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ORIGINAL PAPER

Is the air-breathing organ a significant route for CO_2 excretion during aquatic hypercapnia in the pirarucu, *Arapaima gigas?*

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Abstract
The pirarucu is one of the very few obligate air-breathing fish, employing a gigantic, highly vascularized air-breathing organ (ABO). Traditionally, the ABO is thought to serve mainly for O_2 uptake ($\dot{M}O_2$), with the gills providing the major route for excretion of CO_2 ($\dot{M}CO_2$) and N-waste. However, under aquatic hypercapnia, a common occurrence in its natural environment, branchial $\dot{M}CO_2$ to the water may become impaired. Under these conditions, does the ABO become an important route of $\dot{M}CO_2$ excretion to the air? We have answered this question by measuring $\dot{M}CO_2$ and $\dot{M}O_2$ in both air and water phases, as well as the pattern of air-breathing, in pirarucu under aquatic normocapnia and hypercapnia (3% CO_2). Indeed, $\dot{M}CO_2$ to the air phase via the ABO increased 2- to 3-fold during exposure to high water PCO_2, accounting for 59–71% of the total, with no change in the dominant contribution of the ABO to $\dot{M}O_2$ (71–75% of the total). These adjustments were quickly reversed upon restoration of aquatic normocapnia. During aquatic hypercapnia, $\dot{M}CO_2$ via the ABO became more effective over time, and the pattern of air-breathing changed, exhibiting increased frequency and decreased breath volume. Ammonia-N excretion (86–88% of total) dominated over urea-N excretion and tended to increase during exposure to aquatic hypercapnia. We conclude that the ability of the ABO to take on the dominant role in CO_2 excretion when required may have been an important driver in the original evolution of air-breathing, as well as in the functionality of the ABO in modern air-breathing fish.

Keywords Gills · Lung · Bimodal breathing · N-waste excretion · Water pH · O_2 uptake

Introduction

Traditionally, most theories about the evolutionary origin and present-day functional significance of air-breathing in fish emphasize aquatic hypoxia (low water PO_2) as the primary driver (e.g. Packard 1974; Randall et al. 1981a; Graham 1997; Janis and Farmer 1999; Graham and Lee 2004; Zaccone et al. 2018; Bayley et al. 2018; Damsgaard et al. 2020; Milsom et al. 2021). Under these conditions, the air-breathing organ (ABO) becomes the major site of O_2 uptake (from the air), whereas the gills continue to serve as the major route of CO_2 excretion (to the water). More rarely is aquatic hypercapnia (high water PCO_2) considered in terms of the evolutionary origin and functional significance of air-breathing (Ultsch 1987, 1996; Shartau and Brauner 2014). Yet we know that almost invariably, instances of low PO_2 occur in concert with simultaneous high PCO_2 in aquatic environments where air-breathing fish flourish such as in swamps and ephemeral ponds of the Amazon basin (Val and Almeida-Val 1995). Both are caused by the biological

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respiration of organic material (Dehadrai and Tripathi 1976; Hamilton et al. 1995). Often, CO_2 production exceeds O_2 consumption, and CO_2 outgassing may be limited such that PCO_2 elevation becomes uncoupled from O_2 depletion (Richey et al. 1988, 2002; Hamilton et al. 1995; Fontes et al.2015). It is also known that atmospheric PCO_2 levels were exceptionally high in the Devonian period, when it is believed that air-breathing in vertebrates first evolved, whereas atmospheric PO_2 levels were reasonably high at that time (Budyko et al. 1987).

The pirarucu (*Arapaima gigas*) is an obligate air-breathing fish, which is endemic to the Amazon basin. As an adult, it is one the largest freshwater fish in the world. After a few days of early life where it is an exclusive water-breather, it starts to use a relatively large, highly vascularized lung-like sac as its air-breathing organ (ABO) (Brauner et al. 2004; Pelster et al. 2020; Frommel et al. 2021). When the water is normoxic or hypoxic, the gills perform most of the CO_2 and nitrogenous waste excretion, while the ABO performs most of the O_2 uptake (Randall et al. 1978; Stevens and Holeton 1978; Brauner et al. 2004; Brauner and Val 1996; Gonzalez et al. 2010; Pelster et al. 2020; Wood et al. 2020; Aaskov et al. 2022). Even during virtual anoxia in the water, there is negligible repartitioning of CO_2 excretion from the gills to the ABO (Aaskov et al. 2022). In the present study, we asked whether the ABO could take on a more important role in CO_2 excretion when the PCO_2 in the water was substantially increased to 3% (~3 kPa, ~ 22 mmHg), as indicated by the early findings of Burggren (1979) on another obligate air-breather, the blue gourami. A level of 3% CO_2 is very common in Amazonia waters; indeed, values up to 8% PCO_2 have been recorded (Val and Wood 2022). We hypothesized that aquatic hypercapnia at this level would substantially lower or even reverse the PCO_2 gradient across the gills from blood to water, resulting in reduced branchial CO_2 excretion or even net branchial CO_2 uptake. As a result, blood PCO_2 levels would rise, thereby potentially increasing blood-to-air PCO_2 gradients at the ABO, and/or stimulating air-ventilation, thereby increasing CO_2 excretion via the ABO. Furthermore, shifts in both blood flow distribution between gills and ABO (Farrell 1978; Hulbert et al. 1978) as well, as Bohr, Root and Haldane effects in the blood passing through the two exchange sites (Johansen et al. 1978a, b; Randall et al. 1978; Brauner and Randall 1996; Weber et al. 2022) could also alter O_2 uptake and N-waste excretion. Therefore, in the present study we measured CO_2 and O_2 exchange in both the air and water phases, as well as ammonia-N and urea-N excretion to the water phase in pirarucu in normocapnic normoxic water, and in hypercapnic normoxic water.

Methods

Experimental animals

Experiments were performed at the Laboratory of Ecophysiology and Molecular Evolution (LEEM) of the Brazilian National Institute for Research of the Amazon (INPA) in Manaus, Brazil. All procedures were in compliance with Brazilian national and INPA animal care regulations (protocol 027/2015). Pirarucu (*Arapaima gigas*, 904 ± 44 g, range = 575–1370 g, $N = 28$, approximately 5 months old, sex unknown) were obtained from local commercial aquaculture. At INPA, they were held in a large shaded outdoor tank with free access to air, and fed daily with commercial pellets (NutripeixeTr36, Purina Co., São Paulo, SP, Brazil 36% protein). The composition of the well water, which was also used in all experiments, was $[Na^+] = 60–80$, $[K^+] = 10–20$, $[Ca^{2+}] = 3–7$, $[Mg^{2+}] = 1–3$, $[Cl^-] = 10–30$ μmol L^{-1}, pH = 6.5-7.0 Acclimation and experimental temperature was 27 ± 1 °C. Experiments were performed in May-June 2023, between 8 am and 10 pm.

Experimental chambers and protocols

Two-chambered closed system respirometers of two sizes, similar to those used by Stevens and Holeton (1978) and Pelster et al. (2020), were employed to make simultaneous recordings of O_2 uptake ($\dot{M}O_2$) and CO_2 excretion ($\dot{M}CO_2$) in both air and water phases, as well as ammonia-N and urea-N excretion to the water phase. In the respirometer used for the smallest fish (575–745 g), typical water volumes and air volumes were about 8.0 L and 0.5 L, respectively, whereas the larger fish (760–1370 g) were run in respirometers with typical water and air volumes of 13.0 L and 0.8 L. Exact volumes were adjusted for each individual fish so that they could easily access the surface.

