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Reciprocal modulation of O_2 and CO_2 cardiorespiratory chemoreflexes in the tambaqui

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Abstract

This study examined the effect of acute hypoxic and hypercapnic cardiorespiratory stimuli, superimposed on existing cardiorespiratory disturbances in tambaqui. In their natural habitat, these fish often encounter periods of hypoxic hypercapnia that can be acutely exacerbated by water turnover. Tambaqui were exposed to periods of normoxia, hypoxia, hyperoxia and hypercapnia during which, externally oriented O_2 and CO_2 chemoreceptors were further stimulated, by administration into the inspired water of sodium cyanide and CO_2-equilibrated water, respectively. Hyperoxic water increased the sensitivity of the NaCN-evoked increase in breathing frequency (f_R) and decrease in heart rate. Hypoxia and hypercapnia attenuated the increase in f_R but, aside from blood pressure, did not influence the magnitude of NaCN-evoked cardiovascular changes. Water PO_2 influenced the magnitude of the CO_2-evoked cardiorespiratory changes and the sensitivity of CO_2-evoked changes in heart rate and blood flow. The results indicate that existing respiratory disturbances modulate cardiorespiratory responses to further respiratory challenges reflecting both changes in chemosensitivity and the capacity for further change.

Keywords: Cardiorespiratory reflexes; Oxygen and carbon dioxide chemoreceptors; Environmental hypoxia/hypercapnia; Tambaqui (*Colossoma macropomum*)

1. Introduction

In water-breathing fish, all O_2 and pH/CO_2 cardiorespiratory-related chemoreception occurs at chemoreceptor loci on the gill arches and/or in the orobranchial cavity (e.g. Randall and Jones, 1973; Butler et al., 1977; Smatresk et al., 1986; Burleson and

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Smatresk, 1990; McKendry et al., 2001). These chemoreceptors monitor O_2 and CO_2/pH levels in the water (externally oriented) or arterial blood (internally oriented) and, once stimulated, they initiate cardiorespiratory and hormonal responses including an increase in breathing, a decrease in heart rate (Taylor and Butler, 1982; McKenzie et al., 1995; see Smatresk, 1990; Burleson et al., 1992; Milsom et al., 1999; Perry and Gilmour, 2002 for reviews) and the release of adrenal catecholamines (Perry and Reid, 2002; Reid and Perry, 2003). The gill arch chemoreceptors are innervated by branches of the glossopharyngeal and/or vagus nerves while the chemoreceptors in the oro-branchial cavity are innervated by branches of the trigeminal and/or facial nerves (Butler et al., 1977; Burleson et al., 1992). Those chemoreceptors on the first gill arch and pseudobranch, innervated by the glossopharyngeal nerve, are likely the evolutionary pre-cursor of the mammalian carotid body (Jonz et al., 2004). Unlike mammals (Nattie, 1999), and other air-breathing vertebrates (Milsom, 2002), water-breathing fish, so far studied, do not possess central (brain) respiratory-related pH/CO_2 chemoreceptors (Gilmour, 2001; Milsom et al., 2002).

Tambaqui (*Colossoma macropomum*) are water-breathing, acid-tolerant (Wilson et al., 1999) and hypoxia-tolerant (p50 = 2.4 mmHg O_2; Brauner et al., 2001) fish that perform aquatic surface respiration (ASR) under conditions of severe environmental hypoxia in "varzea" lakes and lagoons of the Amazon River basin (Val and Almeida-Val, 1995; Rantin and Kalinin, 1996). Under these conditions, they remain at the surface of the water where they skim the O_2-rich surface layer. To facilitate this, the lower lip swells and acts as a funnel that directs the surface water into the mouth and over the gills (Saint-Paul, 1988). Recent studies have examined the distribution, innervation and function of the peripheral O_2 and CO_2/pH chemoreceptors in this species (Sundin et al., 2000; Milsom et al., 2002; Reid et al., 2003; Florindo et al., 2004; Gilmour, Milsom, Rantin, Reid and Perry, unpublished). Despite indirect evidence, it is not yet clear whether tambaqui possess separate (or distinct) populations of peripheral O_2 and CO_2/pH chemoreceptors or whether sensing of both stimulus modalities occurs in the same cell.

In mammals and some other vertebrates, hypoxic and hypercapnic stimuli often act synergistically (e.g., Tenney and Brooks, 1966; Lahiri and DeLaney, 1975a,b; Daristotle et al., 1987; West et al., 1987; see reviews by Gonzalez et al., 1994; Lahiri and Forster, 2003). Indeed, for any given level of PO_2, there is a greater amount of carotid sinus nerve discharge when CO_2 levels are increased. Furthermore, the slope of the PCO_2-carotid sinus nerve discharge curve increases as PO_2 levels are lowered.

With the exception of life in underground environments (Boggs and Birchard, 1989), healthy, terrestrial air-breathing animals are rarely, if ever, exposed to simultaneous changes in inspired O_2 and CO_2. Fish, on the other hand, are often exposed to environmental (aquatic) hypoxia and/or hypercapnia (elevated inspired CO_2). This is particularly true for tropical and Neotropical fish, such as tambaqui, who inhabit waters in which O_2 and CO_2 levels can fluctuate on a daily basis due to photosynthetic O_2 production during the day and its absence during the night. Indeed, O_2 levels within these aquatic environments can be supersaturated during the day but severely hypoxic, or completely anoxic during the night (Val and Almeida-Val, 1995). Additionally, hypoxic and/or hypercapnic conditions can occur in stagnant ponds and pools that are formed following the end of the annual flood cycle (Val and Almeida-Val, 1995). There are many adaptations (genetic, physiological and behavioural), including aquatic surface respiration described above, that have evolved to facilitate survival under these conditions (Graham, 1997).

Since O_2 and CO_2 levels can fluctuate rapidly in these aquatic environments, it is likely that severe hypoxic or hypercapnic challenges may arise when the water is already mildly to moderately hypoxic or hypercapnic. Given this, and the potential for interactive and synergistic effects between O_2 and CO_2, we examined how existing respiratory challenges affected the acute cardiorespiratory responses to a further, more severe, respiratory challenge. We hypothesised that simultaneous respiratory challenges would produce augmented cardiorespiratory responses provided that the scope for change was still present.

2. Materials and methods

2.1. Experimental animals

Juvenile tambaqui (*C. macropomum*) (1.37 ± 0.09 kg; $N = 6$) were obtained from CAUNESP (Aqua-

culture Center of the São Paulo State University – UNESP), Jaboticabal, São Paulo, Brazil, and transported to the Federal University of São Carlos. These fish were descendants of native tambaqui taken from the Amazon in 1993 and introduced into the south-eastern region of Brazil for aquaculture. Animals were maintained outdoors in fibreglass aquaria supplied with aerated water from an artesian well. Temperature was maintained at 25 °C, and the animals were fed ad libitum every second day. Experiments were performed between January and March.

2.2. Animal preparation

Animals were anaesthetised in an aqueous solution of benzocaine (100 mg/l) pre-dissolved in 2 ml of 70% ethanol. During surgery the gills were ventilated with a second solution of benzocaine (50 mg/ml) gassed with air. Impedance electrodes were sutured to each operculum to monitor the breath-by-breath displacement of the operculum as a measure of ventilation rate and amplitude. Using a Dremel tool, a hole was drilled in the snout between the nostrils. A flared cannulae (PE160) was fed through the hole and secured. This cannula was used to administer doses of NaCN and CO_2-equilibrated water into the inspired water stream to stimulate externally oriented O_2 and CO_2 chemoreceptors, respectively.

To monitor arterial blood pressure and heart rate, the caudal artery was cannulated as described by Axelsson and Fritsche (1994). Briefly, a lateral incision (2 cm) was made at the level of the caudal peduncle under the lateral line. The underlying epaxial muscle was cut to expose the vertebrae. The caudal artery was cannulated in the anterograde direction by blind puncture. The wound was closed and the cannula was sutured to the body wall. To measure blood flow (cardiac output) a 3S ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the ventral aorta (VA). To accomplish this, the operculum was reflected forward and a small incision was made parallel to the ventral aorta within the opercular chamber. The VA was exposed by blunt dissection and the flow probe was placed around the vessel using lubricating jelly as an acoustic couplant. This approach avoided any damage to the pericardium; the probe lead was attached to the skin with surgical sutures. Fish were allowed to recover for a minimum of 24 h prior to experimentation. During both recovery from surgery and experimentation, fish were housed in individual black Plexiglas boxes supplied with flowing, aerated (or experimentally gassed; see below) water.

2.3. Experimental protocol

2.3.1. The effects of water O_2 and CO_2 status on cyanide-evoked cardiorespiratory reflexes

This series of experiments examined whether the cardiorespiratory reflexes elicited by acute stimulation of externally oriented O_2 chemoreceptors would be altered or modulated by the persistent background level of the water partial pressure of O_2 (PwO_2) or CO_2 ($PwCO_2$). Although internally applied NaCN (into the arterial blood) can also induce cardiorespiratory reflexes in this species, the responses to externally applied NaCN are substantially greater (Sundin et al., 2000). Tambaqui were exposed to the following conditions: (1) normoxia (PwO_2 = 140 mmHg; $PwCO_2$ = 1–2 mmHg); (2) hyperoxia (PwO_2 = 600 mmHg; $PwCO_2$ = 1–2 mmHg); (3) hypoxia (PwO_2 = 80 mmHg; $PwCO_2$ = 1–2 mmHg) and (4) hypercapnia (PwO_2 = 140 mmHg; $PwCO_2$ = 24 mmHg; approximately 3.5% CO_2). The levels of hypoxia and hypercapnia were chosen, based on our previous work (Sundin et al., 2000), to stimulate O_2 and CO_2 chemoreceptors, respectively, whilst producing sub-maximal cardiorespiratory effects. Following a 20-min period under each condition, a series of NaCN injections (0.02, 0.05, 0.1, 0.5 mg/ml NaCN in water) was administered through the snout cannula, into the inspired water, to stimulate externally oriented O_2 chemoreceptors. A control injection of water was also administered. The O_2 and CO_2 levels in the injected NaCN and water were equivalent to the O_2 and CO_2 levels within the experimental chamber at the time of injection. The doses of NaCN were administered at least 10 min apart in a random order and consisted of 2 ml of the appropriate dose injected over 10 s.

