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CO_2 chemoreception and the cardioventilatory effects of hypercarbia in fish

By

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"Personally, I'm always ready to learn,
although I do not always like being taught."

- **Winston Churchill**

"The sort of general malaise that only the genius
possess and the insane lament."

- **Dr. Evil**

"An intellectual is a man who takes more
words than necessary to tell more
than he knows"

- **Dwight D. Eisenhower**

"One fish, two fish,
red fish, blue fish."

- **Dr. Seuss**

Abstract

It has generally been accepted that O_2 (rather than CO_2/H^+) chemoreceptors predominate in controlling physiological function in fishes. Prior to this investigation the existing dogma had been that the physiological responses to hypercarbia (elevated external CO_2) were not directly attributable to changes in PCO_2/pH but were instead an indirect effect of the marked Bohr and Root effects that occur during respiratory acidosis. Recently, the literature in this area had begun to indicate otherwise and thus has inspired this thesis studying putative piscine CO_2/H^+ chemoreception and the cardioventilatory reflexes mediated by it during periods of elevated ambient CO_2.

In situ and *in vivo* experiments were performed on rainbow trout (*Oncorhynchus mykiss*) to examine i) the direct effect of CO_2 on the systemic vasculature and ii) the influence of internal versus external hypercarbic acidosis on a variety of cardiovascular variables. Results from *in situ* saline perfused trunk preparations indicated that CO_2 (0.6, 1.0 or 2.0% CO_2) elicited a significant vasodilation, but only in the presence of pre-existing humoral adrenergic tone (10^{-4} M adrenaline). In contrast, hypercarbia *in vivo* triggered a statistically significant increase in systemic resistance (~70%) that was associated with elevated ventral aortic (~42%) and dorsal aortic (~43%) blood pressures as well as a significant bradycardia (12%); cardiac output was not significantly affected. Separately, the carbonic anhydrase inhibitor acetazolamide (30 mg kg^{-1}) and external hyperoxia were successfully used to elevate the endogenous arterial partial pressure of CO_2 ($PaCO_2$). However, only the presence of elevated external partial pressure of CO_2 (ie. in the water; $PwCO_2$) elicited the aforementioned bradycardia and hypertension,

indicating that the cardiovascular responses to hypercarbia were, most likely, mediated exclusively by externally oriented chemoreceptors.

Adult Atlantic salmon (*Salmo salar*) were exposed to acute, localized environmental hypercarbia (2.0 and 4.0% CO_2 in air) over the gills via injections (60 ml/kg) of equilibrated seawater directly into the buccal cavity. In order to differentiate CO_2-specific cardiorespiratory adjustments from those triggered by pH during environmental hypercarbia a further series of injections were performed using aerated seawater titrated with HCl to the complimentary pH (pH=7.0 and 6.3, respectively). In response to 2.0 and 4.0 % CO_2 in air the salmon displayed significant, dose-dependent increases in ventilatory amplitude (V_{amp}, up to 83%) and frequency (V_f, up to 23%) alongside significant, dose-dependent decreases in cardiac output (V_b, up to 24%) and frequency (H_f, up to 26%), with no effect on arterial blood pressure (P_A). The complimentary series of external injections of seawater titrated to pH = 7.0 and 6.3 had no significant effect on any of the cardiovascular or ventilatory variables measured. These results show that, in Atlantic salmon, changes in PCO_2, and not changes in pH, are responsible for the hypercarbia-induced cardiorespiratory adjustments.

Adult Atlantic salmon (*Salmo salar*) and Pacific spiny dogfish (*Squalus acanthias*) were exposed to acute environmental hypercarbia (approximately 20 min). Experiments were performed to examine the influence of environmental hypercarbia on aspects of cardiorespiratory physiology, and in separate series of experiments the muscarinic antagonist atropine (100 nmol kg^{-1}; both species) and complete branchial denervation (dogfish) were used to investigate putative CO_2-chemoreceptive sites on the gills and their link to the autonomic nervous system. The data suggest that CO_2-sensitive

chemoreceptors located on the gills of both species are not only neuronally linked to the autonomic nervous system but are also responsible for mediating nearly all of the hypercarbia-induced cardioventilatory adjustments observed in this study.

Finally, it had become apparent through pilot studies that the magnitude of the cardioventilatory hypercarbic reflex was not universal across all fish species. The homogeneity of the cardiovascular and ventilatory responses amongst fish to elevated ambient CO_2 was investigated by exposing six species of fish to acute environmental hypercarbia (approximately 20 min). The experiments were performed *in vivo* using two marine teleosts, Atlantic salmon (*Salmo salar*) and Pacific sanddab (*Citharychthus sordidus*); two freshwater teleosts, brown bullhead (*Ictalurus nebulosus*) and American eel (*Anguilla rostrata*); as well as one marine elasmobranch, the Pacific spiny dogfish (*Squalus acanthias*). These results are compared with data previously obtained from rainbow trout (*Oncorhynchus mykiss*; McKendry and Perry, 2001). The results of this investigation revealed that there is large inter-specific variation in the cardiovascular and ventilatory responses of fishes to environmental hypercapnia. These hypercarbia-induced adjustments range from *A. rostrata* which displayed no cardioventilatory adjustments during hypercarbic exposure ($PwCO_2 = 6.33 \pm 0.20$ torr), to *C. sordidus* which experienced a phenomenal bradycardia (~66%; $PwCO_2 = 8.14 \pm 0.50$ torr).

Résumé

Il a longtemps été accepté que des chimiorécepteurs d'O_2 (plutôt que ceux du CO_2/H^+) prédominent dans les fonctions physiologiques des poissons. Préalablement à notre étude, les croyances étaient que les réponses physiologiques de l'hypercarbie (CO_2 externe élevé) n'étaient pas directement attribuables aux changements du PCO_2/pH mais plutôt, à un effet indirect de Bohr et Root qui survient durant l'acidose respiratoire. Récemment, la littérature dans ce domaine commence à indiquer autrement ce qui a inspiré cette thèse étudiant la chimioréception CO_2/H^+ possible chez les poissons et les réflexes cardiorespiratoires médiés durant une période de CO_2 ambiant élevé.

Des expériences *in situ* et *in vivo* ont été effectuées sur des truites arc-en-ciel (*Oncorhynchus mykiss*) pour examiner i) les effets directs du CO_2 sur la circulation systémique et ii) l'effet de l'acidose hypercapnique interne versus externe sur une variété de variables cardiovasculaires. Les résultats d'une perfusion *in situ* du tronc avec du salin ont indiqués que le CO_2 (0.6, 1.0 ou 2.0 % CO_2) a causé une vasodilatation significative mais seulement avec la présence d'un tonus adrénergique hormonal pré-existant (10^{-4} M adrénaline). Au contraire, l'hypercarbie *in vivo* a déclenché une augmentation statistiquement significative de la résistance systémique (~70%) qui est associée à une élévation de la pression sanguine de l'aorte ventral (~42%) et de l'aorte dorsal (~43%) ainsi que d'une bradycardie significative (12%); le débit cardiaque n'était pas significativement affecté. D'autre part, l'acétazolamide (30mg), un inhibiteur de l'anhydrase carbonique, et l'hyperoxie externe ont élevé la pression partielle artérielle de CO_2 ($PaCO_2$). Par contre, seulement la présence d'une pression partielle élevée du CO_2

externe (c'est-à-dire dans l'eau; $PwCO_2$) a causé la bradycardie et l'hypertension souvent mentionnée, ce qui indique que les réponses cardiovasculaires à l'hypercarbie étaient médiées exclusivement par des chimiorécepteurs orientés vers l'extérieur.

Des saumons de l'Atlantique adultes (*Salmo salar*) fut exposés à une hypercarbie environnementale aigue (2.0 et 4.0 % CO_2 dans l'air) localisée au dessus des branchies par une injection (60 ml/kg) d'eau de mer équilibrée directement dans la cavité buccale. Afin de différencier les ajustements cardiorespiratoires spécifiques au CO_2 de ceux causés par le pH durant une hypercarbie environnementale, une autre série d'injections utilisant de l'eau de mer titré avec du HCl au pH complémentaire de (pH = 7.0 et 6.3 respectivement) fut faites. En réponse au 2.0 et 4.0% de CO_2 dans l'air, les saumons ont manifesté une augmentation de l'amplitude respiratoire (V_{amp}, jusqu'à 83%) et de la fréquence respiratoire (V_f, jusqu'à 23%) dépendant de la dose, ainsi qu'une diminution du débit cardiaque (V_b, jusqu'à 24%) et de la fréquence cardiaque (H_f, jusqu'à 26%) dépendant de la dose, sans avoir un effet sur la pression sanguine artériel (P_A). La série complémentaire d'injections externes titrées à un pH de 7.0 et de 6.3, n'a eu aucun effet sur les variables cardiovasculaires et respiratoires mesurées. Ces résultats démontrent que chez les saumons de l'Atlantique, c'est le changement de PCO_2 et non celui du pH qui est responsable pour les ajustements cardiorespiratoires induits par l'hypercarbie.

Des saumons de l'Atlantique adultes (*Salmo salar*) et l'aiguillat commun (*Squalus acanthias*) ont été exposés à une hypercarbie environnementale aigue (approximativement 20 min). Des expériences ont été faites pour examiner l'influence d'une hypercarbie environnementale sur les aspects physiologiques de la cardiorespiration. D'autres séries d'expériences séparées, l'atropine, un antagoniste

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Finalement, il est devenu évident que les études préliminaires sur le réflexe cardiorespiratoire face à l'hypercarbie n'était pas universel chez tous les espèces de poissons. On a étudiés l'homogénéité des réponses cardiovasculaires et respiratoires à une élévation du CO_2 ambiant chez six espèces de poissons, en les exposant à une hypercarbie environnante aigue (approximativement 20 min). Ces expériences ont été faites *in vivo* chez deux téléostéens marins, le saumon de l'Atlantique (*Salmo salar*) et le sanddab du pacifique (*Citharychthus sordidus*); deux téléostéens d'eau douce, la barbotte brune (*Ictalurus nebulosus*) et l'anguille d'Amérique (*Anguilla rostrata*); ainsi qu'un élasmobranche marin, l'aiguillat commun (*Squalus acanthias*). Ces résultats sont comparés aux données obtenues antérieurement avec la truite arc-en-ciel (*Oncorhynchus mykiss*; McKendry and Perry, 2001). Les résultats de cette étude ont révélé une grande variation inter-spécifique des réponses cardiovasculaires et respiratoires face à une hypercapnie environnementale. Les ajustements induits par l'hypercarbie variaient de *A. rostrata* qui ne démontrait aucun ajustement cardiorespiratoire durant l'exposition à l'hypercarbie ($PwCO_2$ = 6.33 ± 0.20 torr), jusqu'à *C. sordidus* qui subissait une bradycardie phénoménale (66% $PwCO_2$ = 8.14 ± 0.50 torr).

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Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The existing dogma

It is generally accepted that O_2 (rather than CO_2/H^+) chemoreceptors predominate in the control of physiological function in fishes (e.g. Cameron, 1989). Certainly, their important role in mediating cardio-respiratory adjustments during hypoxia is universally recognized (for a review see Fritsche and Nilsson, 1993).

That aside, environmental acidosis, hypercapnia (elevation of internal CO_2 levels), hypercarbia (elevation of external CO_2 levels) and the blood acidosis associated with exercise have all been demonstrated to elevate ventilation in fish (Randall and Jones, 1973; Janssen and Randall, 1975; Eddy, 1976; Thomas and LeRuz, 1982; Thomas, 1983; Heisler et al., 1988; Wood et al., 1990; Kinkead and Perry, 1991; Wood and Munger, 1994; Perry and Gilmour, 1996). Because of the inverse relationship between the partial pressure of CO_2 (PCO_2) and pH it has been difficult to distinguish both the nature of the specific ventilatory stimulus and its site of action (Milsom, 1995a & b). General consensus had been that ventilatory responses to hypercarbia-induced changes in arterial blood PCO_2 and pH ($PaCO_2$ and pHa, respectively) in fish were an indirect effect of the marked Bohr and Root effects that can occur in hypercapnic fish blood. By impairing the blood oxygen transport capability (Hughes and Shelton, 1962; Cameron, 1971; Eddy, 1971, 1973), these effects were believed to be indirectly triggering this hypercarbic reflex via receptors sensitive to changes in arterial oxygen content (Smith and Jones, 1982; Perry et al., 1989). Several studies supported this point of view (see review by Milsom, 1995). For example, in trout ventilation volume increased during normoxic hypercapnia,

apparently due to decreased blood oxygen saturation and content (Randall and Jones, 1973; Eddy, 1976; Smith and Jones 1982; Kinkead and Perry, 1991). However, ventilation returns to pre-hypercapnic levels if water PO_2 is increased and blood O_2 content increases, despite sustained increases in $PaCO_2$ (Smith and Jones, 1982). Moreover, during chronic hypercapnia, the oxygen saturation of trout blood returns to pre-hypercapnic values with a similar time course as the decline in ventilation (Eddy and Morgan, 1969; Eddy, 1976). Lastly, ventilation frequency in teleosts was shown to fall drastically during hyperoxia despite rising $PaCO_2$ and falling pHa (Randall and Jones, 1973).

