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ORIGINAL PAPER

Ventilatory responses of the clown knifefish, *Chitala ornata*, to arterial hypercapnia remain after gill denervation

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Abstract

The aim of this study was to corroborate the presence of CO_2/H^+-sensitive arterial chemoreceptors involved in producing air-breathing responses to aquatic hypercarbia in the facultative air-breathing clown knifefish (*Chitala ornata*) and to explore their possible location. Progressively increasing levels of CO_2 mixed with air were injected into the air-breathing organ (ABO) of one group of intact fish to elevate internal PCO_2 and decrease blood pH. Another group of fish in which the gills were totally denervated was exposed to aquatic hypercarbia (pH ~ 6) or arterial hypercapnia in aquatic normocarbia (by injection of acetazolamide to increase arterial PCO_2 and decrease blood pH). Air-breathing frequency, gill ventilation frequency, heart rate and arterial PCO_2 and pH were recorded during all treatments. The CO_2 injections into the ABO induced progressive increases in air-breathing frequency, but did not alter gill ventilation or heart rate. Exposure to both hypercarbia and acetazolamide post-denervation of the gills also produced significant air-breathing responses, but no changes in gill ventilation. While all treatments produced increases in arterial PCO_2 and decreases in blood pH, the modest changes in arterial PCO_2/pH in the acetazolamide treatment produced the greatest increases in air-breathing frequency. These results strengthen the evidence that internal CO_2/H^+ sensing is involved in the stimulation of air breathing in clown knifefish and suggest that it involves extra-branchial chemoreceptors possibly situated either centrally or in the air-breathing organ.

Keywords Air breathing · CO_2 · Gill denervation · Aquatic hypercarbia · Acetazolamide

Introduction

Due to differences in the solubility of O_2 and CO_2 in water and air, ventilation in water-breathing vertebrates is driven primarily by the need to obtain sufficient O_2, while in terrestrial air-breathing vertebrates it is primarily driven by the need to eliminate CO_2 (Rahn 1966; Dejours 1981). Bimodal breathers, those species that exchange gases with both media, include many species of actinopterygian fishes, the sarcopterygian lungfish and the larvae of all amphibian species as well as a few adult amphibians.

Air breathing in the actinopterygian fishes has arisen independently many times and is found in over 400 species (Graham 1997; Bayley et al. 2019). Within this group, adaptations for gas exchange can be found at all sites along the digestive system from the mouth to pharyngeal and branchial diverticula, the gut, and various air-breathing organs that arise as outpockets from the gut. At least 47 species from 24 genera of bony fish breathe air using a lung- or air-breathing organs (Graham 1997). These exchange surfaces represent a wealth of evolutionary experiments that are accompanied by changes in respiratory pumps and valving as well as in the control mechanisms that regulate the flow of air across them. The latter include the respiratory responses to aquatic hypoxia and hypercarbia.

In many species of air-breathing fish, environmental hypercarbia induces an initial increase in gill ventilation

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followed by a switch to air breathing and a fall in gill ventilation if ambient CO_2 levels rise sufficiently (*Amia calva*, Johansen et al. 1970; *Ancistrus chagresi* and *Hypostomus plecostomus,* Graham and Baird 1982; *H. unitaeniatus*; de Lima Boijink et al. 2010; *Notopterus chitala*, Dehadrai 1962). This is also the case in some sarcopterygian lungfishes (*Protopterus aethiopicus*, Jesse et al. 1967; *Neoceratodus forsteri*, Johansen et al. 1967; *Lepidosiren paradoxa*, Sanchez and Glass 2001; Sanchez et al. 2005). However, in some facultative air-breathing species, aquatic hypercarbia fails to produce any changes in gill ventilation (*Symbranchus marmoratus*, Johansen 1966; *Misgurnus anguillicaudatus*, McMahon and Burggren 1987; *Pangasianodon hypophthalmus,* Thomsen et al. 2017) or in air-breathing frequency (*Amphipnous cuchia*, Lomholt and Johansen 1974). Recently, we found that an increase of aquatic CO_2 in the facultative air-breathing species, *Chitala ornata*, induced a significant increase in air-breathing frequency without having any effect on gill ventilation (Tuong et al. 2018a). While many lungfish show no response to increasing levels of CO_2 in inspired air (Sanchez and Glass 2001; Perry et al. 2005, 2008) others do, increasing lung ventilation (Smith 1930; Delaney et al. 1974, 1976, 1977; Babiker 1979). To the best of our knowledge, similar studies have not been conducted on actinopterygian fishes.

Reports of the orientation of the chemoreceptors involved in the cardiorespiratory responses to CO_2 in air-breathing fish species are also varied. de Lima Boijink et al. (2010) found a difference in the distribution of CO_2-sensitive receptors involved in gill versus air-breathing responses in the facultative air-breathing jeju (*Hoplerythrinus unitaeniatus*). The gill ventilatory responses, which were exclusive to aquatic CO_2, were eliminated by complete gill denervation. However, a small but significant air-breathing response to high levels of CO_2 remained after gill denervation and the site and orientation of the receptors producing it could not be determined. More recently, we reported that injections of acetazolamide, to block carbonic anhydrase leading to CO_2 retention and blood acidification, elicited a significant air-breathing response in *Chitala ornata* (Tuong et al. 2018a). Both of these studies indicate that the stimulation of air breathing in these two species was, at least in part, due to stimulation of internally oriented chemoreceptors [i.e., receptors sensitive to internal (arterial blood) changes rather than external (water) changes].

