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RESEARCH ARTICLE

Calorespirometry reveals that goldfish prioritize aerobic metabolism over metabolic rate depression in all but near-anoxic environments

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ABSTRACT

Metabolic rate depression (MRD) has long been proposed as the key metabolic strategy of hypoxic survival, but surprisingly, the effects of changes in hypoxic O_2 tensions (Pw_{O_2}) on MRD are largely unexplored. We simultaneously measured the O_2 consumption rate ($\dot{M}_{O_2}$) and metabolic heat of goldfish using calorespirometry to test the hypothesis that MRD is employed at hypoxic Pw_{O_2} values and initiated just below P_{crit}, the Pw_{O_2} below which $\dot{M}_{O_2}$ is forced to progressively decline as the fish oxyconforms to decreasing Pw_{O_2}. Specifically, we used closed-chamber and flow-through calorespirometry together with terminal sampling experiments to examine the effects of Pw_{O_2} and time on $\dot{M}_{O_2}$, metabolic heat and anaerobic metabolism (lactate and ethanol production). The closed-chamber and flow-through experiments yielded slightly different results. Under closed-chamber conditions with a continually decreasing Pw_{O_2}, goldfish showed a P_{crit} of 3.0±0.3 kPa and metabolic heat production was only depressed at Pw_{O_2} between 0 and 0.67 kPa. Under flow-through conditions with Pw_{O_2} held at a variety of oxygen tensions for 1 and 4 h, goldfish also initiated MRD between 0 and 0.67 kPa but maintained $\dot{M}_{O_2}$ to 0.67 kPa, indicating that P_{crit} is at or below this Pw_{O_2}. Anaerobic metabolism was strongly activated at $Pw_{O_2} \leq 1.3$ kPa, but only used within the first hour at 1.3 and 0.67 kPa, as anaerobic end-products did not accumulate between 1 and 4 h exposure. Taken together, it appears that goldfish reserve MRD for near-anoxia, supporting routine metabolic rate at sub-P_{crit} Pw_{O_2} values with the help of anaerobic glycolysis in the closed-chamber experiments, and aerobically after an initial (<1 h) activation of anaerobic metabolism in the flow-through experiments, even at 0.67 kPa Pw_{O_2}.

KEY WORDS: Calorespirometry, Critical oxygen tension, Environmental hypoxia, Fish, Metabolic depression, Metabolic heat

INTRODUCTION

Aerobic pathways of ATP production yield ~15 times more ATP than anaerobic pathways (Hochachka and Somero, 2002). Consequently, environmental hypoxia and the corresponding shift to anaerobic metabolism seriously threaten energy balance by reducing an animal's ability to generate sufficient ATP to meet metabolic demands. Despite the critical importance of aerobic respiration to the maintenance of metabolic function, many organisms inhabit and thrive in various hypoxic and even anoxic environments (Bickler and Buck, 2007; Ramirez et al., 2007). Fishes are particularly adept at surviving low-oxygen environments, having independently evolved hypoxia tolerance numerous times (Hochachka and Lutz, 2001) owing to the relatively high prevalence of hypoxia among aquatic habitats (Boesch, 2002; Diaz and Breitburg, 2009; Diaz and Rosenberg, 1995; Smith et al., 2006).

Metabolic rate depression (MRD) has been proposed as the hallmark response enabling hypoxic survival in hypoxia-tolerant animals (e.g. Boutilier and St-Pierre, 2000; Hochachka et al., 1996). MRD is achieved through reductions in whole-animal (e.g. locomotion, reproduction, feeding) and cellular (e.g. growth, repair, protein synthesis) processes (Guppy and Withers, 1999; Richards, 2010), reducing ATP demand and rates of anaerobic fuel depletion (glycogen) and waste accumulation (lactate and protons). Although MRD is a well-described response to anoxia exposure in a range of animals including fruit flies (Callier et al., 2015), goldfish (Addink et al., 1991; van Waversveld et al., 1989) and turtles (Jackson, 1968), it has been suggested that MRD would also enhance hypoxic survival (Boutilier and St-Pierre, 2000; Hochachka et al., 1996). Indirect (i.e. non-calorimetric) measurements in common frogs (Donohoe and Boutilier, 1998) and direct (i.e. calorimetric) measurements in goldfish (van Ginneken et al., 1994, 2004) and tilapia (van Ginneken et al., 1997) suggest that MRD may be employed at hypoxic O_2 tensions (Pw_{O_2} for water). Indeed, goldfish reduced metabolic heat by ~31% at ~2.1 kPa Pw_{O_2} compared with normoxia (~21 kPa) (van Ginneken et al., 2004), and tilapia reduced metabolic heat by ~40% at ~1.1 kPa Pw_{O_2} (van Ginneken et al., 1997). However, it is still unknown how these changes in metabolic heat correspond with changes in aerobic and anaerobic metabolism, and how this is affected by Pw_{O_2}.

MRD would be particularly important at Pw_{O_2} values below an animal's critical Pw_{O_2} for O_2 consumption rate ($\dot{M}_{O_2}$), referred to as P_{crit}, which is the Pw_{O_2} at which $\dot{M}_{O_2}$ becomes dependent on environmental P_{O_2}. P_{crit} is largely determined by the O_2 binding affinity of hemoglobin (Hb) (Mandic et al., 2009), and at Pw_{O_2} values below P_{crit} the ability to extract environmental O_2 to saturate Hb is constrained and thus unable to support routine metabolic rate (MR) aerobically. The animal can attempt to sustain ATP production at routine levels through an upregulation of anaerobic glycolysis, but this comes with the depletion of carbohydrate reserves and the accumulation of deleterious anaerobic end-products (Richards, 2009), ultimately limiting hypoxic survival time (Lague et al., 2012; Speers-Roesch et al., 2013). However, if the animal is capable of reducing its energy-consuming processes through a controlled, hypoxia-induced MRD, then it could simultaneously mitigate the negative consequences of reduced ATP production and increased rates of fuel depletion and waste accumulation. We therefore hypothesized that MRD is

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List of symbols and abbreviations

C_{O_2}	oxygen content
F	flow rate
Hb	hemoglobin
M	mass
$\dot{M}_{O_2}$	oxygen consumption rate
MR	metabolic rate
MRD	metabolic rate depression
P_{50}	P_{O_2} at which Hb is 50% saturated with oxygen
P_{crit}	critical partial pressure of oxygen for $\dot{M}_{O_2}$
P_{O_2}	partial pressure of oxygen
Pw_{O_2}	partial pressure of oxygen in water
T	time
V	volume

employed at hypoxic Pw_{O_2} values and is initiated just below P_{crit}, where the negative impacts of reduced aerobic capacity and increased anaerobic reliance begin to accrue.

