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Striped catfish (*Pangasianodon hypophthalmus*) use air-breathing and aquatic surface respiration when exposed to severe aquatic hypercarbia

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Abstract

We investigated the extent to which the facultative air-breathing fish, the striped catfish (*Pangasianodon hypophthalmus*), uses air-breathing to cope with aquatic hypercarbia, and how air-breathing is influenced by the experimental exposure protocol and level of hypercarbia. We exposed individuals to severe aquatic hypercarbia (up to P_wCO_2 = 81 mmHg) using step-wise and progressive exposure protocols while measuring gill ventilation rate, heart rate, mean arterial blood pressure, and air-breathing frequency, as well as arterial blood pH and PCO_2. We confirm that *P. hypophthalmus* is tolerant of hypercarbia. Under both protocols gill ventilation rate, heart rate, and mean arterial blood pressure were maintained near control levels even at very high CO_2 levels. We observed a marked amount of individual variation in the $PwCO_2$ at which air-breathing was elicited, with some individuals not responding at all. The experimental protocol also influenced the onset of air-breathing. Air-breathing began at lower P_wCO_2 in the step-wise protocol (23 ± 4.1 mmHg) compared with the progressive protocol (46 ± 7.8 mmHg). Air-breathing was often followed by aquatic surface respiration, at higher PCO_2 (71 ± 5.2 mmHg) levels. On average, the blood PCO_2 was approximately 43% lower (46 ± 2.5 mmHg) than water P_wCO_2 (~81 mmHg) at our highest tested CO_2 level. While this suggests that aerial CO_2 elimination is an effective, and perhaps critical,

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respiratory strategy used by *P. hypophthalmus* to cope with severe hypercarbia, this observation may also be explained by a long lag time required for equilibration.

KEYWORDS
aerial respiration, blood gases, cardiorespiratory control, CO_2, hypercapnia, Pangasius

1 | INTRODUCTION

Many bodies of freshwater around the world naturally contain high levels of dissolved carbon dioxide (aquatic hypercarbia) well above atmospheric (~0.3 mmHg) PCO_2 levels (Raymond et al., 2013). Within freshwater systems large diel and seasonal fluctuations in PCO_2 occur (McNeil & Matsumoto, 2019; Ultsch, 1987; Wilmer, 1934), and yet, thousands of fish species occupy these variable and CO_2-rich freshwater habitats. For fish, due to high rates of gill ventilation, highly vascularized gill epithelia, and the ease with which CO_2 crosses biological membranes, increases in external aquatic PCO_2 are quickly reflected internally. When CO_2 dissolves into a body fluid it is hydrated to bicarbonate (HCO_3^-) and H^+. The latter, if not completely buffered creates an acid–base disturbance and leads to respiratory acidosis (Brauner et al., 2019). Maintaining acid–base balance is essential, thus, inhabiting waters with high, and variable, CO_2 levels poses a homeostatic challenge for resident fishes.

Sensing, responding, and compensating for changes in environmental CO_2 are essential coping responses for fish (Bayley et al., 2019). Hypercarbia initially results in an increased influx of CO_2 into the body and simultaneously diminishes the outward diffusion gradient for metabolically produced CO_2 to leave the fish. The primary method of pH regulation in fish during hypercarbia is via increased net acid excretion (metabolic compensation; Shartau et al., 2019). However, the secondary contributions of respiratory compensation should not be ignored, even in exclusively gill-breathing fishes where gill ventilation can never lower blood PCO_2 below ambient levels. Three principle cardiorespiratory responses—an increase in gill ventilation, a decrease in heart rate, and an increase in arterial blood pressure—are the common piscine responses to aquatic hypercapnia (Tresguerres et al., 2019). Of the three, only the gill hyperventilation has been shown to enhance gas exchange efficiency by reducing the contact time of inspired water at the gill and facilitating removal of metabolically produced CO_2 (Tresguerres et al., 2019). Orobranchially located, externally oriented, CO_2-sensitive chemoreceptors have overwhelmingly been shown to be responsible for initiating hypercapnia-induced cardiorespiratory responses in fish (Gilmour & Perry, 2006; Milsom, 2012).

The responses detailed above are those commonly displayed in most fully aquatic, exclusively gill-breathing fish studied to date. However, there are some 656 extant species of air-breathing fish (across 22 taxonomic orders) that supplement aquatic respiration with aerial gas-exchange (Damsgaard et al., 2020; Graham, 1997). Regardless of the air-breathing organ (ABO) present (e.g., lung, swimbladder, mouth, skin, and gastrointestinal tract) air-breathing fishes have an alternate, extra-branchial, route for CO_2 elimination during hypercarbia exposure. In South American lungfish (*Lepidosiren paradoxa*) aerial CO_2 elimination via the lung sufficiently exceeds hypercarbia induced CO_2 influx rates such that arterial PCO_2 (P_aCO_2) was lower than water PCO_2 (P_wCO_2) (Sanchez et al., 2005). Such a feat could not be achieved by a fully aquatic fish reliant solely on gill ventilation. Perhaps having an extra-branchial route of CO_2 elimination in addition to more commonly employed cardiorespiratory- and metabolic-compensation mechanisms partly explains why air-breathing fishes are so prevalent in naturally occurring hypercarbic habitats like tropical swamps (Graham, 1997) and in hypercarbic aquaculture conditions (Lefevre et al., 2014).

