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RESEARCH ARTICLE

Rates of hypoxia induction alter mechanisms of O_2 uptake and the critical O_2 tension of goldfish

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ABSTRACT

The rate of hypoxia induction (RHI) is an important but overlooked dimension of environmental hypoxia that may affect an organism's survival. We hypothesized that, compared with rapid RHI, gradual RHI will afford an organism more time to alter plastic phenotypes associated with O_2 uptake and subsequently reduce the critical O_2 tension (P_{crit}) of the rate of O_2 uptake ($\dot{M}_{O_2}$). We investigated this by determining P_{crit} values for goldfish exposed to short (~24 min), typical (~84 min) and long (~480 min) duration P_{crit} trials to represent different RHIs. Consistent with our predictions, long duration P_{crit} trials yielded significantly lower P_{crit} values (1.0–1.4 kPa) than short and typical duration trials, which did not differ (2.6±0.3 and 2.5 ±0.2 kPa, respectively). Parallel experiments revealed these time-related shifts in P_{crit} were associated with changes to aspects of the O_2 transport cascade that took place over the hypoxia exposures: gill surface areas and haemoglobin–O_2 binding affinities were significantly higher in fish exposed to gradual RHIs over 480 min than fish exposed to rapid RHIs over 60 min. Our results also revealed that the choice of respirometric technique (i.e. closed versus intermittent) does not affect P_{crit} or routine $\dot{M}_{O_2}$, despite the significantly reduced water pH and elevated CO_2 and ammonia levels measured following closed-circuit P_{crit} trials of ~90 min. Together, our results demonstrate that gradual RHIs result in alterations to physiological parameters that enhance O_2 uptake in hypoxic environments. An organism's innate P_{crit} is therefore most accurately determined using rapid RHIs (<90 min) so as to avoid the confounding effects of hypoxic acclimation.

KEY WORDS: Critical O_2 tension, Environmental change, Goldfish, Hypoxia, Metabolic responses, Plasticity

INTRODUCTION

Environmental hypoxia is a common characteristic of many aquatic systems and is becoming increasingly prevalent, severe and long-lasting because of anthropogenic and climate change effects (Friedrich et al., 2014; IPCC, 2014; Smith et al., 2006). Many studies have examined the physiological impacts of hypoxia exposure on a diverse array of fish species, but these have focused almost exclusively on either the severity of the hypoxic exposure [i.e. partial pressure of O_2 in water ($P_{w_{O_2}}$)] or its duration. However, a third dimension of hypoxic exposure, the rate of hypoxia induction (RHI), has received very little attention and is rarely even controlled for (or at least reported) when environmental hypoxia is experimentally induced (Rogers et al., 2016). This is unlike other abiotic variables such as temperature, which are typically altered at consistent rates across studies [e.g. 0.2–0.3°C min^{-1} for the determination of critical thermal maxima (CT_{max})] owing to the effects they have on organismal responses (e.g. temperature tolerance in fishes; Mora and Maya, 2006). Similarly, RHIs may influence the physiological responses of fishes to hypoxia, particularly, time-dependent responses related to environmental O_2 extraction.

Most fishes possess mechanisms that enhance O_2 extraction and delivery to tissues as $P_{w_{O_2}}$ is reduced, such as increased haemoglobin (Hb) synthesis (Gracey et al., 2001) and concentration in the blood (Affonso et al., 2002), increased haematocrit (Lai et al., 2006; Turko et al., 2014), increased Hb–O_2 binding affinity (Turko et al., 2014), increased ventilation frequency and amplitude (Holeton and Randall, 1967; Itazawa and Takeda, 1978; Tzaneva et al., 2011; Vulesevic and Perry, 2006), and a redistribution of blood supply to critical tissues (Sundin et al., 1995). Some fishes, including goldfish and numerous other species, also have the ability to dramatically increase lamellar surface area in response to hypoxia exposure through apoptotic reductions to the inter-lamellar cell mass (ILCM; Anttila et al., 2015; Borowiec et al., 2015; Crispo and Chapman, 2010; Dhillon et al., 2013; Ong et al., 2007; Sollid et al., 2003, 2005; Turko et al., 2012). While these modifications to different parts of the O_2 transport cascade function to improve O_2 uptake at low $P_{w_{O_2}}$, the time courses over which these modifications are enacted differ and may potentially impact the critical P_{O_2} (P_{crit}) of the rate of O_2 uptake ($\dot{M}_{O_2}$).

P_{crit} is defined as the $P_{w_{O_2}}$ at which a fish's $\dot{M}_{O_2}$ transitions from being regulated at some stable level independent of $P_{w_{O_2}}$ (i.e. oxyregulation) to being dependent upon $P_{w_{O_2}}$ (i.e. oxyconformation). At P_{crit}, the fish's aerobic scope is theoretically zero and at $P_{w_{O_2}}$ values below P_{crit}, the fish's ability to generate ATP aerobically is limited (Farrell and Richards, 2009). P_{crit} therefore reflects a fish's ability to acquire and use environmental O_2 as a function of $P_{w_{O_2}}$, with a lower P_{crit} indicating a greater ability to extract O_2 to maintain aerobic metabolism in hypoxic environments. A low P_{crit} is beneficial because it allows the animal to maintain a routine level of function and activity in hypoxic environments while avoiding a reliance on anaerobic glycolysis and/or metabolic rate depression. Indeed, we have recently shown that goldfish prioritize their use of aerobic metabolism in hypoxic environments over their exceptional ability to induce metabolic rate depression, which they reserve for anoxic environments (Regan et al., 2017). Goldfish also appear to enhance their ability to extract environmental O_2 over relatively short time periods in hypoxia, which in theory should result in a lowering of their P_{crit} value (Regan et al., 2017). Because this ability is influenced by a suite of O_2 extraction mechanisms that are both plastic and time-dependent, we hypothesized that gradual RHIs would allow fish to induce plastic mechanisms that enhance O_2 extraction, resulting in lower P_{crit} values than those of fish exposed to rapid RHIs.

