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The relative roles of external and internal CO_2 *versus* H^+ in eliciting the cardiorespiratory responses of *Salmo salar* and *Squalus acanthias* to hypercarbia

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Summary

Fish breathing hypercarbic water encounter externally elevated P_{CO_2} and proton levels ($[H^+]$) and experience an associated internal respiratory acidosis, an elevation of blood P_{CO_2} and $[H^+]$. The objective of the present study was to assess the potential relative contributions of CO_2 *versus* H^+ in promoting the cardiorespiratory responses of dogfish (*Squalus acanthias*) and Atlantic salmon (*Salmo salar*) to hypercarbia and to evaluate the relative contributions of externally *versus* internally oriented receptors in dogfish.

In dogfish, the preferential stimulation of externally oriented branchial chemoreceptors using bolus injections (50 ml kg^{-1}) of CO_2-enriched (4 % CO_2) sea water into the buccal cavity caused marked cardiorespiratory responses including bradycardia (–4.1±0.9 min^{-1}), a reduction in cardiac output (–3.2±0.6 ml min^{-1} kg^{-1}), an increase in systemic vascular resistance (+0.3± 0.2 mmHg ml min^{-1} kg^{-1}), arterial hypotension (–1.6± 0.2 mmHg) and an increase in breathing amplitude (+0.3±0.09 mmHg) (means ± S.E.M., *N*=9–11). Similar injections of CO_2-free sea water acidified to the corresponding pH of the hypercarbic water (pH 6.3) did not significantly affect any of the measured cardiorespiratory variables (when compared with control injections). To preferentially stimulate putative internal CO_2/H^+ chemoreceptors, hypercarbic saline (4 % CO_2) was injected (2 ml kg^{-1}) into the caudal vein. Apart from an increase in arterial blood pressure caused by volume loading, internally injected CO_2 was without effect on any measured variable.

In salmon, injection of hypercarbic water into the buccal cavity caused a bradycardia (–13.9±3.8 min^{-1}), a decrease in cardiac output (–5.3±1.2 ml min^{-1} kg^{-1}), an increase in systemic resistance (0.33± 0.08 mmHg ml min^{-1} kg^{-1}) and increases in breathing frequency (9.7±2.2 min^{-1}) and amplitude (1.2±0.2 mmHg) (means ± S.E.M., *N*=8–12). Apart from a small increase in breathing amplitude (0.4±0.1 mmHg), these cardiorespiratory responses were not observed after injection of acidified water.

These results demonstrate that, in dogfish and salmon, the external chemoreceptors linked to the initiation of cardiorespiratory responses during hypercarbia are predominantly stimulated by the increase in water P_{CO_2} rather than by the accompanying decrease in water pH. Furthermore, in dogfish, the cardiorespiratory responses to hypercarbia are probably exclusively derived from the stimulation of external CO_2 chemoreceptors, with no apparent contribution from internally oriented receptors.

Key words: hypercarbia, hypercapnia, ventilation, cardiovascular, gill, dogfish, *Squalus acanthias*, salmon, *Salmo salar*, cardiac output, $[H^+]$, CO_2.

Introduction

In recent years, a growing number of studies have focused on CO_2/H^+ chemoreception in fish (Perry et al., 1999; Burleson and Smatresk, 2000; Sundin et al., 2000; Reid et al., 2000; Crocker et al., 2000; McKendry and Perry, 2001; McKendry et al., 2001). Although earlier studies attributed the physiological responses to hypercarbia (at least in teleosts) to indirect impairment of blood O_2 transport (Randall, 1982; Smith and Jones, 1982), it is clear that elevation of $[CO_2]$ itself, or the accompanying reduction in pH, is able directly to elicit cardiorespiratory responses *via* interaction with specific CO_2/H^+ chemoreceptors (Heisler et al., 1988; Graham et al., 1990; Kinkead and Perry, 1991; Milsom, 1995a,b; Perry and Gilmour, 1996; Gilmour, 2001). Furthermore, while numerous studies have exclusively examined the ventilatory consequences of hypercarbia (Gilmour, 2001), relatively few have addressed the impact of CO_2 on the cardiovascular system. On the basis of these few studies, it would appear that, similar to the response of fish to hypoxia (Fritsche, 1990), there is large interspecific variation in the nature of their cardiovascular responses to hypercarbia. Indeed, although not universally observed (Crocker et al., 2000; Sebert et al., 1976), bradycardia appears to be the most consistent cardiovascular response to hypercarbia amongst the species that have been examined

(Perry et al., 1999; Sundin et al., 2000; Reid et al., 2000; McKendry et al., 2001).

The cardiorespiratory responses to hypercarbia have been studied most thoroughly in the rainbow trout (*Oncorhynchus mykiss*) and Pacific dogfish (*Squalus acanthias*). The cardiovascular response of trout to hypercarbia consists of a reduction in cardiac output ($\dot{V}_b$), a lowering of cardiac frequency (f_H), an increase in systemic vascular resistance (R_S) and an elevation of arterial blood pressure (P_a) (Perry et al., 1999; McKendry and Perry, 2001). Dogfish also exhibit a pronounced bradycardia and reduction in $\dot{V}_b$ in response to hypercarbia but, in contrast to trout, P_a is reduced and R_S is unaltered (McKendry et al., 2001).