Given the abundance and diversity of extant air-breathing fishes it is perhaps not surprising that a variety of responses to hypercarbia have been documented even within facultative air-breathing species that commonly occupy hypercarbic environments and use similar air-breathing organs, that is, swimbladders. In most species of facultative air breathing actinopterygian fishes low levels of aquatic CO_2 stimulate gill ventilation while significant increases in CO_2 inhibit gill breathing and stimulate air breathing (*Amia calva* Johansen et al., 1970; *Ancistrus chagresi* and *Hypostomus Plecostomus* Graham & Baird, 1982; *H. unitaeniatus* de Lima Boijink et al., 2010). There have been some studies, however, in which increases in aquatic CO_2 were without effect on gill ventilation (*Symbranchus marmoratus* Johansen, 1966; *Misgurnus anguillicaudatus* McMahon & Burggren, 1987) in addition to two recent studies on species native throughout the Mekong River drainage (Thomsen et al., 2017; Tuong et al., 2018). Exposure to elevated levels of aquatic CO_2 did not evoke any gill ventilatory response in clown knifefish (*Chitala ornata*) or the striped catfish (*Pangasianodon hypophthalmus*). It did, however, stimulate air-breathing in the clown knifefish but not in the striped catfish (Thomsen et al., 2017; Tuong et al., 2018). There were two notable differences in these studies. The clown knifefish performs air-breathing under control (normocarbic) conditions (Tuong et al., 2018) while the striped catfish only rarely air-breathes unless conditions are hypoxic (Lefevre et al., 2011; Thomsen et al., 2017). In the study on the clown knifefish, fish was exposed to a rapid progressive rise in aquatic CO_2 to levels of roughly 30 mmHg over 30 min and held there for 1 h. The striped catfish were exposed to step-wise increases in CO_2 (1 h each) to a maximum of 37.5 mmHg PCO_2. While it seems reasonable to conclude that there are species-specific differences in the cardiorespiratory responses to hypercarbia amongst air-breathing fishes, we wondered whether methodological differences between these studies could at least be partly responsible for this inter-species disparity. In particular, might there be a difference in the threshold required to elicit air breathing as a function of the rate of hypercarbia exposure? This seems quite possible. In two studies investigating the cardiorespiratory responses to hypoxia of the South American erythrinid, the traira (*Hoplias malabaricus*; Rantin et al., 1993; Sundin et al., 1999) bradycardia was not initiated until

20 mmHg and gill ventilation frequency did not increase until approximately 50 mmHg using a slow step-wise protocol (Rantin et al., 1993) while heart rate declined and gill ventilation rate increased beginning at a PO_2 of 100 mmHg using a rapid progressive hypoxia protocol (Sundin et al., 1999). If PCO_2 chemoreceptors in air-breathing fishes are similarly sensitive to the rate of change of PCO_2 this could explain the differences in the studies just described.

Given these differences, a primary objective of this study was to determine whether the hypercarbia exposure protocol impacts cardiorespiratory responses to aquatic hypercarbia in the striped catfish (*P. hypophthalmus*). We used two different experimental protocols: (A) a graded step-wise increase and (B) a progressive increase, both to levels of CO_2 resulting in a fall in water pH to below 6.0 (81 mmHg CO_2) while continuously measuring gill ventilation rate, heart rate, arterial blood pressure, as well as air-breathing frequency. pH levels in the lower Mekong River range from 6.9 to 8.4 with both seasonal and spatial variations (Li et al., 2013). *P. hypophthalmus* naturally inhabits stagnant tropical waters; such bodies of water are prone to frequent hypercarbia (e.g., in excess of 60 mmHg; Ultsch, 1987). Furthermore, local aquaculture systems experience increases in PCO_2 to levels of 30 mmHg or more towards the end of the growth cycle (Damsgaard et al., 2015a).

2 | MATERIAL AND METHODS

2.1 | Experimental animals

We used a total of 14 striped catfish of undetermined sex (*P. hypophthalmus*, 264 ± 17 g) obtained from a local commercial fish farm. Before experimentation, we group-housed *P. hypophthalmus* in a large outdoor holding tank within a recirculating aquaculture system at the College of Aquaculture and Fisheries, Can Thσ University, Vietnam. Photoperiod matched ambient conditions and water chemistry was routinely monitored: (PCO_2 < 0.5 mg L^{-1}, dissolved O_2 > 95% air saturation, temperature ~ 26°C, pH ~ 7.6, NH_3^+ ~ 0.02 mg L^{-1}, NO_3^- ~ 90 mg L^{-1}). We maintained *P. hypophthalmus* in holding tanks for at least 2 weeks before experimental use. Fish were fed commercial pellets containing 28% protein and 5% fat (Proconco Corporation, Bien Hoa Industrial zone) and were fasted for 2 days before surgery and throughout experimentation. We conducted all experiments indoors at room temperature (~26°C) in December, 2018. All experiments were conducted in accordance with the national guidelines for the protection of animal welfare in Vietnam as well as the guidelines of the University of British Columbia ACUP A13-0253 and Danish legislation.

2.2 | Animal surgeries

The 14 *P. hypophthalmus* were surgically instrumented with dorsal aorta cannulae and impedance electrodes to measure cardiorespiratory responses to elevated CO_2. The fish were anesthetized with benzocaine (0.11 g L^{-1}) and maintained anesthetized throughout by irrigating the gills with a well oxygenated, more dilute (0.05 g L^{-1}) benzocaine solution (Phuong et al., 2017). We followed the cannulation procedure developed by Soivio et al. (1975), which had previously been used successfully in *P. hypophthalmus* by Damsgaard, Gam, et al. (2015). In addition, we sutured disk-type gill impedance electrodes (Grass 10 mm Gold Cup EEG electrodes) to each opercula with nylon sutures. While anaesthetized, the mass (g) of each fish was recorded. Postsurgery, we allowed animals to recover individually for 12–24 h in darkened, aerated, circular tanks (~1 m in diameter) at room temperature. All animals survived surgery and recovered.

2.3 | CO_2 titration curves

We were unable to measure PCO_2 directly in the water. Instead, we continuously monitored water pH. To determine the corresponding PCO_2 level we used a Wöstoff gas-mixer (H. Wösthoff) to bubble known levels of CO_2 (1%, 3%, 5%, 7%, and 9%) into experimental tank water. By measuring water pH at each CO_2 level we created a titration curve (Figure S1) from which we could interpolate the CO_2 levels for each of our experimental pHs: pH 7 = 1.3% CO_2 (9.9 mmHg), pH 6.5 = 2.4% CO_2 (18.2 mmHg), pH 6 = 6.3% CO_2 (47.9 mmHg), and pH 5.75 = 10.7% CO_2 (81.3 mmHg).

2.4 | Experimental protocols

Following recovery, we carefully transferred fish individually into the experimental chamber (210 L) that we had prefilled with water (pH ~ 7.5, PCO_2 < 7.6 mmHg, temperature ~26°C) that was continuously aerated with an air stone and an aquarium pump. The sides of the experimental chamber were opaque to minimize disturbance to the fish except for a strip a few centimetres below the water line on one side that was left unobstructed to enable observation of air-breathing behavior. We connected the impedance leads to an impedance convertor to measure gill ventilation frequency (beats min^{-1}) and the dorsal aorta cannula to a pressure transducer to record heart rate (beats min^{-1}) and blood pressure (cmH_2O). Pressure transducers were calibrated before and after every experiment using a water filled manometer. All outputs were monitored and recorded with a data acquisition system (Dataq DI-720, DATAQ Instruments, Inc.) recording at 125 Hz per channel. We allowed each fish to acclimate undisturbed for 1 h to the experimental chamber.