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List of symbols and abbreviations

C_{O_2}	oxygen content
CT_{max}	critical thermal maxima
Hb	haemoglobin
ILCM	inter-lamellar cell mass
$\dot{M}_{O_2}$	oxygen consumption rate
NTP	organic phosphates (ATP and GTP)
OEC	oxygen equilibrium curve
P_{50}	P_{O_2} at which Hb is 50% saturated with oxygen
P_{CO_2}	partial pressure of carbon dioxide
P_{crit}	critical partial pressure of oxygen for $\dot{M}_{O_2}$
P_{O_2}	partial pressure of oxygen
Pw_{CO_2}	partial pressure of carbon dioxide in water
Pw_{O_2}	partial pressure of oxygen in water
RBC	red blood cell
RHI	rate of hypoxia induction

We tested this hypothesis by determining the P_{crit} values of goldfish exposed to progressive reductions in Pw_{O_2} (from normoxia to near-anoxia; referred to as P_{crit} trials) over different durations: ∼24 min to represent rapid RHI, ∼84 min to represent typical RHI (P_{crit} trials in the literature typically last 60 to 120 min; see Rogers et al., 2016) and ∼480 min to represent gradual RHI. We also ran parallel hypoxic exposures of different RHIs to investigate morphological and physiological traits of goldfish that might play causal roles in a time-related shift in P_{crit}, including (among other traits) gill morphometry and Hb–O_2 binding affinity. Furthermore, our use of different respirometric techniques allowed us to disentangle the effects of time and technique on the determination of P_{crit}, thereby addressing a longstanding concern over the use of closed-chamber respirometry and its associated metabolic end-product accumulation for the determination of P_{crit} (Keys, 1930; Rogers et al., 2016; Snyder et al., 2016; Steffensen, 1989). And finally, we chose goldfish as our study species because they have well-characterized responses to hypoxia exposure (Dhillon et al., 2013; Mitrovic et al., 2009), including a well-resolved P_{crit} as determined by closed-chamber respirometry (Dhillon et al., 2013; Fry and Hart, 1948; Fu et al., 2011; Regan et al., 2017), which could aid our analysis of how RHI might influence the underlying physiology of P_{crit}.

MATERIALS AND METHODS

Animals

Goldfish (*Carassius auratus auratus* Linnaeus 1758; 2.87±0.14 g wet mass; N=84; sex unknown) were purchased from a commercial supplier (The Little Fish Company, Surrey, BC, Canada) and held under a 12 h:12 h light:dark cycle in 100-litre recirculating systems of well-aerated, dechlorinated, 17°C water (replaced weekly) at the University of British Columbia (UBC). Stocking density was <0.3 g l^{-1}. Fish were fed to satiation daily (Nutrafin Max Goldfish Flakes) except for 24 h before transfer to the experimental apparatus, when feeding ceased. UBC's Animal Care Committee approved all procedures (protocol A13-0309).

Respirometry

We exposed goldfish to P_{crit} trials of short (∼24 min), typical (∼84 min, representing a typical closed-chamber P_{crit} trial's duration) and long (∼480 min) durations to represent progressively reduced RHIs. These different RHIs were achieved using different respirometric techniques (details below), while the respirometer chambers, animal transfer protocol, habituation period and mean fish mass remained consistent across all trials. Each fish was used only once.

We used two 32 ml flow-through respirometer chambers made from stainless steel as described in Regan et al. (2013). For each trial, we inserted a fish into the chamber and held it under flow-through conditions for ≥16 h prior to commencing the P_{crit} trial. The fish chamber was supplied with flow-through water at a rate of 190 ml h^{-1} and maintained at 17°C. Inflowing water was drawn from a well-mixed reservoir held at ∼26 kPa (manually controlled using compressed N_2 and O_2) and pumped to the respirometer chamber via a peristaltic pump (Gilson Minipuls 3, Middleton, WI, USA) through a combination of stainless steel tubing and gas-impermeable Tygon peristaltic tubing. The Pw_{O_2} of the inflowing water was maintained slightly hyperoxic to ensure that the outflowing water was always at or slightly above normoxic Pw_{O_2}. Following the habituation period, we conducted our respirometry experiments.

For the typical duration P_{crit} trials (84±8 min), we used closed-circuit respirometry. To start the trial, the inflowing and outflowing water supply lines were short-circuited to create a closed loop, with water recirculating through the chamber by the peristaltic pump at the same rate (190 ml h^{-1}) as during the habituation period to ensure minimal disturbance to the fish and good mixing of the chamber's water volume. Chamber Pw_{O_2} was then allowed to decrease as a result of the fish's respiration. An O_2 optode placed within the chamber (see Regan et al., 2013) continuously measured Pw_{O_2}, and $\dot{M}_{O_2}$ was calculated according to:

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

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 14.485 µmol l^{-1} kPa^{-1} (Boutilier et al., 1984), T is the time period over which the change in C_{O_2} is calculated (5 or 2 min; see below), V is the fish chamber volume (32 ml) plus the volume of the closed-circuit water lines minus the volume displaced by the fish itself, and M is the mass of the fish. The trials were ended when Pw_{O_2} reached 0 kPa, at which point the short-circuit was dismantled and flow-through conditions were reestablished to return chamber Pw_{O_2} to habituation period conditions.

For the short duration P_{crit} trials (24±2 min), we again used closed-circuit respirometry as described for the typical P_{crit} trials. To shorten the trial and hasten the Pw_{O_2} decline, we made initial normoxic $\dot{M}_{O_2}$ readings and then manually replaced the entire water volume of the respirometry chamber and its water supply lines over ∼5 min with water equilibrated to 5.3 kPa Pw_{O_2} using a 60 ml syringe. Pw_{O_2} was therefore reduced from normoxia to ∼5.3 kPa not by the fish's $\dot{M}_{O_2}$, but by the active replacement of the water volume. At this point, we attached the water supply lines to the peristaltic tubing, turned the pump back on to 190 ml h^{-1}, and allowed the fish to deplete the closed system's O_2 through its own respiration (typically over a ∼20 min period). We chose 5.3 kPa as our replacement Pw_{O_2} for two reasons: first, it allowed for reliable $\dot{M}_{O_2}$ measurements starting at ∼4.8 kPa, which provided enough $\dot{M}_{O_2}$ data points above P_{crit} to construct robust P_{crit} traces; and second, the amount of time required for the fish to reduce Pw_{O_2} from 5.3 kPa to anoxia put the overall duration of these P_{crit} trials within our targeted duration of between 20 and 30 min. Although these procedures reduced the overall duration of the P_{crit} trial, we must point out that the RHI below 5.3 kPa was similar to that of the typical duration trials. If mechanisms of enhanced O_2 extraction are

only induced at Pw_{O_2} <5.3 kPa, then these two techniques could result in similar P_{crit} values.