Despite the growing literature on the physiological responses of fish to hypercarbia, there is continuing debate surrounding the physical location of CO_2 chemoreceptors and the precise manner by which these receptors are stimulated; i.e. whether the receptors respond to changes in P_{CO_2} *per se* or to the accompanying decrease in pH. Although there is evidence for central CO_2 chemoreception, at least in air-breathing fish (Wilson et al., 2000), peripheral gill receptors are believed to be the predominant site of CO_2 sensing in fish (Burleson and Smatresk, 2000; Sundin et al., 2000; Reid et al., 2000; McKendry et al., 2001; Gilmour, 2001). It is less clear, however, whether these peripheral receptors are externally oriented so as to monitor the external environment and/or internally oriented to monitor the extracellular fluids (Gilmour, 2001). In rainbow trout, *Oncorhynchus mykiss*, there is evidence both for (Wood and Munger, 1994) and against (McKendry and Perry, 2001) internally oriented CO_2 chemoreceptors. Studies examining the relative roles of CO_2 *versus* pH ($[H^+]$) in mediating the responses to hypercarbia have focused almost exclusively on ventilatory control (Janssen and Randall, 1975; Thomas and Le Ruz, 1982). The results of these and other studies have demonstrated that the stimulation of breathing during hypercarbia is related specifically to the increase in water P_{CO_2} (P_{wCO_2}) with no contribution of the lowered water pH (pHw). The only two studies that have assessed the relative contributions of P_{wCO_2} and pHw in eliciting cardiovascular responses during hypercarbia have demonstrated that changes in pHw, in the absence of changes in P_{CO_2}, are unable to evoke cardiovascular responses (Sundin et al., 2000; Reid et al., 2000).

Despite the robust cardiorespiratory responses elicited by stimulation of branchial chemoreceptors in dogfish during hypercarbia, there are no data on the orientation (internal *versus* external) of these receptors or their specificity for changes in CO_2 *versus* H^+. Rainbow trout appear to rely exclusively on externally oriented chemoreceptors for cardiorespiratory control during hypercarbia, but the specificity of these receptors for CO_2 and H^+ is unknown (at least for the cardiovascular responses). In addition, it is not known whether the reliance of trout on external chemoreceptors for cardiorespiratory control during hypercarbia extends to other salmonid species. Thus, the goals of the present study were to assess the potential relative contributions of CO_2 *versus* H^+ in promoting the cardiorespiratory responses of dogfish (*Squalus acanthias*) and Atlantic salmon (*Salmo salar*) to hypercarbia and to evaluate the relative contributions of externally *versus* internally oriented receptors in dogfish. This was accomplished by comparing their responses to preferential stimulation by CO_2 and H^+ of putative external or internal (dogfish only) receptors.

Materials and methods

Experimental animals

Pacific spiny dogfish (*Squalus acanthias* L.) were collected by net during trawls by local fishermen or angled by hook and line and transported to holding facilities at Bamfield Marine Station (BMS; Bamfield, Vancouver Island, British Columbia, Canada). The dogfish were kept under natural photoperiod in a 75 000 l opaque circular tank provided with aerated full-strength sea water (30–32 ‰) at 12 °C, and they were fed twice weekly with herring. In the present study, 11 dogfish (mass range 960–3000 g) were used within 4 weeks of their capture.

Atlantic salmon, *Salmo salar* L., were obtained from a fish farm (Vancouver Island, British Columbia, Canada) and were transported in oxygenated water to BMS, where they were housed outdoors in fibreglass tanks supplied with fresh flowing full-strength sea water (12 °C). They were fed daily with commercial salmonid pellets and were allowed to acclimate for 2 weeks before use. In the present study, 13 salmon (mass range 300–575 g) were used within 4 weeks of their arrival.

All experimental protocols (including surgical procedures; see below) were previously approved by the University of Ottawa Animal Care Committee in accordance with guidelines provided by The Canadian Council for Animal Care.

Surgical procedures

Dogfish were immersed in an aerated anaesthetic solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g l^{-1}) and transferred to an operating table, where the gills were irrigated continuously with the same anaesthetic solution. A lateral incision was made in the caudal peduncle to expose and cannulate (PE 50; Clay Adams) both the caudal vein and the caudal artery in the anterograde direction (Axelsson and Fritsche, 1994). While the arterial cannula allowed caudal artery blood pressure measurements, the caudal vein cannula permitted injections and/or repeated blood sampling. All cannulae were filled with heparinized (100 i.u. ml^{-1} ammonium heparin; Sigma Chemical, St Louis, MO, USA) dogfish saline (500 mmol l^{-1} NaCl). In addition, the pericardial cavity was exposed with a ventral midline incision and the pericardium was dissected to expose the conus arteriosus. To enable measurement of cardiac output ($\dot{V}_b$), a 3S or 4S ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed non-occlusively around the conus. Lubricating jelly was used with the perivascular flow probe as an acoustic couplant. Silk sutures were used to close the ventral and caudal peduncle incisions and to anchor the cardiac output probe lead and the cannulae to the skin. To assess ventilatory amplitude and

frequency and to inject solutions into the buccal cavity, catheters (Clay Adams PE 160) were inserted into the spiracular openings and sutured to the head. After surgery, dogfish were placed into individual flow-through opaque acrylic or wooden boxes and left to recover for approximately 24 h before experimentation.

Atlantic salmon were anaesthetised using benzocaine (0.1 g l^{-1} ethyl-*p*-aminobenzoate, Sigma) in sea water until breathing movements stopped. The fish were then placed on the operating table, where their gills were force-ventilated with the same oxygenated anaesthetic solution. To permit measurement of arterial blood pressure (P_a), a polyethylene cannula (Clay Adams, PE 50) was implanted into the dorsal aorta *via* percutaneous puncture of the roof of the buccal cavity (Olson et al., 1997).

To record ventilatory frequency and amplitude adjustments *via* changes in buccal cavity pressure, a heat-flared cannula (Clay Adams, PE 160) was threaded through a puncture in the snout of each salmon and secured using a single silk ligature and Vetbond. A second heat-flared cannula (PE 160) was threaded through a separate puncture in the snout of each salmon to permit the injection of treated sea water into the buccal cavity and out over the gills.

Fish were fitted with a cardiac flow probe after all cannulae had been secured. A small ventral incision was made to expose the pericardial cavity, and the pericardium was dissected away to expose the bulbus arteriosus. A 3S or 4S ultrasonic flow probe (Transonics Systems Inc., Ithaca, NY, USA) was placed non-occlusively around the bulbus to enable the measurement of cardiac output (Olson et al., 1997). The incision was sutured closed and sealed with Vetbond, and the flow probe cable was secured externally to the ventral surface of the fish using silk ligatures and Vetbond.