After this acclimation period we began logging data and withdrew ~0.3 ml of arterial blood into a heparinized (50 IU mL^{-1}) syringe for determination of resting arterial PCO_2 and pH using an i-STAT VetScan Analyzer with an CG3+ cartridge (Abaxis) and temperature corrected using the iSTAT manual equation as validated previously (Damsgaard, Phuong, et al., 2015). After 15 min at pH = 7.5 (control; PCO_2 < 7.6 mmHg), we began bubbling with CO_2 gas (99% pure) rather than air. By continuously monitoring water pH and manually adjusting the CO_2 inflow we could control the rate of the CO_2 increase, and corresponding pH decrease, to match the profile of either

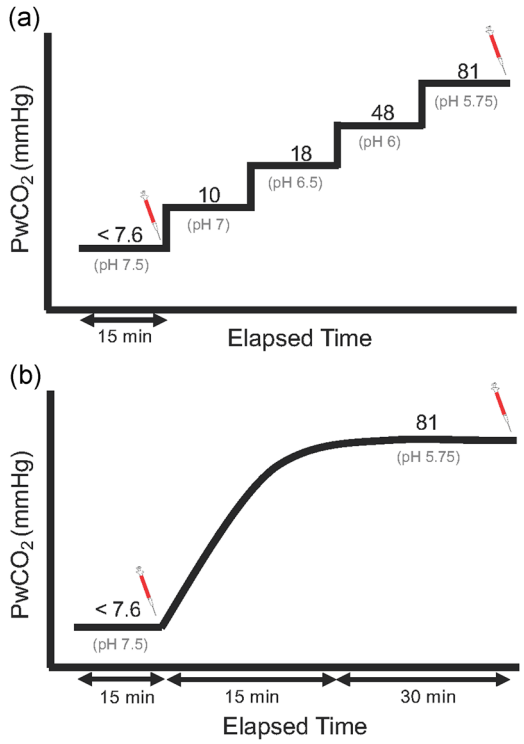

FIGURE 1 Schematic of the two experimental protocols tested. (a) Step-wise increase of water PCO_2 (P_w CO_2) from < 7.6 mmHg to 81 mmHg and corresponding decrease in water pH from 7.5 to 5.75 with a 15 min interval at each level. (b) Progressive increase in P_w CO_2 from < 7.6 mmHg to 81 mmHg and corresponding decline in water pH from 7.5 to 5.75 that was then maintained for 30 min. Corresponding water pH at each P_wCO_2 level is shown in gray. Blood was extracted from instrumented animals at the start and end of the experiment to measure blood chemistry (PCO_2 and pH), indicated on the figure by the syringes. Whilst care was taken to follow these protocols, they represent idealized protocols and in reality there was some variation in duration between trials since the rate of increase in water PCO_2 was manually controlled

the step-wise (Figure 1a) or progressive (Figure 1b) experimental protocol. Although the duration of the step-wise protocol was longer than the progressive protocol the total CO_2 load experienced (area below the curve) in each protocol was similar. Water pH, gill ventilation frequency (f_v), heart rate (f_H), and mean arterial blood pressure (MAP) were recorded continuously throughout the experiments. In addition, we also recorded when the fish took air-breaths (AB) or performed aquatic surface respiration (ASR) along with the corresponding pH. At the end of each protocol (at pH = 5.75; P_wCO_2 = 81 mmHg), we took a second blood sample to measure blood PCO_2 and pH as above.

We exposed all fish to both experimental protocols with at least a 24 h recovery period in their individual holding tanks between experimental protocols. Eight of the fish were exposed to the step-wise protocol first and six to the progressive protocol first. Furthermore, these six fish were exposed to the progressive protocol before being instrumented with the cannula and electrodes to determine whether instrumentation impacted the behavioral response of *P. hypophthalmus* to hypercapnia. Thus, only AB and ASR behavior were measured for these individuals in this run. They were subsequently instrumented and then exposed to the step-wise protocol. There were no differences in the responses of the fish as a result of the order of exposure and thus, both groups were combined for the data analysis.

2.5 | Data and statistical analysis

We used LabChart to view and quantify experimental data traces. In the step-wise protocol we quantified f_v, f_H, and MAP at the end of the 15 min baseline step and subsequently at the end of each pH/CO_2 step. We used a similar approach in the progressive protocol, f_v, f_H, and MAP were quantified at the end of the 15 min baseline step, as soon as each matching pH (7, 6.5, and 6) was reached during the rapid ramp, and at the end of the 30 min holding phase at pH = 5.75. We also measured f_v, f_H, and MAP immediately before and after each AB that occurred. f_v, f_H, and MAP quantitation was performed on seven fish in each protocol.

We note that the reliability of the iSTAT for blood gas measurements in fish run at lower temperature (10–20°C) has been criticized in the past (Harter et al., 2014). However, iSTAT temperature corrected blood PCO_2 values have been validated for *P. hypophthalmus*, using classic CO_2 tonometry in blood run at a temperature (30°C) similar to our experimental conditions (Damsgaard, Gam et al., 2015). We also note that Harter et al., concluded that at values of PCO_2 above 19 mmHg, the measurement error was less than 1% (fig. 4b in their paper) and that the iSTAT system could measure PCO_2 in trout blood accurately at higher PCO_2 levels. Finally, Harter et al. (2014) found that pH was measured accurately with the iSTAT system and we note that the arterial pH values measured in the present study are consistent with the arterial PCO_2 values we report based on estimates from the non-bicarbonate buffer line for *Pangassius*. Based on the sum of these observations, we are confident that our reported blood PCO_2 values measured at higher temperatures and higher levels of CO_2, are reasonably accurate.

We present all data as mean ± standard error of the mean (*SEM*) and illustrate individual responses on all figures. We conducted all statistical analyses using R 3.2.2 (R Core Team 2014). Linear mixed effect models using the package lmerTest (Kuznetsova et al., 2017) followed by a two-way analysis of variance (ANOVA) were used to detect significant changes in heart rate, ventilation rate and mean arterial blood pressure as pH/CO_2 changed. The protocol and pH were included in the model as fixed effects. An interaction term between these two factors was also tested but removed if it did not significantly improve the model. The individual fish ID was included as a random effect to account for the fact that the same fish were tested in both protocols. Linear mixed effect models were also used to analyse changes in cardiorespiratory parameters before and after an air-breath where individual fish ID was included as a random effect to account for multiple measures for each individual. To detect

whether the frequency of air-breathing changed as pH decreased in the step-wise protocol a one-way ANOVA was used, and a paired t-test was used to detect changes in air-breathing frequency between protocols at pH 5.75. To determine whether the onset of air-breathing and ASR were different between protocols we also used paired *t*-tests. Finally, to detect differences in blood PCO_2 and pH from the start and end of the experiment and between protocols, two-way ANOVA's were used.

