



The effects of dissolved organic carbon on the reflex ventilatory responses of the neotropical teleost (*Colossoma macropomum*) to hypoxia or hypercapnia



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HIGHLIGHTS

- This study tested the hypothesis that reflex ventilatory responses would be impaired in Tambaqui in natural blackwater.
- Commercial humic acid impaired the ventilatory response to hypoxia or hypercapnia.
- Natural blackwater from the Rio Negro or humic acid derived from blackwater was without effect.
- The negative effects of commercial humic acid were absent in the presence of DOC (minus the humic acid fraction)

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ABSTRACT

The tambaqui (*Colossoma macropomum*), migrates annually between whitewater and blackwater rivers of the Amazon. Unlike the whitewater, blackwater is characterized by higher levels of dissolved organic carbon (DOC), including humic acids (HA). Because humic substances impair sensory processes, the current study tested the hypothesis that O₂ and/or CO₂ chemoreception is impeded in blackwater owing to the presence of HA. Thus, the ventilatory responses of tambaqui to hypoxia or hypercapnia were assessed in well water transported from Manaus, local blackwater, and in well water containing HA either extracted from Rio Negro water or obtained commercially (Sigma Aldrich; SA). In well water, tambaqui exhibited typical hyperventilatory responses to hypoxia or hypercapnia. These responses were prevented by simultaneously exposing fish to SA HA (20 mg l⁻¹). The negative effects of SA HA on ventilation were prevented when natural DOC (30 mg l⁻¹; extracted from Rio Negro water after first removing the endogenous HA fraction) was added concurrently, indicating a protective effect of this non-humic acid DOC fraction. The hyperventilatory responses were unaffected during acute exposure or after acclimation of fish to Rio Negro water. HA extracted from Rio Negro water did not impair the hyperventilatory responses to hypoxia or hypercapnia. This study, while demonstrating a negative effect of SA HA derived from peat (coal) on the control of breathing in tambaqui, failed to reveal any detrimental consequences of HA (derived from the decomposition of a variety of lignin-rich plants) naturally occurring in the blackwaters of the Rio Negro.

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1. Introduction

The ability of an organism to sense and respond to a change in its surroundings is a fundamental feature underlying environmental adaptation. In fishes, a well-studied physiological response to environmental perturbation is reflex adjustment of ventilation

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(Shelton et al., 1986). Typically, ventilation is adjusted to regulate total water flow over the gills to levels that are optimal for a particular level of activity (Jones and Randall, 1978) and environmental gas composition (Randall and Shelton, 1963). In fishes, ambient O₂ is the predominant determinant of ventilatory water flow with lowered levels (hypoxia) promoting hyperventilation and higher levels (hyperoxia) leading to hypoventilation [reviewed by Perry et al. (2009)]. The effects of CO₂ on ventilation, while specific and significant, normally are secondary to the effects of O₂ (Tresguerres et al., 2019).

Globally, numerous fish species experience natural fluctuations of ambient O₂ and CO₂ but owing to its great biodiversity, the majority of such species are found in the waters of the Amazon flood basin (Val et al., 2005). Thus, the physiological and behavioural adaptations associated with hypoxia and hypercapnia (elevated CO₂) have been extensively studied in the fishes of the Amazon (Val, 1997; Val et al., 1998, 1999) that experience marked fluctuations in ambient gas levels, both spatially and temporally. Among a suite of adaptations that include air-breathing (Johansen, 1970; Graham, 1997) and aquatic surface respiration (Kramer and McClure, 1982), hyperventilation is a common physiological response in Amazon species (Scott et al., 2008) although the onset of the hyperventilation typically occurs at more severe levels of hypoxia and hypercapnia than in many non-tropical species (Perry et al., 2009). The lower levels of O₂ and higher levels of CO₂ required to initiate ventilatory responses presumably reflects the adaptation of the Amazon species to persistent hypoxic and hypercapnic environments. Owing to respiratory breakdown of organic matter, PCO₂ levels in the Rio Negro can reach 3.2–3.6 mm Hg during the period of high water (Rasera et al., 2013; Amaral et al., 2019) or roughly 10X greater than those typically reported for well-aerated temperate waters. Water PCO₂ levels as high as 60 mm Hg have been recorded in Amazon flood plains during flooding (Furch and Junk, 1997). Water O₂ levels mirror those of CO₂; published values for the Anavilhanas archipelago of the Rio Negro are only 54% air saturation or 82 mm Hg (see Table 1 in Oliveira and Ferreira, 2008). It is important to note that water PO₂ and PCO₂ levels in the Rio Negro are subject to marked spatial, seasonal and diurnal fluctuations with the most severe levels of hypoxia and hypercapnia attained during darkness.

In tambaqui (*Colossoma macropomum*), the hyperventilatory responses to hypoxia and hypercapnia are initiated by activation of O₂/CO₂ chemoreceptors associated with the branchial arches and orobranchial cavity (Sundin et al., 2000; Milsom et al., 2002; Florindo et al., 2004). As in other teleost species [reviewed by Zachar and Jonz (2012)], it is assumed that these chemoreceptors are neuroepithelial cells (NECs) (Dunel-Erb et al., 1982) that have been characterised *in vitro* as polymodal chemoreceptors responsive to hypoxia (Jonz et al., 2004; Burleson et al., 2006; Qin et al., 2010), hypercapnia (Qin et al., 2010) and elevated ammonia (Zhang et al., 2011; Porteus et al., 2020).

Tambaqui are distributed throughout the Amazon basin where they can be found in both whitewater and blackwater rivers, which drain distinct geomorphological formations resulting in marked differences in their optical and physico-chemical properties. Unlike whitewater, that contains higher ionic composition and lower levels of dissolved organic carbon (DOC), the blackwater is characterized by low levels of ions and high DOC concentration, of which humic acids (HA) comprise a significant component (Thurman, 1985). However, certain populations of tambaqui migrate annually between whitewater and blackwater rivers, such as the Rio Solimões and the Rio Negro (Goulding and Carvalho, 1982). Previous studies that have examined the ventilatory responses of tambaqui were conducted on fish obtained from

aquaculture operations and maintained in well water. No studies have assessed the respiratory responses of tambaqui in their natural blackwater, yet it is reasonable to expect that the responses might differ markedly from those in well water because of the presence of HA, which are the largest molecules in the DOC fractions, operationally defined by their precipitation at low pH (<2.0) (Thurman, 1985; Gaffney et al., 1996). These represent a subset of the components of DOC originating from soil and the decomposition of land plants (allochthonous DOC) during flooding. Several previous studies have demonstrated that HA can impair sensory function in fishes including olfaction (Hubbard et al., 2002) and chemical communication (Fisher et al., 2006; Fabian et al., 2007; Mobley et al., 2020), while inhibiting membrane-bound receptors (Yang et al., 2002). As a part of its mode of action, it has been suggested that HA may block or damage chemoreceptors (Fisher et al., 2006). Thus, we hypothesised that tambaqui in blackwater would exhibit attenuated ventilatory responses to hypoxia and hypercapnia owing to the presence of HA. Experiments were conducted during a field expedition to the Anavilhanas archipelago of the Rio Negro using tambaqui obtained from a well water source in Manaus. Although HA of commercial origin (Sigma Aldrich; derived from peat or soft coal) diminished the hypoxic and hypercapnic hyperventilatory responses, an effect that was counteracted by the presence of Rio Negro DOC obtained after first removing the endogenous HA fraction, acute transfer or acclimation to blackwater was without effect as was the addition of HA extracted from Rio Negro DOC.