In our earlier study (Pelster et al. 2020) we ran > 1-h tests in these chambers in the absence of the fish and found no detectable O_2 movement between the air phase and water phase under realistic PO_2 gradients from air to water. In the present study we ran similar tests with the water phase equilibrated with 3% CO_2 and a normocapnic air phase, and could detect no movement of CO_2 into the air phase. However, when a fish is present, it periodically breaks the surface to air-breathe and exhales bubbles under water. The convective mixing could promote CO_2 movement from water to air phase during aquatic hypercapnia, whereas the underwater exhalation would do the opposite. We do not know which is the larger influence, but these complications are unavoidable, and should be appreciated.

The fish was allowed to settle in clean water and adjust to the respirometer for several hours. Aeration of both phases

was maintained throughout the adjustment period. The water was changed by several cycles of siphoning (without air exposure of the fish) 15–30 min before the start of the experiment. At this time, the exact water level was set. The water used for the change was either normocapnic, normoxic water (control), or water that had been pre-equilibrated with a 3% CO_2/air mixture.

At the start of an experiment, aeration was suspended, water samples (2 × 10 ml, with replacement) were taken for measurements of initial water PCO_2, ammonia, and urea concentrations, water PO_2 was measured directly in the water phase, and the respirometer was then sealed. Needle-shielded micro-optodes for the continuous measurement of air-phase PCO_2 and PO_2 were inserted through sealed ports into the air chamber at this time. At the end of the experiment (typically 0.7–2 h), the continuous recordings of air phase PCO_2 and PO_2 were stopped, final water PO_2 was measured directly in the chamber, and final water samples (2 × 10 ml, with replacement) were taken for measurements of water PCO_2, ammonia, and urea-N concentrations. The exact volumes of the air and water phases were then measured. Within each experiment, the air in the air-breathing chamber was renewed when the PO_2 declined by about 4–5% O_2 (4–5 kPa, 30–37.5 mmHg) and/or the PCO_2 increased to about 3% CO_2 (3 kPa, 22 mmHg). As a result, within an experiment, there were 1–4 cycles of air-phase measurements, but only one cycle of water-phase measurement spanning the entire duration of the experiment.

In *Series 1*, experiments were performed under either normocapnia in the water phase (i.e. control conditions) or hypercapnia (3% CO_2) in the water phase. The air phase remained normocapnic throughout, though of course air PCO_2 increased and air PO_2 declined in between flushes of the air-breathing chamber due to the fish's respiration. Not all measurements were obtained in all fish, so N was variable for different parameters. These fish had been fasted for 2–4 days before experiments.

In *Series 2*, a repeated measures design was used, and all measurements were obtained in all fish. The same fish ($N = 5$) were studied in sequential experiments in first normoxic normocapnia (control), then normoxic hypercapnia (3% CO_2), and then in recovery when they were returned to normoxic normocapnia. Each experiment lasted about 1 h, with an intervening period of about 15 min during which the experimental water was changed over, and the air-breathing chamber was flushed. These fish had been fasted for 5–7 days before experiments.

Analytical techniques

PO_2 levels in water were measured using a DO 6 + galvanic oxygen electrode and meter (Oakton Instruments, Vernon Hills, IL, USA), and PO_2 in the air was determined using PreSens fiber optic oxygen sensors (PreSens Precision Sensing GmbH, Regensburg, Germany) calibrated with air and pure N_2. PCO_2 levels in both water and air phases were measured using a prototype Presens PCO_2 micro-optode system. Both PCO_2 and PO_2 sensors were protected inside 23-guage hypodermic needles; the fluorophore tips were advanced out of the needles into air or water phases to make measurements. The PCO_2 micro-optodes were calibrated using water samples equilibrated with defined PCO_2 levels created by a gas flow-mixing system (Cellasic ONIX™, Millipore, Burlington, MA, USA). This system was also used to set the water 3% CO_2 level in the hypercapnia treatments. These PCO_2 sensors were originally designed to measure PCO_2 levels only in water. However, as in our earlier study (Pelster et al. 2020), we found that they functioned well and maintained calibration in the humid gas phase (90–100% relative humidity) in the air-breathing chambers of our respirometers. While the PO_2 optode showed an almost instantaneous response, the prototype PCO_2 optode needed 1–2 min to fully stabilize (Pelster et al. 2020). As a result, the stepwise changes observed in the PO_2 signal under control conditions were obscured in the PCO_2 signal.

Ammonia-N and urea-N concentrations in water were determined using the colorimetric assays of Verdouw et al. (1978) and Rahmatullah and Boyde (1980) respectively. Throughout, urea is expressed in units of urea-N, taking into account the two N atoms in a urea molecule in contrast to the 1 N atom in an ammonia molecule.

Calculations

Mass-specific O_2 consumption from the water phase ($\dot{M}O_{2water}$, in mmol kg^{-1} h^{-1}) was calculated as:

$$\dot{M}O_{2-water} = \alpha O_2 * \Delta P_w O_2 * V_w * T^{-1} * BM^{-1} \quad (1)$$

where αO_2 is the physical solubility of oxygen in water at the experimental temperature (mmol L^{-1} kPa^{-1}) (Boutilier et al. 1984), $\Delta P_w O_2$ is the change in O_2 partial pressure (kPa) in the water, V_w is the volume (L) of the water phase, T is the time period (h) over which $\Delta P_w O_2$ was measured, and BM is body mass (kg).

Mass-specific CO_2 excretion to the water phase ($\dot{M}CO_{2water}$, in mmol kg^{-1} h^{-1}) was determined as the sum of two components, as in Pelster et al. (2020). The change in CO_2 partial pressure ($\Delta P_w CO_2$ in kPa) and the solubility of CO_2 (αCO_2 in mmol L^{-1} kPa^{-1}) in the INPA water, as determined by Pelster et al. (2020), yields the concentration of CO_2 dissolved in the water that exerts partial pressure. However, as explained by Pelster et al. (2020) there is a second component, an additional smaller portion of CO_2

excretion that does not exert partial pressure because it is converted to NH_4HCO_3 by the simultaneously measured ammonia excretion (M_{Amm}, in µmol kg^{-1} h^{-1}) of the fish. This is added to the first component:

$$\dot{M}CO_{2water} = [\alpha CO_2 * \Delta P_w CO_2 * V_w * T^{-1} * BM^{-1}] + [M_{amm} * 1000] \quad (2)$$

The rates of ammonia-N and urea-N excretion (both in µmol kg^{-1} h^{-1}) were calculated as:

$$M_{amm-N;urea-N} = (C_F - C_I) * V_w * T^{-1} * BM^{-1} \quad (3)$$

where C_F and C_I are the final and initial concentrations of ammonia-N or urea-N in water and the other symbols are as above.