2.3.2. The effects of water oxygen status on CO_2-evoked cardiorespiratory reflexes

2.3.2.1. Administration of CO_2-equilibrated water. This series of experiments examined whether the cardiorespiratory reflexes elicited by acute stimulation of

externally oriented CO_2 receptors would be altered or modulated by the persistent background level of water PO_2. A recent study (Gilmour, Milsom, Rantin, Reid and Perry, unpublished) demonstrated that injections of hypercapnic saline, into the arterial blood, were not effective in eliciting cardiorespiratory reflexes in this species. On the other hand, CO_2-equilibrated water, injected into the inspired water stream elicited changes in ventilation and cardiovascular reflexes. Furthermore, these evoked responses were CO_2-specific and did not occur if acidified water (titrated to the pH levels obtained with these various levels of CO_2) was injected in place of CO_2-equilibrated water. The fish were exposed to the normoxic ($PwO_2 = 140$ mmHg), hypoxic ($PwO_2 = 80$ mmHg) and hyperoxic ($PwO_2 = 600$ mmHg) conditions described above. Following a 20-min period under each condition, a bolus (50 ml/kg over 20 s) of CO_2 (1, 3, 5 and 10%)-equilibrated water was administered through the snout cannula, into the inspired water, in order to stimulate externally oriented CO_2 chemoreceptors. Under normoxic, hypoxic and hyperoxic conditions the O_2 level in the CO_2-equilibrated water was adjusted to 140, 80 and 600 mmHg, respectively. A control injection of water was also administered. The doses of CO_2-equilibrated water were administered at least 10 min apart in a random order.

2.3.2.2. Hypoxic and hyperoxic hypercapnia. In this series of experiments, fish were exposed to 20 min of either hypoxic hypercapnia ($PwO_2 = 80$ mmHg; $PwCO_2 = 24$ mmHg) or hyperoxic hypercapnia ($PwO_2 = 600$ mmHg; $PwCO_2 = 24$ mmHg). Cardiorespiratory variables were assessed prior to the initiation of hypercapnia (i.e., when the water was normocapnic and either hypoxic or hyperoxic) and during the last 2 min of the hypoxic hypercapnia and hyperoxic hypercapnia periods. We did not expose the animals to normoxic hypercapnia in this study as the comparison between hypoxic hypercapnia and hyperoxic hypercapnia was sufficient to examine the effects of the water O_2 level on the cardiorespiratory responses to aquatic hypercapnia. Our previous studies (Milsom et al., 2002; Reid et al., 2003; Florindo et al., 2004; Gilmour, Milsom, Rantin, Reid and Perry, unpublished) have all examined normoxic hypercapnia in this species.

2.4. Analytical techniques

Using a peristaltic pump, water was withdrawn from the snout cannulae or, during times of injection, from a separate cannula placed in the vicinity of the fish's head. Water was pumped through thermostatted cuvettes containing PO_2, PCO_2 and pH electrodes (Cameron Instruments) to measure these variables within the inspired water. The O_2 electrode was calibrated using a solution of sodium sulphite (2 g/l; $PwO_2 = 0$ mmHg) and air-equilibrated water ($PwO_2 = 140$ mmHg). The CO_2 electrode was calibrated using appropriate gas mixtures produced by a Cameron GF-3/MP gas mixing flow controller. The pH electrode was calibrated by pumping precision buffers across the electrodes.

Blood pressure was measured by connecting the caudal artery cannula to a pressure transducer (Bell and Howard) connected to a BIOPAC DA100 pressure amplifier; calibration was achieved with a static column of water. Blood flow was determined by connecting the ultrasonic flow probe (factory calibrated) to a blood flow meter (Model T106; Transonic Systems). Breathing was measured by impedance by connecting the opercular impedance leads to an impedance converter (UFI, model 2991).

2.5. Data and statistical analysis

All data were recorded using a BIOPAC MP100 data acquisition system in conjunction with Acknowledge software and a PC. The rate of analog to digital conversion was 40 Hz per recorded variable. This acquisition system allowed for continuous recordings of all variables, namely PwO_2, $PwCO_2$, pHw, ventilation amplitude (V_{amp}; determined by subtraction of the breath-by-breath minimum opercular displacement value, measured by the impedance converter, from the maximum value), ventilation frequency (f_R; automatic rate calculation from the raw impedance trace), mass-specific blood flow (V_b), heart rate (f_h; automatic rate calculation from the pulsatile blood flow trace), mean dorsal aortic pressure (P_a; arithmetic mean) and systemic resistance (R_s; P_a/V_b).

The data are reported as the mean ± 1 standard error of the mean (S.E.M.). Data for all variables were compiled as mean values for 1 min preceding an

injection of NaCN or CO_2-equilibratred water through the snout cannula. Mean measurements of each variable were then compiled for 10 s intervals for the first 60 s post-injection and then for 1 min intervals at 2, 3 and 4 min post-injection. Total ventilation was calculated as the product of ventilation amplitude and frequency at a given measurement time. The time course of these responses, for the highest dose of NaCN (0.5 mg/ml; Figs. 2–5) or CO_2-equilibrated water (10% CO_2; Figs. 7–8), is plotted on the left panels of Figs. 2–5, 7 and 8. Given that the maximum response to an injection of NaCN or CO_2-equilibrated water did not always occur at the same time, the maximum response, within the first 2 min post-injection, was also determined and the resultant values (i.e., maximum value minus pre (starting) value) are plotted, as a function of NaCN or CO_2 dose, on the right panels of Figs. 2–5, 7 and 8. For hypoxic and hyperoxic hypercapnia, the values before and 15 min after the initiation of hypercapnia are plotted (Fig. 10). To determine whether or not the sensitivity of the responses to NaCN or CO_2 were altered by the water oxygenation status or hypercapnia, Hill Plots were constructed from each individual dose–response curve (from each animal) to yield an EC_{50} value from each experiment. These values were then compiled into a global mean.

The effects of any given dose of NaCN or CO_2, on all variables, were determined using a one-way repeated (RM) measures analysis of variance (ANOVA) followed by a post hoc multiple comparison test. The effects of the different levels of O_2 within the water (normoxia versus hypoxia versus hyperoxia), on the time course of the responses, were statistically analysed using a two-way RM ANOVA followed by a multiple comparison test. The effects of the different levels of water oxygenation (or hypercapnia) on the maximum response minus the starting value in response to all treatments (NaCN or CO_2) were statistically analysed using a two-way RM ANOVA. All statistical testing, including the determination of normality and equal variance was performed with statistical software (SigmaStat 3.0; SPSS). In all cases, the software executed the appropriate parametric or non-parametric tests, including the most appropriate multiple comparison post hoc test. The limit of statistical significance was taken to be 5% ($p < 0.05$).

3. Results

3.1. The effects of water oxygenation status on NaCN-evoked cardiorespiratory reflexes

Fig. 1 illustrates the effects of external NaCN administration on breathing (impedance), pulsatile blood flow and dorsal aortic blood pressure under normoxic and hyperoxic conditions. NaCN caused, under both conditions, an increase in breathing (Fig. 1A and D), a decrease in heart rate (visible on both the blood flow and blood pressure traces) and a decrease, followed by a slight increase, in blood pressure (which, in this example, was more pronounced in the normoxic trace). In this example, the NaCN-evoked increase in breathing under normoxic conditions was very rapid, whereas the increase was delayed by approximately 1 min under hyperoxic conditions.

3.1.1. NaCN-evoked respiratory reflexes

Fig. 2A, C and E illustrates the time course of the respiratory responses to 0.5 mg/ml NaCN. Fig. 2B, D and F illustrates the magnitude of the response (maximum value minus starting value) as a function of [NaCN]. Under all conditions, NaCN caused an increase in breathing frequency (Fig. 2A; $^{*}p < 0.001$). The breathing frequency response was blunted during hypoxia ($^{\#}p < 0.001$) and augmented during hyperoxia ($^{\#}p = 0.017$), compared to the response during normoxia (Fig. 2A). The breathing frequency response to NaCN was different under hyperoxic compared to hypoxic conditions ($^{\&}p < 0.001$). Based on the dose–response curve (Fig. 2B), the breathing frequency response to NaCN was augmented under hyperoxic conditions compared with normoxic ($^{\#}p = 0.024$) and hypoxic ($^{\&}p = 0.004$) conditions.

