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RESEARCH ARTICLE

Characterizing the metabolic capacity of the anoxic hagfish heart

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ABSTRACT

Pacific hagfish, *Eptatretus stoutii*, can recover from 36 h of anoxia at 10°C. Such anoxia tolerance demands the mobilization of anaerobic fuels and the removal of metabolic wastes – processes that require a functional heart. The purpose of this study was to measure the metabolic response of the excised, cannulated hagfish heart to anoxia using direct calorimetry. These experiments were coupled with measurements of cardiac pH and metabolite concentrations, at multiple time points, to monitor acid–base balance and anaerobic ATP production. We also exposed hagfish to anoxia to compare the *in vitro* responses of the excised hearts with the *in vivo* responses. The calorimetry results revealed a significant reduction in the rate of metabolic heat production over the first hour of anoxia exposure, and a recovery over the subsequent 6 h. This response is likely attributable to a rapid anoxia-induced depression of aerobic ATP-production pathways followed by an upregulation of anaerobic ATP-production pathways such that the ATP production rate was restored to that measured in normoxia. Glycogen-depletion measurements suggest that metabolic processes were initially supported by glycolysis but that an alternative fuel source was used to support the sustained rates of ATP production. The maintenance of intracellular pH during anoxia indicates a remarkable ability of the myocytes to buffer/regulate protons and thus protect cardiac function. Altogether, these results illustrate that the low metabolic demand of the hagfish heart allows for near-routine levels of cardiac metabolism to be supported anaerobically. This is probably a significant contributor to the hagfish's exceptional anoxia tolerance.

KEY WORDS: Anaerobic metabolism, Anoxia, Direct calorimetry, Glycogen, *Eptatretus stoutii*, Heart

INTRODUCTION

Hagfish are benthic fishes that spend considerable periods of time buried in the sediment and the putrefying carcasses of aquatic animals where they feed (Lesser et al., 1997; Martini, 1998). These environments are routinely hypoxic and even anoxic, so it is unsurprising that hagfish display an exceptional tolerance to hypoxia and anoxia. Hansen and Sidell (1983) demonstrated that anoxia and cyanide poisoning did not have an effect on the *in situ* function of the heart from the Atlantic hagfish *Myxine glutinosa*. Similarly, the Pacific hagfish *Eptatretus stoutii* has been shown to tolerate 36 h of anoxia at 10°C with a regular heartbeat and full recovery (Cox et al., 2010, 2011). Long-term anoxic survival like this demands the maintenance of certain organ functions within the animal, and one such critical organ is the heart. The maintenance of cardiac function under anoxic conditions allows for the mobilization of metabolic fuel stores (e.g. glycogen) from storage tissues (e.g. liver) and the removal of waste from metabolically active tissues (Stecyk et al., 2008). These two functions are critical to the anoxic survival of the animal and indeed, Cox et al. (2010) found that these are well maintained in anoxic hagfish: cardiac power output, a proxy for energy usage, decreased by only 25% in hagfish exposed to 36 h of anoxia. The maintenance of anoxic cardiac function in hagfish is thought to be associated with a relatively low cardiac metabolic requirement (Forster, 1991), such that the hagfish heart's routine power output – the lowest ever measured in a fish – is within the heart's maximum glycolytic potential (Farrell and Stecyk, 2007). The importance of glycolytic pathways to cardiac function in hagfish was demonstrated by Hansen and Sidell (1983). Using an *in situ* preparation on Atlantic hagfish, they found that despite inhibition of cardiac mitochondrial function with cyanide or sodium azide, cardiac output was maintained for at least 3 h. However, inhibiting glycolytic function with iodoacetate resulted in a significant decrease in cardiac output. The authors also found that the activity ratio of the enzymes pyruvate kinase (PK) to cytochrome c oxidase (CO) were 5.6-fold higher in the hagfish ventricle compared with that of cod. This was interpreted as the hagfish ventricle having a 'more anaerobically geared' metabolism (Hansen and Sidell, 1983). Together, these results suggest that anaerobic glycolysis plays an important role in maintaining cardiac function, and thus survival, in the anoxic hagfish.

Because the glycolytic capacity of the hagfish heart is thought to be capable of supporting routine cardiac function, and because this cardiac function is well maintained in anoxic environments, we hypothesized that the metabolic rate of the hagfish heart would be similarly maintained in anoxic environments. We tested this hypothesis by performing direct calorimetry on the excised, cannulated hearts of Pacific hagfish, *E. stoutii*, which are capable of beating for at least 24 h post-excision under anoxic conditions (Wilson, 2014). Direct calorimetry – the measurement of heat produced by, in this case, an excised heart – was used because it is the only technique that enables the continuous measurement of metabolic rate under anoxic conditions. In addition to the calorimetry measurements, we ran additional parallel anoxic exposures on excised, cannulated hearts and sampled them at three time points throughout the exposures to measure a variety of cardiac metabolites: lactate and glycogen levels were measured to estimate reliance on anaerobic ATP-production pathways, and intracellular pH (pH_i) was measured to determine how the heart's ability to maintain acid–base balance was influenced by anoxic exposure.

In addition to the excised heart experiments, we exposed two groups of hagfish to 36 h of normoxia and anoxia. Hagfish heart tissue was analysed for the same metabolites described above and blood was analysed for a number of haematological parameters.

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List of symbols and abbreviations

Hct	haematocrit
P_{50}	O_2 tension at which hemoglobin is 50% saturated
P_{CO_2}	partial pressure of dissolved CO_2
P_{O_2}	partial pressure of dissolved O_2
pH_i	intracellular pH
S_{O_2}	haemoglobin oxygen saturation
T_{O_2}	whole blood total O_2 content

This allowed us to compare the response of the *in vitro* heart with that of the *in vivo* heart, which was important because the *in vitro* heart would experience an almost immediate lack of O_2 upon induction of environmental anoxia, whereas *in vivo*, the heart becomes progressively hypoxic until true anoxia is reached when the fish's large O_2 stores are depleted; this could take a considerable period of time given the hagfish's low haemoglobin P_{50} value [~7–12 mmHg P_{O_2} (Wells et al., 1986; Forster et al., 1992)], large blood volume and low metabolic rate (Cox et al., 2010, 2011). This fact made the *in vivo* exposures especially important to testing our hypothesis that metabolic activity is maintained in the hagfish heart during prolonged periods of anoxia.