2. Materials and methods

Experiments were performed in December 2014 aboard a research vessel (the Ana Clara, Manaus, Brazil) or on an adjacent sheltered dock during a field trip to the Anavilhanas archipelago of the Rio Negro [approximately 110 km east (upstream) of Manaus; 2° 43' 10.9"S, 60° 45' 18.8"W; see Johannsson et al. (2017) for map]. Tambaqui (*Colossoma macropomum*; 51.9 ± 4.5 g, N = 32; not all fish were weighed) were obtained from a hatchery in Manaus. Tambaqui raised on aquaculture farms are maintained at a sex ratio of approximately 50/50. The animals used in the current study were young and probably derived from artificial manipulation, which may have slightly unbalanced the sex ratio. The small size of the experimental fish prevented accurate determination of sex. Fish were kept in holding tanks at the Brazilian National Institute for Research of the Amazon (INPA) prior to being transferred to the Ana Clara. At INPA, the fish were held in 3000 L polyethylene tanks supplied with aerated well water at 28 °C. The chemical composition of the INPA water (Wood et al., 2003) resembled the ion-poor water of the Rio Negro blackwater but contained low levels of DOC (in µM; Na⁺ 19, Cl⁻ 21, Ca²⁺ 11, Mg²⁺ 2, K⁺ 16, DOC 0.6 mg l⁻¹, pH 6.0–6.5). Once aboard, all fish were held at a density of 20 fish per tank in 150 L polyethylene tanks at natural photoperiod in recirculating aerated water that was obtained either directly from the Rio Negro, or from INPA via storage in large reservoirs on the Ana Clara. In both cases, the recirculated water flowed continuously through a bio-filter to minimise ammonia accumulation; measured levels of total ammonia (NH₃ + NH₄⁺) never exceeded 250 µM. Partial (~50%) water changes were conducted twice per day. The chemical composition of the Rio Negro water was assumed to be similar to values reported for this same site by Wood et al. (2016) (in µM; Na⁺ 20–30, Cl⁻ 20–30, Ca²⁺ 10–15, Mg²⁺ 3–7, K⁺ 10–15). The DOC measured from this particular location was 8–11 mg l⁻¹ and the pH was 4.0–4.5. Under static holding conditions, the pH of Rio Negro and INPA water midway between water changes was ~6.9 and 6.7, respectively; temperature was 27 °C.

Thus, the fish clearly were modifying the pH of the holding water, presumably owing to ammonia excretion. Fish were unfed for the 12-day duration of the expedition. All procedures complied with Brazilian national and INPA animal care regulations (permit 047/2012).

2.1. Surgical procedures

Depending on their origin (INPA water or Rio Negro water), tambaqui were anaesthetized either in INPA water or Rio Negro water using tricaine methanesulfonate anaesthetic [0.2 g l^{-1} MS-222 (Sigma), adjusted to pH 7.0 with 1.0 g l^{-1} NaHCO_3]. To enable the recording of pressure fluctuations associated with breathing cycles, fish were outfitted either with buccal catheters (PE 160, Clay Adam Intra Medic) implanted into the buccal cavity (secured with surgical silk ligatures) via the anterior region of the snout (Scott et al., 2017) or lower jaw, or the opercular cavity via catheters inserted through an opercular flap. Pilot experiments demonstrated that more reliable data were obtained by measuring buccal cavity pressure changes via a snout catheter, thus the other methods were abandoned, and only data obtained using buccal catheters are reported. During the minor surgery, irrigation of the gills with water was stopped (less than 5 min duration but typically about 1 min). Afterward, fish were allowed to recover either in INPA water or Rio Negro water in opaque covered boxes for at least 2 h prior to experimentation. Admittedly, even such brief periods without gill ventilation coupled with short recovery times, should be avoided in future studies.

2.2. Basic experimental protocol

Except in trials designed to assess breathing in fish acclimated to INPA or Rio Negro water, all experiments utilised a paired design whereby ventilation was monitored first under control conditions and again after manipulation of water chemistry, on the same fish after a recovery interlude of approximately 45 min. With this experimental design, it was important to perform an additional control (sham) experiment in which the measurements were repeated with similar disturbance of the fish but without any alterations to water chemistry. Owing to limited time and relatively few available fish, the effects of hypoxia and hypercapnia were assessed using the same fish. Thus, in 50% of the trials, the fish were exposed first to hypoxia while in the other 50%, fish were exposed first to hypercapnia. Fish were allowed to recover under normoxic/normocapnic conditions for at least 20 min prior to the second trial. In trials using fish acclimated to Rio Negro water, the pH of the water in the holding boxes was adjusted to match the pH of the fish in INPA water. After surgery, these fish were transferred to individual holding boxes (3 L volume) for recovery after which the boxes were moved to the experimental setup; a further waiting period of about 20 min elapsed prior to beginning experiments. Exposures to hypoxia or hypercapnia always were preceded by 10 min of baseline recording.

2.3. Exposures to hypoxia and hypercapnia

After establishing baseline data, fish were exposed to acute hypoxia (final water $\text{PO}_2 = 20 \text{ mm Hg}$) by gassing the boxes with a mixture of O_2 and N_2 provided in the appropriate ratio using precision mass flow controllers (Sierra C100L Smarttrak, SRB Controls, Markham, ON, Canada). Measurements of ventilation during hypoxia were obtained continuously but are reported only after 30 min, at which time ventilation was stable. The fish were allowed to recover under normoxic conditions for 20 min prior to beginning a second period of baseline recording and switching to hypercapnia

($\text{PCO}_2 = 22.5 \text{ mm Hg}$; provided by mixing air and CO_2 with the same precision mass flow controllers) for 30 min. The levels of hypoxia and hypercapnia employed in the present study were similar to those used in previous studies on tambaqui (Sundin et al., 2000; Gilmour et al., 2005). At the conclusion of the experiment, water pH and temperature were recorded from each holding box ($\text{pH} = 6.70 \pm 0.12$; data averaged from 11 different experiments; temperature = $30.3 \text{ }^\circ\text{C} \pm 0.5$; $n = 11$). In trials using HA extracted from Rio Negro water, samples were collected from each box at the end of the experiment and stored at $4 \text{ }^\circ\text{C}$ for subsequent quantification of dissolved organic carbon at INPA (see below).