Prior to actively replacing the water volume, we converted the system to closed-circuit and made a series of normoxic $\dot{M}_{O_2}$ readings between 25 and 19 kPa to aid in our calculation of P_{crit} (see below). Upon reaching 19 kPa, we converted the system back to flow-through, reestablished a normoxic Pw_{O_2} of ∼21 kPa, and then commenced the active water volume replacement.

For the long duration P_{crit} trials, we used three different respirometric techniques to ensure the mean P_{crit} values were the result of P_{crit} trial duration and not respirometric technique per se. These trials varied in average duration from 434 to 562 min depending on the technique used. We chose a time duration of ∼480 min because it was significantly longer than the typical trial durations, but likely shorter than would be required to induce gene expression acclimation responses. It is also in line with some of the longer P_{crit} trial durations observed in the literature (see Rogers et al., 2016).

For our first technique, we used closed-circuit respirometry where we added a 250 ml water reservoir to reduce the rate at which the fish's respiration depleted the system's O_2. This reservoir was a glass bottle placed immediately after the peristaltic pump. Water leaving the respirometer chamber was pumped into the reservoir directly over a stir bar that mixed the water volume to prevent O_2 stratification in the bottle. Water flowed out of the reservoir through a stainless steel line that punctured the bottle's rubber stopper and went directly into the stainless steel line supplying the respirometer chamber. All materials used were gas-impermeable glass or stainless steel. Attaching this reservoir to the closed-circuit system took ∼2 min, after which the peristaltic pump was turned back on and the P_{crit} trial was run according the closed-circuit technique described for the typical duration P_{crit} trials. The average duration for these closed-circuit trials was 434±56 min.

Second, we used flow-through respirometry where $\dot{M}_{O_2}$ was calculated according to:

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

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 (we used a single P_{O_2} optode for these measurements) and f is water flow rate (190 ml h^{-1}). We held fish at approximately 26, 16, 5.3, 2.7, 1.3, 0.7 and 0 kPa, each Pw_{O_2} in series, in that order and for 1 h, and at each Pw_{O_2} we measured $\dot{M}_{O_2}$ at 10, 30 and 60 min (10 min was the minimum time required to ensure Pw_{O_2} had equilibrated across the respirometer and the upstream and downstream Pw_{O_2} measurement chambers). Because the calculated $\dot{M}_{O_2}$ at each Pw_{O_2} was nearly identical at each of the three time points, we averaged across the time points and calculated P_{crit} from those averaged $\dot{M}_{O_2}$ values for each individual. The average duration for these flow-through trials was 562±19 min, including the time required to reach target Pw_{O_2} values.

Third, we used a variation on intermittent flow respirometry that combined flow-through and closed-circuit respirometry. We used flow-through conditions to manually reduce Pw_{O_2} from normoxia to ∼2.8 kPa over ∼430 min and then commenced a period of closed-circuit respirometry, which took an additional ∼15 min. We chose a target Pw_{O_2} of 2.8 kPa to start the closed-circuit portion of the trial based upon our earlier short-term P_{crit} trials (which used the same combined respirometric technique) that suggested we could reliably determine P_{crit} from this Pw_{O_2}. Upon reaching 2.8 kPa, we converted to the closed-circuit setup and allowed the fish's respiration to deplete the remaining O_2 in the closed system as described previously. This combination of techniques allowed for closed-circuit $\dot{M}_{O_2}$ measurements with a reduced accumulation of metabolic end-products. As with the rapid RHI P_{crit} trials, we used closed-circuit respirometry to make a series of normoxic $\dot{M}_{O_2}$ readings between 25 and 19 kPa prior to the active (but in this case gradual) reduction of Pw_{O_2} to aid in our calculation of P_{crit} (see below). Upon reaching 19 kPa, we converted the system back to flow-through, reestablished a Pw_{O_2} of ∼21 kPa, and then commenced the active water volume replacement. The average duration of these combined flow-through and closed-circuit trials was 444±12 min.

Parallel hypoxic exposures for physiological measurements

We ran two separate but identical parallel sets of hypoxic exposures to investigate potentially causal physiological factors in a time-dependent reduction in P_{crit}. These parallel exposures involved manually reducing Pw_{O_2} of aquaria from normoxia to anoxia over 60 and 480 min periods to represent rapid and gradual RHIs, respectively. We also ran normoxic control exposures during which Pw_{O_2} remained normoxic for 480 min following the habituation period. Each exposure was run in two 10 litre aquaria housing four fish each, and each aquarium was fitted with a screen just below the water surface to prevent the fish from accessing the air–water interface. We mimicked the respirometric P_{crit} trials described above as closely as possible, with exposures run at 17°C at the same time of day (each trial commenced at ∼09:00 h) following a ≥16 h habituation period, and in complete darkness. Fish from the first set of parallel exposures were sampled to assess gill morphology and haematological parameters, and fish from the second set of parallel exposures were sampled to measure plasma [lactate].

At the end of each exposure, fish were euthanized by inconspicuously introducing anaesthetic (buffered MS-222, final concentration of 200 mg l^{-1}) to the water. Once fish ceased to respond to a tail pinch, individuals were removed, weighed, and then blood was sampled and gills were dissected. To sample blood from the fish in the first set of parallel exposures, the fish's tail was severed and blood was collected from the caudal preduncle using a 60 µl heparinized capillary tube. Ten microlitres of blood was pipetted into 1 ml Drabkins reagent for determination of [Hb], 20 µl of blood was mixed with 10 µl of heparinized Cortland's saline plus 80 µl of 3% perchloric acid for determination of the concentration of red blood cell (RBC) organic phosphates (ATP and GTP; [NTP]), and 10 µl of blood was mixed with 5 µl of heparinzed Cortland's saline for determination of Hb–O_2 binding affinity. The entire right gill basket was then removed from the fish and immediately immersed in 1 ml of Karnovsky's fixative (25% glutaraldehyde, 16% formaldehyde, 0.15 mol l^{-1} sodium cacodylate, pH 7.4). Twenty-four hours later, the gill basket was transferred to 0.15 mol l^{-1} sodium cacodylate and stored at 4°C until use. This procedure was repeated for all four fish in each tank, and then duplicated for the second tank of four fish, yielding N=8 for each treatment. For the second set of parallel exposures, fish were euthanized and blood was collected in the same manner as before, but the plasma was separated from the red blood cells by centrifugation and immediately assayed for plasma [lactate] (see below).