MATERIALS AND METHODS

Hagfish

Hagfish [*Eptatretus stoutii* (Lockington 1878)] were captured at ~100 m depth in Barkley Sound, British Columbia, Canada and transported to the Department of Fisheries and Oceans-University of British Columbia (UBC) Centre for Aquaculture and Environmental Research, West Vancouver, British Columbia. They were housed in 1100 litre tanks with aerated, flow-through seawater (~30‰ at 10±1°C) and fed squid once a week. Experimental animals were fasted for 1 week prior to use. All procedures were approved by the UBC Animal Care Committee (A08-0312) and conducted in accordance with their guidelines.

Calorimeter

The calorimeter used in our study was built in the lab of Lord Rothschild (Clarke, 1957). The instrument consists of a glass chamber embedded into either end of an aluminium cylinder (140 mm long ×70 mm diameter). One chamber is the experimental chamber the other is the reference chamber. When sealed with a rubber stopper, the volume of each glass chamber was 3.4 ml. Two stainless steel needles (23 gauge) were inserted through each stopper and connected to polyethylene (PE) 50 tubing so that each chamber had inflow and outflow lines. This allowed for physiological saline to be circulated through each chamber and for the cannulated heart to be perfused. A third needle was inserted through the rubber stopper of the experimental chamber for the insertion of a thermocouple so that temperature could be monitored. The PE tubing serving the calorimetry chambers was connected to a single peristaltic pump (Gilson Minipuls 3) that circulated saline from an Erlenmeyer flask through the system. Flow rate was set at 9.4 ml h^{-1} and flow was identical between chambers. An air-stone in the Erlenmeyer flask was used to aerate the saline or to remove oxygen using compressed nitrogen. An oxygen probe (Oakton DO 6+) placed in the Erlenmeyer flask was used to monitor dissolved oxygen concentrations throughout the experiment. The calorimeter was placed within a styrofoam cooler that was held within an insulated ice chest (Coleman 6-Day Xtreme, Golden, CO, USA). Inside the cooler, the calorimeter was placed on top of a layer of aluminium blocks that were used to draw heat from the experimental and reference chambers. The entire system was held within a temperature-controlled (8±1°C) environmental chamber for the duration of the study.

Heat calibration, completed using the procedure described by Regan et al. (2013), showed that 1 mV was equal to 0.67 mW. The calibration also revealed that the calorimeter achieved thermal equilibrium after approximately 2 h. This time lag was factored out of the heat traces using an 'instantaneous correction' (Bartholomew et al., 1981) for flow-through respirometry corrected for calorimetric heat data. The equation used was:

$$Q_{eq} = [(Q_e T - Q_e T_{-1})/(1 - e^{-dT(1/T_{lag})})] + Q_e T_{-1}, \quad (1)$$

where Q_{eq} is the time-corrected data point (in mW), Q_eT is the uncorrected data point at time T, Q_eT_{-1} is the uncorrected data point at time T_{-1}, dT is the time interval between successive data points (in min) and T_{lag} is the calorimeter's time lag (in min).

Heart dissection for calorimetry

The animals were killed using a sharp blow to the head followed by decapitation. The systemic heart was quickly excised and then washed in ice-cold hagfish saline (in mmol l^{-1}: 450 NaCl, 9 KCl, 10 $CaCl_2$, 50 $MgCl_2$ and 15 HEPES-Na buffer, pH 7.8). The bulbus arteriosus was removed so that saline could flow easily from the ventricle. A 3 cm cannula made from PE50 tubing was inserted through the atrium into the ventricle via the atrial ventricular valve. Surgical silk secured the cannula in place and the cannula was attached to the steel needle inserted through the rubber stopper of the experimental chamber. This allowed the heart to be perfused when sealed inside the chamber. Observation of the heart, prior to insertion into the system, revealed that the ventricle would inflate and then contract, releasing saline in a continuous pulsatile manner at a rate of approximately 6 bpm.

Measuring metabolic heat production

Once the heart was mounted inside the experimental chamber, aerated saline at 8°C was pumped through the system. This saline did not contain glucose because we wanted to accurately monitor the use of glycogen stores by the heart. To determine if we could measure the heat of metabolism with the calorimeter, we ran a number of preliminary experiments using conditions that would influence the metabolic rate of the heart. These included alternating periods of anoxia and normoxia, as well as the application of the poison iodoacetate to inhibit anaerobic glycolysis during anoxia exposure. In preliminary experiments we observed that if iodoacetate was not applied, the excised hagfish heart continued to contract for more than 24 h of experimentation, including prolonged periods of anoxia (Fig. S1).

To quantify the change in metabolic heat production with anoxia exposure, the hearts were perfused with aerated saline until a stable baseline was achieved (4.1±0.42 h). At this point, anoxia was induced by bubbling nitrogen into the circulating saline for the following 16 h and then iodoacetate was applied to the anoxic saline. An example of the data from such a protocol is Fig. 1A. The baseline voltage value varied slightly from run to run. It was therefore measured for each run once the output voltage stabilized after the application of iodoacetate. This baseline value was then subtracted from all measurements in the experiment. The dry mass of each heart was also obtained.

Converting metabolic heat production to ATP equivalents

To convert joules to approximate ATP equivalents in the normoxic hearts, we assumed the hearts were oxidizing glucose and yielded 36 moles of ATP per mole of substrate. Next, we calculated the theoretical quantity of waste heat in the entire aerobic ATP generation pathway by summing the standard free energy change for aerobic ATP production (7.3 kcal mol^{-1} ATP×36 moles ATP=+263 kcal) with the standard free energy change of glucose oxidation to CO_2 and H_2O (−686 kcal mol^{-1} glucose). This yielded a total of 423 kcal of heat produced per mole of glucose oxidized or 11.8 kcal of waste heat per mole of ATP produced via aerobic metabolism (423 kcal ×36 mol^{-1} ATP=11.8 kcal mol^{-1} ATP) equating to 49.2 kJ mol^{-1} ATP produced. We then used this number to convert the kJ detected by our calorimetry experiments to ATP equivalents produced by the normoxic hearts.