The Root effect

The Root effect is described as the reduction in hemoglobin (Hb) oxygen-carrying capacity caused by a decrease in blood pH (pHa; Scholander and Van Dam, 1954). This drop in blood pH is observed under several different situations. For example, metabolic activity of the tissues produces and releases either CO_2 or lactic acid during aerobic or anaerobic energy metabolism, respectively. Either one of these metabolites is capable of acidifying the blood (Pelster and Randall, 1998). Lowering of blood pH may also accompany increased $PaCO_2$, and perhaps most pertinent to this thesis, occurs when fish are exposed to elevated ambient CO_2 (hypercarbia). Increasing PCO_2 in the water ($PwCO_2$) reverses what is typically an outwardly directed CO_2 diffusion gradient, causing a net inward diffusion of CO_2 through the gills, thus resulting in an elevated $PaCO_2$ (hypercapnia). Figures 1-1 outlines the sequence of diffusive processes and chemical reactions involved in the release of O_2 from the hemoglobin via the Root effect.

It should also be noted that the Root effect is generally considered to be exclusive to teleost hemoglobins; however, it has been observed in mollusc hemocyanins (Bridges et al, 1984; Miller and Magnum, 1988). Interestingly, Brix (1982) reported a reversed Root effect in the blood of the marine gastropod, *Buccinum undatum*, effectively allowing these organisms to increase their oxygen carrying capacity without increasing hematocrit concentration.

The Bohr effect

The Bohr effect describes the change (Δ) in Hb-O_2 affinity that results from a change in proton concentration ($[H^+]$), often expressed numerically as $\Delta \log P_{50}/\Delta pH$ – where P_{50} represents an expression of oxygen affinity; the partial pressure of O_2 a which a respiratory pigment is 50% saturated with O_2 (i.e. Bohr coefficient; Riggs, 1988; Brauner and Randall, 1998). One method of measuring the magnitude of the Bohr effect is by measuring the change in Hb-O_2 affinity when pH is changed by adding a strong acid or base at fixed PCO_2.

In nearly all species of teleost fish studied to date CO_2 excretion, other than dissolved CO_2, depends on HCO_3^- dehydration. Carbonic anhydrase, which catalyzes HCO_3^- dehydration, is not accessible to the plasma in teleost fish (Henry *et al.*, 1988; Perry *et al.*, 1997), but exists in very high concentrations in the red blood cell, allowing virtually all bicarbonate dehydration to occur in the red blood cell.

Elasmobranchs have carbonic anhydrase activity in (or accessible to) their plasma, with relatively lower carbonic anhydrase activity in the red blood cell, virtually eliminating the Bohr effect in these species (Wood et al., 1994). Figures 1 - 2 illustrates

the production and consumption of Bohr protons within the red blood cell during gas exchange at the gills.

Cracks in the dogma

There were some difficulties, however, with the theory that the hypercarbia-induced cardiorespiratory responses in fish were simply a reflection of reduced blood O_2-transport capability. First and foremost, virtually no data exist to support a direct link between CO_2 and cardiovascular adjustments observed during periods of elevated ambient CO_2. With respect to ventilation, a review article by Milsom (1995) points out that goldfish blood, for example, shows Bohr and Root effects but these fish have been shown to exhibit no respiratory response to hypercarbia or acid exposure (Dejours, 1972, 1973). Furthermore, pronounced hyperventilatory responses to CO_2 have been reported in elasmobranchs (Heisler *et al.*, 1988; Wood *et al.*, 1990; Graham *et al.*, 1990; Perry and Gilmour, 1996), species that lack Root effects (Butler and Metcalfe, 1988). Other studies have demonstrated significant respiratory responses in teleosts to the respiratory acidosis accompanying hyperoxia, mild hypercarbia or exercise, independent of changes in arterial O_2 or other potential respiratory stimulants (Heisler *et al.*, 1988; Wood *et al.*, 1990; Kinkead and Perry, 1991; Perry and Gilmour, 1996; Burleson and Smatresk, 2000).

More recently, Perry et al. (1999) clearly demonstrated a significant dose-dependent cardio-respiratory response to step-wise increases in ambient CO_2 in rainbow trout (*Oncorhynchus mykiss*), while clearly disassociating these hypercarbic reflexes from any physiologically significant hypoxaemia. This study represented the first definitive

separation of hypercarbia-specific cardio-respiratory effects from those typically attributed to hypoxaemia.

The exact mechanism(s) by which CO_2 exerts its effects on ventilation and cardiovascular function is not known, but recent pharmacological evidence alludes to externally oriented CO_2/H^+ receptors linked to the autonomic nervous system. Perry et al. (1999) demonstrated that prior treatment of *O. mykiss*, with the α–adrenoreceptor antagonist yohimbine prevented the increased blood pressures and systemic vascular resistance observed during acute hypercarbia but did not influence the CO_2-induced bradycardia.

In response to the aforementioned evidence and inconsistencies, this study aimed to answer a number of questions concerning CO_2/H^+ chemoreception in fish, including the presence, orientation, localization and function of the proposed chemoreceptors, as well as the universality of response to CO_2/H^+ and their mechanism of action.

Goals

1. To confirm the hypercarbia-induced cardiovascular effects observed by Perry et al. (1999).
2. To investigate the potential direct role of CO_2 acting on the systemic vasculature, thereby contributing to the systemic hypertension observed during acute hypercarbic exposure.
3. To evaluate whether the cardiovascular effects of hypercarbia are attributable to external and/or internal elevation of PCO_2.

4. To evaluate the relative contributions of external CO_2 versus those of external pH (H^+) towards the piscine hypercarbic cardiorespiratory response.
5. To investigate, *in vivo*, via pharmacological means the putative link between the suggested branchial CO_2 chemoreceptors and the autonomic nervous system.
6. To investigate, *in vivo*, via neuronal transection, a putative link between the suggested branchial CO_2 chemoreceptors and the autonomic nervous system.
7. To investigate the variability among fish species with respect to their CO_2/H^+ chemoreceptive abilities and their cardiovascular and ventilatory responses to CO_2.

Overall hypothesis:

Externally oriented branchial chemoreceptors sensitive to changes in ambient CO_2, linked to the antonomic nervous system, contribute to the mediation of cardioventilatory parameters, observed in select piscine species, during acute hypercarbic exposure.

Figure 1-1. The sequence of diffusional processes and chemical reations involved in the release of oxygen from hemoglobin (Hb) via the Root effect (CA, carbonic anhydrase). Adapted from Pelster et al (1992).

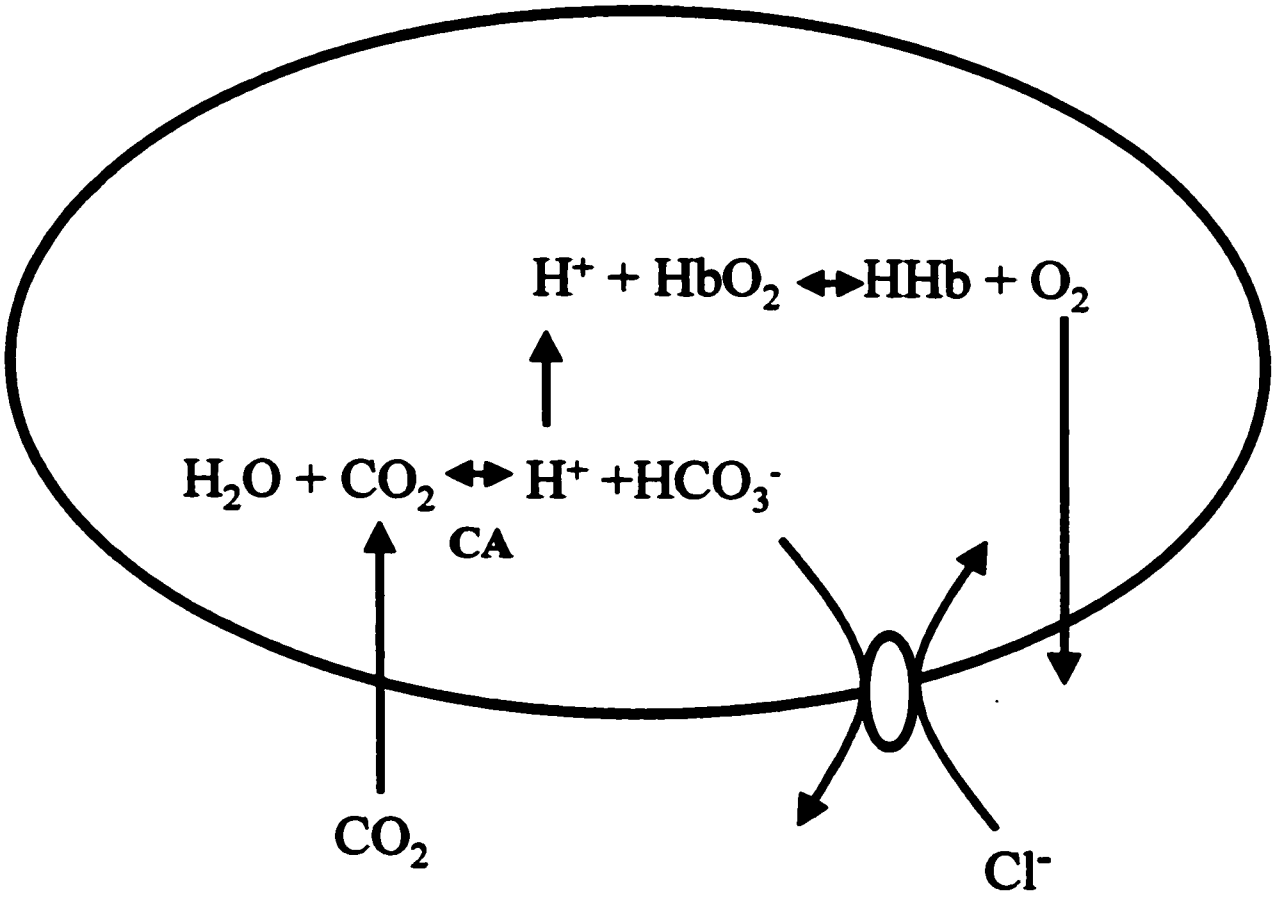
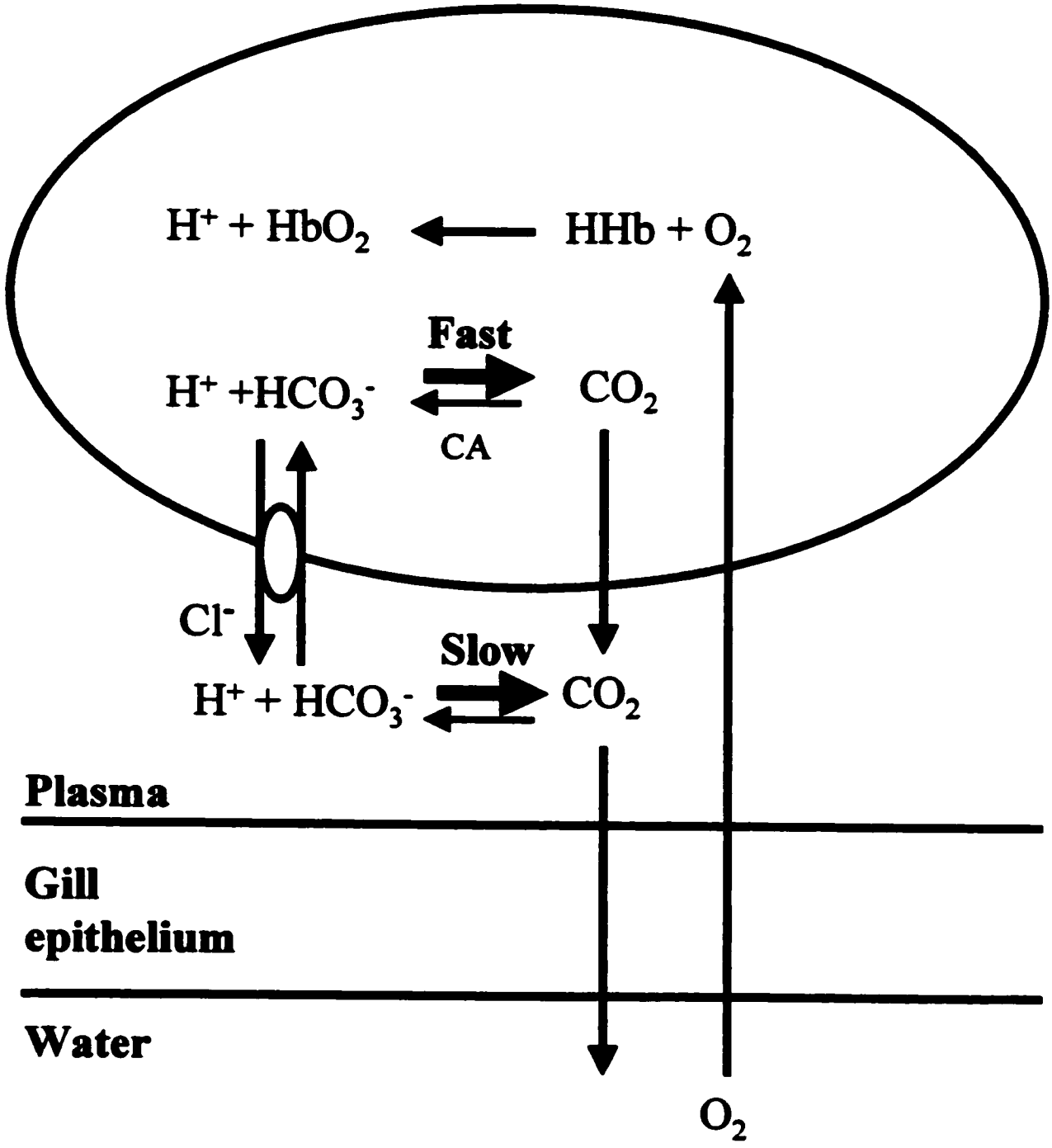

H+ + HbO2 ↔HHb + O2
H2O + CO2 ↔ H+ +HCO3-
CA
CO2
Cl-

Figure 1-2. Diagrammatic representation of gas exchange at the gills. Oxygen diffuses into the red cell and binds to hemoglobin (Hb), releasing Bohr protons (H^+). CA, carbonic anhydrase. Adapted from Brauner and Randall (1998).