We tested this hypothesis using closed-chamber and flow-through calorespirometry to simultaneously measure O_2 consumption rates, MRD (via metabolic heat) and anaerobic glycolysis (via excretion rates of the anaerobic end-product ethanol) in goldfish held at Pw_{O_2} values ranging from normoxia to anoxia. We also performed terminal sampling experiments on goldfish exposed to the same Pw_{O_2} values as used in the calorespirometry experiments to fully quantify whole-body anaerobic metabolism. Goldfish were chosen owing to their exceptional hypoxia tolerance and well-documented ability to induce MRD (e.g. Addink et al., 1991; van Waversveld et al., 1989; van Ginneken et al., 2004), something not all fish species are capable of (Stangl and Wegener, 1996). We used calorespirometry because it is the 'gold standard' of MR measurements and the only way to accurately measure MR on hypoxemic animals in real time (see Kaiyala and Ramsay, 2011; Nelson, 2016). Despite the superiority of calorespirometry, only a few studies have measured the metabolic heat of fishes (Addink et al., 1991; Regan et al., 2013; Stangl and Wegener, 1996; van Ginneken et al., 1994, 1997, 2004; van Waversveld et al., 1989), and only three of these (van Ginneken et al., 1994, 1997, 2004) have measured metabolic heat at Pw_{O_2} other than normoxia and anoxia. While the data from these studies suggest that MRD is employed at hypoxic Pw_{O_2} values, they exposed their organisms to progressive hypoxia over sometimes prolonged periods of time and they did not relate their measurements to P_{crit} nor directly assess the contributions of anaerobic metabolism at various hypoxic Pw_{O_2} values. Furthermore, other studies that have attempted to examine the role of MRD and other metabolic and respiratory responses to hypoxia have not attempted, to our knowledge, to directly assess the relative contributions of MRD, aerobic respiration and anaerobic metabolism at different hypoxic Pw_{O_2} values over time. Consequently, we still do not have a comprehensive picture of the hypoxic survival strategies of fishes.

MATERIALS AND METHODS

Study organisms

We obtained adult goldfish [*Carassius auratus auratus* (Linnaeus 1758); 2.06±0.39 g wet mass; n=264; sex unknown] from a commercial supplier (Delta Aquatics, Burnaby, BC, Canada) and held them under a 12 h:12 h light:dark cycle in a 76 litre recirculating system of aerated, dechlorinated, 17°C water at the University of British Columbia (Vancouver, BC, Canada). Stocking density was <0.4 g l^{-1} and water in the recirculating system was replaced weekly. We fed the fish to satiation daily (Nutrafin Max Goldfish Flakes) except for 24 h before transfer to the experimental apparatus, when feeding ceased. The University of British Columbia's Animal Care Committee approved all procedures.

Calorespirometer

We used a differential calorespirometer to simultaneously measure metabolic heat and $\dot{M}_{O_2}$ under closed and flow-through conditions. The design and operation of the calorespirometer are described in detail in Regan et al. (2013). Briefly, the metabolic heat of a fish is detected as a voltage by a collection of Peltier units (Custom Thermoelectric Peltier module 12711-5L31- 03CQ, Bishopville, MD, USA) via the Seebeck effect and converted to wattage using an empirically determined calibration coefficient (see Regan et al., 2013). The design of the calorespirometer allows for the simultaneous measurements of metabolic heat and $\dot{M}_{O_2}$ using P_{O_2} optodes (Ocean Optics OR125, Dunedin, FL, USA) placed on the inflowing and outflowing water lines as well as in the fish chamber. To determine $\dot{M}_{O_2}$ under closed-chamber conditions, a P_{O_2} optode within the fish chamber measured the change in Pw_{O_2} over sequential 5 min intervals and was then corrected for chamber volume and fish mass according to:

$$\dot{M}_{O_2} = (\Delta C_{O_2} \cdot \Delta T^{-1} \cdot V) M^{-1}, \quad (1)$$

where C_{O_2} is the O_2 content of the water converted to µmol l^{-1} from Pw_{O_2} using the solubility factor of 1.9312 µmol l^{-1} $mmHg^{-1}$ (Boutilier et al., 1984), T is the time period over which the change in C_{O_2} is calculated (5 min), V is the fish chamber volume (32 ml) minus the volume displaced by the fish itself, and M is the mass of the fish. To determine $\dot{M}_{O_2}$ under flow-through conditions, the difference in Pw_{O_2} between inflowing and outflowing water lines supplying the fish chamber was measured using the same P_{O_2} optode and corrected for flow rate and fish mass according to:

$$\dot{M}_{O_2} = [(Ci_{O_2} - Co_{O_2}) \cdot F] M^{-1}, \quad (2)$$

where Ci_{O_2} and Co_{O_2} are O_2 content of inflowing and outflowing water, respectively, converted from Pw_{O_2} as described above, F is water flow rate (22 ml h^{-1}) and M is the mass of the fish. Under flow-through conditions, the chamber Pw_{O_2} could be held constant for extended time periods, allowing us to measure $\dot{M}_{O_2}$ and metabolic heat at different time points at any desired Pw_{O_2}.