All cardiovascular cannulae were filled with heparinised (50 i.u. ml^{-1} sodium heparin) saline (0.9 % NaCl). After surgery, fish were revived and placed in opaque flow-through acrylic boxes and left to recover for approximately 24 h prior to experimentation.

Experimental protocol

Cardiorespiratory effects of external CO_2 versus *H^+*

Experiments commenced with a 10 min recording period under normoxic, normocarbic resting conditions. After this 'pre' period, a 'randomised' series of treated seawater injections (50 ml kg^{-1}) commenced, with 10 min intervals between injections. The injections were delivered over a 20 s period into the spiracular (dogfish) or snout (salmon) cannulae; fish continued to breathe during the injection period. After mixing and dilution by inspired water ($P_{W CO_2} \approx 0.5$ mmHg; 1 mmHg=0.133 kPa), it was estimated that the P_{CO_2} of the water flowing over the lamellae would be approximately 10 mmHg in both species (see Discussion for further details). Pilot experiments demonstrated that local sea water equilibrated with 4 % CO_2 in air had a pH of 6.3. To differentiate between CO_2-induced cardiorespiratory effects and those triggered by the decrease in pH normally observed during periods of elevated ambient [CO_2], treated seawater injections consisted of sea water equilibrated with 4 % CO_2 or sea water titrated with HCl to a pH of 6.3. The sea water was vigorously aerated before and after addition of HCl to ensure removal of CO_2. Aerated seawater injections of the same volume (50 ml kg^{-1}) were used in control experiments.

Cardiorespiratory effects of internal CO_2 in dogfish

In a separate series of experiments, dogfish were injected *via* the caudal vein cannula with saline (500 mmol l^{-1} NaCl; pH 7.2; 2 ml kg^{-1}) pre-equilibrated with 4 % CO_2 (pH 5.4). Assuming negligible interconversion between $CO_2/HCO_3^-/H^+$ within the brief period of transit to the gill, mixing of the injected saline with the venous blood was estimated to yield a final P_{CO_2} of 9.0 mmHg (see Discussion for further details). Because these injections were without effect on any measured variable (see below), there was no further attempt to dissect the relative roles of CO_2 *versus* H^+. Control fish were injected with 2 ml kg^{-1} of saline.

Analytical procedures

Measurement of water gas levels

A pump-driven loop continuously withdrew inspired water and passed it over P_{O_2} and P_{CO_2} electrodes (Radiometer) housed in temperature-controlled cuvettes (12 °C) and connected to a Radiometer blood gas analyser. The O_2 electrode was calibrated by pumping a zero solution (2 g l^{-1} sodium sulphite) or air-saturated water continuously through the electrode sample compartments until stable readings were recorded. The CO_2 electrode was calibrated in a similar manner using mixtures of 0.5 % and 1.0 % CO_2 in air provided by a gas-mixing flow meter (Cameron Instruments). The electrodes were calibrated prior to each individual experiment.

Measurement of cardiorespiratory variables

Ventilation amplitude (V_{AMP}) was assessed by monitoring pressure changes in the spiracle (dogfish) or mouth (salmon) associated with each breathing cycle. The ventilation catheter was filled with sea water, connected to pressure transducer (Bell and Howell) and linked to an amplifier (Harvard Biopac DA 100). The pressure transducer was calibrated daily against a static column of water.

The dorsal aortic (trout) or caudal artery (dogfish) cannula was flushed with heparinised saline (100 i.u. ml^{-1}) to prevent clotting and then connected to a pressure transducer (Bell and Howell) pre-calibrated against a static column of water. Analog blood pressure signals were measured using Harvard Biopac amplifiers (DA 100). Cardiac output was determined by attaching the ultrasonic flow probe to a Transonic T106 single-channel blood flow meter. All flow probes were pre-calibrated in the factory using diluted mammalian blood (haematocrit 25 %) at 13 °C.

All analog signals (water gas levels, blood and ventilation pressures and $\dot{V}_b$) were converted to digital data by interfacing with a data-acquisition system (Biopac Systems Inc.) using Acknowledge data-acquisition software (sampling rate set at

10 Hz) and a Pentium PC. Thus, continuous data recordings were obtained for mass-specific $\dot{V}_b$, cardiac frequency (f_H; automatic rate calculation from the pulsatile $\dot{V}_b$ trace), cardiac stroke volume (V_S; $\dot{V}_b/f_H$), ventilation frequency (f_V; automatic rate calculation from the raw ventilation pressure traces), V_{AMP} (the difference between maximum and minimum ventilation pressures), mean blood pressure (arithmetic mean) and systemic vascular resistance (R_S; mean $P_a/\dot{V}_b$).

Statistical analyses

All data are represented as means ± 1 S.E.M. Absolute changes in measured and calculated variables were determined by subtracting the value at the injection point (time zero) from all data points. The data obtained during the period of seawater or saline injection (20 s) were not analysed. Time series data were analysed using repeated-measures one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* multiple-comparison tests. When violations of normality occurred (detected automatically by the software package), the data were analysed using a non-parametric repeated-measures ANOVA (Friedman) followed by Dunnett's multiple-comparison test. When data from only a single time point were compared with the pre-injection value, a paired Student's *t*-test (parametric data) or Wilcoxon signed-rank test (non-parametric data) was used. Differences among the various treatments (control, pH-adjusted, CO_2-enriched) were analysed using one-way ANOVA (parametric data) or Kruskal–Wallis one-way ANOVA on ranks (non-parametric data). $P<0.05$ was considered to be statistically significant. Calculations were performed using the SigmaStat (SPSS; version 2.03) software package.

