ORIGINAL PAPER

Anoxic survival of the Pacific hagfish (*Eptatretus stoutii*)

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Abstract It is not known how the Pacific hagfish (*Eptatretus stoutii*) can survive extended periods of anoxia. The present study used two experimental approaches to examine energy use during and following anoxic exposure periods of different durations (6, 24 and 36 h). By measuring oxygen consumption prior to anoxic exposure, we detected a circadian rhythm, with hagfish being active during night and showing a minimum routine oxygen consumption (RMR) during the daytime. By measuring the excess post-anoxic oxygen consumption (EPAOC) after 6 and 24 h it was possible to mathematically account for RMR being maintained even though heme stores of oxygen would have been depleted by the animal's metabolism during the first hours of anoxia. However, EPAOC after 36 h of anoxia could not account for RMR being maintained. Measurements of tissue glycogen disappearance and lactate appearance during anoxia showed that the degree of glycolysis and the timing of its activation varied among tissues. Yet, neither measurement could account for the RMR being maintained during even the 6-h anoxic period. Therefore, two independent analyses of the metabolic

responses of hagfish to anoxia exposure suggest that hagfish utilize metabolic rate suppression as part of the strategy for longer-term anoxia survival.

Keywords Anoxia · Routine metabolic rate ·
Excess post-anoxic oxygen consumption ·
Metabolic rate suppression · Lactate · Glucose · ATP

Abbreviations

ANOVA	Analysis of variance
°C	Degree celsius
EPAOC	Excess post-anoxic oxygen consumption
$\dot{M}O_2$	Metabolic rate (rate of O_2 consumption)
MRS	Metabolic rate suppression
PO_2	Partial pressure of O_2
RMR	Routine metabolic rate

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Introduction

Many of the nearly 60 extant species of hagfishes (*Myxinidae*) routinely inhabit hypoxic and likely anoxic environments when they burrow into either hypoxic sediments (Lesser et al. 1997; Martini 1998) or the bodies of dead or moribund animals on which they feed. In this regard, hagfishes are well known for having the lowest routine metabolic rate (RMR) among fishes (Brauner and Berenbrink 2007; Forster 1990) and a glycogen store that can support glycolytic activity for many hours (Baldwin et al. 1991; Davison et al. 1990; Forster 1991; Hansen and Sidell 1983). Despite this potential for anoxia tolerance, relatively little is known about the whole animal metabolic responses of hagfishes during anoxia, in part because

studies have rarely exposed hagfish to severe hypoxia or anoxia for more than a few hours.

The majority of the available literature on anaerobic function in hagfish centres on the heart, which is typically regarded as an anoxia-intolerant vertebrate tissue. Yet hagfish responses to short-term exposures to anoxia and severe hypoxia are not profound. For example, Hansen and Sidell ([1983]) showed that when the gills of an anaesthetized hagfish (*Myxine glutinosa*) were perfused with nitrogen-equilibrated water (to create severe hypoxia) the gross mechanical activity (frequency and force of heart beat) of an in situ heart was maintained for 3 h. Gross mechanical activity was also maintained for 3 h when the heart was metabolically poisoned to stop mitochondrial respiration using either cyanide or azide. Axelsson et al. ([1990]) showed that cardiac output was maintained in *M. glutinosa* exposed to severe hypoxia (1.5–2.2 kPa), as did Forster et al. ([1992]) for the New Zealand hagfish, *Eptatretus chirratus,* (5.3 kPa) for 15 min (Bernier et al. [1996a], [b]; Forster [1990], [1991]; Forster et al. [1991]; Foster and Forster [2007]). Perry et al. ([1993]) showed that a 15-min anoxic exposure elevated circulating noradrenaline in *M. glutinosa* to the same degree as a 30-min exposure to severe hypoxia (1.1–1.6 kPa). Furthermore, the only in vivo study of prolonged (36 h) anoxia showed that Pacific hagfish (*E. stoutii*) reduced cardiac output by only 33% and cardiac power output (a measure of cardiac ATP requirement) by only 25%, although heart rate was halved (Cox et al. [2010]). These changes were completed by the 3rd hour of anoxia, after which cardiac performance remained unchanged for over 33 h.

These results come as a surprise because the strategy employed by most vertebrates capable of surviving prolonged anoxia is to reduce energy consumption through the controlled suppression of metabolic rate. Metabolic rate suppression (MRS) extends the time that glycogen stores can last and slows the rate of accumulation of noxious anaerobic wastes (Farrell and Stecyk [2007]). Champions in this regard are the anoxia-tolerant freshwater turtles (*Trachemys*, *Pseudemys* and *Chrysemys* species), which are capable of suppressing metabolic rate by up to 99% when submerged in anoxic water (Arthur et al. [1997]; Herbert and Jackson [1985]; Jackson and Ultsch [2010]). Freshwater turtles manage glycolytic wastes using their shell and skeleton to buffer accumulating metabolic wastes such as lactate and hydrogen ions (Jackson [2000]). Hypoxia-tolerant fish also utilize MRS to extend hypoxic or anoxic survival, but their ability to suppress metabolic rate is highly dependent on temperature and the extent of suppression is limited to roughly 60–70% reduction in RMR (Jackson [2000]; Speers-Roesch et al. [2010]; van Ginneken et al. [1999], [2001]; van Waversveld et al. [1988], [1989]). Even the anoxia-tolerant crucian carp, *Carassius carassius*, which converts lactate to

ethanol as a waste management strategy, suppresses brain activity by 31% (Johansson et al. [1995]; Shoubridge and Hochachka [1980]), although it does maintain cardiac output during chronic anoxia (Stecyk et al. [2004]).