Hypoxic exposures

Individual fish were transferred to a flow-through calorespirometer held at 17°C and a flow rate of 22 ml h^{-1}, and in this apparatus we performed both closed-chamber and flow-through calorespirometry experiments following a 16 h normoxic habituation period. For the closed-chamber experiments (n=8), the trial began by stopping water flow and allowing the fish to reduce Pw_{O_2} from normoxia to anoxia over 60–90 min. The experiment was ended when the chamber Pw_{O_2} reached anoxia, at which point we introduced a lethal dose of anaesthetic (buffered MS-222, final chamber concentration of 150 mg l^{-1}) to determine the calorespirometer's baseline heat signature. For the flow-through experiments, inflowing Pw_{O_2} was manually adjusted to yield one of four chamber Pw_{O_2} values over a ~60 min period (20, 1.3, 0.67 or 0 kPa; n=3–6 for each) and the animals were maintained at one of these Pw_{O_2} values for up to 4 h (referred to as the experimental period). We measured metabolic heat over the full 21 h period (16 h normoxia habituation, 1 h transition to exposure Pw_{O_2}, 4 h experimental period) and collected effluent water samples either before (time 0) or at 1 and 4 h during

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the experimental period for measurements of ethanol (a glycolytic end-product excreted across goldfish gills). Following the experiment, we introduced a lethal dose of anaesthetic (buffered MS-222, see above) to determine the calorespirometer's baseline heat signature. At the end of each experiment, we recalibrated the P_{O_2} optodes to determine any drift that had occurred over the course of the experiment (up to ~10%) for the purpose of later correction, and then washed the calorespirometer and its water lines with a 10% bleach solution. The flow-through and closed-chamber calorespirometry experiments were performed in fall 2014 and winter 2015, respectively.

Comparison of closed-chamber and flow-through calorespirometry

To more directly compare the results of the closed-chamber and flow-through calorespirometry experiments, we conducted a back-to-back comparison of the two techniques using the same fish. This was required because our first experiments (presented in Figs 1 and 2) using these techniques were conducted at different times of year and yielded different routine normoxic $\dot{M}_{O_2}$ values, which could affect our determination of P_{crit}. We measured routine $\dot{M}_{O_2}$ using both techniques and determined P_{crit} during closed-chamber respirometry (P_{crit} was not determined via flow-through calorespirometry because it would require the fish to undergo multiple runs at different Pw_{O_2} values). Briefly, fish were introduced to the calorespirometer and allowed to habituate under the same conditions as the calorespirometry experiments described above. Following the habituation period, we first measured the fish's routine $\dot{M}_{O_2}$ under normoxia using flow-through respirometry (as described above), then immediately closed off the respirometer chamber and measured $\dot{M}_{O_2}$ using closed-chamber respirometry (as described above). We repeated this three times for each of six fish, and the Pw_{O_2} was not allowed to drop below 16 kPa during these closed-chamber measurements. Following the final closed-chamber measurement, we allowed the fish to deplete the chamber's O_2 content so as to determine its P_{crit}. Metabolic heat was not measured during these back-to-back experiments.

Terminal sampling experiments

To better estimate the effects of Pw_{O_2} on anaerobic metabolism, we ran parallel hypoxia exposures where we euthanized animals to measure whole-body concentrations of lactate and ethanol. For each Pw_{O_2}, we exposed 24 goldfish spread across six tanks (four fish per tank) and sampled two replicate tanks at each of 0, 1 and 4 h to match the experimental periods of the calorespirometry experiments (n=8 per time point). We sampled fish by inconspicuously introducing a lethal dose of anaesthetic (buffered MS-222, see above), weighing the individual fish, freezing them immediately in liquid N_2, and then storing them at −80°C for later metabolite analyses. We ensured that the conditions between these experiments and the calorespirometry experiments were similar by including a 16 h habituation period followed by a 1 h transition period to the desired Pw_{O_2}, conducting exposures in the dark, and by preventing the fish in the tank from accessing the air–water interface (which was not available to the calorespirometry fish). Thus, the main difference between this experiment and the calorespirometer experiment was vessel size (calorespirometer chamber was 32 ml and exposure tanks were 10 litres), which could affect the ability of the fish to move during our hypoxia exposures and yield different levels of lactate and ethanol accumulation. We are, however, confident that fish movement is minimal in the calorespirometer based on the relatively smooth heat traces observed over the habituation and experimental periods, and periodic visual inspection of the fish in the 10 litre tanks revealed little to no movement, especially during the hypoxia exposures. Thus, despite the differences in exposure regimes, the fish from both the calorespirometry and the terminal sampling experiments likely responded to hypoxia in a similar manner.

Lactate and ethanol analyses

To link our whole-body calorespirometry measurements of MR to the activation of anaerobic metabolism, we measured whole-body concentrations of lactate and ethanol. Entire goldfish from the 10 litre tank exposures were ground into a fine powder using a liquid N_2-chilled mortar and pestle. To extract the metabolites (lactate and ethanol) from the powder, an aliquot of powder was weighed and transferred to a 2 ml centrifuge tube containing 1 ml of ice-cold 30% $HClO_4$ and immediately homogenized at 0°C using a Polytron homogenizer set to the highest setting for 30 s. The resulting homogenate was then centrifuged at 20,000 ***g*** for 5 min at 4°C and the supernatant was transferred to a new 1.5 ml centrifuge tube and neutralized using 3 mol l^{-1} Tris base to avoid the volatilization of ethanol that occurs in association with vigorous CO_2 production when $HClO_4$ is neutralized with K_2CO_3. We confirmed that neutralization with Tris base does not affect our enzymatic analysis. We measured ethanol immediately following neutralization using a commercial kit designed for biological ethanol analysis (Diagnostic Chemical Ltd., PEI, Canada), and then froze the unused portion of