Under normoxic conditions, 0.5 mg/ml NaCN caused an increase in breath amplitude (Fig. 2C; $^{*}p = 0.016$). The increases observed during hyperoxia and hypoxia were significant, compared to the pre value, based on the one-way RM ANOVA ($p = 0.019$ and 0.022, respectively) although the multiple comparison test did not identify specific differences. The response under hypoxic conditions was significantly less than under hyperoxic conditions (Fig. 2C; $^{\&}p = 0.006$). When the data are plotted as a dose–response curve, there were increases, under all three conditions (Fig. 2D; *), in breath amplitude at the higher doses

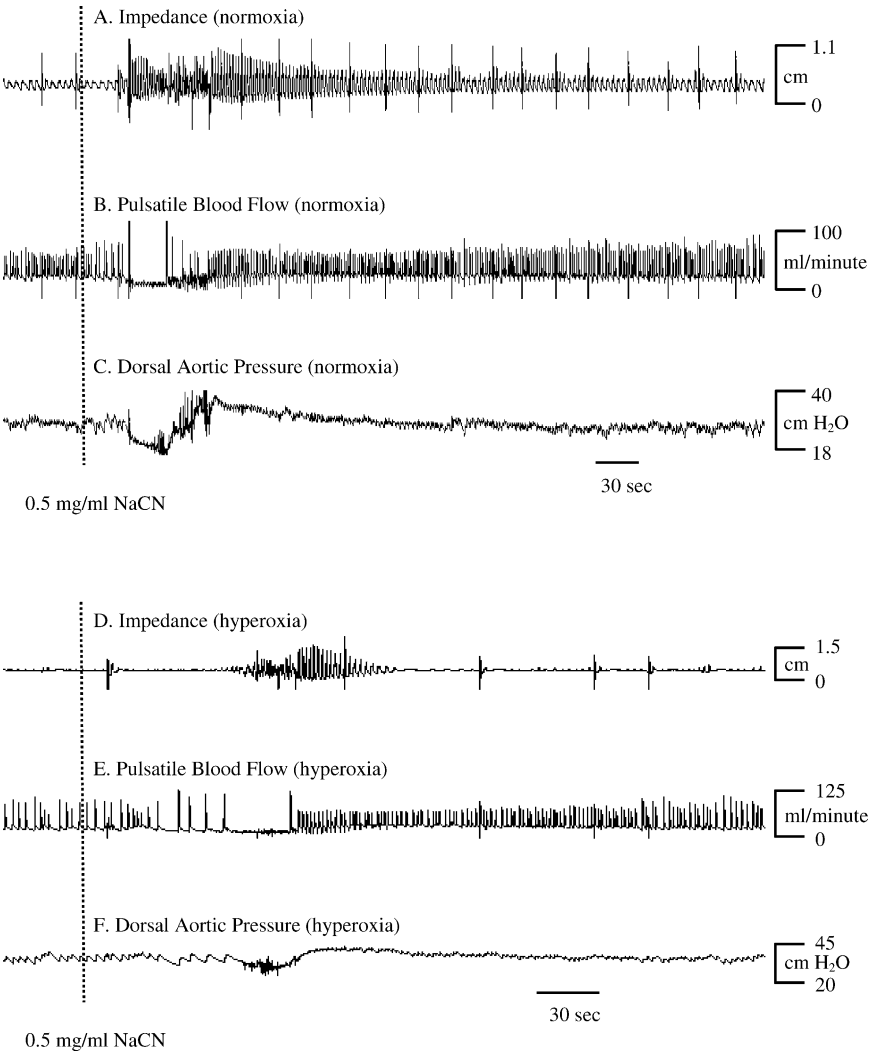

Fig. 1. Traces illustrating the changes in breathing (impedance, cm; A and D), pulsatile blood flow (ml/min; B and E) and dorsal aortic pressure (cmH_2O; C and F) in response to externally administered NaCN (0.5 mg/ml) under normoxic (A–C; PwO_2 = 140 mmHg) and hyperoxic (D–F; PwO_2 = 600 mmHg) conditions. In each trace, the dashed line represents the time at which NaCN was administered. The large regular deflections (panels A and B) are likely coughs that are being detected as disturbances by both the impedance converter and the blood flow probe.

of cyanide (normoxia, $p < 0.001$; hyperoxia, $p = 0.030$; hypoxia, $p = 0.019$). The increase was less under hypoxic conditions compared to the response during normoxia ($^{\#}p = 0.002$; Fig. 2D).

In response to 0.5 mg/ml NaCN (Fig. 2E) total ventilation increased (*) under all conditions (normoxia, $p = 0.003$; hyperoxia, $p = 0.005$; hypoxia, $p < 0.001$). The overall response, to 0.5 mg/ml NaCN, during hypoxia was less than the response during normoxia ($^{\#}p = 0.027$) and hyperoxia ($^{\&}p = 0.005$). There was a trend for the magnitude of the overall ventilatory response to NaCN to be augmented during hyperoxia and attenuated during hypoxia (Fig. 2F).

The EC_{50} value for the NaCN-evoked increase in breathing frequency (Table 1) was less under hyperoxic conditions (0.029 ± 0.004 mg/ml NaCN) compared to hypoxic conditions (0.055 ± 0.008 mg/ml) but

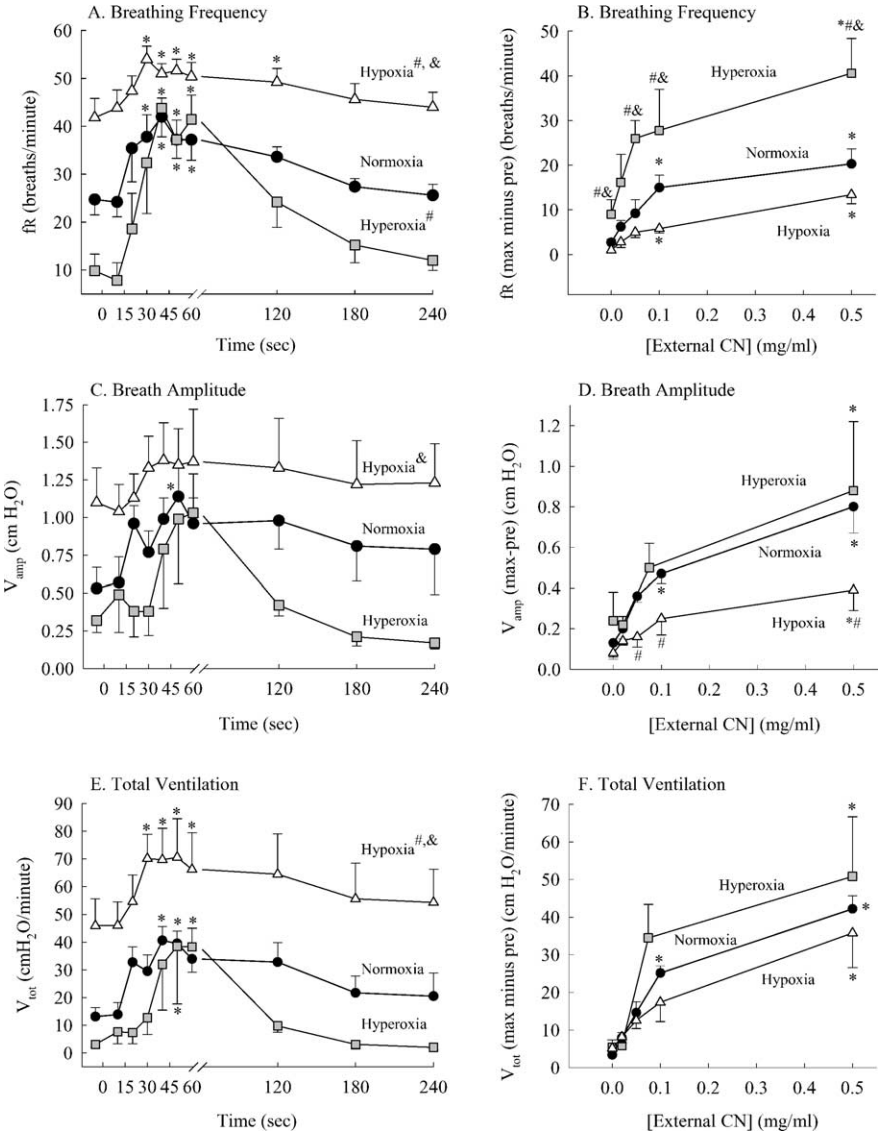

Fig. 2. External cyanide-evoked respiratory chemoreflexes in tambaqui exposed to normoxic (140 mmHg PwO_2; black circles), hypoxic (80 mmHg PwO_2; open triangles) and hyperoxic (600 mmHg PwO_2; grey squares) water. Panels A (breathing frequency; breaths/min), C (breath amplitude; cmH_2O) and E (total ventilation; cmH_2O/min) illustrate the time course of changes in response to administration of 2 ml of 0.5 mg/ml NaCN through the external cannula, into the buccal cavity. The injection was initiated immediately following the first data point. Panels B, D and F are dose–response curves for the maximum response minus the starting value of breathing frequency (B), breath amplitude (D) and total ventilation (F) for external NaCN doses of 0, 0.02, 0.05, 0.1 and 0.5 mg/ml under normoxic, hypoxic and hyperoxic conditions. In panels A, C and E, an asterisk (*) represents a difference from the starting value (the first data point) in each of the individual groups (one-way repeated measures (RM) ANOVA). A number sign (#) illustrates that the time course for changes during hypoxia or hyperoxia were different from those during normoxia (two-way RM ANOVA). An ampersand (&) sign illustrates a difference in the time course during hypoxia and hyperoxia (two-way RM ANOVA). In panels B, D and F, an asterisk (*) represents a difference from the value at [external NaCN] = 0. A number sign (#) denotes a significant difference from the value under normoxic conditions; an ampersand (&) sign indicates a significant difference between hyperoxia and hypoxia. The data are shown as the mean ± 1 standard error of the mean.

