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RESEARCH ARTICLE

Exceptional cardiac anoxia tolerance in tilapia (*Oreochromis* hybrid)

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SUMMARY

Anoxic survival requires the matching of cardiac ATP supply (i.e. maximum glycolytic potential, MGP) and demand (i.e. cardiac power output, PO). We examined the idea that the previously observed *in vivo* downregulation of cardiac function during exposure to severe hypoxia in tilapia (*Oreochromis* hybrid) represents a physiological strategy to reduce routine PO to within the heart's MGP. The MGP of the ectothermic vertebrate heart has previously been suggested to be ~70 nmol ATP s^{-1} g^{-1}, sustaining a PO of ~0.7 mW g^{-1} at 15°C. We developed an *in situ* perfused heart preparation for tilapia (*Oreochromis* hybrid) and characterized the routine and maximum cardiac performance under both normoxic (>20 kPa O_2) and severely hypoxic perfusion conditions (<0.20 kPa O_2) at pH 7.75 and 22°C. The additive effects of acidosis (pH 7.25) and chemical anoxia (1 mmol l^{-1} NaCN) on cardiac performance in severe hypoxia were also examined. Under normoxic conditions, cardiac performance and myocardial oxygen consumption rate were comparable to those of other teleosts. The tilapia heart maintained a routine normoxic cardiac output ($\dot{Q}$) and PO under all hypoxic conditions, a result that contrasts with the hypoxic cardiac downregulation previously observed *in vivo* under less severe conditions. Thus, we conclude that the *in vivo* downregulation of routine cardiac performance in hypoxia is not needed in tilapia to balance cardiac energy supply and demand. Indeed, the MGP of the tilapia heart proved to be quite exceptional. Measurements of myocardial lactate efflux during severe hypoxia were used to calculate the MGP of the tilapia heart. The MGP was estimated to be 172 nmol ATP s^{-1} g^{-1} at 22°C, and allowed the heart to generate a PO_{max} of at least ~3.1 mW g^{-1}, which is only 30% lower than the PO_{max} observed with normoxia. Even with this MGP, the additional challenge of acidosis during severe hypoxia decreased maximum ATP turnover rate and PO_{max} by 30% compared with severe hypoxia alone, suggesting that there are probably direct effects of acidosis on cardiac contractility. We conclude that the high maximum glycolytic ATP turnover rate and levels of PO, which exceed those measured in other ectothermic vertebrate hearts, probably convey a previously unreported anoxia tolerance of the tilapia heart, but a tolerance that may be tempered *in vivo* by the accumulation of acidotic waste during anoxia.

Key words: heart, cardiovascular, hypoxia, energy metabolism, acidosis, fish.

INTRODUCTION

Environmental hypoxia occurs commonly in aquatic environments. Despite the significant physiological challenge this represents for fishes, many fishes have evolved the ability to survive extended periods of exposure to hypoxia (i.e. low levels of oxygen) and even anoxia (i.e. total lack of oxygen) (Nikinmaa and Rees, 2005). An adaptive strategy considered central to hypoxia and anoxia tolerance in fishes is the ability to match ATP demand to the reduced capacity for ATP supply during hypoxia despite an increased reliance on anaerobic glycolysis (Boutilier, 2001; Farrell and Stecyk, 2007).

Sustained cardiac function is central to hypoxia and anoxia tolerance in fishes, even if it occurs at a depressed rate. During exposure to either hypoxia or anoxia, convection is still required to distribute fermentable fuel and hormones, and remove waste (Driedzic and Gesser, 1994; Farrell and Stecyk, 2007). However, the heart is also one of the most sensitive organs to oxygen deprivation. Under hypoxic conditions, the heart must sustain its activity by relying considerably upon anaerobic glycolysis, which is fuelled by endogenous glycogen stores and blood-borne glucose (Gamperl and Driedzic, 2009). In anoxia, a total energetic reliance on anaerobic glycolysis is required, where the maximum glycolytic potential of the cardiac muscle (MGP; the maximum ATP production solely from glycolysis) must be able to supply sufficient ATP to satisfy the ATP demand of the heart's operating power output (PO), which has a minimum level. Survival time under anoxia then becomes limited either by the size of the glycolytic fuel stores or by waste accumulation (lactate and hydrogen ions). Therefore, cardiac anoxia tolerance in the first instance requires that cardiac ATP demand, as set by PO, can be accommodated by MGP (Farrell and Stecyk, 2007).

The few vertebrate hearts that are known to be anoxia tolerant possess an *in vivo* cardiac ATP demand during anoxia exposure that is below their cardiac MGP. These species include crucian carp [*Carassius carassius* (Stecyk et al., 2004; Vornanen et al., 2009)], hagfishes [*Myxine glutinosa* (Axelsson et al., 1990), *Eptatretus cirrhatus* (Forster et al., 1992) and *Eptatretus stoutii* (Cox et al., 2010)] and some species of North American freshwater turtles, such as the painted turtle [*Chrysemys picta* (Jackson, 2002)]. Two mechanisms have emerged for achieving a cardiac ATP demand below the MGP during anoxia: (1) exhibiting a naturally low routine cardiac ATP demand that can be sustained by anaerobic glycolysis [e.g. hagfish and crucian carp (Farrell and Stecyk, 2007; Gesser and Overgaard, 2009)] or (2) substantially downregulating cardiac ATP demand to a level sustainable by anaerobic glycolysis [e.g. painted turtle and common carp (*Cyprinus carpio*) (Jackson, 2002; Stecyk

and Farrell, 2006)]. Cardiac downregulation is typically achieved by slowing heart rate (f_H) (Farrell, 2007), the mechanical equivalent of spike arrest seen in the anoxic turtle brain (Lutz and Nilsson, 1997). Based on all available studies, but on less than a dozen species, Farrell and Stecyk proposed that the MGP for the ectothermic vertebrate heart is ~70 nmol ATP s^{-1} g^{-1} at 15°C, and that this will support a PO of 0.7 mW g^{-1} (Farrell and Stecyk, 2007).

Tilapia are hypoxia-tolerant cichlid fishes that inhabit environments that can experience regular bouts of hypoxia. Tilapia lack a coronary circulation (Pieperhoff et al., 2009), and they downregulate routine cardiac activity in hypoxia *in vivo* (Speers-Roesch et al., 2010). Speers-Roesch and colleagues showed that tilapia (*Oreochromis* hybrid: *Oreochromis niloticus* × *mossambicus* × *hornorum*) exposed to 8 h at a water partial pressure of oxygen (P_{O_2}) of 1 kPa decreased f_H, cardiac output ($\dot{Q}$) and PO by 50–60% (Speers-Roesch et al., 2010), and affirmed previous claims that bradycardia is an important component of cardiac hypoxia tolerance (Farrell, 2007; Farrell and Stecyk, 2007). Further, they reported that these changes in cardiac function were associated with increases in heart lactate content and decreases in cardiac intracellular pH, and also suggested that depression of PO was necessary to match ATP demand with the limited capacity for ATP supply from anaerobic glycolysis (Speers-Roesch et al., 2010).

The purpose of the present study was to characterize the MGP and maximum cardiac power output (PO_{max}) of the *in situ* tilapia heart under conditions of severe hypoxia (<0.20 kPa, pH 7.75), chemical anoxia (1 mmol l^{-1} NaCN with <0.20 kPa, pH 7.75), and severe hypoxia with extracellular acidosis (<0.20 kPa, pH 7.25). This allowed us to test the hypothesis that the downregulation of cardiac function seen in tilapia during exposure to low oxygen *in vivo* (Speers-Roesch et al., 2010) is a physiological strategy to reduce routine PO to within the MGP (Farrell and Stecyk, 2007). To accomplish our objective, an *in situ* perfused heart preparation was developed that generated maximum cardiac performance comparable to *in vivo* levels, permitting tight control of perfusion conditions and cardiac workload (Farrell et al., 1988; Farrell et al., 1989), and allowing for measurement of lactate efflux and myocardial oxygen consumption rate ($\dot{V}_{O_2}$). These last values were required to define ATP turnover rate and the MGP of the tilapia heart.

MATERIALS AND METHODS

Animals

Adult male tilapia (*Oreochromis niloticus* × *mossambicus* × *hornorum*; strain origin: Ace Developments, Burneau, ID, USA) were purchased from Redfish Ranch (Courtenay, BC, Canada). The fish were held in well-aerated 400 l aquaria with recirculating freshwater (22°C) at the University of British Columbia. Fish (N=20, 475±6 g) were fed daily with commercial trout pellets (FirstMate, Taplow Aquaculture, North Vancouver, BC, Canada) for at least 3 months before experimentation. All experimental procedures were conducted according to guidelines approved by the Animal Care Committee at the University of British Columbia, and the Canadian Council on Animal Care.

Surgical procedures

Fish were anaesthetized in aerated water containing buffered ethyl 3-aminobenzoate methane sulphonate (MS-222; Sigma-Aldrich, Oakville, ON, Canada; 0.2 g l^{-1} MS-222 buffered with 0.2 g l^{-1} $NaHCO_3$), weighed, and placed on a surgery table where their gills were continuously irrigated *via* the mouth with chilled, aerated water containing buffered MS-222 (0.15 g l^{-1} MS-222 buffered with 0.2 g l^{-1} $NaHCO_3$). They were then injected with 1 ml kg^{-1} of heparinized saline (200 i.u. ml^{-1}) *via* the caudal vessels. Because of anatomical differences between tilapia and rainbow trout (*Oncorhynchus mykiss*), the *in situ* perfused tilapia heart preparation involved slight modifications to the protocol previously described for rainbow trout (Farrell et al., 1986; Farrell et al., 1989).