Results

Dogfish

Injection over the external surface of the gills of seawater-equilibrated with 4% CO_2 caused a rapid and transient lowering of f_H (Fig. 1). At the peak of the response (20 s after beginning the injection), f_H was decreased by 4.1±0.9 min^{-1} (N=11) (Fig. 1C). Although analysis of the entire time series did not reveal any statistically significant effects of control injections or injections of acidified water (Fig. 1C), a subsequent analysis of only the 20 s time point (when the maximal response to CO_2 was achieved) revealed a significant effect of control and acidified sea water on lowering f_H (Fig. 1D). This injection effect (–1.0±0.3 to –1.5±0.4 min^{-1}) could explain no more than 36% of the overall response to O_2 and, importantly, the f_H response to CO_2-enriched water was statistically larger than the responses to control injections or to injections of acidified water (Fig. 1D). Further, there was no statistical difference in the f_H response of dogfish to control sea water *versus* acidified sea water.

The effects of external injections on the remaining measured or calculated cardiovascular variables are shown in Table 1. Injection of CO_2-enriched sea water caused a significant lowering of $\dot{V}_b$ (approximately 15%) that was caused solely by the reduction in f_H; V_s was unaffected (data not shown). Systemic resistance was significantly increased (34%) after injection of CO_2-enriched sea water. Control injections or injections of acidified sea water did not affect $\dot{V}_b$ or R_S (Table 1).

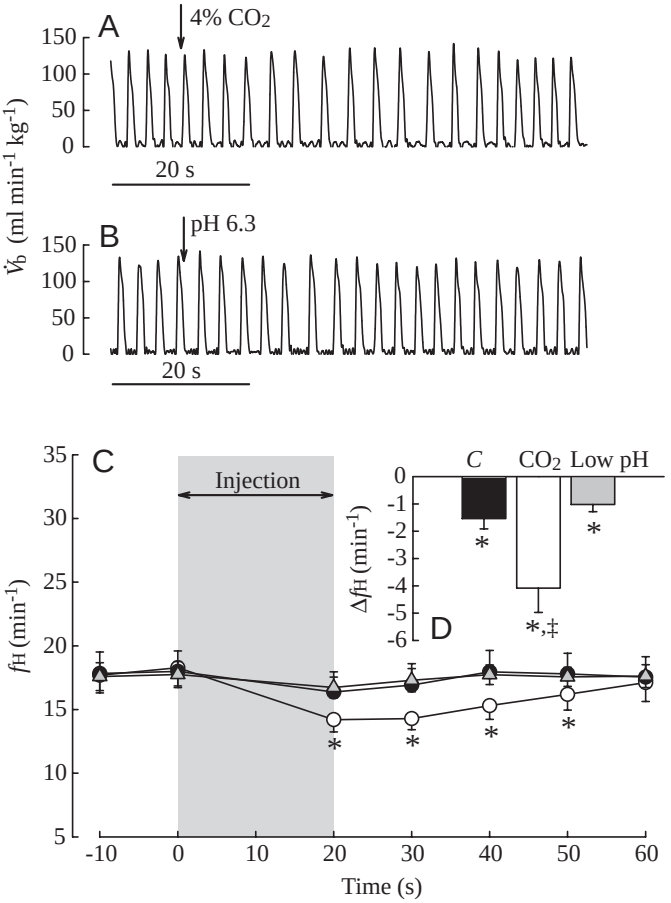


Fig. 1. The effects of external injections of CO_2-enriched sea water (4% CO_2; white symbols; N=11) or CO_2-free acidified sea water (pH 6.3; grey symbols; N=7) on cardiac output ($\dot{V}_b$) and cardiac frequency (f_H) in dogfish (*Squalus acanthias*). The effects of (A) CO_2 or (B) acidified water are depicted as representative traces and as mean values (C). Control fish (black symbols; N=11) received bolus injections of sea water. The shaded box represents the 20 s period of injection. (D) The changes in f_H (Δf_H) at the 20 s time point. Data are shown as means +1 standard error of the mean (S.E.M.). An asterisk denotes a statistically significant difference from the final pre-injection value (time zero). A double dagger denotes a statistically significant difference from the control and low-pH seawater groups. *C*, control. 1 mmHg=0.133 kPa.

Injection of CO_2-enriched sea water over the gills caused a significant increase in V_{AMP} (0.3±0.09 mmHg) that was not observed in control fish or in fish injected with acidified sea water (Fig. 2); f_V was not affected by any treatment (Table 1).

Apart from an increase in arterial blood pressure (3.3±0.9 mmHg) and R_S (0.22±0.13 mmHg ml min^{-1} kg^{-1}) caused by volume loading itself (i.e. a similar increase was also observed in the control group), internally injected CO_2 was without effect on any measured variable (Table 2).

Table 1. *The effects in dogfish (*Squalus acanthias*) of external injections of CO_2-enriched sea water (4 % CO_2) or CO_2-free acidified sea water (pH 6.3) on selected cardiorespiratory variables including ventilation frequency, cardiac output, arterial blood pressure and systemic vascular resistance*

	Control			4 % CO_2			pH 6.3		
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ
f_V (min^{-1})	35.0±1.2 (9)	35.6±1.1 (9)	0.58±0.42 (9)	34.6±1.0 (9)	34.5±1.2 (9)	–0.03±0.56 (9)	33. 3±1.6 (6)	34.4±1.5 (6)	1.15±0.79
$\dot{V}_b$ (ml min^{-1} kg^{-1})	25.3±2.9 (10)	24.6±2.8 (10)	–0.66±0.49 (10)	23.4 ±3.1 (10)	20.2±2.9* (10)	–3.15±0.64‡ (10)	32.0±6.2 (6)	30.5±5.6 (6)	–1.50±0.88 (6)
P_a (mmHg)	16.9±1.3 (10)	16.6±1.2 (10)	–0.38±0.28 (10)	18.6±1.0 (11)	17.1±0.9* (11)	–1.6±0.22‡ (11)	18.1±2.2 (7)	17.5±2.1 (7)	–0.62±0.15 (7)
R_S (mmHg ml min^{-1} kg^{-1})	0.76±0.2 (10)	0.76±0.02 (10)	0.00±0.02 (10)	0.82±0.1 (10)	1.10±0.2* (10)	0.28±0.17‡ (10)	0.77±0.2 (6)	0.85±0.3 (6)	0.08±0.05 (6)

f_V, ventilation frequency; $\dot{V}_b$, cardiac output; P_a, arterial blood pressure; R_S, systemic resistance.