This experimental design was applied to six separate experimental series that were performed in no particular order. In each case, four fish were examined simultaneously. Note that in all experiments, once the fish had been transferred to their experimental boxes, all further experimental exposures were performed by changing over the water, rather than by moving the fish. The six series were:

2.3.1. Effects of hypoxia and hypercapnia on ventilation in INPA water with and without Sigma Aldrich HA

In these experiments, fish were transferred from holding tanks containing INPA water into boxes containing aerated INPA water. After a first series of measurements using unaltered INPA water, 20 mg L^{-1} (total) of Sigma Aldrich HA was added to the water and a second series of measurements was performed.

2.3.2. Effects of hypoxia and hypercapnia on ventilation in INPA water with and without Sigma Aldrich humic acid in the presence or absence of Rio Negro DOC

In these experiments, fish were transferred from holding tanks containing INPA water into boxes containing aerated INPA water. After a first series of measurements using unaltered INPA water, 20 mg L^{-1} (total) of Sigma Aldrich HA and 30 mg L^{-1} DOC (minus the endogenous HA component) extracted from Rio Negro water (see below) were added to the water and a second series of measurements was performed.

2.3.3. Effects of hypoxia and hypercapnia on ventilation in fish acutely transferred from INPA water to Rio Negro water

In these experiments, fish were transferred from holding tanks containing INPA water into boxes containing aerated INPA water. After a first series of measurements in the unaltered INPA water, the boxes were refilled with pH-adjusted Rio Negro water and a second series of measurements was performed.

2.3.4. Effects of hypoxia and hypercapnia on ventilation in Rio Negro water

In these experiments, fish were transferred from holding tanks containing Rio Negro water into boxes containing aerated Rio Negro water where ventilation was monitored.

2.3.5. Effects of hypoxia and hypercapnia on ventilation in INPA water with and without HA extracted from Rio Negro water

In these experiments, fish were transferred from holding tanks containing INPA water into boxes containing aerated INPA water. After a first series of measurements using unaltered INPA water, HA extracted from Rio Negro water was added to the water and a second series of measurements was performed. The volume of freshly prepared Rio Negro HA that was added was based on an estimate of 640 mg L^{-1} DOC in the reverse osmosis concentrates (see below). Four experiments were completed using HA concentrations of 5.2, 10.0, 19.2 and 35 mg L^{-1} Rio Negro HA; detailed data for the 19.2 mg L^{-1} group are presented.

2.3.6. Control experiments to assess the effects of repeated exposure to hypoxia and hypercapnia

In these experiments, fish were transferred from holding tanks containing INPA or Rio Negro water into boxes containing aerated INPA or Rio Negro water. Two sets of measurements were carried out so as to assess possible effects of trial repetition, with similar disturbance to the fish between trials as per series 2.3.1–2.3.5 (see above).

2.4. Analytical procedures

2.4.1. Breathing measurements

Ventilation frequency (f_V) and amplitude (V_{amp}) were determined by recording pressure oscillations in the buccal cavity using physiological pressure transducers (TSD104A, Biopac Systems, Harvard Apparatus, Montreal, QC, Canada) calibrated against a static column of water and data were logged to a laptop computer using AcKnowledge (Biopac System) data acquisition software (20 Hz data acquisition rate). Average values for f_V (determined manually by counting pressure peaks) and V_{amp} were determined over 20 s intervals just prior to switching to hypoxia or hypercapnia (baseline data) and during the final 60 sec of the 30-min hypoxia or hypercapnia exposure.

2.4.2. Extraction and characterization of DOC from Rio Negro water

Concentrates of DOC were prepared aboard the Ana Clara according to Duarte et al. (2016) by pumping Rio Negro water through a reverse-osmosis unit (Vontronv® ULP21-4021 polyamide membrane, Permutation, model PEOS-0001, Curitiba, Brazil). After collection, the concentrates were treated with a cation exchange resin (Amberlite IR⁻¹18 (H), Sigma-Aldrich, St. Louis, USA), to remove cations that had accumulated during reverse-osmosis. Concentrates were filtered (0.45 μm pore size; Acrodisc™, Pall, Ann Arbor, USA) and stored in opaque bottles at 4 °C prior to use in experiments, extraction of HA or for later determination of DOC concentration.

2.4.3. Extraction of HA

DOC concentrate (~1 L) prepared by reverse osmosis was adjusted to pH 7.0 with 1 M KOH. An aliquot was centrifuged (10,000 rpm \times 10 min) to ensure no solids were present. Upon confirming no (or negligible) suspended solids, the remainder of the DOC concentrate was acidified to pH 1.5 using 65% nitric acid and centrifuged in aliquots (10,000 rpm \times 10 min). The supernatant (containing DOC minus HA) was removed and stored for later use and analysis. The pellets (HA) were re-suspended in distilled water by repeated up-and-down pipetting while raising pH to 7.0. The re-suspended HA solutions either were used in experiments or stored at 4 °C for subsequent analysis of total organic carbon.

2.4.4. Analysis of DOC in Rio Negro DOC concentrates, re-suspended HA solutions and supernatants containing DOC minus HA

All samples were pre-filtered (0.45 μm) and DOC concentration was determined in duplicate through the combustion and oxidation of organic carbon in samples, with subsequent generation of CO₂ that was measured by a non-dispersive infra-red (NDIR) detector using a Total Organic Carbon (TOC) Analyzer (Apollo 900, Teledyne Tekmar). DOC concentrates were previously diluted (100x) in Milli Q water prior the analysis. The equipment was calibrated using a manufacturer-provided stock solution of potassium hydrogen phthalate (KHP) diluted in Milli Q water. After every four samples (in duplicate) a measurement of blank (Milli Q water) and standard solution (10 or 25 mg l⁻¹) was performed to check the accuracy of measurements.

2.5. Statistical analysis

Data are presented as means \pm 1 standard error (SEM). Data were analyzed by two-way repeated measures analysis of variance (RM ANOVA) using exposure to hypoxia or hypercapnia as one factor and treatment as the second factor; separate analyses were carried out for hypoxia and hypercapnia. Where data failed to meet the assumptions of normality and equal variance, they were transformed, or if they could not be transformed to meet the assumption, the analysis was carried out on ranked data. All statistical analyses were carried out using SigmaPlot v 13.0 (Systat Software, San Jose, CA, USA).

3. Results

3.1. The effects of Sigma Aldrich HA on the ventilatory response to hypoxia/hypercapnia

Exposure of tambaqui to hypoxia or hypercapnia using INPA water (low DOC; 0.79 mgL⁻¹ measured in triplicate from a single sample) caused standard hyperventilatory responses (Fig. 1). In both cases, f_V and V_{amp} were increased by 1.5- to 2.2-fold. In a control set of experiments, repeating the sequence of hypoxia/hypercapnia exposures did not affect the hyperventilatory responses (Fig. S1). Although the data were not quantified, there was no obvious effect of exposing fish to hypercapnia on the response to hypoxia and vice-versa. Regardless, in 4 of the 8 trials, the fish were first exposed to hypoxia and the other 4 fish were exposed first to hypercapnia. The acute addition of 20 ml L⁻¹ HA (Sigma Aldrich) prevented the usual hyperventilatory responses with f_V and V_{amp} remaining constant during hypoxia or hypercapnia (Fig. 1). Because there was no significant interaction between the effects of treatment (humic acid) and exposure (hypoxia or hypercapnia), it was not possible to assess the statistical significance of differences within treatment groups in all cases (Fig. 1). It is clear, however, that the overall significant effects of treatment were driven by ventilatory increases occurring within the INPA water group.