The goal of the present study was to characterize whole animal and tissue metabolic responses when hagfish were exposed to prolonged anoxia to test the hypothesis that hagfish, similar to other hypoxia-tolerant fish species studied to date, utilize some form of MRS during anoxia. Whole animal measurement of metabolic rate using calorimetry for vertebrates with low metabolic rates, such as hagfish or anoxic freshwater turtles, is difficult if not impossible (Jackson and Ultsch [2010]). Therefore, metabolic rate was indirectly assessed during anoxia using two independent methods. One approach compared the normoxic oxygen consumption $(\dot{M}O_2)$ with excess post-anoxic $\dot{M}O_2$ (EPAOC), as performed in metabolic studies of intense anaerobic exercise (Gaesser and Brooks [1984]; Hill and Lupton [1923]). The assumption was that, in the absence of metabolic suppression during anoxia, EPAOC must mathematically account for RMR being maintained during the period of anoxia. The second approach used measurements of tissue glycogen disappearance and lactate appearance during anoxia to estimate rates of glycolysis in hagfish tissues known to be metabolically important.

Materials and methods

Adult, sexually immature hagfish (*Eptatretus stoutii* Lockington 1878) were captured at ∼100 m depth in Barkley Sound, BC, Canada (48°50′N, 125°08′W) and transported to the Centre for Aquaculture and Environmental Research (CAER), West Vancouver, BC, Canada. They were housed year round in 1,100 L tanks with aerated, flow-through seawater (temperature 10 ± 1°C; salinity ∼30 ppt). Food was withheld for a minimum of 2 weeks prior to experimentation. All of the following procedures were approved by the University of British Columbia Animal Care Committee (A07-0680) and conducted in accordance with the Canadian Council of Animal Care guidelines.

Experimental protocols

Two separate studies were conducted in order to characterize metabolic responses to anoxia in hagfish. In one, $\dot{M}O_2$ was monitored before and after anoxia (termed respirometry study) and, in the other, tissues were sampled to measure metabolic fuel use and metabolic waste accumulation (termed metabolite study). The two studies shared the same exposure protocol (anoxic periods lasting 6, 24,

36 or 48 h). The respirometry study used 22 hagfish (body mass = 158 ± 3 g) and the tissue metabolite study used 64 hagfish (body mass = 80 ± 3 g). An additional eight hagfish were rapidly killed and blotted dry with paper towel to determine the percentage of body mass of the heart, liver, tongue and parietal muscle.

Respirometry study

Individual hagfish were weighed and placed in one of four identical 2.5 L Loligo respirometer chambers (Loligo Systems, Tjele, Denmark) for at least a 24-h period while RMR was recorded (Fig. 1). After this recovery from handling to starting at around 11:00 for each experiment, the respirometer was flushed (1 L min^{-1}) with nitrogen-saturated seawater from a gas exchange column until our MINI-DO oxygen meters (Loligo Systems, Tjele, Denmark) read 0.0 kPa (usually after ~1 h), at which time the anoxic period started. The point at which hagfish reached whole body anoxia could not be directly measured as it was not possible to measure blood oxygen levels due to the hagfish's tendency to tie knots in attached leads, therefore blocking cannula and destroying fibreoptic oxygen probes. However, cardiovascular measurements (Cox et al. 2010) do inform us about the possibility of a transition period for the initial 3 h of aquatic anoxia. The regularity of the heartbeat and the level of immobility (even at night) for the ensuing 33 h of anoxia convinced us that the last molecule of oxygen had been titred out by the animal's metabolism well before the 6th hour of aquatic anoxia.

Following each of the three anoxic exposures (6, 24 and 36 h; each N = 8 fish), introducing air-saturated seawater restored normoxic conditions within ~10 min. $\dot{M}O_2$ measurements were resumed throughout the recovery period until RMR was restored in all hagfish (Fig. 2). The relatively large volume of the respirometer allowed the hagfish to adopt their relaxed, curled position during
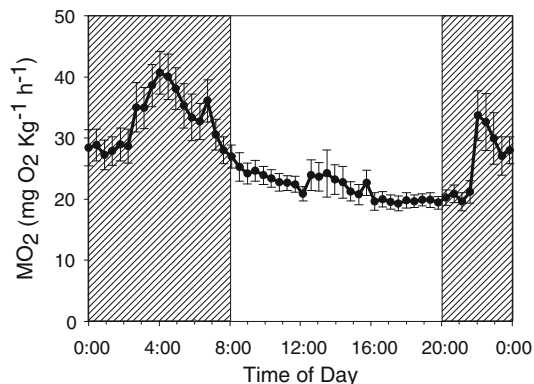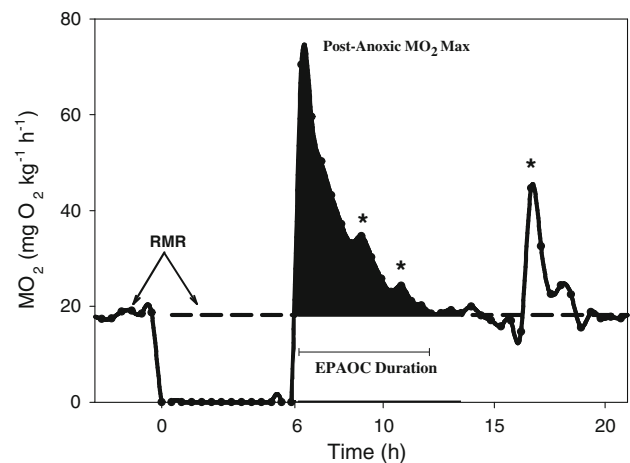

Fig. 2 Representative oxygen consumption trace of a hagfish taken from normoxia, exposed to 6 h of anoxia and then allowed to recover in normoxia. *Asterisk* indicates periods of spontaneous activity. *Shading* indicates measured EPAOC

normoxia and their typical uncurled position during anoxia. A similar experimental protocol was used for a 48-h anoxic period, but the data are not reported because four of the eight hagfish did not recover afterwards.

$\dot{M}O_2$ was determined using intermittent flow respirometry (Steffensen 1989). Each measurement cycle consisted of flushing the respirometer with aerated seawater (10 ± 1°C, 30 ppt) at a flow rate of 1 L min^{-1} for 5 min, followed by a 22-min measurement period during which the chamber was closed and water was recirculated inside the respirometer. Water partial pressure of oxygen (PO_2) was reported each second using MINI-DO probes (Loligo Systems, Tjele, Denmark). The initial 2-min period was discarded because of nonlinear oxygen depletion, while the subsequent 20-min linear period resulted in at least a 10% decrease in water PO_2 (Steffensen 1989), from which $\dot{M}O_2$ was calculated using LoliResp6 software (Loligo Systems Tjele, Denmark). The oxygen probes were calibrated prior to each trial using aerated seawater and an oxygen-free sodium sulphite solution.