The primary cardiorespiratory chemoreceptors in fish are believed to be the neuroepithelial cells (NECs) that are found throughout the gills and are innervated by the ninth (glossopharyngeal) and tenth (vagus) cranial nerves (Milsom 2012; Jonz et al. 2015). A subpopulation of O_2-sensitive NECs also responds to changes in CO_2 as well as to ammonia (Qin et al. 2010; Zhang et al. 2015; Abdallah et al. 2015). There appear to be subpopulations of NECs that sense external (water) versus internal (blood) levels of O_2 and some that detect both (Milsom and Brill 1986; Burleson and Milsom 1993; Burleson 2009). While it is clear that some of these receptors also monitor changes in CO_2 in ambient water, their role in monitoring changes in internal (blood) CO_2 levels remains uncertain (see Gilmour 2001; Perry and Gilmour 2002; Milsom 2012 for reviews).

In jeju (*Hoplerythrinus unitaeniatus*), the internally oriented receptors sensing changes in O_2 levels in the blood play a large role in eliciting air breathing in response to hypoxia and these receptors are exclusively located in the gills (Lopes et al. 2010). This could also be the case for internally oriented CO_2 receptors in *Hoplerythrinus* and *Chitala*. Furthermore, while the presence of central CO_2/H^+ chemoreceptors in water-breathing fish is equivocal, there is strong evidence for their presence in sarcopterygian lungfish (Smith 1930; Delaney et al. 1974, 1977; Babiker 1979; Sanchez et al. 2001). Within the air-breathing actinopterygian fishes, they have been reported to be present in gar (*Lepisosteus oculatus*) (Wilson et al. 2000) and Siamese fighting fish (*Beta splendens*) (Corcoran et al. 2007).

Given the equivocal nature of the data, the aim of the present study was to confirm the existence of internally oriented CO_2 chemoreceptors involved in eliciting cardiorespiratory responses in *Chitala ornata* and to determine their location. We measured ventilation, heart rate and arterial blood pressure while introducing varying levels of CO_2 into the air-breathing organ to load CO_2 into the systemic circulation while the fish swam in normoxic, normocarbic waters. We also monitored the response to aquatic hypercarbia and injections of acetazolamide, both before and following gill denervation to determine the role of gill neuroepithelial cells (both internally and externally oriented) in these responses.

Materials and methods

Experimental animals

Chitala ornata (400–900 g) were obtained from a farmer in Hau Giang province, Vietnam. They were held in 2 m^3 tanks in a recirculating aquaculture system in which water flow was maintained at a rate of 0.19 $m^3\ h^{-1}$. Water parameters were: dissolved oxygen > 95% air saturation, $PCO_2 < 0.5$ mgL^{-1}, pH ≈ 7.6, $NH_3^+ \approx 0.02$ mg L^{-1}, $NO_3^- \approx 90$ mgL^{-1} and temperature ≈ 27 °C. Fish were fed commercial floating pellets (43% protein) (Stella S3, Nutreco company, Ho Chi Minh city, Viet Nam) twice a day and fasted for 2 days before experiments.

Chitala ornata is a facultative air-breathing fish (Dehadrai 1962; Tuong et al. 2018b) that occurs naturally throughout the Mekong River drainage in Thailand, Laos, Cambodia and Vietnam (Poulsen et al. 2004; Vidthayanon

2012). In Vietnam, they are produced in large numbers in aquaculture systems in the Mekong delta (Viet 2015). Their air-breathing organ is large with a unique shape, reflecting both the exotic body form of this species and the organ's functions for respiratory gas exchange, buoyancy control as well as both sound production and reception (Dehadrai 1962; Graham 1997). In its native habitat, *Chitala ornata* inhabits large, deep pools of the mainstreams of the Mekong River where PCO_2 levels range from 0.02 to 0.6%, and pH levels vary between 6.9 and 8.4 with obvious monthly and spatial variations (Li et al. 2013). However, this species is also naturally found in the more stagnant waters of the delta flood plain where plant cover and organic enrichment can lead to severe hypercarbia with PCO_2 levels in excess of 60 mmHg (Ultsch 1987). They are also exposed to PCO_2 levels in excess of 30 mmHg toward the end of the growth cycle in local aquaculture systems (Damsgaard et al. 2015).

Fish preparation

Fish ($n = 12$) were anesthetized by immersion in benzocaine (0.35 ml L^{-1}) and then gently moved to a surgical table where their gills were irrigated with a dilute aerated anesthetic solution (0.2 ml L^{-1}). The dorsal aorta of each fish was catheterized with fine bore polythene tubing (PE 50; I.D. 0.4 mm, O.D. 0.8 mm) containing 50 IU/ml of heparinized saline and the cannulae fed out of the buccal cavity through a hole in the rostrum and secured with nylon suture (Soivio et al. 1975). Two impedance electrodes were then attached, one to each operculum and secured by nylon suture.

In a subgroup of fish ($n = 6$), the air-breathing organ was also cannulated with a polythene cannula (PE 100) fed into the air-breathing organ through the buccal cavity, esophagus and pneumatic duct (physostome). The tip of this cannula was fed out of the buccal cavity through a hole in the skin between the corner of the mandible and maxilla and was secured in place by nylon suture.

In the other subgroup ($n = 6$), branches of cranial nerves IX and X projecting to the gills on both sides were exposed and transected. A retractor was used to open the gill operculum and cranial nerves IX and X of both sides were exposed using iris scissors to make two individual incisions in the skin just above the gills. Cranial nerve IX is situated near the origin of first gill arch, while the branches of cranial nerve X can be easily located between the third and fourth gill arches. Each nerve branch was dissected free of connective tissue and blood vessels and cut. Fish were then returned to a holding tank and allowed to recover after surgery for 24 h (Phuong et al. 2016; Tuong et al. 2018a, b).