To determine whether non-HA components of DOC could protect against the negative effects of Sigma Aldrich HA on ventilation, these experiments were repeated in the presence of DOC extracted from Rio Negro water after the removal of the endogenous HA component. The results clearly demonstrated that the non-HA components of Rio Negro DOC prevented the otherwise attenuating effects of Sigma Aldrich HA on the hyperventilatory responses to hypoxia (Fig. 2A and B) and hypercapnia (Fig. 2C and D). The slightly higher f_V ($P = 0.039$) observed in fish tested in INPA water in the hypoxia trial (Fig. 2A) was largely a result of the lower baseline value in the fish after addition of Rio Negro DOC. Regardless of treatment, all fish exhibited similar hyperventilatory responses.

3.2. The effects of acute exposure or acclimation to Rio Negro water on the ventilatory responses to hypoxia/hypercapnia

The ventilatory responses to hypoxia and hypercapnia in fish acclimated to INPA water were assessed first in INPA water and again after its rapid replacement with Rio Negro (high DOC) water. The hyperventilatory responses to hypoxia were virtually identical regardless of which water was used (Fig. 3A and B). The ventilatory responses to hypercapnia (Fig. 3C and D) also were unchanged by water composition although it is noteworthy that i) the baseline f_V was unusually high in fish preceding hypercapnia after acute addition of Rio Negro water (Fig. 3C) and ii) a statistically significant effect of hypercapnia in increasing V_{amp} was not observed in this experimental series (Fig. 3D).

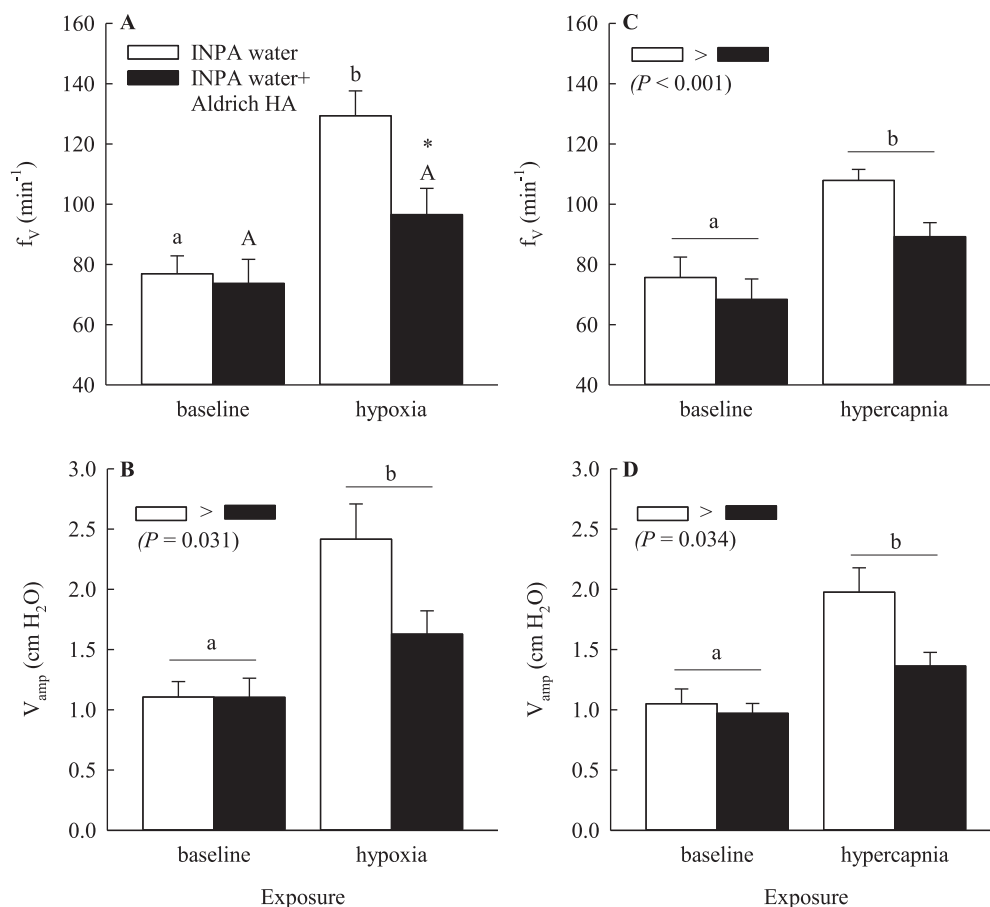


Fig. 1. The effects of Sigma Aldrich HA on the ventilatory responses of tambaqui (*Colossoma macropomum*) to acute hypoxia or hypercapnia. (A, C) Ventilation frequency (f_V) and (B, D) amplitude (V_{amp}) were measured in fish ($N = 8$) exposed to A, B) hypoxia (20 mm Hg) or C, D) hypercapnia (22.5 mm Hg) in INPA well water (white bars) and again after acute addition of Sigma Aldrich HA (20 mg L^{-1} ; black bars). Data are shown as means \pm 1 SEM. Data were analyzed by 2-way RM ANOVA with treatment and exposure as the two factors. For A), $P_{\text{treatment} \times \text{hypoxia}} = 0.028$; different letters indicate a significant effect of exposure within a treatment group and an asterisk indicates a significant effect of treatment within an exposure level. For B-D), effects of treatment and exposure were significant but the interaction term was not (although trends were apparent for B and D); different letters indicate a significant effect of exposure and the fill symbols are used to indicate a significant effect of treatment, i.e. lower ventilation in fish treated with Sigma Aldrich HA (log-transformed data, $P_{\text{treatment}} = 0.031$, $P_{\text{hypoxia}} < 0.001$, $P_{\text{treatment} \times \text{hypoxia}} = 0.077$ for B; rank-transformed data, $P_{\text{treatment}} = 0.001$, $P_{\text{hypercapnia}} < 0.001$ for C; $P_{\text{treatment}} = 0.034$, $P_{\text{hypercapnia}} < 0.001$, $P_{\text{treatment} \times \text{hypoxia}} = 0.057$ for D).

Similar to the fish acutely exposed to Rio Negro water, fish acclimated to Rio Negro water and then assessed in Rio Negro water displayed similar ventilatory responses to the fish acclimated to and assessed in INPA water (Fig. 4A–D). Interestingly, like the fish acutely transferred, the fish in Rio Negro water exhibited increased baseline f_V prior to hypercapnia exposure (Fig. 4C). Fish in this series of experiments also did not exhibit a significant increase in V_{amp} upon exposure to hypercapnia, although the trend was close to statistical significance (Fig. 4D; $P_{\text{hypercapnia}} = 0.059$).