Metabolite study

To examine metabolic fuel usage during anoxia, the apparatus and exposure protocol were the same as for the respirometry experiment described above. Hagfish were carefully and rapidly extracted from the respirometers and terminally sampled after 6-, 24- and 36-h anoxic periods (each N = 10 fish). Because hagfish became relatively unresponsive during anoxia, they rarely moved when removed from the respirometer. Fish were rapidly euthanized by concussion and a blood sample was collected immediately from the caudal sinus. The heart, liver, tongue and a dorsal muscle chunk were promptly extracted, in that

Fig. 1 Average $\dot{M}O_2$ during the 24-h pre-anoxic acclimation period (N = 22). *Shading* indicates dark, night time period

order, and immediately freeze-clamped in liquid nitrogen. The tissue sampling procedure took <4 min. Even so, this sampling method resulted in unusually low tissue levels of ATP and creatine phosphate (CrP). Since ATP and CrP analysis required a faster tissue sampling protocol an additional experiment ($N = 6$) was performed using a faster tissue sampling protocol. Fish were similarly removed from the respirometers and euthanized, but were then immediately freeze-clamped whole (<5 s) using two liquid nitrogen cooled aluminium plates. Tissues were extracted from the frozen fish while submerged in liquid nitrogen. There was no statistical difference in glycogen or lactate levels between these two tissue sampling protocols and so reported glycogen and lactate values used the first sampling protocol and reported ATP and CrP values used the second protocol.

Blood samples were immediately analysed for pH, glucose and lactate. Whole blood pH was measured in triplicate using a Radiometer micro-capillary electrode (G299A) thermostated (10°C) in a Radiometer BMS-2 system and displayed on a Radiometer PHM 73 (Radiometer, Copenhagen, Denmark). The whole blood sample was then centrifuged in a microcentrifuge tube at $7,000g$ for 5 min to collect plasma, which was stored on ice for immediate analysis of glucose and lactate using a YSI 2300 Stat plus analyzer (YSI Life Sciences, Yellow Springs, OH, USA).

All tissue samples were stored whole and again after being ground into a fine powder (using a mortar and pestle cooled with liquid nitrogen) at −80°C until analysis. For glycogen, glucose and lactate analysis, approximately 100 mg of ground tissue was weighed into pre-cooled micro centrifuge tubes and ice-cold 1 mol L^{-1} $HClO_4$ was added. Samples were immediately homogenized at the highest speed of a Polytron homogenizer (PT1200C, Kinematica, Lucerne, Switzerland) for 15 s at 0°C and then stored on ice. Each sample was vortexed and 200 µL of the homogenate slurry was transferred into a microcentrifuge tube, placed in liquid nitrogen and stored at −80°C until glycogen content was measured. The remaining homogenate was centrifuged for 5 min at 4°C at $20,000g$, the supernatant was neutralized with 3 mol L^{-1} K_2CO_3 and assayed for lactate and glucose (after Bergmeyer 1983). Tissue homogenates were thawed on ice and partially neutralized with 3 mol L^{-1} K_2CO_3 to measure glycogen content as free glucose following digestion with amyloglucosidase (Bergmeyer 1983).

Calculations and statistics

Excess post-anoxic oxygen consumption (EPAOC) was determined as the area between the $\dot{M}O_2$ recovery curve and the measured minimum RMR for individual fish (Fig. 2; see results) and is referred to as measured EPAOC. Fish activity during recovery may have resulted in a slight overestimate of measured EPAOC for some individuals. We assumed that if *E. stoutii* maintain minimum RMR throughout anoxia, then measured EPAOC must at least equal or exceed (depending on the cost to replenish metabolite and oxygen stores) the estimate of EPAOC as calculated from the product of minimum RMR and the time in anoxia (6, 24 or 26 h). To make comparisons, ATP turnover rates were calculated using the equivalents of 4.5 mol of ATP to 1 mol of O_2, 3 mol of ATP to 1 mol of glucose from glycogen, and 0.67 mol of ATP to 1 mol of lactate (Busk and Boutilier 2005; Rolfe and Brand 1996).

Statistical differences in EPAOC and measurements of tissue metabolites among different anoxic exposures were tested using a one-way ANOVA, followed by a Holm–Sidak post-hoc test. Holm–Sidak is recommended as the most appropriate post-hoc test by the statistical package Sigmastat (Aspire Software International, Ashburn, VA, USA). Comparisons between measured and estimated EPAOC values for a given anoxic exposure were tested using t-tests. Statistical significance was set at $p \leq 0.05$ and all values are reported as means ± SEM.

Results

Normoxic metabolic rate

During daytime, hagfish were quiescent (curled and inactive), with $\dot{M}O_2$ being its lowest between 16:00 and 20:00 (Fig. 1). Minimum RMR, taken as the mean for daylight $\dot{M}O_2$ measurements, was 20.6 ± 1.0 mg O_2 kg^{-1} h^{-1} ($N = 22$) at 10°C. Hagfish were nocturnally active, particularly around 22:00 and 04:00 (visual observations), which was reflected in their elevated $\dot{M}O_2$ (Fig. 1). This spontaneous activity could triple RMR to 64.7 ± 3.0 mg O_2 kg^{-1} h^{-1}. The highest recorded $\dot{M}O_2$ (up to 80 mg O_2 kg^{-1} h^{-1}) was measured immediately following handling during the transfer to the respiration chambers.