Experimental protocol

At around 24 h post-surgery, fish were moved to the experimental tank. Impedance leads (Harvard Instruments, Hollliston, Massachusetts) were connected to an impedance convertor (UFI Model 2991, Morrow Bay, California) to measure gill ventilation rate (min^{-1}) and air-breathing frequency (h^{-1}). The dorsal aorta cannula was connected to a pressure transducer and amplifier to measure heart rate (min^{-1}) and blood pressure (cm H_2O), and also to draw blood for blood gas (PCO_2, pH and HCO^{3-}) analysis using an i-STAT VetScan Analyzer (Abaxis, Union City, CA, USA) and/or for injection of acetazolamide. A data acquisition system (Dataq DI-720, DATAQ Instruments, Inc., Akron, OH, USA) was used to record all signals at 125 Hz per channel.

The group of six fish (566 ± 120.23 g) with ABO catheters was used to study the effects of increasing blood PCO_2 by injecting CO_2 gas into the air-breathing organ. During the surgery to feed the catheter into the ABO, the air inside the gas bladder was withdrawn through the catheter to yield a reference of gas bladder volume. Gas was withdrawn through the air bladder catheter until the air bladder was deflated, but avoiding complete collapse to prevent damage to the vasculature in the bladder wall (2.80 ± 0.26 ml withdrawn and injected per 100 g fish). Fish were moved to the experimental tank and allowed to rest for at least 1 h. Then, after a minimum 15 min of control recording, a blood sample was taken. Following this, a volume equal to the reference volume was withdrawn from the air-breathing organ and replaced with 50% CO_2 gas mixed with air. The air above the experimental tank was also replaced with a gas mixture of the same composition as that injected into the air-breathing organ. Measurements were made for 15 min, after which a second blood sample was drawn from the dorsal aorta catheter for blood gas analysis. The gas in the air-breathing organ (and in the air above the tank) was then progressively replaced with 75%, 90% and 100% CO_2 gas mixtures (balance air). The injection of similar volumes of low levels of CO_2 into ABO did not induce significant AB and thus served as controls for the injections of higher levels of CO_2, demonstrating that volume changes per se did not stimulate air breathing.

The group of six denervated fish (761.67 ± 42.46 g) was exposed to normoxic/normocapnic water, pH = 7.8 and 95% O_2 saturation. Once the fish were relaxed and in a steady state, recordings were made for at least 30 min. Water temperature and pH were measured continuously. A blood sample was then drawn from the dorsal aorta to measure pH and PCO_2 at the end of the control period. Following this, CO_2 gas (99% pure) was bubbled into the experimental tank to make the water progressively hypercarbic until the water pH reached 6.0 (equivalent to 40–50 torr PCO_2) as monitored continuously by pH meter

(Toledo micro pH meter, Mettler Toledo, Columbus, OH, USA). The water pH level was held stable at pH 6 for 30 min whereupon a further blood sample was drawn and the fish moved back to the resting tank for 24 h of recovery before the subsequent acetazolamide trial.

The following day, the fish that was recovered from the hypercarbia trial of the previous day was returned to the experimental tank. Once it was relaxed and in a steady state, recordings were made for at least 30 min. Water temperature and pH were measured continuously. A blood sample was then drawn from the dorsal aorta to measure pH and PCO_2 prior to treatment. Acetazolamide dissolved in DMSO (dimethyl sulfoxide) at 30 mg ml^{-1} kg^{-1} was injected through the dorsal aorta catheter. After 15 min, allowing for the acetazolamide to distribute throughout the body, recordings were made for a minimum of 30 min followed by a blood sample for blood gas analysis. The water was air saturated throughout the trial.

In an earlier study (Tuong et al. 2018a, b), it was shown that injection of DMSO alone had no effect on AB frequency in *C. ornata*. Denervated fish were examined at necropsy, post-experiment, to confirm that the nerve sections were complete.

Data and statistical analysis

Gill ventilation frequency, heart rate and air-breathing frequency were measured over the last 10 min of each control run and each air-breathing organ injection. They were recorded over the first (0–10), middle (11–20) and last (21–30) 10 min of the hypercarbic exposure and acetazolamide injections. Data are presented as mean ± S.E.M. Differences between initial levels of each variable and those during CO_2 injections into the air-breathing organ were determined with a paired samples *t* test. A one-way ANOVA for repeated measurement followed by an LSD post hoc test was used to test for significant differences in blood pH and PCO_2 in the ABO injection experiment. Linear regressions were applied to the entire data set for each of these variables. Differences between initial levels of each variable and those during the hypercarbic or acetazolamide exposures were determined using a two-way repeated-measures analysis of variance (ANOVA) followed by a Holm–Sidak post hoc test to check the effects of hypercarbia, acid exposures and acetazolamide injections on the cardiorespiratory variables at control, 20, 40 and 60 min of exposure. We used a one-way ANOVA for repeated measures followed by a Student–Newman–Keuls post hoc test to verify changes in heart rate and mean arterial pressures before and after an air breath. Statistical significance was assigned as $p < 0.05$.

Results

Injection of CO_2 into the air-breathing organ

Replacing the air in the ABO with CO_2 of different concentrations had no effect on gill ventilation or heart rate (Fig. 1a, b). However, it caused a large increase in air-breathing frequency. Thus, there was a strong correlation between injected CO_2 fraction and air-breathing frequency ($y = e^{\wedge(0.0364*x)}$, $R^2 = 0.93$) (Fig. 1c). Furthermore, this alteration in air-breathing frequency was reflected in the arterial pH and PCO_2 (Fig. 2).