H+ + HbO2
HHb + O2
Fast
H+ +HCO3-
CO2
CA
Cl-
Slow
H+ + HCO3-
CO2
Plasma
Gill
epithelium
Water
O2

Chapter 2

Cardiovascular effects of hypercarbia in rainbow trout (*Oncorhynchus mykiss*): A role for externally oriented chemoreceptors

ABSTRACT

In situ and *in vivo* experiments were performed on rainbow trout (*Oncorhynchus mykiss*) to examine i) the direct effect of CO_2 on the systemic vasculature and ii) the influence of internal versus external hypercarbic acidosis on cardiovascular variables including blood pressure, cardiac output and systemic vascular resistance. Results from *in situ* saline perfused trunk preparations indicated that CO_2 (0.6, 1.0 or 2.0% CO_2) elicited a significant vasodilation, but only in the presence of pre-existing humoral adrenergic tone. In the absence of pre-existing vascular tone, CO_2 was without effect on systemic resistance. In contrast, hypercarbia *in vivo* triggered a statistically significant increase in systemic resistance (~70%) that was associated with elevated ventral aortic (~42%) and dorsal aortic (~43%) blood pressures as well as a significant bradycardia (~12%); cardiac output was not significantly affected.

To determine the potential roles of internal versus external chemoreceptors in mediating the cardiovascular responses to hypercarbia, experiments were performed that elevated arterial partial pressure of CO_2 ($PaCO_2$) without ncreasing in external PCO_2 ($PwCO_2$). In one series, trout were given a bolus injection of the carbonic anhydrase inhibitor acetazolamide (30 mg kg^{-1}) to inhibit CO_2 excretion and thus raise $PaCO_2$ 5-7 h prior to being exposed to an acute increase in $PwCO_2$ (maximum $PwCO_2 = 6.3 \pm 0.4$ mm Hg). Despite a marked increase in $PaCO_2$ (~7 mm Hg) after injection of acetazolamide, there was no increase in dorsal aortic blood pressure (P_{DA}) or systemic resistance (R_S). The ensuing exposure to hypercarbia, however, significantly increased P_{DA} (~20%) and R_S (~35%). A second series of experiments used a 5 - 7 h period of exposure to

hyperoxia (PwO_2 = 643 ± 16 mm Hg) to establish a new, elevated baseline $PaCO_2$ (7.8 ± 1.1 mm Hg) without any change in $PwCO_2$. Despite a steadily increasing $PaCO_2$ during the 5 - 7 h of hyperoxia there was no associated increase in P_{DA} or R_S. Ensuing exposure to hypercarbia, however, significantly increased P_{DA} (~20%) and R_S (~150%). Plasma adrenaline levels were increased significantly during exposure to hypercarbia and thus likely contributed to the accompanying cardiovascular effects that were observed.

These findings demonstrate that the cardiovascular effects associated with hypercarbia in rainbow trout are unrelated to any direct constrictory effects of CO_2 on the systemic vasculature and are unlikely to be triggered by activation of internally oriented receptors. Instead, the data suggest that the cardiovascular responses associated with hypercarbia are mediated exclusively by externally oriented chemoreceptors.

INTRODUCTION

It is generally accepted that O_2 (rather than CO_2/H^+) chemoreceptors predominate in controlling physiological function in fishes (e.g. Cameron, 1989). Certainly, their important role in mediating cardio-respiratory adjustments during hypoxia is universally recognized (for a review see Fritsche and Nilsson, 1993). An equivalent role for CO_2/H^+ receptors is less obvious. Although there is extensive documentation on the effects of hypercarbia on modulating ventilation (e.g. Janssen and Randall, 1975; Thomas and Le Ruz, 1982; Thomas, 1983; Wood and Munger, 1994; Burleson and Smatresk, 2000), the effects generally have been attributed to changes in blood O_2 status arising from Bohr and Root effects, rather than to specific effects of CO_2 (e.g. Randall, 1982; Smith and Jones 1982). This traditional view, however, has been challenged (see Perry and Wood, 1989) for a variety of reasons. These include pronounced hyperventilatory responses to CO_2 in i) elasmobranchs (Heisler *et al.*, 1988; Wood *et al.*, 1990; Graham *et al.*, 1990; Perry and Gilmour, 1996), species that lack Root effects (Butler and Metcalfe, 1988), and ii) teleosts displaying no significant impairment of blood O_2 status (e.g. during mild hypercarbia or hyperoxic hypercarbia; Kinkead and Perry, 1991; Burleson and Smatresk, 2000). Thus, models of the control of breathing in fishes have been revised extensively in recent years to include a role for CO_2/H^+ receptors (Milsom, 1995a,b). Experiments incorporating denervation techniques (Burleson and Smatresk, 2000; Reid *et al.*, 2000; Sundin *et al.*, 2000) have revealed that the gill is a significant site of CO_2 chemosensitivity.

In contrast, there is a paucity of data concerning the effects of CO_2 on piscine cardiovascular physiology. Recently, however, Perry *et al.* (1999) demonstrated that hypercarbia specifically elicits profound cardiovascular adjustments in rainbow trout that include increased blood pressure and systemic resistance. Although Perry *et al.* (1999) successfully identified CO_2/H^+ as the stimulus for the cardiovascular responses during hypercarbia, that study was unable to discern whether the effects were caused by the environmental hypercarbia acting on externally oriented chemoreceptors or by the ensuing rise in endogenous levels of CO_2 influencing internally oriented receptors. Moreover, the possibility that the pressor response to hypercarbia was related to specific constrictory effects on the systemic vasculature, though suggested by Perry *et al.* (1999), has not been tested.

The goals of this study, therefore, were i) to evaluate whether the cardiovascular effects of hypercarbia in rainbow trout are attributable to external and/or internal elevation of PCO_2 and ii) to investigate the potential role of CO_2 acting directly on the systemic vasculature. The experiments were performed *in situ* using a saline-perfused trunk preparation (Wood and Shelton, 1975) and *in vivo* using an extracorporeal arterial blood shunt (Thomas, 1994).

MATERIALS AND METHODS

Experimental animals

Rainbow trout, *Oncorhynchus mykiss*, of either sex were obtained from Linwood Acres Trout Farm (Cambellcroft, Ontario) and were transported in oxygenated water to the University of Ottawa. Trout were maintained on a 12L:12D photoperiod in 1300 l fiberglass tanks supplied with flowing, aerated and dechlorinated City of Ottawa tap water (14°C). They were fed a commercial pelleted diet and given at least 2 weeks to acclimate to the holding conditions prior to experimentation.

In situ experiments

An *in situ* pump-driven trunk perfusion preparation (Wood and Shelton, 1975) was used to investigate the possible direct effects of CO_2 on the systemic vasculature, that could potentially attenuate or contribute to *in vivo* increases in R_S during hypercarbia. Rainbow trout, weighing between 182 and 271 g (experimental N = 39) were euthanised by anaesthetic overdose using 2 g l^{-1} ethyl *m*-aminobenzoate (MS-222) buffered with 4 g l^{-1} $NaHCO_3$. The fish was placed ventral side up on an operating table, the heart was exposed and 50 i.u. kg^{-1} of sodium heparin was injected into the ventricle over a 1 min period and allowed to circulate for 4 min. The animal was then completely transected at the anterior end of the heart, the atrium and ventricle were removed, and the dorsal aorta was cannulated using polyethylene tubing (Clay Adams, PE 160). A circular incision was made (~0.5 cm in depth) around the spine and dorsal aorta to allow for a sunken silk ligature to secure the cannula and prevent leakage due to back-pressure. The

preparation was then immediately submersed in a constant-level 0.9% NaCl saline bath (14^{O} C) and perfused with aerated Cortland saline (Wolf, 1963).

The reservoir of perfusate was contained in a temperature controlled constant-level water bath atop a Mettler top-loading balance. The preparation was supplied with a constant flow of perfusate via an adjustable speed polystaltic pump (Buchler Instruments) preset to supply approximately 5 ml kg total body weight min^{-1}; the change in weight of the saline reservoir was used to calculate actual flow rates. The pump output was noticeably pulsatile, so an inverted 500 ml Erlenmeyer flask containing an air bubble (300 - 400 ml) was inserted as a Windkessel to dampen the pulsatility between the trunk and the pump (Wood and Shelton, 1975). The entire system, from trunk to perfusate was connected using polyethylene tubing (PE 160). Perfusate back-pressure from the trunk was monitored via a T-joint accessing a UFI model 1050BP pressure transducer pre-calibrated daily against a static column of water. Analog pressure signals were converted to digital data and collected at 10 samples sec^{-1} on a computer accompanied by a data-acquisition system (Biopac) using AcqKnowledge 3.03 software.

Series I: Investigation of potential direct constrictory effects of CO_2 on the systemic vasculature

Environmental hypercarbia *in vivo*, was recently shown to elicit an increase in systemic resistance (Perry *et al.* 1999). This experimental series was designed to assess whether this effect was due, at least in part, to CO_2 acting directly on the systemic vasculature.

After the trunk preparation had stabilized and a constant baseline pressure was established (approximately 30 min), experimental recordings were begun. After an additional 10 min of perfusing the trunk with aerated saline, the perfusate was switched (via a three-way valve) to saline gassed with either 0.6%, 1.0% or 2% CO_2 to yield PCO_2's of approximately 4.5, 7.5 and 15 mm Hg. The preparation was perfused with hypercapnic saline for 20 min before switching back to aerated saline for the remaining 10 min. As the perfusate left the water bath which sat on the top-loading scale weight/flow measurements were recorded manually at regular intervals to facilitate calculations of R_S.

Series II: Investigation of potential direct dilatory effects of CO_2 on the systemic vasculature

Initial procedures were identical to those used in Series I. However, after the initial 10 min of data recording using aerated saline, the perfusate was switched (via a three-way valve) to aerated saline containing 10^{-4} mol l^{-1} adrenaline (bitartrate salt) to induce vasoconstriction. Within 40 min, the preparation had reached a new stable, elevated baseline level of vasoconstriction and the perfusate was switched once more, to saline containing 10^{-4} mol l^{-1} adrenaline that was gassed either with air (control), 0.6%, 1.0% or 2% CO_2. Experiments were terminated after a further 70 min.

In vivo experiments

Surgical procedures

Rainbow trout, weighing between 707 and 1450 g (experimental N = 27) were anaesthetized using benzocaine (0.10 g l^{-1} ethyl-*p*-aminobenzoate, Sigma) until breathing movements stopped. The fish were then placed on an operating table where their gills were force-ventilated with the same oxygenated anaesthetic solution. To permit drug injections and measurement of dorsal aortic pressure (P_{DA}) a polyethylene cannula (Clay Adams, PE 50) was implanted into the dorsal aorta via percutaneous puncture of the roof of the buccal cavity (Soivio *et al.*, 1975). To allow an extracorporeal blood shunt, a lateral incision was made in the caudal peduncle immediately below the lateral line to expose, separate from the surrounding tissue, and cannulate (PE 50) the caudal artery and vein in the anterograde direction. The incision was closed using sutures and both cannulae were secured to the caudal peduncle independently via silk ligatures.

A small ventral incision was made to expose the pericardial cavity, and the pericardium was dissected to expose the bulbus arteriosus. To allow measurement of central venous pressure (P_{VEN}), a non-occlusive silicone cannula (0.51 mm ID; 0.94 mm OD) was implanted into the right horn of the ductus of Cuvier and secured with cyanoacrylate glue (Olson et al., 1997). The silicone cannula was then attached to a polyethylene tube (Clay Adams, PE 90), approximately 75 cm in length, filled with heparinized (50 i.u. ml^{-1} sodium heparin) Cortland saline. To allow measurement of ventral aortic pressure (P_{VA}) the ventriculo-bulbar junction was temporarily clamped shut to restrict blood flow while the bulbus arteriosus was cannulated with silicone tubing. The clamp was removed and 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 ($\dot{V}b$; Olson *et al.*, 1997). The incision was sutured and

sealed with Vetbond™ (3M Animal Care Products, MN). The bulbus and Ductus cannulae as well as the flow probe cable were secured externally to the ventral surface of the fish using silk ligatures and Vetbond™. After surgery, fish were revived and placed in opaque acrylic boxes supplied with flowing water where they were left to recover for ~24 h prior to experimentation.