Respirometry study

No mortality was observed for anoxic periods lasting up to 36 h and all hagfish recovered back to their RMR. EPAOC is reported for the 6-, 24- and 36-h anoxic periods (Table 1). The peak, post-anoxic $\dot{M}O_2$ value was reached within the first 20 min of the recovery period. This peak $\dot{M}O_2$ was approximately four times higher than minimum RMR and unaffected by the duration of anoxia (Table 1). Thereafter, $\dot{M}O_2$ declined towards minimum RMR (Figs. 2, 3), with EPAOC lasting approximately as long as the anoxic period (Fig. 3, Table 1). Spontaneous activity sometimes created minor spikes in $\dot{M}O_2$ (Fig. 2) after

Table 1 Mean pre-anoxic routine metabolic rate and post-anoxic recovery variables

Anoxic period (h)	Pre-anoxic RMR (mg O_2 kg^{-1} h^{-1})	Peak post-anoxic $\dot{M}O_2$ (mg O_2 kg^{-1} h^{-1})	Duration of EPAOC (h)	EPAOC (mg O_2 kg^{-1})	N
6	20.8 ± 1.8	82.7 ± 8.2[a]	8.6 ± 1.7[a]	133.2 ± 31.1[a]	8
24	21.3 ± 2.5	83.1 ± 6.9[a]	22.0 ± 3.5[b]	418.5 ± 97.0[b]	7
36	19.7 ± 2.3	73.3 ± 4.0[a]	36.6 ± 5.7[c]	371.7 ± 41.4[b]	7

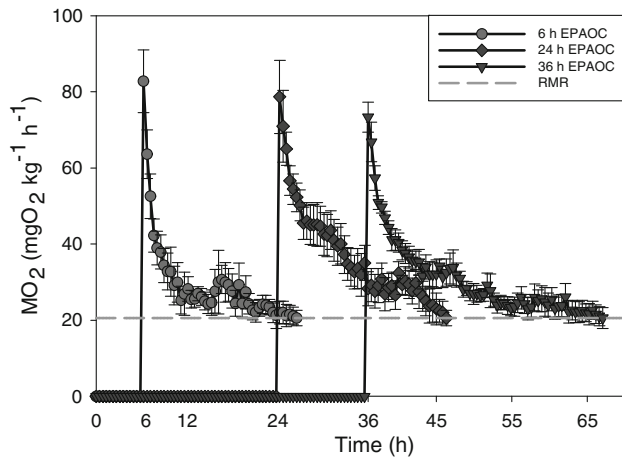Different letters indicate significant differences within columns ($p \leq 0.05$)

Fig. 3 Average measured EPAOC for the three anoxic treatment groups. Individual fish underwent one of the three anoxic treatments. Time 0 represents transfer of fish from normoxia into anoxia. Normoxia was restored at 6 h ($N = 8$), 24 h ($N = 7$), and 36 h ($N = 7$), and EPAOC was measured. Average routine metabolic rate (RMR) was calculated from the preceding normoxic period (see "Materials and methods")

Table 2 Mean ATP equivalents for measured and estimated EPAOCs

Time in anoxia (h)	Estimated ATP equivalents of EPAOC (mmol kg^{-1})	Measured ATP equivalents of EPAOC (mmol kg^{-1})
6	17.5 ± 1.5[a]	18.7 ± 4.4[a]
24	71.7 ± 8.6[b]	58.9 ± 9.4[b]
36	99.9 ± 11.6[c]	52.3 ± 5.8[b*]

Different letters indicate significant differences within columns ($p \leq 0.05$)

* Significant difference between estimated and measured values ($p \leq 0.05$)

several hours into recovery during night-time, but not during daytime. Such spikes were most evident after a 6-h anoxic period (Fig. 3). Therefore, EPAOC following 6 h of anoxia may have been slightly over estimated.

Measured EPAOC values for the 6- and 24-h anoxic periods were not significantly different from their estimated EPAOC values (Table 2). Even so, the 3-fold increase in measured EPAOC between the 6- and 24-h anoxic periods was less than the theoretical 4-fold increase expected had metabolic rate remained unchanged during the 24-h anoxic exposure. Furthermore, measured EPAOC did not change significantly between the 24- and 36-h anoxic periods (Table 2). As a result, the measured 3-fold increase in EPAOC between the 6- and 36-h periods was less than the theoretical 6-fold increase had metabolic rate remained unchanged. Consequently, the measured EPAOC for the 36-h anoxic period was half of the estimated EPAOC (Table 2).

Metabolic study

Metabolite concentrations in heart, liver, tongue and skeletal muscle are shown in Figs. 4 and 5 for the normoxic

state. By comparison, glycogen in all four tissues was significantly lower and lactate significantly higher following the 36-h anoxic period (Figs. 4 and 5).

The rates of glycogen depletion and lactate production varied among tissues. Skeletal and tongue muscle, which represent 35 and 6.6% of body mass, respectively, did not decrease their glycogen levels significantly until after 24 h of anoxia (Fig. 4a) and more than 50% of these glycogen stores remained after 36 h of anoxia. Skeletal muscle lactate did not change over the 24-h anoxic exposure, but increased 3-fold after 36 h (Fig. 5a). Similarly, lactate levels in tongue muscle did not change significantly until the 24 h measurement, but then increased by 8-fold after 36 h of anoxia (Fig. 5a).

In contrast to skeletal and tongue muscle, liver and heart, which together compose 3 and 0.1% of body mass, respectively, nearly completely depleted their glycogen during the initial 6 h of anoxia, without further change thereafter. However, their lactate levels increased progressively over the entire 36-h anoxic period (Figs. 4b, 5b).