The goal of these parallel exposures was to assess the effects of RHI on physiological adjustments that may explain differences in P_{crit}, but there are differences between the P_{crit} trials and the parallel exposures that the reader should be made aware of. The main difference was vessel size (respirometer chamber was 32 ml and exposure aquaria were 10 litres), which could have affected the ability of the fish to move throughout the exposure. However,

observations of the fish in the 10-litre aquaria suggest that goldfish do not increase activity during progressive hypoxia exposure. Furthermore, the parallel exposures were terminated when Pw_{O_2} reached 0 kPa. As the samples were taken at this point, the haematology and gill morphology measurements were not taken precisely at the point at which we observed differences in P_{crit}, and this could affect our ability to relate the two studies. However, the fish used for the haematology and gill morphology analyses were only exposed to an additional ∼7 to ∼15 min of progressively deepening hypoxia (for rapid and gradual RHI, respectively) beyond what they were exposed to by the time P_{crit} had been reached. Thus we do not believe these relatively minor differences in time would affect our ability to directly relate these components of our study.

Gill morphometrics

Gill samples were randomly assigned an alphanumeric code by an independent party so analysis could be performed blindly. The second gill arch of each gill basket was isolated and its anterior hemibranch imaged using light microscopy (Olympus Stereomicroscope SZX10; 6.3× magnification, 10× zoom; image capture using cellSens Software). The images were used in combination with ImageJ v2.0.0 software to measure filament length and number, and lamellar height (distance from base to the distal edge of the lamellae), length (distance lamellae runs along the filament) and frequency (number of lamellae per unit distance of filament). We made the lamellar measurements by dividing the length of the gill arch into five sections, and then isolating a filament from each of these sections. Each filament was imaged from the top and the side, providing clear views of the height and length of its lamellae that we later measured. Specifically, we measured the height, length and width of three lamellae per filament (one from the filament's base, one from its middle and one from its tip), as well as the width of inter-lamellar channels in these three regions. We then estimated each filament's lamellar frequency (lamellae μm^{-1}) by dividing filament length by the sum of that filament's average channel and lamellar widths. Total lamellar surface area ($SA_{L,total}$) for each fish was then calculated according to:

$$SA_{L,total} = SA_F \cdot F \cdot 16, \quad (3)$$

where SA_F is the mean lamellar surface area of the five analyzed filaments, F is the number of filaments per gill arch, and 16 is the product of two hemibranchs per gill arch, four gill arches per gill basket and two gill baskets per individual fish (according to Wegner, 2011).

Blood analyses

Hb–O_2 binding affinity was determined within 60 min of blood sampling by constructing an oxygen equilibrium curve (OEC) using the thin film spectrophotometric technique (Lilly et al., 2013) and a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A Wostoff gas mixing pump (H. Wösthoff Messtechnik GmbH, Bochum, Germany) mixed compressed O_2 and N_2 to each of nine P_{O_2} values between 0 and 21 kPa P_{O_2}, always starting with 0 kPa and working toward 21 kPa, and each P_{O_2} was maintained for 20 min, during which Hb–O_2 saturation was determined spectrophotometrically. A sigmoidal OEC was fit through the % Hb–O_2 saturation versus P_{O_2} data for each fish, and Hb P_{50} (the P_{O_2} at which Hb is 50% saturated with O_2) was determined using SigmaStat 11.0.

We measured whole blood [Hb] spectrophotometrically at 17°C and 540 nm after conversion to cyanomethemoglobin using Drabkin's reagent (Sigma-Aldrich). The measurements were made using a Shimadzu UV-160 spectrophotometer and a millimolar extinction coefficient of 11.

We measured RBC [NTP] spectrophotometrically at 17°C using the GAPDH- and PGK-catalyzed reactions converting glycerate 3-phosphate to glyceraldehyde 3-phosphate, where the oxidation of NADH to NAD^+ was measured at 340 nm (Bergmeyer et al., 1983). Finally, we measured plasma [lactate] spectrophotometrically at 17°C using the LDH-catalyzed reaction converting lactate to pyruvate, where the reduction of NAD^+ to NADH was measured at 340 nm (Bergmeyer et al., 1983). [NTP] and [lactate] were measured using a 96-well microplate spectrophotometer (Molecular Devices).

CO_2 and nitrogenous end-product measurements

The accumulation of metabolic end-products during closed-chamber/circuit respirometry is regarded as a major shortcoming of the technique (e.g. Keys, 1930; Rogers et al., 2016; Snyder et al., 2016; Steffensen, 1989), but measurements of metabolic end-products in the respirometer chamber are rarely, if ever, reported. To address this knowledge gap, we ran a separate set of closed-circuit P_{crit} trials (91±10 min) to measure accumulated levels of CO_2 and nitrogenous end-products (NH_3+NH_4^+). For each of four fish, we took water samples from the respirometer chamber at three time points: start of the habituation period; end of a 16 h habituation period immediately prior to starting the P_{crit} trial; and end of the P_{crit} trial as soon as the respirometer's Pw_{O_2} reached 0 kPa. Pw_{CO_2} was determined using the Henderson–Hasselbalch equation and measurements of total CO_2 content in the water (CO_2+HCO_3^-; Corning 965 Carbon Dioxide Analyzer, Corning, NY, USA) and pH (probe: SaS gK2401C, Radiometer Analytical, France; meter: VWR Symphony SB70P, VWR, Radnor, PA, USA). Total ammonia (NH_3+NH_4^+) was measured using an API ammonia test kit, and unionized ammonia (NH_3) was calculated from this value in combination with the particular trial's water pH and temperature (17°C).

P_{crit} calculation

P_{crit} is defined as the Pw_{O_2} at which an organism's stable $\dot{M}_{O_2}$ transitions from being independent of to being dependent upon Pw_{O_2}. There are different methods to calculate P_{crit}, but analyses performed by Mueller and Seymour (2011) suggest that most of the methods used yield comparable values. We therefore decided to use a variation on a two-segment linear regression model (details below) to identify P_{crit} as the Pw_{O_2} at which the two linear trend lines (one representing the Pw_{O_2} range of oxyregulation, the other of oxyconformation) intersect on a graph plotting $\dot{M}_{O_2}$ as a function of Pw_{O_2} (BASIC program of Yeager and Ultsch, 1989). This method is employed widely throughout the literature (see Rogers et al., 2016) and has been used on goldfish (Fu et al., 2011; Dhillon et al., 2013; Regan et al., 2017).