Experimental protocol

Measurement of blood/water respiratory variables

The extracorporeal blood shunt employs a peristaltic pump to continuously pull blood from the caudal artery, passing it over PO_2, pH and PCO_2 electrodes before returning it to the fish via the cannulated caudal vein (Perry and Gilmour, 1996). O_2, CO_2 and pH electrodes (Radiometer) were housed in temperature controlled cuvettes and connected to a Radiometer blood gas analyzer. To reduce the chance of blood clotting in the tubing or electrodes, the entire loop was flushed for 10 min with a sodium heparin solution (1000 i.u. ml^{-1} in saline) immediately prior to experimentation. A similar pump-driven loop continuously withdrew water from just in front of the fishes mouth and passed it over a separate set of temperature controlled of PO_2, PCO_2 and pH electrodes that were connected to a Radiometer and Cameron Instruments meters. All electrodes were calibrated prior to each individual experiment (Perry *et al.* 1999).

Measurement of cardiovascular variables

The dorsal aorta, Ductus of Cuvier and bulbus arteriosus cannulae were connected to UFI model 1050BP pressure transducers pre-calibrated against a static column of water. Mean blood pressure was calculated as (systolic pressure + diastolic pressure)/2. Cardiac output was determined by connecting the 3S or 4S ultrasonic flow probe to a Transonic T106 small animal blood flow meter. Analog signals were converted to digital data and collected at 40 samples sec^{-1} on a computer accompanied by a data-acquisition system (Biopac Systems) using AcqKnowledge 3.03 software. Recordings began upon stabilization of blood and water levels.

Series I: Cardiovascular effects of hypercarbia

Once blood pressures and water/cardiovascular values had stabilized, an initial blood sample (0.5 ml) was drawn, for plasma catecholamine content (see below), from the return line of the extracorporeal blood shunt. After 10 min of normocarbic normoxia, the water supplying the fish box was rendered hypercarbic for 20 min by gassing a water equilibration column with 1.5% CO_2 in air (Cameron flowmeter model GF-3/MP). A second blood sample (0.5 ml) was drawn at maximum hypercarbic exposure, immediately prior to switching the inflow water back to normocarbic normoxia. In this series systemic resistance was calculated using the formula: $R_S = (P_{DA} - P_{VEN})/(Vb)$; gill resistance was calculated using the formula: $R_g = (P_{VA} - P_{DA})/(Vb)$.

Series II: Cardiovascular effects of hypercarbia in acetazolamide treated fish

The carbonic anhydrase inhibitor acetazolamide was used to investigate the cardiovascular effects of external versus internal hypercapnia. Once stable blood

pressures and water/cardiovascular values were achieved, the fish were administered a bolus injection of acetazolamide (30 mg kg^{-1}) via the return line of the extracorporeal blood shunt and data were recorded for 5 - 7 h, at which time $PaCO_2$ had risen to a new elevated baseline level. At this point an initial blood sample (0.5 ml) was drawn from the return line of the extracorporeal blood shunt. After 10 min of normocarbic normoxia, the water supplying the fish box was rendered hypercarbic for 20 min (see above). A second blood sample (0.5 ml) was drawn at maximum hypercarbic exposure, immediately prior to switching the inflow water back to normocarbic. Owing to potential duress of the substantial surgical load during these prolonged experiments and the lack of effect of CO_2 on R_g in *series I*, ventral aortic and Ductus of Cuvier cannulae were not implanted for *series II* and *III*. In turn, R_g was not calculated and R_S was calculated as $R_S = P_{DA}/(Vb)$ for *series II & III*.

Series III: Cardiovascular effects of hypercarbia in hyperoxic fish

Because of potential non-specific effects of acetazolamide on cardiorespiratory function, a second 'non-drug induced' method (i.e. hyperoxia) was used to elevate endogenous levels of CO_2. Once blood pressures and water/cardiovascular values had stabilized, the water supplying the fish box was rendered hyperoxic by gassing a water equilibration column with 100% O_2 for 6 - 8 h, at which time $PaCO_2$ had established a new elevated baseline level. At this point an initial blood sample (0.5 ml) was drawn from the return line of the extracorporeal blood shunt. Following 10 min of normocarbic normoxia, the water supplying the fish was rendered hypercarbic for 20 min as *in series I*

- *II.* A second blood sample (0.5 ml) was drawn at maximum hypercarbic exposure, immediately prior to switching the inflow water back to hyperoxic normocarbia.

Analytical techniques

All blood samples collected for measurements of catecholamines were centrifuged immediately at 12 000g for 1 min, and flash-frozen in liquid N_2 before being placed in storage at -80°C. Plasma noradrenaline and adrenaline levels were determined on alumina-extracted samples (200 µl) using high pressure liquid chromatography (HPLC) with electrochemical detection (Bernier and Perry 1997). The HPLC consisted of a Varian Star 9012 solvent delivery system (Varian Chromatography Systems) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments). The extracted samples were passed through an Ultratechsphere ODS-C_{18}5 µm column (HPLC technology Ltd.) and the separated amines were integrated with the Star Chromatography software program (version 4.0, Varian). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations.

Statistical analyses

Data are presented as mean values ± one standard error of the mean (SEM). For the *in situ* experiments (Figure 2-1), the statistical significance of the observed effects of a given treatment over time were tested using a one-way repeated measures ANOVA followed by a Dunnett post-hoc pairwise comparison with the final control point (t = 10 min). For the *in vivo* experiments (Table 2-1 and Figures 2-2, 2-4, 2-6 and 2-7), the

statistical significance of the observed effects was determined using paired t-tests or one-way repeated measures ANOVA followed by a Dunnett's post-hoc pairwise comparison. All statistical analyses were performed using SigmaStat 2.0 commercial software package (SPSS Inc.) with a fiducial limit of 5%. All figures were plotted using the Sigmaplot 4.0 commercial graphics software package (SPSS Inc.).

RESULTS

In situ experiments

Direct effects of CO_2 on systemic vasculature

Owing to a large degree of variability amongst the initial R_S values of the different perfused preparations, the absolute change in R_S is depicted in Figs 2-1. Absolute changes were determined by subtracting the value at the time of switching perfusate CO_2 composition (10 min) from all previous and subsequent data points. In the absence of humoral and neuronal tone, perfusing the systemic vasculature of decapitated trout with saline equilibrated with 0.6 - 2.0% CO_2 had no vasoconstrictory effect. In contrast, trunks that were pre-perfused with aerated saline containing 10^{-4} mol l^{-1} adrenaline (creating an increase in R_S of 56.4 ± 3.2 mm Hg ml min^{-1} kg^{-1}) displayed a significant decrease in R_S upon adding 0.6 - 2.0% CO_2 to the perfusate (Fig 2-1B - D). In the absence of CO_2 (i.e. continued perfusion with aerated saline containing 10^{-4} mol l^{-1} adrenaline), R_S remained stable (Figure 2-1A). While this CO_2-induced dilation of the systemic vasculature was not dose-dependent in the range studied (0.6 - 2.0%), the overall direct effect of CO_2 clearly was vasodilatory.

In vivo experiments

Series I. Cardiovascular effects of hypercarbia

Exposing rainbow trout to an acute increase in $PwCO_2$ (from 0.63 ± 0.18 to 6.15 ± 0.40 mm Hg) caused a statistically significant increase in P_{DA} and P_{VA} with no effect on gcardiac output despite a significant decrease in heart frequency (Fig 2-2A - D).

Furthermore, there was no increase in central venous pressure (normocarbia: $P_{VEN} = 3.9 \pm 1.3$ mm Hg; maximum hypercarbia: $P_{VEN} = 4.7 \pm 1.2$ mm Hg). The significant increases in arterial pressure with virtually no change in venous pressure or cardiac output during hypercarbia resulted in stable gill resistance but a significant increase in systemic resistance of approximately 70% (Fig 2-2E & F).

Series II. Cardiovascular effects of hypercarbia in acetazolamide treated fish

Figure 2-3 depicts typical data acquisition traces from a single representative fish and illustrates the effects of increasing internal $PaCO_2$ in the absence of elevated external PCO_2 as well as the cardiovascular response to hypercarbia in these carbonic anhydrase inhibited trout. The single bolus injection of acetazolamide triggered an immediate drop in R_S that was followed by a rapid and sustained elevated level of Rs over the ensuing 7 h. Importantly, the increase in R_S was not preceded by any changes in $PaCO_2$ and there clearly was no correlative relationship between $PaCO_2$ and R_S as long as $PwCO_2$ remained low. However, upon exposure to hypercarbia, there was a marked and progressive increase in R_S that was correlated with both $PwCO_2$ and $PaCO_2$.

The quantitative data from these experiments are shown in Fig 2-4. Acetazolamide injection and the ensuing rise in $PaCO_2$ did not statistically affect P_{DA}, Vb or R_S; H_f was significantly reduced by ~25 – 30%. The decrease in H_f occurred within a few minutes of acetazolamide injection and was not correlated with $PaCO_2$ (data not shown). Rainbow trout subjected to virtually the same degree of hypercarbia as in *series I* (Table 1) were still able to increase P_{DA} (~20%) and R_S (~35%) (Figure 2-4A & D, respectively) despite the pre-existing acetazolamide-induced elevated $PaCO_2$ (Table 2-1).

It is worth noting (see Table 2-1) that the mean increase in $PaCO_2$ during hypercarbia (3.3 mm Hg) was much less than the increase caused by acetazolamide (7.0 mm Hg).

Series III. Cardiovascular effects of hypercarbia in hyperoxic fish

Figure 2-5 represents excerpts from a typical data acquisition trace, illustrating both the lack of hyperoxia-induced cardiovascular adjustments as well as the vasoconstrictory response to hypercarbia in these hyperoxic trout. Although there was no change in $PwCO_2$, $PaCO_2$ gradually increased during hyperoxia with no correlative increase in R_S. The quantitative data from these experiments are shown in Fig 2-6. Rainbow trout subjected to the same degree of hypercarbia as in *series I* increased their P_{DA} (~20%) and R_S (~150%) (Figure2-6A & D, respectively) despite the hyperoxia-induced elevated $PaCO_2$ (Table 2-1), which, in itself, did not elicit any cardiovascular changes. There was no effect of hypercarbia on heart rate or cardiac output (Figure 2-6B & C).

Water/blood gases and catecholamines during hypercapnia

During each of the hypercarbic periods in the three aforementioned series, the significant increase in $PwCO_2$ triggered a statistically significant rise in $PaCO_2$. Although PaO_2 appeared to increase during hypercarbia in each of the three treatments, the increase was statistically significant only in the control group. There was no change in PwO_2 in switching from normocarbia to hypercarbia within each treatment (Table 2-1).

Neither acetazolamide nor hyperoxia elicited a significant increase in resting/normocapnic plasma levels of noradrenaline and adrenaline (Figure 2-7 A&B).

However, while hypercarbia elicited a slight, albeit statistically insignificant increase in noradrenaline in each of the three treatments, adrenaline levels rose significantly with hypercarbia for each group (i.e. control, acetazolamide-treated, and hyperoxia exposed) with hypercarbia.

Figure 2-1. Effects of acute elevations of perfusate PCO_2 on systemic resistance (R_S) in pre-contracted perfused trunk preparations of rainbow trout (*Oncorhynchus mykiss*). In all preparations, adrenaline (10^{-4} mol l^{-1}) was first added to the perfusate to attain an increased baseline R_S prior to raising PCO_2 in the continued presence of adrenaline. The interval of elevated PCO_2 begins with the dashed vertical line. A) Control (air only; N = 5), B) 0.6% CO_2 (N = 6), C) 1.0% CO_2 (N = 5) D) 2% CO_2 (N = 5). Values are means ± 1 SEM; significant differences ($P < 0.05$) from the normocarbic values (time = 10 min) are indicated by horizontal double arrows.