To convert joules to approximate ATP equivalents in the anoxic hearts, we assumed an entire reliance on anaerobic glycolysis and a range of glycolytic ATP yields, from 2 to 3 moles ATP per mole of substrate. Using the method described above and assuming a glucose-to-lactate free energy change of −47 kcal mol^{-1} glucose, we calculated 67.8 kJ mol^{-1} ATP (2 moles ATP per mole substrate) and 35.2 kJ mol^{-1} ATP (3 moles ATP per

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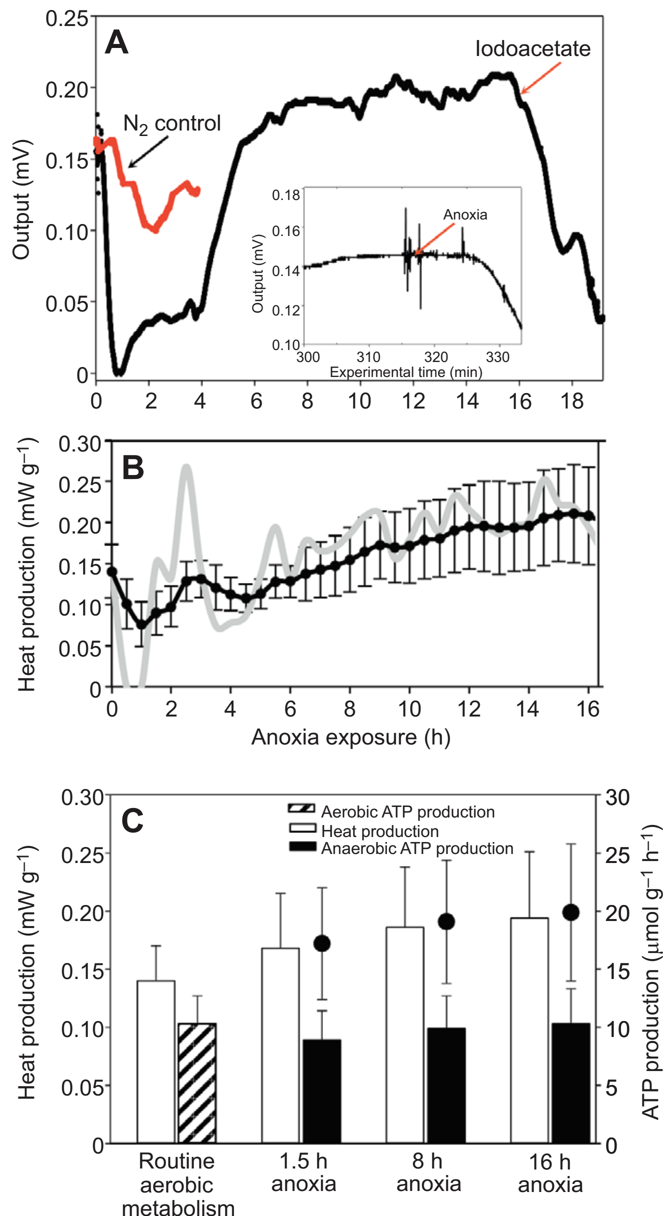

Fig. 1. Metabolic heat production by excised hagfish hearts during anoxia exposure. (A) Representative voltage trace from a single experiment with an excised heart. Inset is an expansion of the trace at the onset of anoxia exposure. The superimposed red line on the panel shows how the voltage measurement (in this case, from a control run) is affected by compressed N_2 being bubbled into the saline. Time is in hours of anoxia exposure or minutes following mounting and perfusion of the heart with aerated saline (inset). Iodoacetate was applied to the anoxic heart at 16 h to inhibit glycolysis. (B) Average metabolic heat production of the excised hearts during 16 h of anoxia. The black line represents the averaged data prior to correction for the time lag of the calorimeter. The grey line is generated using Bartholomew et al.'s (1981) 'instantaneous correction' for flow-through respirometry, adapted for calorimetry. (C) Metabolic heat production and ATP production by excised hagfish hearts during normoxia and anoxia. The striped bar is aerobic ATP production while the black bars are glycolytic ATP yields calculated assuming that 2 moles of ATP are generated per mole of substrate. The black circles are the values calculated for each time point assuming that 3 moles of ATP are generated per mole of substrate. Routine aerobic metabolism refers to the rate of metabolism just prior to the start of anoxia. Values were calculated using metabolic heat production data that was time-corrected and are reported as means±s.e. There are no statistical differences between any measurements. N=5 hearts.

mole substrate) produced via anaerobic metabolism, which we used to convert the kJ detected by our calorimetry experiments to ATP equivalents produced by the anoxic hearts.

Heart preparation for tissue metabolite analysis

We used the same exposure protocols as described for the calorimetry studies to monitor changes in tissue pH and metabolite concentrations in the excised hearts at multiple time points during anoxia exposure. The only difference was that the glass test tubes containing the cannulated hearts were held in a beaker of saline instead of the calorimeter. The hearts were sampled after 6 h of normoxia, and after 1.5 h and 8 h of anoxia: 6 h of normoxia was chosen because this was the time required for the excised hearts to reach an initial thermal equilibrium in normoxia; 1.5 h of anoxia was chosen because the maximum decrease in heat production was seen at 2.38±0.83 h of anoxia and we wanted to identify the basis for suppression of aerobic metabolism; and 8 h of anoxia was selected as the third sampling time because the rate of metabolic heat production was still increasing at this point. It is important to note that all hearts were observed to be beating when sampled. To preserve the tissues, hearts were quickly removed from the system, rinsed in ice-cold saline, blotted dry and then quickly frozen on a ∼2 kg piece of aluminium stored at −80°C. These samples were then kept at −80°C until analysis.