In brief, an incision was made through the body wall from the anal opening to the posterior edge of the pectoral girdle to expose the viscera and permit access to the 1–5 hepatic veins (some of the seemingly larger vessels were composed of several smaller vessels). Then, all of the vessels but the largest, continuous, ventrally located hepatic vein were tied off using 3/0 silk thread (Seraflex, Serag Wiessner, Naila, Germany), and a stainless steel input cannula was inserted into the remaining hepatic vein *via* a shallow incision into the most anterior portion of the vessel. This was done so that advancing the input cannula along the hepatic vein placed the cannula tip inside the sinus venosus. With the input cannula secured by silk thread and the pericardium left intact, saline (see below for composition) perfusion of the heart was started immediately from a temporary input reservoir containing adrenaline bitartate salt (5 nmol l^{-1}) and sodium heparin (10 i.u. ml^{-1}). Outflow from the heart was collected in a stainless steel output cannula inserted and secured into the ventral aorta between the second and third gill arches with two 0/0 silk threads (16S, Deknatel, Teleflex Medical, Research Triangle Park, NC, USA). The insertion of the output cannula was facilitated by removal of the lower jaw and gills, until a point where the tip of the cannula rested confluent with the bulbus arteriosus. The total time required to complete the perfused heart preparation was approximately 30 min.

Following surgery, the fish were transferred to and immersed in a physiological saline bath (0.7% NaCl) maintained at 22°C. The input cannula was immediately connected to an adjustable constant-pressure reservoir, and the output cannula was connected to a separate constant pressure head set at a routine output pressure of 2.5 kPa. The height of the input pressure reservoir was adjusted to set cardiac filling pressure and routine $\dot{Q}$ to approximately 12 ml min^{-1} kg^{-1}, simulating *in vivo* $\dot{Q}$ under resting, normoxic conditions (Speers-Roesch et al., 2010). $\dot{Q}$ was measured by an in-line electromagnetic flow probe in the output line (SWF-4, Zepeda Instruments, Seattle, WA, USA). Input (P_{in}) and output (P_{out}) pressures were measured through saline-filled side arms (PE50 tubing) connected to pressure transducers (DPT 6100, Smiths Medical, Kirchsecon, Germany). Hearts were allowed to equilibrate for approximately 20 min under routine conditions (see below) before the experimental trials commenced.

Perfusion composition

The perfusate contained (in mmol l^{-1}) 124.1 NaCl, 2.50 KCl, 0.93 $MgSO_4 \cdot 7H_2O$, 2.52 $CaCl_2 \cdot 2H_2O$, 5.55 glucose, 3.87 TES acid and 6.13 TES salt (Sigma-Aldrich). At 22°C the perfusate was pH 7.75; *in vivo* blood pH for resting, normoxic tilapia is ~7.7–7.8 at 22°C (B.S.-R., unpublished results). Regardless of the experimental trial, the perfusate contained 5 nmol l^{-1} of adrenaline, which was replenished every 20 min to offset rapid degradation. This level of adrenaline is within the range measured *in vivo* in tilapia plasma under routine conditions (Vianen et al., 2001; Chen et al., 2002), and was chosen based on the results of preliminary experiments, which were performed because of the variable results for the effect of adrenergic stimulation of the teleost heart. For example, while *in situ* perfused hearts of sea raven (*Hemitripterus americanus*), ocean pout (*Zoarces americanus*) and rainbow trout required elevated catecholamine levels (i.e. adrenaline concentration up to 500 nmol l^{-1}) to achieve maximal *in situ* performance (Farrell et al., 1983; Hanson et al., 2006), there is little effect of adrenaline on the

heart of Atlantic cod [*Gadus morhua* (Petersen and Gamperl, 2010)]. In our preliminary experiments, cumulative dose–response trials (in mol l^{-1}: 0, 5×10^{-10}, 5×10^{-9}, 5×10^{-8} and 5×10^{-7}) with adrenaline (N=3) were performed on *in situ* tilapia hearts under normoxic conditions, and maximum cardiac performance was assessed at each concentration following a 10 min equilibration period of routine work. In the absence of adrenaline, maximum cardiac output ($\dot{Q}_{max}$; 39±6 ml min^{-1} kg^{-1}) and PO_{max} (4.4±0.4 mW g^{-1}) were similar (one-way repeated measures ANOVA with Holm–Sidak *post hoc* tests) to the values obtained with the 5×10^{-9} mol l^{-1} adrenaline routinely used in all experiments (~39 ml min^{-1} kg^{-1} and ~5 mW g^{-1}), and similar to those obtained using 5×10^{-7} mol l^{-1} adrenaline (38±6 ml min^{-1} kg^{-1} and 4.6±0.3 mW g^{-1}). Similarly, preliminary measurements with high levels of noradrenaline (5×10^{-6} mol l^{-1}; N=2), comparable to those seen *in vivo* (Vianen et al., 2001; Chen et al., 2002), appeared to have no impact on maximum cardiac performance during exposure to severe hypoxia with acidosis or following recovery from this treatment. During an insult of severe hypoxia with acidosis (<0.20 kPa O_2, pH 7.25), hearts were able to achieve values of $\dot{Q}_{max}$ and PO_{max} with 5×10^{-6} mol l^{-1} noradrenaline of 23±1 ml min^{-1} kg^{-1} and 2.3±0.8 mW g^{-1}, respectively, compared with 21±2 ml min^{-1} kg^{-1} and 2.4±0.3 mW g^{-1} in the severe hypoxia with acidosis trial with 5×10^{-9} mol l^{-1} adrenaline (see below; N=7). Further, after recovery from severe hypoxia with acidosis these values were 28±1 ml min^{-1} kg^{-1} and 3.4±0.2 mW g^{-1} with 5×10^{-6} mol l^{-1} noradrenaline, compared with 30±2 ml min^{-1} kg^{-1} and 3.1±0.2 mW g^{-1} with 5×10^{-9} mol l^{-1} adrenaline. Therefore, we feel confident that any major influences of adrenergic stimulation on the heart's mechanical performance were not overlooked by using 5×10^{-9} mol l^{-1} adrenaline in the perfusate.

The control, normoxic perfusate was aerated with medical-grade compressed air (Praxair, Vancouver, BC, Canada) for at least 1 h prior to use. Previous studies with rainbow trout have demonstrated no significant difference in *in situ* cardiac performance between air-saturated and hyperoxic (95.5% O_2, 0.5% CO_2) perfusate (Hanson et al., 2006). The severe hypoxia exposure (<0.20 kPa O_2, typically ~0.07 kPa O_2, pH 7.75) was achieved by gassing the perfusate with compressed medical-grade N_2 (Praxair). The water P_{O_2} achieved for severe hypoxia exposure was 7-fold lower than the haemoglobin-O_2 P_{50} of tilapia (1.45 kPa) (Speers-Roesch et al., 2010), and below the venous P_{O_2} of tilapia exposed to severe environmental hypoxia of <1 kPa O_2 *in vivo* (B.S.-R., unpublished results). Acidotic perfusate (<0.20 kPa O_2, pH 7.25) required altering the ratio of TES acid (to 5.91 mmol l^{-1}) and TES salt (to 4.09 mmol l^{-1}) in the saline in accordance with the Henderson–Hasselbalch equation. This level of acidosis is comparable to the venous blood pH observed in tilapia and other fishes exposed to severe environmental hypoxia *in vivo* (Scott et al., 2008) (B.S.-R., unpublished results). The effect of chemically arresting oxidative phosphorylation was also tested by adding 1 mmol l^{-1} NaCN to the severely hypoxic perfusate. This level of NaCN is commonly used to inhibit cardiac oxidative metabolism (Driedzic, 1983; Arthur et al., 1992; Xie et al., 1998). Ingress of oxygen into the cardiac tissues during the severe hypoxia exposure was impeded by the surrounding tissues and pericardium, by using glass connectors and tubes for the input perfusate, and by bubbling the bath saline with N_2 for 20 min prior to the onset of and during exposure to severe hypoxia and trapping the N_2 gas over the preparation with a plastic lid.

Experimental trials

Three separate experimental trials were used: severe hypoxia (<0.20 kPa O_2, pH 7.75), severe hypoxia with acidosis (<0.20 kPa O_2, pH 7.25) and chemical anoxia (severe hypoxia with 1 mmol l^{-1} NaCN, <0.20 kPa O_2, pH 7.75). Each trial consisted of measuring maximum cardiac performance during each point in the following sequence of exposures (Fig. 1A): normoxia, severe hypoxia (alone or with additional treatment of chemical anoxia or acidosis) and recovery in normoxia (except following exposure to NaCN). Thus, each preparation acted as its own control. For all preparations, lactate efflux and $\dot{V}_{O_2}$ were also measured at strategic intervals during each exposure (Fig. 1A) to assess the contributions of aerobic metabolism and anaerobic glycolysis to cardiac work under conditions of routine and maximum cardiac performance. At the conclusion of each trial, the heart (N=20, ventricular mass=0.163±0.005 g) was rapidly excised while performing routine $\dot{Q}$, blotted to remove excess liquid and weighed.

Severe hypoxia trial (N_2 at pH 7.75)

This trial (N=6) quantified the effects of N_2-induced severe hypoxia (<0.20 kPa O_2, and typically ~0.07 kPa O_2). The tilapia heart was first exposed to 20 min of routine performance in normoxia, followed by a 10 min test of normoxic maximum cardiac performance, and 20 min of routine normoxic performance. The heart was then exposed to a progressive decline in P_{O_2} from normoxia to severe hypoxia over a period of 10–20 min, and severe hypoxia (<0.20 kPa O_2) was maintained for at least 50 min before reassessing maximum cardiac performance. The total exposure to severe hypoxia lasted 70 min. Afterwards, the bath cover was removed, N_2 bubbling of the bath stopped and the hearts were allowed to recover in normoxic saline for 20 min. It took <2 min for the perfusate P_{O_2} to reach 20.0–21.3 kPa O_2. Following a 10 min reassessment of maximum cardiac performance under normoxic conditions, and a subsequent 10 min of routine performance in normoxia, the heart was quickly excised and weighed.