Blood lactate levels also increased progressively during the 36-h anoxic period, while blood pH decreased progressively (Figs. 4c, 5c). Glucose concentrations were maintained in heart, liver and skeletal muscle during anoxia, but increased significantly in tongue and blood after 36 h of anoxia (Table 3). With the exception of a significant and sustained decrease in cardiac ATP after 6 h, ATP and CrP levels were maintained during anoxia in all tissues (Table 2).
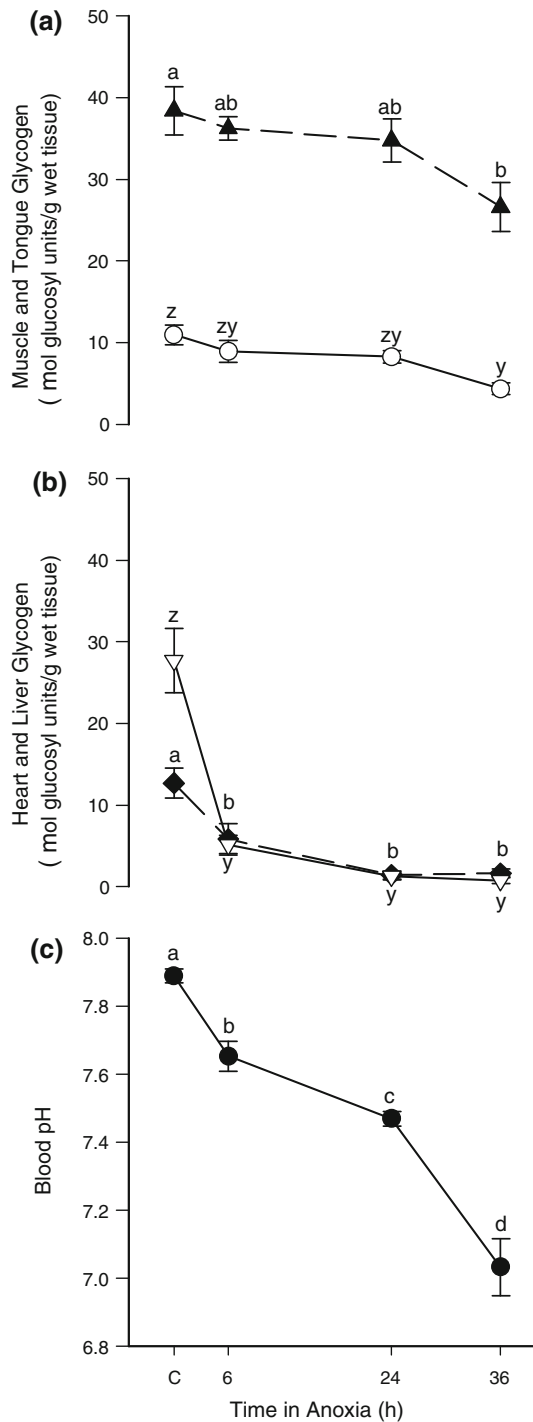
Fig. 4 Glycogen concentrations in **a** tongue (*filled triangle*) and skeletal muscle (*circle*), **b** heart (*inverted triangle*) and liver (*filled diamond*), and **c** blood pH (*filled circle*). *Dissimilar letters* denote statistical differences ($p \leq 0.05$) between time points within a specific tissue. *N* values are 6–10 and SEM is indicated by *vertical bars*

Fig. 5 Lactate concentrations in **a** tongue (*filled triangle*) and skeletal muscle (*circle*), **b** heart (*inverted triangle*) and liver (*filled diamond*), and **c** blood (*filled circle*). *Dissimilar letters* denote statistical differences ($p \leq 0.05$) between time points within a specific tissue. *N* values are 6–10 and SEM is indicated by *vertical bars*

Discussion

The present study provides information on the anoxia tolerance of the Pacific hagfish, which were shown to fully
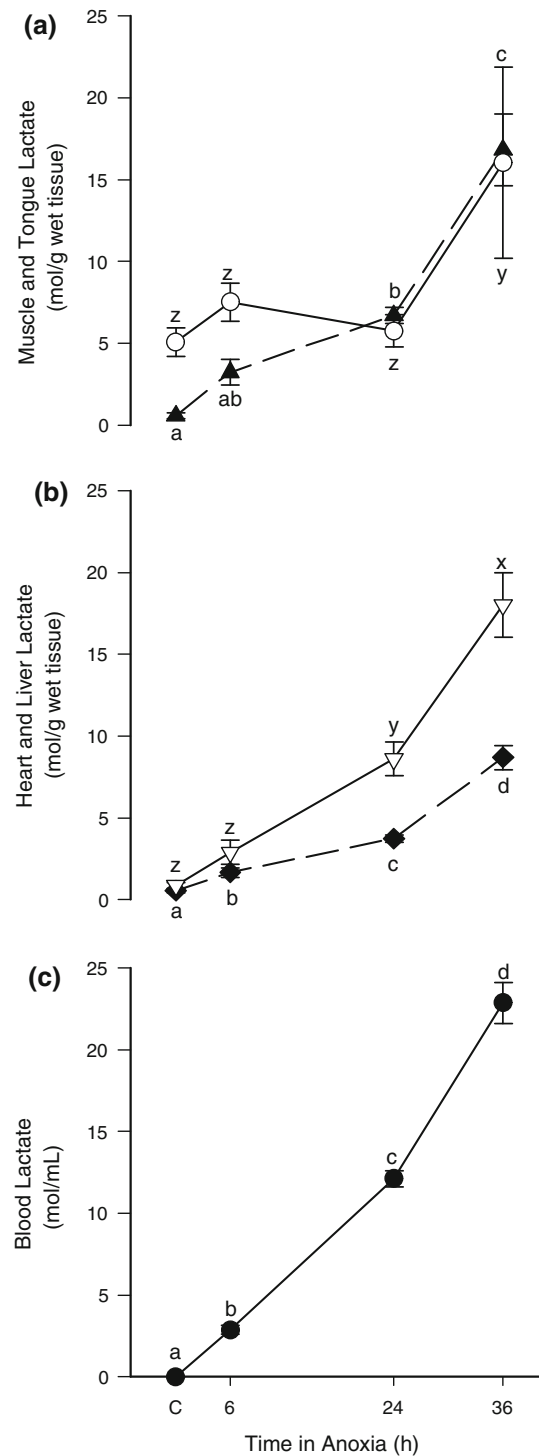
recover from a 36-h anoxic exposure at 10°C. This result agrees with previous work on this species (Cox et al. 2010). While such an impressive anoxia tolerance was anticipated

Table 3 Tissue glucose, CrP, ATP and blood glucose following exposure to normoxia (control) and for varying durations of anoxia