1 mmHg=0.133 kPa.

Control fish were given a bolus external injection of sea water.

'Pre' refers to the data immediately before injection and 'Post' refers to the data 20 s later.

Data are reported as means ± 1 S.E.M. (*N*).

Differences between pre and post values within any group are denoted by asterisks; differences in delta values from corresponding values in the control group are denoted by double daggers.

Table 2. *The effects in dogfish (*Squalus acanthias*) of internal injections of CO_2-enriched saline (4 % CO_2) or saline alone (controls) on selected cardiorespiratory variables including cardiac frequency, ventilation amplitude, ventilation frequency, cardiac output, arterial blood pressure and systemic vascular resistance*

	Control			4 % CO_2		
	Pre	Post	Δ	Pre	Post	Δ
f_H (min^{-1})	18.3±1.4 (8)	18.6±1.3 (8)	0.29±0.52 (8)	17.1±1.2 (10)	17.2±1.2 (10)	0.10±0.53 (10)
V_{AMP} (mmHg)	1.64±0.2 (8)	1.59±0.2 (8)	–0.05±0.03 (8)	1.56±0.1 (10)	1.50±0.1 (10)	–0.06±0.05 (10)
f_V (min^{-1})	35.1±1.6 (7)	33.9±1.6* (7)	–1.14±0.36 (7)	34.0±1.6 (9)	33.6±1.7 (9)	–0.44±0.45 (9)
$\dot{V}_b$ (ml min^{-1} kg^{-1})	27.1±4.6 (7)	26.5±4.1 (7)	–0.58±0.76 (7)	28.4±4.5 (9)	26±4.0 (9)	–2.32±1.76 (9)
P_a (mmHg)	18.3±1.5 (6)	22.5±1.7* (6)	4.21±1.10 (6)	17.1±2.1 (9)	20.4±2.8* (9)	3.30±0.89 (9)
R_S (mmHg ml min^{-1} kg^{-1})	0.92±0.2 (6)	1.12±0.2* (6)	0.20±0.12 (6)	0.77±0.1 (8)	0.99±0.2* (8)	0.22±0.13 (8)

f_H, cardiac frequency; V_{AMP}, ventilation amplitude; f_V, ventilation frequency; $\dot{V}_b$, cardiac output; P_a, arterial blood pressure; R_S, systemic resistance.

1 mmHg=0.133 kPa.

'Pre' refers to the data immediately before injection and 'Post' refers to the data 20 s later.

Data are reported as means ± 1 S.E.M. (*N*).

Significant differences between pre and post values within any group are denoted by asterisks.

Salmon

Injection of CO_2-enriched water into the buccal cavity of salmon (Fig. 3) caused a marked bradycardia (peak response –13.9±3.8 min^{-1}, *N*=11). The decrease in f_H led to a significant 16 % decrease in $\dot{V}_b$ (Table 3). Systemic resistance was significantly increased after injection of external CO_2, yet dorsal aortic blood pressure was unaffected (Table 3). No significant changes in any cardiovascular variable were observed in the control group or in the fish that received acidified sea water.

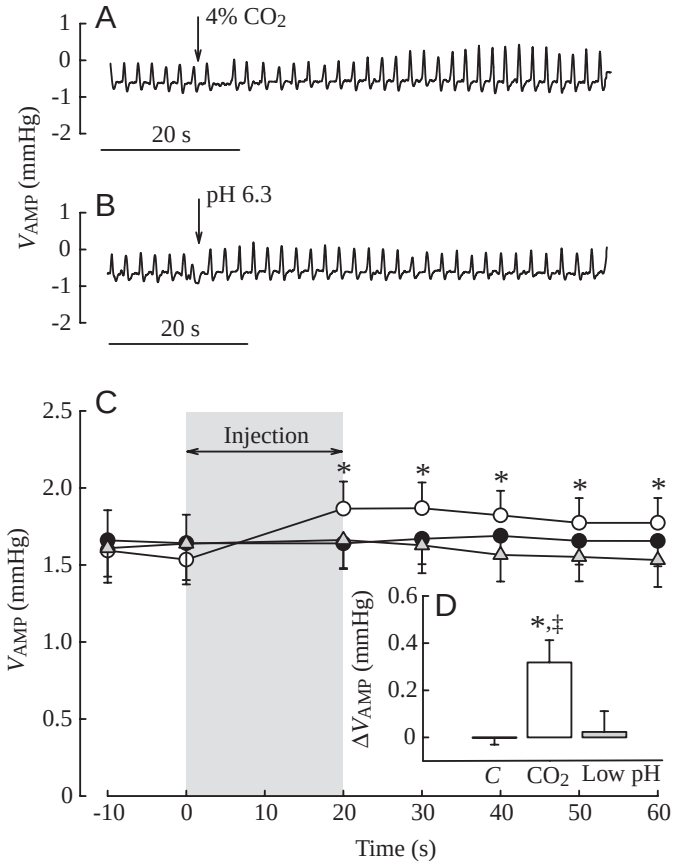


Fig. 2. The effects of external injections of CO_2-enriched sea water (4 % CO_2; white symbols; N=9) or CO_2-free acidified sea water (pH 6.3; grey symbols; N=6) on ventilation amplitude (V_{AMP}) in dogfish (*Squalus acanthias*). The effects of (A) CO_2 and (B) acidified water are depicted as representative traces and as mean values (C). Control fish (black symbols; N=10) received bolus injections of sea water. The shaded box represents the 20 s period of injection. (D) The changes in V_{AMP} (ΔV_{AMP}) at the 20 s time point. Data are shown as means +1 standard error of the mean. An asterisk denotes a statistically significant difference from the final pre-injection value (time zero). A double dagger denotes a statistically significant difference from the control and low-pH seawater groups. *C*, control. 1 mmHg=0.133 kPa.