We calculated $\dot{M}_{O_2}$ values by measuring the change in Pw_{O_2} over sequential time intervals: 5 min between 25 and 5.3 kPa and 2 min between 5.3 and 0 kPa. To standardize our estimates of a stable, oxyregulated $\dot{M}_{O_2}$, we used the mean of each fish's calculated $\dot{M}_{O_2}$ values between 21 and 18.7 kPa Pw_{O_2}. This represented a normoxic routine $\dot{M}_{O_2}$ that was likely close to standard $\dot{M}_{O_2}$ as a result of it being made following a habituation period that was ≥16 h. We then determined P_{crit} as the intersection of this horizontal line with a linear regression through the $\dot{M}_{O_2}$ values that were >15% below the mean routine $\dot{M}_{O_2}$ value. This technique was carried out according to McBryan et al. (2016).

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Data analysis and statistics

We compared all average values of P_{crit}, normoxic $\dot{M}_{O_2}$, blood properties, gill morphometrics and accumulated Pw_{CO_2} and nitrogenous end-products using one-way, two-tailed ANOVAs with a critical α=0.05 (repeated measures for the water pH, Pw_{CO_2} and nitrogenous end-products comparisons). *Post hoc* Tukey tests were used to test for differences between treatment groups. Any data set that did not meet the assumptions of normality or equal variance were log-transformed prior to analysis. All analyses were performed using SigmaStat 11.0. Values reported in the text are presented as means±s.e.m.

RESULTS

Respirometry

Long duration P_{crit} trials resulted in P_{crit} values that were approximately half those of short and typical duration P_{crit} trials (ANOVA, P<0.001; Fig. 1A, Fig. S1). P_{crit} values determined by short and typical trial durations did not differ from one another, nor did the P_{crit} values determined by the three respirometric techniques used for the long duration trials (Fig. 1A). Each of the five respirometric techniques yielded statistically similar normoxic $\dot{M}_{O_2}$ values (ANOVA, P=0.276; Fig. 1B).

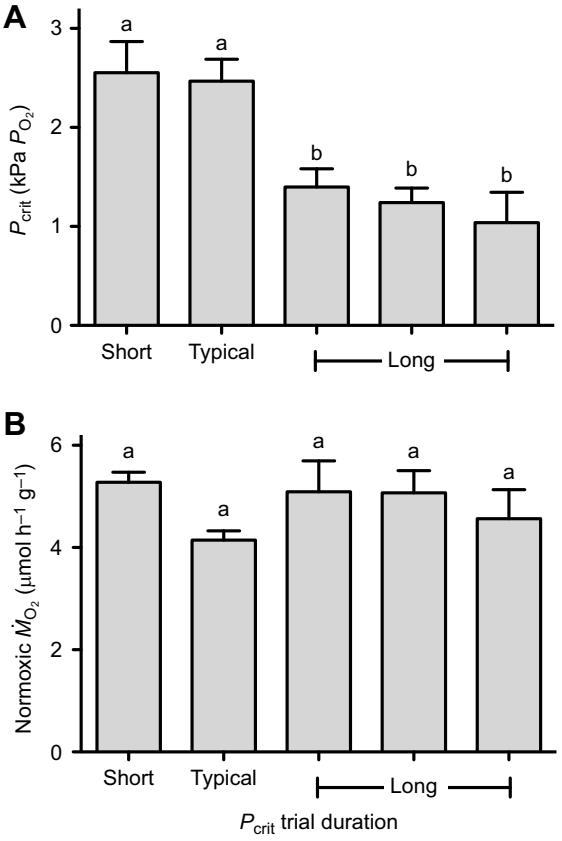

Fig. 1. The effect of P_{crit} trial duration on the average P_{crit} and normoxic $\dot{M}_{O_2}$ values of goldfish. (A) Average P_{crit} values of the individual fish comprising each set of respirometry experiments. (B) Average normoxic $\dot{M}_{O_2}$ values of the individual fish comprising each set of respirometry experiments while those fish were exposed to normoxic Pw_{O_2} (18 to 26 kPa). Details on the respirometric techniques are included in the Materials and methods, but briefly, 'short' used combined flow-through/closed-circuit intermittent flow respirometry (N=5; 24±2.2 min), 'typical' used closed-circuit respirometry (N=6; 84±8 min) and 'long' from left to right used closed-circuit respirometry (N=6; 434±56 min), combined flow-through/closed-circuit intermittent flow respirometry (N=4; 444±12 min) and flow-through respirometry (N=6; 562±19 min). Error bars are s.e.m.; bars that share a letter are not significantly different (one-way ANOVA, [a]P<0.001, [b]P=0.276).

Effect of RHI on gill morphology

RHI significantly affected the mass-specific lamellar surface areas of goldfish (ANOVA, P=0.004; Fig. 2), whereby fish exposed to gradual RHIs had ∼60% larger lamellar surface areas than fish exposed to rapid RHIs and normoxic controls, which did not differ.

Effect of RHI on Hb–O_2 binding affinity, [Hb] and RBC [NTP]

RHI significantly affected Hb–O_2 binding affinity (ANOVA, P=0.007; Fig. 3). Goldfish exposed to rapid RHIs had ∼60% higher Hb P_{50} values than goldfish exposed to gradual RHIs and normoxic controls, which did not differ.

RHI did not affect whole-blood [Hb] (ANOVA, P=0.334; Fig. 4A), but it did affect RBC [NTP] (ANOVA, P=0.001; Fig. 4B), whereby gradual RHI fish had RBC [NTP] values that were approximately half those of the rapid RHI and normoxic control fish, which did not differ.

Effect of RHI on plasma lactate

Goldfish exposed to rapid and gradual RHIs both accumulated similar concentrations of plasma lactate to a level significantly higher than that observed in normoxic control fish (ANOVA, P=0.001; Fig. 4C).

Metabolic end-product accumulation

Respirometer chamber Pw_{CO_2} doubled over the course of a 16 h habituation period under flow-through conditions, and increased 6.5-fold over the course of a typical duration closed-circuit P_{crit} trial (91±10 min; ANOVA, P<0.001; Fig. 5A). Water pH was concomitantly reduced from 7.61 to 6.93 over the course of the P_{crit} trial (ANOVA, P<0.001; Fig. 5B). The concentration of total ammonia (NH_3+NH_4^+) in the chamber also increased (ANOVA, P<0.001; Fig. 5C). Unionized ammonia (NH_3) accumulated in a different way because of pH changes of the water, with [NH_3] tripling over the 16 h habituation period, then falling to an intermediate value by the end of the P_{crit} trial (ANOVA, P<0.001; Fig. 5D).