3.3. The effects of HA extracted from Rio Negro water on the ventilatory responses to hypoxia/hypercapnia

The addition of HA extracted from Rio Negro water to a final concentration of 19.2 mg carbon L^{-1} (a level comparable to the final concentration of Sigma Aldrich HA), had no effect on the hyperventilatory responses of tambaqui to hypoxia (Fig. 5A and B) or hypercapnia (Fig. 5C and D). Lower (5.2 mg L^{-1} ; $N = 8$) or higher levels (35 mg L^{-1} ; $N = 8$) of humic acid also were without effect on the hyperventilatory responses to hypoxia or hypercapnia (data not shown).

4. Discussion

The tambaqui, native to the Amazon River watershed, is of great socio-economic and cultural importance to Brazil (Isaac and Ruffino, 1996; Lima et al., 2020). Through extensive aquaculture and capture fisheries, it has emerged as a dominant resource in Amazonas state (Lima et al., 2020). The tambaqui is also becoming recognized as an important model tropical species in fish physiology research [e.g. Wood et al. (2018)] owing to its capacity to thrive in harsh and unstable environments characterised by large fluctuations in water chemistry (Val et al., 1999). In designing the current study, we were particularly interested by the natural annual migration (Goulding and Carvalho, 1982) of tambaqui between the whitewater and blackwater rivers of the Amazon drainage basin. Specifically, we hypothesized that O_2 and CO_2 chemosensory capacities would be diminished in blackwater owing to the presence of HA (Thurman, 1985), substances known to interfere with other chemosensory processes in fishes (Hubbard et al., 2002; Fisher et al., 2006; Fabian et al., 2007; Mobley et al., 2020). In contrast to our predictions of reduced ventilatory responses to hypoxia and hypercapnia in tambaqui in blackwater, the results clearly demonstrated no differences between fish examined

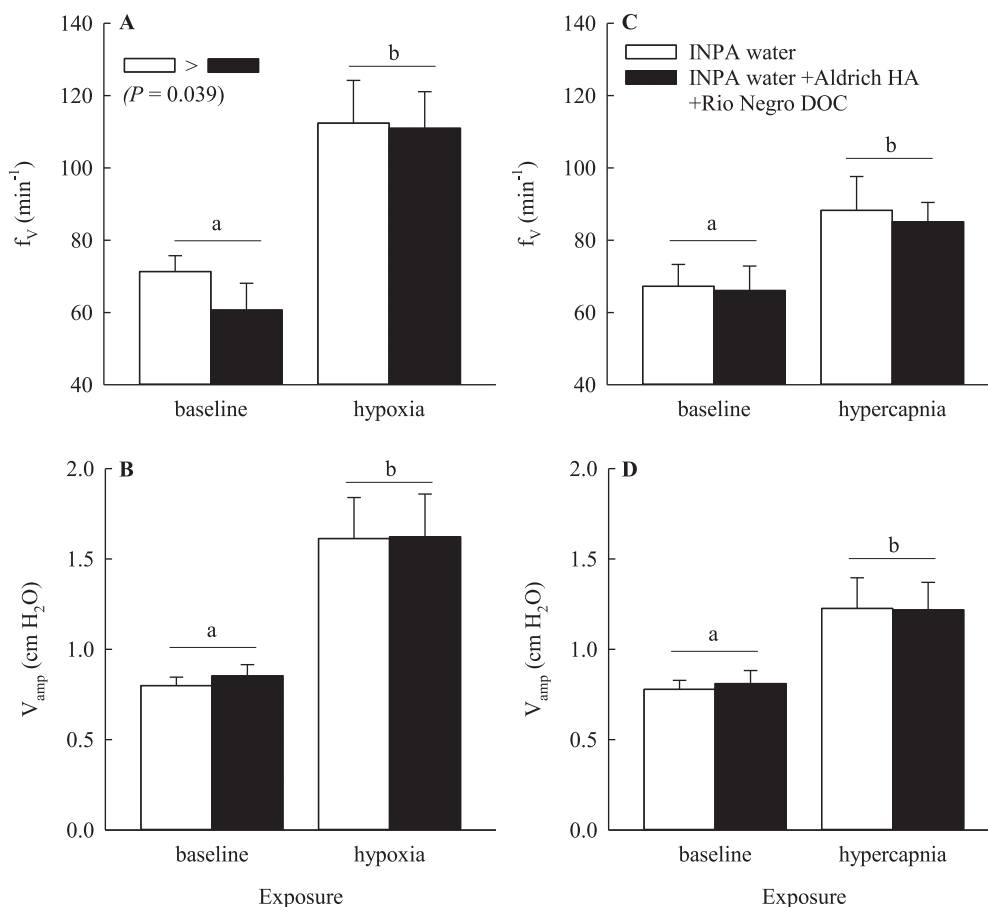


Fig. 2. The non-HA fraction of Rio Negro DOC prevents the inhibitory effects of Sigma Aldrich HA on the ventilatory responses of tambaqui (*Colossoma macropomum*) to acute hypoxia or hypercapnia. (A, C) Ventilation frequency (f_V) and (B, D) amplitude (V_{amp}) were measured in fish ($N = 8$) exposed to A, B) hypoxia (20 mm Hg) or C, D) hypercapnia (22.5 mm Hg) in INPA well water (white bars) and again after acute addition of Sigma Aldrich HA (20 mg L^{-1}) in the presence of DOC extracted from Rio Negro water after first removing the endogenous HA fraction (black bars). Data are shown as means \pm 1 SEM. Data were analyzed by 2-way RM ANOVA with treatment and exposure as the two factors. In A), effects of treatment and hypoxia were significant, but the interaction was not; different letters indicate a significant effect of exposure and the fill symbols are used to indicate a significant effect of treatment, i.e. lower ventilation in fish treated with Sigma Aldrich HA + Rio Negro DOC ($P_{\text{treatment}} = 0.039$, $P_{\text{hypoxia}} = 0.002$). In all other panels, only the effect of exposure was significant, as indicated by the use of different letters ($P_{\text{hypoxia}} = 0.005$ for B; $P_{\text{hypercapnia}} = 0.002$ for C; $P_{\text{hypercapnia}} = 0.012$ for D).

in blackwater or in well water that resembled whitewater but without DOC/HA. These results were particularly intriguing given that HA obtained commercially (Sigma Aldrich) exhibited a distinct inhibitory influence on the ventilatory responses to hypoxia and hypercapnia.