Effects of gill denervation

Gill denervation produced a decrease in arterial PCO_2 and a rise in pH_a (11–6 mmHg and pH 7.7–7.85, respectively). There was also an associated drop in gill ventilation frequency (27–21 min^{-1}), but air-breathing frequency remained unaffected (4–3 min^{-1}). This procedure caused a significant rise in heart rate (22.11–44.46 beats/min) (Table 1).

Cardiorespiratory responses to aquatic hypercarbia and acetazolamide post-gill denervation

Exposure of denervated fish to aquatic hypercarbia (water pH = 6.0; PCO_2 = 40–50 mmHg) had little effect on gill ventilation (Fig. 3), but was associated with a slight but non-significant reduction in heart rate. Similarly, the blood hypercapnia from injection of AZ did not affect gill ventilation frequency in this group, but did cause a significant fall in heart rate (Fig. 3). However, both of these treatments caused large and significant increases in air-breathing frequency (Fig. 4). When these effects are compared to those seen in fish with intact gills (data taken from Tuong et al. 2018a), it appears that gill denervation reduces the air-breathing response to hypercarbia by roughly 40%, but more than triples the response to acetazolamide (Fig. 4). The increase in air-breathing frequency with acetazolamide was particularly notable.

The changes in arterial pH and PCO_2 recorded at the end of the hypercarbia and acetazolamide treatments are plotted against the air-breathing rates in Fig. 5. Note that while the increase in plasma PCO_2 and the decrease in blood pH were greater following aquatic hypercarbia than following acetazolamide, injection of AZ produced a greater increase in air-breathing frequency (Fig. 5).

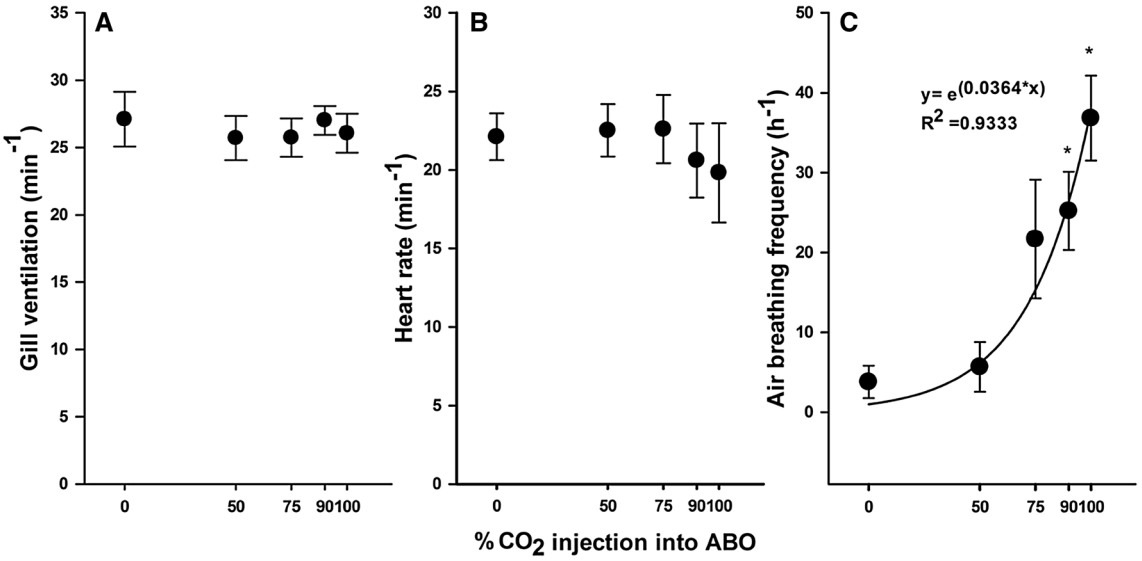

Fig. 1 **a** Gill ventilation frequency, **b** heart rate and **c** air-breathing frequency of intact *Chitala ornata* in response to injections of different CO_2 air mixtures into the air-breathing organ. Data are presented as mean ± SEM. "Asterisk" indicates significant difference from control conditions (one-way ANOVA for repeated measurement, $p < 0.05$). The mean data in **c** were fitted with a regression equation

Discussion

Injection of CO_2 into the air-breathing organ

The injection of increasing concentrations of CO_2 into the air-breathing organ in fish in normocarbic water produced progressive increases in arterial PCO_2, deceases in arterial pH and increases in air-breathing frequency. It had no effect on gill ventilation or on heart rate. These changes were very similar to those previously produced by aquatic hypercarbia in intact fish (Tuong et al. 2018a). When combined with the results of the acetazolamide injections in Tuong et al. (2018a), as well as the acetazolamide injections in the present study, both of which produced a respiratory acidosis in fish in normocarbic water, and increases in air-breathing, there is clear evidence for the existence of internally oriented CO_2/H^+ chemoreceptors in this species.