Metabolite analyses

We measured cardiac concentrations of lactate, glycogen and glucose by first sonicating ∼40 mg of frozen ground cardiac tissue in 10 volumes of a metabolic inhibitor solution consisting of 150 mmol l^{-1} KF and 6 mmol l^{-1} nitrilotriacetic acid (Portner, 1990). We then measured the pH_i of this homogenate using an Accumet microcombination pH electrode (Fisher Scientific, 13-620-96). Next, we prepared the remaining homogenate for metabolite measurements by acidifying it in 30% perchloric acid, neutralizing it in 3 mol l^{-1} potassium carbonate, then centrifuging it at 10,000 ***g*** for 10 min (4°C). We extracted the resulting supernatant and stored it at −80°C for future analyses. Once thawed, the supernatant was centrifuged at 10,000 ***g*** for 10 min (4°C) before measuring lactate, glycogen and glucose levels according to Bergmeyer et al. (1983).

In vivo anoxia exposure

To examine the influence of anoxia exposure on the blood composition and heart metabolites of hagfish, live animals were exposed to either 36 h of normoxia or anoxia at 8°C, as previously described (Cox et al., 2011). Following treatment or control exposure, samples of mixed venous blood were taken from the large caudal blood sinus, as previously described (Cox et al., 2011) and placed on ice. The fish were then killed and their hearts were quickly frozen. The blood was analysed immediately after sampling for haemoglobin (Hb) concentration, haematocrit (Hct), partial pressure of dissolved CO_2 (P_{CO_2}), Hb O_2 saturation (S_{O_2}) and whole blood pH. Plasma was also frozen and later analysed for glucose and lactate. The hearts were stored at −80°C until they were used to determine tissue metabolites and pH_i.

Measurement of plasma composition

Hct was measured in triplicate using micro Hct tubes (10 µl) after centrifuging at 17,000 ***g*** for 3 min. Hb concentration was measured in triplicate with a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) using the cyanomethaemoglobin method and was calculated based on absorption measurements at 540 nm and using an extinction coefficient of 11. Whole-blood pH measurements were performed using a Radiometer BMS 3 Mk2 system, maintained at 5°C with a Radiometer acid–base analyzer PHM71 (Copenhagen, Denmark). Whole-blood total O_2 content (T_{O_2}) was measured according to Tucker (1967). S_{O_2} was calculated from T_{O_2} after subtracting physically dissolved O_2 according to Boutilier et al. (1984). Total CO_2 in the blood plasma was measured using a Corning 965 CO_2 analyzer (Corning, NY, USA), after centrifuging whole blood for 5 min at 5000 ***g***. Plasma HCO_3^- concentration and blood P_{CO_2} were calculated from the plasma pH and T_{CO_2} using the Henderson–Hasselbalch equation and the physicochemical parameters reported by Boutilier et al. (1984). The concentrations of plasma lactate and glucose were measured using a YSI 2300 Stat Plus analyzer (Yellow Springs, OH, USA).

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Statistical analysis

The differences in blood parameters, plasma components, metabolites in the hearts and pH_i between the control and anoxia-exposed animals were tested using an unpaired, two tailed *t*-test ($P<0.05$). The differences in tissue metabolites, pH_i, heat loss and ATP turnover rates from the calorimetry studies were tested using a single-factor ANOVA followed by a Bonferroni *post hoc* test ($P<0.05$).

RESULTS

Influence of anoxia exposure on the metabolic rate of the excised heart

Fig. 1A is a representative trace from one of the calorimetry experiments. The measured voltage is directly proportional to the heat generated by the heart and detected by the thermopiles surrounding the experimental chamber. This figure demonstrates that perfusion of the heart with anoxic saline causes an initial decrease in metabolic heat production, but that this recovers with time. This trace also demonstrates a decrease in metabolic heat production caused by the application of iodoacetate during anoxia exposure, supporting the idea that the heat being detected by the calorimeter was indeed metabolic. The inset in Fig. 1A shows the voltage before and after the heart was perfused with anoxic saline, and demonstrates that the heart had reached a steady heat output before it was made anoxic. The time required for the excised hearts to reach an aerobic steady state prior to the anoxia exposure was 4.1±0.42 h. The red line on Fig. 1A is the voltage output from a control experiment where anoxic saline was perfused through the chamber in the absence of a heart. The rapid deoxygenation of the chamber has an observable and consistent effect on the calorimeter's thermal equilibrium, probably because of a slight decrease in the temperature of the circulating saline caused by the compressed N_2 bubbling in the Erlenmeyer flask. All voltage traces were corrected to compensate for this experimental artefact. The black line on Fig. 1B is the average metabolic heat produced by the hearts during anoxia exposure calculated directly from the voltage output and corrected for the initial decrease in output voltage caused by the application of anoxic saline to the system. The grey line in Fig. 1B is that generated when the average metabolic heat production data are de-smeared to compensate for the time lag of the calorimeter. The average rates of ATP and metabolic heat production, at discrete time points in Fig. 1C, result from this correction.

The metabolic heat production of the excised hearts at the initial aerobic steady state condition was 0.14±0.03 mW g^{-1}. This equalled an estimated ATP turnover rate of 10.3±2.4 µmol g^{-1} h^{-1} (Fig. 1C). The de-smeared data shown in Fig. 1B illustrate that the application of anoxic saline causes metabolic heat production to rapidly drop to zero. After approximately 1 h, metabolic processes began to recover, so that by 1.5 h of anoxia, metabolic heat production was 0.168±0.05 mW g^{-1}, which, depending on ATP yield per mole substrate, was equal to an estimated ATP turnover rate of 8.9±2.5 µmol g^{-1} h^{-1} (2 moles ATP per mole substrate) to 17.2±4.9 µmol g^{-1} h^{-1} (3 moles ATP per mole substrate) (Fig. 1C). Metabolic heat production continued to recover during the anoxia exposure so that by 8 h it was 0.18±0.05 mW g^{-1}, equal to an estimated ATP turnover rate of 9.9±2.8 µmol g^{-1} h^{-1} (2 moles ATP per mole substrate) to 19.1±5.3 µmol g^{-1} h^{-1} (3 moles ATP per mole substrate) (Fig. 1C). At 16 h of anoxia exposure, just prior to the application of iodoacetate, heat production was 0.19±0.06 mW g^{-1}, which is equal to an estimated ATP turnover rate of 10.3±3.04 µmol g^{-1} h^{-1} (2 moles ATP per mole substrate) to 19.9±5.9 µmol g^{-1} h^{-1} (3 moles ATP per mole substrate) (Fig. 1C). It is important to note that these heat-derived ATP-turnover calculations are estimations based on standard free energy changes and assumptions provided in the Materials and methods. Cellular concentrations of reactants and products will affect these estimations of ATP turnover.

