Journal of Comparative Physiology B (2019) 189:199–211
https://doi.org/10.1007/s00360-019-01208-w

ORIGINAL PAPER

# Contractile function of the excised hagfish heart during anoxia exposure

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Received: 26 September 2018 / Revised: 24 January 2019 / Accepted: 29 January 2019 / Published online: 6 February 2019


## Abstract

Pacific hagfish, *Eptatretus stoutii*, can recover from 36 h of anoxia and their systemic hearts continue to work throughout the exposure. Recent work demonstrates that glycogen stores are utilized in the *E. stoutii* heart during anoxia but that these are not sufficient to support the measured rate of ATP production. One metabolic fuel that could supplement glycogen during anoxia is glycerol. This substrate can be derived from lipid stores, stored in the heart, or delivered via the blood. The purpose of this study was to determine the effect of glycerol on the contractile function of the excised *E. stoutii* heart during anoxia exposure. When excised hearts, perfused with metabolite free saline (mf-saline), were exposed to anoxia for 12 h, there was no difference in heart rate, pressure generation (max-dP), rate of contraction (max-dP/$dt_{sys}$), or rate of relaxation (max-dP/$dt_{dia}$) compared to hearts perfused with mf-saline in normoxia. However, hearts perfused with saline containing glycerol (gly-saline) in anoxia had higher max-dP, max-dP/$dt_{sys}$, and max-dP/$dt_{dia}$ than hearts perfused with mf-saline in anoxia. Tissue levels of glycerol increased when hearts were perfused with gly-saline in normoxia, but not when perfused with gly-saline in anoxia. Anoxia exposure did not affect the activities of triglyceride lipase, glycerol kinase, or glycerol-3-phosphate dehydrogenase. This study suggests that glycerol stimulates cardiac function in the hagfish but that it is not derived from stored lipids. How glycerol may stimulate contraction is not known. This could be as an energy substrate, as an allosteric factor, or a combination of the two.

**Keywords** Contractile function · Anaerobic metabolism · Pressure generation · Working heart preparation · Glycerol

## Introduction

The Pacific hagfish, *E. stoutii*, has a remarkable ability to survive long-term exposure to environmental anoxia. Cox et al. (2010) demonstrated that this species can completely recover from 36 h of anoxia. This ability is likely relevant to their behaviors of burrowing into large, dead, animals on the bottom of the ocean to feed, as well as remaining buried in the mud on the ocean floor (Sidell and Beland 1980; Martini 1998; Bucking et al. 2011). The study by Cox et al. (2010) also demonstrated that the function of the systemic heart is maintained in *E. stoutii* during anoxia exposure. In hagfish, the systemic heart, one of four hearts, is comprised of the sinus venosus located upstream of a single atrium and ventricle (Satchell 1991), and blood moves from the jugular vein (Forster et al. 1991), to the ventral aorta towards the gill slits (Johansen 1960). During anoxia exposure, the heart rate ($f_H$) of the systemic heart was found to decrease in *E. stoutii* by 50% in the first 6 h, while stroke volume was more than doubled (Cox et al. 2010). As a result, cardiac output decreased by only 33% (Cox et al. 2010). This continued function of the heart in anoxia must be supported through anaerobic glycolysis.

Recent work using an excised heart preparation demonstrated that glycogen stores are utilized in the systemic heart of *E. stoutii* during anoxia exposure, but also suggests that other substrates support the measured rate of ATP production (Gillis et al. 2015). Specifically, Gillis et al. (2015),

Communicated by H. V. Carey.

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using a perfused heart preparation functioning inside a calorimeter, demonstrated that ATP production is sustained throughout a 16 h anoxic perfusion at the same level as was measured under normoxic conditions. Glycogen stores were also found to have decreased by 85% after the first 8 h of anoxia exposure (Gillis et al. 2015). The remaining glycogen stores would not have been sufficient to support the measured metabolic rate for the remaining 8 h (Gillis et al. 2015). As there were no energy substrates present in the saline perfusing these hearts, it was suggested that they were using other stored metabolic fuels during chronic anoxia exposure (Gillis et al. 2015).

One potential source of metabolic fuel in the hagfish heart during anoxia exposure is lipid droplets (LDs) found in the myocardium. Recently, Icardo et al. (2016) published electron micrographs illustrating the presence of LDs embedded throughout the myocardium of the systemic heart of *E. stoutii* and that of the Atlantic hagfish, *Myxine glutinosa*, another anoxia-tolerant species. Similar LDs can be found in vertebrate skeletal muscle but are not typically seen in healthy myocardium (Wittels and Spann 1968; van der Vusse et al. 1992). For these LDs to be utilized as a fuel source during anoxia exposure, triglycerides derived from the LDs would need to be hydrolyzed to free fatty acids (FFAs) and glycerol. Glycerol would then have to be enzymatically transformed through a series of intermediates to glyceraldehyde-3-phosphate (GAP) to enter glycolysis. More specifically, the LD's would be hydrolyzed by triglyceride lipase (TGL) and the resultant glycerol can then be phosphorylated into glycerol-3 phosphate (G3P) by glycerol kinase (GK). G3P can then be converted into dihydroxyacetone phosphate (DHAP) with the reduction of $NADH^+$ by glyceraldehyde 3-phosphate dehydrogenase (G3PDH). Finally, the isomerization of DHAP into GAP would occur during glycolysis by the ubiquitous triose-phosphate isomerase (Dhar-Chowdhury et al. 2005). These reactions, followed by the remaining half of the glycolytic cycle, would generate 1 mol of ATP per mole of glycerol.

In addition to being an intermediate in the chemical pathway by which LDs could be utilized to generate ATP during anoxia, glycerol can also be stored in the heart, generated via glycolysis, and in a living animal, delivered via the circulation from the liver. This substrate may, therefore, be utilized by the hagfish heart to help maintain ATP production during anoxia exposure. To determine the influence of glycerol on the contractile function of the hagfish heart during anoxia exposure, the current study examined how the function of the systemic heart of *E. stoutii* during anoxia exposure is affected by the addition of this substrate into the circulating saline. In addition, we examined how anoxia exposure affects the activity of TGL, GK, and G3PDH, predicting that their activity would increase with anoxia exposure. We also characterized the metabolic intermediates, triglycerides, glycerol, and glucose in the hearts and whether these are affected by anoxia and the addition of glucose or glycerol to the circulating saline.

## Materials and methods

### Experimental animals

Pacific hagfish, *E. stoutii*, were collected from a depth of approximately 100 m from Barkley Sound, British Columbia, Canada and housed at Bamfield Marine Station (Bamfield, British Columbia, Canada) for approximately 1 week before transport. Hagfish were transported to the University of Guelph Hagen Aqualab (Guelph, Ontario, Canada) and maintained until experimentation. Hagfish were housed in an environmentally controlled aquatic recirculating system (ECARS) with aerated, flow-through artificial seawater (~30‰ at 10 °C) and fed squid once per week. Experimental animals were removed from the tank individually, anesthetized using clove oil (1.7 mL $L^{-1}$ seawater) and then held at 10 °C under anesthetic until no autonomic nerve response to a tail pinch was observed (approximately 45 min). The hagfish were then decapitated and prepared for dissection. All experiments were approved by the University of Guelph Animal Care Committee (AUP #2710) and conducted in accordance with their guidelines.