Mass-specific O_2 consumption from the air phase ($\dot{M}O_{2air}$, in mmol kg^{-1} h^{-1}) was calculated as:

$$\dot{M}O_{2air} = \beta O_2 * \Delta P_a O_2 * V_a * T^{-1} * BM^{-1} \quad (4)$$

Mass-specific CO_2 excretion into the air phase ($\dot{M}CO_{2air}$, in mmol kg^{-1} h^{-1}) was calculated by an analogous equation:

$$\dot{M}CO_{2air} = \beta CO_2 * \Delta P_a CO_2 * V_a * T^{-1} * BM^{-1} \quad (5)$$

where ΔP_aO_2 and ΔP_aCO_2 were the measured changes in respective partial pressures in the air phase, values for βO_2 and βCO_2 were taken from Dejours (1981), V_a is the volume of the air phase, and the other symbols are as above.

Respiratory exchange rate (RER) values were calculated as:

$$RER = \dot{M}CO_2 * \dot{M}O_2^{-1} \quad (6)$$

separately for aerial and aquatic gas exchange and also for total gas exchange.

Nitrogen quotient (NQ) values were calculated as:

$$NQ = \dot{M}_{total-N} * \dot{M}O_2^{-1} \quad (7)$$

where $\dot{M}_{total-N}$ represents total urea-N ($\dot{M}_{urea-N}$) + ammonia-N ($\dot{M}_{amm-N}$) excretion into the water.

Minimal breath volume (Bv) required to explain the amount of oxygen taken up from the air was calculated from the number of breaths taken and the fractional decrease in aerial oxygen content over a given period of time:

$$Bv\ (ml) = (P_iO_2 - P_fO_2) * P_mO_2^{-1} * V_a * breaths^{-1} * BM^{-1} \quad (8)$$

where P_iO_2 and P_fO_2 were the measured initial and final partial pressures of O_2 in the air phase, P_mO_2 gives the partial pressure of oxygen present in the air phase midway during the recording period ($PmO_2 = (P_iO_2 + P_fO_2)*0.5$), and V_a is the volume of the air phase.

This calculation assumes that all oxygen taken in with a breath is consumed, which is not necessarily true. Some oxygen may be present in the exhaled air, resulting in larger breath volumes required to obtain the oxygen consumed. Therefore, the value obtained is the minimum breath volume required to cover the recorded aerial oxygen consumption.

Statistics

Data are expressed as mean ± SEM, and N gives the number of fish used for the measurement. Student's unpaired two-tailed t-test was performed to identify significant differences in various parameters between the two treatments in *Series 1* where different fish in each treatment were employed. To test for significant differences among parameters measured in *Series 2*, where three treatments on the same fish were employed sequentially, one-way repeated measures ANOVA followed by Tukey's test was used. If normality and homogeneity of variance checks failed, the data were appropriately transformed. If standard transformations (arc-sine, log, square root) did not work, then Friedman's non-parametric equivalent of repeated measures ANOVA was used. Statistical analyses were performed using Graphpad 10.0 and SigmaPlot 14.0; statistical significance was accepted for $p < 0.05$.

Results

Series 1

In this series, different fish were used in the control and 3% CO_2 treatments, and not all measurements were obtained in all fish. As a result, N numbers varied according to the parameter and treatment (see Figure Legends), and gas flux means in the two phases did not add up perfectly to total flux means. Nevertheless, under both control and 3% aquatic CO_2, the ABO was clearly the dominant route of O_2 uptake, and total $\dot{M}O_2$ remained unchanged (Fig. 1). Comparing only those fish in which O_2 uptake by both routes was measured simultaneously, the partitioning did not change (control = 77% air, $N = 6$; hypercapnia = 82% air, $N = 9$; Fig. 1). CO_2 excretion exhibited very different patterns (Fig. 2). Total $\dot{M}CO_2$ remained unchanged, but the water phase dominated under control conditions, and the air phase dominated under aquatic hypercapnia, with a significant two-fold increase in the absolute rate of CO_2 excretion for the latter. Again, comparing only those fish in which CO_2 excretion by both routes was measured simultaneously, the partitioning

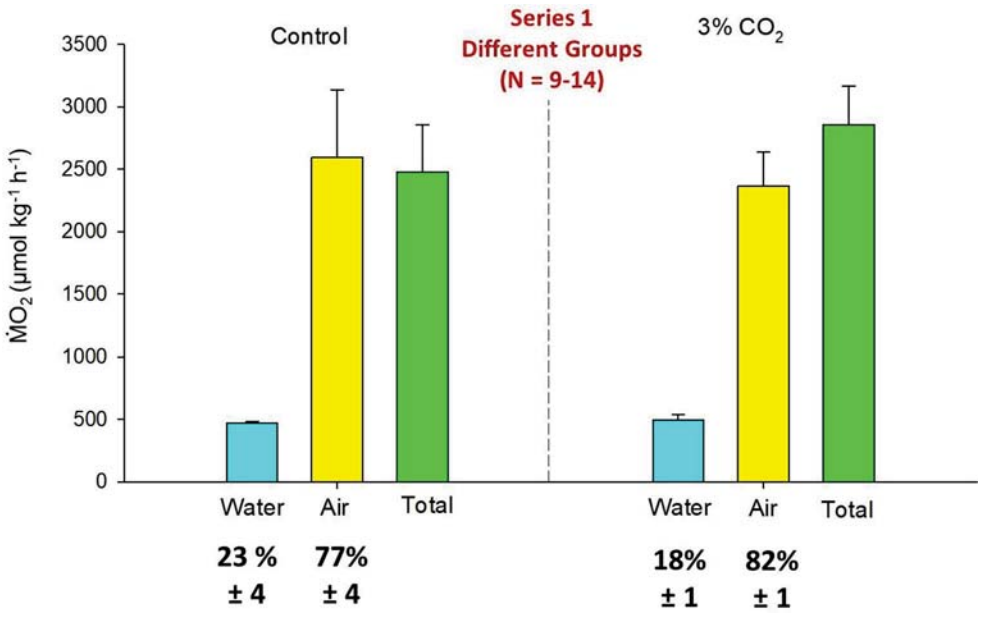

Fig. 1 The rate of O_2 uptake ($\dot{M}O_2$) from water and air phases, and total $\dot{M}O_2$, in pirarucu of *Series 1* under control conditions and under aquatic hypercapnia (3% CO_2). Control: water $N=14$, air $N=9$, total $N=6$. Hypercapnia: water $N=10$, air $N=9$, total $N=9$. Means ± SEM. Water and air means do not add up perfectly to total means because of differing N numbers. Percentage partitioning was calculated only for those fish for which total measurements were made. There were no significant differences $P<0.05$) between treatments