4.1. The basic ventilatory responses to hypoxia and hypercapnia – a critique of the experimental design

In comparison to previous studies that have examined ventilatory responses of tambaqui to hypoxia or hypercapnia (Sundin et al., 2000; Milsom et al., 2002; Gilmour et al., 2005), the baseline breathing frequencies of tambaqui acclimated and assessed in INPA water reported in the current study were markedly higher. The higher baseline f_V in the present study presumably reflects the higher temperatures (approximately 5 °C higher in the present study) of the holding water during experimentation given the well-known relationship between temperature and breathing frequencies in fish (Rantin et al., 2007). Additionally, it is likely that the relatively brief period allowed for fish to recover (necessitated by field conditions) from anaesthesia and handling stress also contributed to the higher baseline f_V . Regardless of the higher f_V , the fish assessed in INPA water exhibited similar ventilatory responses to hypoxia and hypercapnia as reported previously for

tambaqui in well water (Sundin et al., 2000; Milsom et al., 2002; Gilmour et al., 2005). The hyperventilatory responses consisted of increases in both f_V and V_{amp} , although, as reported by Sundin et al. (2000), the V_{amp} response to hypercapnia was highly variable and in some experiments, not statistically significant.

An additional constraint of the current experimental design was the need to use the same fish for both hypoxia and hypercapnia exposures and to repeat these exposures after modifying the water chemistry. Although the period allowed for recovery between exposures was brief (20 min), baseline levels of f_V and V_{amp} clearly were re-established and the magnitude of the ventilatory changes were similar regardless of the order in which the trials were performed (in half the cases, hypercapnia preceded hypoxia and vice versa). Although it is unlikely that resting blood acid-base status was re-established in the 20 min recovery period (Gilmour et al., 2005), f_V and V_{amp} in tambaqui closely track water PCO_2 rather than blood acid-base status (Gilmour et al., 2005). Because the experimental design incorporated a second round of hypoxia and hypercapnia exposures after altering water chemistry (e.g. after adding HA), an additional series of control experiments was performed. In these control experiments, the trials were repeated with similar disturbance of the fish but without any alterations to water chemistry; the ventilatory responses in the second trial were unaltered from the first (Fig. S1). For future experiments, it would be

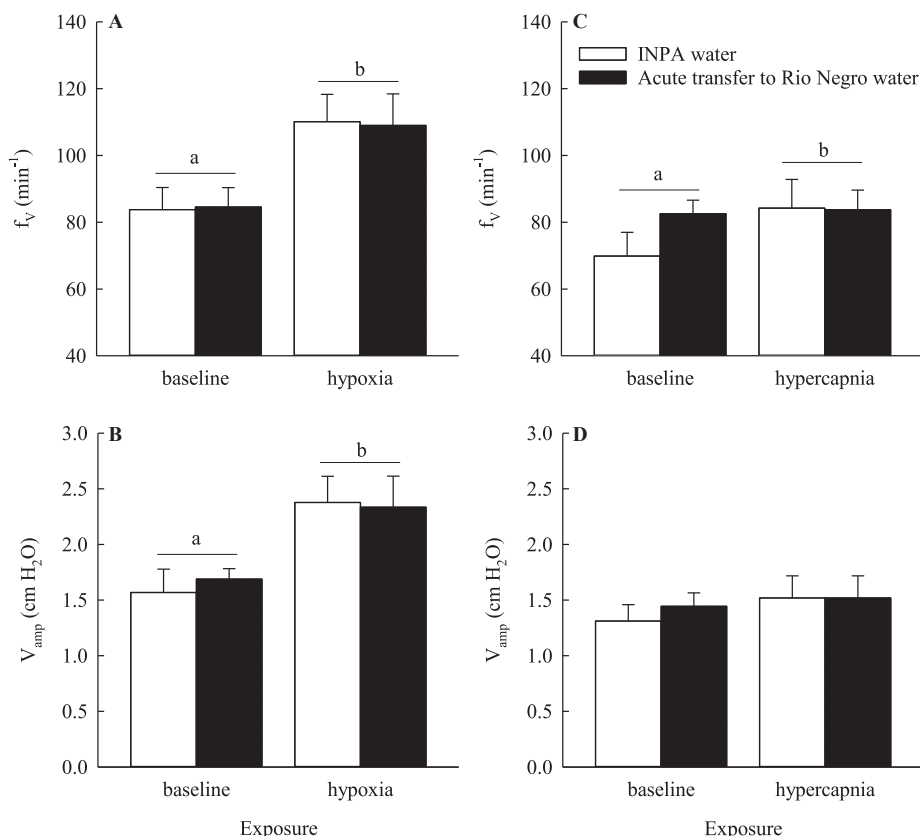


Fig. 3. Acute switch to Rio Negro water does not affect the ventilatory responses of tambaqui (*Colossoma macropomum*) to acute hypoxia or hypercapnia. (A, C) Ventilation frequency (f_v) and (B, D) amplitude (V_{amp}) were measured in fish ($N = 8$) exposed to A, B) hypoxia (20 mm Hg) or C, D) hypercapnia (22.5 mm Hg) in INPA well water (white bars) and again after acutely replacing INPA water with Rio Negro water (black bars). Data are shown as means \pm 1 SEM. Data were analyzed by 2-way RM ANOVA with treatment and exposure as the two factors. Only the effect of exposure was significant, as indicated by the use of different letters ($P_{hypoxia} = 0.002$ for A; $P_{hypoxia} = 0.006$ for B; $P_{hypercapnia} = 0.013$ for C).

preferable to provide fish with longer periods to recover between experimental treatments.

4.2. The interactive effects of Sigma Aldrich HA, and the HA versus non-HA fractions of Rio Negro DOC on chemosensory responses

Initial experiments adopted protocols from previous studies examining the potentially disruptive effects of humic substances on chemosensory processes. Thus, a commercial source of HA (Sigma Aldrich) was used at a concentration comparable to these previous studies (Hubbard et al., 2002; Fisher et al., 2006; Fabian et al., 2007; Mobley et al., 2020). Commercially available HAs such as Sigma Aldrich HA, however, are known to be markedly different than the humic substances present in natural waters (Thurman, 1985) owing to their treatment with chelating agents, elevated ash content, high cation binding capacity and possibly increased reactivity with biological membranes (Morris et al., 2021). Despite such differences in the chemical properties between Rio Negro and Sigma Aldrich HA, it was nevertheless important to perform these initial experiments to enable comparison with the existing literature.