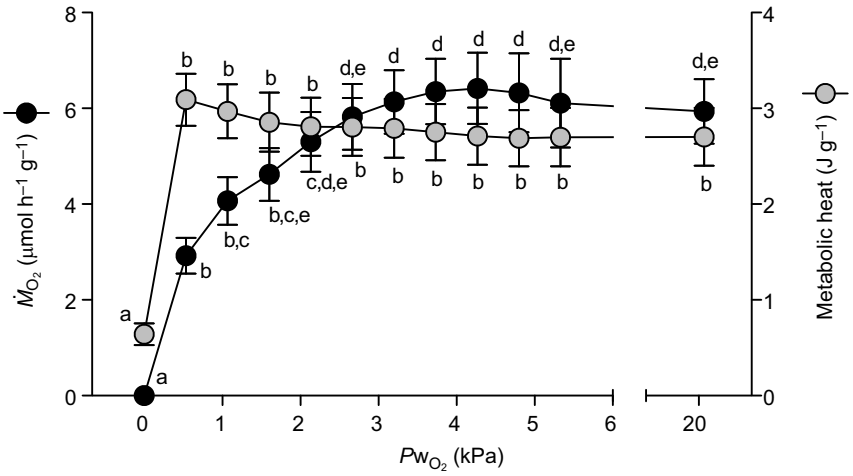

Fig. 1. Closed-chamber calorespirometry measurements of oxygen consumption rate ($\dot{M}_{O_2}$) and metabolic heat in goldfish. Partial pressure of oxygen in water (Pw_{O_2}) was reduced from normoxia to anoxia over 60 to 90 min because of the fish's O_2 consumption. Data are means±s.e.m, n=8. Data points sharing a letter are not significantly different (one-way ANOVA, P>0.05).

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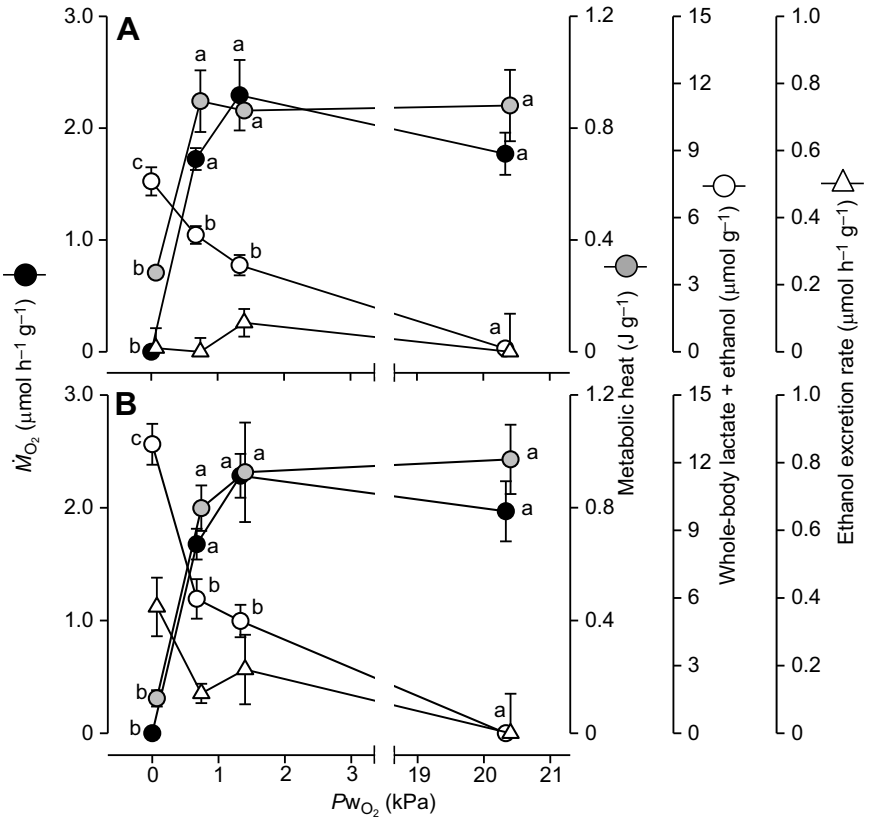

Fig. 2. Flow-through calorespirometry measurements of $\dot{M}_{O_2}$, metabolic heat and glycolytic end-products in goldfish held at different $P_{W_{O_2}}$ values for different periods of time. (A) 1 h; (B) 4 h. Data are means±s.e.m, *n*=3–6, and data points are offset slightly on the *x*-axis for clarity. Data points sharing a letter are not significantly different (one-way ANOVA, *P*>0.05).

the sample extract for later lactate analysis. Lactate concentration was measured using the LDH reaction according to the protocols outlined in Bergmeyer (1983).

O_2 equilibrium curves

To understand how the goldfish's Hb–O_2 affinity is related to P_{crit} and MRD, we constructed O_2 equilibrium curves for the whole blood of five normoxia-acclimated goldfish using the thin film spectrophotometric technique (Lilly et al., 2013). Blood was collected from the caudal artery of anaesthetized fish using 60 µl heparinized capillary tubes. We then centrifuged the tubes and resuspended the red blood cells in HEPES buffer (pH 7.8) to ensure a consistent blood pH across all samples. A Wöstoff gas mixing pump (H. Wösthoff Messtechnik GmbH, Bochum, Germany) mixed compressed O_2 and N_2 to each of seven P_{O_2} values for the construction of the O_2 equilibrium curves, and Hb P_{50} values (the P_{O_2} at which Hb is 50% saturated with O_2) were calculated using the equation of each sigmoidal curve as calculated by SigmaStat 11.0.