### Tissue collection

Following decapitation, the systemic heart was rapidly removed from each animal and washed in ice-cold metabolite-free saline [(mf-saline) in mM: 450 NaCl, 10 $CaCl_2$ dihydrate, 9 KCl, 50 $MgCl_2$, 15 HEPES (pH = 7.8)] (Gillis et al. 2015). Please note that the osmolality of hagfish plasma is very high compared to most vertebrates (Robertson 1976) and this is reflected in the composition of the saline used. Samples of dorsal muscle and liver were also collected. Systemic hearts were also collected from a number of individuals right after decapitation to quantify baseline metabolite concentrations and enzyme activities prior to perfusions. The rest of the isolated hearts were exposed to one of six, 12-h working heart perfusion protocols (described below), then frozen quickly using a dry ice slurry and stored at − 80 °C. The average mass of the intact animals and systemic hearts were 157.7 ± 6.1 g and 0.2 ± 0.01 g, respectively. The systemic hearts of six animals were used for each of the six treatments.

### Plasma glycerol concentrations

The concentration of glycerol was measured in plasma separated from blood that was sampled from hagfish held in

aerated water. Blood was sampled as previously described (Cox et al. 2011). Glycerol was measured using a commercial analytical test kit (F6428) from Sigma–Aldrich (St. Louis, MO).

## Working heart preparation

A cannula, made from a 20-gauge needle, was inserted into the atrium of the systemic heart via the sinous venosus and secured with surgical knots. Initially, the hearts were continuously perfused at a pressure of 7 mmHg at 10 °C with recirculating hagfish saline at a rate of 3.5 mL $min^{-1}$. There were three different salines used for the trials to test the effect of glucose and glycerol supplementation during anoxia. These were the mf-saline containing no fuel sources (Gillis et al. 2015); the glucose saline [(glu-saline) in mM: 425 NaCl, 10 $CaCl_2$ dihydrate, 9 KCl, 50 $MgCl_2$, 20 Glucose, 15 HEPES (pH = 7.8)]; and the glycerol saline [(gly-saline) in mM: 445 NaCl, 10 $CaCl_2$, 9 KCl, 50 $MgCl_2$, 5 glycerol, 15 HEPES (pH = 7.8)]. Glu-saline contained 20 mM glucose as this is the concentration measured previously in *E. stoutti* plasma (Inui et al. 1978). Measurements made in the current study demonstrate that the glycerol concentration in the plasma from *E. stoutii* held in normoxia was 1.3 ± 0.17 mM, $n = 6$. It is not currently known what plasma glycerol levels are during anoxia. To keep the osmolality of the saline constant across the treatments, the concentrations of NaCl were adjusted accordingly. After 15 min of acclimation, a small water-filled balloon attached to a pressure transducer (PowerLab 4/30, ADInstruments, Dunedin, New Zealand) was inserted into the ventricle via the ventral aorta and inflated by approximately 30 µL to enable ventricular pressure and heart rate ($f_H$) to be recorded during perfusion (Klaiman et al. 2014). For baseline function, the balloon was inflated so that ventricular end diastolic pressure was 0 mmHg and developed pressure was greater than 7 mmHg. Previous studies utilizing in situ cardiac preparations for studies of teleost fish set input pressure close to 0 for baseline conditions (Graham and Farrell 1989; Farrell et al. 1996; Mendonca et al. 2007). Hearts were perfused for 12 h at atmospheric $O_2$ levels (~ 21%) or 0% $O_2$, and $O_2$ concentration was constantly recorded using a Witrox 4 $O_2$ meter mini with dipping probe (Loligo® Systems, Viborg, Denmark). The oxygen probe was calibrated to 0% oxygen with $Na_2SO_3$ at 10 °C. Normoxic conditions were maintained with an air stone in the perfusate reservoir connected to an aquarium air pump (Tetra® Whisper 10) while anoxic conditions were created by air stones placed in both the perfusate reservoir and the tissue bath connected to a compressed nitrogen tank and bubbled with 60 mmHg (1.2 PSI) of nitrogen gas. Dissolved oxygen levels in the tissue bath during anoxia exposure remained at 0% saturation throughout the experimental periods. Developed ventricular pressure (dP) data were recorded during the trials and this was used to determine the maximum rate of pressure development during systole and maximum rate of pressure development during diastole. Here we report the average maximum dP (max-dP), average maximum rate of pressure development during systole (max-$dP/dt_{sys}$), and average maximum rate of pressure development during diastole (max-$dP/dt_{dia}$). These three variables were calculated from measurements made between hours 6 and 12 of the perfusion after rates had stabilized from the treatment application. Once the recording period was over, the hearts were removed from the system, flushed with fresh, chilled mf-saline, and flash frozen using liquid nitrogen for later analysis. The concentration of glycerol, glucose, and triglyceride were measured in these tissue samples, as well as the activity of glycerol kinase (GK), glycerol-3-phosphate dehydrogenase (G3PDH), and triglyceride lipase (TGL).

## Preparation of tissue for enzyme and stable metabolite analysis

Heart, liver, and white muscle samples were placed in a pre-chilled mortar, covered in liquid nitrogen, cracked into small pieces and then placed in a pre-weighed Precellys® tube (Bertin Technologies, Paris, FR) with 200 mg of homogenizer beads and 250 µL of ice cold homogenization buffer [in mM: 3 Mercaptoethanol, 20 EDTA (pH = 7.6)] The samples were then homogenized with a Precellys® Evolution (Bertin Technologies) using a set protocol of 6800 RPM, with 2 × 25 s cycles separated by a 5 s pause. The homogenized samples were then centrifuged at 11,000×*g* for 2 min at 4 °C and the supernatant was collected (Ryan 1996). The supernatant was used for the enzyme assays and metabolite measurements.

## Bradford assay

The protein concentration of the supernatants was measured using a BioRad Protein Assay (Bio-Rad Laboratories Ltd, Mississauga, ON) modified for a 96-well plate and read using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). Samples were kept on ice until utilized for the enzyme and metabolite assays.

## Enzyme assays

Each enzyme assay (TGL, GK, and G3PDH) was standardized to total protein and optimized for maximal activity. Conditions are described below.

Triglyceride lipase (TGL) activity was measured using an indirect assay adapted from Young et al. (1988). Enzyme conditions were 7.5 mM emulsified triolein, 5 mg $mL^{-1}$ bovine serum albumin (BSA), 0.345 mM sodium dodecylsulphate, 1.5 U $mL^{-1}$ heparin in 5× PBS. Reaction was

halted after 30 min with 30 µL of perchloric acid and each sample was assayed for glycerol.

Glycerol kinase (GK, EC 2.7.1.30) activity was determined as in Hansen and Sidell (1983) and modified for a 96-well plate (Perez-Jimenez et al. 2009). Reaction conditions were (in mM) 5 $MgCl_2$, 10 PEP, 0.75 NADH, 5 ATP, 4 U $mL^{-1}$ PK and 4 U $mL^{-1}$ LDH, 10 glycerol, pH = 7.6.

Glycerol-3 phosphate dehydrogenase (G3PDH, EC 1.1.1.8) activity was measured as described by Crabtree and Newsholme (1972). Assay conditions, in mM, were 0.2 NADH, 1 KCN, 0.4 dihydroxyacetone phosphate, 70 mM Tris, pH = 7.5. The maximum velocity of the reaction ($V_{max}$) was calculated using SoftMax Pro® 6.2.2.

### Tissue glucose

Glucose concentration in the plasma samples were measured in duplicate using a plasma glucose kit (GAGO20-1KT) from Sigma–Aldridge. Glucose concentrations in the tissues were measured as in Bergmeyer et al. (1983), where samples are added to a glucose assay reagent [in mM: 1 β-nicotinamide adenine dinucleotide phosphate (β-NADP), 1 ATP and 1.5 U $mL^{-1}$ glucose-6 phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) in phosphate buffer (70 mM $NaH_2PO_4$, 2.5 mM $MgCl_2$ dihydrate, pH = 7.7)] and incubated at 37 °C for 5 min before being read by the spectrophotometer at 340 nm. Hexokinase (EC 2.7.1.1) was then added at a concentration of 2.3 U per well and incubated for another 20 min at 37 °C before the final spectrophotometer reading.