Severe hypoxia with acidosis trial (N_2 at pH 7.25)

This trial (N=7) quantified the additive effects of acidosis (pH 7.25 compared with pH 7.75) in combination with N_2-induced, severe hypoxia (<0.20 kPa O_2). The experimental protocol was identical to that of the severe hypoxia trial, except that after a 40 min period of severe hypoxia the heart was additionally perfused with severely hypoxic acidotic saline for 30 min (20 min of routine cardiac function and 10 min for assessment of maximum performance), bringing the total treatment time to 70 min. The heart was then allowed to recover in normoxic saline (pH 7.75) for 20 min, followed by a 10 min reassessment of maximum cardiac performance under these conditions, before being returned to routine levels for 10 min, and then excised and weighed.

Chemical anoxia trial (N_2 + NaCN at pH 7.75)

This trial (N=7) tested the additional effects of NaCN when combined with N_2-induced, severe hypoxia (<0.20 kPa O_2). The experimental protocol was identical to that of the severe hypoxia trial, except that after a 40 min period of severe hypoxia, 1 mmol l^{-1} NaCN (Sigma-Aldrich) was added to the perfusate and perfusion continued for 30 min (20 min of routine cardiac function and 10 min for assessment of maximum performance), bringing the total treatment time to 70 min. Following the test of maximum cardiac performance, the heart was returned to routine $\dot{Q}$ in chemical anoxia without being returned to normoxic conditions. It was then excised, blotted, weighed and frozen in liquid nitrogen for subsequent analysis of myocardial lactate content (see below). It was elected that the chemically anoxic hearts not be recovered for a number of reasons. First, while reversing N_2-induced hypoxia is relatively simple upon reperfusion with normoxic saline,

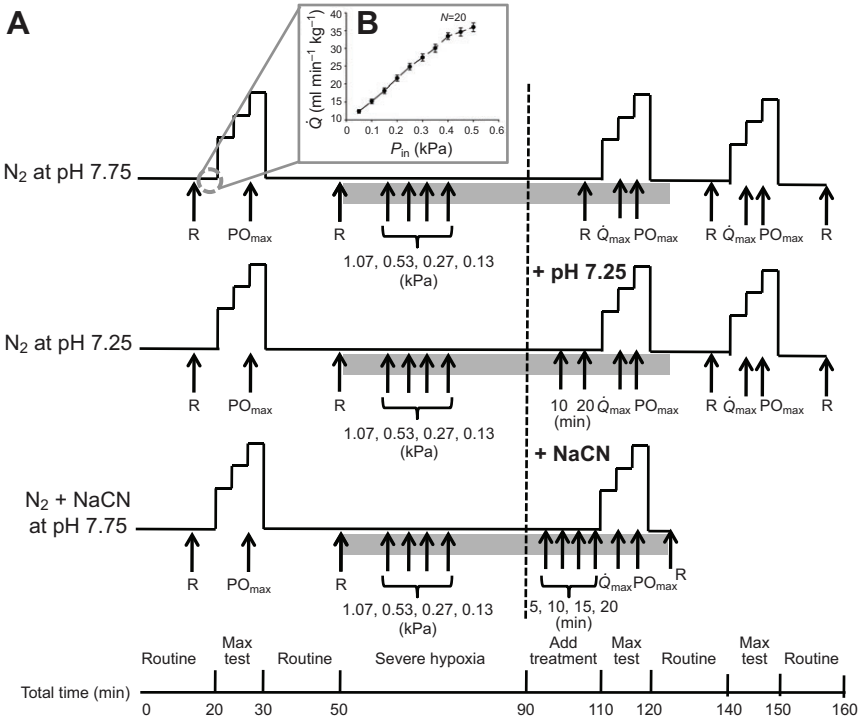

Fig. 1. (A) Graphical representation of the three trials (severe hypoxia, N_2 at pH 7.75; severe hypoxia with acidosis, N_2 at pH 7.25; and chemical anoxia, N_2+NaCN at pH 7.75) used to assess routine and maximum cardiac performance of tilapia hearts. Each preparation was first tested under normoxic conditions so that it acted as its own control. Output pressure (P_{out}) is indicated by the solid black line (routine levels set at 2.5 kPa), and tests of maximum cardiac performance (max tests) are represented by the stepwise increases in P_{out}. The grey shaded area represents the common exposure to severe hypoxia (<0.20 kPa O_2), and the vertical dashed line indicates the addition of treatment (pH 7.25 for acidosis or 1 mmol l^{-1} NaCN for chemical anoxia) to the severely hypoxic perfusate. The arrows indicate the 30 s period over which perfusate was sampled for lactate, and when $\dot{V}_{O_2}$ measurements were taken in normoxia. The numbers labelling the arrows grouped by the bracket indicate the particular oxygen tensions at which lactate efflux was measured, while the numbers after the addition of treatment (on the right side of the vertical dashed line) represent the time after the beginning of the treatment (acidosis, pH 7.25; chemical anoxia, 1 mmol l^{-1} NaCN) at which lactate was sampled. R represents a lactate sample after 15 min of routine cardiac output ($\dot{Q}$; 12 ml min^{-1} kg^{-1}) in a given exposure. $\dot{Q}_{max}$ and maximum cardiac power output (PO$_{max}$) both indicate lactate samples after 1 min of maximum cardiac performance. (B) A normoxic Starling curve (cumulative values, N=18) curve for the *in situ* perfused tilapia heart. Each value is the mean (±s.e.m.) $\dot{Q}$ at a particular input pressure (P_{in}) within a particular trial.

we could not guarantee that NaCN would be completely and quickly washed out. Also, recovery from N_2 has greater biological significance than recovery from NaCN.

Assessment of cardiac performance

Characterization of maximum cardiac performance

Maximum cardiac performance was quantified before and during all exposure treatments by measuring $\dot{Q}_{max}$ and PO$_{max}$. Starling curves were generated by examining the relationship between filling pressure (P_{in}) and $\dot{Q}$ when P_{in} was raised in 0.05 kPa increments and allowed to stabilize at each increment. P_{in} was increased until $\dot{Q}_{max}$ was determined. PO typically increased from routine levels by 2- to 3-fold and in proportion with $\dot{Q}$, after which P_{out} was increased in 0.3 kPa increments until PO$_{max}$ was reached. Perfusate samples for lactate analysis were taken after 1 min stabilization at both $\dot{Q}_{max}$ and PO$_{max}$. Each assessment of maximum cardiac performance lasted about 10 min, after which P_{out} and P_{in} were restored to routine levels.

Oxygen consumption and lactate efflux

Two in-line oxygen probes (Microx TX3 fiber-optic oxygen meter, PreSens, Regensburg, Germany) continuously monitored and logged the P_{O_2} entering and leaving the heart so that $\dot{V}_{O_2}$ could be calculated at any point during the experiment. $\dot{V}_{O_2}$ (µl O_2 s^{-1} g^{-1} ventricular mass) of the *in situ* tilapia heart was calculated as follows (Graham and Farrell, 1990):

$$\dot{V}_{O_2} = (\Delta P_{O_2} \times \alpha_{O_2} \times \dot{Q}) / (M_v \times 60) , \quad (1)$$

where ΔP_{O_2} is the decrease in P_{O_2} between the input and output perfusate (kPa), α_{O_2} is the solubility of oxygen in the perfusate [0.2946 µl O_2 ml^{-1} perfusate kPa^{-1} at 22°C (Graham, 1987)], $\dot{Q}$ is in ml min^{-1}, M_v is ventricular mass (g) and 60 is the conversion from minutes to seconds.

The outflow perfusate was sampled at strategic intervals to measure lactate efflux from the heart (Fig. 1A). The sampling line was first cleared for 30 s and then 0.5 ml samples were removed over a 30 s period. These samples were taken at routine and maximum levels of cardiac function, including while hypoxia was developing (1.07, 0.53, 0.27 and 0.13 kPa O_2). Heart and perfusate samples were frozen and stored at –80°C until lactate analysis was performed.

Perfusate [lactate] was measured in duplicate using spectrophotometric methods described previously (Bergmeyer, 1983). Lactate efflux rate (nmol lactate min^{-1} g^{-1} ventricular mass) was calculated as:

$$\text{Lactate efflux rate} = ([\text{lactate}]_{\text{perfusate}} \times \dot{Q} \times 1000) / M_v , \quad (2)$$

where $[\text{lactate}]_{\text{perfusate}}$ is the lactate concentration in the outflow perfusate sample (nmol l^{-1}), $\dot{Q}$ is in ml min^{-1}, M_v is in g (Arthur et

al., 1992), and 1000 is the conversion factor to litres from millilitres. Inflow perfusate was periodically sampled during every experiment and always contained non-detectable levels of lactate. The MGP was taken as the lactate efflux rate when the heart was performing at PO_{max} in severe hypoxia (see Discussion for a consideration of the O_2 present in perfusate during severe hypoxia).

A comparison of the myocardial lactate contents was necessary to determine whether lactate efflux could be used to accurately compare glycolytic capacity and MGP between trials. In other words, we wanted to confirm that the level of lactate accumulated in the heart was similar between trials; a discrepancy in these values between trials would indicate that differences in lactate efflux might be due to differences in lactate handling rather than differences in glycolytic capacity. Complementing our sampled hearts from the NaCN trial, we performed additional experiments where hearts were sampled during exposure to normoxia (N=3) or during exposure to severe hypoxia (N_2 at pH 7.75; N=3). In both cases, the protocol was identical to the NaCN trial (i.e. each trial lasted a total of 120 min including two PO_{max} tests) except no NaCN was used and the hearts remained either normoxic throughout the trial before sampling or severely hypoxic before sampling. Hearts were harvested shortly after the end of the second PO_{max} test. Lactate content was $<0.01\,\mu mol\,g^{-1}$ ventricular mass in normoxic hearts, but increased significantly (one-way ANOVA, $P<0.05$) in hearts under severe hypoxia ($2.1\pm0.2\,\mu mol\,g^{-1}$) and chemical anoxia ($2.2\pm0.3\,\mu mol\,g^{-1}$). However, the myocardial lactate contents under NaCN and severe hypoxia were not significantly different from each other (one-way ANOVA, $P>0.05$), indicating that lactate efflux could be accurately used to compare MGP between these treatments. To obtain myocardial lactate content, frozen heart tissue was broken into small pieces under liquid N_2 using an insulated mortar and pestle. For lactate extraction, 0.8 ml of ice-cold $1\,mol\,l^{-1}$ $HClO_4$ was added to a microcentrifuge tube containing 50–100 mg of tissue and the mixture was immediately sonicated on ice for three bursts of 10 s using a Kontes sonicator on its highest setting. The homogenate was centrifuged at 10,000 ***g*** for 10 min at 4°C and the supernatant neutralized with $3\,mol\,l^{-1}$ K_2CO_3. Neutralized extracts were assayed spectrophotometrically for lactate content following previous methods (Bergmeyer, 1983).