The concentrations of CO_2 injected into the air-breathing organ were high. This might suggest that while CO_2 can be loaded into the arterial system through the air-breathing organ, diffusion is slow and equilibrium is incomplete. However, while under normocarbic conditions, less than 20% of metabolic O_2 uptake occurs across the air-breathing organ, when in hypoxic water, up to 50% of O_2 extraction is from the air-breathing organ (Tuong et al. 2018b). Furthermore, under these conditions, it has been shown that the amount of CO_2 excreted into the swim bladder is almost equal to the amount of O_2 extracted (Dehadrai 1962) suggesting that the diffusion capacity of the organ can be high. Thus, it was surprising that individual arterial post-injection PCO_2 levels never rose above 60 mmHg (mean of 38.1 mmHg with 90% CO_2 injection). However, although the gas injected into the air-breathing organ contained 50, 75, 90 and 100% CO_2, we do not know the ABO volume and hence the initial post-injection mixed concentration. It is also possible that the high levels of CO_2 are vasoactive and reduce blood flow and diffusion capacity under these conditions. The only other studies that have examined the effects of increasing levels of CO_2 in air-breathing organs were made on the sarcopterygian lungfishes. The outcomes following manipulation of CO_2 in the ABO in this group were inconsistent. While increasing levels of CO_2 in inspired air increased air breathing in *Protopterus anectens* and *P. aethiopicus* (Smith 1930; Delaney et al. 1974, 1976, 1977; Babiker 1979), they had no effect in *P. dolloi* and *Lepidosiren paradoxa* (Sanchez and Glass 2001; Perry et al. 2008) and even led to a decrease in air breathing in one study on *P. aethiopicus* (Jesse et al. 1967).

Effects of gill denervation

Gill denervation gave rise to a drop in PCO_2, a rise in pH_a, a slight drop in gill ventilation frequency and a significant rise in heart rate. The rise in heart rate reflects denervation of the cardiac branch of the vagus nerve, and the drop in PCO_2 and rise in pH_a likely reflect the higher heart rate and

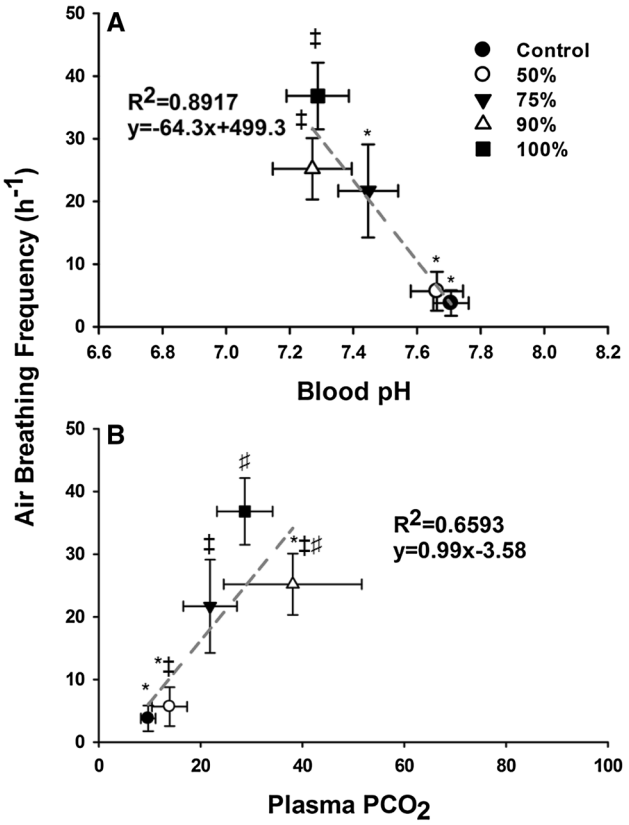

Fig. 2 Air-breathing frequency expressed as function of **a** the arterial pH or **b** the arterial PCO_2. The data are shown as mean ± SEM. A one-way ANOVA for repeated measurement followed by an LSD post hoc test was used to test for differences in blood pH and PCO_2. Mean data points with similar symbols are not significantly different. Linear regressions have been applied to the entire data set for each variable

increased gill perfusion. Similar results have been obtained in several exclusively water-breathing fish (traira, Sundin et al. 1999; Reid et al. 2000; tambaqui, Sundin et al. 2000) as well as in the facultative air-breathing jeju (de Lima Boijink et al. 2010). In the gar, total gill denervation significantly reduced gill ventilation frequency and amplitude. In other studies, however, gill denervation produced no significant changes in gill ventilation frequency or heart rate (channel catfish, Burleson and Smatresk 1990; tambaqui, Florindo et al. 2004; *Amia*, McKenzie et al. 1991). Thus, the general picture that emerges is that gill chemoreceptors provide little tonic stimulation of gill ventilation under normoxic, normocarbic conditions.

Similarly, gill denervation in *Chitala* had no effect on air-breathing frequency, which remained at the same low levels for fish in normocarbic/normoxic water. The jeju does not take air breaths under these conditions and hence no effect was seen in this species either (Lopes et al. 2010). *Amia* also take only infrequent air breaths in aquatic normoxia, and while denervated animals did not breathe air at all during 15 min of observation (McKenzie et al. 1991), normal levels of air breathing were observed over a longer time period (Hedrick and Jones 1999). In gar, complete branchial denervation severely reduced resting air breathing. Thus in some species, there is a low level of drive arising from the gills that stimulates occasional air breathing, under normoxic, normocarbic conditions, while in other species there is not.

Cardiorespiratory responses to aquatic hypercarbia and acetazolamide post-gill denervation

Gill ventilation and heart rate

We have previously shown that exposure of intact *Chitala* to hypercarbic water produced a no significant fall in heart rate, while arterial hypercapnia produced by injection of acetazolamide produced a significant bradycardia (Tuong et al. 2018a). In the present study, we obtained identical results following gill denervation. In contrast, CO_2 injection into the air-breathing organ did not give rise to an attending bradycardia. Unfortunately, these results do not correlate with either the degree of change in arterial PCO_2/pH (the fall in heart rate is greater with the acetazolamide treatment even though the increase in PCO_2 is less), or with the source of the stimulus (both acetazolamide and CO_2 injection into the air-breathing organ produce internal changes in PCO_2/pH). However, to the extent that bradycardia is present, the results suggest that the receptors involved lie outside the gills.