### Tissue glycerol

The concentration of glycerol in each tissue sample was measured using an assay adapted from Wakayama and Swanson (1977). In brief, the reaction conditions, in mM, were 3.6 ATP, 2.2 NAD, 1.5 DTT, 4 U $mL^{-1}$ glycerol phosphate dehydrogenase (G3PDH) (EC 1.1.1.8)) and 6.5 U $mL^{-1}$ GK (EC 2.7.1.30)) in buffer (in mM), 516 hydrazine hydrate, 268 glycine, 3.6 $MgCl_2$, pH = 9.2.

### Tissue triglycerides

Triglyceride concentration in the tissue supernatants was measured using an indirect assay from Sigma–Aldrich glycerol and triglyceride assay kit. After samples were assayed for glycerol, warmed (30 °C) lipoprotein triglyceride lipase reagent (EC 3.1.1.34) (Sigma–Aldrich Canada, Oakville, ON) was added to each well, and the plates were incubated for 10 min and reread with the spectrophotometer to determine the concentration of glycerol created by the breakdown of TG.

## Statistical analysis

A two-way repeated measures ANOVA was completed on the data collected over the length of the experiment (12 h) for each of the cardiac functional parameters for the hearts exposed to normoxia and anoxia, while perfused with MF-saline, to test for the effects of oxygen and time as well as their interaction. Two-way ANOVAs followed by Holm–Sidak tests for multiple comparisons were used to test the effects of anoxia and metabolite supplementation, as well as their interaction, on the function of the hearts, and on the measured enzyme activities and tissue metabolite concentrations. Differences in metabolite concentrations and enzyme activities between tissues from each species were tested using a one-way ANOVA. Any data set that failed to conform to the assumption of normality were log transformed prior to analysis. All data are presented as mean ± standard error. All values were evaluated for significant differences at an alpha level of 0.05.

## Results

### The influence of anoxia on the functional parameters of excised hearts from *E. stoutii*

The max $f_H$, max $dP/dt_{sys}$, max $dP/dt_{dia}$ and max dP of the excised hearts from *E. stoutii* exposed to normoxia, perfused with mf-saline, decreased over the first 4–5 h of the experiment but then stabilized for the remaining 7–8 h (Fig. 1a, c, e). The results of the two-way repeated measures ANOVA used to analyze the functional parameters of the hearts exposed to normoxia or anoxia while perfused with mf-saline, indicate that there is no interaction between the factors tested (oxygen and time) and that there was no effect of oxygen on the functional parameters. This indicates that any effect of time was independent of oxygen content. There were, however, significant differences within each treatment for the data collected over the first 4–5 h of the perfusion experiments and that collected subsequently, but that there were no differences in the data collected between 6 and 12 h. The two-way repeated measures ANOVA was completed on the data from these two groups as these hearts demonstrated the greatest change in function over the 12-h experimental period and had the lowest variability at each time point (Fig. 1). As a result of this statistical analysis, the values we present in Fig. 2, and compare statistically using two-way ANOVAs followed by Holm–Sidak tests, are averages of the data collected between hours 6 and 12 of the perfusion for all treatments.

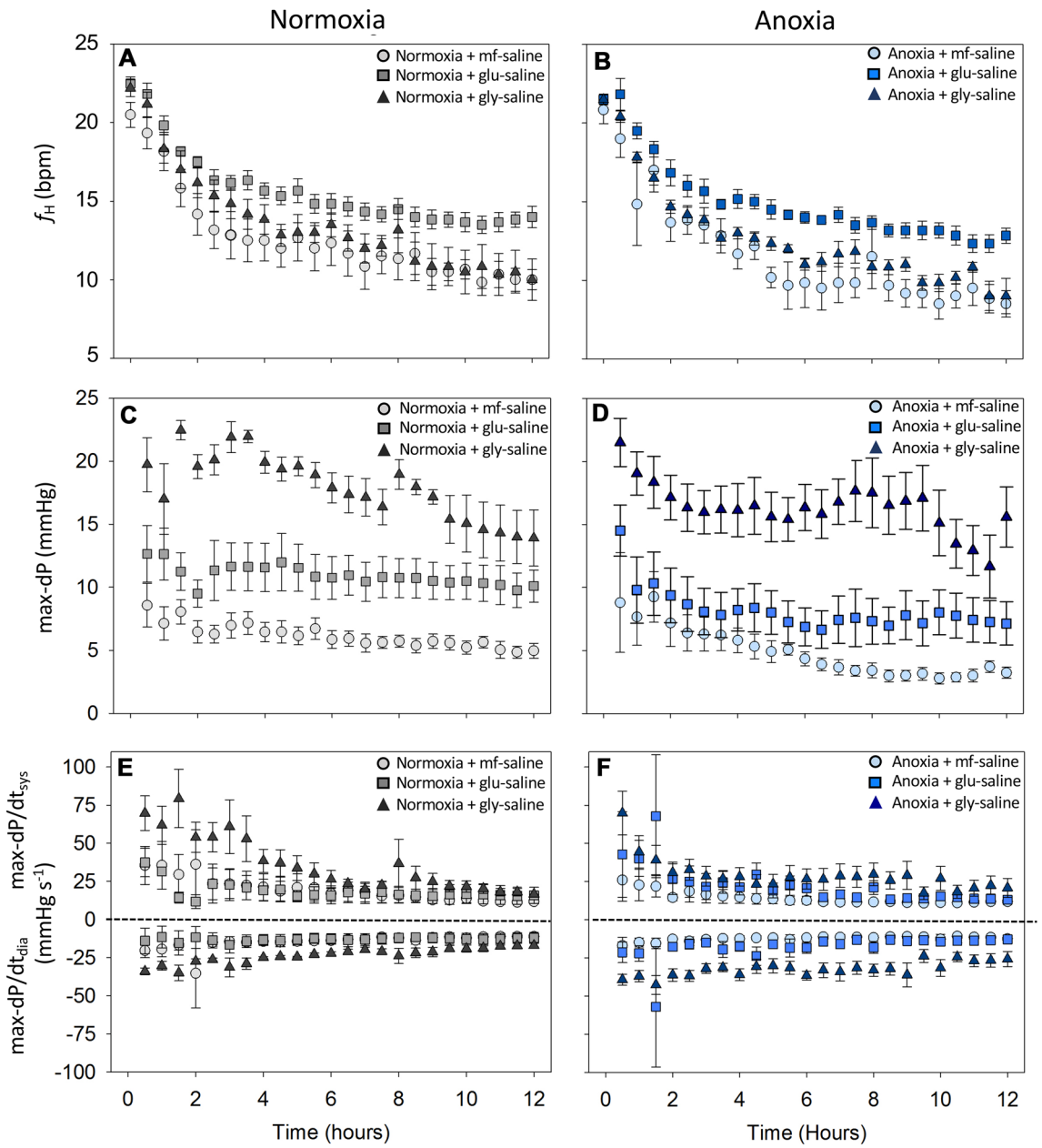


**Fig. 1** The influence of supplemental glycerol or glucose on the function of hearts from *E. stoutii* during 12 h of normoxia or anoxia. **a, b** Average heart rate ($f_H$) of hearts from *E. stoutii* exposed to normoxia (**a**) and anoxia (**b**) and perfused with metabolite free saline (mf-saline), saline containing 20 mM glucose (glu-saline), or saline containing 5 mM glycerol (gly-saline). **c, d** Average maximum developed pressure (max-dP) of hearts from *E. stoutii* exposed to normoxia (**c**) and anoxia (**d**) and perfused with mf-saline, glu-saline, or gly-saline. **e, f** Average maximal rate of contraction during systole (max-dP/dt$_{sys}$), and average maximal rate of relaxation during diastole (max-dP/dt$_{dia}$) of hearts from *E. stoutii* exposed to normoxia (**e**) and anoxia (**f**). $f_H$ is measured in beats per minute; max-dP is measured in mmHg, dP/dt$_{sys}$ and dP/dt$_{dia}$ are measured in mmHg s$^{-1}$. $N = 6$ hearts per treatment. Values are shown as mean ± standard error. The scaling of the *y*-axis of figures in the same row is identical and the scaling of the *x*-axis (time) of all panels is identical. mf-saline, metabolite free saline