Cardiac ATP turnover rate ($nmol\,ATP\,s^{-1}\,g^{-1}$) was estimated from $\dot{V}_{O_2}$ and lactate efflux. In normoxia and recovery, ATP turnover rate was primarily a result of $\dot{V}_{O_2}$ (assuming that 1 mol O_2 results in the formation of 6 mol ATP) (Reeves, 1963; Ferguson, 1987; Mast and Elzinga, 1990), with a minor contribution of lactate efflux (assuming that 1 mol lactate yields 1.5 mol ATP, and that lactate is derived from either glucose or glycogen). ATP turnover rate was plotted against routine and maximum PO ($mW\,g^{-1}$). ATP turnover rate in severe hypoxia, severe hypoxia with acidosis, and chemical anoxia was estimated from lactate efflux only, and were plotted against PO values that corresponded to routine $\dot{Q}$, $\dot{Q}_{max}$ and PO_{max}.

Data acquisition and data analysis

Real-time measurements of f_H, P_{in}, P_{out}, $\dot{Q}$ and PO were recorded throughout the experiment using data acquisition software (Labview version 5.1, National Instruments, Austin, TX, USA). The oxygen probes were calibrated with a temperature sensor at two points, 0% O_2 ($1\,g\,l^{-1}$ Na_2SO_3; Sigma-Aldrich) and 21% O_2 (air-saturated water), before each experiment. Pressure transducers were similarly calibrated before each experiment and their signals were amplified with a Senselab amplifier (Somedic Sales AB, Hörby, Sweden). The ventral aortic flow signal from the in-line flow probe was amplified (Flowmeter SWF-4, Zepeda Instruments, Seattle, WA, USA) and regularly calibrated volumetrically with known flow rates of perfusate. Cannulae resistances were calibrated by measuring the pressure drop across the cannulae at known flow rates and these resistances were used to adjust the measured P_{in} and P_{out} for the relevant flow (subtracting the pressure drop from the measured P_{in} and adding the pressure drop to the measured P_{out}). Thus, reported P_{in} and P_{out} values represent accurate pressures in the sinus venosus and in the bulbus arteriosus, respectively. Reported values for f_H, P_{in}, P_{out}, $\dot{Q}$ and PO represent 30 s averages either immediately prior to taking a perfusate sample or after 1 min of stabilization during an assessment of maximum performance, and were acquired using the LabView perfused heart data acquisition and analysis program written by Drs M. Axelsson (University of Gothenburg) and J. Altimiras (Linköping University). f_H was calculated from the pulsatile pressure trace. PO ($mW\,g^{-1}$ ventricle) was calculated as:

$$PO = \dot{Q} \times (P_{out} - P_{in}) \times 0.0167 / M_v , \quad (3)$$

incorporating $\dot{Q}$ ($ml\,min^{-1}$), P_{out} and P_{in} (kPa), M_v (g) and 0.0167 (the conversion factor to mW). Stroke volume (V_S) was calculated as:

$$V_S = (\dot{Q} / f_H) / M_b , \quad (4)$$

where $\dot{Q}$ was measured in $ml\,min^{-1}$, f_H in $beats\,min^{-1}$ and M_b (body mass) in kg (Overgaard et al., 2004).

Statistical analysis

Values for $\dot{Q}_{max}$ during normoxia, severe hypoxia or severe hypoxia with treatment, and recovery were compared within experimental trials using a one-way ANOVA with Holm–Sidak *post hoc* tests. The same procedure was carried out for PO_{max}, routine $\dot{Q}$ and routine PO within trials. One-way ANOVA with Holm–Sidak *post hoc* tests were used to compare parameters (including routine $\dot{Q}$, routine PO, routine f_H, $\dot{Q}_{max}$ and PO_{max}) within exposures (normoxia, severe hypoxia or severe hypoxia with treatment, and recovery) across different experimental trials (i.e. normoxia values were compared between the severe hypoxia, severe hypoxia with acidosis, and chemical anoxia trials). Two-way ANOVA was used to compare parameters such as $\dot{V}_{O_2}$, lactate efflux and ATP turnover rate among different treatments (i.e. at different points in the experiment) and experimental trials. Repeated measures were not possible in these data sets because of variable N values. For statistical comparisons, α=0.05 was used to determine statistical significance. Statistical analyses were carried out using SigmaStat 3.0.

RESULTS

Cardiac performance in normoxia

Normoxic tilapia hearts exhibited a typical Starling curve with $\dot{Q}$ increasing 3-fold from routine levels when P_{in} was increased from ~0.05 to 0.50 kPa (Fig. 1B). $\dot{Q}_{max}$ (~39 $ml\,min^{-1}\,kg^{-1}$) and PO_{max} (~5 $mW\,g^{-1}$) in normoxia did not differ across trials. During tests of maximum performance, f_H remained unchanged (data not shown).

Routine cardiac performance

Routine levels of $\dot{Q}$ (~12 $ml\,min^{-1}\,kg^{-1}$) and PO (~1.4 $mW\,g^{-1}$) were unaffected by any of the three severe hypoxia treatments (severe hypoxia alone, or severe hypoxia with either acidosis or chemical anoxia; Fig. 2A,B). Despite a sustained routine performance, after a 40 min exposure to severe hypoxia, the intrinsic f_H of the perfused heart was significantly decreased by ~13% from ~55 $beats\,min^{-1}$ in normoxia (Fig. 2C). No further change in f_H occurred with a subsequent 20 min exposure to either severe hypoxia alone or with chemical anoxia, but a subsequent 20 min exposure to severe

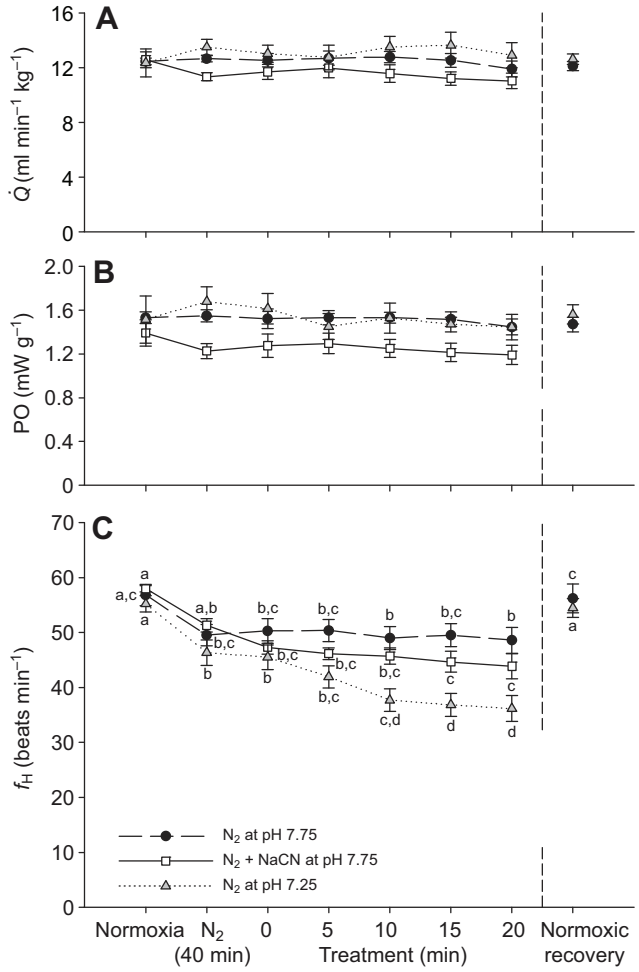

Fig. 2. Routine cardiac function (A, $\dot{Q}$; B, PO; and C, heart rate f_H) of the *in situ* tilapia heart during normoxia, after 40 min severe hypoxia, throughout 0–20 min of treatment exposures (treatments: N_2 at pH 7.75, N=6; N_2 at pH 7.25, N=7; or N_2 + NaCN at pH 7.75, N=6), and after 20 min of normoxic recovery. Values are means ± s.e.m. The vertical dashed line represents the 10 min during which a maximum cardiac function assessment was performed under treatment conditions. Different letters indicate statistically significant differences (P<0.05) for f_H at different time points in a particular trial; there was no significant change in $\dot{Q}$ or PO within any of the trials (P>0.05).