Aquatic hypercarbia has also been shown to produce bradycardia in many, but not all species of water-breathing fish (see Milsom 2012 for review). In some species, it arises from receptors located on all gill arches (Reid et al.

Table 1 Blood gas and cardiorespiratory variables measured in normoxia/normocarbia in control animals from each subgroup (ABO injection fish had intact gill innervation; aquatic CO_2 and acetazolamide fish had their gills denervated)

Experiment	Denervation/intact (D/I)	PCO_2 (mmHg)	pH	fGill (min^{-1})	HR (min^{-1})	fAB (h^{-1})
ABO injection	I	9.71 ± 1.43	7.71 ± 0.06	27.11 ± 2.5*	22.11 ± 1.8*	3.80 ± 2.03
Aquatic CO_2	D	6.74 ± 0.55	7.84 ± 0.03	21.39 ± 1.6*‡	44.46 ± 6.5	3.66 ± 1.83
Acetazolamide	D	7.31 ± 0.49	8.00 ± 0.04	19.78 ± 1.6‡	40.79 ± 10.9	2.73 ± 2.48

One-way ANOVA following Tukey SHD analysis for significance. Different symbols indicate significant differences

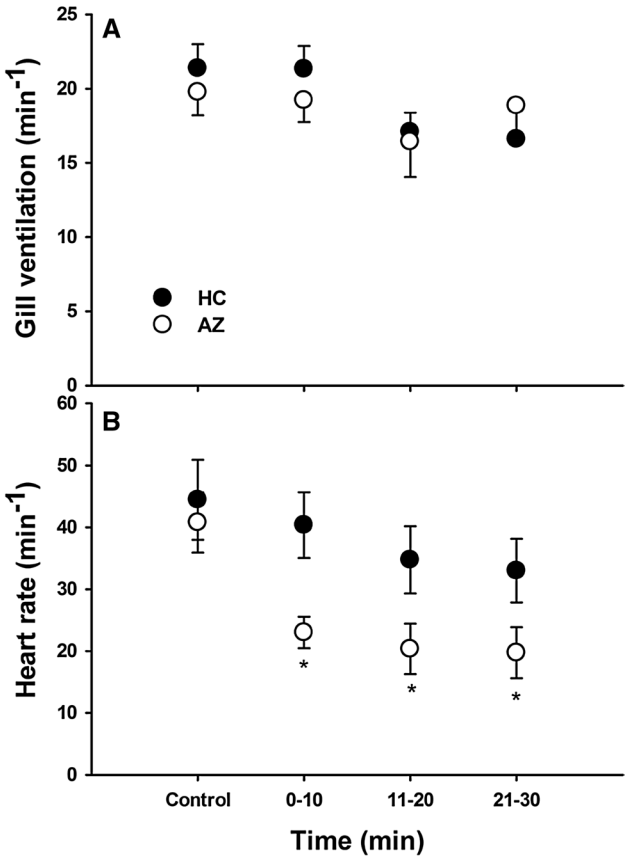

Fig. 3 **a** Gill ventilation frequency and **b** heart rate of denervated *Chitala ornata* exposed to aquatic hypercarbia (to pH 6.0) and acetazolamide. (One-way ANOVA for repeated measurement followed by LSD post hoc test; $p < 0.05$.) Data were presented as mean ± SEM. "Asterisk" indicates values significantly different from the control value

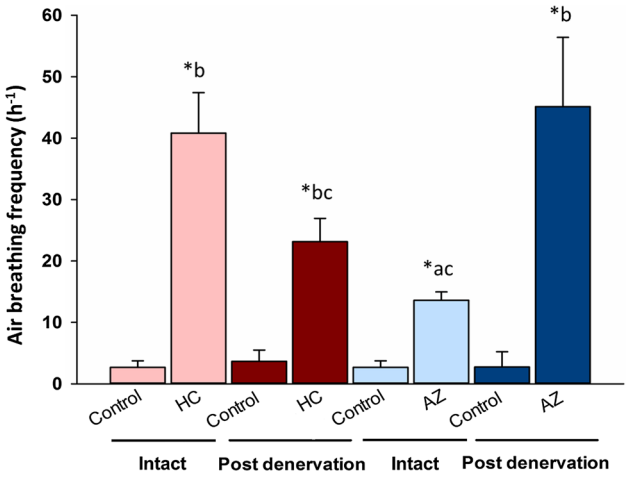

Fig. 4 Air-breathing frequency under control conditions and following exposure to aquatic hypercarbia or following injection of acetazolamide in intact and denervated fish. "Asterisk" indicates a significant difference of values compared to their control, and pair sample *t* test was used. One-way ANOVA followed by Tukey SHD was used to test significant difference of air-breathing frequency among HC, HC post-denervation, AZ and AZ post-denervation; the different alphabet letters indicate significant differences. Values for intact fish (pink bars and light blue bars) were taken from Tuong et al. (2018a, b), while those post-denervation (maroon and dark blue bars) were taken from the present study

2000), while in others it is mediated exclusively by receptors located in the first gill arch (Sundin et al. 2000; McKendry and Perry 2001; Perry and Reid 2002). Aquatic hypercarbia also produces bradycardia in the facultative air-breathing jeju mediated by receptors distributed across all gill arches (de Lima Boijink et al. 2010).