P_{crit} calculation

P_{crit} is defined as the $P_{W_{O_2}}$ at which an organism's routine $\dot{M}_{O_2}$ transitions from being independent of to being dependent upon $P_{W_{O_2}}$. We determined P_{crit} for each individual in the closed-chamber calorespirometry experiments using the BASIC program (Yeager and Ultsch, 1989), which uses a two-segment linear regression model to determine P_{crit} as the $P_{W_{O_2}}$ at which the two linear trend lines intersect on a graph plotting $\dot{M}_{O_2}$ as a function of $P_{W_{O_2}}$. Some individuals' $\dot{M}_{O_2}$ values increased above routine $\dot{M}_{O_2}$ levels at hypoxic $P_{W_{O_2}}$ values close to P_{crit}, and including these $\dot{M}_{O_2}$ values would overestimate P_{crit}. To prevent this, we excluded from our routine $\dot{M}_{O_2}$ estimation any $\dot{M}_{O_2}$ value that exceeded 1.5 times the standard deviation of an individual's average $\dot{M}_{O_2}$ between 13 and 21 kPa $P_{W_{O_2}}$.

Data analysis and statistics

$\dot{M}_{O_2}$ and ethanol production rates were calculated at each time point, while metabolic heat was represented by averaging the continual heat measurements made over the 20 min straddling the time point (e.g. 50–70 min for 1 h time point). All data are presented as means± s.e.m. The effects of $P_{W_{O_2}}$ on each variable were determined using one-way ANOVA (SigmaStat 11.0).

RESULTS

Closed-chamber calorespirometry experiments

We used closed-chamber calorespirometry to measure P_{crit} and to characterize the effects of a progressive reduction in $P_{W_{O_2}}$ on $\dot{M}_{O_2}$ and metabolic heat. $P_{W_{O_2}}$ in the closed-chamber experiments was decreased from normoxia to anoxia by the fish's own $\dot{M}_{O_2}$ over 60–90 min (depending on the fish's $\dot{M}_{O_2}$). P_{crit} was calculated to be 3.0±0.3 kPa (Fig. 1). At $P_{W_{O_2}}$ values above P_{crit}, there were no significant effects of changes in $P_{W_{O_2}}$ on the average routine $\dot{M}_{O_2}$, while at $P_{W_{O_2}}$ values below P_{crit} (at which the fish spent ~30 min), $\dot{M}_{O_2}$ progressively fell to zero as the goldfish depleted the available oxygen. Metabolic heat was maintained at routine levels at all $P_{W_{O_2}}$ values between 20 and 0.5 kPa (Fig. 1) but was depressed upon reaching anoxia, eventually stabilizing at ~21% of routine normoxic values (an MRD of 79%; Fig. 1) after ~20 min.

Flow-through calorespirometry experiments

We used flow-through calorespirometry to characterize the effects of $P_{W_{O_2}}$ and time on $\dot{M}_{O_2}$, metabolic heat and excreted ethanol. We held individuals at one of four $P_{W_{O_2}}$ values (20, 1.3, 0.67 or 0 kPa) for 1 and 4 h. For 1 h exposures, $\dot{M}_{O_2}$ and metabolic heat were

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maintained at routine levels to $P_{W_{O_2}}$ of 0.67 kPa, while at $P_{W_{O_2}}$ values below this, $\dot{M}_{O_2}$ fell to zero and metabolic heat fell to ~32% of routine levels (an MRD of 68%; Fig. 2A). Similarly, for 4 h exposures, $\dot{M}_{O_2}$ and metabolic heat were maintained at routine levels to $P_{W_{O_2}}$ of 0.67 kPa, while at $P_{W_{O_2}}$ values below this, $\dot{M}_{O_2}$ fell to zero and metabolic heat fell to ~20% of routine levels (an MRD of 80%; Fig. 2B).

Ethanol excretion rates were undetectable following 1 h exposure at all $P_{W_{O_2}}$ values (Fig. 2A). These rates increased following 4 h exposure, and higher rates were generally detected at lower $P_{W_{O_2}}$ values (Fig. 2B), but these increases were not statistically significant (Fig. 2B).

Whole-body anaerobic end-product concentrations

Whole-body concentrations of lactate significantly increased over time 0 values following 1 and 4 h at 1.3 kPa, 0.67 kPa and anoxia (Table 1). Whole-body concentrations of ethanol significantly increased over time 0 values following 1 and 4 h of anoxia exposure (Table 1). The total anaerobic end-product concentrations at 1.3 and 0.67 kPa following 4 h were similar to those following 1 h, suggesting that the rate of anaerobic end-product accumulation fell to near-zero levels after 1 h (Fig. 2). A similar result was observed for the anoxia-exposed fish, though to a lesser extent, with anaerobic end-product concentrations being ~1.8-fold higher following 4 h exposure than following 1 h exposure (Fig. 2).

Closed-chamber versus flow-through calorespirometry

Individual $\dot{M}_{O_2}$ values determined in the same fish in a back-to-back comparison of closed-chamber and flow-through respirometry were positively correlated (n=18, r=0.925, P<0.0001; Fig. 3A) and yielded similar mean $\dot{M}_{O_2}$ values (t=0.423, P=0.678; Fig. 3B). The closed-chamber portion of these experiments yielded a P_{crit} of 2.7±0.2 kPa (n=6).

Hb–O_2 equilibrium curves

The Hb of goldfish displayed a very high affinity for O_2, resulting in a steep O_2 equilibrium curve and an average whole-blood P_{50} of 0.49±0.12 kPa (Fig. 4).