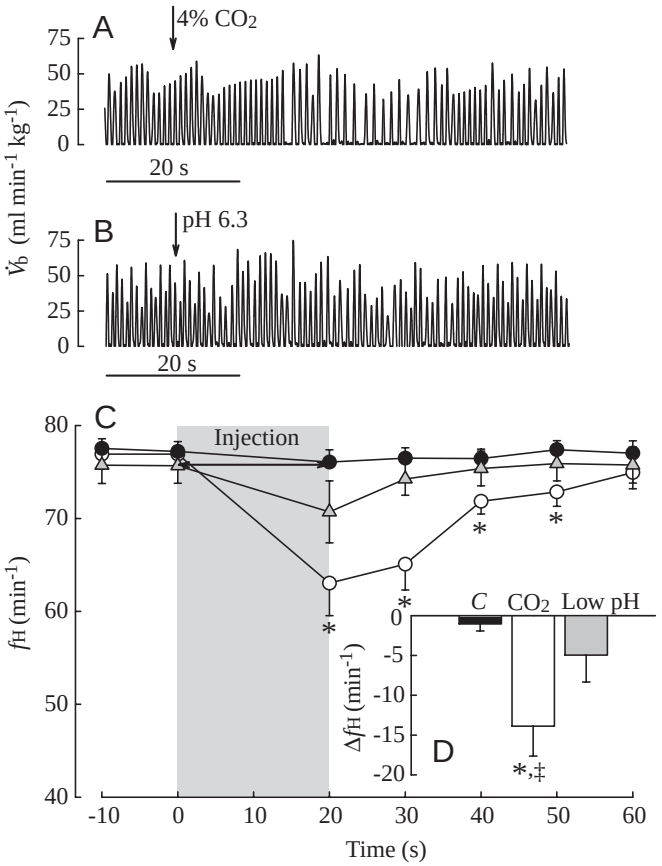


Fig. 3. The effects of external injections of CO_2-enriched sea water (4 % CO_2; white symbols; N=11) or CO_2-free acidified sea water (pH 6.3; grey symbols; N=11) on cardiac output ($\dot{V}_b$) and cardiac frequency (f_H) in Atlantic salmon (*Salmo salar*). The effects of (A) CO_2 and (B) acidified water are depicted as representative traces and as mean values (C). Control fish (black symbols; N=12) received bolus injections of sea water. The shaded box represents the 20 s period of injection. (D) The changes in f_H (Δf_H) at the 20 s time point. Data are shown as means +1 standard error of the mean. An asterisk denotes a statistically significant difference from the final pre-injection value (time zero). A double dagger denotes a statistically significant difference from the control and low-pH seawater groups. *C*, control. 1 mmHg=0.133 kPa.

At the peak of the response (20 s), V_{AMP} increased by 52 % in fish receiving CO_2-enriched sea water (Fig. 4). Although smaller in comparison (P<0.05), injection of acidified water over the gills also caused a significant elevation of V_{AMP} (12 %) (Fig. 4C). Injection of CO_2-enriched water also caused a significant increase in f_V of approximately 10 min^{-1} (Table 3). Ventilation frequency was unchanged in fish receiving control injections (sea water) or injections of acidified sea water (Table 3) (Fig. 4).

Discussion

Methods

The goal of the present study was to assess the relative roles of external and internal CO_2 *versus* H^+ in eliciting the cardiorespiratory responses of dogfish and salmon to hypercarbia. Although distinguishing between the effects of CO_2 and H^+ is straightforward, it is somewhat more problematic to isolate the potentially separate roles of changes in external *versus* internal CO_2/H^+. The experimental approach used in the present study was to compare the physiological responses of fish to external injections of sea water with their responses to internal injections of saline. It was assumed that the injection of CO_2-enriched or acidified water into the buccal cavity would preferentially stimulate putative external gill chemoreceptors, whereas injections into the caudal vein would preferentially activate putative internal chemoreceptors. Clearly, however, the injection of CO_2-enriched water over the external surface of the gills would lead to some diffusive entry of CO_2 into the fish and thus cause the possible stimulation of both external and internal

Table 3. *The effects in salmon (*Salmo salar*) of external injections of CO_2-enriched sea water (4 % CO_2) or CO_2-free acidified sea water (pH 6.3) on selected cardiorespiratory variables including ventilation frequency, cardiac output, arterial blood pressure and systemic vascular resistance*

	Control			4 % CO_2			pH 6.3		
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ
f_V (min^{-1})	67.7±1.7 (12)	68.7±1.5 (12)	1.08±0.66 (12)	67.5±1.2 (10)	77.2±2.5* (10)	9.70±2.15 (10)	65.8±1.7 (9)	68.1±2.3 (9)	2.30±1.06 (9)
$\dot{V}_b$ ($ml\,min^{-1}\,kg^{-1}$)	35.5±2.7 (11)	35.7±2.7 (11)	0.20±0.31 (11)	32.7±1.6 (9)	27.4±2.4* (9)	–5.33±1.20 (9)	35.6±3.9 (9)	33.4±4.6 (9)	–2.19±1.82 (9)
P_a (mmHg)	29.2±1.4 (11)	29.2±1.4 (11)	–0.01±0.60 (11)	27.6±1.1 (9)	27.8±1.4 (9)	0.18±0.74 (9)	26.3±1.7 (11)	26.6±1.6 (11)	0.30±0.88 (11)
R_S ($mmHg\,ml\,min^{-1}\,kg^{-1}$)	0.86±0.1 (9)	0.86±0.1 (9)	0.00±0.02 (9)	0.92±0.1 (8)	1.25±0.1* (8)	0.33±0.08 (8)	0.84±0.1 (9)	0.98±0.2 (9)	0.14±0.13 (9)

f_V, ventilation frequency; $\dot{V}_b$, cardiac output; P_a, arterial blood pressure; R_S, systemic resistance.