Table 1
EC_{50} values (mg/ml NaCN) for external cyanide-evoked cardiorespiratory reflexes under normoxic/normocapnic, hypoxic, hyperoxic and hypercapnic conditions

	Normoxia	Hypoxia	Hyperoxia	Hypercapnia
f_R	0.035 ± 0.008	0.055 ± 0.008	$0.029 \pm 0.004^{\&}$	0.035 ± 0.012
V_{amp}	0.038 ± 0.005	0.030 ± 0.004	0.033 ± 0.001	0.042 ± 0.009
V_{tot}	0.040 ± 0.004	0.041 ± 0.003	0.038 ± 0.008	0.035 ± 0.006
f_h	0.043 ± 0.006	0.037 ± 0.005	$0.021 \pm 0.004^{\#,\&}$	0.033 ± 0.006
P_a	0.046 ± 0.006	0.073 ± 0.022	0.057 ± 0.011	0.053 ± 0.011
V_b	0.051 ± 0.009	0.047 ± 0.006	0.048 ± 0.012	0.041 ± 0.004
R_s	0.120 ± 0.005	0.120 ± 0.014	0.091 ± 0.005	0.150 ± 0.011

EC_{50} values were determined from Hill plots.
$^{\#}$ Difference from normoxia ($P = 0.009$).
$^{\&}$ Difference between hypoxia and hyperoxia ($P = 0.033$ and 0.004 for f_R and f_h, respectively).

not different from the value under normoxic conditions (0.035 ± 0.008 mg/ml).

3.1.2. NaCN-evoked cardiovascular reflexes

In response to 0.5 mg/ml NaCN, heart rate decreased under normoxic, hypoxic and hyperoxic conditions (Fig. 3A; $p < 0.001$). Water oxygenation status did not affect the magnitude (Fig. 3A) of the heart rate changes in response to NaCN injections (0.5 mg/ml), nor the dose–response curve (Fig. 3B; $p = 0.173$). Bolus injections of 0.5 mg/ml NaCN also caused a decrease in dorsal aortic blood pressure under all three conditions (Fig. 3C; normoxia, $p = 0.016$; hyperoxia, $p < 0.001$; hypoxia, $p < 0.001$). Under hypoxic conditions, the NaCN-evoked decrease in dorsal aortic blood pressure was attenuated at the lower doses of cyanide (Fig. 3D; $^{\#}p = 0.048$). NaCN caused a decrease in blood flow (Fig. 3E) and an increase in systemic vascular resistance (Fig. 3G) under all three conditions. The decrease in blood flow (Fig. 3F; $p = 0.951$) and increase in systemic resistance ($p = 0.067$; Fig. 3H) were not affected by the water oxygenation status. The EC_{50} value for the NaCN-evoked decrease in heart rate (Table 1) was less under hyperoxic conditions compared to the value under both hypoxic and normoxic conditions.

3.2. The effects of aquatic hypercapnia on NaCN-evoked cardiorespiratory reflexes

3.2.1. Respiratory chemoreflexes

Under hypercapnic conditions, breathing frequency (Fig. 4A), breath amplitude (Fig. 4C) and total ventilation (Fig. 4E) were elevated (prior to NaCN) compared to the normoxic normocapnic control animals. All respiratory variables increased ($^{*}p < 0.001$) with cyanide treatment under hypercapnic conditions. Overall, the magnitude of the breathing frequency response (Fig. 4A) to NaCN was less ($^{\#}p = 0.019$) under hypercapnic conditions while the magnitude of the amplitude response (Fig. 4C) was not different ($p = 0.094$). Hypercapnia reduced the breathing frequency response to the two highest doses of NaCN (Fig. 4B; $^{\#}p = 0.031$). Hypercapnia did not reduce the magnitude of the increase in breath amplitude (Fig. 4D; $p = 0.409$) and total ventilation (Fig. 4F; $p = 0.928$). The EC_{50} values (Table 1) for NaCN-evoked respiratory reflexes were not altered by aquatic hypercapnia.

3.2.2. Cardiovascular chemoreflexes

During hypercapnia, NaCN caused a decrease ($p < 0.001$) in heart rate (Fig. 5A), dorsal aortic blood pressure (Fig. 5C) and blood flow (Fig. 5E) but an increase in systemic vascular resistance (Fig. 5G; $p = 0.007$). The changes in these variables were not different from those seen under normocapnic conditions. Aquatic hypercapnia did not alter the EC_{50} for NaCN-evoked cardiovascular reflexes (Table 1).

3.3. The effects of water oxygenation status on CO_2-evoked cardiorespiratory reflexes

An example of the effects of external CO_2 administration on breathing, pulsatile blood flow and dorsal aortic blood pressure under normoxic conditions are illustrated in Fig. 6. The CO_2 treatment caused a very transient and slight increase in breathing, a transient decrease in heart rate and a small decrease in blood pressure.

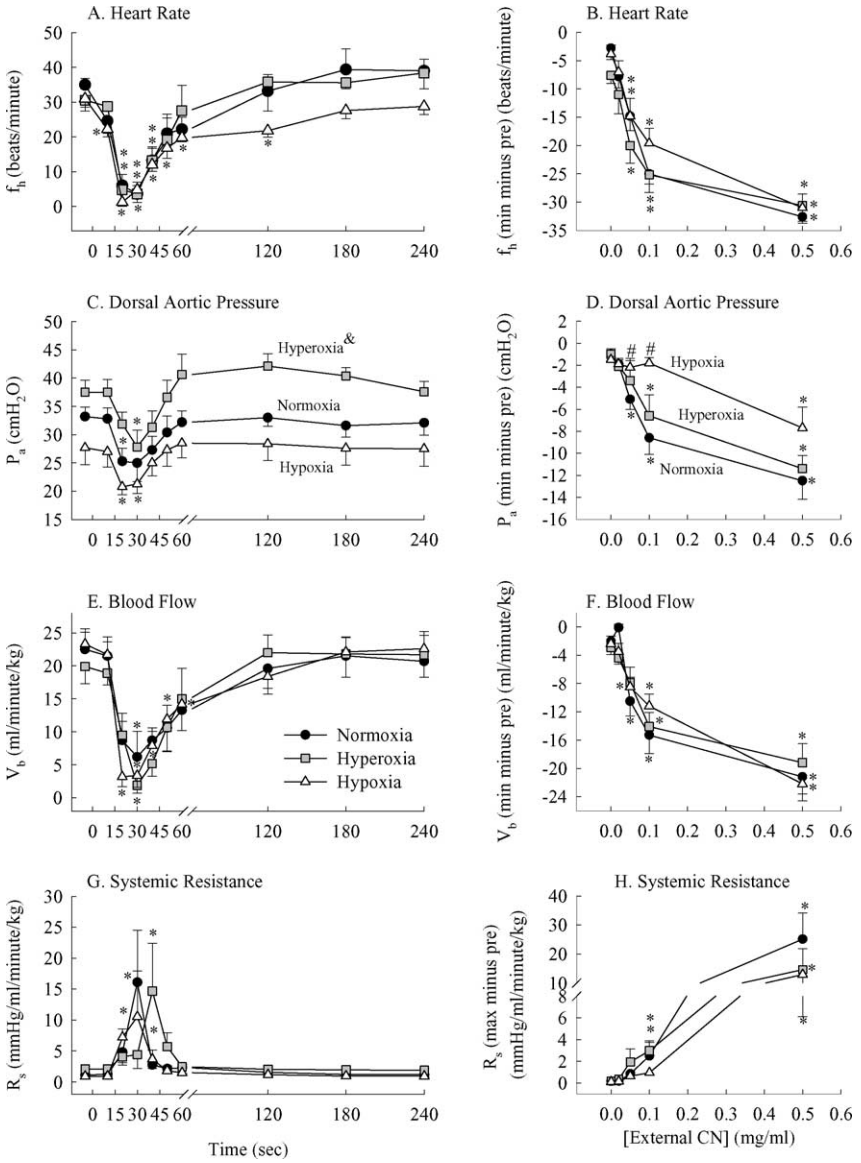

Fig. 3. External cyanide-evoked cardiovascular chemoreflexes in tambaqui exposed to normoxic, hypoxic and hyperoxic water. Panels A, C, E and G illustrate the time course of change in response to administration of external NaCN (0.5 mg/ml). Panels B, D, F and H are dose–response curves for the maximum response minus the starting value as a function of the external NaCN dose. The symbols are the same as those in Fig. 2.

3.3.1. *Respiratory chemoreflexes*

Externally injected CO_2-equilibrated water had no significant effect on breathing frequency under any condition when the data are plotted as the time course response to the highest dose of CO_2 (Fig. 7A). When the data are plotted as the difference between the maximum and starting values (i.e., the dose–response curve; Fig. 7B) the response under hyperoxic conditions was augmented compared to the response during normoxia (#) and hypoxia ($^{\&}p = 0.026$).