DISCUSSION

We hypothesized that goldfish employ MRD at hypoxic $P_{W_{O_2}}$ values and initiate it at $P_{W_{O_2}}$ values just below P_{crit}. This hypothesis predicted that metabolic heat would decrease from routine levels at a $P_{W_{O_2}}$ below P_{crit}, when the fish's ability to take up environmental O_2 to support a routine $\dot{M}_{O_2}$ was compromised. Our closed-chamber calorespirometry experiments yielded a P_{crit} of 3.0±0.3 kPa (Fig. 1), consistent with the P_{crit} values reported in other studies on goldfish (Fry and Hart, 1948; Fu et al., 2011). However, contrary to our hypothesis that MRD is initiated at hypoxic

Table 1. Whole-body concentrations (μmol g^{-1}) of lactate and ethanol in goldfish exposed to different $P_{W_{O_2}}$ (partial pressure of oxygen in water) values for 1 and 4 h following a 16 h normoxic habituation period (0 h)

		0 kPa	0.67 kPa	1.3 kPa	20 kPa
[Lactate]	0 h	1.20±0.40^a	0.22±0.18^a	0.53±0.47^a	0.21±0.20
	1 h	6.42±0.54^b	4.21±0.34^b	3.11±0.48^b	0.15±0.14
	4 h	10.6±0.93^c	4.89±0.86^b	4.52±0.72^b	0.11±0.15
[Ethanol]	0 h	0.61±0.11^a	0.74±0.14	0.87±0.20	–
	1 h	1.20±0.17^b	1.02±0.10	0.77±0.21	–
	4 h	2.24±0.20^c	1.07±0.10	0.45±0.08	–

Different letters indicate significant differences between time points within a $P_{W_{O_2}}$ exposure (P<0.05). [Ethanol] measurements at 20 kPa were not taken.

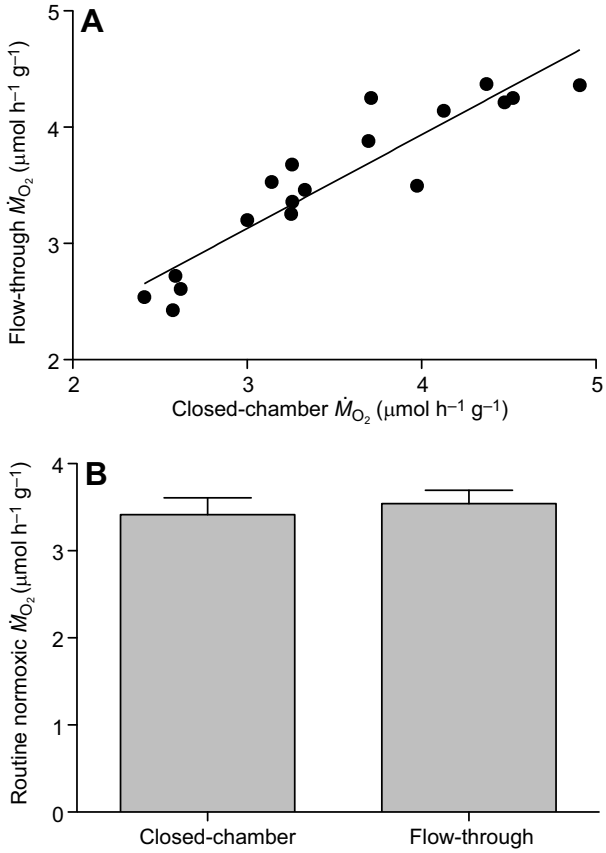

Fig. 3. A comparison of $\dot{M}_{O_2}$ measurements of goldfish made using closed-chamber and flow-through calorespirometry. Both respirometric techniques were performed in the same apparatus following a ≥16 h habituation period, at the same time of day (~10:00 h PST), and at $P_{W_{O_2}}$ ≥16 kPa. (A) $\dot{M}_{O_2}$ values resulting from closed and flow-through techniques, with measurements made back-to-back on the same fish (n=18, r=0.925, P<0.0001). (B) Average $\dot{M}_{O_2}$ measurements for each technique, with error bars representing s.e.m. (n=18, t=0.423, P=0.678).

$P_{W_{O_2}}$ values just below P_{crit}, metabolic heat was maintained at routine normoxic levels to a $P_{W_{O_2}}$ of 0.67 kPa, and MRD was only evident in goldfish exposed to anoxia. The magnitude of the anoxia-induced MRD [79% depression in closed-chamber experiments; 68% (1 h) and 80% (4 h) in flow-through experiments; Figs 1, 2] was very similar to what has been shown previously for anoxia-exposed goldfish using calorimetry (Addink et al., 1991; Stangl and Wegener, 1996; van Ginneken et al., 1994).

Metabolic responses to hypoxia

Goldfish maintained routine MR at severely hypoxic $P_{W_{O_2}}$ values under both closed-chamber and flow-through conditions (Figs 1, 2), but appear to have used different strategies to do so. In the closed-chamber experiments, metabolic heat was maintained at routine normoxic levels to 0.67 kPa despite a decrease in $\dot{M}_{O_2}$ at 3.0 kPa (Fig. 1), suggesting that anaerobic glycolysis was upregulated to support MR (though lactate and ethanol could not be measured in closed-chamber experiments as a function of $P_{W_{O_2}}$). In the flow-through experiments, metabolic heat was similarly maintained at routine normoxic levels to 0.67 kPa at both 1 and 4 h, but unlike the closed-chamber experiments, $\dot{M}_{O_2}$ was maintained at near-routine levels at all hypoxic $P_{W_{O_2}}$ values tested. This suggests that MR was supported aerobically even at severely hypoxic $P_{W_{O_2}}$ values and that MRD is reserved for all but severely hypoxic (<0.67 kPa) or

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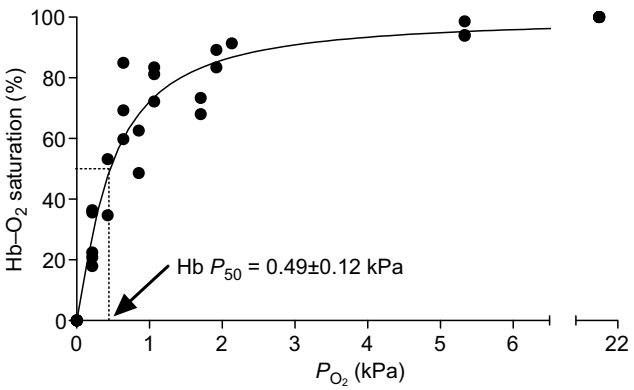