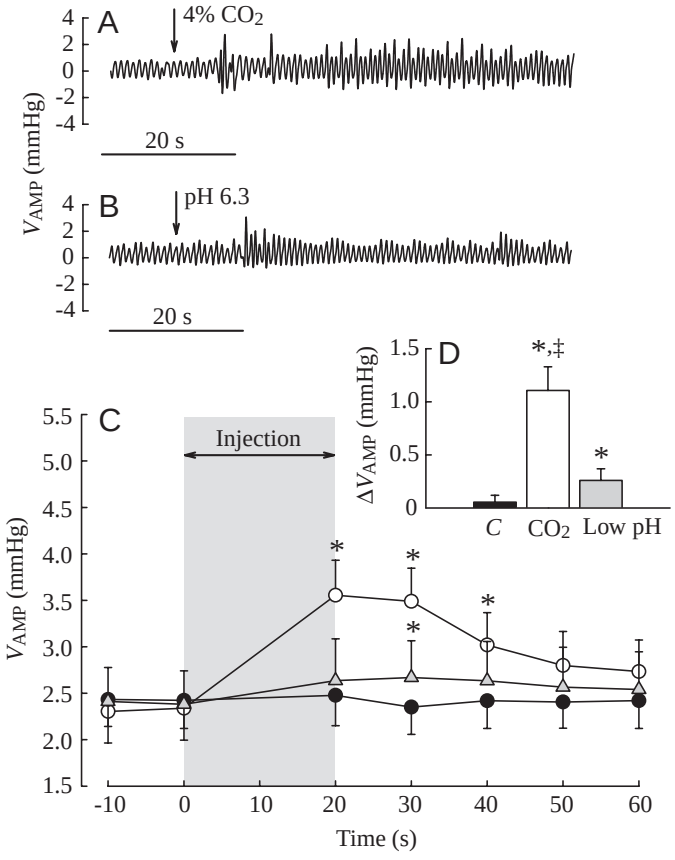
1 mmHg=0.133 kPa.

Control fish were given a bolus external injection of sea water. 'Pre' refers to the data immediately before injection and 'Post' refers to the data 20 s later.

Data are reported as means ± 1 S.E.M. (*N*).

Differences between pre and post values within any group are denoted by asterisks; differences in delta values from corresponding values in the control group are denoted by double daggers.

chemoreceptors. Similarly, the injection of CO_2-enriched saline into the caudal vein could also potentially activate both internal and external receptors as a result of additional excretion of CO_2 into the ventilatory water flowing past the gills.



Despite the improbability of exclusively stimulating a single population of receptors (external or internal), we are confident that preferential stimulation of receptor populations did occur in the present study. In other words, the extent of activation of putative internal receptors would be trivial during external injections in comparison with internal injections and *vice versa*. Assuming a ventilation volume of 327 $ml\,min^{-1}\,kg^{-1}$ (Lenfant and Johansen, 1966), we estimated that the external injections (50 $ml\,kg^{-1}$ of 4 % CO_2 delivered over 20 s) would yield a final P_{CO_2} of approximately 10 mmHg in the vicinity of the gill lamellae (assuming complete mixing with inspired water at P_{CO_2}=0.5 mmHg). Similarly, assuming complete mixing with venous blood (P_{CO_2}=1.5 mmHg) (K. M. Gilmour, unpublished observations) and a cardiac output of 25 $ml\,min^{-1}\,kg^{-1}$ in dogfish (Short et al., 1979; Bernier et al., 1999; Gilmour et al., 2001), internal injections (2 $ml\,kg^{-1}$ of 4 % CO_2 delivered over 20 s) would yield a final P_{CO_2} of approximately 7.3 mmHg. This estimation does not account for potential interconversion of $CO_2/HCO_3^-/H^+$ in the blood plasma prior to reaching the gill.

Fig. 4. The effects of external injections of CO_2-enriched sea water (4 % CO_2; white symbols; *N*=10) or CO_2-free acidified sea water (pH 6.3; grey symbols; *N*=9) on ventilation amplitude (V_{AMP}) in Atlantic salmon (*Salmo salar*). The effects of (A) CO_2 and (B) acidified water are depicted as representative traces and as mean values (C). Control fish (black symbols; *N*=12) received bolus injections of sea water. The shaded box represents the 20 s period of injection. (D) The changes in V_{AMP} (ΔV_{AMP}) at the 20 s time point. Data are shown as means +1 standard error of the mean. An asterisk denotes a statistically significant difference from the final pre-injection value (time zero). A double dagger denotes a statistically significant difference from the control and low-pH seawater groups. *C*, control. 1 mmHg=0.133 kPa.

Despite the presence of carbonic anhydrase in dogfish plasma (Wood et al., 1994), its catalytic capacity is low (Henry et al., 1997) and, thus, chemical interconversions within the plasma would be expected to be minor. It is unclear whether catalysed chemical reactions within the red blood cells further modified the plasma P_{CO_2} during transit of blood to the gills. Regardless, it is unlikely that external injection of CO_2-enriched sea water would yield higher levels of internal P_{CO_2} than after internal injection of CO_2-enriched saline and *vice versa*. Assuming a standard salmonid ventilation volume (approximately 200 ml min^{-1} kg^{-1}), the P_{CO_2} of the water flowing over the lamellae of Atlantic salmon would be approximately 13 mmHg.

Thus, the final CO_2 levels flowing over the gills (see above) are within the range encountered by many fish species and of levels experienced by salmonids in aquaculture facilities. For example, when fish are densely stocked and supplied with recirculating ground water, CO_2 tensions of approximately 20 mmHg are not uncommon (Eddy et al., 1977). Salmon generally inhabit well-aerated waters that are likely to exhibit low and constant levels of CO_2. It has been pointed out (Perry et al., 1999) that significant elevations in ambient CO_2 could potentially occur in large bodies of water subject to thermal stratification or in smaller lakes or rivers fed by carbonate- or bicarbonate-rich water derived from underground springs. With these conditions in mind, the Pw_{CO_2} values experienced by salmon in this study appear to be in line with putative acute hypercarbic exposure in the wild and/or in commercial holding facilities.