There was no significant increase in ventilation amplitude (Fig. 7C) and total ventilation (Fig. 7E) under any of the gas conditions in response to the boluses of 10% CO_2-equilibrated water (time course data). When

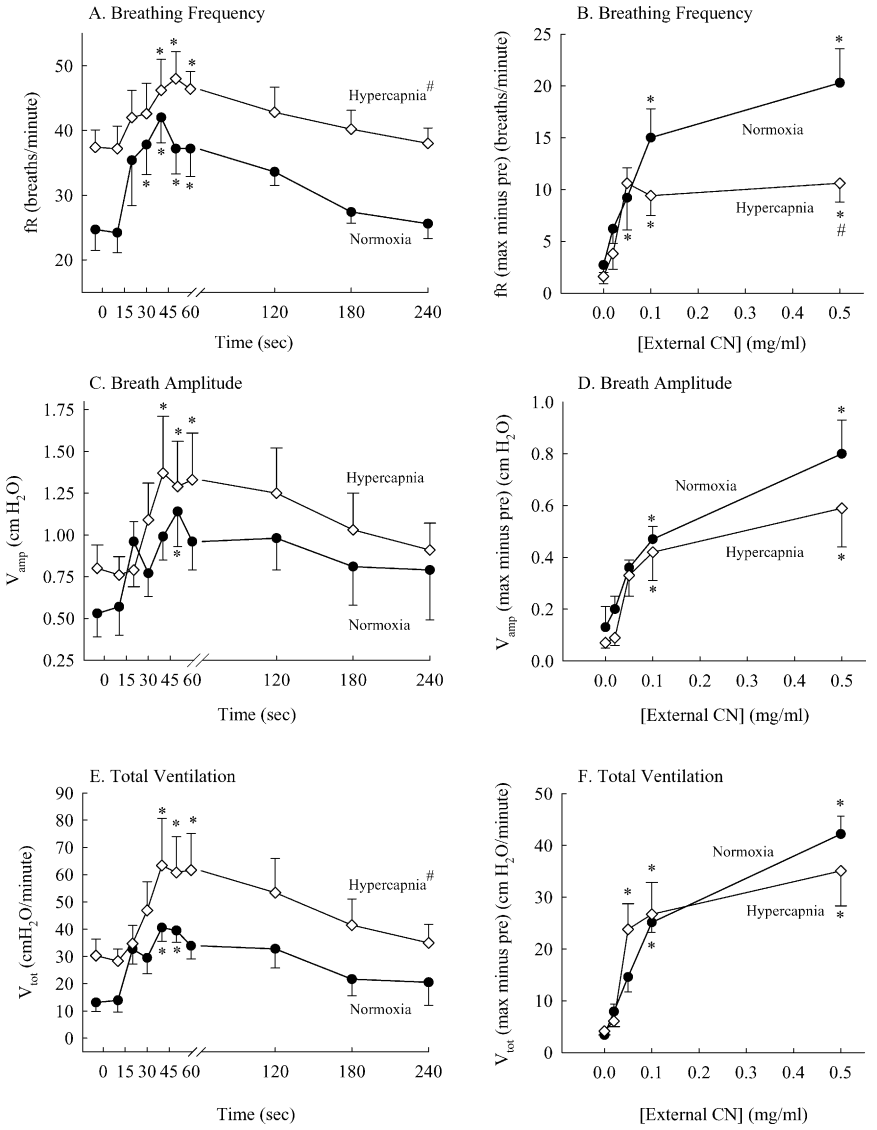

Fig. 4. External cyanide-evoked respiratory chemoreflexes in tambaqui exposed to normoxic normocapnic (140 mmHg PwO_2; 0 mmHg $PwCO_2$; black circles) and hypercapnic (water gassed with 3.5% CO_2; approximately 24 mmHg $PwCO_2$; open diamonds) water. Panels A, C and E illustrate the time course of change in response to administration of external NaCN (0.5 mg/ml). Panels B, D and F are dose–response curves for the maximum response minus the starting value as a function of the external NaCN dose. In panels A, C and E, an asterisk (*) represents a difference from the starting value (the first data point) in each of the individual groups (one-way RM ANOVA). A number sign (#) denotes a significant difference from the normoxic normocapnic conditions. In panels B, D and F, an asterisk (*) represents a difference from the value at [external NaCN] = 0. A number sign (#) denotes a significant difference from the value under normoxic normocapnic conditions.

the breath amplitude and total ventilation data are expressed as the maximum value post-CO_2 minus the starting pre-CO_2 value (Fig. 7D and F), there was an increase (*) in both amplitude and total ventilation under hyperoxic, but not normoxic or hypoxic conditions. It was not possible to calculate EC_{50} values for external CO_2-evoked respiratory chemoreflexes from Fig. 7.

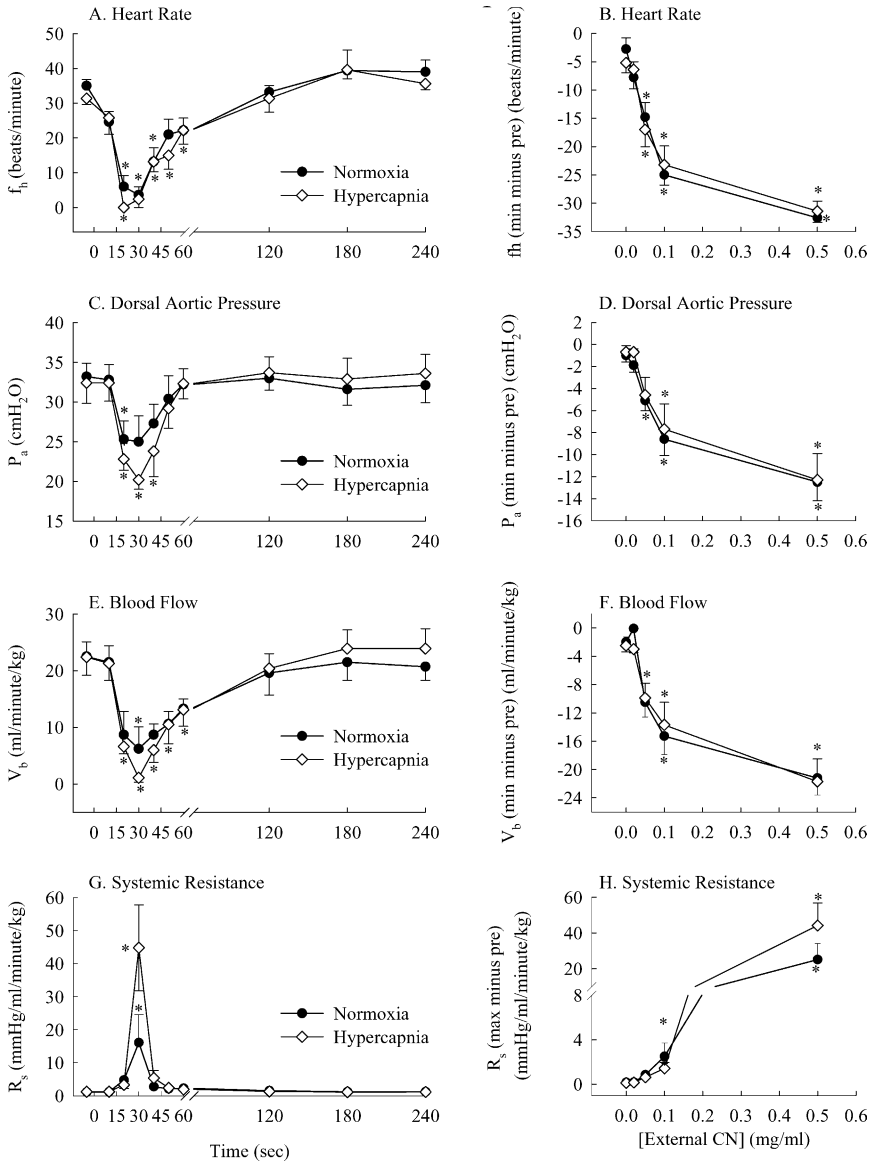

Fig. 5. External cyanide-evoked cardiovascular chemoreflexes in tambaqui exposed to normoxic normocapnic and hypercapnic water. Panels A, C, E and G illustrate the time course of change in response to administration of external NaCN (0.5 mg/ml). Panels B, D, F and H are dose–response curves for the maximum response minus the starting value as a function of the external NaCN dose. The symbols are the same as those in Fig. 4.

3.3.2. *Cardiovascular chemoreflexes*

External administration of 10% CO_2-equilibrated water caused a decrease in heart rate (Fig. 8A) during normoxia ($p = 0.006$), hyperoxia ($p < 0.001$) and hypoxia ($p = 0.006$). Hypoxia blunted the external CO_2-evoked bradycardia (Fig. 8B; $^{\&}p = 0.008$). The EC_{50} for the CO_2-evoked decrease in heart rate (Table 2) was significantly less during hyperoxia than during normoxia.

Following the injection of external CO_2, there was a trend for dorsal aortic pressure to decrease and then increase (Fig. 8C) although these changes were not statistically significant (normoxia,

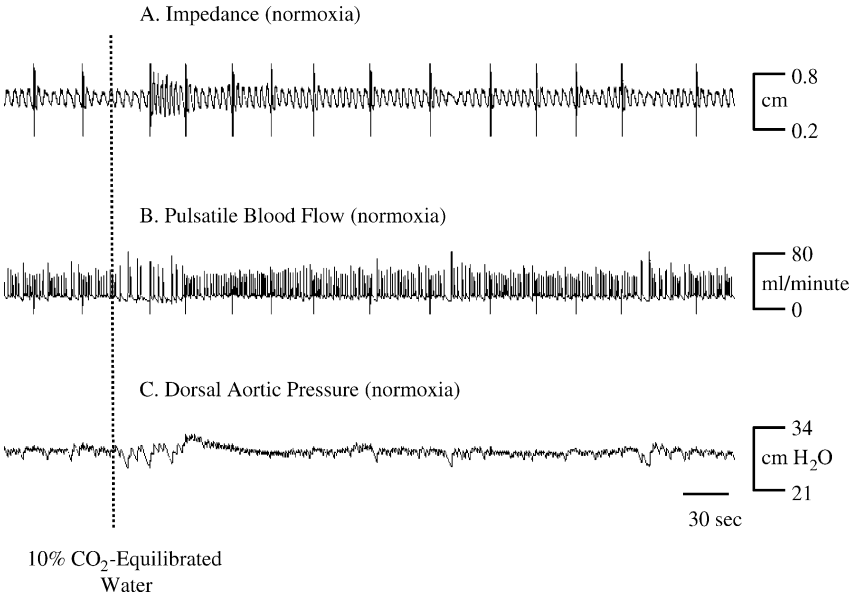

Fig. 6. Traces illustrating the changes in breathing (impedance, cm; A), pulsatile blood flow (ml/min; B) and dorsal aortic pressure (cmH_2O; C) in response to externally administered 10% CO_2-equilibrated water during normoxic (PwO_2 = 140 mmHg) conditions. In each trace, the dashed line represents the time at which CO_2-equilibrated water was administered.