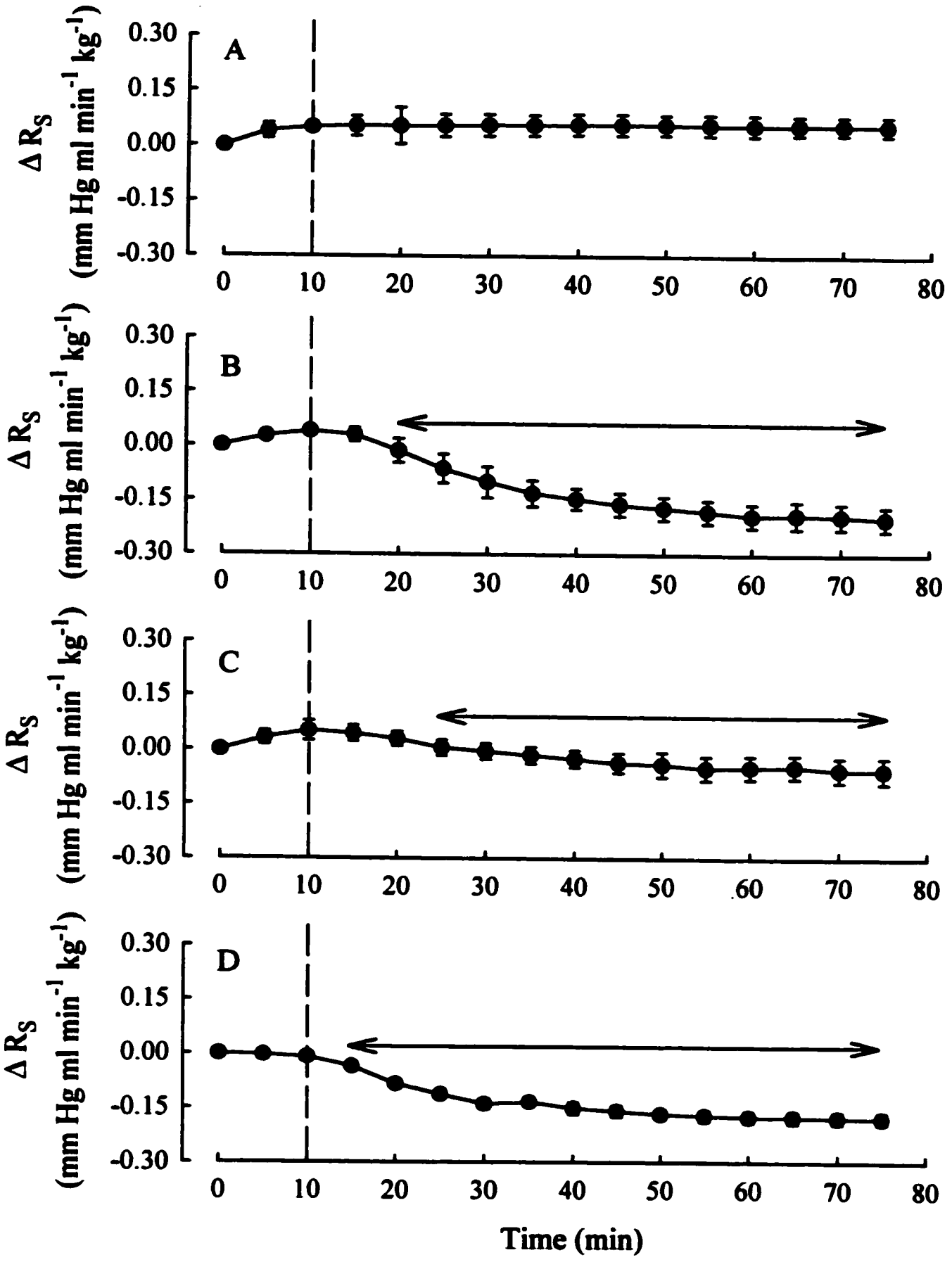

A
B
C
D
0.30
0.15
0.00
-0.15
-0.30
0
10
20
30
40
50
60
70
80
Δ R_S (mm Hg ml min^-1 kg^-1)
Time (min)

Figure 2-2. The effects of hypercarbia ($PwCO_2$ = 6.2 ± 0.4 mm Hg) on cardiovascular variables in the rainbow trout (*Oncorhynchus mykiss*) including A) dorsal aortic blood pressure (P_{DA}; N = 9), B) ventral aortic pressure (P_{VA}; N = 6), C) heart rate (H_f; N = 9), D) cardiac output (Vb; N = 8), E) gill resistance (R_g; N = 6) and F) systemic resistance (R_S; N = 6). Black bars represent pre-hypercarbic (i.e. normocarbia) values; white bars represent maximal responses obtained during hypercarbia. Values are means ± 1 SEM; * denotes a significant difference between normocarbia and hypercarbia ($P < 0.05$).

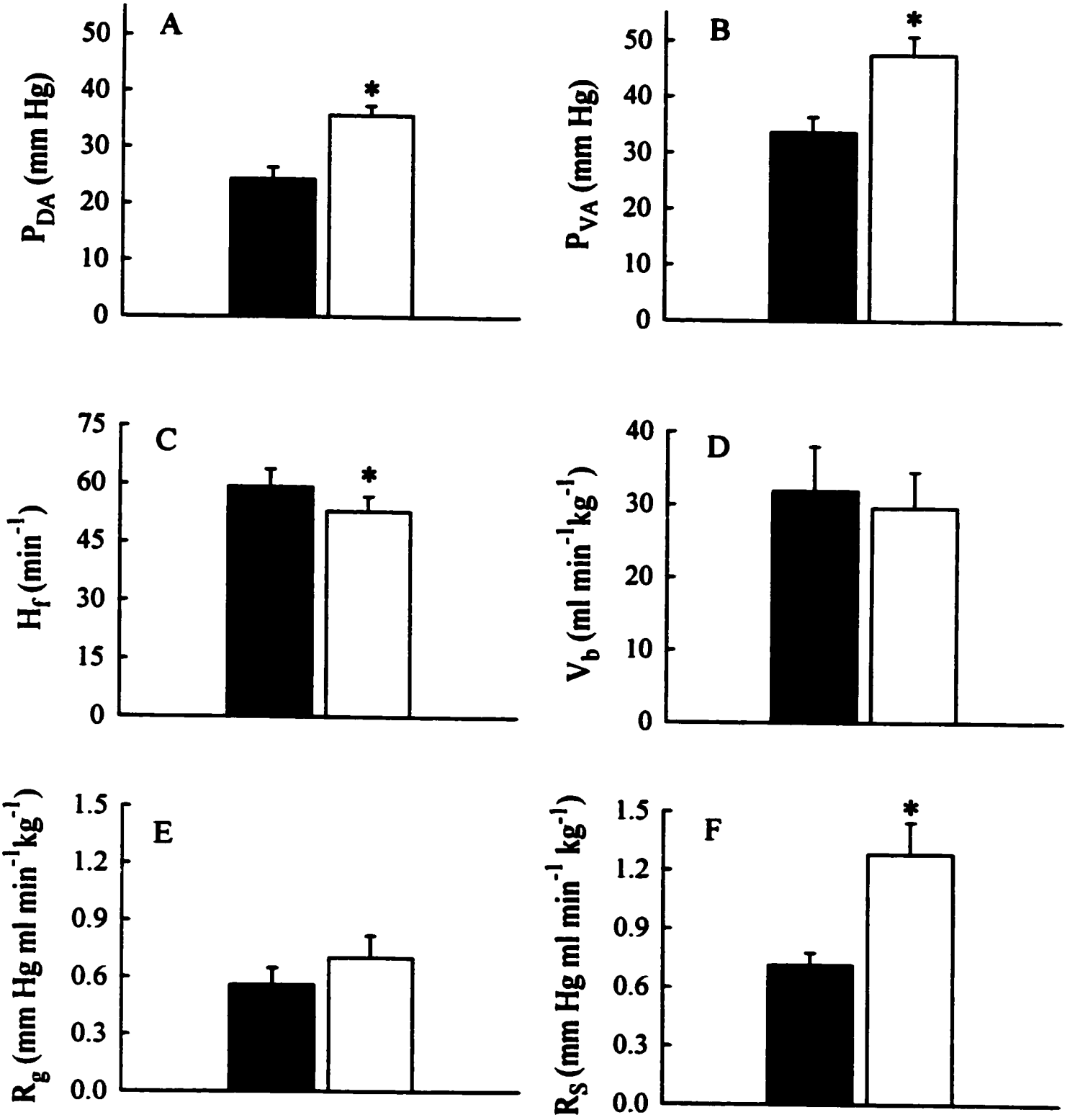

A
P_{DA} (mm Hg)
50
40
30
20
10
0
*
B
P_{VA} (mm Hg)
50
40
30
20
10
0
*
C
H_f (min^{-1})
75
60
45
30
15
0
*
D
V_b (ml min^{-1}kg^{-1})
40
30
20
10
0
E
R_g (mm Hg ml min^{-1}kg^{-1})
1.5
1.2
0.9
0.6
0.3
0.0
F
R_S (mm Hg ml min^{-1} kg^{-1})
1.5
1.2
0.9
0.6
0.3
0.0
*

Figure 2-3. Representative data acquisition traces illustrating the typical effects of acetazolamide injection (dashed line) followed by hypercarbia (shaded area) on A) water PCO_2 ($PwCO_2$), B) arterial PCO_2 ($PaCO_2$) and C) systemic resistance (R_S) in rainbow trout, *Oncorhynchus mykiss*.

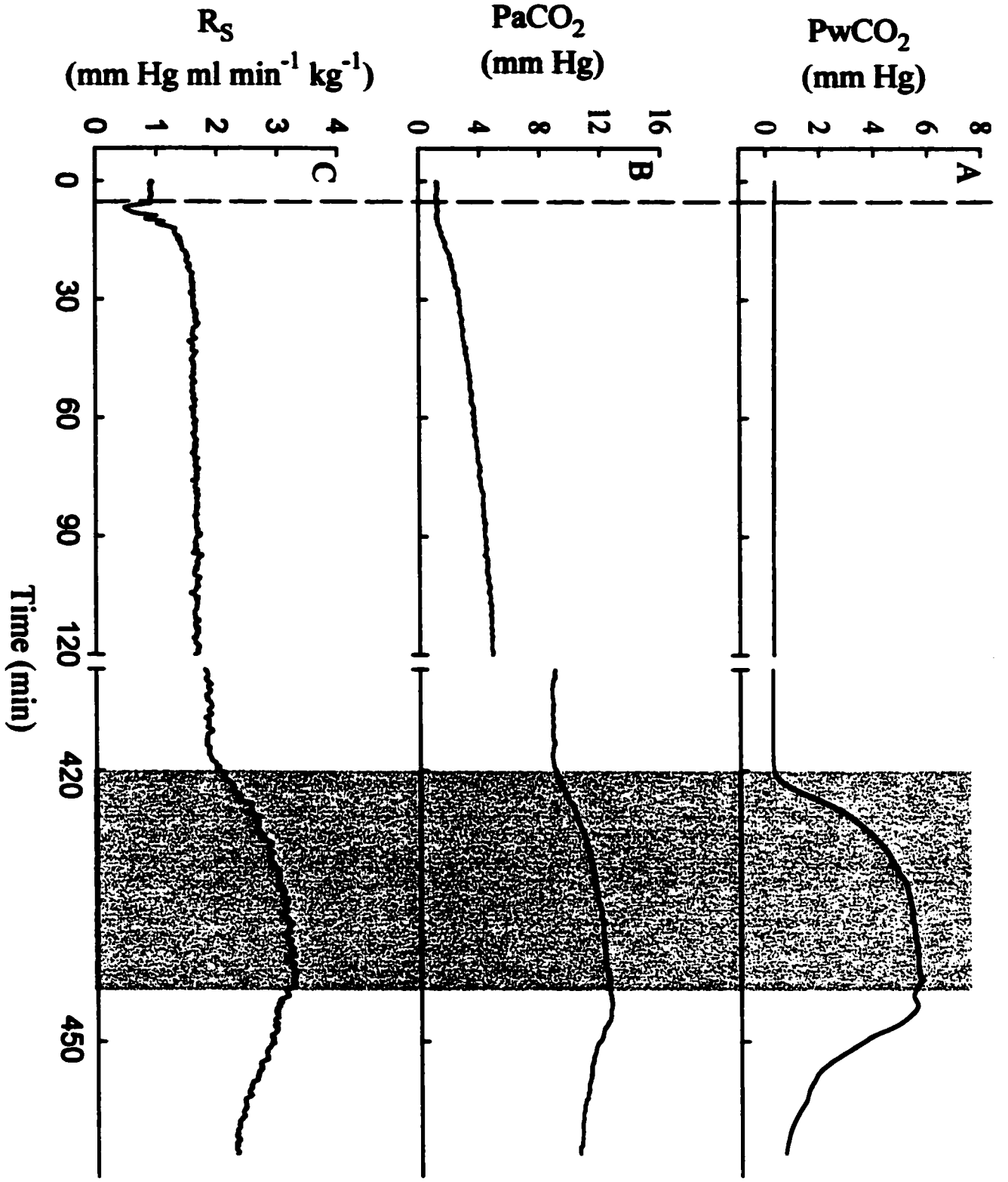

R_S
(mm Hg ml min^{-1} kg^{-1})
$PaCO_2$
(mm Hg)
$PwCO_2$
(mm Hg)
0 1 2 3 4
C
0 4 8 12 16
B
0 2 4 6 8
A
Time (min)
0
30
60
90
120
420
450

Figure 2-4. The effects of acetazolamide injection followed by hypercarbia ($PwCO_2$ = 6.3 ± 0.4 mm Hg) on cardiovascular variables in rainbow trout including A) dorsal aortic pressure (P_{DA}; N= 9), B) heart rate (H_f; N = 7), C) cardiac output (Vb; N = 8) and D) systemic resistance (R_S; N = 7). Black bars represent pre-acetazolamide normocarbia, light gray bars represent post-acetazolamide normocarbic values, white bars represent values after 20 min of hypercarbia. Values are means ± 1 SEM. * denotes a significant difference from pre-acetazolamide normocarbia; † denotes a significant difference between post-acetazolamide normocarbia and hypercarbia ($P < 0.05$).