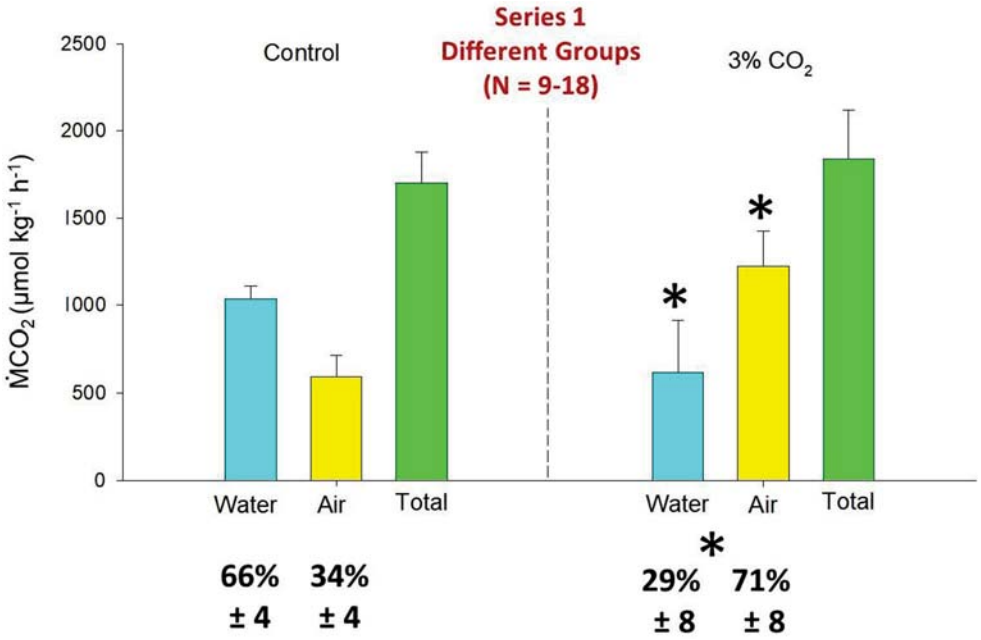

Fig. 2 The rate of CO_2 excretion ($\dot{M}CO_2$) into water and air phases, and total $\dot{M}CO_2$, in pirarucu of *Series 1* under control conditions and under aquatic hypercapnia (3% CO_2). Means ± SEM. Control: water $N=18$, air $N=9$, total $N=9$. Hypercapnia: water $N=9$, air $N=9$, total $N=9$. Water and air means do not add up perfectly to total means because of differing N numbers. Percentage partitioning was calculated only for those fish for which total measurements were made. Asterisks represent significant differences ($P<0.05$) between treatments

Table 1 Respiratory quotients (RER) and Nitrogen quotients (NQ) in pirarucu of *Series 1* under control conditions and under aquatic hypercapnia (3% CO_2). Means ± SEM (N)

	Control	3% CO_2 Water
RER-water	2.34 ± 0.18 (14)	1.31 ± 0.71 (9)
RER-air	0.34 ± 0.16 (8)	0.51 ± 0.04 (9)
RER-total	0.93 ± 0.22 (6)	0.68 ± 0.14 (9)
NQ-water	0.55 ± 0.09 (14)	0.96 ± 0.13* (9)
NQ-total	0.13 ± 0.03 (6)	0.17 ± 0.03 (9)

Asterisks indicate significant differences ($P<0.05$) between treatments

changed significantly (control = 34% air, $N=9$; hypercapnia = 71% air, $N=9$; Fig. 2). Although the data were highly variable, the decrease in CO_2 excretion to the water phase during hypercapnia was significant. Supplementary Fig. S1 explores the variability, showing that $\dot{M}CO_2$ to the water was maintained at a very high rate in one fish, two others actually took up CO_2 from the water at substantial rates (i.e. negative $\dot{M}CO_2$), while the other six showed lower positive rates.

Under control conditions the total respiratory exchange rate (RER) averaged 0.93, and this fell to 0.68 under aquatic hypercapnia (Table 1). The RER for the water phase was about 7-fold higher than for the air phase in the control

animals, reflecting the preferential CO_2 excretion to the water versus the air. This declined to about a 2.5-fold difference under aquatic hypercapnia, reflecting the significant increase in $\dot{M}CO_2$ to the air phase and reduction to the water phase (Fig. 2). However, none of the RER changes were significant in light of the variability noted above.

Under control conditions, ammonia-N was the predominant N-waste (88% of total-N) excreted to the water while urea-N accounted for 12% (Fig. 3). Under aquatic hypercapnia, total-N excretion to the water increased significantly by 1.7-fold, and this was entirely due to a significant 1.8-fold rise in ammonia-N excretion. Urea-N excretion did not change, so ammonia excretion now accounted for 92% of the total-N excretion (Fig. 3). Notably, the water pH dropped precipitously from 6.3 – 6.5 under control conditions to 4.1–4.3 with 3% CO_2 equilibration, and these fish had been fasted 2–4 days, important observations for interpreting the increased ammonia-N efflux (see Discussion).

The nitrogen quotient (NQ) for the water phase was 0.55 while the total NQ was 0.13 under control conditions (Table 1), reflecting the fact that all ammonia-N and urea-N excretion occurred into the water, whereas this route accounted for only a minor percentage of the total $\dot{M}O_2$ (Fig. 1). Exposure to 3% CO_2 in the water resulted in a significant 1.7-fold increase in NQ to the water, which was entirely due to the increased rate of ammonia-N excretion. The much smaller increase in the total NQ was not significant (Table 1).

One fish of *Series 1* in the 3% CO_2 treatment was returned to control conditions while recording continued for 60 min. $\dot{M}CO_2$ to the water phase quickly changed from a negative value to a positive value, $\dot{M}CO_2$ to the air phase quickly dropped by 65%, ammonia excretion to the water decreased by 20%, while urea-N excretion to the water and $\dot{M}O_2$ from each phase did not change appreciably (data not shown). This suggested that the physiological adjustments implemented during aquatic hypercapnia were quickly reversed upon return to normocapnia.

Series 2

In this series, a repeated measures design was used in which the same five fish were followed through sequential treatments, and all measurements were obtained on all fish. To follow up on the recovery issue, a final measurement period was incorporated in which the fish was returned to normocapnic water.

As in *Series 1*, the ABO was the dominant route of O_2 uptake, and total $\dot{M}O_2$ remained unchanged throughout the three experimental treatments (Fig. 4). $\dot{M}O_2$ from the air accounted for 71–75% of the total. Again, CO_2 excretion exhibited very different patterns. Total $\dot{M}CO_2$ remained unchanged throughout, but the dominance (86%) of the water phase during normocapnia was replaced by dominance (57%) of the air phase during exposure to 3% CO_2 in the water. This was due to a 2.8-fold increase in CO_2 excretion to the air phase (Fig. 5). The original partitioning was almost completely restored during the recovery period (77% of $\dot{M}CO_2$ to the water), confirming that physiological readjustments were rapid.

Supplementary Fig S2 explores the variability in the $\dot{M}CO_2$ responses, showing that the changes in CO_2 excretion to the air phase were consistent among fish (panel A), yet those for the water phase were highly variable (panel B), as in *Series 1* (cf. Supplementary Fig. S1). The fish with the highest $\dot{M}CO_2$ to the water phase during the normocapnic control period was actually able to slightly increase its rate of aquatic CO_2 excretion during exposure to 3% CO_2 in the