$p = 0.740$; hypoxia, $p = 0.140$; hyperoxia, $p = 0.068$). Water oxygenation status did not significantly influence the effects of external CO_2 on P_a (Fig. 8D; Table 2).

External CO_2 (10%) caused a decrease in blood flow under all three conditions (Fig. 8E). There was a trend for hyperoxia to augment, and hypoxia to blunt, the magnitude of the CO_2-evoked decrease in blood flow (Fig. 8F). The EC_{50} value (Table 2) for the CO_2-induced decrease in blood flow was significantly less under hypoxic conditions compared to normoxic conditions.

Table 2
EC_{50} values (%CO_2) for external CO_2-evoked cardiovascular reflexes under normoxic/normocapnic, hypoxic and hyperoxic conditions

	Normoxia	Hypoxia	Hyperoxia
f_h	2.08 ± 0.27	1.14 ± 0.46	1.10 ± 0.19[#] ($p = 0.045$)
P_a (max)	2.16 ± 0.40	2.88 ± 0.59	2.85 ± 0.17
V_b	2.34 ± 0.41	1.01 ± 0.26[#] ($p = 0.027$)	1.57 ± 0.12
R_s	3.37 ± 0.59	1.96 ± 0.21	2.56 ± 0.01

EC_{50} values were determined from Hill plots.
[#] Significant difference from normoxia.

Systemic resistance increased in response to administration of 10% external CO_2 under all three conditions (Fig. 8G; $p < 0.001$). The EC_{50} values (Table 2) for the change in R_s during hypoxia and hyperoxia were less than during normoxia (hyperoxia, $p = 0.056$; hypoxia, $p = 0.054$).

3.4. Hypoxic hypercapnia versus hyperoxic hypercapnia

Fig. 9 illustrates breathing (Fig. 9A), pulsatile blood flow (Fig. 9B) and dorsal aortic pressure (Fig. 9C) before, during and after exposure to hypercapnic conditions imposed upon hyperoxic conditions. Breathing frequency (Fig. 10A) and total ventilation (Fig. 10C) increased (*) during hyperoxic hypercapnia compared to hyperoxia alone but hyperoxic hypercapnia did not cause a change in breath amplitude or in any of the cardiovascular variables (Fig. 9B and C; Fig. 10B and D–G). During hypoxic hypercapnia there was no change in any respiratory or cardiovascular variable beyond those observed during hypoxia alone (Fig. 10). Breathing frequency (Fig. 10A), breath amplitude (Fig. 10B) and total ventilation (Fig. 10C) were greater during hypoxic hypercapnia than during hyperoxic hypercapnia (+).

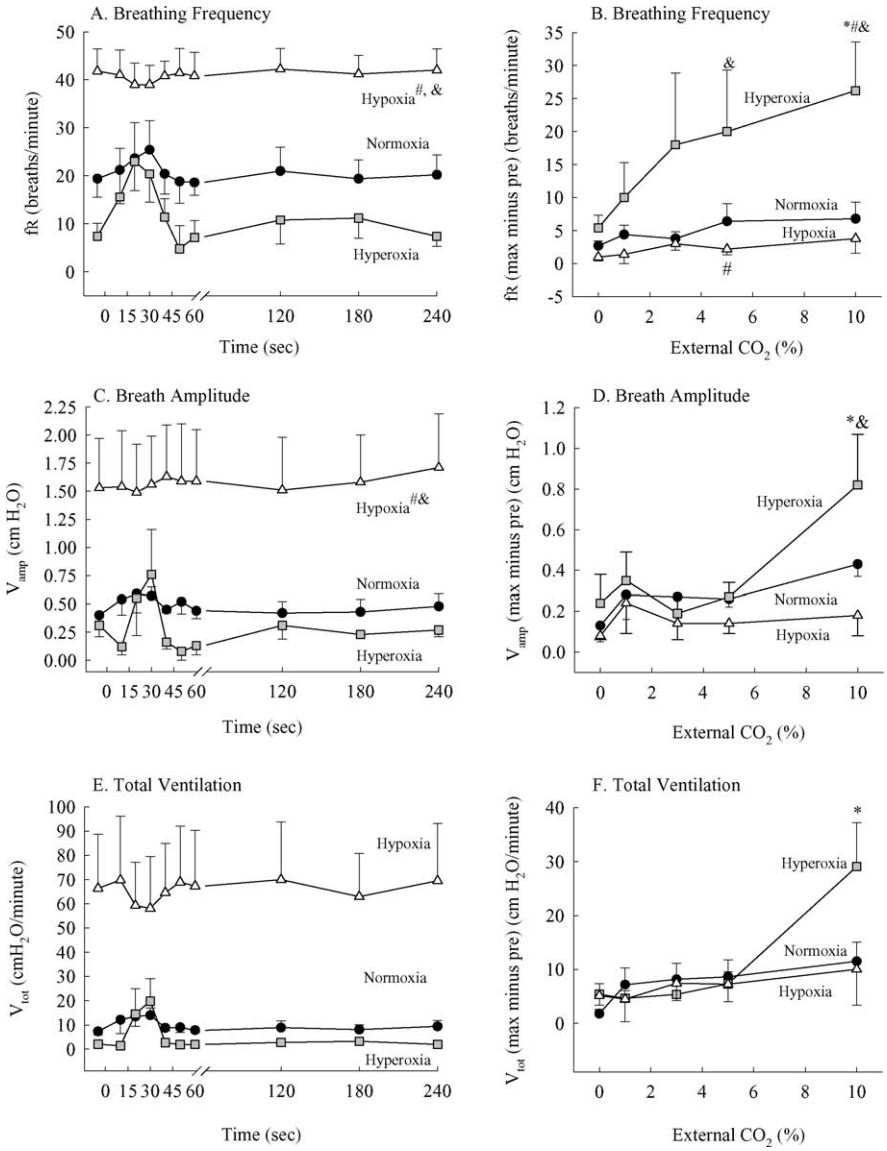

Fig. 7. Respiratory chemoreflexes evoked by the external administration of CO_2-equilibrated water in tambaqui exposed to normoxic (140 mmHg PwO_2; black circles), hypoxic (80 mmHg PwO_2; open triangles) and hyperoxic (600 mmHg PwO_2; grey squares) water. Panels A (breathing frequency; breaths/min), C (breath amplitude; cmH_2O) and E (total ventilation; cmH_2O/min) illustrate the time course of changes in response to administration of 10% CO_2-equilibrated water through the external cannula, into the buccal cavity. The injection was initiated immediately following the first data point. Panels B, D and F are dose–response curves for the maximum response minus the pre (starting) value for breathing frequency (B), breath amplitude (D) and total ventilation (F) as a function of external doses of 0, 1, 3, 5 and 10% CO_2-equilibrated water under normoxic, hypoxic and hyperoxic conditions. In panels A, C and E, an asterisk (*) represents a difference from the starting value (the first data point) in each of the individual groups (one-way RM ANOVA). A number sign (#) indicates that the time course for hypoxia or hyperoxia was different from that during normoxia (two-way RM ANOVA). An ampersand (&) sign indicates a difference in the time course during hypoxia and hyperoxia (two-way RM ANOVA). In panels B, D and F, an asterisk (*) represents a difference from the value at external CO_2 = 0%. A number sign (#) indicates a significant difference from the value under normoxic conditions; an ampersand (&) sign denotes a significant difference between hyperoxia and hypoxia.