The $f_H$ of the hearts perfused with mf-saline in normoxia was initially 20.5 ± 0.1 bpm and the average $f_H$, measured between 6 and 12 h, was 10.9 ± 0.9 bpm (Fig. 1a). The max-dP for these control hearts was initially 8.6 ± 1.7 mmHg and the average max-dP was 6.0 ± 0.2 mmHg (Fig. 1b). The max-dP/dt$_{sys}$ was initially + 35.5 ± 12.5 mmHg s$^{-1}$ and the average max-dP/dt$_{sys}$ was + 14.4 ± 3.2 mmHg s$^{-1}$ (Fig. 1c). The max-dP/dt$_{dia}$ for the excised hearts under these control conditions was initially − 20.4 ± 4.6 mmHg s$^{-1}$ and the average max-dP/dt$_{dia}$

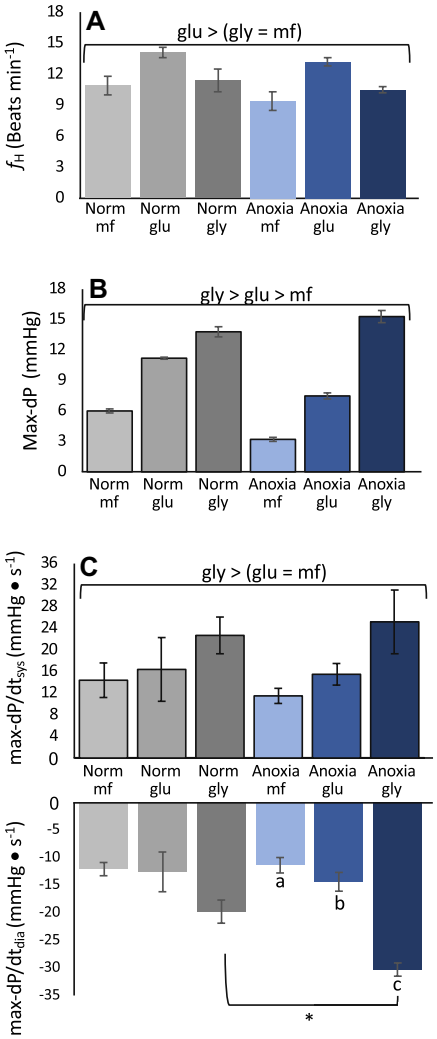


**Fig. 2** The influence of supplemental glycerol or glucose on the averaged functional parameters of excised hearts from *E. stoutii* measured between hours 6 and 12 of normoxia or anoxia exposure. Differences in functional parameters were determined using a two-way ANOVA, and values are mean ± standard error ($N=6$). Differences in the main effect of metabolite supplementation are indicated above the bars except where there was an interaction between anoxia and metabolite supplementation ($P<0.05$). **A** Average heart rate ($f_H$) was higher in hearts perfused with glu-saline in normoxia and anoxia than perfused with gly-saline and mf-saline in normoxia and anoxia. **B** Average maximum developed pressure (max-dP) was higher in hearts perfused with gly-saline in normoxia and anoxia than perfused with glu-saline and mf-saline in normoxia and anoxia. max-dP was higher in hearts perfused with glu-saline in normoxia and anoxia than perfused with mf-saline in normoxia and anoxia. **C** Average maximum rate of contraction during systole (max-dP/dt$_{sys}$) was higher in hearts perfused with gly-saline in normoxia and anoxia than perfused with glu-saline and mf-saline in normoxia and anoxia. The average rate of relaxation (max-dP/dt$_{dia}$) was significantly affected by oxygen and metabolite supplementation (interaction $P<0.05$), with max-dP/dt$_{dia}$ being higher in hearts perfused with glu-saline in anoxia than in hearts perfused mf-saline in anoxia. max-dP/dt$_{dia}$ was also higher in hearts perfused with gly-saline in anoxia than in hearts perfused with mf-saline and glu-saline in anoxia. Finally, max-dP/dt$_{dia}$ was higher in hearts perfused with gly-saline in anoxia than in hearts perfused with gly-saline in normoxia. Bars indicated with different letters within oxygen treatment are statistically different while the asterix indicates an effect of oxygen treatment within metabolite treatment ($P<0.05$)

was $-12.1 \pm 1.2$ mmHg s$^{-1}$ (Fig. 1c). As determined by the two-factor ANOVA there was no difference between these functional parameters measured in the hearts perfused with mf-saline in normoxia and those perfused with mf-saline in anoxia (average $f_H$, average max-dP, average max-dP/dt$_{sys}$, average max-dP/dt$_{dia}$) ($P<0.05$) (Fig. 2).

## The influence of supplemented fuels on the functional parameters of the excised *E. stoutii* hearts

As demonstrated by measurements of $f_H$, max-dP, max-dP/dt$_{sys}$, and max-dP/dt$_{dia}$, the addition of glucose and glycerol to the saline perfusing the hearts caused changes in cardiac function but these effects were metabolite dependent (Figs. 1, 2). For example, the average $f_H$ of hearts perfused with glu-saline, in both normoxia and anoxia, were higher than those perfused with mf-saline or gly-saline ($P<0.05$) (Fig. 2A). Interestingly, the addition of glycerol to the saline perfusing the hearts in normoxia and anoxia, had a larger effect on the average max-dP, average max-dP/dt$_{sys,}$ and average max-dP/dt$_{dia}$ than did the addition of glucose (Fig. 2B, C). For example, the average max-dP of hearts perfused with gly-saline in normoxia was 2.3-, and 1.2-fold that of hearts perfused with mf-saline in normoxia and with glu-saline in normoxia, respectively ($P<0.05$) (Fig. 2B). Similarly, the max-dP of hearts perfused with gly-saline in anoxia was 4.8-fold that of hearts perfused with mf-saline in anoxia and twofold that of hearts perfused with glu-saline in anoxia ($P<0.05$) (Fig. 2B). In addition, while there was no difference in average max-dP/dt$_{sys}$ between hearts perfused with mf-saline or glu-saline in normoxia, and anoxia, this parameter was significantly higher in the hearts perfused with gly-saline in normoxia and anoxia ($P<0.05$). Finally, there was no difference in the average max-dP/dt$_{dia}$ between hearts perfused with mf-saline, glu-saline, and gly-saline in normoxia. There was a significant interaction between the effects of anoxia exposure and metabolite supplementation on the average max-dP/dt$_{dia}$ ($P<0.05$) with this parameter being higher in the hearts perfused with gly-saline in anoxia than in hearts perfused with gly-saline in normoxia. The presence of an interaction between the two factors indicates that a change in both oxygen and metabolite is needed to cause a significant change in average max-dP/dt$_{dia}$. The average max-dP/dt$_{dia}$ was also higher in the hearts perfused with gly-saline in anoxia than with mf-saline and glu-saline in anoxia ($P<0.05$) (Fig. 2C).