3 | RESULTS

3.1 | Experimental protocol

While the duration of each step-wise protocol was longer than the progressive protocols (89 ± 3.8 min vs. 52 ± 2.1 min, respectively) the CO_2 load accumulated (area below the PCO_2 profile curve) during each protocol was on average not significantly different between groups (Figure S2). Specifically, the mean CO_2 load for the step-wise protocol trials was 472 ± 26.7 CO_2 units and 413 ± 15.58 CO_2 units for the progressive protocols ($t(18) = 1.94$, $p = 0.07$). Therefore, the rate of CO_2 increase was the primary difference between the two experimental protocols. While overall the rate of change in the progressive protocol (~3 mmHg CO_2 min^{-1}) was faster than in the step-wise protocol (~1 mmHg CO_2 min^{-1}), the step-wise protocol involved periods of stasis punctuated by rapid increases in P_wCO_2.

3.2 | Cardiorespiratory responses

All measured cardiorespiratory variables except air breathing frequency were insensitive to hypercarbia, even at very high levels (Figure 2). While gill ventilation rate decreased significantly at the highest CO_2 level (Figure 2a; $F_{4,60} = 2.97$, $p = 0.03$) in the stepwise protocol (Estimate (contrast with $PCO_2 < 7.6$ mmHg): −16.42), the change in gill ventilation rate in the progressive protocol across the same CO_2 range (Estimate: −3.60) was not significant. Overall, there was no significant difference in gill ventilation rate between experimental protocols ($F_{1,62} = 1.32$, $p = 0.26$). Neither heart rate (Figure 2b; $F_{4,60} = 1.37$, $p = 0.26$) or mean arterial blood pressure (Figure 2c; $F_{4,60} = 0.34$, $p = 0.85$) were affected by increased CO_2 in either protocol. Heart rate did not differ between protocols ($F_{1,63} = 0.01$, $p = 0.96$) but mean arterial blood pressure was lower in the progressive protocol compared with the stepwise protocol ($F_{1,63} = 10.15$, $p = 0.002$, Estimate: −5.27). Individual variance accounted for 48% of the total variance in ventilation between protocols. Individuals were relatively consistent in heart rate (27% of total variance) but less

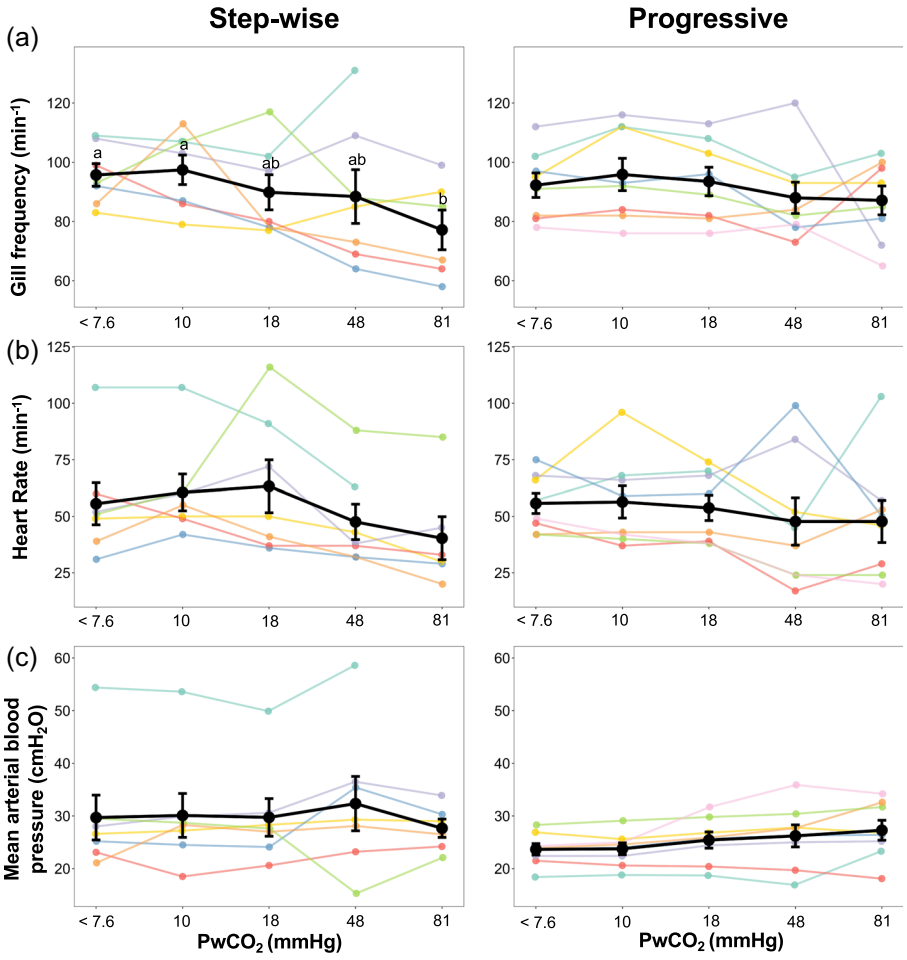

FIGURE 2 Cardiorespiratory responses are largely insensitive to stepwise and progressive increases in aquatic CO_2. (a) Gill ventilation frequency (ventilations per minute), (b) heart rate (beats per minute), and (c) mean arterial blood pressure (cmH_2O) during exposure to progressive and step-wise increases in water CO_2 levels. In all panels individual fish are shown by thin colored lines and translucent points and the mean and standard error of all individuals are shown by black points, error bars and a thick black line and dissimilar letters show significant differences ($p < 0.05$)

consistent in their mean arterial blood pressure (18% of total variance) between protocols. This inconsistency is likely due to the one individual with the highest blood pressure in the stepwise protocol but the lowest in the progressive protocol (Figure 2c).