The inhibitory effects of Sigma Aldrich HA on the reflex ventilatory responses to hypoxia or hypercapnia were consistent with previous studies that have examined other sensory responses in fish [e.g. Hubbard et al. (2002)]. The mechanisms underlying the inhibitory effects of Sigma Aldrich HA are unclear. However given that O_2 and CO_2 chemoreception in fish are thought to be initiated by activation of gill chemosensory cells (presumed to be NECs) that can monitor the external and internal environments, the inhibitory

effects of Sigma Aldrich HA are likely mediated by its interference with the detection of external hypoxia and hypercapnia because HA was unlikely to enter the circulatory system within the timeframe of our experiments. It is possible that HA directly impedes the chemosensory properties of these cells, possibly by its interaction with the plasma membrane (Campbell et al., 1997; Al-Reasi et al., 2013b), and ensuing downstream effects on cell signalling. The results of the current study provide additional indirect evidence that the hyperventilatory reflexes associated with hypoxia and hypercapnia in tambaqui are mediated, at least in part, by externally oriented respiratory chemoreceptors as previously suggested (Sundin et al., 2000). In support of a direct effect of Sigma Aldrich HA on gill O_2/CO_2 chemoreceptors was the observation that its effects were counteracted by the presence of DOC extracted from Rio Negro water after removal of the endogenous HA component. This non-HA fraction of Rio Negro DOC would contain fulvic acids (FA, also classified as “humic substances” but with lower molecular weight than HA) and a wide variety of smaller, more hydrophilic molecules (proteins, amino acids, carbohydrates, fatty acids, small organic acids and violacein) (Leenheer, 1980; Thurman, 1985). Thus, these non-HA components of Rio Negro DOC exerted a protective function during exposure of fish to Sigma Aldrich HA. Such protective effects of DOC on physiological processes in fish subjected to environmental stressors have been documented previously (Wood et al., 2011). In particular, DOC is well recognised for its protective influence against metal toxicity (Paquin et al., 2002). While part of this protection is due to the complexation of cationic metals by DOC, reducing their bioavailability, another part of this protection

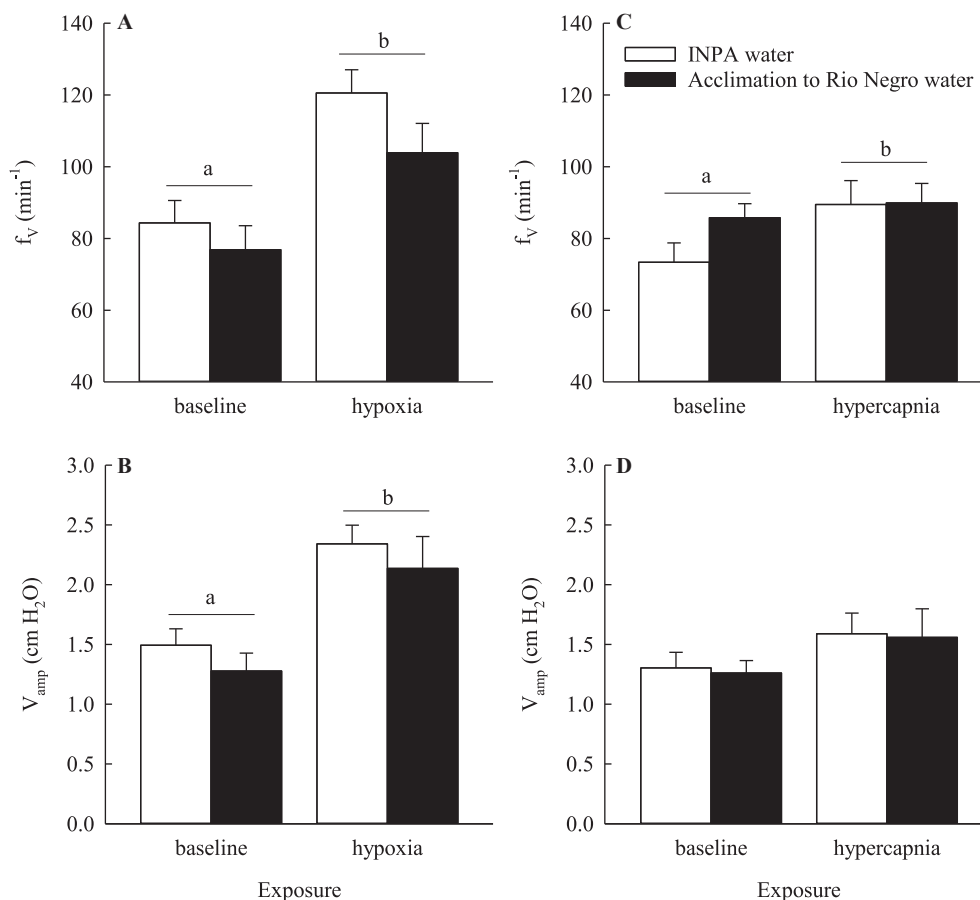


Fig. 4. Acclimation to Rio Negro water does not affect the ventilatory responses of tambaqui (*Colossoma macropomum*) to acute hypoxia or hypercapnia. (A, C) Ventilation frequency (f_v) and (B, D) amplitude (V_{amp}) were measured in fish exposed to A, B) hypoxia (20 mm Hg) or C, D) hypercapnia (22.5 mm Hg) in INPA well water (N = 12–16; white bars) and in a separate group of fish acclimated to Rio Negro water (N = 11; black bars). The data for fish in INPA water represent a collective of the pre-treatment data from Figs. 1–3, 5 and S1). Data are shown as means \pm 1 SEM. Data were analyzed by 2-way RM ANOVA with treatment and exposure as the two factors. Only the effect of exposure was significant, as indicated by the use of different letters ($P_{\text{hypoxia}} < 0.001$ for A; $P_{\text{hypoxia}} < 0.001$ for B; $P_{\text{hypercapnia}} = 0.022$ for C; $P_{\text{hypercapnia}} = 0.059$ for D).

appears to be due to direct positive effects of DOC on ionoregulation, as seen for responses of native cardinal tetras to Cu in Rio Negro water (Crémazy et al., 2016). Indeed, there is now considerable evidence that natural DOCs can also minimize the disruptive effects of acidic soft water on ion homeostasis (Gonzalez et al., 2002, 2005; Wood et al., 2003; Duarte et al., 2016, 2018). Such beneficial effects, however, are not always consistent. Interestingly, Sadauskas-Henrique et al. (2019) reported that DOC extracted from the Rio Negro not only failed to protect tambaqui against ion losses induced by exposure to acidic ion-poor water, but actually exacerbated the ionoregulatory disturbances. In that case however, the chemistry of the DOC appeared to have changed during prolonged storage, thereby complicating interpretation. For this reason, we worked with natural DOC, fresh from the river, or freshly extracted DOC, for all experiments.

Simultaneous to our study, the DOC present at the same site in the Rio Negro was extensively characterized by Johannsson et al. (2017). In brief, their analyses revealed an autochthonous overall nature, high in aromatic (phenolic) compounds (humic substances) of high molecular weight, with approximately equal contributions from HA-like and FA-like components, but the surprising presence of a significant tryptophan-like component as well. The FA and tryptophan-like components, as well as a variety of small hydrophilic molecules, would have been present in our non-HA DOC fraction.