## The effect of anoxia on the maximal enzymatic activity of TGL, GK, and G3PDH in the excised hearts of *E. stoutii*

The maximal enzymatic activity of TGL, GK, and G3PDH in the hearts at time of sacrifice was $38.3 \pm 5.2$ mU mg

**Table 1** The activity of triglycerides lipase, glycerol kinase; and glycerol-3 phosphate dehydrogenase, and the concentrations of glucose, glycerol and triglycerides in the hearts of *E. stoutii* sampled at time of sacrifice and after 12 h of perfusion with metabolite free saline in normoxia

| | $t=0$ | $t=12$ h (norm mf-saline) |
|---|---|---|
| TGL | 38 ± 5* | 4 ± 2* |
| GK | 0.28 ± 0.08 | 0.31 ± 0.13 |
| G3PDH | 0.33 ± 0.05* | 0.06 ± 0.02* |
| Glucose | 0.06 ± 0.02 | 0.08 ± 0.02 |
| Glycerol | 5.5 ± 1.9 | 3.8 ± 1.0 |
| Triglycerides | 2.1 ± 1.1 | 2.6 ± 0.7 |

$N=6$ hearts per treatment. Values are shown as mean ± standard error

$T=0$, sampled at time of sacrifice; norm mf-saline, hearts perfused with metabolite free saline for 12 h in normoxia. Enzyme activity is shown as mU mg protein$^{-1}$; and metabolite concentration in µmol g$^{-1}$

*TGL* triglycerides lipase, *GK* glycerol kinase, *G3PDH* glycerol-3 phosphate dehydrogenase

Values in the same row indicated with an "*" are significantly different ($P<0.05$)

protein$^{-1}$, 0.3 ± 0.1 mU mg protein$^{-1}$, and 0.3 ± 0.1 mU mg protein$^{-1}$, respectively (Table 1). After 12 h of perfusion in normoxia with mf-saline, the maximal activity of TGL and G3PDH measured in the hearts, was significantly lower than the initial values ($P<0.05$) but there was no significant change in the activity of GK (Table 1). There was no difference in the activities of TGL, GK, and G3PDH in hearts perfused with mf-saline in normoxia and those perfused with mf-saline in anoxia ($P<0.05$) (Fig. 3).

## The effect of supplemented fuels on the activity of enzymes associated with glycerol metabolism in the *E. stoutii* hearts

There was no difference in the maximal activities of TGL, GK, and G3PDH in the hearts perfused with glu-saline or gly-saline in normoxia, or anoxia, and those perfused with mf-saline in normoxia or anoxia ($P<0.05$) (Fig. 3).

## The effect of anoxia on the concentrations of metabolites associated with glycerol metabolism in the excised hearts of *E. stoutii*

The concentration of glycerol in the hearts from *E. stoutii* at time of sacrifice was 5.5 ± 1.9 µmol g$^{-1}$ and there was no difference between this value and that measured after 12 h of perfusion in normoxia with mf-saline (Table 1). There was no difference in the glycerol levels measured in the hearts perfused with mf-saline in normoxia and those perfused with mf-saline in anoxia ($P<0.05$) (Fig. 4A).

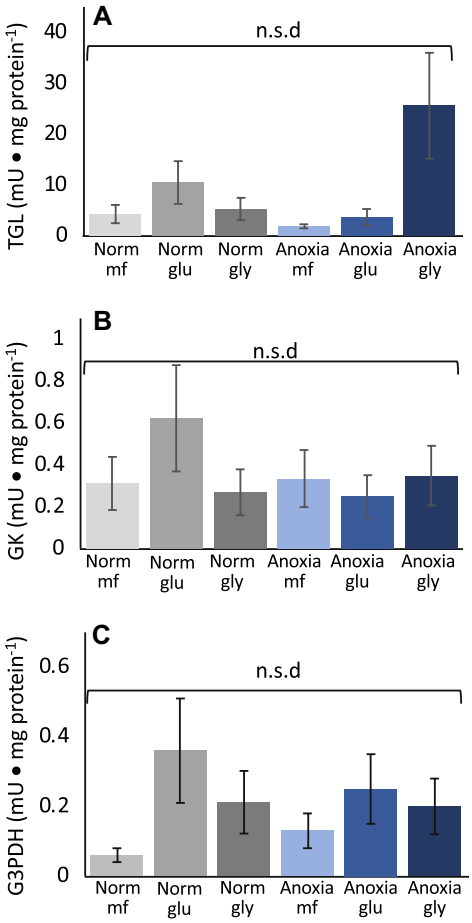


**Fig. 3** The influence of 12 h of anoxia exposure and/or metabolite supplementation on the maximal activity of triglyceride lipase (**a**), glycerol kinase (**b**) and glycerol-3 phosphate dehydrogenase (**c**) measured in excised hearts from *E. stoutii* after treatment. Within species differences in functional parameters were determined using a two-way ANOVA, and values are mean ± standard error ($N=6$). There were no significant differences between any treatments (n.s.d.) ($P<0.05$). *Norm* normoxia, *mf* mf-saline, *glu* glu-saline, *gly* gly-saline, *TGL* triglycerides lipase, *GK* glycerol kinase, *G3PDH* glycerol-3 phosphate dehydrogenase. $N=6$ hearts per treatment

The concentration of glucose in the hearts sampled at time of sacrifice was 0.06 ± 0.03 µmol g$^{-1}$ and there was no difference between this value and that measured after 12 h of perfusion in normoxia with mf-saline (Table 1). There was no difference in the glucose concentrations in the hearts perfused with mf-saline in normoxia and those perfused with mf-saline in anoxia (Fig. 4B).

The concentration of TGs in the hearts sampled at time of sacrifice was 2.1 ± 1.1 µmol g$^{-1}$ and there was no difference between this value and that measured after 12 h of perfusion in normoxia with mf-saline (Table 1). There was no difference in the concentration of TGs in hearts