3.3 | Alternate routes of gas-exchange

While rates of gill ventilation remained relatively stable in response to elevated CO_2, exposure to aquatic hypercarbia increased air-breathing frequency in *P. hypophthalmus*. As aquatic CO_2 levels increased, air-breathing frequency increased and was significantly higher than baseline levels once the PCO_2 exceeded 48 mmHg (Figure 3a; $F_{4,55} = 6.20$, $p = 0.0003$, Estimate (contrast with $P_wCO_2 < 7.6$ mmHg): $P_wCO_2 = 48$ mmHg + 0.09, $P_wCO_2 = 81$ mmHg + 0.16). Due to the rapid rate of CO_2 increase in the progressive protocol air-breathing frequency was only calculated at the final CO_2 level (81 mmHg). However, at this high CO_2 level, protocol had no effect on air-breathing frequency (Figure 3a, $t(9) = 0.08$, $p = 0.94$) and it was the same in the step-wise and progressive protocols (0.18 ± 0.05 vs. 0.19 ± 0.07 respectively). However, the onset P_wCO_2 for air-breathing was different between protocols. In the step-wise protocol the first air-breath tended to occur earlier at 23 ± 4.1 mmHg, whereas in the progressive protocol air-breathing began at 46 ± 7.8 mmHg ($t(8) = 2.296$, $p = 0.051$; Figure 3b). As there was often quite a gap between the first air-breath and the second, after which air-breathing was more regular, we also calculated the onset P_wCO_2 of the second air-breath which was different between protocols (Figure 3c). In the step-wise protocol the second air-breath occurred at 37 ± 4.8 mmHg, whereas in the progressive protocol air-breathing did not begin until 63 ± 4.7 mmHg ($t(6) = 2.98$, $p = 0.025$). In addition to an increase in the frequency of air-breathing at high CO_2 levels we also observed the fish in both experimental protocols performing aquatic surface respiration (ASR; Supplemental video). Aquatic surface respiration was more prevalent in the progressive protocol (13/14 individuals, 94%) than in the step-wise protocol where only 7/13 (54%) of individuals performed ASR. Although ASR was variable in duration (range 3–85 min), ASR almost always followed the onset of air-breathing, and so was initiated at slightly higher CO_2 levels overall

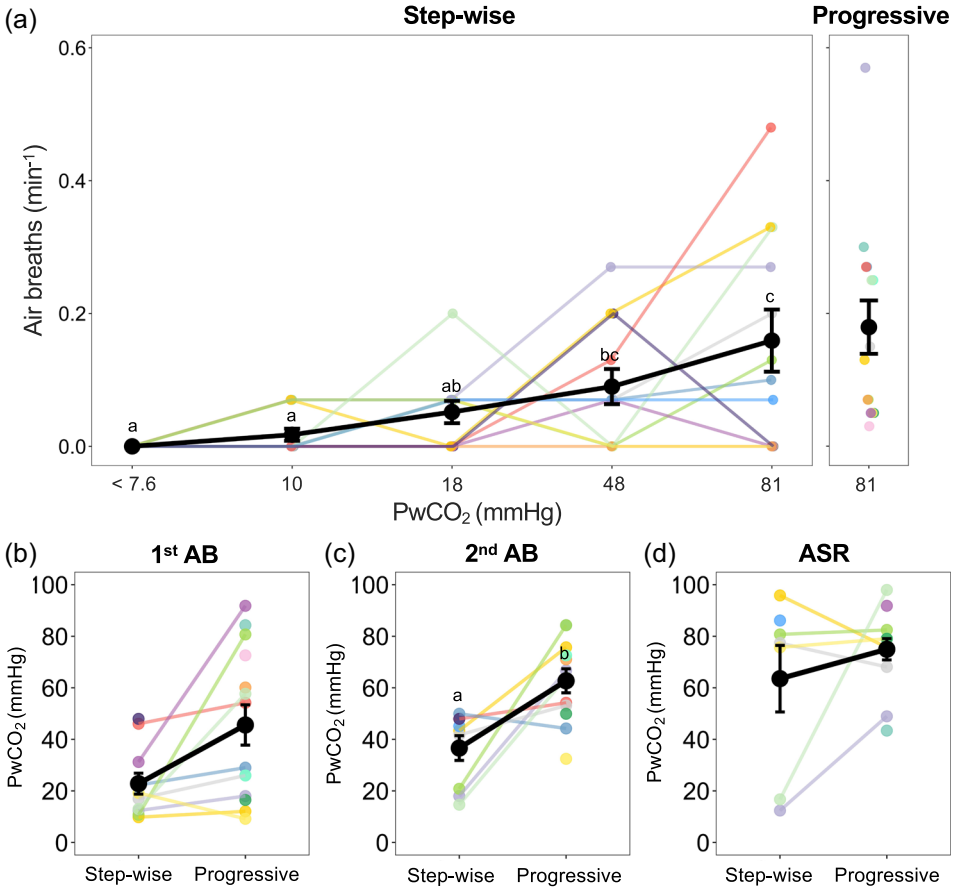

FIGURE 3 Air-breathing frequency and onset threshold are impacted by increasing aquatic CO_2levels. (a) Air-breathing frequency (breaths per minute) of individuals exposed to increasing CO_2 levels during the step-wise protocol (left) and at the highest CO_2 level achieved in the progressive protocol (right). (b–d) Onset P_wCO_2 threshold for the first air-breath taken (b), second air-breath taken (c), and onset of aquatic surface respiration, and (d) in both step-wise and progressive protocols. In all panels individual fish are show by thin colored lines and translucent points and the mean and standard error of all individuals are shown by black points, error bars and a thick black line. Dissimilar letters show significant differences ($p < 0.05$)

(71 ± 5.2 mmHg) than air-breathing. Unlike air-breathing, the P_wCO_2 at the onset of ASR was not different between protocols (Figure 3d; progressive: 75 ± 4.1 mmHg, step-wise: 64 ± 12.9 mmHg t (5) = 1.03, $p = 0.35$).

Overall, we observed a lot of interindividual variation in the air-breathing and ASR responses to aquatic hypercarbia in both protocols. Some animals did not respond, others only demonstrated air-breathing, others both air-breathing and ASR. Furthermore, the frequency, duration, and onset thresholds were also variable. Representative profile traces detailing the variety of responses observed are shown in Figure 4 for reference.