DISCUSSION

We hypothesized that gradual RHIs would allow goldfish to induce time-dependent plastic phenotypes that enhance O_2 uptake. This

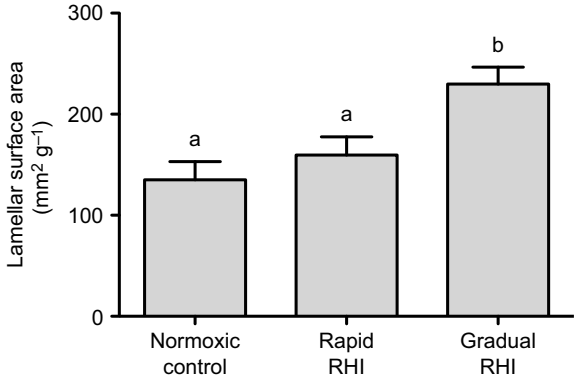

Fig. 2. The effect of the rate of hypoxia induction (RHI) on the mass-specific lamellar surface area of goldfish. Mass-specific lamellar surface areas of goldfish exposed to rapid and gradual RHIs (normoxia to anoxia in 60 and 480 min, respectively) and normoxic controls (N=8 for each; one-way ANOVA, P=0.004). Error bars are s.e.m.; bars that share a letter are not significantly different.

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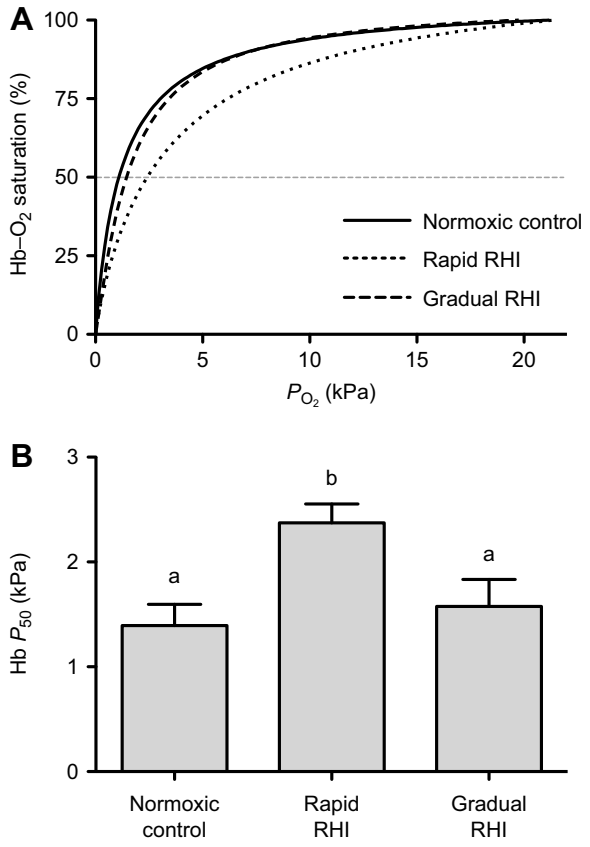

Fig. 3. The effect of RHI on the oxygen equilibrium curve (OEC) and the Hb P_{50} value of goldfish. (A) OECs for the extracted whole blood of goldfish exposed to rapid and gradual RHIs (normoxia to anoxia in 60 and 480 min, respectively) and normoxic controls. The blood was collected from the fish immediately upon Pw_{O_2} reaching ∼0 kPa (for rapid and gradual RHIs), and the spectrophotometric determination of Hb–O_2 saturation was begun as soon thereafter as possible (<1 h). Each OEC is a trendline through the data points of eight blood samples exposed to nine Pw_{O_2} values between 0 and 21 kPa P_{O_2}. Grey horizontal dashed line highlights the 50% Hb–O_2 saturation point. (B) Average Hb P_{50} values (P_{O_2} at which Hb is 50% saturated with O_2) for each treatment group (one-way ANOVA, P=0.007). Error bars are s.e.m.; bars that share a letter are not significantly different.

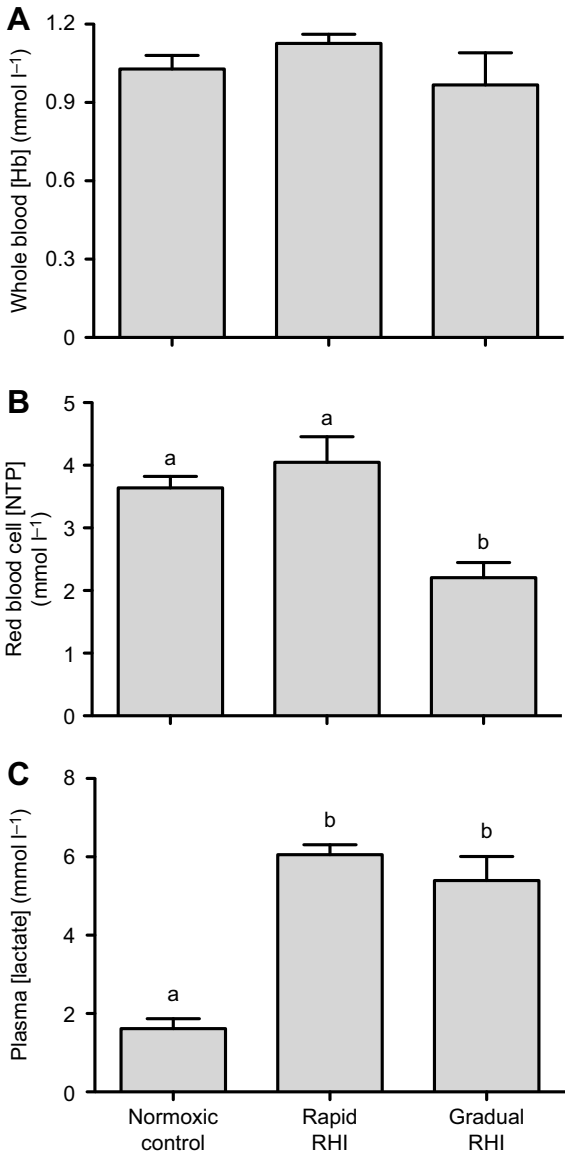

Fig. 4. The effect of RHI on blood parameters of goldfish. (A) Average values for whole blood [Hb] measured spectrophotometrically using Drabkins reagent (N=6–8; one-way ANOVA, P=0.334). (B) Average values for red blood cell [NTP] (N=6–8; one-way ANOVA, P=0.001). (C) Average values for plasma [lactate] (N=6–8; one-way ANOVA, P=0.001). Error bars are s.e.m.; bars that share a letter are not significantly different.