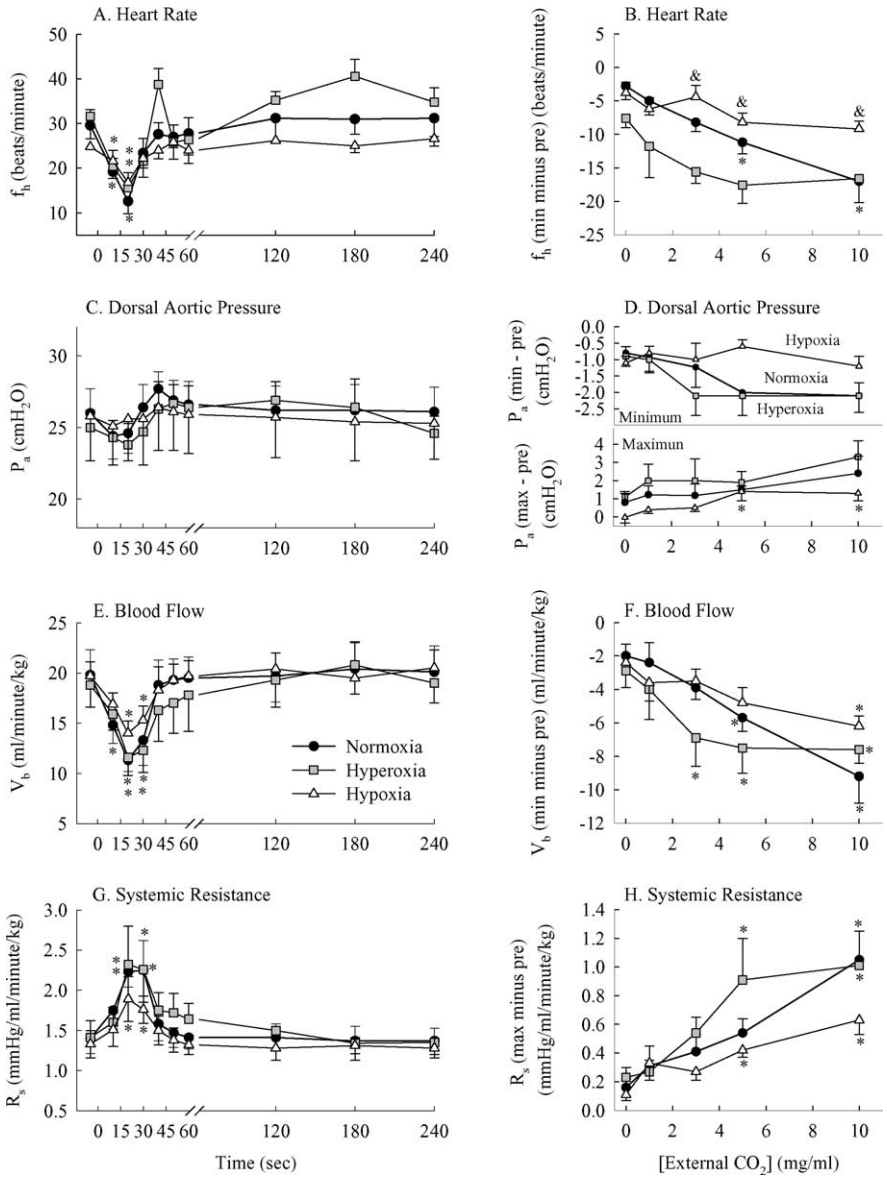

Fig. 8. External CO_2-equilibrated water evoked cardiovascular chemoreflexes in tambaqui exposed to normoxic, hypoxic and hyperoxic water. Panels A, C, E and G illustrate the time course of changes in response to administration of 10% CO_2-equilibrated water though the external cannula, into the buccal cavity. Panels B, D, F and H are dose–response curves for the maximum response minus the starting value as a function of external doses of CO_2. The symbols are the same as those in Fig. 7.

4. Discussion

4.1. Effects of hyperoxia, hypoxia and hypercapnia on NaCN-evoked chemoreflexes

Under hyperoxic conditions, the magnitude of the increase in breathing frequency in response to NaCN was augmented but the magnitude of the breath amplitude response was unaltered (Fig. 2B and D). Hypoxia, on the other hand, attenuated the magnitude of the increase in breath amplitude in response to NaCN but did not alter the magnitude of the breathing frequency response (although there was a trend towards

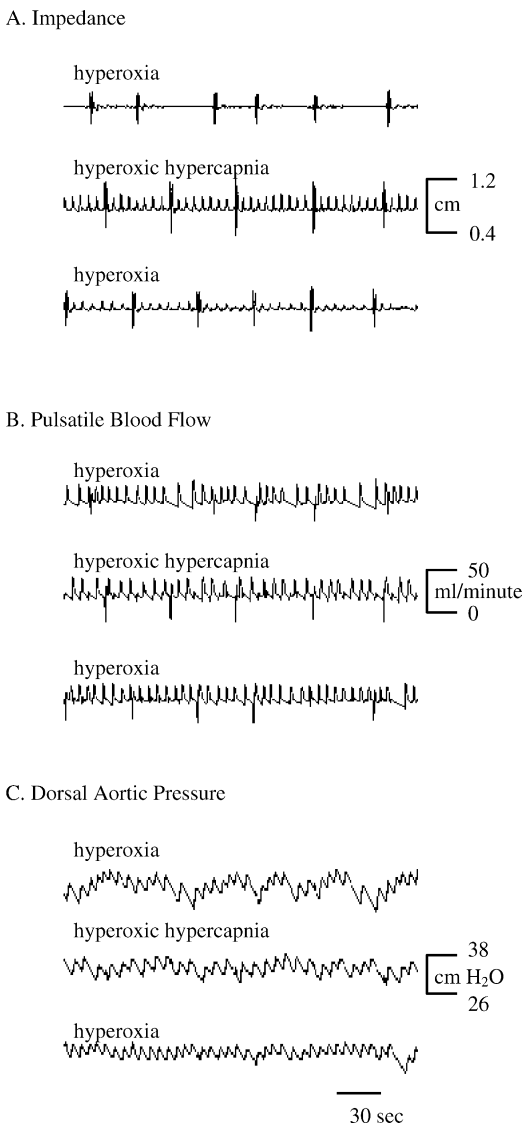

Fig. 9. Traces of breathing (impedance, cm; A), pulsatile blood flow (ml/min/kg; B) and dorsal aortic pressure (cmH_2O; C) before (upper trace; hyperoxia), during (middle trace; hyperoxic hypercapnia) and after (bottom trace; hyperoxia) exposure to hyperoxic hypercapnic conditions.

attenuation). Although speculative, these differential effects of water oxygenation status on ventilation frequency and amplitude are consistent with the notion of separate populations of O_2 chemoreceptors controlling breathing frequency and breath amplitude in tambaqui (Sundin et al., 2000; Milsom et al., 2002).

Using artificially ventilated, anaesthetised cats, Mulligan and Lahiri (1981) demonstrated that the increase in carotid body chemoreceptor discharge, induced by NaCN, was augmented by decreasing PO_2 from 90 to 60 to 40 mmHg while raising PO_2 to 400 mmHg reduced, and in some cases abolished, chemoreceptor discharge. Furthermore, carotid chemoreceptor discharge, in response to NaCN, was augmented by hypoxia and almatrine, which mimics tissue hypoxia and causes persistent excitation of the carotid body (Lahiri et al., 1989). These authors demonstrated that the slope of the NaCN dose–response curves became steeper as the PO_2 was lowered (i.e., increased sensitivity). Although there was greater baseline activity under hypoxic conditions compared to hyperoxic conditions, the maximum response attainable was the same under hyperoxic and hypoxic conditions (Mulligan and Lahiri, 1981). The difference was that the maximum value was reached at a lower NaCN dose under the hypoxic conditions. The net result of this phenomenon in the carotid body was the opposite of what we saw in the present study albeit we were measuring final cardiorespiratory responses rather than direct chemoreceptor neural activity.

Burleson and Milsom (1993) demonstrated, using an isolated gill preparation and recordings from afferent fibres, that fish chemoreceptors are stimulated by both external (water) and internal (perfusate) hypoxia as well as by NaCN. These authors did not, however, examine the effects of cyanide under hypoxic conditions and thus we have no insight into the mechanistic basis of the difference between the interactive effects observed in the mammalian carotid body and the tambaqui cardiorespiratory responses of the present study.

When breathing frequency and breath amplitude were combined into total ventilation, there was no significant effect of water oxygenation status on the response to NaCN although there was a trend for hypoxia and hyperoxia to attenuate and augment, respectively, the response to NaCN (Fig. 2F). This observation suggests that in the face of persistent aquatic hypoxia, tambaqui are still likely to be able to respond to a further bout of acute hypoxia by increasing ventilation, albeit this ability to mount an additional response is likely to be blunted with increasing levels of background hypoxia.

The water oxygenation status had little effect on the cardiovascular responses to NaCN. Hyperoxia did,

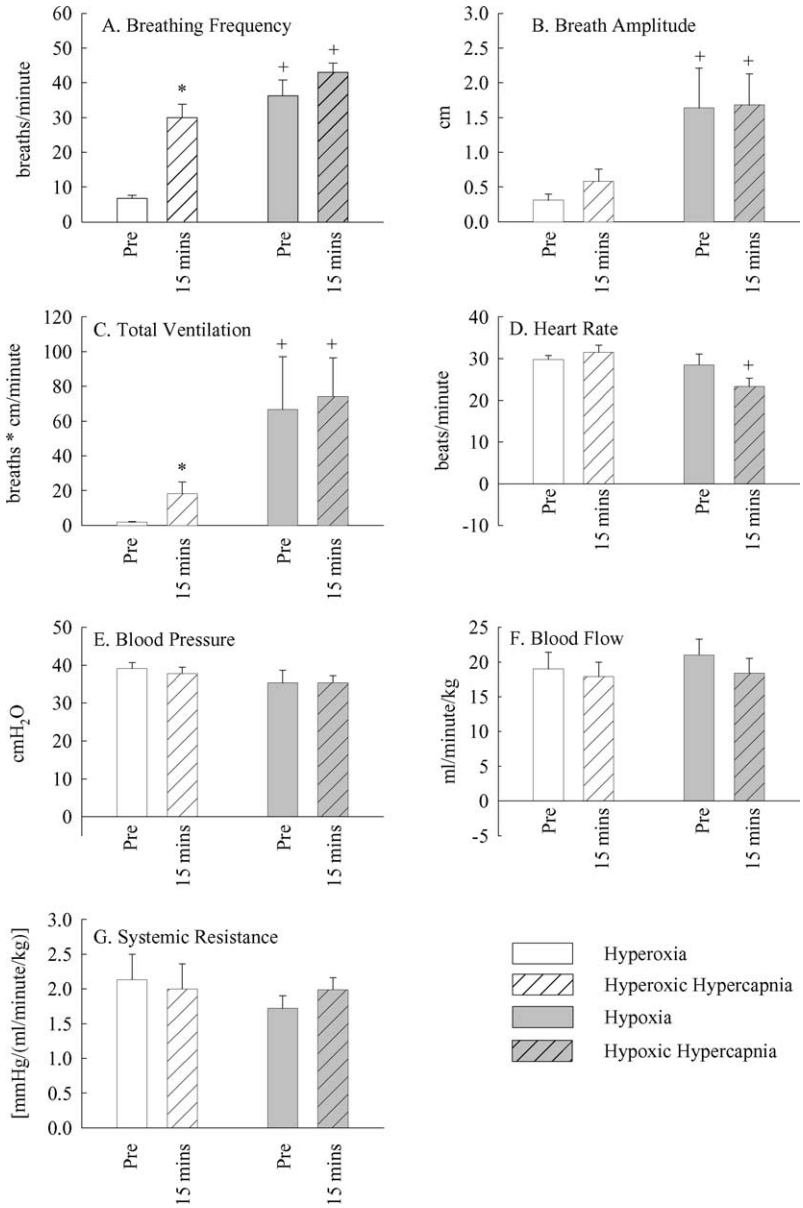

Fig. 10. Respiratory (A–C) and cardiovascular (D–G) variables before (pre) and 15 min into exposure to either hyperoxic hypercapnia (water equilibrated with 3.5% CO_2 in oxygen; open bars) or hypoxic hypercapnia (water equilibrated with 3.5% CO_2 at a PwO_2 of 80 mmHg; closed bars). An asterisk (*) indicates that the 15-min value is different from the pre value (paired *t*-test) while a plus sign (+) signifies that the hypoxic hypercapnic value is different from the hyperoxic hypercapnic value (two-way ANOVA).

