **274**, E773-E778.
Rodnick, K. J., Bailey, J. R., West, J. L. and Driedzic, A. (1997). Acute regulation of glucose uptake in cardiac muscle of the American eel *Anguilla rostrata*. *J. Exp. Biol.* **200**, 2871-2880.
Rodnick, K. J., Gamperl, A. K., Lizars, K. R., Bennett, M. T., Rausch, R. N. and Keeley, E. R. (2004). Thermal tolerance and metabolic physiology among redband trout populations in south-eastern Oregon. *J. Fish Biol.* **64**, 310-335.
Rousseau, E., Smith, J. S. and Meissner, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *Am. J. Physiol.* **253**, C364-C368.
Schramm, M., Klieber, H. G. and Daut, J. (1994). The energy expenditure of actomyosin-ATPase, Ca^{2+}-ATPase and Na^{+},K^{+}-ATPase in guinea-pig cardiac ventricular muscle. *J. Physiol.* **481**, 647-662.
Shiels, H. A. and Farrell, A. P. (1997). The effect of temperature and adrenaline on the relative importance of the sarcoplasmic reticulum in contributing Ca^{2+} to force development in isolated ventricular trabeculae from rainbow trout. *J. Exp. Biol.* **200**, 1607-1621.
Shiels, H. A. and White, E. (2005). Temporal and spatial properties of cellular Ca^{2+} flux in trout ventricular myocytes. *Am. J. Physiol.* **288**, R1756-R1766.
Tada, M., Kirchberger, M. A. and Katz, A. M. (1975). Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **250**, 2640-2647.
Teerijoki, H., Krasnov, A., Pitkänen, T. I. and Mölsä, H. (2000). Cloning and characterization of glucose transporter in teleost fish rainbow trout (*Oncorhynchus mykiss*). *Biochim. Biophys. Acta* **1494**, 290-294.
Teerijoki, H., Krasnov, A., Gorodilov, Y., Krishna, S. and Mölsä, H. (2001). Rainbow trout glucose transporter (OnmyGLUT1): functional assessment in *Xenopus laevis* oocytes and expression in fish embryos. *J. Exp. Biol.* **204**, 2667-2673.
Tetens, V. and Christensen, N. J. (1987). Beta-adrenergic control of blood oxygen affinity in acute hypoxia exposed rainbow trout. *J. Comp. Physiol. B* **157**, 667-675.
Tetens, V., Lykkeboe, G. and Christensen, N. J. (1988). Potency of adrenaline and noradrenaline for β-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J. Exp. Biol.* **134**, 267-280.
Tibbits, G. F., Kashihara, H., Thomas, M. J., Keen, J. E. and Farrell, A. P. (1990). Ca^{2+} transport in myocardial sarcolemma from rainbow trout. *Am. J. Physiol.* **259**, R453-R460.
Tibbits, G. F., Moyes, C. D. and Hove-Madsen, L. (1992). Excitation-contraction coupling in the teleost heart. In *Fish Physiology: The Cardiovascular System*, Vol. XIIA (ed. W. S. Hoar, D. J. Randall, and A. P. Farrell), pp. 267-304. San Diego, CA: Academic Press
Tse, C. M. and Young, J. D. (1990). Glucose transport in fish erythrocytes: variable cytochalasin-B-sensitive hexose transport activity in the common eel (*Anguilla japonica*) and transport deficiency in the paddyfield eel (*Monopterus albus*) and rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **148**, 367-383.
Van Raaij, M. T. M., Van den Thillart, G. E. E. J. M., Vianen, G. J., Pit, D. S. S., Balm, P. H. M. and Steffens, A. B. (1996). Substrate mobilization and hormonal changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. *J. Comp. Physiol. B* **166**, 443-452.
West, T. G., Arthur, P. G., Suarez, R. K., Doll, C. J. and Hochachka, P. W. (1993). In vivo utilization of glucose by heart and locomotory muscles of exercising rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **177**, 63-79.
Whitesell, R. R., Regen, D. M., Beth, A. H., Pelletier, D. K. and Abumrad, N. A. (1989). Activation energy of the slowest step in the glucose carrier cycle: break at 23°C and correlation with membrane lipid fluidity. *Biochemistry* **28**, 5618-5625.
Wood, C. M. and Shelton, G. (1980). The reflex control of heart rate and cardiac output in the rainbow trout: interactive influences of hypoxia, haemorrhage, and systemic vasomotor tone. *J. Exp. Biol.* **87**, 271-284.
Wood, C. M., Pieprzak, P. and Trott, J. N. (1979). The influence of temperature and anaemia on the adrenergic and cholinergic mechanism controlling heart rate in rainbow trout. *Can. J. Zool.* **57**, 2440-2447.
Xu, K. Y. and Becker, L. C. (1998). Ultrastructural localization of glycolytic enzymes on sarcoplasmic reticulum vesticles. *J. Histochem. Cytochem.* **46**, 419-427.
Zar, J. H. (1999). *Biostatistical Analysis*, pp. 994. Upper Saddle River, NJ: Prentice Hall.
Zima, A. V., Kockskämper, J. and Blatter, L. A. (2006). Cytosolic energy reserves determine the effect of glycolytic sugar phosphates on sarcoplasmic reticulum Ca^{2+} release in cat ventricular myocytes. *J. Physiol.* **577**, 281-293.