Cardiorespiratory variables were largely unperturbed by air-breathing. Heart rate increased, albeit marginally, after an air-breath (Figure 5a; $F_{1,175} = 6.70$. $p = 0.01$, Estimate: + 6.69). While, for both gill ventilation frequency (Figure 5b; $F_{1,174} = 1.24$, $p = 0.26$) and mean arterial blood pressure (Figure 5c; $F_{1,175} = 0.01$, $p = 0.94$) there were no significant differences before and after an air-breath. Whilst there is a large amount of variation between individuals, 30% of the total variance in heart rate is explained by the individual. Therefore, there is some consistency within an individual's response (Figure 5a). A similar amount of individual consistency is seen for changes in mean arterial blood pressure before and after an air-breath with 34% of the variance explained by the individual (Figure 5c), yet only 11% of the variance in gill ventilation rate is due to the individual suggesting the ventilatory response lacks consistency between individuals (Figure 5b).

3.4 | Blood values

In both protocols, arterial PCO_2 increased (Figure 6a; $F_{1,34} = 252.02$. $p < 0.0001$) and blood pH decreased (Figure 6b; $F_{1,33} = 386.54$. $p < 0.0001$) from the start ($P_wCO_2 < 7.6$ mmHg) until the end of the trial ($P_wCO_2 = 81$ mmHg). Blood PCO_2 did not differ between protocols (Figure 6b; $F_{1,34} = 1.01$, $p = 0.32$), whereas blood pH did differ (Figure 6b; $F_{1,33} = 5.91$ $p = 0.02$) at the start of the trial (+0.16 in step-wise protocol) but was similar between protocols by the end of the trial (+0.02 in step-wise protocol). Despite the increase in blood PCO_2 during the trial, the PCO_2 at the end was 43% lower (46 ± 2.5 mmHg) than the PCO_2 (~81 mmHg) in the surrounding water. This is suggestive of an increase in gas exchange efficiency associated with the air breathing, and consistent with this, the mean arterial PO_2 increased from 66 ± 6 in fish breathing normocarbic water to 113 ± 10 mmHg in fish at the end of the CO_2 protocol, despite no changes in gill ventilation.

4 | DISCUSSION

We asked whether the facultative air-breathing fish, *P. hypophthalmus*, responded differently to aquatic hypercarbia depending upon the exposure protocol used. We observed the same overall responses to severe levels of hypercarbia in both protocols (i.e., air-breathing and aquatic surface respiration), but we found that the onset P_wCO_2 for air-breathing was lower when the increases in CO_2 exposure were rapid but

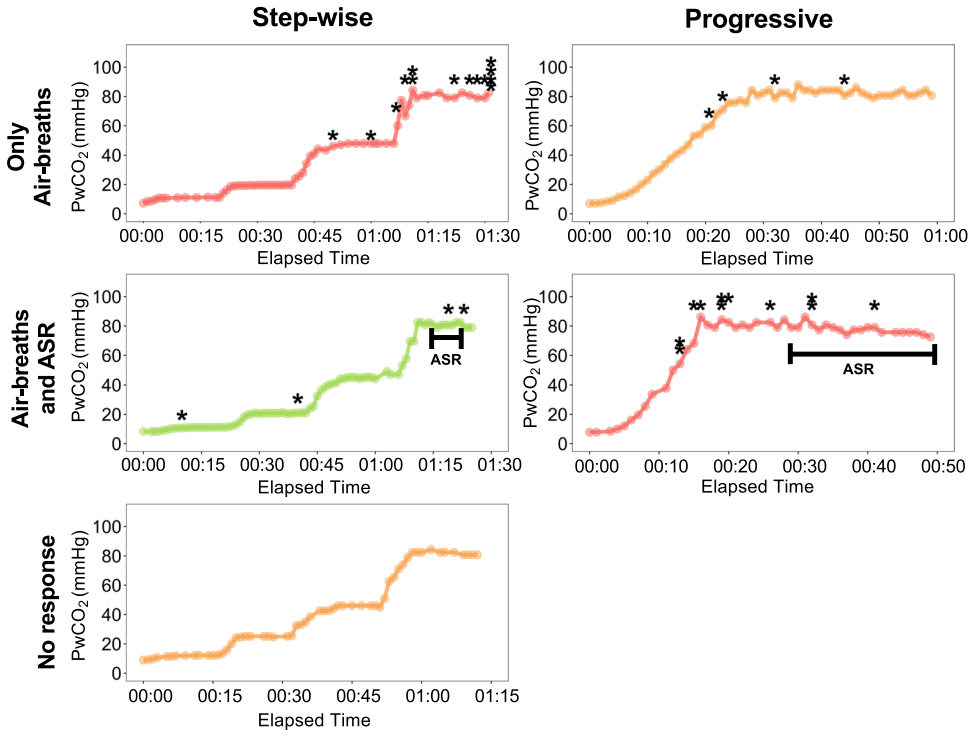

FIGURE 4 Representatives profiles of each type of documented response to both step-wise and progressive hypercarbia. Raw traces of increasing aquatic CO_2 (mmHg) are shown by the colored points. Colors correspond to individual fish ID and match those on all other figures. Asterisks indicate individual air-breaths and the black bars denote the onset and duration of aquatic surface respiration (ASR)

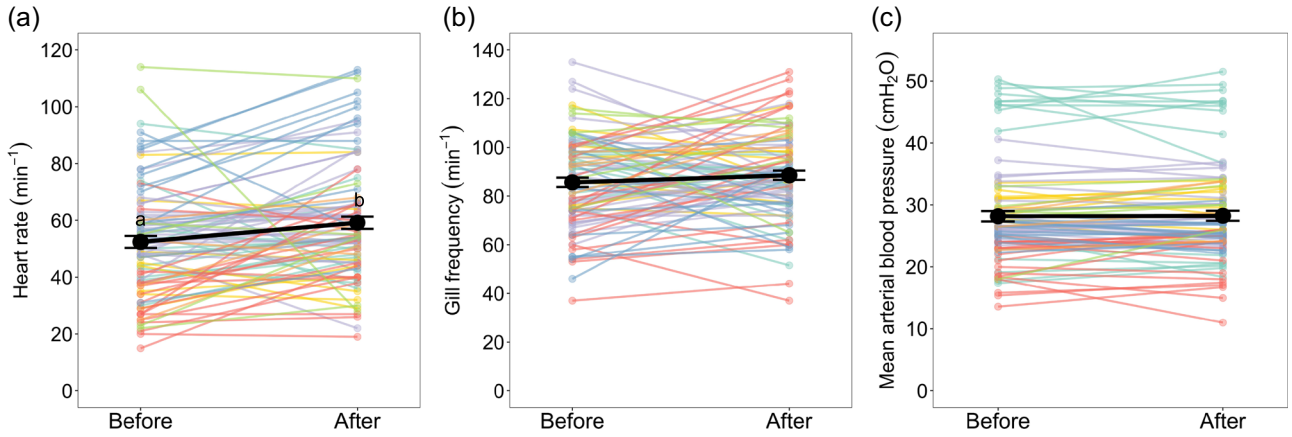

FIGURE 5 Cardiorespiratory responses before and after air-breathing are highly variable in *P. hypophthalmus*. (a) Heart Rate (beats per minute), (b) gill ventilation frequency (ventilations per minute), and (c) mean arterial blood pressure (cmH_2O). In all panels individual fish are show by thin colored lines and translucent points and the mean and standard error of all individuals are shown by black points, error bars and a thick black line. Dissimilar letters show significant differences ($p < 0.05$)