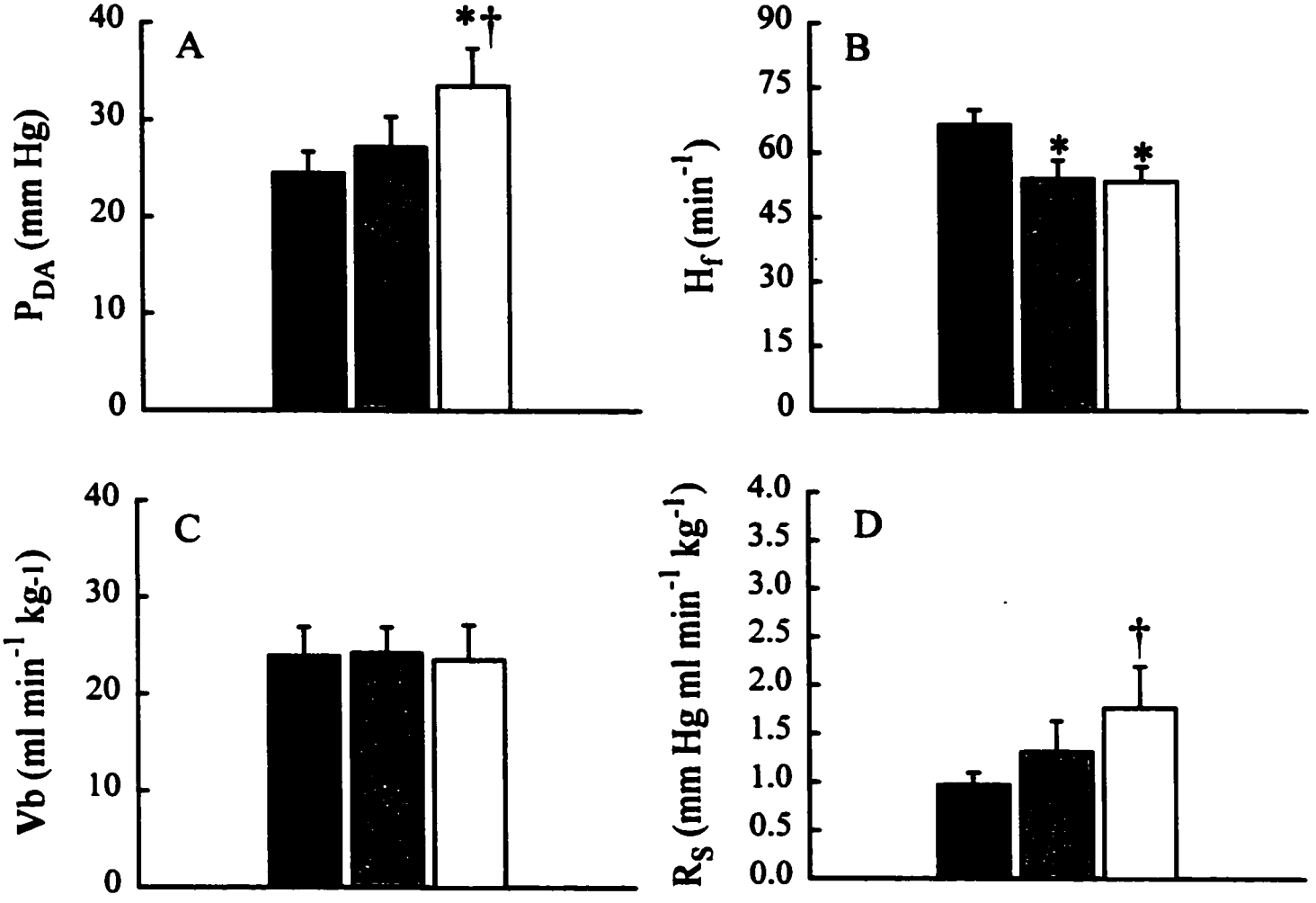

A
B
C
D
P_{DA} (mm Hg)
H_f (min^{-1})
Vb (ml min^{-1} kg-1)
R_S (mm Hg ml min^{-1} kg^{-1})
40
30
20
10
0
90
75
60
45
30
15
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*†
*
*
†

Figure 2-5. Representative data acquisition traces illustrating the typical effects of hyperoxia (dashed line) followed by hypercarbia (shaded area) on A) water PO_2 (PwO_2) B) water PCO_2 ($PwCO_2$), C) arterial PCO_2 ($PaCO_2$) and D) systemic resistance (R_S) in rainbow trout, *Oncorhynchus mykiss*.

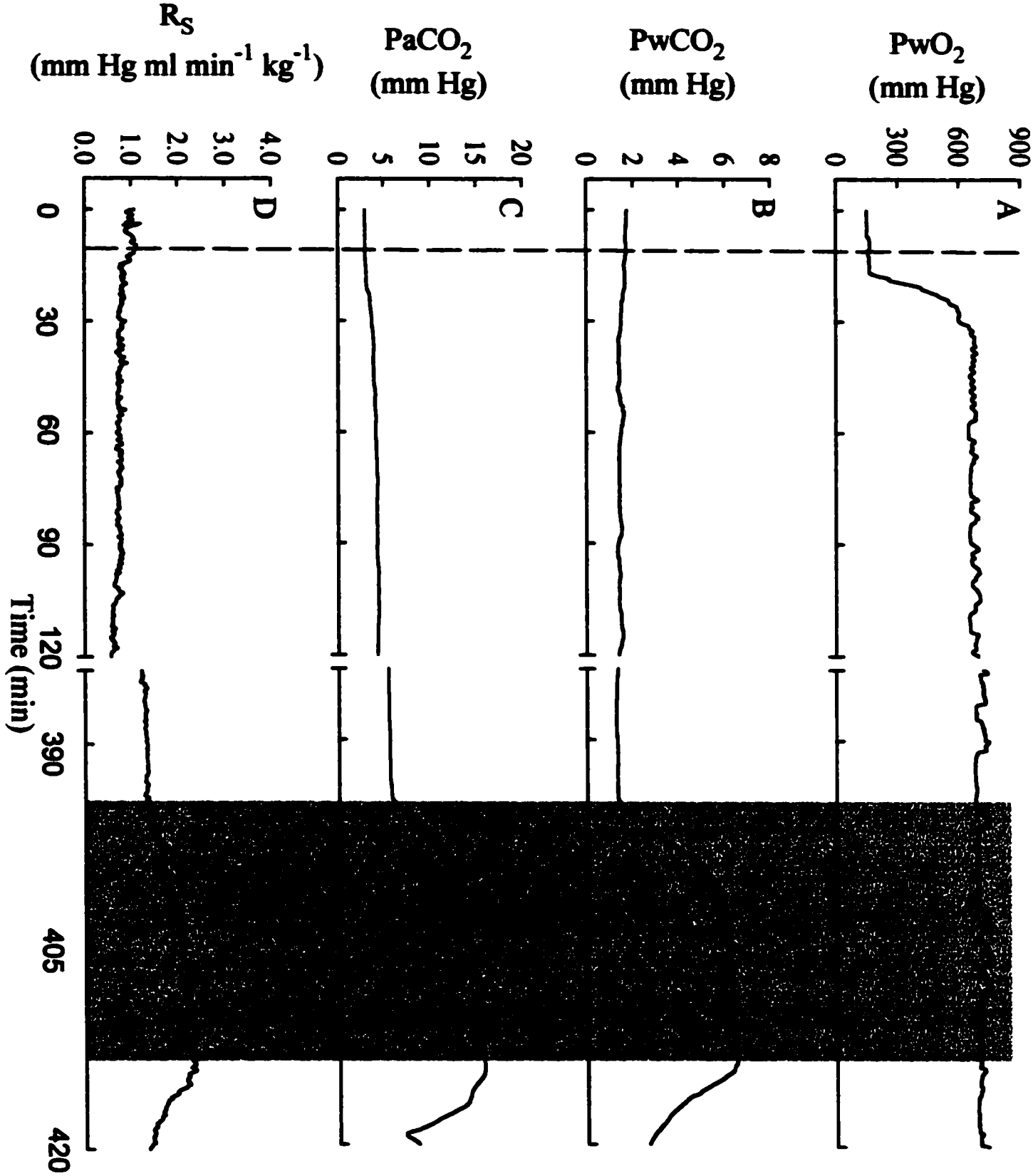

R_S
(mm Hg ml min^{-1} kg^{-1})
$PaCO_2$
(mm Hg)
$PwCO_2$
(mm Hg)
PwO_2
(mm Hg)
0.0
1.0
2.0
3.0
4.0
D
0
5
10
15
20
C
0
2
4
6
8
B
0
300
600
900
A
0
30
60
90
120
390
405
420
Time (min)

Figure 2-6. The effects of hyperoxia (PwO_2 = 643.2 ± 16.4 mm Hg) followed by hypercarbia ($PwCO_2$ = 7.2 ± 0.7 mm Hg) on cardiovascular variables in rainbow trout including A) dorsal aortic pressure (P_{DA}; N = 9), B) heart rate (H_f; N = 7), C) cardiac output (Vb; N = 9) and D) systemic resistance (R_S; N = 7). Black bars represent normoxic normocarbia values, light gray bars represent hyperoxic normocarbic values, white bars represent values after 20 min of hypercarbia. Values are means ± 1 SEM. * denotes a significant difference from pre-hyperoxia normocarbia; † denotes a significant difference between post-hyperoxia normocarbia and hypercarbia ($P < 0.05$).

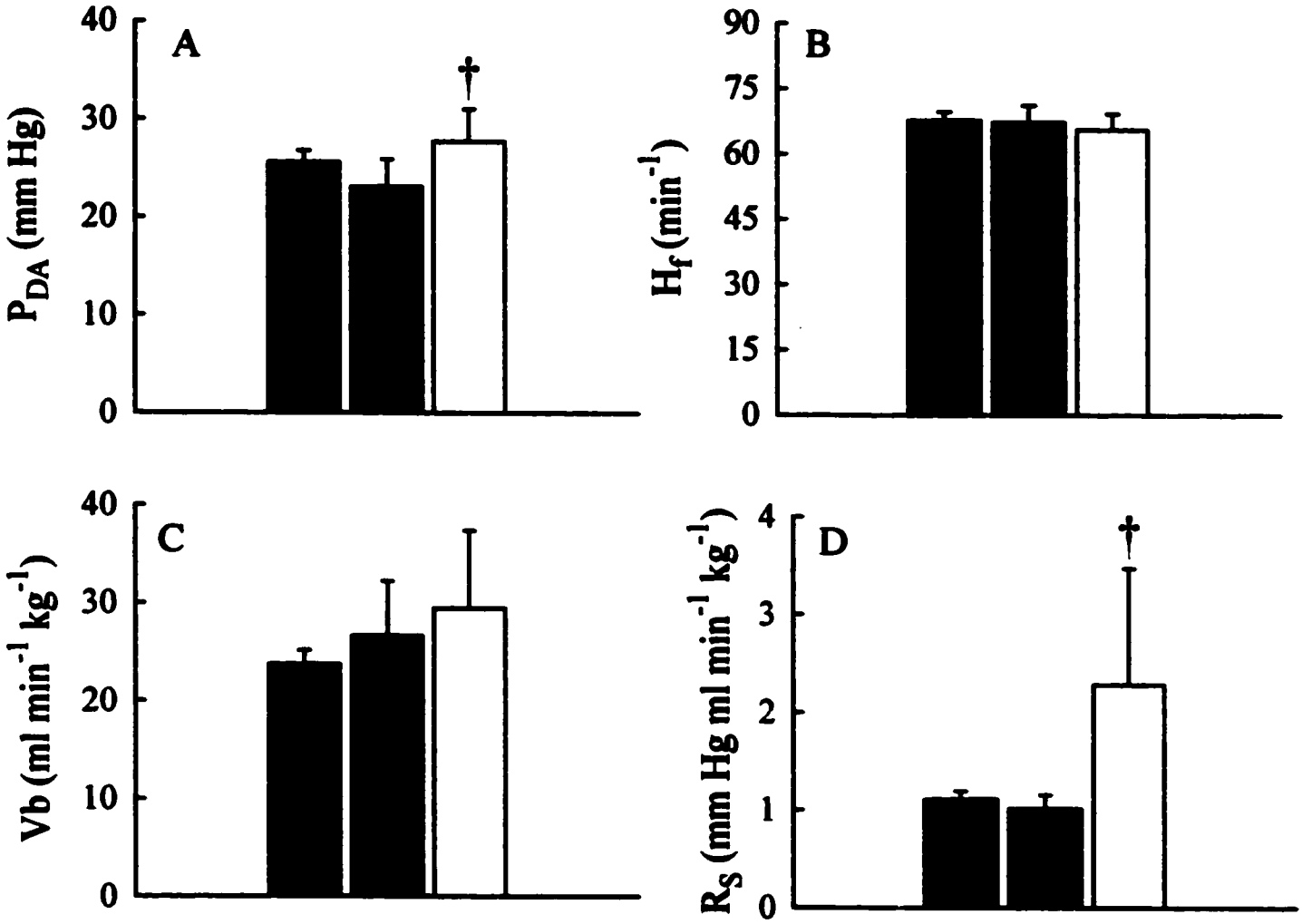

A
P_{DA} (mm Hg)
40
30
20
10
0
†
B
H_f (min^{-1})
90
75
60
45
30
15
0
C
Vb (ml min^{-1} kg^{-1})
40
30
20
10
0
D
R_S (mm Hg ml min^{-1} kg^{-1})
4
3
2
1
0
†

Figure 2-7. The effects of external hypercarbia ($PwCO_2$ = 6.2 ± 0.4 mm Hg) on plasma levels of A) noradrenaline and B) adrenaline in rainbow trout. Black bars represent the untreated, control, group (n = 7); light gray bars represent the acetazolamide treated group (n = 6); white bars represent the hyperoxia-exposed group (n = 6). 'Pre' = immediately prior to hypercarbia, 'max' = at maximum hypercarbia reached. Values are means ± 1 SEM; * denotes a significant difference between normocarbia and maximum hypercarbia ($P < 0.05$).