Neither aquatic hypercarbia nor arterial hypercapnia produced by intra-arterial injection of acetazolamide produced any changes in gill ventilation frequency in either intact (Tuong et al. 2018a) or gill denervated *Chitala*. Similarly, injection of CO_2 into the air-breathing organ did not affect gill ventilation. This was also the case in intact *Symbranchus* (Johansen 1966) and *Misgurnus anguillicaudatus* (McMahon and Burggren 1987). Our finding is in contrast to an earlier study on *Chitala chitala* (Dehadrai 1962) and *Hoplerythrinus unitaeniatus* (de Lima Boijink et al. 2010), in which, as levels of aquatic CO_2 increased, aquatic respiration became slower and shallower and eventually stopped with fish resorting to purely aerial respiration. In most obligate water-breathing fishes, however, exposure to aquatic hypercarbia produces increases in gill ventilation (see Milsom 2012 for review). This response arises from receptors that appear to be exclusively branchial, externally oriented and respond specifically to changes in CO_2 and not H^+ (Gilmour et al. 2005).

The putative O_2 chemoreceptors in the gills are the neuroepithelial cells and it has now been shown that while some NECs are sensitive to both O_2 and CO_2, others are sensitive to only O_2 or CO_2 (Qin et al. 2010). This suggests there are different subsets of NECs with different properties. There have been few studies of the location, innervation and orientation of these CO_2-sensitive chemoreceptors. The lack of any changes in gill ventilation in response to internal or external CO_2 raises questions about the presence of any CO_2-sensitive NECs on the gills of *Chitala ornata*.

Air breathing

In several studies on sarcopterygian lungfishes, increasing levels of aquatic CO_2 (> 2–10%) led to inhibition of gill breathing and stimulated air breathing (Johansen et al. 1967, 1970; Graham and Baird 1982; Jesse et al. 1967; Graham 1997; Sanchez and Glass 2001; Sanchez et al. 2005). This was also the case in *Chitala chitala* (Dehadrai 1962). In the jeju (de Lima Boijink et al. 2010) where, following complete gill denervation, there was still a very small but

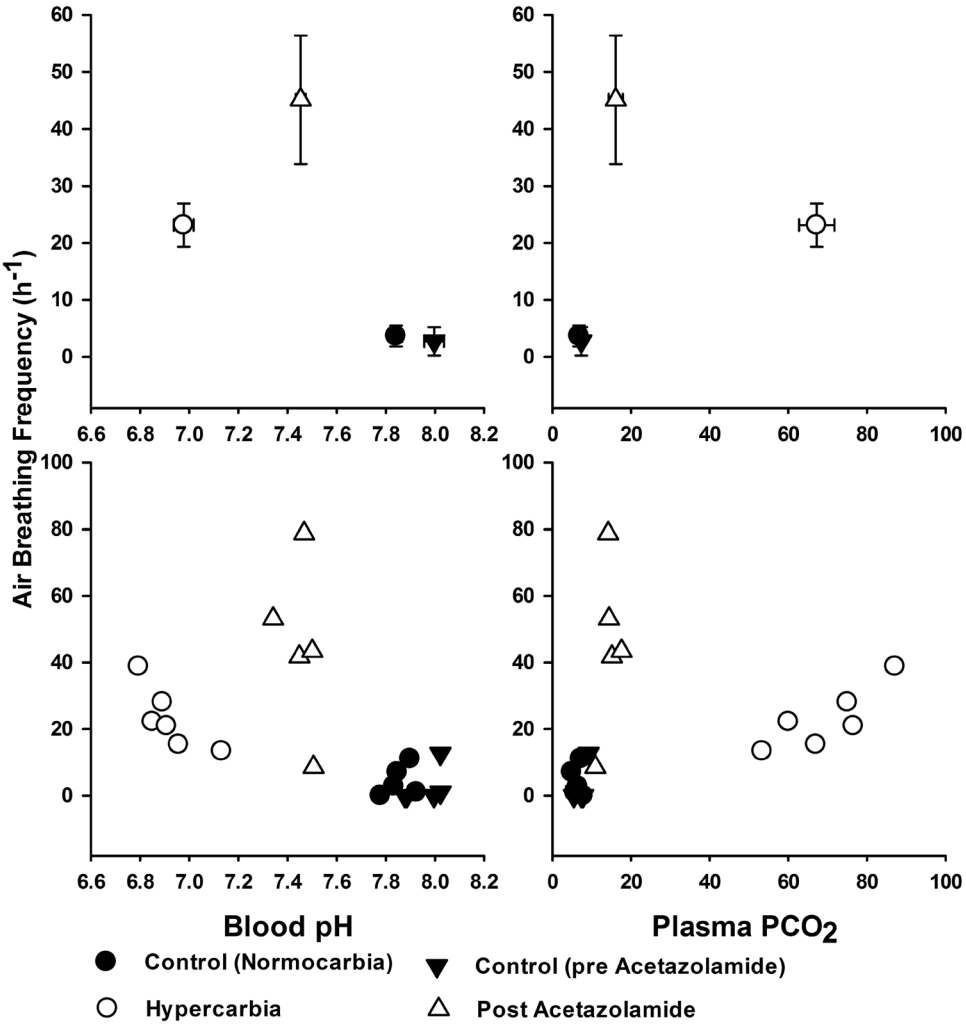

Fig. 5 Air-breathing frequency expressed as function of the arterial pH (left hand panel) or PCO_2 (right hand panel) with all fish gill nerves denervated. The data are shown as mean ± SEM. The two upper panels present mean values (mean ± SEM), while the two lower panels show individual data

significant air-breathing response (one breath every 10 min or 20% of the response seen in intact fish) in five out of ten fish. It was concluded that a small percentage of the air breathing in response to hypercarbia was either mediated by extra-branchial chemoreceptors at some unknown location or by some non-specific stimulus. Bolus injections of CO_2-equilibrated saline that were administered to determine the relative roles of internal versus external chemoreceptors were only administered to fish without access to air raising the possibility that the extra-branchial sites involved in producing the remaining air-breathing response could be internal. In *Chitala*, denervation of the gills also reduced, but did not eliminate the air-breathing response to hypercarbia. In the case of *Chitala*, the reduction was modest (40%) and most of the response remained. Gill denervation also appeared to enhance the air-breathing response to acetazolamide even though the increase in plasma PCO_2 and the decrease in blood pH were greater in hypercarbia than following acetazolamide. The mechanistic basis of this is not at all clear and awaits further investigation. Since fish were exposed to water CO_2 and then acetazolamide, but not the reverse, it is possible that the history of exposure could contribute to the greater responses to the second stressor.