Although acute exposure or acclimation of tambaqui to Rio

Negro water did not affect the hyperventilatory responses to hypoxia or hypercapnia, we reasoned that potential inhibitory effects of endogenous Rio Negro HA might be offset by the non-HA DOC components of the water, as witnessed for Sigma Aldrich HA. Thus, a critical experiment was to isolate the HA from Rio Negro water and to specifically test its effects on ventilation. The results of this particular experiment revealed that Rio Negro HA alone, when used at essentially the same concentration as Sigma Aldrich HA ($\sim 20 \text{ mg L}^{-1}$), was without effect on the ventilatory responses to hypoxia or hypercapnia. Clearly, there are important differences between Rio Negro and Sigma Aldrich HA that allows one to inhibit chemosensory responses while the other is benign. Previous studies have similarly reported differing effects of natural DOC and Sigma Aldrich HA on the physiology of aquatic organisms (Wood et al., 2003; Glover and Wood, 2005). As discussed by Al-Reasi et al. (2013a) and Morris et al. (2021), although Sigma Aldrich HA exhibits high aromaticity (as quantified by its specific absorbance at 340 nm) and high chemical reactivity (as quantified by a Proton Binding Index based on titration), both of which are hallmark signatures of positive DOC effects on ionoregulation and protection against metals, it is chemically different from natural aquatic humic substances. This point was originally noted by Malcolm and MacCarthy (1986) and Chio et al. (1987). Extracted from peat (soft coal), commercial HA has an unusually high ash content and likely has been treated with strong chelating agents to strip away metals. As a result, it may have an unusually high cation binding capacity

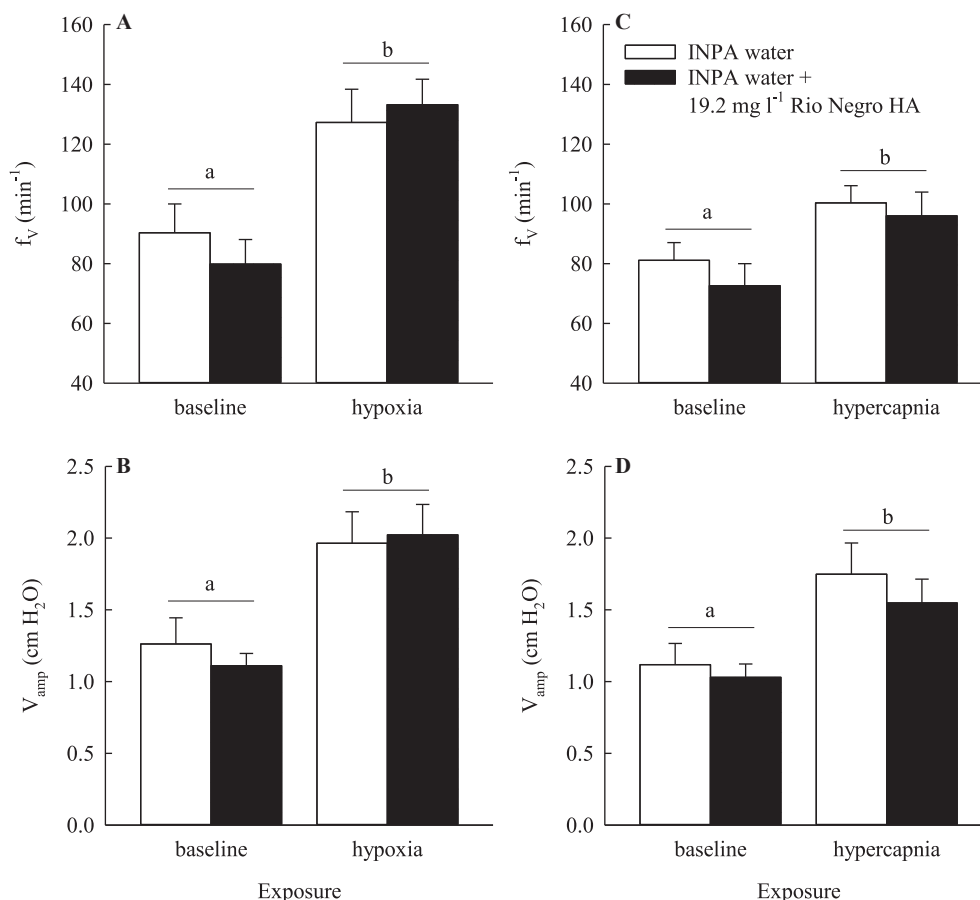


Fig. 5. Acute addition of HA extracted from Rio Negro water does not affect the ventilatory responses of tambaqui (*Colossoma macropomum*) to acute hypoxia or hypercapnia. (A, C) Ventilation frequency (f_v) and (B, D) amplitude (V_{amp}) were measured in fish ($N = 8$) exposed to A, B) hypoxia (20 mm Hg) or C, D) hypercapnia (22.5 mm Hg) in INPA well water (white bars) and again after acutely adding humic acid (19.2 mg L^{-1}) extracted from Rio Negro water (black bars). Data are shown as means \pm 1 SEM. Data were analyzed by 2-way RM ANOVA with treatment and exposure as the two factors. Only the effect of exposure was significant, as indicated by the use of different letters ($P_{\text{hypoxia}} < 0.001$ for A; $P_{\text{hypoxia}} = 0.001$ for B; $P_{\text{hypercapnia}} < 0.001$ for C; $P_{\text{hypercapnia}} = 0.001$ for D).

and reactivity with biological membranes. Therefore, its biological actions may be very different from those of naturally occurring aquatic HA.

The absence of any effect of Rio Negro blackwater or extracted Rio Negro HA on the chemosensory response of tambaqui to hypoxia or hypercapnia does not necessarily eliminate possible deleterious effects at other locations of the Rio Negro or at other times of the year. For example, other regions of the Rio Negro are known to contain markedly higher levels of DOC (and thus humic acid), reaching 30 mg L^{-1} (Walker, 1995)(R.M. Mendonca, unpublished data). Seasonally, DOC levels and chemistry will vary according to precipitation rates (Leenheer, 1980; Holland et al., 2017). Clearly, however, at the particular location on the Rio Negro of our field study (coordinates $2^{\circ}23'41''\text{S}$, $60^{\circ}55'14''\text{W}$; approximately 110 km upstream of Manaus, Brazil) and time of year (December), there is no apparent impediment of the naturally occurring HA on the control of breathing in tambaqui (*C. macropomum*). One could argue that the lack of any detrimental effects of natural DOC (in particular HA) on O_2 and CO_2 chemoreception is an expected result arising from natural selection in which tambaqui became adapted to the constraints of their environment by developing chemoreceptors with insensitivity to natural DOC.

5. Conclusions

The neotropical teleost fish *Colossoma macropomum* (tambaqui)

migrates annually through whitewater and blackwater rivers of the Amazon basin, the latter being enriched with DOC, including HA. The aim of this study was to determine whether high levels of HA impaired O_2 and/or CO_2 chemoreception in light of previous studies reporting deleterious effects of HA on sensory processes in fish. Although commercial humic acid caused an attenuation of the ventilatory response to hypoxia or hypercapnia, natural blackwater or humic acid derived from blackwater was without effect. Thus, it is concluded that the capacity of tambaqui to respond to these environmental stimuli likely is unaltered during migrations.

Author statement

Steve F. Perry: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Funding acquisition; Kathleen M. Gilmour: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Funding acquisition; Rafael M. Duarte: Methodology, Formal analysis, Investigation, Writing – review & editing; Chris M. Wood: Methodology, Writing – review & editing, Validation; Vera M. F. Almeida-Val: Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing; Adalberto L. Val: Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.130314>.

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