Influence of anoxia on pH_i and metabolic substrates

As the saline used to perfuse the excised hearts did not contain glucose, it is not surprising that tissue glycogen

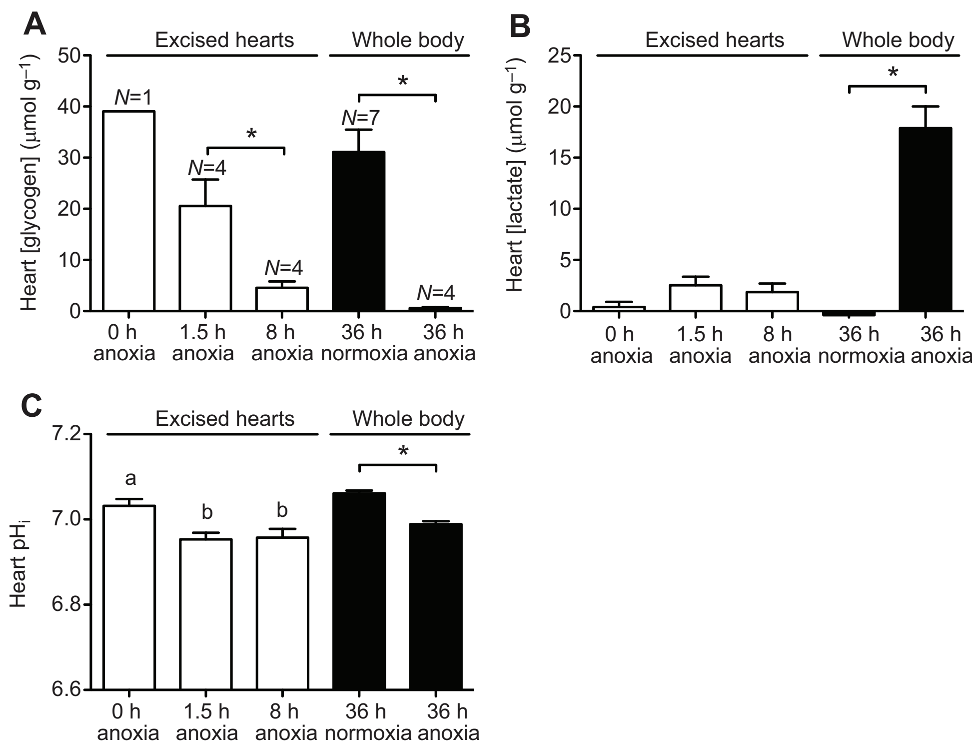

Fig. 2. The influence of anoxia exposure on metabolic fuels and intracellular pH in the isolated hagfish heart or in the heart of hagfish exposed to anoxia for 36 h. (A) Tissue glycogen; (B) tissue lactate; (C) intracellular pH (pH_i). 'Excised hearts' (open bars) indicates *in vitro* exposure to anoxia, and 'Whole body' (closed bars) indicates hearts sampled from live animals (*in vivo*) at the reported times. '0 h anoxia' represents hearts sampled after perfusion for 6 h with aerated saline. '1.5 h/8 h anoxia' represents hearts sampled after perfusion for 6 h with aerated saline and then 1.5 h or 8 h, respectively, with anoxic saline. Statistically significant differences are either indicated by letters that differ or by a bracket and asterisk ($P<0.05$). *N* (number of hearts) =5 for the excised heart studies and *N*=9 for the *in vivo* studies. Values are means±s.e.

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Table 1. Blood parameters in mixed venous blood of hagfish exposed to 36 h of anoxia

Parameter	36 h normoxia	36 h anoxia
Hb (mmol l^{-1})	1.52±0.14	2.87±0.37*
Haematocrit (%)	10.35±0.82	17.78±1.21*
P_{CO_2} (mmHg)	1.91±0.16	13.43±0.70*
pH	7.81±0.03	6.97±0.07*
S_{O_2} (%)	68.55±2.44	−0.31±0.9*
Lactate (mmol l^{-1})	0.009±0.001	28.03±1.29*
Glucose (mmol l^{-1})	0.78±0.13	2.60±0.11*

Hb, haemoglobin; haematocrit calculated as proportion of plasma, by volume, of red blood cells. P_{CO_2}, partial pressure of dissolved CO_2; S_{O_2}, haemoglobin oxygen saturation. *P<0.05, significant difference between the means of treatment and control values. N=9 for all values.

decreased during the anoxia exposure. This also confirms that there was metabolic activity. Between 1.5 h and 8 h of anoxia, glycogen stores decreased by 78% (Fig. 2A). These levels are lower than that measured after 6 h of perfusion with aerated saline (i.e. normoxia on Fig. 2A). These differences could not be tested, as we were only able to measure glycogen in a single heart that had been perfused with aerated saline. However, the glycogen level measured in this heart was similar to that measured in the hearts of the control animals in the *in vivo* study (Fig. 2A). The glycogen levels in the hearts sampled after 8 h of anoxia in the *in vitro* study were 87% lower than those in the hearts sampled prior to anoxia exposure, whereas the levels of glycogen in the hearts of the fish exposed to anoxia for 36 h in the *in vivo* study were 97% lower than those in the control fish (Fig. 2A).