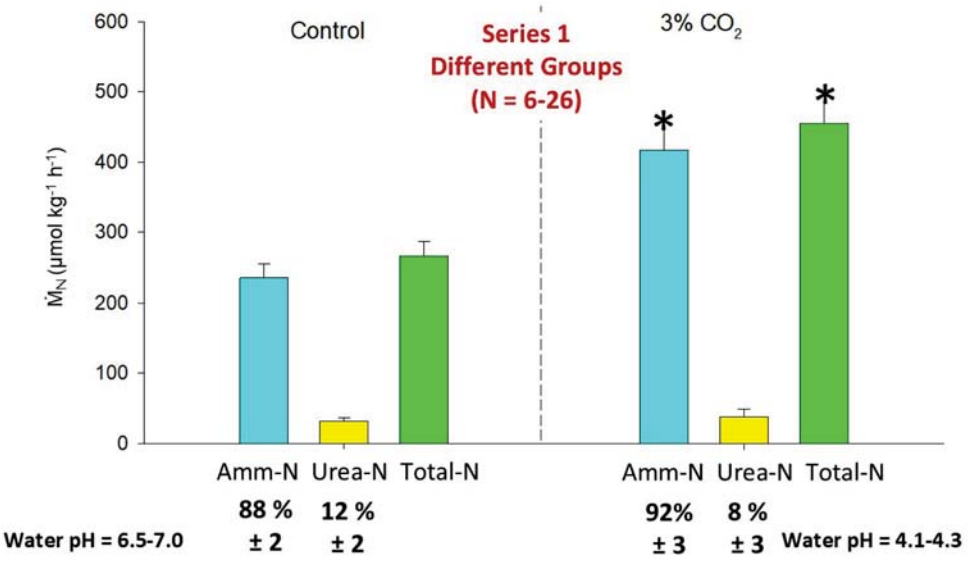

Fig. 3 The rates of ammonia-N, urea-N, and total-N excretion to the water phase in pirarucu of *Series 1* under control conditions and under aquatic hypercapnia (3% CO_2). Control: $N = 26$: Hypercapnia: $N = 9$. Means ± SEM. Percentage partitioning was calculated only for those fish for which total measurements were made. Asterisks represent significant differences ($P < 0.05$) between treatments. The water pH is also shown below the panel for each treatment. Note the much lower water pH under aquatic hypercapnia

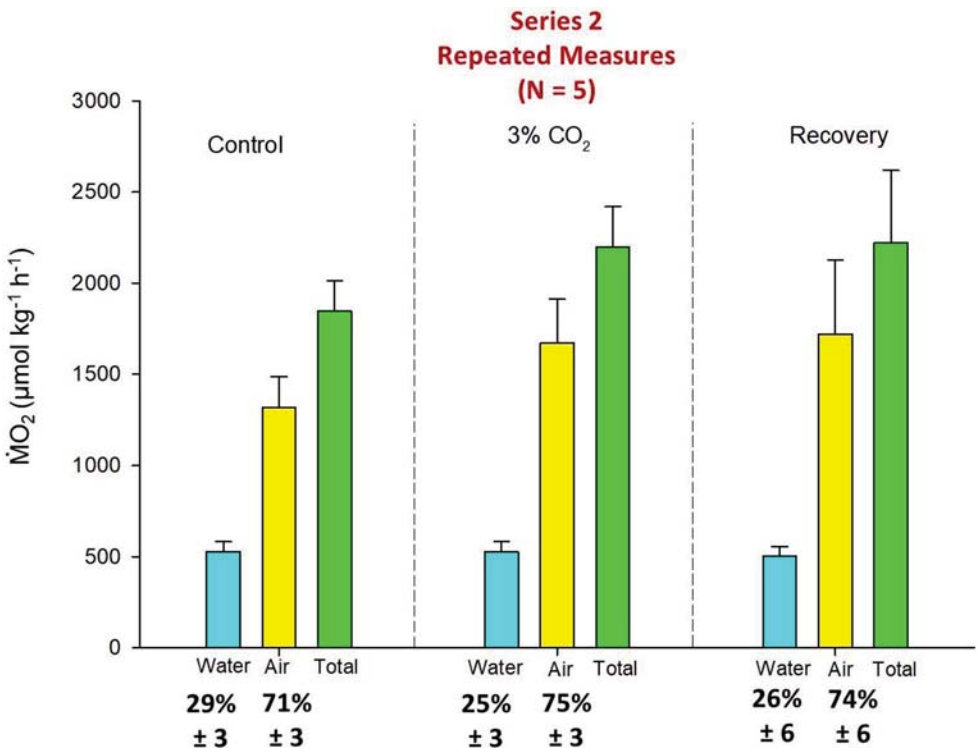

Fig. 4 (**A**) The rate of O_2 uptake ($\dot{M}O_2$) from water and air phases, and total $\dot{M}O_2$, in pirarucu of *Series 2* under control conditions, under aquatic hypercapnia (3% CO_2), and during recovery upon restoration of control conditions. A repeated measures design was employed, in which the same 5 fish were examined successively under the same conditions. Means ± SEM. Percentage partitioning between air and water phases is also shown. There were no significant differences $P < 0.05$) between treatments

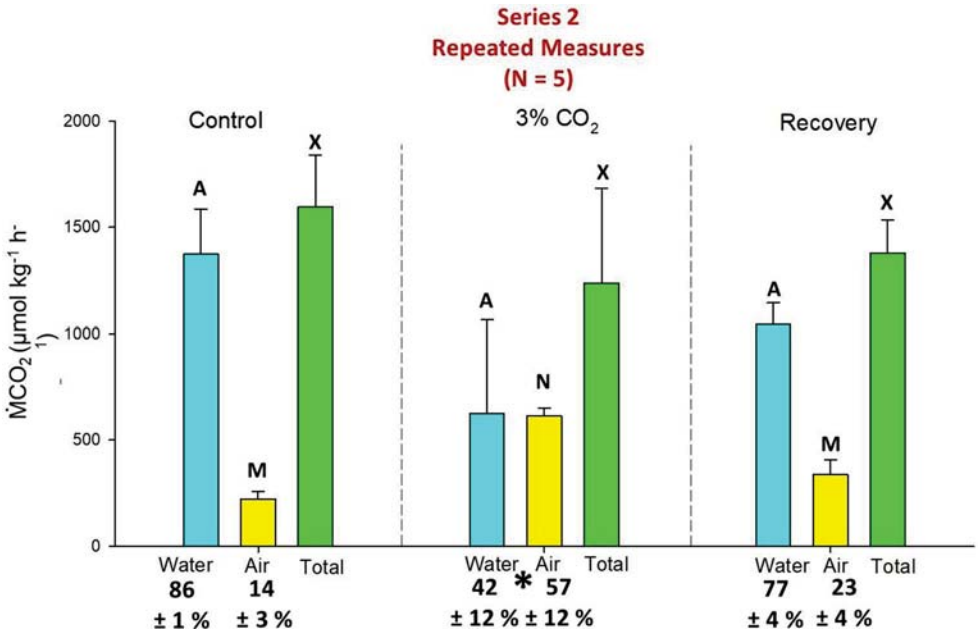

Fig. 5 The rate of CO_2 excretion ($\dot{M}CO_2$) into water and air phases, and total $\dot{M}CO_2$, in pirarucu of *Series 2* under control conditions, under aquatic hypercapnia (3% CO_2)., and during recovery upon restoration of control conditions. A repeated measures design was employed, in which the same 5 fish were examined successively under the same conditions. Means ± SEM. Percentage partitioning between air and water phases is also shown. Means sharing the same letter were not significantly different within a phase at $P < 0.05$. The asterisk indicates a significant difference in the percentage partitioning during 3% CO_2 exposure from both the control and recovery treatments

water, yet the fish with the second highest control rate exhibited negative $\dot{M}CO_2$ (i.e. net CO_2 uptake from the water) during aquatic hypercapnia. The other three decreased their $\dot{M}CO_2$ to the water by 30–60% but did not take up CO_2 from the water (Supplementary Fig. S2).