hypothesis predicted that the P_{crit} of goldfish exposed to long duration P_{crit} trials would be lower than those of goldfish exposed to short or typical duration P_{crit} trials, and our results agree with these predictions regardless of the respirometric technique used. Furthermore, fish exposed to gradual RHIs developed greater lamellar surface areas and Hb–O_2 affinities over their exposures than fish exposed to rapid RHIs, both characteristics that would enhance O_2 extraction under hypoxia and therefore likely explain the lower P_{crit} associated with the long duration trials. Taken together, our results suggest that time (more precisely, RHI) is a significant determinant of P_{crit} in goldfish.

The majority of P_{crit} measurements are made using closed-chamber respirometry over the course of 60 to 120 min (Rogers et al., 2016). Here, our representative closed-circuit P_{crit} trials lasted ∼84 min and resulted in a P_{crit} of 2.5±0.2 kPa (Fig. 1A). Our values are in general agreement with values previously reported for goldfish [∼3.6 kPa (Fry and Hart, 1948); 3.0 kPa (Fu et al., 2011); 3.3 kPa (Dhillon et al., 2013); and 3.0 kPa (Regan et al., 2017)], though slightly lower owing to a possible combination of experimental temperature differences and the fact that our study used closed-circuit respirometry as opposed to static closed-chamber respirometry. Reducing the trial duration to ∼24 min did not affect P_{crit} (Fig. 1A), which may not be surprising considering the RHI below a Pw_{O_2} of 5.3 kPa was similar between the short and typical duration P_{crit} trials (see Materials and methods for details). However, our results clearly indicate that increasing the trial duration from ∼84 min (i.e. reducing its RHI) to ∼480 min resulted in significantly lower P_{crit} values. The reasons for this variation could be related to time, technique or some combination of the two, and we will explore these possibilities below.

Effects of time on the physiology of O_2 uptake

Goldfish exposed to gradual RHIs developed significantly larger lamellar surface areas than those of normoxic controls and goldfish exposed to rapid RHIs, which did not differ. Hypoxia-induced gill remodeling was first observed in goldfish and the closely related

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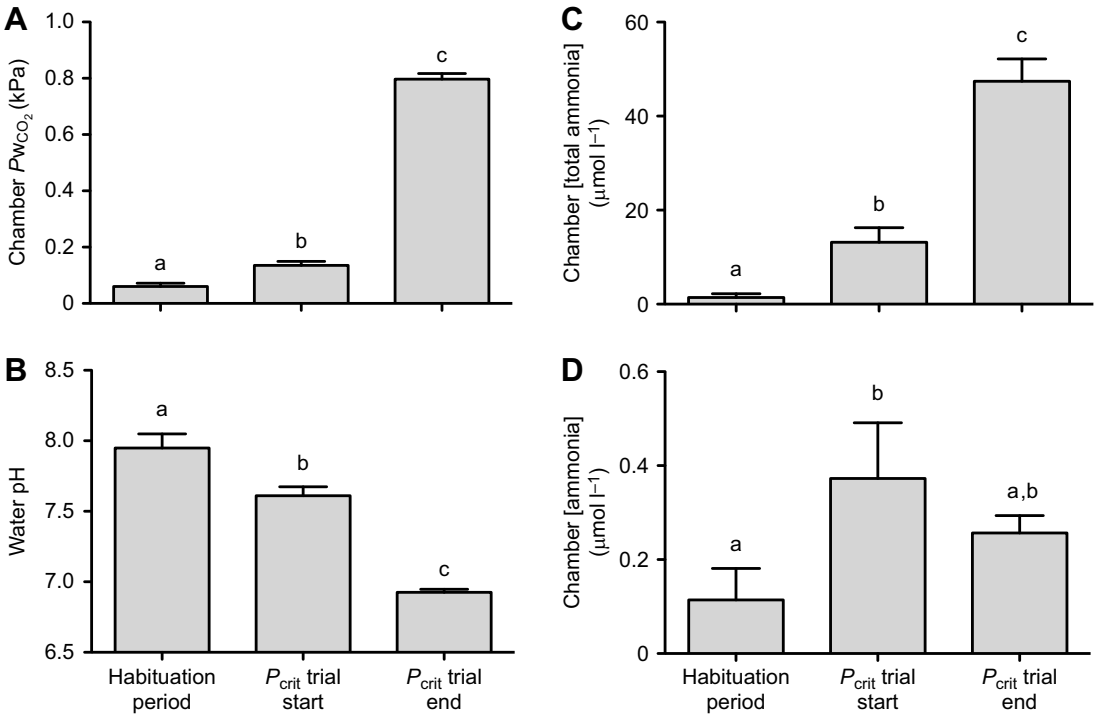

Fig. 5. The effects of closed-circuit respirometry on water chemistry and the buildup of metabolic end-products. (A) Chamber Pw_{CO_2}, measured as total CO_2 and converted to Pw_{CO_2} using water pH and the Henderson–Hasselbalch equation (N=4; one-way ANOVA, P<0.001). (B) Water pH (N=4; one-way ANOVA, P<0.001). (C) Total ammonia concentration (NH_3+NH_4^+; N=4; one-way ANOVA, P<0.001). (D) Unionized ammonia concentration, calculated using water pH and temperature (N=4; one-way ANOVA, P<0.001). Error bars are s.e.m.; bars that share a letter are not significantly different.