intermittent in the step-wise protocol. At the end of both protocols blood PCO_2 was similar, but was well below ambient P_wCO_2. The differential between high-ambient and lower-internal PCO_2 may be due to significant aerial CO_2 elimination via air-breathing, or could be due to an equilibration lag-time. Our primary objective was to investigate the impact of differing methods of hypercarbia exposure upon *P. hypophthalmus* cardiorespiratory responses. We conclude that *P. hypophthalmus* are remarkably tolerant to hypercarbia and that the method of hypercarbia exposure impacts only select cardiorespiratory responses (i.e., the onset of air-breathing). We also document the first report of aquatic surface respiration and air-breathing in response to aquatic hypercarbia in *P. hypophthalmus* and conclude that aerial respiration may contribute significantly to survival in high CO_2 aquatic environments.

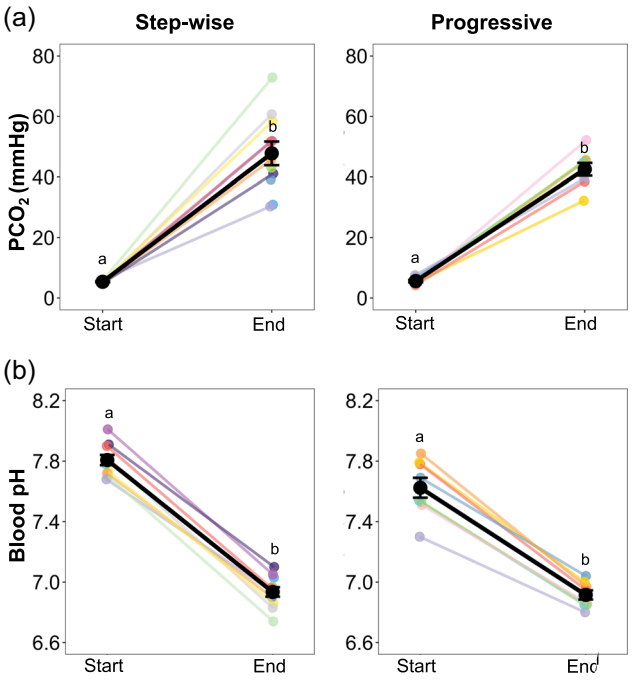

FIGURE 6 Exposure to aquatic hypercarbia increases blood PCO_2 and decreases blood pH in both experimental protocols. (a) Blood PCO_2 (mmHg), (b) blood pH. In all panels individual fish are show by thin colored lines and translucent points and the mean and standard error of all individuals are shown by black points, error bars and a thick black line. Dissimilar letters show significant differences ($p < 0.05$)

4.1 | Air breathing

Arterial PCO_2 increased as aquatic PCO_2 rose with a concomitant decrease in arterial pH. If branchial respiration was the sole route for CO_2 elimination, then P_wCO_2 and blood P_aCO_2 would be predicted to be similar—this was not the case. Instead, we observed frequent air-breathing and blood PCO_2 was much lower (−43%) than ambient water PCO_2 at the end of both experimental protocols. While this suggests that air-breathing is effective at eliminating CO_2 it should be noted that a similar differential was found in the one fish that did not air breathe nor performed aquatic surface respiration and could simply reflect the lag time required for full equilibration (McKenzie et al., 2002). That mean arterial PO_2 almost doubled in fish at the end of the CO_2 protocol, despite no changes in gill ventilation, however, suggests that the difference was at least in part a result of the air breathing.

Hypercarbia induced air-breathing has been documented in several facultative air-breathing species (e.g., de Lima Boijink et al., 2010; Sanchez & Glass, 2001; Sanchez et al., 2005; Tuong et al., 2018). However, increased air-breathing has only been documented previously in response to aquatic hypoxia in *P. hypophthalmus* (Lefevre et al., 2011; Thomsen et al., 2017) and not hypercarbia (Thomsen et al., 2017). Given the hypercarbic natural habitat of *P. hypophthalmus* and the air-breathing propensity of this species we were not surprised to observe hypercarbia-induced air-breathing at high P_wCO_2. However, we were surprised that we observed some air-breathing before the maximum CO_2 level

(37.5 mmHg; ~5% CO_2) tested by Thomsen et al. (2017). Instrumentation can be eliminated as an explanatory variable as the fish in both studies were cannulated. Furthermore, we observed no difference in air-breathing responses between instrumented and noninstrumented animals in our study. One possible explanation is that the slow rate of CO_2 exposure (~0.08 mmHg CO_2 min^{-1}) used in the study of Thomsen et al. (2017) did not trigger air-breathing, whereas our faster 3 mmHg CO_2 min^{-1} (progressive) and 1 mmHg CO_2 min^{-1} (step-wise) rates did. That the onset of air-breathing occurred at a lower P_wCO_2 in the step-wise protocol might seem at odds with this, but while the rate of increase in CO_2 exposure was slower overall in the step-wise protocol the increases in CO_2 during the steps were rapid and at a much faster rate than the mean increases in the progressive protocol. These rapid and intermittent increases in CO_2 could have triggered an earlier onset of air-breathing than the slower constant increase in the progressive protocol. These responses are reminiscent of ectotherm thermal responses that also differ when produced at very slow and fast rates of change due to acclimation responses and sensory processing lag-times, respectively (Åsheim et al., 2020; Kovacevic et al., 2019; Terblanche et al., 2007). Clearly, and in support of our research question, both the rate of change of P_wCO_2 and the method of exposure appear to be important factors for the initiation of air-breathing by PCO_2/H^+ sensitive chemoreceptors in *P. hypophthalmus* and further studies are warranted to resolve this issue.