however, result in a lower EC_{50} value for the NaCN-evoked bradycardia suggesting a sensitisation either of the O_2 chemoreceptors that control heart rate or changes in the vagal tone to the heart itself. Since we did not measure chemoreceptor discharge directly, it is not possible to distinguish between the possibilities. Interestingly, hyperoxia lowered the EC_{50} of both rate responses (breathing frequency and heart rate) to NaCN. Although tambaqui are often faced with hypoxic conditions, they can also encounter environmental hyperoxia

during the daytime when plants are undergoing photosynthesis. Despite the lack of effect of hypoxia on the magnitude of the heart rate response to NaCN, hypoxia did blunt the blood pressure response to several of the lower doses of NaCN (Fig. 3D). Neither hypoxia nor hyperoxia altered the magnitude or sensitivity of the blood flow or systemic resistance changes to NaCN. The attenuated decrease in blood pressure, in response to the low NaCN doses during hypoxia, may have resulted from an increase in stroke volume and cardiac output that compensated for the NaCN-evoked bradycardia.

Gamperl et al. (2001) reported that hearts of hypoxia-intolerant rainbow trout can be preconditioned such that an initial 5-min period of acute severe hypoxia can abolish the myocardial dysfunction induced by a further 30-min period of hypoxia. However, Gamperl et al. (2004) and Overgaard et al. (2004) also demonstrated, in hypoxia-tolerant rainbow trout, that such hypoxia pre-conditioning had no protective effect to a further bout of severe hypoxia. Given their hypoxia-tolerance, tambaqui may respond to hypoxic pre-conditioning in a similar manner to hypoxia-tolerant trout. In other words, the pre-existing hypoxia may not exert many beneficial effects owing to the animal's already high level of hypoxia tolerance. If this is indeed the case, then it is possible that the relative hypoxia tolerance of tambaqui, compared to mammals in general, may explain the difference in the effects of hypoxia on NaCN-evoked chemoreflexes (i.e., carotid body output in mammals versus the changes in breathing/cardiovascular function in tambaqui) mentioned above.

Aquatic hypercapnia attenuated the magnitude of the increase in breathing frequency in response to acute stimulation with NaCN but did not modify the changes seen in breath amplitude, total ventilation or any of the cardiovascular variables. The effects of hypercapnia on breathing frequency and breath amplitude are opposite to those of aquatic hypoxia. These differences are consistent with the idea that there are separate O_2 and CO_2 chemoreceptors in this species. The EC_{50} values for all NaCN-evoked responses were the same during hypercapnia and normocapnia (normoxia) suggesting that there was no CO_2-induced sensitisation of the cardiorespiratory responses to NaCN. Although the magnitude of the breathing frequency response to NaCN was attenuated, the continued increase in breath amplitude, in response to NaCN during hypercapnia, appeared to be capable of maintaining the overall hypoxic ventilatory response during superimposed hypercapnia. The data suggest that aquatic hypercapnia likely would not compromise the ability of this fish to respond to a superimposed bout of acute hypoxia. This is important as hypoxia is a greater threat to normal physiological processes in fish than is hypercapnia.

4.2. Effects of hyperoxia and hypoxia on acute or persistent CO_2-evoked chemoreflexes

Under normoxic and hypoxic conditions, the ventilatory responses to a bolus injection of CO_2-equilibrated water were minimal but under hyperoxic conditions there was a significant increase in ventilation in response to CO_2-equilibrated water (Fig. 7B, D and F). Thus, hypoxia blunted the increase in breathing frequency compared to that seen in hyperoxia in response to externally applied CO_2-equilibrated water. Tambaqui are capable of breathing at a significantly greater rate than 40 breaths per minute (the level observed during hypoxia; Fig. 7A) and it is unlikely that the O_2 chemoreceptors would be firing at a maximal rate under the hypoxic conditions in this study (water $PO_2 = 80$ mmHg). Previous studies (Milsom et al., 2002; Florindo et al., 2004) have recorded values between 70 and 100 breaths per minute under moderate hypercapnia and severe hypoxia. Thus, unless constrained by the elevated breath amplitude, the absence of a frequency response to a bolus of 10% CO_2-equilibrated water under hypoxic conditions must have resulted from a blunting of either the chemoreceptor input or the response to the input, and not a reduced scope for response. The blunting of the acute CO_2 response by hypoxia is the reciprocal of the blunting of the NaCN-evoked frequency response by hypercapnia. Hypoxic conditions also blunted the CO_2-induced bradycardia, despite the absence of differences in pre-injection heart rate under the different conditions of water oxygenation, suggesting the potential for modification of CO_2 receptor function by the level of O_2 in the water.

Under conditions of hyperoxic hypercapnia there was an elevation in breathing frequency and total ventilation, compared to values recorded under hyperoxia alone. Hyperoxic hypercapnia did not result in any change in cardiovascular function compared to hyperoxia alone. On the other hand, there was

no change in any variable during hypoxic hypercapnia compared to hypoxia alone. Therefore, hypoxia blunted the hypercapnic-induced increases in breathing that occurred when O_2 levels were high. This result is consistent with the effects of hypoxia and hyperoxia on the responses to 10% CO_2-equilibrated water.

4.3. Potential mechanisms underlying interactive effects between O_2 and CO_2

In mammals, interactive effects of O_2 and CO_2 are detectible at the level of ventilation (Nielsen and Smith, 1952), at the level of carotid sinus nerve discharge (Tenney and Brooks, 1966; Daristotle et al., 1987; Lahiri and Delaney, 1975a) and to a very small extent at the level of the increase in intracellular [Ca^{2+}], within the glomus cells, associated with chemoreceptor activation (Bamford et al., 1999; Roy et al., 2000). High levels of CO_2 increase the sensitivity of the carotid body chemoreceptors to low O_2; conversely, low levels of O_2 increase the sensitivity of the ventilatory response to hypercapnia (Bamford et al., 1999). From a control systems perspective, the PO_2-chemoreceptor activity curve shifts rightward in the presence of elevated CO_2 levels while hypoxic conditions cause an increase in the slope of the PCO_2-chemoreceptor activity curve (Lahiri and Delaney, 1975a). The net result, however, is the opposite of what we observed in the present study. We found a less than additive effect as opposed to an augmented effect.

Clearly, in our study, resting levels of breathing were elevated during hypoxia and reduced during hyperoxia. Therefore, it is reasonable to suspect that the augmented increase in breathing frequency seen during hyperoxia and the attenuated increase in breath amplitude seen during hypoxia, in response to NaCN, may have resulted, at least in part, from an altered scope, or capacity, for change. A high existing level of respiratory drive may have precluded any further increase in breathing or changes in cardiovascular function as the scope for change would be significantly reduced due to already high levels of breathing or a low heart rate. However, the EC_{50} value for the increase in breathing frequency was significantly less during hyperoxia than during hypoxia, suggesting that there was an increase in the sensitivity of the respiratory control system that regulates breathing frequency, during hyperoxia and a reduction in the sensitivity of the system controlling amplitude during hypoxia. These changes in sensitivity indicate that the drive-dependent changes in ventilation seen here were due to more than just a change in scope. Thus (less than additive) interactive effects must occur and it is not clear whether these take place at the level of the chemoreceptors, the central integration of chemoreceptor inputs, or at respiratory motor neurons and muscles, downstream of the chemoreceptor loci.

4.4. Summary

The results of this study indicate the following: (1) Water oxygenation status influenced the magnitude of acute respiratory responses, and the sensitivity of breathing frequency, to NaCN. (2) With the exception of blood pressure, water oxygenation status did not influence the magnitude of the cardiovascular responses to NaCN but did alter the sensitivity of the heart rate response to cyanide. (3) Aquatic hypercapnia attenuated the magnitude of the increase in breathing frequency in response to acute stimulation with NaCN but did not modify breath amplitude, total ventilation or any of the cardiovascular responses. (4) The water oxygenation status affected the magnitude of the ventilatory and heart rate responses to externally applied CO_2-equilibrated water. (5) Aquatic hypoxia abolished the ventilatory responses to hypercapnia; aquatic hyperoxia did not. The results indicate that existing respiratory disturbances modulate cardiorespiratory responses to further respiratory challenges reflecting both changes in chemosensitivity and the capacity for further change.

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