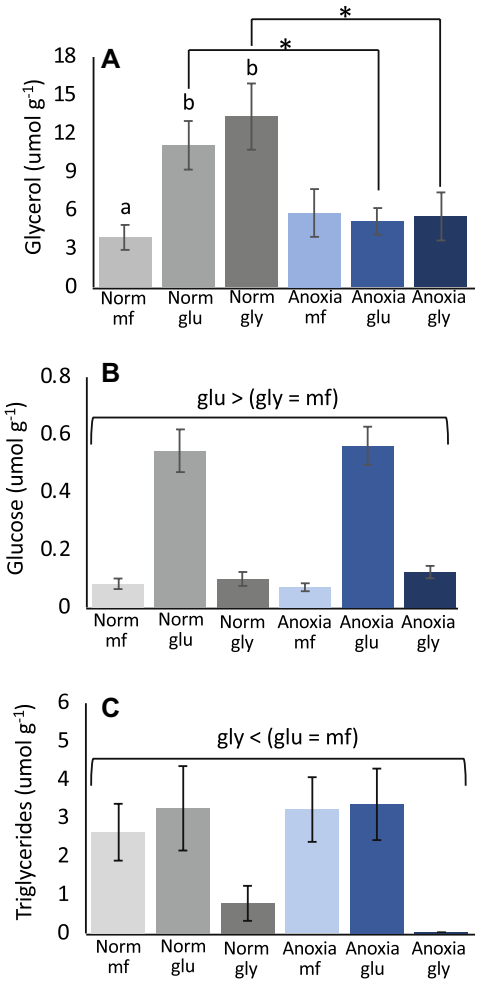


**Fig. 4** The influence of anoxia exposure and/or metabolite supplementation on metabolic fuels in excised hearts from *E. stoutii* after 12 h of treatment. Differences in tissue metabolite concentrations were determined using a two-way ANOVA and values are mean ± standard error ($N=6$). Differences in the main effect of metabolite supplementation are indicated above the bars ($P<0.05$) except where there was an interaction between anoxia and metabolite supplementation. **A** Tissue glycerol concentrations were significantly affected by oxygen and perfusate supplementation (Interaction $P<0.05$), with glucose and glycerol supplementation increasing tissue glycerol in normoxia but not anoxia. Glycerol concentrations were also higher in hearts perfused with glu-saline in normoxia then with glu-saline in anoxia as well as in hearts perfused with gly-saline in normoxia than with gly-saline in anoxia. **B** Tissue concentrations of glucose were higher in hearts perfused with glu-saline, in anoxia and normoxia than those perfused with gly-saline or mf-saline, in anoxia and normoxia. **C** Tissue concentrations of triglycerides were lower in hearts perfused with gly-saline in normoxia and anoxia than those perfused with glu-saline and mf-saline in anoxia and normoxia. *Norm* normoxia, *MF* metabolite-free saline, *glu* saline containing 20 mM glucose, *gly* saline containing 5 mm glycerol. $N=6$ hearts per treatment. Values are shown as mean ± standard error. Bars indicated with different letters within oxygen treatment are statistically different while the asterix indicates an effect of oxygen treatment within metabolite treatment ($P<0.05$)

perfused with mf-saline in normoxia and those perfused with mf-saline in anoxia.

## The effect of supplemented glucose and glycerol on the concentrations of metabolites associated with glycerol metabolism in the excised hearts of *E. stoutii*

There was a significant interaction between the effects of anoxia and metabolite supplementation on the concentration of glycerol measured in the hearts ($P<0.05$). The concentration of glycerol in hearts perfused with gly-saline in normoxia was 2.4-fold higher than that in hearts perfused with gly-saline in anoxia ($P<0.05$); and the concentration of glycerol in hearts perfused with glu-saline in normoxia were 2.1-fold higher that in hearts perfused with glu-saline in anoxia ($P<0.05$). Additionally, the concentration of glycerol in hearts perfused with gly-saline in normoxia was 3.4-fold that in hearts perfused with mf-saline in normoxia ($P<0.05$) (Fig. 4A). There was, however, no difference in the concentrations of glycerol in the hearts perfused with mf-saline in anoxia, glu-saline in anoxia or gly-saline in anoxia (Fig. 4A).

The concentration of glucose in hearts perfused with glu-saline in normoxia was 6.6-fold that in hearts perfused with mf-saline in normoxia ($P<0.05$) (Fig. 4B). However, there was no difference in glucose concentrations between hearts perfused with mf-saline in normoxia and those perfused with gly-saline in normoxia. The glucose concentration in the hearts perfused with glu-saline in anoxia was eightfold that in hearts perfused with mf-saline in anoxia ($P<0.05$) (Fig. 4B).

There was no difference in the concentration of TGs in the hearts perfused with mf-saline in normoxia and those perfused with glu-saline in normoxia (Fig. 4C). However, the concentration of TGs in the hearts perfused with gly-saline in normoxia was 70% less than that in the hearts perfused with mf-saline in normoxia ($P<0.05$). There was no difference in the concentration of TGs in the hearts perfused with mf-saline in anoxia and those perfused with glu-saline in anoxia (Fig. 4C). There were, however, only trace amounts of TGs in the hearts perfused with gly-saline in anoxia. These levels were significantly lower than that in hearts perfused with mf-saline in anoxia ($P<0.05$) (Fig. 4C).

## Tissue-specific differences in glycerol concentrations and maximal activities of TGL, GK, and G3PDH

The concentration of glycerol in the liver samples was 14-fold, and ninefold that measured in the heart and skeletal muscle, respectively ($P<0.05$) (Fig. 5). The maximal activity of GK and G3PDH was significantly higher in the

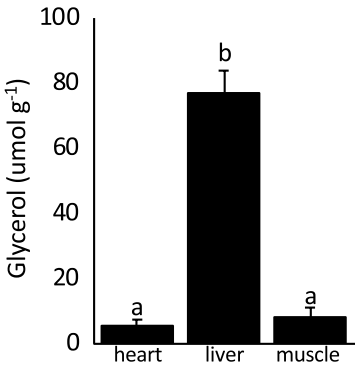


**Fig. 5** Endogenous levels of glycerol in the heart, liver, and skeletal muscle of *E. stoutii*. $N=6$ for each tissue. Values are shown as mean ± standard error. Between tissue difference in glycerol concentration was tested using a one-way ANOVA. Bars that do not share a letter are statistically different ($P<0.05$)

liver compared to that in either cardiac muscle or skeletal muscle ($P<0.05$) (Fig. 6).

## Discussion

This study demonstrates for the first time that the excised systemic hearts of *E. stoutii* can maintain contractile function during at least 12 h of anoxia exposure without supplemental fuel sources or external pacing. Interestingly, the addition of glycerol to the saline perfusing the hearts during anoxia exposure caused a significant increase in average max-dP, average max-dP/$dt_{sys,}$ and average max-dP/$dt_{dia}$. These increases in function were larger than that seen with the addition of glucose to the saline. This suggests that glycerol stimulates cardiac function in the hagfish but that it is not derived from stored lipids. There was also an increase in glycerol in hearts provided with glycerol during normoxia exposure but there was no such increase in tissue concentrations of glycerol in the hearts provided with glycerol during anoxia exposure. This suggests that glycerol is being utilized to fuel the measured increase in contractile function. However, it is not possible at this time, to rule out that glycerol could be increasing ATP production and/or contractile function via an allosteric effect.

### Contractile function of an isolated hagfish heart

The initial levels of pressure generation in the excised hearts were higher than that measured in live *E. stoutii* but then decreased and remained relatively constant within a physiological range (Cox et al. 2010). The higher levels of function at the start of the experiment may reflect the heart responding to its removal from the animal and perfusion in the working heart preparation. Such manipulation involves both

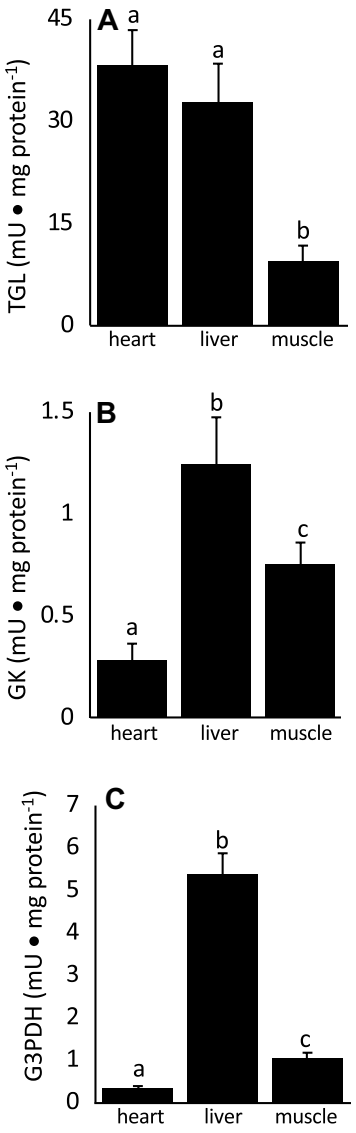


**Fig. 6** Comparison of the maximal activities of triglyceride lipase, glycerol kinase, and glycerol-3 phosphate dehydrogenase measured in the heart, liver, and skeletal muscle of *E. stoutii*. Between tissue difference in enzyme activity was tested using a one-way ANOVA. Bars that do not share a symbol letter are statistically different ($P<0.05$). **A** The activity of triglyceride lipase (TGL) was significantly higher in the heart and liver than in the muscle. **B** The activity of glycerol kinase (GK) was significantly higher in the liver than in the heart and muscle. The activity of GK was also significantly higher in the muscle than in the heart. **C** The activity of glycerol-3 phosphate dehydrogenase (G3PDH) was significantly higher in the liver than in the heart and muscle. The activity of G3PDH was also significantly higher in the muscle than in the heart. $N=6$ tissues. Values are shown as mean ± standard error