The total RER averaged 0.89 under control conditions, fell to 0.61 under aquatic hypercapnia, and was only partially restored to 0.68 during normocapnic recovery, though none of the changes were significant (Table 2). The RER for the water was much higher than for the air phase, though the latter more than doubled during exposure to 3% CO_2 in the water, a significant increase. This was due to a greatly increased $\dot{M}CO_2$ but unchanged $\dot{M}O_2$ via the ABO (Fig. 3). These changes were largely reversed during recovery (Table 2). Overall, the RER patterns of *Series 2* were in agreement with those of *Series 1* (cf. Table 1).

As in *Series 1*, ammonia-N accounted for the majority (86%) of total N-excretion while urea-N accounted for only

Table 2 Respiratory quotients (RER), nitrogenous waste excretion rates, % ammonia-N, % urea-N, and Nitrogen quotients (NQ) in pirarucu of *Series 2* under control conditions, under aquatic hypercapnia (3% CO_2), and during normocapnic recovery. A repeated measures design was used, with each fish undergoing the three treatments sequentially. Means ± SEM ($N = 5$)

	Control	3% CO_2 Water	Recovery
RER-water	2.56 ± 0.16	1.00 ± 0.78	2.18 ± 0.29
RER-air	0.18 ± 0.04^A	0.39 ± 0.04^B	0.21 ± 0.04 ^{A}
RER-total	0.89 ± 0.15	0.61 ± 0.22	0.68 ± 0.09
Ammonia-N excretion rate (μmol-N kg^{-1} h^{-1})	357 ± 47	314 ± 54	254 ± 35
Urea-N excretion rate ((μmol-N kg^{-1} h^{-1})	56 ± 18	19 ± 7	33 ± 6
Total-N excretion rate (μmol-N kg^{-1} h^{-1})	413 ± 55	333 ± 58	287 ± 13
% Ammonia-N excretion	86 ± 3^A	95 ± 2^B	88 ± 2^A
% Urea-N excretion	14 ± 3^A	5 ± 2^B	12 ± 2^A
NQ-water	0.84 ± 0.17	0.65 ± 0.12	0.62 ± 0.11
NQ-total	0.24 ± 0.05	0.16 ± 0.03	0.14 ± 0.02

For parameters where letters are shown, means not sharing the same letter are significantly different ($P < 0.05$) between treatments

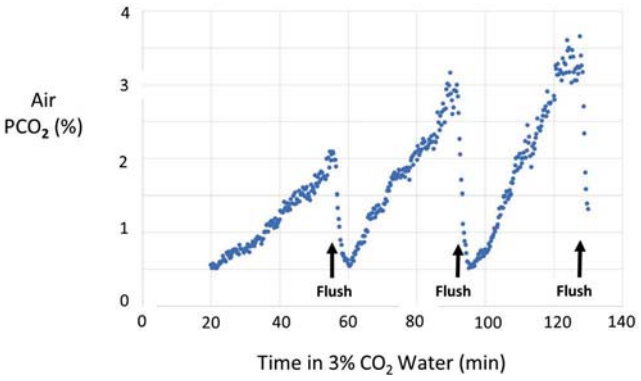

Fig. 6 Original recordings of air PO_2 in the air-chamber, illustrating the change in breathing pattern in the same pirarucu between (**A**) the control condition and (**B**) exposure to aquatic hypercapnia (3% CO_2). Note the clear step changes in PO_2 upon every exhalation in (**A**) in contrast to less clearly defined and smaller, more frequent changes in (**B**)

14% under control conditions. This shifted significantly to 94% ammonia-N and 6% urea-N during exposure to 3% CO_2 in the water, with reversal during normocapnic recovery (Table 2), similar to the non-significant trend in *Series 1* (cf. Figure 3). However, the patterns of absolute ammonia-N and urea-N excretion rates differed from *Series 1*. There were no significant changes overall, and the percentage shift towards ammonia-N during aquatic hypercapnia was largely due to a non-significant decrease in urea-N excretion rate; ammonia-N excretion rate did not increase but rather tended to fall at this time (Table 2).

Table 3 The effect of exposure to aquatic hypercapnia (3% CO_2 in the water phase) on air-breath frequency, minimal air-breath volume, and total air ventilation in pirarucu. Data from both *Series 1* and *2*. Means ± SEM. See text for additional details

	Control ($N = 12$)	3% CO_2 Water ($N = 13$)
Air-breath frequency (h^{-1})	11.5 ± 1.2	17.7 ± 1.9*
Air-breath volume (ml kg^{-1})	24.5 ± 2.0	17.5 ± 1.6*
Total air-ventilation (ml kg^{-1} h^{-1})	276.4 ± 31.4	291.7 ± 31.7

Asterisks indicate significant differences ($P < 0.05$) between treatments

Respiratory patterns during exposure to aquatic hypercapnia

The recordings from the O_2 optodes in the air chamber yielded clear step changes for every breath, with the sharp declines in PO_2, each corresponding to an exhalation (Fig. 6A). As explained in Methods, we could use these to monitor air-breathing frequency and to provide estimates of minimum breath volume needed to account for the observed O_2 consumption. In general, during exposure to 3% CO_2 in the water, air-breathing frequency increased, and the step changes in air-chamber PO_2 became smaller and less sharp. Indeed, in a number of fish, it was not possible to pick out the individual air-breaths during aquatic hypercapnia, and it appeared as though the fish was "panting". Figure 6B illustrates an extreme example. The data in Table 3 are based only on those fish, from both series, where it was possible to calculate frequency and minimum breath volume from the recordings. These show that air-breathing frequency increased significantly by 53% and minimum air-breath volume decreased significantly by 29% during exposure to 3% CO_2 in the water. Total air ventilation did not change significantly.

CO_2 excretion through the ABO appeared to become more effective over time during exposure to aquatic hypercapnia. Figure 7 shows PCO_2 recordings from the air-chamber of a fish where there were three air-flush and recording cycles. Clearly, the rate of PCO_2 rise increased progressively. Overall, we collected data from 8 fish from both series where there were two successive recording cycles, and from 4 fish where there were three successive cycles. These indicate that $\dot{M}CO_2$ into the air phase increased significantly by 30% on the second cycle, with no further change on the third cycle (Table 4). $\dot{M}O_2$ from the air phase remained the same through the three cycles.