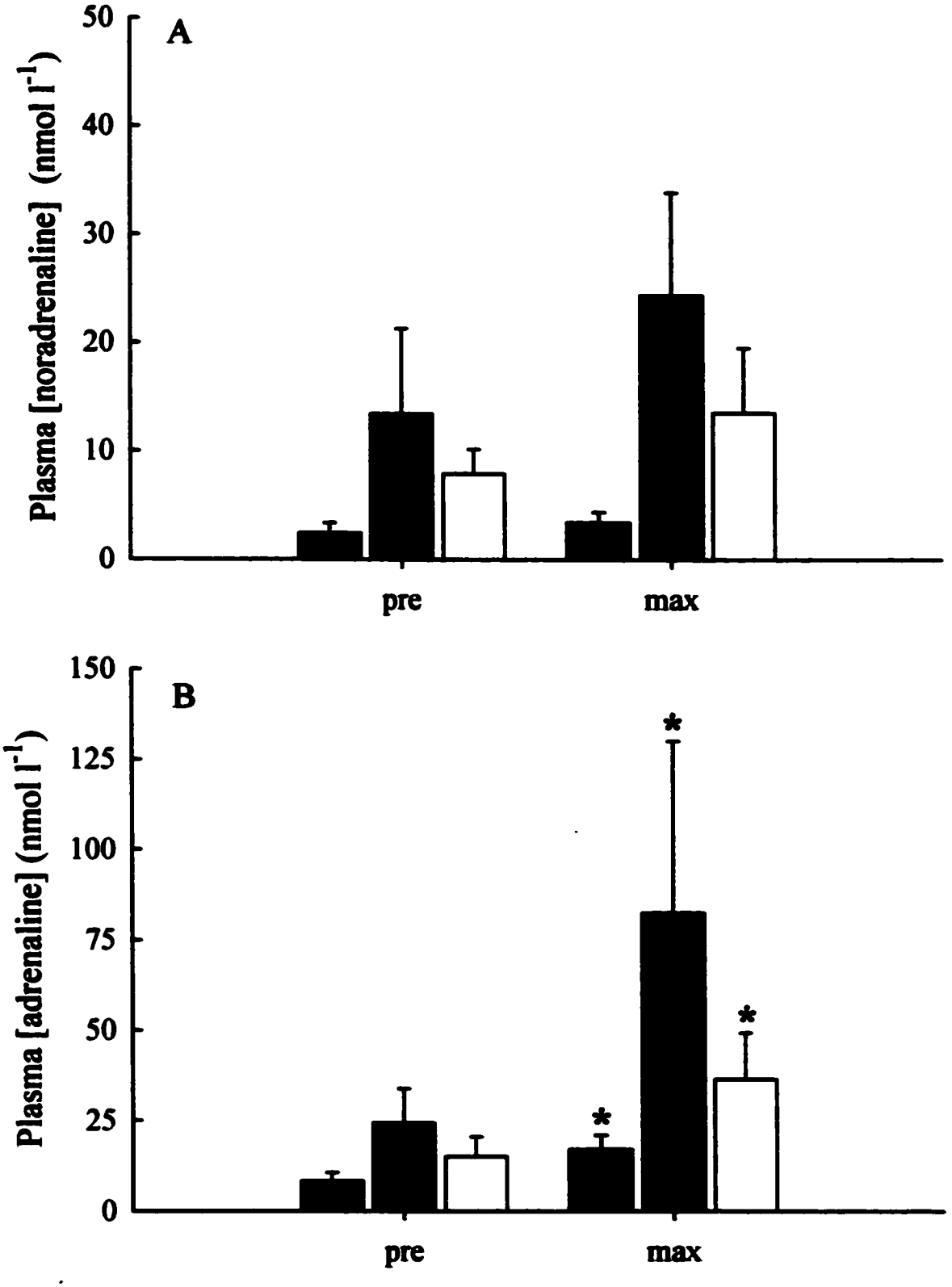

A
Plasma [noradrenaline] (nmol l-1)
50
40
30
20
10
0
pre
max
B
Plasma [adrenaline] (nmol l-1)
150
125
100
75
50
25
0
*
*
*
pre
max

Table 2-1. The effects of an increase in external $PwCO_2$ on water/blood gases in rainbow trout (*Oncorhynchus mykiss*) in each of the three treatment groups. * denotes a statistically significant difference from the pre-value within that treatment; † denotes a significant difference between post-acetazolamide/hyperoxia normocarbia and hypercarbia, n = 5 - 8 ($P < 0.05$).

	control		ACTZ			Hyperoxia		
	Normocarbia	Hypercarbia	Pre-ACTZ	Normocarbia	Hypercarbia	Pre-hyperoxia	Normocarbia	Hypercarbia
PwO_2	153.7 ± 2.2	151.2 ± 2.4	162.7 ± 7.8	153.6 ± 2.1	150.9 ± 1.4	140.6 ± 7.7	643.2 ± 16.4*	643.2 ± 17.6*
$PwCO_2$	0.63 ± 0.18	6.15 ± 0.4*	0.33 ± 0.01	0.38 ± 0.06	6.25 ± 0.39*†	0.77 ± 0.22	1.59 ± 0.8	7.20 ± 0.7*†
PaO_2	121.4 ± 3.2	138.0 ± 2.8*	121.4 ± 5.2	128.8 ± 4.3	133.7 ± 2.8	123.7 ± 7.6	349.4 ± 10.3*	367.6 ± 2.7*
$PaCO_2$	1.96 ± 0.32	6.19 ± 0.70*	1.85 ± 0.20	8.86 ± 0.70*	12.19 ± 0.80*†	3.21 ± 0.6	7.76 ± 1.1*	10.22 ± 1.22*
pHa	7.83 ± 0.05	7.43 ± 0.05*	7.82 ± 0.03	7.26 ± 0.04*	7.16 ± 0.03*	7.73 ± 0.06	7.60 ± 0.06	7.46 ± 0.08

DISCUSSION

The results of the present study confirm that elevated levels of ambient CO_2 evoke significant increases in arterial blood pressure and systemic resistance in conjunction with a significant bradycardia in rainbow trout. Two significant new findings are i) that these effects cannot be attributed to any direct action of CO_2 on the systemic vasculature and ii) that the cardiovascular effects are attributable to hypercarbia (ie. elevation of CO_2 in water), not internal hypercapnia. The results of this investigation provide the strongest support to date for the involvement of external CO_2 chemoreceptors linking the cardiovascular and autonomic nervous systems in fish.

Direct effects of CO_2 on the systemic vasculature

In situ saline-perfused trunk perfusions have been used previously to investigate the direct effects of vasoactive agents on the systemic vascular resistance (e.g. Wood and Shelton, 1975). It was recently suggested (Perry *et al.*, 1999) that increases in systemic resistance observed during hypercarbia in rainbow trout were potentially the result of, or at least enhanced by CO_2/H^+ acting directly on the blood vessels. However, the three levels of CO_2/H^+ examined in this study (0.6, 1.0 and 2.0%) did not elicit any vasoconstrictory effects in preparations devoid of neuronal or humoral tone. The suitability of the preparation and its ability to display substantial increases in systemic resistance was confirmed by a second series of *in situ* experiments (Fig 2-1). Adrenaline (10^{-4} mol l^{-1}) evoked a profound vasoconstriction of the vasculature and thus was used as a tool to pre-contract the preparations. Under such pre-contracted conditions, a clear

vasodilatory effect of CO_2/H^+ was observed. Because the systemic vasculature, *in vivo*, also displays vasomotor tone, hypercapnia, alone, would be expected to lower systemic resistance. Thus, the net increase in systemic resistance that occurs during hypercarbia *in vivo* must reflect the opposing influences of direct CO_2/H^+-induced vasodilation and adrenergic (neuronal and/or humoral) vasoconstriction (Perry *et al.*, 1999). Indeed, it is tempting to speculate that the reflex adrenergic vasoconstriction associated with hypercarbia (Perry *et al.*, 1999) evolved as a mechanism to prevent potentially severe reductions in arterial blood pressure. The mammalian-like vasodilatory response of the systemic vasculature to elevated CO_2 is in marked contrast to the branchial vasculature of rainbow trout which displays a significant vasoconstrictory response to elevated CO_2 in saline-perfused gill preparations (Perry *et al.*, 1984). However, because the study of Perry *et al.* (1984) used non-physiological levels of CO_2 (37.5 Torr), additional studies are required before any meaningful comparisons can be drawn between CO_2 sensitivity of the gill vasculature and the pulmonary vasculature of mammals.

In vivo response to hypercarbia: Internal versus external chemoreceptors

In this study, exposing rainbow trout to hypercarbia caused cardiovascular adjustments similar to those observed by Perry *et al.* (1999). Both studies reported a significant bradycardia as well as increases in arterial blood pressures (both P_{VA} and P_{DA}) and systemic resistance with no effect on cardiac output or gill vascular resistance. This series of experiments not only confirmed the cardiovascular potency of hypercarbia but also, more importantly, provided a basis for comparing the cardiovascular effects elicited

by hypercarbia with those elicited by hypercarbia after treatment with acetazolamide or hyperoxia exposure.

The enzyme carbonic anhydrase (CA) is responsible for the production of molecular CO_2 via the rapid catalyzed dehydration of H_2CO_3 in the red blood cell (reviewed by Henry and Heming, 1998). The CA inhibitor acetazolamide (ACTZ), administered at the same dose as in this study (30 mg kg^{-1}) was previously shown to slow the dehydration reaction to its uncatalysed rate thereby increasing $PaCO_2$ (e.g. Gilmour *et al.*, 1994). As $PaCO_2$ gradually rises, the increased PCO_2 gradient across the gill is able to reset CO_2 excretion to normal levels. Thus, 5 - 7 h after the injection of ACTZ, a new elevated stable baseline $PaCO_2$ was established ($PaCO_2$ increased from 1.9 ± 0.2 to 8.9 ± 0.7 mm Hg). Despite this large increase in $PaCO_2$, R_S and P_{DA} were unaffected. These results, therefore, do not support a role for internal CO_2/H^+ chemoreceptors in the cardiovascular responses associated with hypercarbia. In some individual fish (e.g. see Fig 2-3), the injection of ACTZ caused a rapid small increase in R_S. It is unlikely that this was related to inhibition of CA, *per se*, because it preceded any increase in $PaCO_2$. Moreover, R_S remained relatively constant for the ensuing 7 h despite continuing elevation of $PaCO_2$. Similarly, in accordance with previous data obtained using channel catfish, the bradycardia that was initiated by injection of ACTZ also preceded the rise in $PaCO_2$. However, unlike the transient bradycardia that was observed in catfish (Henry *et al.*, 1988), the decrease in H_f persisted after ACTZ treatment in the present study.

Because CA is known to play a role in mammalian carotid body CO_2 chemoreception (see review by Iturriaga, 1993), it is conceivable that the inability of fish to respond to hypercapnia reflected an impairment of chemoreceptor function. This

seems unlikely, however, because hypercarbia in the CA-inhibited fish elicited similar cardiovascular responses as those observed in the untreated group of trout (i.e. significant increases in P_{DA} and R_S). These responses occurred despite the likelihood of inhibition of CA within any CO_2/H^+ chemoreceptor (internally or externally oriented) owing to the high degree of permeability of ACTZ across biological membranes (e.g. Swenson and Maren, 1987). Furthermore, it is unlikely that CO_2 chemoreception would be fully impaired by CA inhibition unless the rate of intracellular pH change were the key factor determining the sensitivity to CO_2. Regardless, owing to the possibility of chemoreceptor impairment and the non-specific side effects of ACTZ (e.g. bradycardia), exposure to hyperoxic water was used as an additional mechanism to elevate endogenous levels of CO_2.

Hyperoxia has previously been shown to cause a significant hypoventilatory response in rainbow trout (e.g. Wood and Jackson, 1980). The hypoventilation impedes CO_2 excretion and causes an increase in $PaCO_2$ (Wood and Jackson, 1980). As during ACTZ treatment, $PaCO_2$ eventually (6 - 8 h) reached levels great enough to increase the outward driving force for CO_2 diffusion, allowing a new elevated stable baseline to be established ($PaCO_2$ increased from 3.2 ± 0.6 to 7.8 ± 1.1 mm Hg). Once again, despite elevated levels of endogenous CO_2, there was no increase in P_{DA} or R_S. These results are consistent with those of a previous study (Wilkes *et al.* 1981) in which hyperoxia-induced endogenous hypercapnia failed to increase P_{DA} in the white sucker (*Catostomus commersoni*).

Recent branchial dennervation studies with the tropical fish, traira (*Hoplias malabaricus*) suggest that the hypercarbic bradycardia and increase in ventilation

frequency arise from receptors exclusively within the gills but present on more than one gill arch (Reid *et al.*, 2000). Furthermore, these CO_2/pH chemoreceptors seem to be more sensitive to changes in CO_2 than they are to changes in pH. Sundin et al. (2000) performed a similar investigation using the tambaqui (*Colossoma macroponum*) and suggested that the hypercarbic bradycardia reflex is elicited, at least in that species, by branchial receptors confined to the first gill arch. In contrast, selective branchial dennervation of tambaqui demonstrated that chemoreceptors mediating the hypercarbia-induced increase in ventilation frequency were confined exclusively to the gills but not only on the first gill arch. The authors of these recent studies point out the wide array of potential combinations for the distribution of cardiorespiratory chemoreceptors and suggest that many more species will need to be examined before any reliable trends emerge.