physical stimulation and changes to the physiological and biochemical environment in which the heart is working. In addition, the hearts in this study are not functioning within the pericardium and, therefore, potentially able to become

more distended during diastole. Such an effect would cause greater stretch of the myocardium and, therefore, potentially greater systolic function due to the Frank–Starling response. Such an effect could explain the initial high levels of contractile function. However, the relatively constant functional parameters of the heart after 5 h of function, as determined by the 2-way repeated measures ANOVA, and the fact that these functional parameters are within the range measured in live animals suggests that relevant physiological data were being collected.

Cox et al. (2010) measured a $f_H$ of 10.4 ± 1.3 bpm in *E. stoutii* in normoxia at 10 °C and then found that this decreased by half after 4 h of anoxia exposure. Recently, Wilson et al. (2016) demonstrated that the spontaneous $f_H$ of excised hagfish hearts at 10 °C was 13.4 ± 1.1 bpm under normoxic conditions and 5.1 ± 0.5 bpm after 2 h of anoxia. The average $f_H$ measured in the current study between hours 6 and 12 of perfusion under normoxic conditions with mf-saline is similar to that of these previous studies made during normoxia exposure and there was no difference in this parameter with anoxia exposure. It is suggested by Wilson et al. (2016) that tonic adrenergic stimulation of hagfish $f_H$ is mediated by transmembrane adenylyl cyclase (tmAC) activation and that the loss of this tonic stimulation with anoxia exposure leads to a decrease in $f_H$. One potential reason why there was no difference in $f_H$ between the hearts perfused with mf-saline in normoxia and anoxia in the current study is that they were continuously perfused at 7 mmHg and there was a balloon in the ventricle. The resultant ventricular pressure would offer resistance throughout the cardiac cycle and as a result cause the myocardium to experience mechanical stretch. The hearts in the Wilson et al. (2016) study were not perfused and did not contain a balloon. As described by the Frank–Starling response, stretch increases force production by the myocardium and in the current study, the maintenance of a minimum level of stretch by the balloon under normoxia and anoxia would ensure a minimal level of baseline contractile function. Examination of the influence of stretch on the function of the hagfish heart should be the focus of future studies to see if this explains the results of the current study. However, as all hearts were treated identically in the current study any effect of stretch on function would have been similar between the different treatments.

## Influence of anoxia exposure and the addition of metabolic substrates on contractile function

Pressure generation by the heart as measured by max-dP, the rate of force generation as measured by max-dP/$dt_{sys}$, and the rate of relaxation as measured by max-dP/$dt_{dia}$, are all dependent on cellular ATP levels as this controls the number of cross bridges formed in the muscle, the rate of cross bridge formation, as well as the rate that $Ca^{2+}$ can be pumped back into the sarcoplasmic reticulum (SR) via sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) after contraction. The finding of the current study that there was no difference in the ability of the excised hearts to function when perfused with mf-saline in anoxia or normoxia suggests that there is no change in ATP utilization by the contractile machinery. If exposure to anoxia did cause a decrease in ATP production by the myocytes, the lack of change in contractile function suggests that other ATP consuming processes such as protein synthesis were downregulated so as to maintain ATP supply to the contractile element. Another possibility, supported by previous experiments using a calorimeter and excised *E. stoutti* hearts, is that ATP production/utilization is not affected by anoxia exposure (Gillis et al. 2015).

The increase in multiple functional parameters that occurred when the excised hearts were perfused with saline containing either glycerol or glucose suggests that the hearts have the capacity to absorb these metabolites, and that this simulates energy usage by the contractile machinery. The ability of isolated vertebrate hearts to take up glycerol present in the perfusate was established with rat hearts using radiolabeled glycerol (Gambert et al. 2007). It was also demonstrated in this previous study that 3.3 mM glycerol in the perfusate caused increased glycerol oxidation, and an increase in the synthesis of triacylglycerides (Gambert et al. 2007). Glycerol diffuses into cells directly through the membrane as well as through glycerol-selective aquaporins (Hibuse et al. 2009). Hibuse et al. (2009) demonstrated that knockdown of glycerol-selective aquaporin 7 (AQP7) in rat H9c2 cardiomyotubes caused a ~26% decrease in glycerol content. This suggests that direct transmembrane diffusion is the primary mode of entry. However, gene transcripts for glyceroaquaporins have been cloned in *E. stoutii* (Herr et al. 2014) and *E. burgeri* (Cutler 2006) suggesting that these could also play a role in glycerol transport in the hagfish heart. In the current study, the higher glycerol concentration in the hearts perfused with gly-saline in normoxia than in hearts perfused with mf-saline in normoxia, suggests an increase in glycerol diffusion into the hearts.

The increase in mechanical function with substrate supplementation indicates that there is an increase in ATP utilization by the contractile machinery. This could be due to the substrates being directly utilized for energy production, or by stimulating a greater allocation of stored energy reserves to the contractile machinery. The addition of glycerol to the saline during normoxia and anoxia exposure caused a larger increase in cardiac function than did the addition of glucose, with significant increases in average max-dP/$dt_{sys}$, average max-dP/$dt_{dia}$, and average max-dP. The increase in average max-dP/$dt_{sys}$, and average max-dP indicates an increase in the rate of cross-bridge cycling and in the total number of cross-bridges being formed, respectively. The increase in

average max-dP/$dt_{dia}$ was only seen in the hearts perfused with gly-saline in anoxia and this suggests an increase in the activity of SERCA, as this would result in an increase in the rate of removal of cytosolic $Ca^{2+}$ following each contraction. These results suggest that glycerol has a larger stimulatory effect on contractile function in anoxia than does glucose. Why this would only occur with glycerol supplementation in anoxia, and not normoxia, is not known.

The average $f_H$ was higher in hearts perfused with glu-saline in both normoxia and anoxia. This response was not seen in the hearts perfused with gly-saline during anoxia or normoxia exposure. As the hearts were not paced, this response suggests that the function of pacemaker cells found in the sinus venosus and atrioventricular canal in fish hearts (Farrell and Smith 2017) are affected by the addition of glucose.