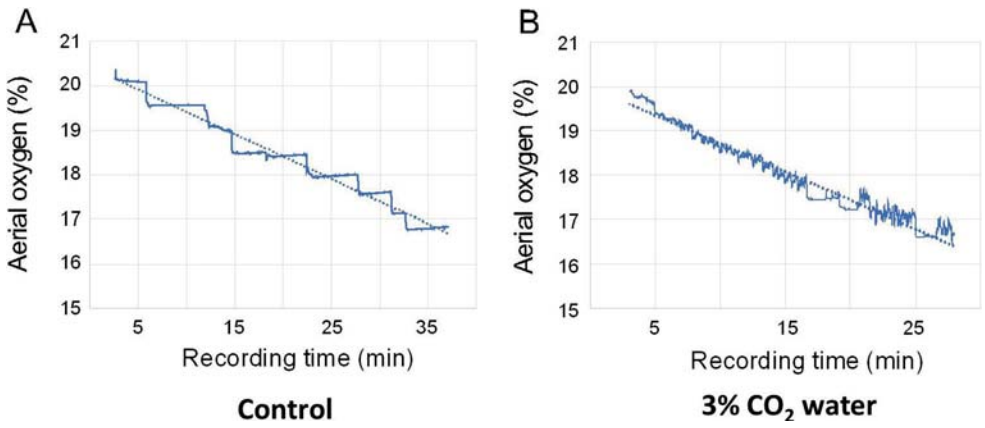

Fig. 7 Original recordings of PCO_2 in the air-chamber in the same pirarucu exposed to 3% CO_2 in the water during three successive air-flush and recording cycles. Note the increasing slope of the PCO_2 rise in each successive cycle

Table 4 Rates of CO_2 excretion ($\dot{M}CO_2$) to the air phase and O_2 uptake ($\dot{M}O_2$) from the air phase in pirarucu on three successive air-flush and recording cycles during exposure to 3% CO_2 in the water. Data from both *Series 1* and *2*. A repeated measures design was used with 8 fish followed through two cycles, and 4 fish followed through three cycles. Means ± SEM (N)

	Cycle 1	Cycle 2	Cycle 3
Air $\dot{M}CO_2$ (μmol kg^{-1} h^{-1})	1067 ± 212^{A} (8)	1384 ± 218^{B} (8)	1325 ± 356^{AB} (4)
Air $\dot{M}O_2$ (μmol kg^{-1} h^{-1})	2293 ± 556^{X} (8)	2474 ± 362^{X} (8)	2084 ± 567^{X} (4)

Means not sharing the same letter are significantly different ($P < 0.05$) between treatments

Discussion

CO_2 excretion via the ABO

Under control normocapnic conditions, the partitioning of $\dot{M}O_2$ (71–77% ABO, 23–29% gills) and $\dot{M}CO_2$ (14–34% ABO, 66–86% gills) in the pirarucu of *Series 1* (Figs. 1, 2) and *Series 2* (Figs. 4, 5) agreed with our own previous study (Pelster et al. 2020) and those of many others on *Arapaima* of comparable size (Randall et al. 1978; Stevens and Holeton 1978; Brauner et al. 2004; Brauner and Val 1996; Gonzalez et al. 2010; Pelster et al. 2020; Aaskov et al. 2022). Aaskov et al. (2022) have provided a useful summary Table. However, during exposure to aquatic hypercapnia, the pattern for $\dot{M}CO_2$ shifted dramatically to 57–71% ABO, 29–43% gills, with negligible change in the partitioning of $\dot{M}O_2$ (75–82% ABO, 18–25% gills). Thus, to answer the question raised in the title and Introduction, the ABO does become a significant route for CO_2 excretion during aquatic hypercapnia.

It is now clear that *Arapaima* (Aaskov et al. 2022), like many other fish that rely on air-breathing during severe aquatic hypoxia (Randall et al. 1981a, b; Smatresk and Cameron 1982; Scott et al. 2017; Aaskov et al. 2023), are able to minimize O_2 loss across the gills to the hypoxic water, though most do not absolutely prevent it. At the same time, at least in *Arapaim* a and the striped catfish (*Pangasianodon hypophthalmus*), CO_2 excretion to the water continues more or less unabated (Aaskov et al. 2022, 2023). The mechanism is unclear, but suggestions include blood shunting in the gills and the fact that Krogh's diffusion coefficient for CO_2 is so much higher than that for O_2.

The present results suggest a rather different situation for CO_2 excretion during aquatic hypercapnia. The gills are unable to maintain the control rate of CO_2 excretion to the water (Figs. 2 and 5), and indeed may allow net CO_2 entry into the fish (Supplementary Figs. S1 and S2), but the ABO can compensate by more than doubling CO_2 excretion to the air phase, with negligible impact on $\dot{M}O_2$ partitioning. Perhaps the inability of the gills to maintain CO_2 excretion to the water is due to a collapse or reversal of the blood-to-water PCO_2 gradient, confounded by the high Krogh's diffusion coefficient for CO_2. Alternately or additionally, in our experiments the maintenance of aquatic normoxia during exposure to 3% CO_2 in the water may have prevented the general down- regulation of gill permeability which is thought to occur during aquatic hypoxia in the pirarucu (Wood et al. 2020) and other hypoxia-tolerant Amazon fish (Wood et al. 2009; Robertson et al. 2015; Wood and Eom 2021). Clearly, it will be of interest to repeat the present experiments under conditions in which the water phase is made simultaneously hypoxic and hypercapnic. Will the ABO become a more or less important route of CO_2 excretion? We anticipate the former, but the experiments remain to be done.

In contrast to water, the solubilities of CO_2 and O_2 in air are very similar (Dejours 1981), Therefore CO_2 excretion by the ABO is more difficult than by the gills per unit O_2 uptake. At least in part, this may explain why the overall RER was not restored to control values (0.89–0.93) during the short-term exposures to aquatic hypercapnia, but rather remained depressed at 0.61–0.68 (Tables, 1, 2). The fish were likely in a state of net CO_2 accumulation resulting in uncompensated respiratory acidosis, both because of this issue, as well as because of CO_2 uptake from the water (discussed above). However, Gonzalez et al. (2010) sampled

pirarucu of comparable size by caudal puncture after 72 h exposure to a higher water PCO_2 level (40 Torr, ~ 5% CO_2, 5 kPa), and found no evidence of internal CO_2 accumulation or respiratory acidosis relative to normocapnic controls, suggesting that the ABO becomes more efficient at CO_2 excretion over time. Clearly, much longer studies of blood gases and pH by indwelling catheter, as well measurements of CO_2 excretion and RER by gills and ABO will be informative.

Nitrogenous waste excretion and metabolic fuel use under normocapnia and hypercapnia

Under control conditions, the partitioning of N-waste excretion was very similar between *Series 1* (Fig. 3) and *Series 2* (Table 2) with ammonia-N accounting for 86–88% and urea-N for 12–14% of the total, in reasonable agreement with several previous studies on pirarucu of similar size (Gonzalez et al. 2010; Pelster et al. 2020; Wood et al. 2020). Using measured RER and NQ values, fractional urea-N excretion values, and classic metabolic theory (Kleiber 1965; Lauff and Wood 1996), it was possible to calculate the percentage contributions of different metabolic fuels to aerobic metabolism under control conditions. In *Series 1*, this yielded estimates of 48% protein, 36% carbohydrate, and 16% lipid, very close to the data of two previous studies on *Arapaima* [53% protein, 27% carbohydrate, 20% lipid by Aaskov et al. (2022), and 61% protein, 16% carbohydrate, 23% lipid by Pelster et al. (2020)]. Interestingly, in *Series 2*, while the control RER and fractional urea-N excretion values were similar to those of *Series 1*, the control NQ values were much higher (0.24 versus 0.13; Tables 1 and 2), resulting in a very different fuel usage profile (89% protein, 11% lipid). The difference was likely due to the longer fasting period (5–7 days in *Series 2* versus 2–4 days in *Series 1*). Pelster et al. (2020) have discussed the characteristic of many Amazonian fish, including the pirarucu, to switch to a heavy reliance on protein oxidation during starvation in contrast to the protein conservation strategy seen in most other fish.