Tissue	Metabolite (μmol g^{-1} wet tissue)	Control	Time in anoxia (h)		
			6	24	36
Heart	Glucose	0.31 ± 0.12 (8)	0.56 ± 0.22 (9)	0.54 ± 0.07 (9)	0.29 ± 0.15 (7)
	CrP	1.14 ± 0.05 (6)	0.81 ± 0.36 (3)	0.66 ± 0.31 (3)	0.71 ± 0.07 (4)
	ATP	6.93 ± 0.43[a] (6)	3.45 ± 1.51[b] (3)	2.47 ± 1.13[b] (3)	4.04 ± 0.47[ab] (4)
Liver	Glucose	0.95 ± 0.32 (10)	1.43 ± 0.38 (9)	1.33 ± 0.11 (9)	1.33 ± 0.32 (8)
	CrP	0.74 ± 0.09 (6)	5.33 ± 3.28 (6)	7.26 ± 3.88 (6)	2.97 ± 1.89 (6)
	ATP	2.25 ± 0.58 (6)	2.71 ± 0.31 (6)	2.79 ± 0.11 (6)	2.40 ± 1.08 (7)
Tongue	Glucose	0.003 ± 0.03[a] (10)	0.15 ± 0.06[a] (9)	0.75 ± 0.12[a] (9)	1.73 ± 0.20[b] (8)
	CrP	5.07 ± 0.79 (6)	8.59 ± 1.68 (6)	7.01 ± 1.08 (6)	5.27 ± 1.23 (7)
	ATP	10.10 ± 1.35 (5)	11.95 ± 3.33 (6)	11.00 ± 1.71 (7)	14.82 ± 1.56 (7)
Muscle	Glucose	0.21 ± 0.04 (9)	0.42 ± 0.04 (10)	0.51 ± 0.06 (10)	0.58 ± 0.17 (5)
	CrP	5.58 ± 0.52 (6)	7.88 ± 1.17 (5)	7.22 ± 0.56 (7)	5.63 ± 0.86 (7)
	ATP	9.37 ± 0.96 (6)	10.87 ± 1.63 (6)	14.08 ± 1.48 (7)	13.95 ± 2.82 (7)
Blood	Glucose (μmol ml^{-1})	0.55 ± 0.05[a] (10)	0.61 ± 0.06[a] (10)	1.36 ± 0.07[b] (10)	1.16 ± 0.22[b] (10)

Different letters indicate significant differences ($p \leq 0.05$) between time points within a specific tissue

given their deep-sea habitat and feeding strategy, 48 h may be close to the limit of anoxic survival time at this temperature. Anoxia tolerance in hagfish comes about in part through a combination of an extremely low RMR and partial depletion of their fermentable glycogen at 36 h of anoxia. A telling result in this regard was the level of blood acidosis at a 36-h anoxic period, i.e., pH 7.03, which approached the pH regarded as lethal for anoxic freshwater turtles (pH < 6.8; Herbert and Jackson 1985). Clearly, the anoxic survival time for *E. stoutii* falls well short of that for crucian carp, which can survive many weeks of anoxia at 8°C, and is the only other reported anoxia-tolerant fish (Johnston and Bernard 1983; Nilsson 1990; Stecyk et al. 2004). Even so, *E. stoutii* is far more anoxia-tolerant than the hypoxia-tolerant common carp (*Cyprinus carpio*), which survive 6 h in anoxia at 15°C (Johnston and Bernard 1983), and tilapia, which survive 24 h in severe hypoxia (5 kPa) at 22°C aided by MRS (Speers-Roesch et al. 2010).

The results of this respirometry experiment lead us to accept our hypothesis that hagfish, similar to other anoxia tolerant vertebrates, utilize MRS during anoxic periods of up to 36 h in duration. Measured EPOAC for a 36-h exposure was clearly well below that required to account for RMR being sustained. As shown below, the results for the metabolite experiment also lead to the same conclusion. Even so, both experimental approaches are indirect estimates of the animal's metabolic rate during anoxia and so the assumptions associated with our measurements are scrutinised below.

One possibility is that RMR was overestimated. This is unlikely since we made the novel observation of a diurnal variation in RMR. Therefore, RMR was assigned to the minimum daytime $\dot{M}O_2$ measurements, when hagfish were coiled and immobile and $\dot{M}O_2$ changed very little. Hagfish were nocturnally active, which corresponded to peaks in $\dot{M}O_2$. This spontaneous activity tripled RMR, confirming 2- to 3-fold increases observed for *E. cirrhatus* during activity (Forster 1990). RMR also quadrupled during post-anoxic recovery and reached levels comparable with RMR of the lamprey *Geotria australis* (64 mg O$_2$ kg^{-1} h^{-1}; Macey et al. 1991) and the Atlantic cod *Gadus morhua* (76 mg O$_2$ kg^{-1} h^{-1}; Bushnell et al. 1994). The RMR (21 mg O$_2$ kg^{-1} h^{-1}) measured here is mid-range for previous RMR measurements reported for hagfishes. The highest RMR reported is 48 mg O$_2$ kg^{-1} h^{-1} for *Myxine glutinosa* at 7°C (Steffensen et al. 1984) and the lowest is 12 mg O$_2$ kg^{-1} h^{-1} for *E. cirrhatus* at 11°C (Forster 1990). Munz and Morris (1965) calculated RMR for *E. stoutii* as 11–14 mg O$_2$ kg^{-1} h^{-1} at 10°C. Thus, our RMR measurement is consistent with hagfishes possessing the lowest metabolic rates among fishes (Brauner and Berenbrink 2007).

Given the EPOAC measured after the 36-h anoxic period, metabolic rate would have had to be maintained at about half of the measured minimum RMR. Therefore, we conclude that our respirometry experiment points to metabolic suppression averaging around 50% of the RMR during a 36-h anoxic period. However, most of this suppression occurred in the final third of this 36-h period given that we could fully account for the measured EPAOC over the first 24-h anoxia exposure through the maintenance of the minimum RMR. This suggestion is quantitatively supported by the results of the metabolite experiment.