Fig. 4. O_2 equilibrium curve for the whole blood of five normoxia-acclimated goldfish. Red blood cells were separated from plasma and resuspended in HEPES buffer (pH 7.8). O_2 levels were achieved using a Wösthoff gas mixing pump attached to cylinders of compressed O_2 and N_2.

near-anoxic environments. This is different than the results of van Ginneken and colleagues (1994, 2004), who showed moderate 27% and 33% decreases in heat production along with lower $\dot{M}_{O_2}$ in goldfish exposed to 3.5 and 2.1 kPa, respectively. These incongruent results are likely due to differences in experimental design and study goals. van Ginneken et al. (1994, 2004) exposed each fish in their studies to progressive hypoxia over prolonged periods of time (e.g. 8.4, 4.2, 2.1 and finally 0.63 kPa over a 16 h period in van Ginneken et al., 2004), which does not allow the authors to disentangle the effects of $P_{W_{O_2}}$ and time on metabolic heat and $\dot{M}_{O_2}$. In contrast, our flow-through calorespirometry experiments exposed goldfish to only a single hypoxic $P_{W_{O_2}}$ (after a 1 h adjustment period) for up to 4 h and we assessed the effects of varying hypoxic $P_{W_{O_2}}$ values using different individuals, allowing us to independently assess the effects of $P_{W_{O_2}}$ and time on metabolic responses. Using this approach, we clearly show that within 1 h of exposure, goldfish are capable of maintaining oxygen uptake under severely hypoxic conditions (0.67 kPa), obviating the need for hypoxia-induced MRD.

Elevated levels of lactate and ethanol at $P_{W_{O_2}} \leq 1.3$ kPa at 1 and 4 h indicate that anaerobic glycolysis also contributed to maintaining MR, though in slightly different ways in anoxia and hypoxia. In anoxia, lactate and ethanol levels continued to increase throughout the 4 h exposure, but their rate of accumulation decreased from 5.81 µmol h^{-1} g^{-1} during the first hour to 1.73 µmol h^{-1} g^{-1} during the subsequent 3 h. These results are consistent with those observed in tissues from anoxia-exposed turtles (*Trachemys scripta elegans*), where lactate production rates were elevated during the first hour of anoxia exposure and subsequently decreased between 1 and 5 h anoxia in brain, liver and white muscle (Kelly and Storey, 1988). Combined, these results suggest that there is an initial reliance on anaerobic metabolism upon anoxia exposure that may compensate for the anoxia-induced limitations on aerobic ATP production while MRD is initiated. In hypoxia, the early reliance on anaerobic metabolism was temporally even more profound than in anoxia. Lactate and ethanol accumulation was confined entirely to the first hour of hypoxia exposure at 1.3 and 0.67 kPa, while $\dot{M}_{O_2}$ was concurrently maintained at routine normoxic levels throughout the hypoxic exposures. Taken together, these data suggest that total ATP turnover is higher over the first hour of hypoxia exposure than in normoxia. Indeed, whole-body estimates of total ATP turnover during this period indicate that it increases from ~10 µmol h^{-1} g^{-1} in normoxia to ~17 and 15 µmol h^{-1} g^{-1} at 1.3 and 0.67 kPa, respectively (assuming P:O_2 of 6 and ATP:lactate/ethanol of 1), while heat production does not change. These inconsistencies are likely a consequence of not being able to temporally match our measurements of anaerobic metabolism (taken as the delta accumulation of lactate and ethanol over the entire hour plus the $P_{W_{O_2}}$ adjustment period) with those of $\dot{M}_{O_2}$ and heat, which were taken at the end of the 1 h (between 50 and 70 min exposure). As such, it is possible there are temporal shifts in fuel selection within the first hour of hypoxia exposure, with lactate and/or ethanol accumulating during the initial descent towards the target $P_{W_{O_2}}$ as $\dot{M}_{O_2}$-sustaining mechanisms are upregulated. Finer-scale studies are needed to confirm this idea.

The P_{crit} values derived from the closed-chamber and flow-through calorespirometry experiments differed substantially, with P_{crit} shifting from 3.0 kPa in the closed-chamber experiments to somewhere between 0 and 0.67 kPa in the flow-through experiments (the exact value cannot be determined). These technique-specific differences in P_{crit} are consistent with a recent study comparing closed and intermittent-flow respirometry (Snyder et al., 2016), which attributed the higher P_{crit} in closed respirometry to metabolic waste accumulation and a faster decline in $P_{W_{O_2}}$. Similar factors may be at play in our closed-chamber calorespirometry experiments, resulting in an overestimation P_{crit}. Another possible explanation might be that the routine normoxic $\dot{M}_{O_2}$ (and heat) in the closed-chamber experiments was approximately twofold higher than in the flow-through experiments (cf. Figs 1 and 2). All else being equal, this would necessitate the fish from the closed-chamber experiments adopting an oxyconforming strategy at a higher $P_{W_{O_2}}$, yielding a higher P_{crit}. However, our back-to-back comparison of calorespirometry techniques suggests that technique per se does not explain the twofold change in $\dot{M}_{O_2}$ and heat production, which might instead be explained by the time of year. In any case, the differences in $\dot{M}_{O_2}$ do not appear to affect P_{crit} and therefore do not explain why P_{crit} is higher in the closed-chamber experiments than in the flow-through experiments.