Lactate concentrations in the excised hearts were 3- and 5-fold higher at 1.5 h and 8 h anoxia than they were at 0 h anoxia (Fig. 2B). However, this level of lactate accumulation is small compared with that of the *in vivo* hearts and that expected from the measured rates of glycogen depletion (Fig. 2A), and is probably a function of lactate loss to the perfusate. In the hearts from the *in vivo* study, the concentration of lactate was 0 in the normoxic group but 17.9±1.3 µmol g^{-1} in the hearts of the anoxic group (Fig. 2B).

The excised hearts from the *in vitro* study had pH_i values of 7.03, 6.95 and 6.96 at 0 h, 1.5 h and 8 h of anoxia, respectively (Fig. 2C). While statistically significant, an absolute change in pH of 0.08 pH units over 8 h is relatively small for a completely anoxic tissue and suggests the heart was buffering and/or extruding the protons resulting from anaerobic glycolysis. Similarly, the hearts of the anoxia-exposed hagfish from the *in vivo* study were 0.07 pH units lower compared with those of the normoxia-exposed hagfish (Fig. 2C).

Influence of anoxia on behaviour and blood composition of hagfish

Normoxic hagfish remained in a coiled position within the exposure chamber and periodically changed position. Under anoxic conditions, the hagfish were uncoiled and displayed no body movement, consistent with what has been seen previously (Cox et al., 2010, 2011).

Thirty-six hours of anoxia significantly altered blood composition (Table 1). For example, haemoglobin content was 1.9-times higher in anoxic hagfish than in normoxic hagfish (Table 1), probably largely due to the 1.7-times higher haematocrit of anoxic hagfish (Table 1). Haemoglobin oxygen saturation was 0% in the anoxic hagfish and 68.6% in the normoxic hagfish, suggesting that 36 h of anoxia was enough to exhaust the blood's oxygen stores (Table 1). P_{CO_2} was 7-times higher in the anoxic fish and plasma lactate increased from ~0 mmol l^{-1} in the normoxic hagfish to 28.0 mmol l^{-1} in the anoxic hagfish. Also, blood pH was reduced from 7.81 in the normoxic fish to 6.97 in the anoxic fish. Finally, the 3.3-times higher plasma glucose concentrations of anoxic fish suggest they were mobilizing glucose stores (Table 1).

DISCUSSION

The results of this study illustrate the significant ability of the Pacific hagfish heart to maintain metabolic function during prolonged periods of anoxia both *in vitro* and *in vivo*. Excised hearts initially responded to anoxia with a significant reduction in metabolic heat production, but recovered after 1.5 h to levels that translated to ATP production rates similar to those of normoxic control hearts. These metabolic rates confirm the hypothesis that the low metabolic demand of the hagfish heart allows routine levels of cardiac metabolism to be supported anaerobically.

Influence of anoxia exposure on the metabolic rate of excised hearts

The general description of cardiac function in the hagfish is 'low and slow'. This is supported by the comparatively low activity of enzymes associated with both aerobic and anaerobic metabolism (Hansen and Sidell, 1983), ventricular blood pressures that are 5- to 10-times lower in hagfish than in any other fish (Cox et al., 2010; Forster et al., 1988; Johnsson and Axelsson, 1996) and a resting heart rate of *E. stoutii* in normoxia of approximately 8 bpm (Cox et al., 2010). In trout, heart rate is 31 bpm at 15°C (Altimiras and Larsen, 2000) and 45 bpm in bluefin tuna under routine conditions (Clark et al., 2013). This low level of cardiac work in the hagfish – the lowest of any fish species studied to date – led Forster (1991) to predict that it could be supported solely by the tissue's glycolytic potential. Such a strategy, fuelled by glycogen stores, would enable the maintenance of cardiac function during anoxia. The results of our study support this idea.

Integration of the heart glycogen measurements into the calorimetry results provides some interesting insight into fuel utilization by the excised hearts during anoxia exposure. First, it appears that the rate of glycogen utilization decreased during anoxia exposure. This is suggested by comparing the rate of glycogen utilization between sampling points. If we assume that the average amount of glycogen in the excised hearts at time 0 was similar to that measured in the normoxic hearts from the *in vivo* experiment (31.1±4.35 µmol g^{-1}), the rate of glycolysis between time 0 and 1.5 h of anoxia was approximately 7 µmol glucosyl units g^{-1} h^{-1} whereas that between 1.5 h and 8 h of anoxia was 2.5 µmol glucosyl units g^{-1} h^{-1}. These rates of glycogen utilization translate into ATP production rates of 14.0–21.0 (2 and 3 moles of ATP, respectively) and 4.9–7.4 µmol g^{-1} h^{-1}, respectively (Fig. 3). Interestingly then, these calculated rates also suggest that as the period of anoxia continues, the rate of anaerobic metabolism estimated by calorimetry cannot be supported solely by the glycogen stored in the heart. The metabolic heat measurements at times 1.5 h, 8 h and 16 h of anoxia translate into ATP production rates of 8.9±2.5 (to 17.2±4.9), 9.9±2.8 (to 19.1±5.3) and 10.3±3.0 (to 19.9±5.9) µmol g^{-1} h^{-1}, respectively. In addition, based on the assumption that the glycogen in the heart at time 0 was 31.1±4.35 µmol g^{-1}, the amount measured in the hearts at 8 h indicates an 85% decrease in glycogen content. If we then assume that the glycogen stores were completely depleted over the next 8 h (8–16 h anoxia), the rate of ATP production

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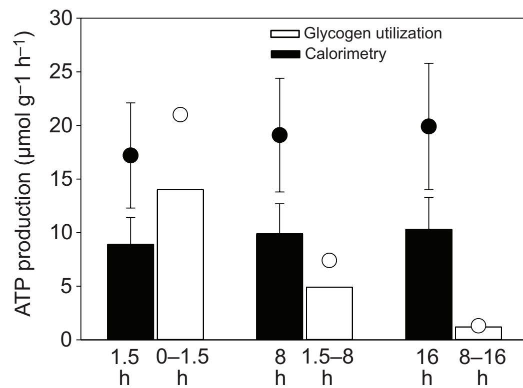