Glycogen and lactate were measured in the hagfish's metabolically most active tissues (heart, liver, tongue and muscle), which composed just over 40% of body mass. Total glycogen for these four metabolically active tissues totalled 90 $\mu mol \ g^{-1}$, which could support RMR for 40 h. Metabolically less active tissues, namely skin, gut and cartilage, were omitted from the tissue analysis. Conservatively assuming a similar amount of glycogen in the unmeasured tissues (i.e., likely over estimating total glycogen), the glycogen store could support RMR for 93 h. We found that glycogen in the metabolically active tissues was depleted by only 60% over the 36-h anoxic period, suggesting that exhaustion of glycogen stores had not become a limiting factor for anoxic survival. In fact, there was no significant change in glycogen content in any tissue during the final 30 h of anoxia exposure. This finding is consistent with studies on freshwater turtles, which show that the energetic costs of overwintering are not limiting factors for anoxic survival whereas waste accumulation may be (Jackson and Ultsch 2010). The measured glycogen utilization rate suggests either a 63% MRS, or an alternative fuel source, or a more efficient use of glycogen than used in the calculations. Similar results were obtained when lactate appearance was used to estimate how long glycogen stores would support RMR during anoxia, a 75% MRS during the 36-h anoxic period.

Some uncertainty exists regarding our quantitative estimates of MRS because hagfish do not become immediately anoxic although the water is anoxic. There is a transition state of progressively worsening hypoxia while the animal titres down its heme oxygen stores at a rate determined by their overall metabolic rate. We estimated that the hagfish's heme oxygen stores could sustain RMR under anoxic conditions for 20 min. Hemoglobin oxygen stores would last ~ 15 min assuming _E. stoutii_, like _E. cirrhatus,_ has a blood oxygen carrying capacity of 0.022 ml O_2 per ml of blood (Wells et al. 1986) and a 15% blood volume (Forster et al. 2001). Myoglobin stores would add a further 5 min, assuming myoglobin binds 1.34 ml of oxygen per gram of myoglobin and _E. stoutii_ myoglobin concentration is similar to that of _E. cirrhatus_ (0.0078 g of myoglobin per gram of muscle tissue) (Davison et al. 1990; Hedrick et al. 1986; Wells and Forster 1989). Uncertainty will remain regarding this transition state until experiments are performed using calorimetry or continuous measurement of blood oxygen partial pressure. Cox et al. (2010) continuously measured cardiac output and heart rate during this transition period and it is interesting to note that they found heart rate dropped within 3 h to a value that was half of that seen in normoxic animals. Heart rate remained at this low level for an additional 33 h during anoxic exposure. Thus, a transition state of some sort exists for the first 3 h at this temperature. Regardless, the

consequence of this transition period is that the measured EPAOC will be proportionally lower relative to the estimated EPAOC and that this bias is obviously greatest for the 6-h anoxic period. However, the bias introduced by the heme oxygen stores amounts to <1% of the measured EPAOC for the longer 36-h anoxic period, an error that is clearly insufficient to discount our conclusion of a 50% MRS. Our estimates of ATP turnover from glycogen turnover and lactate appearance suffer from the same bias, but this error would be larger since the ATP equivalence for anoxic glycolysis and oxidative phosphorylation is an over 10-fold difference.

Additional evidence for the use of MRS in anoxia survival is found in the constant tissue ATP levels during anoxia (Table 3, Fig. 4) (Bickler and Buck 2007; Hochachka et al. 1996; Richards et al. 2008). While tissue freezing was as rapid as possible, tissue ATP or CrP levels may have been under-estimated for normoxic hagfish with a sampling time of about 5 s. Normoxic hagfish tended to tighten their coiled bodies and produced slime prior to being freeze-clamped. However, anoxic hagfish were unresponsive during sampling and remained immobile and therefore we are more confident of the representative nature of these measurements for anoxic hagfish. Muscle ATP levels in normoxic hagfish are comparable to those found in rainbow trout white muscle (Richards et al. 2002). Conversely normoxic hagfish muscle CrP levels are up to 8-fold lower when compared to rainbow trout white muscle (Richards et al. 2002).

While the above considerations focus on the potential to overestimate the degree of metabolic suppression, an overarching (and potentially offsetting) assumption for the post-anoxic recovery of oxygen and metabolite stores was that there is no "recovery tax". This is unlikely to be the case since excess post-exercise oxygen consumption following intense exercise routinely exceeds the product of RMR and exercise duration (Borsheim et al. 1998; Gaesser and Brooks 1984; Hancock and Gleeson 2008). While future studies of whole body calorimetry with hagfish may be required to fully resolve these quantitative issues, the metabolic suppression suggested by the prolonged anoxia experiments performed here place hagfish among the company of other anoxia- and hypoxia-tolerant vertebrates.

As impressive as the 36-h anoxic tolerance was for _E. stoutii_, it is clear from both the EPAOC and metabolite measurements that the response to anoxia is both staged and tissue-dependent, with the heart and liver showing a rapid glycolytic response and the skeletal and tongue muscle showing a slower one. During the first 6 h of anoxia, metabolic rate, as estimated from EPAOC, was not altered profoundly whereas glycogen stores in the heart and liver were nearly depleted. As the liver in fish is considered a major glucose source (Nilsson and Östlund-Nilsson

Table 4 Total depletion of glycogen and accumulation of lactate between control values and following 36 h of anoxia

Delta	Liver	Heart	Muscle	Tongue	Blood
Glycogen (μmol g^{-1} glycosyl units g^{-1} wet tissue)	11.1 ± 1.9	26.9 ± 4.2	6.59 ± 1.4	11.8 ± 4.2	N/A
Lactate (μmol g^{-1} wet tissue or μmol ml^{-1} blood)	8.13 ± 0.8	17.1 ± 2.0	11.0 ± 5.9	16.3 ± 2.2	22.86 ± 1.2

2008), glycogen depletion in the liver may be linked to glucose supply to other tissues. In terms of the heart, its routine normoxic ATP needs can be estimated as 0.23 mmol ATP g^{-1} h^{-1} based on a measured power output of 0.26 mW during prolonged anoxia (Cox et al. 2010) and a conversion to an cardiac O$_2$ consumption of 1.5 mg O$_2$ g^{-1} h^{-1} (Forster 1991). Hagfish reduce cardiac power output by only 25% after 3 h into the same anoxic protocol as used here (Cox et al. 2010), therefore it is hardly surprising that cardiac glycogen was almost entirely depleted by the 6th hour of anoxia. Furthermore, because cardiac power output remains unchanged for the remainder of the 36-h anoxic period, the heart must be supplied with glucose from other tissues by the blood. The situation for cardiac glucose supply is similar in freshwater turtles, which can switch from endogenous glycogen to exogenous glucose with no impact on cardiac function (Reeves 1963). In contrast, glycogen depletion by the tongue and skeletal muscle was minor.