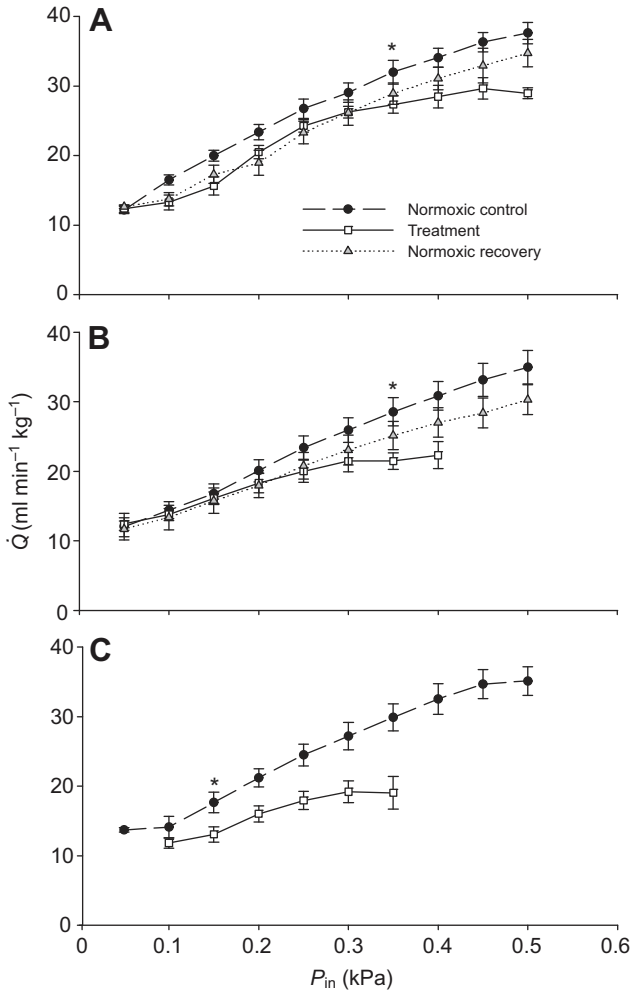

Fig. 3. Starling curves for *in situ* perfused tilapia hearts for all exposures of each trial (A, N_2 at pH 7.75, N=6; B, N_2 at pH 7.25, N=7; and C, N_2 + NaCN at pH 7.75, N=7). Starling curves of performance in each trial are included for the normoxic control, the treatment period and the normoxic recovery (except following exposure to NaCN). Each value is the mean ± s.e.m. $\dot{Q}$ at a particular P_{in} within a particular trial. An asterisk indicates the beginning of statistically significant differences (P<0.05) between the treatment line and its normoxic control.

hypoxia with acidosis led to an additional decline in f_H such that f_H fell to ~35% below the normoxic level (Fig. 2C). In all trials, f_H was restored to the normoxic level after 5 min of recovery.

Maximum cardiac performance

Severe hypoxia

Severe hypoxia made the tilapia heart less sensitive to filling pressure (as shown by a downward shift in the upper arm of the Starling curve compared with normoxia), which resulted in a significant 22% reduction in $\dot{Q}_{max}$ (Fig. 3A, Fig. 4A). PO_{max} was also reduced significantly (by 30%) when compared with normoxia (Fig. 4B). Normoxic saline produced a partial but nearly complete recovery, with $\dot{Q}_{max}$ and PO_{max} not being significantly different from normoxia.

Severe hypoxia with acidosis

Acidosis had an additive effect on cardiac function with severe hypoxia, further decreasing $\dot{Q}_{max}$ and PO_{max}. $\dot{Q}_{max}$ declined by 41% and PO_{max} by 50% compared with normoxia (Fig. 4A,B), again with a decreased sensitivity to filling pressure (Fig. 3B). Normoxic recovery was also lower than that from severe hypoxia alone, with $\dot{Q}_{max}$ and PO_{max} ~20% lower than normoxia (P<0.05).

Chemical anoxia (severe hypoxia with NaCN)

NaCN (1 mmol l^{-1}) had an additive effect on cardiac function with severe hypoxia by right-shifting the Starling curve (Fig. 3C), and decreasing sensitivity of the heart to increased filling pressure. Consequently, $\dot{Q}_{max}$ in chemical anoxia was 49% lower than in normoxia (Fig. 4A) and PO_{max} was 65% lower than in normoxia (Fig. 4B). Both $\dot{Q}_{max}$ and PO_{max} were significantly lower than maximum cardiac performance in severe hypoxia alone (P<0.05), but similar to performance during the additive treatment of acidosis. Recovery was not attempted in these hearts, as previously explained.

ATP turnover rate (myocardial $\dot{V}_{O_2}$ and lactate efflux rate)

Normoxia

Myocardial $\dot{V}_{O_2}$ in all normoxic trials increased with increasing PO up to ~2.5-fold compared with routine values (Fig. 5A). Normoxic $\dot{V}_{O_2}$ values were not significantly different across trials, and lactate

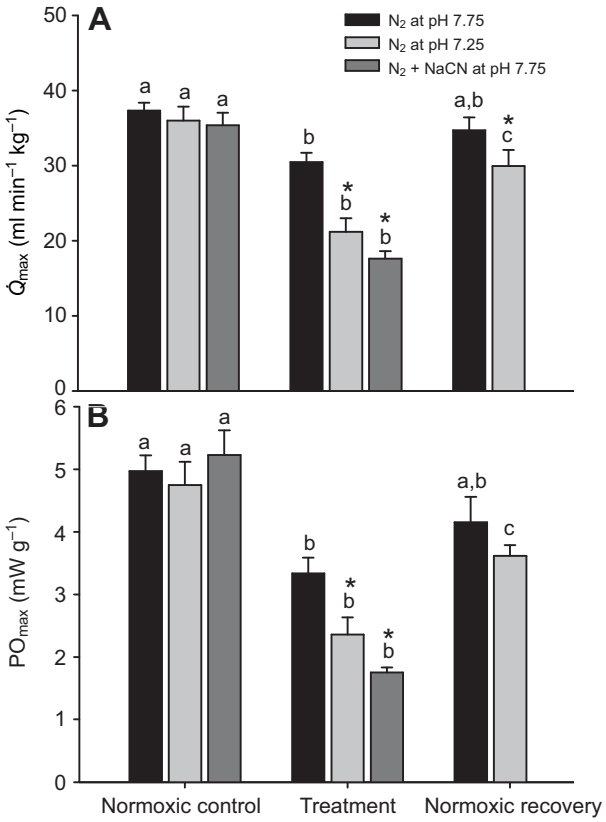

Fig. 4. Maximum cardiac function (A, $\dot{Q}_{max}$; B, PO_{max}) for each trial organized by exposure (normoxia, treatment and normoxic recovery). All tests of maximum cardiac performance were carried out at the end of an exposure. Trials include N_2 at pH 7.75 (N=6), N_2 at pH 7.25 (N=7), and N_2 + NaCN at pH 7.75 (N=6). Values are means ± s.e.m. Different letters indicate statistically significant differences (P<0.05) between exposures within each experimental trial, and asterisks indicate statistically significant differences between trials within each exposure. Values sharing an asterisk within an exposure are not significantly different from one another, with the severe hypoxia trial used as a basis of comparison in the treatment and recovery exposures.

production was minimal (Fig. 6). Therefore, the relationship between ATP turnover rate and PO was similar to that between $\dot{V}_{O_2}$ and PO (Fig. 5B), and both normoxic routine and maximum ATP turnover rate (~135 and ~300 nmol ATP s^{-1} g^{-1}) was similar across trials.

Severe hypoxia

The lactate efflux rate associated with routine cardiac performance was increased by as much as ~3000-fold compared with normoxia under conditions of severe hypoxia alone, with acidosis or with NaCN (Fig. 6). The lactate efflux produced at routine PO in severe hypoxia with extracellular acidosis was significantly lower than that produced during severe hypoxia with NaCN, but not significantly different from that in severe hypoxia alone. Similarly, there was no significant difference in routine lactate efflux between severe hypoxia alone and chemical anoxia (Fig. 7).

ATP turnover rate during severe hypoxia alone and with acidosis increased with PO (Fig. 8), but not to the same extent as during normoxia (Fig. 5B) because of lower PO_{max} values. Maximum ATP turnover rate in severe hypoxia alone (172 nmol ATP s^{-1} g^{-1}; i.e. Fig. 8 at a PO of 3.3 mW g^{-1} for the N_2 at pH 7.75 group) was approximately 43% less than that achieved in normoxia. However, ATP turnover rate did not increase significantly during chemical anoxia, and remained at ~110 nmol ATP s^{-1} g^{-1}, a value that was similar to that achieved during severe hypoxia with acidosis (~120 nmol ATP s^{-1} g^{-1}; Fig. 8). Thus, the MGP under these two conditions generated about a third of the maximum normoxic ATP turnover rate, while during severe hypoxia the ATP turnover rate was slightly greater than half of normoxic values.

Recovery

$\dot{V}_{O_2}$ and ATP turnover rate increased with increasing PO during recovery from severe hypoxia and severe hypoxia with extracellular acidosis (Fig. 5C,D). Significant increases in $\dot{V}_{O_2}$ reflect those in $\dot{Q}_{max}$ and PO_{max}. No significant difference was present between trials. Lactate efflux returned to a minimal level with normoxic recovery and did not increase significantly with PO (Fig. 6).

DISCUSSION

The present study is the first to characterize the *in situ* cardiac performance of tilapia, and offers novel findings regarding cardiac anoxia tolerance in ectothermic vertebrates. Prior to the present study, the tilapia heart was considered hypoxia tolerant. Cardiac activity was known to be downregulated to a steady state level for at least 8 h at 22°C, a level that was ~50% lower than the routine normoxic activity level, achieved largely through hypoxic bradycardia (Speers-Roesch et al., 2010). In the present study, the *in situ* tilapia heart was shown to have an impressive, though unexpected, anoxia tolerance by maintaining normoxic routine levels of cardiac performance and energy demand ($\dot{Q}$ and PO) in severe hypoxia (with and without acidosis) and with chemical anoxia. In addition, the hearts recovered remarkably well following the challenge of severe hypoxia and tests of maximum performance. As such, the hypoxic downregulation of *in vivo* cardiac performance in tilapia is not needed to balance cardiac energy supply and demand. Rather, it may simply reflect the circulatory needs of a general hypometabolic state (whole-animal oxygen uptake is reduced by ~80% and heat production by 45% in hypoxia-exposed tilapia) (van Ginneken et al., 1997; Speers-Roesch et al., 2010) that limits the rate of use of glycogen stores and metabolic waste accumulation. As a result, our data suggest that hypoxic downregulation of cardiac performance *in vivo* is not a necessary strategy to match cardiac energy demand to reduced energy supply during O_2 limitation, as suggested previously (Farrell and Stecyk, 2007; Speers-Roesch et al., 2010). Furthermore, by having a MGP of 172 nmol ATP s^{-1} g^{-1} and a PO_{max} of at least ~3.1 mW g^{-1} (and substantial values even after mitochondrial respiration was blocked by cyanide), this particular tilapia hybrid should be placed among those ectotherms considered to be anoxia tolerant and listed in Table 1 (with the exception of the inclusion of the hypoxia-sensitive rainbow trout). Indeed, maximum cardiac performance and MGP are greater than previous measurements for aquatic ectotherms, albeit at a slightly higher temperature (see below for further discussion).