Fig. 3. A comparison of ATP production rates calculated using calorimetry measurements and the utilization of tissue glycogen between sampling points. The black bars are glycolytic ATP yields calculated from the calorimetry measurements assuming that 2 moles of ATP are generated per mole of substrate. The black circles are the values calculated for each time point assuming that 3 moles of ATP are generated per mole of substrate. The white bars are glycolytic ATP yields calculated from the change in glycogen concentrations assuming that 2 moles of ATP are generated per mole of substrate. The white circles are the values calculated for each time point assuming that 3 moles of ATP are generated per mole of substrate. The rate of ATP production between 8 and 16 h determined using glycogen stores was calculated assuming that there was no glycogen in the heart at 16 h. Values are means±s.e.

during this time would have been approximately 1.2 μmol g^{-1} h^{-1}. However, the sustained levels of metabolic heat over the 8–16 h time frame suggest that no such metabolic depression occurs (Fig. 3). We therefore think that another fuel, in addition to glycogen, may have been supporting metabolic activity during this time frame. Cox et al. (2010, 2011) have previously suggested that the hagfish heart may use an alternative fuel source during anoxia to supplement glycogen stores, which must be the case given that the metabolic rate is maintained during anoxia in the current study (Fig. 3). One alternative fuel may be glycerol, which could be sourced from the breakdown of triglycerides stored in the heart and enter glycolysis via the enzyme glycerol kinase. We are currently examining this possibility.

The red-eared slider turtle *Trachemys scripta* and the crucian carp *Carassius carassius* are highly tolerant of anoxia but employ different survival strategies (Galli et al., 2013; Hicks and Farrell, 2000; Lau and Richards, 2011; Stecyk et al., 2008; van Waversveld et al., 1989). In the turtle, metabolic rate and cardiac output decrease rapidly with anoxia exposure and then remain low for the duration of the anoxia exposure (Galli et al., 2013; Hicks and Farrell, 2000; Lau and Richards, 2011; Stecyk et al., 2008). In the crucian carp, anoxia exposure causes a rapid decrease in cardiac output (metabolic rate has not been measured) that returns to pre-anoxia levels with the upregulation of anaerobic glycolysis, allowing the animal to remain active for at least 5 days (Stecyk et al., 2004). The anoxic response of the hagfish heart in this study is therefore similar to the anoxia response of the crucian carp, where metabolic rate is initially depressed and then recovers as a result of the upregulation of anaerobic ATP production pathways. Our results suggest that this metabolic recovery is quicker in the hagfish than in the crucian carp.

Cox et al. (2010) demonstrate that exposure of *E. stoutii* to anoxia causes heart rate to decrease by 55%, but that stroke volume nearly doubles. The net result is that cardiac output only decreases by ∼33% with anoxia exposure. This work also demonstrated that power output from the heart decreased by only 25%. As power output can be used as a surrogate for energy requirement, this demonstrates that there are still relatively high energy demands of the heart during anoxia exposure.

There are a number of factors that must be taken into account when considering the results of the calorimetry study. First, it was not possible to monitor the rate of contraction, but we did see the hearts contracting throughout the metabolite study under identical conditions and previous work has found that the excised heart of Pacific hagfish continues to beat during 24 h of anoxia exposure (Wilson, 2014). We did not set pre- or after-load on the excised heart because of the technical limitations associated with the calorimetry system. However, *in vivo*, the hagfish heart works at extremely low pressures with ventral aortic pressure being ∼1 kPa during anoxia exposure (Cox et al., 2010). Therefore, it would not take much resistance within the tubing to generate a physiological afterload for the cardiac contractions. The fact that we did not control the afterload on the heart may not be that significant an issue. The energy requirements of the hearts in our excised experiments, however, may not have been as high as that found *in vivo*. This may explain why Cox et al. (2010) observed a mass-specific cardiac power output (the product of cardiac output and ventral aortic blood pressure, expressed as mW g^{-1}) that was approximately 50% higher than the metabolic rates (expressed in mW g^{-1}) measured here in both normoxia and 12 h anoxia. Interestingly, while the excised hearts in our study showed a significant reduction in metabolic rate over the first hour of anoxia, the *in vivo* hearts measured in the Cox et al. (2010) study showed a significant increase in cardiac power output. This increased power output was primarily the result of an increase in ventral aortic blood pressure. Since the excised hearts in our system were held at a constantly low pressure, this may in part explain the different findings of the two studies.

The *in vivo* heart becomes progressively rather than abruptly anoxic. For example, Forster et al. (2001) estimated that the large blood volume of the hagfish has an O_2 storage capacity of 137.9 ml O_2 kg^{-1} for *E. stoutii*, a number twice that of any other fish. This represents a significant reserve of on-board oxygen that has been estimated to support the hagfish's routine metabolic rate [assuming an O_2 consumption rate of 20.6±1.0 mg O_2 kg^{-1} h^{-1} (Cox et al., 2010)] in complete anoxia for approximately 25 min; for a hagfish with a reduced aerobic metabolism, this would be even longer, with estimations between 3 and 6 h for the final molecules of O_2 to be used by the animal (Cox et al., 2010). Certainly by 36 h of anoxia, as indicated by the Hb O_2 saturation levels measured in the *in vivo* anoxia-exposed fish in our study, the last of the blood O_2 stores had been used up. Thus, the initial response of the excised heart to anoxia is indeed an initial response to anoxia; there are no behavioural responses that could influence cardiac function as has been suggested for previous *in vivo* studies (Cox et al., 2010; Forster, 1990; Forster et al., 1992). Furthermore, apart from the relatively small myoglobin-bound O_2 reserves in the cardiac cells themselves (Davison et al., 1990), there are no large stores of O_2 that could obfuscate the onset of cardiac anoxia. The initial metabolic response of the hagfish heart to anoxia is therefore an immediate depression in metabolic rate – probably as a result of rapid depression of aerobic ATP production – followed by a re-establishment of metabolic rate to approximately routine levels, which is probably due to upregulation of anaerobic glycolysis .