Due to the high accumulated blood PCO_2 levels, and the possible effects on hemoglobin-O_2 affinity, we questioned whether air-breathing and ASR may be driven by low blood PO_2 (hypoxemia) and not high PCO_2. While we did not measure hemoglobin P_{50} directly in this study, previous work has demonstrated that *P. hypophthalmus* hemoglobin is relatively insensitive to CO_2/H^+ perturbations (Damsgaard, Phuong, et al., 2015). The hemoglobin P_{50} of *P. hypophthalmus* is high (4.61 mmHg at pHe 7.6 at 25°C) and the Bohr coefficient is average (−0.7 at pHe 7.6°C and 25°C). Furthermore, *P. hypophthalmus* hemoglobins demonstrate no Root effect down to pHe = 6.7, which would maintain effective O_2 transport at high CO_2/H^+ levels (Damsgaard, Phuong, et al., 2015). The lowest blood pH we measured in any experimental fish at the end of the experimental protocol was pH = 6.7. Therefore, O_2 transport was likely not diminished during our experimental protocol. Our measurements of blood PO_2 at the start versus end of the trials are consistent with this and we conclude that air-breathing and ASR were most likely not induced by hypoxemia but were the result of high CO_2.

A rise in air-breathing frequency was associated with a decrease in gill ventilation frequency at elevated P_wCO_2, particularly in the step-wise protocol. In the clown knifefish, *Chitala ornata*, environmental hypercarbia stimulated air breathing but had no effect on gill ventilation (Tuong et al., 2018). However, an inhibition of gill breathing at the onset of air breathing has also been observed in several other species although in most there was an initial increase in gill ventilation before the onset of air breathing (Graham & Baird, 1982; Johansen & Lenfant, 1968; Johansen et al., 1967, 1970; Sanchez & Glass, 2001; Sanchez et al., 2005). The decline in gill ventilation may serve to minimize CO_2 influx into the blood of *P. hypophthalmus*. Lefevre et al. (2011) note that a membranous flap along the opercula edges in *P. hypophthalmus* effectively seals off the branchial cavity and reduces gill-water contact. The temporal concordance between decreased gill ventilation and increased air-breathing demonstrates that significant reductions in gill ventilation are not possible without an alternate route for gas-exchange.

Cardiovascular parameters (heart rate and blood pressure) also appear largely insensitive to hypercarbia, regardless of exposure protocol, in *P. hypophthalmus*. Bradycardia and increased MAP might be considered the typical piscine hypercapnic cardiovascular responses. However, it is clear from tab. 3.2 in Gilmour and Perry (2006) that a lack of cardiovascular response is not uncommon in response to hypercarbia. The presumed benefits of bradycardia and increased blood pressure are to increase diffusion capacity at the gill via increased blood residence time within the gill and increased lamellar recruitment (Gilmour & Perry, 2006). Since O_2 uptake and transport are not threatened in our experiments, increasing diffusion capacity at the gill would only serve to enhance CO_2 influx. Therefore, it is perhaps not surprising that we did not observe either bradycardia or increased blood pressure in our experiments.

Depending on the species, air-breaths themselves may have noticeable, but transient impacts on cardiovascular and gill ventilatory parameters. For example, in *Chitala ornata* blood pressure and gill ventilation may fall after an air-breath (Tuong et al., 2018). The transient cessation of gill ventilation is presumed to prevent O_2 loss across the gills. Neither we, nor Armelin et al. (2019) observed such a gill ventilatory response following air-breathing in *P. hypophthalmus*. Air-breaths are also often associated with post air-breath tachycardia (e.g., Belão et al., 2015; Lopes et al., 2010; McKenzie et al., 2007), presumably to increase perfusion to the ABO. Both we and Armelin et al. (2019) document post air-breath tachycardia in *P. hypophthalmus* although the magnitude of the tachycardia was larger (~3-fold) in the study of Armelin et al. (2019). Overall, air-breathing did not much impact cardioventilatory responses in this study. However, as Figure 5 demonstrates, there was a large amount of variability between air-breaths, even within the same individual, which makes general statements about air-breathing cardio-ventilatory responses in *P. hypophthalmus* difficult.

4.2 | Aquatic surface respiration

Our observation of aquatic surface respiration in *P. hypophthalmus* was surprising. As with hypoxia driven ASR, that is typically initiated later, at lower aquatic PO_2 levels than air-breathing (Kramer et al., 1983), hypercarbia-induced ASR typically commenced after air-breathing at a higher P_wCO_2. The air-breathing response in *P. hypophthalmus* is unmistakeable and involves air-bubble release from the opercula and a post air-breath dive (Browman & Kramer, 1985; Lefevre et al., 2011), presumably to help aid air flow into the respiratory swimbladder. The behavior we have classified as aquatic surface respiration (video) is clearly distinct from air-breathing. During ASR *P. hypophthalmus* actively swim at the water surface and move their heads sinusoidally allowing the mouth to skim the surface water. Shallow, rapid jaw movements appear to move small volumes of surface water in- and out- of their buccal cavity. The benefit

derived from ASR by *P. hypophthalmus* is unclear, While CO_2 off-gassing was undoubtedly occurring from our experimental tank water to the lower PCO_2 ambient air, the pH of the surface water was only ≤ 0.1 pH unit or 16 mmHg PCO_2 different from the bulk water. The ASR behavior does not appear to be a response to low O_2 as experimental tank water was not hypoxic, and as noted above, this species lacks strong Root and Bohr effects (Damsgaard, Phuong, et al., 2015) suggesting this behavior could not be due to reductions in Hb-O_2 binding. Furthermore, studies that have directly examined hypoxic ventilatory responses in *P. hypophthalmus* (e.g., Lefevre et al., 2011) have not observed ASR. This is in stark contrast to other species where hypoxia induces ASR while hypercarbia does not (Florindo et al., 2004).

5 | CONCLUSIONS

We conclude that the rate of CO_2 exposure influences the onset threshold for, and expression of, both air-breathing and aquatic surface respiration in *P. hypophthalmus*. Other cardiorespiratory parameters were relatively insensitive to CO_2 exposure. *P. hypophthalmus* frequently occupies hypercarbic and hypoxic habitats in both nature and in aquaculture (Damsgaard et al., 2015). Aquatic CO_2 levels are often very high (above 60 mmHg) in slow moving tropical habitats with plant surface cover and high organic loading. Here surface diffusion is impaired and the nonequilibrium CO_2 levels presumably come from facultative anaerobic bacteria (Wilmer, 1934; Ultsch, 1987). Our data suggest that *P. hypophthalmus* under such conditions uses air-breathing when exposed to both types of ecologically relevant stressors.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

All data are available from the corresponding author on request.

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