Normoxic cardiac performance

Routine normoxic cardiac performance was stable at a level equivalent to that measured *in vivo* (Speers-Roesch et al., 2010). Tilapia hearts exhibited a Starling curve similar to others described for *in situ* perfused hearts of rainbow trout (Farrell et al., 1991), spiny dogfish [*Squalus acanthias* (Davie and Franklin, 1992)] and sea bass [*Dicentrarchus labrax* (Farrell et al., 2007)]. Routine $\dot{Q}$ required a slightly positive filling pressure. The intrinsic f_H for the *in situ* tilapia heart in normoxia (55–60 beats min^{-1}) was greater than

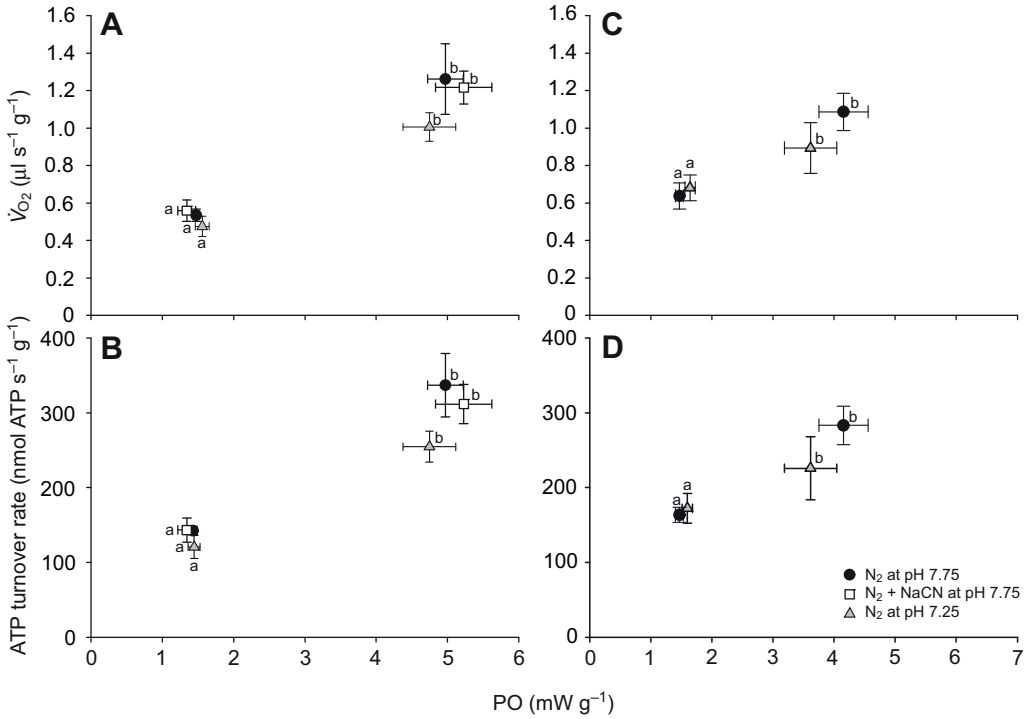

Fig. 5. The relationship between $\dot{V}_{O_2}$ or ATP turnover rate and PO in normoxic control (A and C) and normoxic recovery (B and D) at routine and maximum (PO_{max}) levels of cardiac PO (means ± s.e.m.) for N_2 at pH 7.75 (N=6), N_2 at pH 7.25 (N=7), and N_2 + NaCN at pH 7.75 (N=6). (A) $\dot{V}_{O_2}$ in the normoxic control, (B) ATP turnover rate in the normoxic control, (C) $\dot{V}_{O_2}$ in normoxic recovery and (D) ATP turnover rate in normoxic recovery. Different letters indicate statistically significant differences within a trial (P<0.05). There were no significant differences in $\dot{V}_{O_2}$ or ATP turnover rate between trials at routine PO or PO_{max} levels.

that *in vivo* (~35 beats min^{-1}), suggesting that routine vagal tone probably exists *in vivo*. The normoxic $\dot{Q}_{max}$ and PO_{max} (~39 ml min^{-1} kg^{-1} and ~5 mW g^{-1} at 22°C) of the *in situ* tilapia heart reflect the relatively low activity lifestyle of this species as they are lower than those observed for active fishes such as sea bass [~91 ml min^{-1} kg^{-1} and ~11 mW g^{-1} at 18°C (Farrell et al., 2007)] or salmonids [e.g. rainbow trout: 66 ml min^{-1} kg^{-1} and 8 mW g^{-1} at 15°C (Farrell et al., 1996)]. In contrast, winter flounder (*Pseudopleuronectes americanus*) have a $\dot{Q}_{max}$ of 39.2±4.0 ml min^{-1} kg^{-1} at 10°C (Joaquim et al., 2004) and, like tilapia, are also hypoxia tolerant (Cech et al., 1977) and have only a spongy myocardium lacking coronary arteries. All the same, $\dot{Q}_{max}$ and PO_{max} for tilapia are 3-fold higher than routine normoxic levels, a range that represents an appreciable scope.

Myocardial $\dot{V}_{O_2}$ and ATP turnover rate have never been measured before in tilapia. Routine myocardial $\dot{V}_{O_2}$ in tilapia (0.52 µl s^{-1} g^{-1}, 22°C) falls within the range reported for other fishes [sea raven: 0.32 µl s^{-1} g^{-1}, 10°C (Farrell et al., 1985); rainbow trout: 0.71 µl s^{-1} g^{-1}, 15°C (Graham and Farrell, 1990)]. As expected, the increase in PO produced an equivalent increase in myocardial $\dot{V}_{O_2}$ (Fig. 5). The absence of a significant lactate efflux rate under routine cardiac performance in normoxia suggests that oxygen supply by the perfusate was adequate, though this situation was probably stressed when determining PO_{max} because lactate efflux rate increased very modestly, but not significantly, under these conditions (Fig. 6). At comparable PO values, routine and maximum ATP turnover rate for tilapia is similar to those for rainbow trout (Graham and Farrell, 1990). Circulating catecholamines stimulate cardiac activity in fish under normoxic conditions [rainbow trout (Ask et al., 1980) and sea bass (Farrell et al., 1996)], protect cardiac performance during hypoxia (Farrell et al., 1983; Hanson et al., 2006), and increase in concentration during severe hypoxia (Reid and Perry, 1994; Vianen et al., 2001). Although no apparent effect of catecholamines on tilapia cardiac performance was noted in this present study, more comprehensive studies than those performed here are needed to further investigate these findings.

Effects of severe hypoxia on routine cardiac performance

Routine normoxic levels of $\dot{Q}$ and PO were maintained throughout ~60 min of severe hypoxia exposure even when the final 30 min included acidosis or NaCN. Only f_H decreased in severe hypoxia (by ~13%, and a little more with acidosis), but the heart intrinsically compensated for the decreased f_H by increasing V_S to maintain routine $\dot{Q}$. Cardiac slowing with hypoxia and acidosis has been reported previously (Farrell, 1984; Aberra et al., 2001), and is probably the result of direct effects of hypoxia and H^+ on the pacemaker.

The ability to maintain routine cardiac PO during severe hypoxia or anoxia is a feat demonstrated for few species thus far. Anoxia-tolerant ectothermic vertebrates tend to either have very low routine

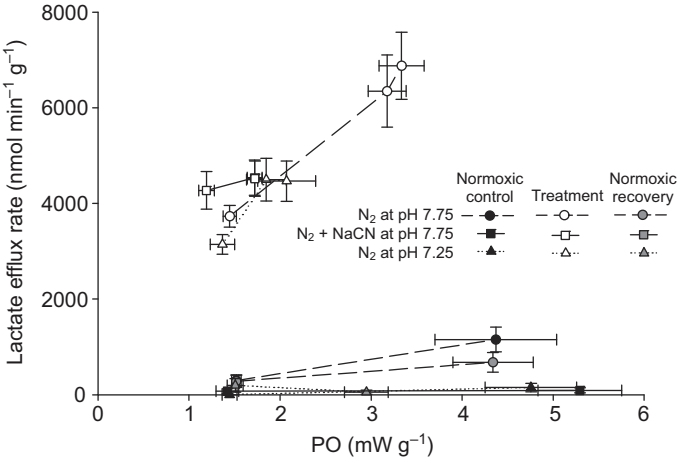

Fig. 6. The relationship between lactate efflux and PO at routine levels, $\dot{Q}_{max}$ and PO_{max} (means ± s.e.m.) in each exposure (normoxic control, treatment and normoxic recovery) of all three trials [N_2 at pH 7.75 (N=6), N_2 at pH 7.25 (N=7) and N_2 + NaCN at pH 7.75 (N=7)]. Treatment values for $\dot{Q}_{max}$ and PO_{max} in the N_2 + NaCN at pH 7.75 trial overlap at PO ~1.8 mW g^{-1}. Statistics were omitted for clarity (see text for details).