Along with data published by Tuong et al. (2018a), the present data strengthen the evidence for the existence of extra-branchial CO_2 receptors involved in air breathing. Given that both hypoxia and increasing temperature lead to increases in both gill ventilation and air breathing in *Chitala* (Gam et al. unpublished data), this suggests that the responses to hypoxia and to hypercapnia may arise from separate populations of chemoreceptors. Given that these receptors respond to internal changes in PCO_2/pH, and are not associated with blood vessels in the gills, and that there is no pseudobranch, it raises the possibility that the extra-branchial site of CO_2/[H+] chemosensing is within the central nervous system. Central CO_2/H^+ chemoreceptor involved in the production of air-breathing responses have been demonstrated in facultative air-breathing fish species including gar and Siamese fighting fish (Wilson et al. 2000; Corcoran et al. 2007) as well as in the South American

lungfish (Sanchez et al. 2001), but not in the bowfin or the Alaska blackfish (Hedrick et al. 1991; Hoffman et al. 2009). Another extra-branchial site that could be involved is the air-breathing organ itself. Neuroepithelial cells (NECs) that are the putative O_2-sensitive chemoreceptors in fish are also found in the air-breathing organs and lungs of *Amia, Protopterus* and *Polypterus* (Zaconne et al. 2018).

It is not clear whether the internal CO_2/pH-sensitive receptors in *Chitala* respond to changes in the CO_2 or the pH of arterial blood. The data from previous studies suggest that the receptors responding to external stimuli respond specifically to changes in CO_2 in the water (not pH) (Thomas and Le Ruz 1982; Reid et al. 2000; Sundin et al. 2000) while, to the extent that there is evidence for ventilatory responses arising from receptors responding to internal stimuli, there is a better correlation between the changes in ventilation and changes in arterial pH rather than arterial PCO_2 (Heisler et al. 1988; Graham et al. 1990; Wood et al. 1990; Wood and Munger 1994). This also appeared to be the case in our previous study on *Chitala*. While it was not possible to draw any firm conclusions about whether it was the changes in arterial blood PCO_2 or pH that gave rise to the increase in air breathing, there was a slightly better correlation with the changes in pH. The data from the present study yield similar results. However, while the correlations between air breathing and arterial PCO_2 and pH post-denervation for the fish exposed to hypercarbic water versus those injected with acetazolamide are significantly different, they do not provide any further insight.

Conclusions

The air-breathing responses to injection of CO_2 into the air-breathing organ of *Chitala ornata* provide convincing support for the existence of internally oriented cardiorespiratory chemoreceptors monitoring changes in arterial CO_2/H^+. Further, the air-breathing responses of denervated fish to hypercarbia and acetazolamide provide convincing evidence that the responses arise from receptors at an extra-branchial site. While the sum of the data are suggestive that this site could be within the central nervous system, further studies will be required to determine whether or not this is the case. The data do suggest that the responses to hypoxia and to hypercapnia may arise from separate populations of chemoreceptors in *Chitala ornata* and the lack of any changes in gill ventilation in response to internal or external CO_2 raises questions about the presence of any CO_2-sensitive NECs in their gills.

It has now been shown that many species of strictly water-breathing fish show cardiorespiratory responses to aquatic CO_2 (see Milsom 2012 for review). In most, the CO_2-ventilatory reflexes arise exclusively from stimulation of branchial chemoreceptors by CO_2 in the water and not the blood. The physiological significance of this remains obscure, as increased ventilation in the presence of aquatic hypercarbia would enhance, not reduce CO_2 uptake. With the transition to air breathing, CO_2/pH takes on a much larger role in modulating ventilation. Air breathing imposes a rise in arterial PCO_2 compared with water breathing (Rahn 1966). In the air-breathing fishes, the elevation in arterial PCO_2 depends on the degree of isolation of the blood from the water phase. Hence, the facultative forms with large branchial surfaces show arterial PCO_2 levels that are similar to water breathers, whereas the obligate fishes show arterial PCO_2 levels that are significantly elevated toward terrestrial levels (Bayley et al. 2019). These authors further argued that the resting arterial PCO_2 level has a significant impact on the effectiveness of respiratory adjustments of blood pH. With bimodal breathing fish, many species show significant increases in gill ventilation in response to low levels of aquatic CO_2 (< 3%), but further increases in aquatic CO_2 (> 2–10%) lead to inhibition of gill breathing and usually, stimulation of air breathing (de Lima Boijink et al. 2010). The latter should serve to help reduce levels of arterial PCO_2 during episodes of aquatic hypoxia. The *Chitala* in the present study appear to have taken this one step further mounting only an air-breathing response to CO_2 (aquatic or ABO). Interestingly, this has evolved in a facultative air breather with arterial PCO_2 levels that are hardly elevated above those seen in water breathers (Gam et al. 2018).

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