crucian carp (*Carassius carassius*) 13 years ago (Sollid et al., 2003, 2005) and in numerous fish species since [e.g. mangrove killifish, *Kryptolebias marmoratus* (Ong et al., 2007; Turko et al., 2012); African cichlids (Crispo and Chapman, 2010); various carp species (Dhillon et al., 2013); Atlantic salmon *Salmo salar* (Anttila et al., 2015); and Atlantic killifish *Fundulus heteroclitus* (Borowiec et al., 2015)]. Dhillon et al. (2013) observed a near-doubling in the lamellar surface area of goldfish following 8 h acclimation to a constant Pw_{O_2} of 0.7 kPa, but to our knowledge, the present study is the first time gills have been shown to remodel over such short time scales under progressively decreasing Pw_{O_2}. Increases to lamellar surface area are typically the result of apoptotic reductions to the ILCM (Sollid et al., 2003). ILCM reductions per se also enhance the gill's diffusion capacity (Bindon et al., 1994; Greco et al., 1995) and contribute to a reduced P_{crit} in crucian carp (Sollid et al., 2003) and Atlantic killifish (Borowiec et al., 2015). However, a study that examined (among other things) O_2 diffusion across the gills of two groups of goldfish with temperature-induced differences in gill surface area found that the differences in gill surface area had no effect on arterial P_{O_2} when acutely exposed to hypoxia (Tzaneva et al., 2011). Although this seems to run counter to what Fick's first diffusion law would predict, the authors speculated that the goldfish that started hypoxia exposure with a smaller gill surface area may have been rapidly remodeling their gills to increase lamellar surface area over the course of the acute hypoxia exposure. Our gill morphometric results lend empirical support to this speculation.

The mean Hb P_{50} value of gradual RHI goldfish (1.6 kPa, similar to long duration P_{crit} of ∼1.2 kPa) was significantly lower than that of rapid RHI goldfish (2.4 kPa, similar to typical duration P_{crit} of 2.5 kPa), but no different than normoxic controls (1.4 kPa). This implies that rapid hypoxia induction reduces Hb–O_2 binding affinity, but gradual induction does not. The underlying mechanism(s) might involve RBC [NTP] and/or pH. Nucleoside triphosphates (ATP and GTP, collectively NTPs) reduce Hb–O_2 binding affinity by binding to sites on the Hb tetramer that stabilize its deoxygenated conformation and consequently increase the P_{50} (Jensen et al., 1998; Wood and Johansen, 1972). The significantly lower [NTP] values of our gradual RHI fish (Fig. 4B) at least partly explain their lower Hb P_{50} values compared with those of the rapid RHI fish (Fig. 3), but the similar [NTP] values in the rapid RHI and normoxic control fish exclude this mechanism as the cause of the rapid RHI fish's elevated Hb P_{50} values. Another possible mechanism for the rapid RHI fish's reduced Hb–O_2 binding affinity is RBC pH. We did not measure RBC pH, but if protons accumulated to higher concentrations in the RBCs of rapid RHI goldfish than gradual RHI goldfish, then this would likely have reduced Hb–O_2 binding affinity via goldfish's Bohr/Root effect (Rodewald and Braunitzer, 1984). Regardless of the causal mechanism(s), the different Hb–O_2 binding affinities of the rapid and gradual RHI fish are likely to at least partly explain their different P_{crit} values.

Respirometric technique and end-product accumulation

Respirometric techniques can be broadly categorized as closed (closed-circuit or static closed-chamber), flow-through or intermittent flow. Though none of these techniques are ideal for all experimental scenarios, intermittent flow respirometry is generally regarded as superior because it avoids the potential accumulation of metabolic end-products presumed to occur in closed respirometry and it has greater temporal resolution than flow-through respirometry (reviewed by Clark et al., 2013; Steffensen, 1989; Svendsen et al., 2016). It has been suggested that the choice of respirometric technique used to determine P_{crit} may influence the results, and indeed P_{crit} in shiner perch (*Cymatogaster aggregata*) shifted from ∼9.9 kPa to ∼6.1 kPa when using closed-chamber versus intermittent flow respirometry,

respectively (Snyder et al., 2016). The authors attribute this to technique, but also discuss the possibility that duration of the P_{crit} trials (~1 h for closed-chamber, ~5 h for intermittent flow) may play a role (Snyder et al., 2016). In the present study, we used modified versions of all three respirometric techniques for our long duration P_{crit} trials, and, despite technique specific-differences and challenges (e.g. flow-through trials demanded a step-wise reduction in Pw_{O_2}; closed-circuit trials resulted in higher $\dot{M}_{O_2}$ values in the mid-Pw_{O_2} range; Fig. S1), each technique yielded nearly identical P_{crit} values, which were all lower than the typical or short duration P_{crit} trials. This suggests that the different P_{crit} values observed between our short and typical duration P_{crit} trials and those of the long duration trials are the result of RHI rather than technique, and this may also be the case with the results of Snyder et al. (2016).

The fact remains that closed respirometry leads to end-product accumulation (Fig. 5), and this could theoretically influence P_{crit} (Keys, 1930; Snyder et al., 2016; Steffensen, 1989). However, within our long duration P_{crit} trials, closed-circuit trials (where CO_2 accumulated) and combined flow-through/closed-circuit trials (where CO_2 did not accumulate) resulted in nearly identical P_{crit} values (Fig. 1A). This suggests that the levels of metabolic end-products that accumulate with closed-circuit respirometry are not high enough per se to have a significant effect on P_{crit}.

Conclusions

Our results demonstrate that RHI significantly alters the P_{crit} of goldfish, whereby long duration P_{crit} trials (i.e. gradual RHIs) yield lower P_{crit} values than short duration P_{crit} trials (i.e. rapid RHIs). These reduced P_{crit} values are caused by time-dependent effects on mechanisms that enhance environmental O_2 extraction, including gill morphology and Hb–O_2 binding affinity. Fishes generally possess numerous time-dependent mechanisms that enhance O_2 extraction in response to hypoxia, so RHI is likely an important factor to consider in all fish species when carrying out P_{crit} trials and experimental hypoxia exposures. Because longer duration P_{crit} trials allow for some degree of acclimation that may consequently reduce P_{crit}, shorter duration P_{crit} trials are likely to best represent the innate abilities of a hypoxia-exposed fish to extract and use O_2 at the time of analysis. Thus, similar to the standardized rate of temperature change used when determining a fish's CT_{max}, an RHI should be chosen that is fast enough to avoid acclimation during the trial. Our data suggest that P_{crit} trials of <90 min are probably sufficient to achieve this.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.D.R.; Methodology: M.D.R., J.G.R.; Formal analysis: M.D.R.; Investigation: M.D.R.; Resources: M.D.R.; Writing - original draft: M.D.R.; Writing - review & editing: M.D.R., J.G.R.; Funding acquisition: J.G.R.

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Data availability

Data are available from the Dryad Digital Repository (Regan and Richards, 2017): http://dx.doi.org/10.5061/dryad.bp20p

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.154948.supplemental

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