Another factor possibly contributing to the lower P_{crit} values obtained from the flow-through experiments versus those from the closed-chamber experiments is time. Fishes possess many mechanisms that enhance O_2 uptake with decreasing $P_{W_{O_2}}$, including increases to gill surface area (Sollid et al., 2003), Hb synthesis (Gracey et al., 2001) and concentration in the blood (Affonso et al., 2002), hematocrit (Lai et al., 2006; Turko et al., 2014), Hb–O_2 affinity (Turko et al., 2014), ventilation frequency and amplitude (Holeton and Randall, 1967; Itazawa and Takeda, 1978; Tzaneva et al., 2011; Vulesevic and Perry, 2006), as well as redistributed blood supply to critical tissues (Sundin et al., 1995). While these mechanisms effectively enhance the uptake of environmental O_2 and its distribution throughout the body, their induction takes time, varying from minutes to days depending on the physiological response examined. Because the fish in the flow-through experiments had spent 1 or 4 h at each $P_{W_{O_2}}$ when their $\dot{M}_{O_2}$ was measured (in addition to the ~1 h required to reduce the $P_{W_{O_2}}$ from normoxia to the target $P_{W_{O_2}}$), they may have had additional time to initiate some of these mechanisms of enhanced O_2 uptake compared with the closed-chamber fish that saw only ~30 min of continually decreasing sub-P_{crit} hypoxic conditions. If P_{crit} is in fact influenced by the rate and duration of hypoxia induction over relatively short time scales, then it becomes important to apply similar methodology both within and between studies (something that is not currently done; see Rogers et al., 2016) to ensure P_{crit} values are comparable. This is especially true

when P_{crit} is used as a reference point for models that, for example, predict how climate change will reshape the distribution of fishes around the world (Deutsch et al., 2015).

Hb–O_2 affinity and initiation of MRD

Our results show that MRD is initiated in goldfish at a Pw_{O_2} somewhere between 0 and 0.67 kPa. Interestingly, our analysis of whole-blood Hb–O_2 affinity reveals a Hb P_{50} value of 0.49±0.12 kPa (Fig. 4; consistent with Burggren, 1982), within the Pw_{O_2} range that goldfish appear to reduce $\dot{M}_{O_2}$ in the flow-through experiments and initiate MRD. It is therefore tempting to think of a causal link between the supply of O_2 to the tissues and the initiation of MRD. Considerable debate exists regarding the signal for MRD, with some data supporting signals residing on the energy production side of the cellular energy flux pathways (de Zwaan and Wijsman, 1976; Hochachka, 1982, 1985; Plaxton and Storey, 1984; Rees and Hand, 1991; Bishop and Brand, 2000; Bishop et al., 2002) and some data supporting signals on the energy consumption side (Caligiuri et al., 1981; Flanigan and Withers, 1991; Robin et al., 1979; Sick et al., 1982; see reviews by Guppy, 2004; Guppy and Withers, 1999; Storey and Storey, 1990). If Hb–O_2 affinity were in fact a signal for MRD, this would place the signal on the energy production side, consistent with some of the more recent views in the field (see Guppy, 2004). Similarly, Coulson (1977) postulated that MR was directly proportional to the circulatory system's ability to supply the tissues with O_2, and this idea gained empirical support when van Ginneken et al. (2004) showed a correlation between hypoxia-induced decreases in MR and heart rate. All told, it is not unreasonable to speculate that a signal for hypoxia-induced MRD involves the supply of O_2 to the tissue. The association between Hb P_{50} and the Pw_{O_2} of MRD initiation is therefore enticing and worth further investigation.

Ecological implications of MRD

The fact that goldfish appear to initiate MRD only near anoxia and maintain $\dot{M}_{O_2}$ without a long-term activation of anaerobic metabolism is well suited to the goldfish's (and the closely related crucian carp's, *Carassius carassius*) natural lake habitat. While these lakes become ice-covered in winter and eventually anoxic, they are severely hypoxic (Vornanen, 2004) for most of the winter at Pw_{O_2} values at which our study reveals goldfish remain aerobic. Goldfish can therefore maintain routine MR for most of the winter without relying on anaerobic glycolysis and/or MRD until it is entirely necessary. This strategy conserves the goldfish's finite anaerobic fuel stores (glycogen), reduces the accumulation of deleterious anaerobic end-products (lactate, protons and ethanol), and allows the goldfish to retain routine function and behaviour under most natural conditions.

Another benefit of a near-anoxic induced MRD is a delayed accumulation of MRD's inherent physiological and ecological costs (Humphries et al., 2003). These include oxidative stress resulting from the production of reactive O_2 species (Carey et al., 2000), impaired immunocompetence resulting from reduced lymphocyte production (Burton and Reichman, 1999), impaired cognitive and memory function resulting from reductions in synaptic contacts and dendritic branching (Popov et al., 1992), and significant reductions in sensory and motor activity (Choi et al., 1998) that increase predation susceptibility. Because the costs associated with each of these likely accumulate with time, a hypoxic survival strategy that involves an extended bout of MRD is likely to cause significant damage regardless of its effectiveness to balance cellular energy supply and demand. The goldfish's predominant reliance on aerobic respiration is therefore the ideal strategy for surviving long-term hypoxic bouts because it minimizes the time the fish is forced to rely on MRD and the associated physiological costs.

Taken together, the goldfish's overall hypoxia tolerance strategy appears finely tuned to its particular hypoxic environment, which is characterized by long, protracted descents into eventual anoxia. This may be the case with other species too; because hypoxic environments vary greatly in severity, duration and rate of hypoxic induction, the hypoxia tolerance strategies employed by organisms native to these different environments are likely to be just as variable.

Conclusions

By demonstrating that goldfish prioritize O_2 uptake over MRD in all but nearly anoxic environments, our results suggest two things. First, the exceptional hypoxia tolerance of goldfish owes more to its O_2 extraction abilities than to MRD. Second, MRD is not necessarily a key mechanism of hypoxic survival, as has been hypothesized (Hochachka et al., 1996), but of anoxic survival. While MRD is an effective means of balancing energy supply and demand, the potential costs associated with reducing cellular and whole-body processes may threaten organismal fitness and preclude its selection in all but the most extreme environments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.D.R. and J.G.R. designed the study. M.D.R. performed all experiments and data analyses. I.S.G. assisted with metabolite assays. M.D.R. drafted the manuscript and all authors commented.

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Data availability

Data have been deposited in the Dryad Digital Repository (Regan et al., 2017): http://dx.doi.org/10.5061/dryad.719bt

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