Anaerobic metabolites of the heart and blood

Cardiac glycogen depletion in both the anoxia-exposed excised and *in vivo* hearts indicates that anaerobic glycolysis is taking place. The rate of glycogen depletion appears to be faster in the excised hearts than the *in vivo* hearts. This is likely attributable to two aspects of

the calorimetry set-up: first, as mentioned above, the onset of anoxia in the excised hearts was probably quicker than in the *in vivo* hearts; second, the cardiac glycogen stores in the *in vivo* hearts were probably augmented by the mobilization of glycogen stores from elsewhere in the animal, as seen by the elevated glucose levels of anoxic hagfish.

Tissue lactate levels did not increase significantly in the anoxia-exposed excised hearts, but they did in the *in vivo* hearts. This could be because the lactate produced by the myocytes of the excised heart was lost into the perfusing saline and thus diluted by its relatively large volume (approximately 25-fold that of the blood volume *in vivo*). The lactate therefore never accumulated in the excised heart itself, but rather continually moved down its concentration gradient and into the saline over the course of the experiment. In the *in vivo* exposures, the accumulation of lactate in the plasma from all of the glycolytically active tissues of the body might decrease the rate at which it could move from the heart into the blood. It would therefore accumulate in the heart, and this could be why our results show an accumulation of lactate in the *in vivo* hearts but not in the excised hearts.

In addition to this accumulation of lactate, the *in vivo* hearts showed significantly lower glycogen concentrations compared with the normoxic control hearts. These concentrations were so low by the 36 h anoxic time point as to be essentially depleted, a result consistent with Cox et al. (2011), who found that cardiac glycogen stores of *in vivo* hagfish hearts were depleted after 24 h of anoxia. Like our fish, their fish survived until the 36 h time point, suggesting that the glycogen required by the hearts was sourced from storage tissues such as the liver for at least 12 h and/or was augmented by another fuel besides glycogen.

pH of the heart and blood

Reductions in blood and intracellular pH are concomitant with anaerobic glycolysis (Robergs et al., 2004). Consistent with this, the blood pH of *in vivo* anoxia-exposed fish was 6.97, 0.84 pH units lower than the blood of the normoxia-exposed fish. Despite this reduction, the pH_i of the anoxic hearts was only 0.07 pH units lower than the normoxic hearts, and the pH_i of the excised hearts sampled at 1.5 h and 8 h anoxia was almost identical to that seen in the hearts from the *in vivo* experiment after 36 h of anoxia exposure. This suggests an ability of the hagfish cardiac myocytes to buffer and/or actively regulate intracellular H^+ concentrations during a metabolic acidosis, and is consistent with regulation of pH_i in the hagfish heart in response to a respiratory acidosis (Baker et al., 2015). The contractile element in cardiac myocytes is extremely sensitive to pH change (Gillis et al., 2000), so this defence of pH_i is essential to the maintenance of cardiac function during anoxia exposure.

There may have been more than just glycolytically sourced H^+ contributing to the blood acidosis measured in the *in vivo* anoxia-exposed fish. Although we did not measure it, it is possible that the chambers that held the fish for the duration of the anoxia exposures became hypercarbic (i.e. elevated environmental CO_2 levels). Plasma P_{CO_2} measurements of approximately 13 mmHg for the anoxia-exposed fish (compared with approximately 2 mmHg for the normoxia-exposed fish at the same 36 h time point) support this possibility. We believe the source of this CO_2 was the fish within the chambers. More precisely, as the flow of water through the chamber was only stopped when P_{O_2} measurements reached 0 mmHg, the source of this CO_2 must have been the fish's O_2 reserves. Interestingly, by combining an estimation for O_2 storage capacity of *E. stoutii* (137.9 ml O_2 kg^{-1}) (Forster et al., 2001) with the total mass of tissue in the chambers, we calculate a P_{CO_2} for the 2.5 litre chambers of 15.1 mmHg, which is very similar to the P_{CO_2} of 13.4 mmHg that we measured in the blood of the anoxic fish. So the hagfish's large O_2 stores probably caused the chambers' hypercarbia. But how did the hypercarbia influence the hagfish's response to anoxia? It is possible that an accumulation of H^+ in the blood stemming from the hydration of CO_2 slowed the rate of H^+ diffusion from the tissues into the blood. This would slow the production of lactate in the tissues, and subsequently, the production rates of glycolytically sourced ATP (e.g. Cochran and Burnett, 1996). In any case, the preferred *E. stoutii* habitats of sandy, muddy burrows (Martini, 1998) and their penchant for feeding within the rotting remains of large, dead vertebrates (Martini, 1998) suggest that when these animals encounter anoxia in the natural environment, they very likely encounter hypercarbia too. Anoxia together with hypercarbia may then be the most ecologically relevant anoxic exposure for these animals.

Summary and perspective

The hagfish heart appears to be an incredibly robust organ capable of maintaining metabolic activity during prolonged periods of anoxia. These characteristics, coupled with a large blood volume to store metabolic wastes, enable cardiac function in the animals to be maintained during extended periods of anoxia with minimal detrimental effects to the animal.

This study represents the first time direct calorimetry has been performed on an excised, functional organ. This technique allowed us to measure the direct and immediate response of the heart to complete anoxia, and highlighted two results in the process that we might not have predicted from *in vivo* experiments alone. First, there was a significant and immediate depression in cardiac metabolic rate with the induction of anoxia, followed by a re-establishment of metabolic rate to near-routine levels when expressed as ATP production rates. This finding, illustrating that metabolic activity is sustained in the heart during anoxia exposure, is supported by substrate use and the accumulation of metabolic wastes. We suspect that the initial metabolic depression is the result of an immediate arrest of aerobic respiration, while the re-establishment of metabolic rate is the result of the activation of anaerobic metabolism supported by glycogen. And second, calorimetry revealed a maintenance of metabolic heat long after the cardiac glycogen levels were likely to have been depleted, suggesting that the hagfish heart utilizes an alternative fuel during prolonged periods of anoxia. This is clearly an area worthy of further study.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
T.E.G., M.D.R. and A.P.F. designed the study. T.E.G., M.D.R., G.K.C. and T.S.H. completed the experiments. M.D.R. performed metabolite analyses. T.E.G., M.D.R. and T.S.H. analysed the data and all authors wrote the paper.

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Supplementary information

Supplementary information available online at
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