CO2 versus H^+

Dogfish

Previous studies on elasmobranchs have revealed pronounced effects of hypercarbia on ventilation (Randall et al., 1976; Heisler et al., 1988; Graham et al., 1990) and cardiovascular function (McKendry et al., 2001). The results of the present study, obtained using external injections of CO_2-enriched water, are consistent with previous studies that have demonstrated pronounced hyperventilation during hypercarbia that is largely (Graham et al., 1990; McKendry et al., 2001) or exclusively (Randall et al., 1976) caused by increases in breathing amplitude. The cardiovascular responses to external CO_2 injection (decreased $\dot{V}_b$, f_H and P_a and increased R_S) were also similar to those previously reported by McKendry et al. (2001) for dogfish exposed to hypercarbia. To our knowledge, this is the first study to attempt to distinguish between the effects of CO_2 *versus* H^+ on eliciting cardiorespiratory effects in any elasmobranch. The results clearly demonstrated that CO_2 itself was the single important variable controlling cardiorespiratory function. Although the injection of acidified sea water did elicit a bradycardia, a similar result was obtained using control sea water, indicating that injection itself was influencing cardiac frequency (albeit the effect was minor).

Salmon

While numerous previous studies using teleosts have focused on the effects of CO_2/H^+ on ventilation, comparatively few have examined cardiovascular responses. Even fewer have attempted to evaluate the relative contributions of CO_2 and H^+ to the cardiovascular responses to CO_2 (Sundin et al., 2000; Reid et al., 2000). Indeed, the present study is the first study to examine the relative roles of CO_2 and H^+ in promoting cardiovascular responses in a salmonid species. As in dogfish (see above), CO_2 was the predominant variable influencing cardiorespiratory function during external injections of hypercarbic water. The only variable directly affected by acidified water was V_{AMP}, and this effect was small (12 % increase) in comparison wiith the effect of CO_2-enriched water (52 %). Nevertheless, this result is in contrast to previous studies that have failed to demonstrate any direct effect of H^+ on ventilation (Janssen and Randall, 1975; Sundin et al., 2000; Reid et al., 2000). This discrepancy may simply reflect the fact that the present experiments were conducted in sea water rather than in fresh water. Sea water contains significantly higher levels of HCO_3^- than fresh water and thus the acidification of the inspired water may have led to significant formation of CO_2 as a result of the titration of HCO_3^- with H^+. Therefore, the minor effect of acidified water on ventilation observed in the present study may actually have been caused by an associated rise in P_{CO_2}.

Although these results (for both dogfish and salmon) suggest that changes in external H^+, *per se*, do not evoke cardiorespiratory responses, they do not exclude a role for H^+ in the overall response to hypercarbia. Indeed, it is likely that the production of H^+ within the chemoreceptor following the inward diffusion of CO_2 is a crucial mechanism in the signal transduction process underlying the cardiorespiratory responses to hypercarbia.

External versus *internal CO2 chemoreceptors*

A previous study (McKendry et al., 2001), using a branchial denervation technique *in vivo*, identified the gills as sites of CO_2 chemoreception in dogfish. Similar results were obtained using channel catfish [(*Ictalurus punctatus*) (Burleson and Smatresk, 2000)], tambaqui [(*Colossoma macropomum*) (Sundin et al., 2000)] or traira [(*Hoplias malabaricus*) (Reid et al., 2000)]. Although clearly identifying the gill as a CO_2 chemoreceptive site, these previous studies were unable to distinguish between external and internal orientation of the receptors. The results of the present study convincingly demonstrate that there is a population of branchial CO_2 chemoreceptors that are externally oriented and that monitor the CO_2 composition of the ventilatory water. Further, it would appear that there are no pre-branchial or afferent branchial internal CO_2/H^+ chemoreceptors, at least none that is activated at the levels of CO_2 used in the present study.

Because of the possibility that the CO_2 within the CO_2-enriched saline injected into the caudal vein was excreted during transit through the gills, the present results cannot exclude the possible involvement of CO_2/H^+ chemoreceptors within the efferent branchial or post-branchial circulation. However, the results of previous studies, in which arterial P_{CO_2} (Pa_{CO_2}) was elevated in dogfish by using acetazolamide injection to inhibit red blood cell carbonic anhydrase, did not support a role for efferent branchial or post-branchial internal CO_2/H^+ chemoreceptors in

stimulating ventilation (for a review, see Gilmour, 2001) or eliciting bradycardia (K. M. Gilmour, unpublished observations). In contrast, Heisler et al. (1988) and Graham et al. (1990) suggested that hyperventilation during hypercarbia in dogfish (*Scyliorhinus stellaris*) or skate (*Raja ocellata*) was triggered by arterial blood respiratory acidosis. Unlike the direct approach used in the present study, however, their conclusion was based on indirect correlative relationships between ventilation and blood acid–base status.

Although the orientation of the branchial CO_2 chemoreceptors in salmon was not examined in the present study, previous research on the involvement of internal *versus* external CO_2 receptors in salmonids has yielded conflicting results. For example, Wood and Munger (1994) reported that injection of trout with carbonic anhydrase reduced the extent of post-exercise CO_2 accumulation while also reducing the magnitude of the post-exercise hyperventilation. In contrast, experimental elevation of $P_{a}CO_2$, either by inhibiting CO_2 excretion using the carbonic anhydrase inhibitor acetazolamide or by exposing fish to hyperoxia, did not affect cardiorespiratory function in trout (McKendry and Perry, 2001). Clearly, further research is required before any general models can be formulated concerning the relative contributions of external and internal CO_2 receptors.

To summarize, the results of this study suggest that the vast majority, if not all, of the hypercarbic reflex can be explained by stimulation of externally oriented branchial chemoreceptors, activated by changes in $P_{w}CO_2$ itself, not by $[H^+]$. Similar studies are required in diverse fish species before CO_2 can be unequivocally adopted as the proximate trigger for hypercarbia-induced cardiorespiratory adjustments in fish.

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