Relative to the 6-h anoxic data, changes in EPAOC and tissue glycogen at 24 h of anoxia were not statistically different. Nevertheless, blood pH continued to decrease, lactate accumulated in all tissues including blood, and blood glucose levels were maintained. These results suggest a steady state of glycolytic activity, but introduce the possibility of an alternative fermentable fuel(s) to glycogen. Possibilities are gluconeogenic amino acids that could be converted into glucose in the liver and then transported to other organs for glycolysis (Hochachka and Somero 2002). This possibility should be explored in future studies, especially since alternative fermentable fuels to glycogen are common among invertebrate animals (Hochachka 1980). Regardless of the fermentable fuel used, metabolic rate was diminished below RMR because the measured EPAOC was less than the estimated EPAOC.

The final third of the 36-h anoxic period was characterised by substantial depletion of glycogen stores in skeletal and tongue muscle, suggesting that these stores are temporarily conserved during the early part of the anoxic exposure. There were corresponding sharp rises in tissue and blood lactate concentrations and a decrease in blood pH. Measured EPAOC was significantly lower than the estimated EPAOC, indicating MRS.

Theoretically, glycogen depletion should quantitatively match lactate appearance at the level of each tissue. However, there were quantitative discrepancies, much of which can be explained by the translocation of cellular lactate (and H$^+$) into the blood. This result suggests that hagfish use their very large blood volume to dilute anoxic metabolic wastes from tissues, assuming the glycolytic activity of the blood is relatively minor (Table 4). Similarly, blood lactate increases and blood pH decreases following exhaustive exercise in *E. cirrhattus* (Davison et al. 1990; Ruben and Bennett 1980). However, this translocation was clearly tissue-specific during anoxia. Calculations from glycogen depletion suggest that the tongue and muscle retained 80 and 70%, respectively, of the lactate produced, whereas the liver and heart only retained 37 and 32%, respectively, of the lactate produced (Table 4). The possibility that hagfish excreted lactate into the surrounding water seems unlikely as lactate was undetectable in water samples taken at the end of the 36-h anoxic period (data not shown). Hansen and Sidell (1983) measured the increase in lactate in the anoxic hagfish heart to be roughly half that expected from cardiac glycogen depletion. Forster (1991) measured lactate removal from perfused, hypoxic hagfish hearts. Furthermore, Davison et al. (1990) showed blood lactate and H$^+$ equilibrate freely with the voluminous subcutaneous sinus, thereby removing concerns that our blood samples taken from the caudal sinus were unreliable indicators of the blood status.

Hagfish glycogen stores do not appear to be particularly high relative to other anoxia-tolerant vertebrates. Liver glycogen values were >100-times lower than those in the crucian carp during winter (Hyvärinen et al. 1985) and 10- to 50-times lower than those in anoxia-tolerant freshwater turtle (Daw et al. 1967; Packard and Packard 2005; Warren and Jackson 2008). Cardiac glycogen stores were comparable to those of rainbow trout, but again were substantially less than in crucian carp and freshwater turtles (Beall and Privitera 1973; Gesser 2002; Vornanen 1994; Warren and Jackson 2008). Normoxic cardiac glycogen, glucose and lactate measured here were comparable to those measured by Hansen and Sidell (1983) in the ventricle of *M. glutinosa*. Furthermore, 20 h of severe hypoxia resulted in comparable levels of cardiac glycogen and lactate to those measured here following 24 h of anoxia (Hansen and Sidell 1983). Normoxic liver glycogen levels measured here compare with those in *M. glutinosa* (Foster et al. 1993). However, cardiac glycogen ($\sim$60 μmol g^{-1} wet tissue) and tongue ($\sim$85 μmol g^{-1} wet tissue) of *E. cirrhatus*, are nearly triple and double, respectively, those of *M. glutinosa*

ventricle (Forster 1991; Baldwin et al. 1991; Hansen and Sidell 1983). Clearly, rather than high glycogen stores, the anoxic tolerance of *E. stoutii* lies in its very low RMR and also its ability to undergo MRS during prolonged anoxia.

Measurements of glycolytic rates in hagfishes include those of the tongue muscle (~7% of total body mass) during active feeding, which are comparable to those of tuna white muscle during burst swimming (Baldwin et al. 1991; Guppy et al. 1979). Baldwin et al. (1991) reported lactate concentrations in retractor muscles of *E. cirrhatus* following 1.5- to 6.5-min bursts of continuous feeding to be among the highest ever reported for muscle work by unrestrained animals. During the early stages of anoxia, however, the tongue's glycogen stores appear to be preserved.

In summary, we present two compelling, independent, but indirect lines of evidence to suggest *E. stoutii* undergo metabolic suppression during a 36-h anoxic exposure to prolonged anoxia. EPAOC measurements suggest an average 50% suppression of ATP turnover, while calculation of ATP turnover from tissue glycogen depletion and lactate accumulation suggests 63–75% suppression, respectively. The various assumptions used in arriving at these quantitative estimates will require verification using whole body calorimetry and further experiments are needed to evaluate alternative fuels to glycogen. In addition, there is a suggestion that MRS was staged and this resulted in variable rates and timing of glycogen depletion among tissues. Neither the level of anoxic MRS nor the liver glycogen stores in *E. stoutii* approached those reported for freshwater turtles. Instead, the degree of MRS appears to be in line with that seen so far in all other hypoxia-tolerant fishes (goldfish, tilapia and European eel), which reduce metabolic rate ~70% during anoxia (Jackson 2000; Speers-Roesch et al. 2010; van Ginneken et al. 1999, 2001; van Waversveld et al. 1988, 1989). Thus, the anoxia tolerance of hagfishes centres on their normally very low RMR. This conclusion conforms with the suggestion that an inherently low RMR can be functionally adaptive for hypoxic and anoxic survival (Mandic et al. 2009; Stecyk et al. 2004).

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