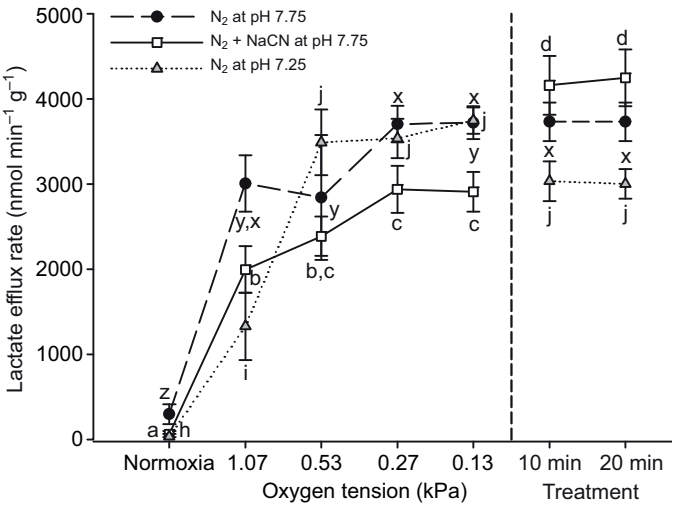

Fig. 7. Trends in lactate efflux with decreasing oxygen tension from normoxia (~20.0–21.3 kPa O_2) to severe hypoxia (0.13 kPa O_2), as well as throughout the 20 min treatment exposure (N_2 at pH 7.75, N_2 + NaCN at pH 7.75, or N_2 at pH 7.25). The dashed vertical line represents the beginning of the 20 min treatment exposure. The values are means ± s.e.m. at particular oxygen tensions (normoxia to 0.13 kPa O_2), and at two time points during the treatment exposure (10 and 20 min). Different letters indicate statistically significant differences (P<0.05) within a trial: N_2 + NaCN at pH 7.75 (a–d), N_2 at pH 7.25 (h–j) or N_2 at pH 7.75 (x–z). There was no statistically significant difference between the severe hypoxia and chemical anoxia trials.

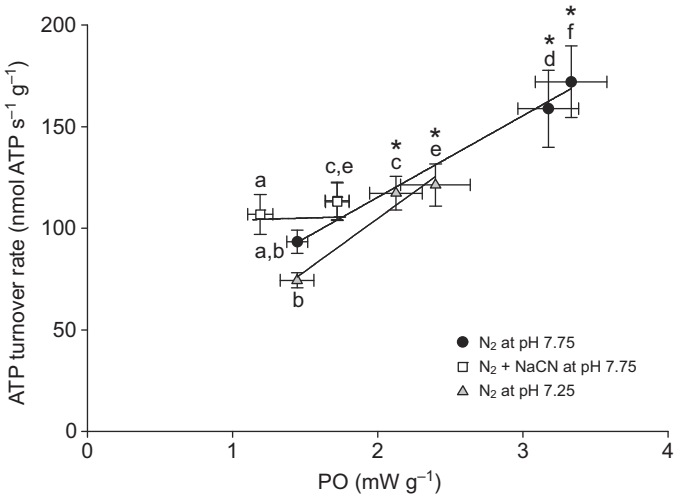

Fig. 8. The relationship between ATP turnover rate in the N_2 treatment exposure and PO at routine levels, $\dot{Q}_{max}$ and PO_{max} (means ± s.e.m.) of all three trials [N_2 at pH 7.75 (N=6), N_2 at pH 7.25 (N=7) and N_2 + NaCN at pH 7.75 (N=6)]. Values for $\dot{Q}_{max}$ and PO_{max} in the N_2 + NaCN at pH 7.75 trial overlap at PO ~1.8 mW g^{-1}. Asterisks indicate statistically significant differences (P<0.05) within each trial compared with its routine performance. Different lowercase letters indicate statistically significant differences (P<0.05) across trials at each PO level (routine, $\dot{Q}_{max}$, PO_{max}); the letters used for each given PO value are as follows: a,b – routine; c,d – $\dot{Q}_{max}$; and e,f – PO_{max}.

cardiac PO in normoxia, such as hagfishes [*in vivo* studies: *M. glutinosa*, 0.1 mW g^{-1} (Axelsson et al., 1990); *E. cirrhatus*, 0.4 mW g^{-1} (Forster et al., 1992); and *E. stoutii*, 0.2 mW g^{-1} (Cox et al., 2010)], and crucian carp (*in situ* perfused heart study: 0.44 mW g^{-1}; J. A. W. Stecyk, K. O. Stensløkken, L. M. Hanson, A.P.F. and G. E. Nilsson, unpublished results) or reduce cardiac PO through a combination of bradycardia and arterial hypotension (Farrell and Stecyk, 2007). The tilapia heart is unique in being able to maintain a comparatively high routine PO (1.4 mW g^{-1}) in severe hypoxia exposures (<0.20 kPa O_2) of ~60 min, including a 30 min period of chemical anoxia. Hypoxia-sensitive hearts, such as rainbow trout hearts, rapidly fail when P_{O_2} is <2.7 kPa (Hanson et al., 2006), because their routine PO lies above the capability of their MGP.

Effects of severe hypoxia on maximum cardiac performance and maximum glycolytic potential

The *in situ* tilapia heart showed an impressive maximum performance during severe hypoxia exposure. The 30% decrease in PO_{max} with severe hypoxia is similar to that observed in crucian carp (32%; J. A. W. Stecyk, K. O. Stensløkken, L. M. Hanson, A.P.F. and G. E. Nilsson, unpublished results), and the 65% decrease in PO_{max} with NaCN is not much greater than that for the red-eared slider turtle under severe hypoxia alone [*Chrysemys scripta*, 51% (Farrell et al., 1994)]. These decreases are a direct result of decreased energy availability due to the switch from oxidative phosphorylation to anaerobic glycolysis. With the *in situ* perfused heart preparation, the accumulation of lactate and H^+ from glycolysis normally seen in plasma does not occur with severe hypoxia *in vivo*. Therefore, in order to simulate the negative effects of acidosis on cardiac contractility, which occurs *via* decreased sensitivity of the myofilament to Ca^{2+} as well as the depressive effect of acidosis on the pacemaker (Orchard and Kentish, 1990; Crampin and Smith, 2006; Gesser and Overgaard, 2009), we examined the additional effect of low pH (7.25) during severe hypoxia. An additive effect of acidosis during severe hypoxia exposure was evident in the significant decreases in maximum cardiac performance ($\dot{Q}_{max}$ and PO_{max}) as well as in f_H. In most fishes, the additive and negative inotropic effects of acidosis (Farrell et al., 1983; Driedzic and Gesser, 1994; Gesser and Overgaard, 2009) markedly decrease cardiac twitch force and *in situ* cardiac performance (Poupa et al., 1978; Gesser and Poupa, 1983; Overgaard et al., 2005). In our study, acidosis diminished the increases in cardiac V_S and $\dot{Q}$ when P_{in} was increased (Fig. 3B). Severe acidosis has also been hypothesized to inhibit glycolysis; for example, a drop of 0.4 pH units was associated with a 70% decrease of anaerobic metabolism in mammalian cardiac tissue (Williamson et al., 1975). For tilapia hearts, we could not clearly discern such an effect because the routine lactate efflux and ATP turnover rate were generally similar for all trials during severe hypoxia. In addition, the lower maximum ATP turnover rate with acidosis was associated with a lower cardiac PO, which means that we cannot distinguish between effects of acidosis on contractility and glycolysis.

A challenge with any study of the effects of anoxia, especially with anoxia-tolerant species, is the complete removal of oxygen. In the present study, severe hypoxia represented a P_{O_2}<0.20 kPa, and more typically ~0.07 kPa when maximum cardiac performance was tested. However, we cannot exclude the possibility that mitochondrial respiration was contributing towards the ATP generation that supported the mechanical activity of the heart in severe hypoxia. An important observation in this regard is that, under routine PO conditions, lactate efflux rate had increased dramatically and rapidly when perfusate P_{O_2} reached 1.07 kPa (Fig. 7). Routine lactate efflux rate did not increase substantially beyond that level with further decreases in P_{O_2}, or even following the addition of NaCN to block

Table 1. Maximum *in situ* cardiac ATP turnover rate and *in situ* cardiac PO_{max} in anoxia for tilapia and other ectothermic vertebrates

Species	Temperature (°C)	PO_{max} (mW g^{-1})	Maximum cardiac ATP turnover rate (nmol ATP s^{-1} g^{-1})	Source
Tilapia (*Oreochromis* hybrid)	22	3.34±0.25	172±18	Severe hypoxia trial (current study)
	22	1.75±0.08	113±9	Chemical anoxia trial (current study)
Hagfish (*Eptatretus cirrhatus*)	18	0.28	62[a]	Forster, 1991
Red-eared slider turtle (*Chrysemys scripta*)	15	0.76	73	Farrell et al., 1994; Arthur et al., 1997
Rainbow trout (*Oncorhynchus mykiss*)	16	0.54	107[b]	Arthur et al., 1992
Crucian carp (*Carassius carassius*)	8	1.13	79	J. A. W. Stecyk, K. O. Stensløkken, L. M. Hanson, A.P.F. and G. E. Nilsson, unpublished results

PO, cardiac power output.
All values for ATP turnover rate were calculated from cardiac lactate efflux rate, assuming that 1 mol lactate yields 1.5 mol ATP.
[a]Calculated from the reported lactate efflux rate.
[b]Adjusted from the reported ATP turnover rate, which assumed that 1 mol lactate yields 1 mol ATP.

mitochondrial respiration, indicating that the severe hypoxia exposure (<0.20 kPa O_2) either approached or reached functional anoxia. However, both the additive treatments of acidosis and NaCN limited maximum lactate efflux rate to just below 5000 nmol min^{-1} g^{-1} and maximum ATP turnover rate to 110–120 nmol ATP s^{-1} g^{-1}, unlike the turnover rate of 172 nmol ATP s^{-1} g^{-1} with severe hypoxia alone. These lower ATP turnover rates were also associated with a lower PO_{max}, making it unclear whether there was an inhibition of glycolysis, contractility or both. Thus, while severe hypoxia alone represented just over a 40% decrease in maximum ATP turnover rate, blocking of mitochondrial respiration magnified this decrease to nearly 65% (Fig. 9). One interpretation of these results is that a small amount of oxygen could still have been available during severe hypoxia and contributed to ATP turnover rate while most of the heart was still working at near-maximal levels using anaerobic glycolysis. An obvious difficulty with such an interpretation is that the maximum lactate efflux rate was substantially greater during severe hypoxia alone than with the addition of NaCN. Therefore, the maximum lactate efflux rate observed during severe hypoxia must represent the MGP, which is equal to an ATP turnover rate of 172 nmol ATP s^{-1} g^{-1}. Further support for this conclusion is provided by considering the input P_{O_2} in severe hypoxia, which never exceeded 0.13 kPa O_2 and has the potential to yield an ATP turnover rate of only 17 nmol ATP s^{-1} g^{-1}. Yet, the decrease in ATP turnover rate produced by NaCN is 4-fold greater. If there was an aerobic ATP turnover rate of 17 nmol ATP s^{-1} g^{-1} during severe hypoxia, then it would generate a PO of 0.26 mW g^{-1} (using the same PO to ATP generation ratio observed with chemical anoxia), which then would reduce the PO_{max} generated by an MGP of 172 nmol ATP s^{-1} g^{-1} to 3.08 mW g^{-1}. In view of this, we are left with the conclusion that the MGP can be represented by 172 nmol ATP s^{-1} g^{-1} supporting a PO_{max} of at least ~3.1 mW g^{-1}, which is higher than the 113 nmol ATP s^{-1} g^{-1} supporting a PO_{max} of 1.75 mW g^{-1} indicated after the addition of NaCN during severe hypoxia.