We did not calculate fuel use profiles during exposure to hypercapnic water or during recovery, because the fish were very likely not in steady state under these conditions. In this regard, the large increases in ammonia-N excretion, total-N excretion (Fig. 3) and aquatic NQ (Table 1) seen during exposure to high water PCO_2 in *Series 1*, did not occur in *Series 2* (Table 2). In *Series 1*, we attribute these changes not to increased protein oxidation, but rather to a much improved PNH_3 gradient for branchial ammonia excretion, with the much lower pH of the water (4.1–4.3), caused by 3% CO_2, acting as an "NH_3 trap". It is unclear why this did not occur in *Series 2* where the water pH changes were the same. However, the % ammonia-N excretion again increased and the % urea-N excretion decreased during aquatic hypercapnia (Table 2). Perhaps the prolonged fasting period was a contributing factor.

The control of air-breathing during aquatic hypercapnia

It is well-established that O_2-sensitive chemoreceptors responsive to low aquatic PO_2 stimulate air-breathing in a variety of bimodally breathing fish (reviewed by Graham 1997; Milsom 2012; Zaccone et al. 2018; Milsom et al. 2021). Indeed, putative candidates have been found by immunohistochemistry in the epithelia of the gills and orobranchial cavity of *Arapaima* (Zaccone et al. 2020). The present study has shown that chemoreceptors sensitive either directly or indirectly to high aquatic PCO_2 also stimulate air-breathing in this species. This has been seen in a few other air-breathing fish species, particularly lungfish (reviewed by Milsom et al., 2021), but not previously in *Arapaima*, to our knowledge. Notably, higher vertebrates similarly exhibit hyperventilation in the face of elevated PCO_2, a response mediated through both central and peripheral chemoreceptors (Dejours 1981; Milsom et al., 2021). In *Arapaima*, it remains to be determined whether the responsible chemoreceptors are the same as the aquatic PO_2 chemoreceptors (i.e. polymodal sensors), or are different receptors sensitive to increased PCO_2 and/or decreased pH in the water, the arterial blood, the ABO, or brain as all of these compartments will be affected when water PCO_2 rises. Notably, Farrell and Randall (1978) in *Arapaima* and Tuong et al. (2018) in the clown knifefish (*Chitala ornata*) reported that injections of CO_2 gas into the ABO stimulated air-breathing frequency. A follow-up study (Tuong et al. 2019) pointed to increased arterial PCO_2 as the proximate stimulus in *Chitala*; complete denervation of the gills did not eliminate the response.

The pattern and effectiveness of air-breathing during exposure to aquatic hypercapnia

Certainly, in our experiments, the net effect of aquatic hypercapnia was to change the pattern of air- breathing by increasing the frequency and decreasing the individual volumes of air-breaths. We emphasize that this change in pattern during aquatic hypercapnia was likely more extreme than indicated by the data in Table 3, which are based only on those recordings where it was possible to discern the frequency. As illustrated in Fig. 6B, in certain sections of many recordings, the breathing became so shallow and frequent that individual breaths could not be reliably detected. Furthermore, as explained in Methods, our calculation of breath volume yielded only the minimum volume needed

to account for $\dot{M}O_2$ from the air. By way of comparison, air-breath volumes in pirarucu under control conditions reported by Pelster et al. (2020), where expired PO_2 was also known, yielded more accurate estimates that were approximately twice as high as those reported in Table 3.

While the increase in air-breathing frequency during aquatic hypercapnia should be helpful in clearing CO_2, the adaptive significance of the reduced air-breath volumes is unclear. Decreased breath volumes would be expected to increase fractional dead-space and provide a lower turnover of air on each breath. Regardless, it is clear that the effectiveness of CO_2 excretion increased with the time of exposure to aquatic hypercapnia (Fig. 7; Table 4). Possibly this simply reflected increased loading of the fish with CO_2 as exposure to aquatic hypercapnia was prolonged, such that the blood PCO_2 level continued to increase, improving the blood-to-air PCO_2 gradient in the ABO. Alternately and/or additionally, changes in blood flow distribution, air-flow distribution, red cell function, and breathing patterns in the ABO may also have improved over time.

To answer these and other questions, future studies should employ chronic indwelling catheters to measure blood and ABO gas levels, as originally done by Randall et al. (1978) in *Arapaima*. For example, we suspect that the great variability in CO_2 excretion to the water during aquatic hypercapnia seen in both *Series 1* (Supplementary Fig S1) and *Series 2* (Supplementary Fig. S2) reflected variability in the blood PCO_2 levels regulated by different individuals. Randall et al. (1978) reported a mean arterial PCO_2 of 26 mmHg in pirarucu under normocapnia, whereas we exposed our fish to water with a PCO_2 ~ 22 mmHg (3% CO_2). We would expect that fish that took up CO_2 from the water regulated blood PCO_2 below 22 mmHg, and those that continued to excrete CO_2 to the water regulated blood PCO_2 well above 22 mmHg.

Perspectives

As reviewed comprehensively by Milsom et al. (2021), there have been numerous studies on $\dot{M}O_2$ partitioning between the gills and the ABO in air-breathing fish. However, there have been far fewer on $\dot{M}CO_2$ partitioning, and the present investigation is one of only a very few that have examined $\dot{M}CO_2$ partitioning in the face of aquatic hypercapnia. We have shown that the ABO can play a substantial role in CO_2 excretion under these conditions, without compromising O_2 uptake. While this is a new finding for *Arapaima gigas*, one of the world's largest freshwater fish, it simply parallels a classic study done 45 years ago on another obligate air-breather, the very small (8 g) blue gourami (*Trichogaster trichopterus*) (Burggren 1979). The gourami has a very different type of ABO – labyrinth organs lying within a suprabranchial chamber. When the gourami was exposed to 3% CO_2 in the water, the frequency of air-breathing increased greatly. $\dot{M}CO_2$ to the water fell from 85% to less than 40%, and $\dot{M}CO_2$ to the air phase increased from 15% to more than 60% of the total. There was no impact on the partitioning of $\dot{M}O_2$. Clearly the functional responses of these two very different species to aquatic hypercapnia are very similar.

To conclude, the ABO is not exclusively dedicated to just taking up O_2! In nature, the air is normally normoxic and normocapnic. However, the water is often hypoxic and/or hypercapnic in tropical ecosystems, and the ability of the ABO to take on the dominant role in CO_2 excretion when required may have been a critical driver in the original evolution of air-breathing, as well as in the functionality of the ABO in modern air-breathing fish.

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Author contributions All authors contributed to the study conception and design. Chris Wood and Bernd Pelster performed the experiments and analyzed the samples and data. Adalberto Val provided advice and logistical support. The first draft of the manuscript was written by Chris Wood. All authors commented on earlier versions of the manuscript, and approved the final version.

Data availability From the authors, upon reasonable request.

Declarations

Ethical approval All procedures were in compliance with Brazilian national animal care regulations, and those of the Brazilian National Institute for Research of the Amazon (INPA) in Manaus, Brazil under protocol 027/2015.

Competing interests Bernd Pelster is an editor of this journal, but had no role in the reviewing or publication process.

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