## Metabolic fuel utilization during anoxia exposure

Previous work has demonstrated that exposure of excised *E. stoutii* hearts, perfused with mf-saline, to anoxia causes an initial decrease in metabolic heat production but that this fully recovers by 4 h of exposure and remains constant for at least 16 h (Gillis et al. 2015). This result was interpreted as a decrease, and then the recovery, of ATP utilization. In this previous study, ATP utilization was estimated to be ~ 10 μmol $g^{-1}$ $h^{-1}$ throughout the 16-h anoxia exposure (Gillis et al. 2015). Using this rate as a guide, it is then estimated that ~ 18 μmol of ATP is required to fuel the ~ 150 mg hearts, in the current study, over the 12-h anoxia exposure. We have previously determined that the glycogen content of the *E. stoutii* heart is ~ 31 μmol $g^{-1}$ (Gillis et al. 2015). In anoxia, this amount of glycogen would produce either 11 or 17 μmol of ATP depending on if it is assumed that 2 or 3 moles of ATP are produced per mole substrate. This suggests that the amount of glycogen stored in the heart would not support the estimated ATP requirements. In the current study, the lack of change in the concentration of glycerol, triglyceride or glucose in the hearts perfused with mf-saline and exposed to anoxia for 12 h suggests that they were not being used as metabolic fuels. These results, therefore, indicate that another metabolic fuel was being utilized in the hearts perfused with mf-saline in anoxia, but what this is, remains to be determined.

In the current study, there was no difference in the activity of TGL, GK, or G3PDH in hearts perfused in normoxia for 12 h compared to hearts perfused in anoxia for 12 h. This suggests that there was no difference in lipid hydrolysis (Fig. 4). The lower TGL and G3PDH activity in hearts perfused in normoxia with mf-saline for 12 h compared to in hearts sampled immediately following sacrifice, suggests a decrease in glycolytic activity (Table 1). However, caution must be taken when interpreting a change in the activity of an enzyme as indicting an alteration in the function of an ATP-producing pathway.

One relevant question, from a biological perspective, is if the measured rate of GK and G3PDH activity would generate adequate ATP from glycerol to support the requirements of the anaerobic hagfish heart. As mentioned above, we have previously estimated that a 150 mg hagfish heart would utilize ~ 18 μmol of ATP during 12 h of anoxia (Gillis et al. 2015). In the current study, the measured activity rates of GK and G3PDH in the perfused hearts were each ~ 0.3 mU mg $protein^{-1}$. This indicates that these enzymes could catalyze the conversion of 0.3 μmol of substrate $min^{-1}$ mg $protein^{-1}$. Walsh et al. (1983) demonstrated that the activity of G3PDH in eel red muscle, expressed as mg $protein^{-1}$, was approximately twofold that measured per gram wet weight of tissue. There are no such measurements from the hearts of any fish, but if we assume a similar conversion in hagfish myocardium, then we can estimate whether or not glycerol could indeed support the energetic requirements of the anoxic hagfish heart. In a 150 mg hagfish heart, if 1 mol of ATP is produced per mol of glycerol, as suggested in the proposed pathway, the measured enzymatic activity would translate into ~ 0.023 μmol ATP generated per min, or ~ 16 μmol ATP generated in 12 h. Therefore, with adequate glycerol, this pathway could supply the ATP requirements of the tissue.

The higher average max-dP, and average max-dP/$dt_{sys}$ measured in the hearts perfused with gly-saline in normoxia and anoxia than in hearts perfused with mf-saline, suggests that glycerol is diffusing into these hearts, and then stimulating ATP production and greater contractile function. Interestingly, the concentration of glycerol measured in the hearts perfused with gly-saline in anoxia was less than half that measured in the hearts perfused with gly-saline in normoxia but the hearts were functioning similarly. This result could reflect the hearts in normoxia using more efficient aerobic pathways to generate ATP and/or that the anoxic hearts were using glycerol at a faster rate than the normoxia hearts. Work by Hibuse et al. (2009) supports the suggestion that glycerol can be used to generate ATP in the heart as these authors found that a ~ 26% decrease in cellular glycerol, caused by the knockdown of AQ7 in H9c2 cardiomyotubes, was accompanied by a ~ 29% decrease in ATP content. The levels of glycerol measured in the hagfish heart in the current study are an order of magnitude greater than that measured in rat cardiac H9c2 cardiomyotubes (~ 5 umol/g vs 0.48 umol/g) by Hibuse et al. (2009). This difference may suggest a greater reliance on glycerol for ATP production in the hagfish heart as a decrease in available glycerol can lead to a decrease in cellular ATP (Hibuse et al. 2009). In support of this suggestion, a recent meta-analysis by Areta and Hopkins (2018) indicates that higher levels of

carbohydrate availability in the skeletal muscle of human males were associated with moderately higher levels of resting glycogen.

The level of glycerol in the hearts perfused with glu-saline in anoxia were the same as those perfused with mf-saline in anoxia. This result is not surprising as there was no endogenous glycerol available. The higher levels of glycerol in the hearts perfused with glu-saline in normoxia compared to those perfused with mf-saline in normoxia suggest that glucose was in excess and was being used to synthesize glycerol. This idea is supported by work completed by Driedzic and Ewart (2004) that demonstrated that glycerol synthesized in the liver of the rainbow smelt, comes from carbon that originates as glucose/glycogen. Further study is required to determine if a similar pathway is functioning in the hagfish heart.

The concentrations of glycerol measured in the liver of *E. stoutii* at time of sacrifice were higher than that measured in either the heart or skeletal muscle. In addition, the measured levels of liver glycerol are relatively high compared to most other fish species. For example, the glycerol concentrations measured in the livers of *E. stoutii*, were $76.8 \pm 6.9$ µmol $g^{-1}$ while that measured in the liver of the smooth flounder, *Pleuronectes putnami*, was 21.4 µmol $g^{-1}$ and that of the Atlantic tomcod, *Microgadus tomcod*, was $< 1.0$ µmol $g^{-1}$ (Driedzic et al. 1998). Only the liver of rainbow smelt, *Osmerus mordax*, a fish that uses glycerol generated by the liver as an antifreeze in the winter has higher liver glycerol levels than *E. stoutii*. Levels measured in the rainbow smelt in December were 50 µmol $g^{-1}$, while those measured in February were 150 µmol $g^{-1}$ (Treberg et al. 2002). The activity levels of GK and G3PDH, enzymes that could be used to make glycerol, were also relatively high in the liver of *E. stoutii* compared to in the heart and muscle. This along with the high endogenous levels of glycerol in the liver suggest that this organ could be a significant source of glycerol in the animal.

Excised hearts perfused with gly-saline in normoxia and anoxia had > 70% less TGs compared to those from all other experimental groups. This result suggests that the supplemental glycerol stimulated an increase in TG hydrolysis. However, how the presence of glycerol in the saline would stimulate TG hydrolysis is not known. As hagfish blood contains glycerol, future studies using live animals are required to study how TG hydrolysis in the heart is affected by anoxia.

## Summary and perspectives

The greater contractile function of the excised hearts perfused with gly-saline in both normoxia and anoxia, compared to those perfused with mf-saline and glu-saline, suggests that glycerol stimulates ATP utilization thereby causing an increase in contractile function. If this is a physiological response, there are many questions to be answered. The first is if glycerol is being utilized by the heart, or is it stimulating the use of another metabolic substrate? The second is what is the source of the glycerol? This study did not find any evidence that anoxia causes an increase in the use of lipid droplets as a source of metabolic fuel during 12 h of anoxia exposure. However, the high concentration of glycerol in the liver suggests that this organ could be a source. Clearly studies of the metabolic response of live hagfish to anoxia, specifically focused on the mobilization, and selection, of metabolic fuels are required.

**Acknowledgements** The Authors would like to thank C.R. Freedman and Dr. D.S. Fudge for comments on an earlier draft, as well as Dr. I. Lorenzen-Schmidt and A. Pierce for technical assistance.

**Funding** This work was supported by an operating grant from the Canadian Institutes of Health Research to W.G.P, and a Discovery Grant, and Discovery Accelerator Supplement, from the National Sciences and Engineering Research Council of Canada to T.E.G.

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