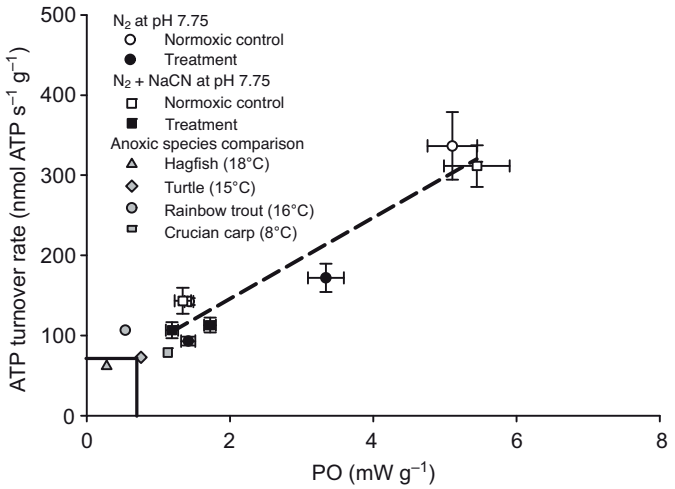

Fig. 9. Trends in ATP turnover rate as a function of PO from normoxia to severe hypoxia/anoxia in the N_2 at pH 7.75 and N_2 + NaCN at pH 7.75 trials. The box outlined in black plots the current accepted maximum glycolytic potential of the ectothermic heart (Farrell and Stecyk, 2007). Points from this study represent mean ± s.e.m. ATP turnover rate at a given PO in different exposures and trials. The figure displays routine and maximum ATP turnover rate at both routine PO and PO_{max} for tilapia, where the dashed line represents the general trend from normoxia to anoxia within these points. Grey filled points indicate mean values for maximum severely hypoxic or anoxic cardiac performance in other species (see Table 1). Sources for the comparative points are as follows: hagfish, *Eptatretus cirrhatus* (Forster, 1991); red-eared slider turtle, *Chrysemys scripta* (Arthur et al., 1997; Farrell et al., 1994); rainbow trout, *Oncorhynchus mykiss* (Arthur et al., 1992); and crucian carp, *Carassius carassius* (J. A. W. Stecyk, K. O. Stensløkken, L. M. Hanson, A.P.F. and G. E. Nilsson, unpublished results). Statistics were omitted for clarity.

These conclusions also suggest that NaCN may be exerting confounding effects, limiting the heart's ability to either increase PO or possibly generate ATP anaerobically. Previous studies examining the effects of NaCN on fish hearts (Hansen and Sidell, 1983; Arthur et al., 1992) only did so under conditions of routine function (where we observed no effect of NaCN). Aside from inhibition of oxidative phosphorylation by binding to cytochrome *c* oxidase, NaCN can alter nitric oxide flux (Kiang et al., 2003), bind to iron-containing metallo-enzymes such as myoglobin (Antonini and Brunori, 1971), and potentially alter the redox state of mitochondria. Also, the production of reactive oxygen species is associated with blockage of the electron transport chain (Ambrosio et al., 1993), which in turn has been shown to contribute to cardiac failure and contractile dysfunction in mammals (Ide et al., 1999; Ide et al., 2000). Thus, NaCN treatment may not simply induce anoxia, which means that the MGP obtained for the tilapia heart in the severe hypoxia exposure (172 nmol ATP s^{-1} g^{-1}) is a more reliable estimate of MGP.

A comparison of maximum glycolytic potential across species

The results of the present study suggest that the MGP of the tilapia heart at 22°C is at least 172 nmol ATP s^{-1} g^{-1}, which supports a PO_{max}

of at least ~3.1 mW g^{-1}. This estimate (and even that for NaCN) exceeds the previously proposed upper limits of ~70 nmol ATP s^{-1} g^{-1} and ~0.7 mW g^{-1} for the anoxic ectothermic vertebrate heart at ~15°C (Farrell and Stecyk, 2007). There are tremendous difficulties with making cross-species comparisons to reach such generalizations, however, and this earlier upper limit was based on animals that inhabit a narrow thermal window (3–15°C) during extended periods of anoxia.

Correspondingly, such measurements are often made under very different experimental conditions; for example, temperature is a significant variable with such comparisons because all previous measurements of *in situ* cardiac performance have been made at colder temperatures (see Table 1). While it is possible to use a Q_{10} value to normalize each result to a common temperature, often the correct Q_{10} value is unknown, or the biological relevance of such an adjustment is not well understood. For example, in any given species, acclimation to another species' temperature may not be tolerated *in vivo*; it is unlikely that normoxic hagfish tolerate 22°C or that normoxic tilapia of this strain tolerate 5°C. Furthermore, anoxia tolerance in lower vertebrates is usually associated with cold rather than the 22°C used with tilapia. However, a Q_{10} value of 2.1 is known for lactate efflux, and thus for glycolytic capacity in rainbow trout heart (Overgaard et al., 2004). Using this Q_{10} value, the measured cardiac MGP of the anoxia-tolerant crucian carp at 8°C (79 nmol ATP s^{-1} g^{-1}; J. A. W. Stecyk, K. O. Stensløkken, L. M. Hanson, A.P.F. and G. E. Nilsson, unpublished results) can be normalized to 22°C (223.2 nmol ATP s^{-1} g^{-1}). Thus, the cardiac MGP may be similar for crucian carp and tilapia at a common temperature of 22°C. However considering that whole-animal and cardiac anoxia tolerance of crucian carp have not been quantified at 22°C, the biological relevance of such a comparison remains tenuous and is in need of experimental verification. Until such experiments are performed, tilapia hearts have the highest cardiac MGP recorded to date, with the acknowledgement that similar measurements have been performed at different, but biologically relevant, temperatures.

In addition, a reconsideration of cardiac MGP using a larger species pool representing a range of thermal tolerances (especially species from warmer environments) and life history exposure to low oxygen (e.g. hibernators, short-term survivors, permanently exposed, etc.) would greatly refine and expand the characterization of cardiac anoxia tolerance in ectothermic vertebrates. With regard to tilapia we propose that, in response to ecological pressure, tilapia may have evolved a high cardiac MGP and anoxic PO_{max} to permit short-term high-energy function in severe hypoxia or anoxia. For example, tilapia may rely upon burst exercise to escape to hypoxic refugia or to evade predators while in hypoxic refugia, as seen in tilapia in Lake Victoria to avoid Nile perch (Chapman et al., 2002). Given that tilapia typically, and not temporarily or seasonally, reside in hypoxic habitats (e.g. swamps and stagnant eutrophic ponds), it would be reasonable to hypothesize that the capacity for greater anoxic levels of cardiac function may enable them to better cope with the physiological challenges presented by these environments.

CONCLUSION

We have shown that the *in situ* perfused tilapia heart is capable of maintaining normoxic routine levels of *in vivo* cardiac performance in severe hypoxia and chemical anoxia. The scope for maximum cardiac performance of the *in situ* tilapia heart in severe hypoxia and chemical anoxia remained sizeable, although lower than in normoxia. These results suggest that oxygen limitation is not a major constraint on the ability of the tilapia to maintain routine cardiac function, which apparently can be sustained by anaerobic energy production alone at least in the short term. Thus, downregulation of cardiac performance as observed in tilapia during hypoxia exposure *in vivo* (Speers-Roesch et al., 2010) is not needed to balance cardiac energy supply and demand, contrary to previous suggestions (Farrell and Stecyk, 2007; Speers-Roesch et al., 2010). Instead, such downregulation may be more important for minimizing fuel use and waste production, especially considering that the additive effect of acidosis on *in situ* cardiac performance was significant and that severe acidosis cannot be avoided during hypoxia by tilapia *in vivo* (Speers-Roesch et al., 2010), unlike in crucian carp (Farrell and Stecyk, 2007). The impressive MGP and anoxic PO_{max} of the *in situ* tilapia heart suggest that tilapia should be placed among the anoxia-tolerant ectothermic vertebrates.

LIST OF SYMBOLS AND ABBREVIATIONS

f_H	heart rate
MGP	maximum glycolytic potential
M_v	ventricular mass
P_{in}	input pressure
PO	cardiac power output
P_{O_2}	partial pressure of oxygen
PO_{max}	maximum cardiac power output
P_{out}	output pressure
$\dot{Q}$	cardiac output; flow
$\dot{Q}_{max}$	maximum cardiac output; maximum flow
$\dot{V}_{O_2}$	oxygen consumption rate
V_S	stroke volume
α_{O_2}	solubility of oxygen

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