To ensure that the absence of a response to internal CO_2 did not reflect an inhibitory influence of elevated PO_2, hyperoxic fish were exposed to hypercarbia. Reassuringly, hypercarbia during hyperoxia elicited similar cardiovascular responses as those observed during normoxic hypercarbia. Thus, in the present study, significant relationships between $PaCO_2$ and cardiovascular variables (R_S and P_{DA}) were observed exclusively in the presence of elevated external PCO_2. Clearly, these results do not support a role for internally oriented CO_2/H^+ chemoreceptors but instead support the involvement of externally directed receptors that likely reside within the gill (Burleson and Smatresk, 2000).

The increase in R_S during hypercarbia in trout results from the stimulation of α-adrenoceptors and can be blocked using the antagonist yohimbine (Perry *et al.*, 1999).

The stimulation of α-adrenoceptors can result from increased discharge of sympathetic adrenergic neurons and/or by increased levels of circulating catecholamines. In the present study, plasma adrenaline levels increased during hypercarbia in all three treatments (i.e. untreated, acetazolamide-treated, and hyperoxia-exposed), while circulating noradrenaline levels were not significantly elevated. Previous studies have also noted an increase in circulating catecholamine levels during hypercapnia in trout (Perry *et al.* 1987, Perry and Kinkead, 1989; Kinkead *et al.*, 1993; Thomas *et al.*, 1994; Perry and Gilmour, 1996) and a similar ratio of adrenaline: noradrenaline (~2:1; Reid *et al.*, 1998). However, the role of circulating catecholamines in the cardiovascular responses to hypercarbia is likely secondary to the role of the neuronal component of the autonomic nervous system. In a recent study, Perry *et al.* (1999) reported stable levels of plasma catecholamines during similar hypercarbic exposure. However, even in the absence of elevated plasma catecholamine levels, fish still displayed the characteristic significant increases in P_{DA} and R_S, as well as decreased H_f. Previous studies also have reported stable catecholamine levels during mild to moderate hypercapnia (Kinkead and Perry, 1991; Julio *et al.*, 1998). It has been suggested that increases in circulating plasma catecholamines may be indicative of the severity of the hypercarbic assault and may reflect the extent of hypoxaemia elicited by the ensuing respiratory acidosis (Julio *et al.*, 1998). In this regard, it is puzzling that fish experiencing hyperoxia displayed a significant elevation of plasma adrenaline concentration during hypercarbia. Previous studies demonstrated that hyperoxia is capable of preventing catecholamine secretion during hypercarbia (Perry *et al.*, 1989) or metabolic acidosis (Aota *et al.*, 1990). Indeed, it was argued that hyperoxia was able to offset the effects of acidosis on lowering blood

O_2 content and thereby prevent the requirement for catecholamine secretion (Perry *et al.*, 1989).

In summary, this study has demonstrated that elevated CO_2/H^+ has a vasodilatory effect on the systemic vasculature of rainbow trout. This direct effect likely reduces the net extent of the hypercarbia-induced vasoconstriction observed *in vivo* or indeed may be the origin or stimulus that initiates the reflex vasoconstriction. While the physiological benefits of the cardiovascular responses to hypercarbia are unclear, the results of this study indicate that these changes are triggered by increased external CO_2/H^+ levels and are likely mediated via externally located CO_2/H^+ chemoreceptors linked to the neuronal and humoral components of the autonomic nervous system. Further studies should focus on determining whether the responses observed in rainbow trout are indeed representative of teleosts in general. In addition, differentiating between CO_2 and H^+ induced effects may help clarify the mechanisms involved in CO_2/H^+ chemoreception in fish.

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Chapter 3

CARDIOVENTILATORY EFFECTS OF EXTERNAL CO_2 VERSUS pH IN ATLANTIC SALMON (*Salmo salar*)

ABSTRACT

Adult Atlantic salmon (*Salmo salar*) gills were exposed *in vivo* to acute, localized environmental hypercarbia (2.0 or 4.0% CO_2 in air) via injections (60 ml kg^{-1}) of CO_2 - equilibrated seawater directly into the buccal cavity. To differentiate CO_2-specific cardiorespiratory adjustments from those triggered by pH/H^+, a further series of injections were performed using aerated seawater titrated with HCl to the complimentary pH (pH = 7.0 and 6.3, respectively). In response to 2.0 and 4.0 % CO_2 in air, fish displayed significant increases in ventilatory amplitude (V_{AMP}, up to 83%) and frequency (V_f, up to 23%) alongside significant decreases in cardiac output (V_b, up to 24%) and frequency (H_f, up to 26%), with no effect on arterial blood pressure (P_A). The complimentary series of external injections using acidified water (no CO_2) did not significantly affect any of the cardiovascular or ventilatory variables measured, except for a slight decrease in cardiac output (~3% at pH = 6.3). These results show that, in Atlantic salmon, changes in the partial pressure of CO_2 in the water ($PwCO_2$), and not changes in pH, are responsible for the hypercarbia-induced cardiorespiratory adjustments.

INTRODUCTION

Recently, a growing number of studies has focused on CO_2/H^+ chemoreception in fish. Perry *et al.* (1999) demonstrated that a hypercarbia-induced bradycardia and systemic hypertension occur in rainbow trout, *Oncorhynchus mykiss*. It was later shown that these cardiovascular effects were caused by the environmental hypercarbia and not the associated rise in internal levels of CO_2 (McKendry and Perry, 2001). Prior blockade of the α-adrenoceptors eliminated the pressor responses to hypercarbia but did not eliminate the bradycardia (Perry *et al.* 1999). Although specific evidence is lacking, it was suggested (Perry *et al.* 1999) that the hypercarbic bradycardia involves an increase in parasympathetic (cholinergic) vagal tone, which is the predominant mechanism of controlling heart rate in most teleosts (Farrell and Jones, 1992).

Selective branchial denervations of the neotropical tambaqui (*Colossoma macroponum*) suggest that, at least in that species, the hypercarbia-induced bradycardia is mediated via receptors located exclusively on the first gill arch (Sundin *et al.*, 2000). Furthermore, the distribution of receptors responsible for producing the hypercarbic bradycardia appears to be different from that of the receptors mediating the hypoxic bradycardia, strengthening arguments that changes in CO_2/pH may act as a cardiac stimuli in fish, independent from the hypercarbia-mediated hypoxaemia (Sundin *et al.*, 2000).

As the focus on CO_2-chemoreception in fish intensifies, the investigation into the relative importance of elevated ambient CO_2 itself versus the accompanying decrease in pH has only recently begun. Early experiments specifically aimed at separating the

effects of $[H^+]$ (ie. changes in pH) from those of CO_2 lead to the conclusion that, for salmonids, changes in external or internal $[H^+]$ did not affect ventilation unless accompanied by hypercapnia (Janssen and Randall, 1975; Neville 1979; Thomas and LeRuz, 1982). However, because the hypercarbia-induced cardiorespiratory adjustments had, until very recently (Perry *et al.*, 1999; McKendry and Perry, 2001), been attributed to an impairment in blood O_2 transport capability (e.g. Randall, 1982; Smith and Jones, 1982), and not the elevated ambient CO_2 itself, they had received much less attention than the hypercarbic ventilatory reflex.

Branchial denervation studies have indicated that, in the traira (*Hoplias malabaricus*) extra-branchial receptors may be involved in mediating the increase in ventilatory amplitude observed during hypercarbic exposure, however, the bradycardia and elevated ventilatory frequency arise from receptors exclusively within the gills (Reid *et al.*, 2000). Moreover, acid injections (internal and external) did not mimic the cardiorespiratory responses observed during hypercarbia (Reid *et al.*, 2000) suggesting that CO_2 may be more important than pH in triggering these cardioventilatory adjustments.

Thus, by injecting, directly over the gills, seawater equilibrated with a preset percentage of CO_2 in air, or titrated to an equivalent pH this study will evaluate the relative contributions of CO_2 itself versus those of pH/H^+ in mediating the cardiorespiratory responses to hypercarbia in Atlantic salmon.

MATERIALS AND METHODS

Experimental animals

Atlantic salmon, *Salmo salar*, of either sex were obtained from a fish farm (Vancouver Island, British Columbia) and were transported in oxygenated water to Bamfield Marine Station where they were housed outdoors in fiberglass tanks supplied with fresh flowing full strength seawater (12° C); they were fed daily with commercial salmonid pellets

Surgical procedures

Atlantic salmon, weighing between 300 g and 575 g (experimental N = 12) were anaesthetized using benzocaine (0.1g l^{-1}ethyl-*p*-aminobenzoate, Sigma) in seawater 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 (Sovio *et al.*, 1975; Olson *et al.* 1997).

In order 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 seawater into the buccal cavity.

Fish were then fitted with a cardiac flow (V_b) 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 closed using sutures and sealed with Vetbond™, the flow probe cable was secured externally to the ventral surface of the fish using silk ligatures and Vetbond™.

The cardiovascular cannula were filled with heparinized (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 ~24 h prior to experimentation.

Experimental protocol

Measurement of water respiratory variables

A pump-driven loop continuously withdrew inspired water from the box, immediately in front of the buccal cavity, and passed it over PO_2 and PCO_2 electrodes (Radiometer). O_2 and CO_2 electrodes were housed in temperature controlled cuvettes and connected to a Radiometer blood gas analyzer. All electrodes were calibrated prior to each individual experiment (Perry et al. 1999).

Measurement of cardiovascular and ventilation variables

The arterial and buccal cannulae were connected to UFI model 1050BP (UFI, Morro Bay, CA) pressure transducers that were pre-calibrated against a static column of water. Mean arterial blood pressure was calculated as: (systolic pressure + diastolic

pressure)/2. Cardiac output was determined by connecting the 3S or 4S ultrasonic flow probe to a Transonic T106 small animal blood flow meter. Analog signals were converted to digital data and collected at 40 samples sec^{-1} using a computer linked to a data-acquisition system (Biopac System Inc., Goleta, CA) using AcqKnowledge 3.0 software. Recordings began once all parameters had stabilized.

Cardioventilatory effects of external CO_2 vs. pH

Experiments commenced with a 10 min recording period under normoxic, normocarbic resting conditions. After this 'pre'-period, a randomized series of treated seawater injections (60 ml kg^{-1}) commenced, with 10 min intervals between injections. Prior pilot experiments had shown that local seawater equilibrated with 2% CO_2 in air had a pH of 7.0, while seawater equilibrated with 4% CO_2 had a pH of 6.3. In order to differentiate between CO_2-induced cardiorespiratory effects and those triggered by a decrease in pH, normally observed during periods of elevated ambient CO_2, treated seawater injections consisted of seawater equilibrated with either 2% or 4% CO_2, and aerated seawater titrated to a pH of either 7.0 or 6.3 using HCl. Aerated seawater injections, of the same volume (60 ml kg^{-1}) were used as the control.

Statistical analyses

Data are presented as mean values ± one standard error of the mean (SEM). Mean values were compiled by sampling over a 20 sec period. Vascular resistance (R_S) was calculated using the formula: $R_S = P_A/Vb$. For all experiments, the statistical significance of the observed effects was determined using paired t-tests. One-way ANOVA and Dunn's post hoc comparison was used across treatments in Table 3-1. All

statistical analyses were performed using SigmaStat 2.0 commercial software package (SPSS Inc.) with a fiducial limit of 5%. All figures were plotted using the Sigmaplot 4.0 commercial graphics software package (SPSS Inc.).

RESULTS

Cardioventilatory effects of external CO_2 versus pH in Atlantic salmon

Injections of seawater equilibrated with 2% or 4% CO_2 in air into the buccal cavity of Atlantic salmon, *Salmo salar*, caused a significant increase in ventilation amplitude, V_{AMP} (27% and 45%, respectively), and frequency, V_f (11% and 19%, respectively; Figure 1A and B). Injecting the same volume of aerated seawater titrated to pH values that were complimentary to seawater gassed with 2% and 4% CO_2 (i.e. pH = 7.0 and 6.3, respectively) did not significantly affect ventilation amplitude or frequency. Despite an apparent dose-dependent trend with external injections of CO_2 only the 4% CO_2 treatment elicited a significantly greater ventilatory response than untreated seawater.

Injections of seawater equilibrated with 2% or 4% CO_2 in air triggered a significant decrease in heart rate, (H_f; 18% and 21%, respectively), and cardiac output, (V_b; 17% and 19%, respectively; Figure 3-2A and B), while systemic vascular resistance, R_S, rose significantly (45% and 64%, respectively; Figure 3-2D). Injections of aerated seawater titrated to either pH = 7.0 or pH = 6.3 did not significantly affect H_f or R_S (Figure 3-2 A and D). However pH = 6.3 did elicit a slight (~3%) but statistically significant decrease in V_b (Figure 3-2B). Blood pressure (P_A) was not affected by either CO_2 or pH (Figure 3-2C). It should also be noted that control injections of aerated seawater had no effect on any of the measured or calculated variables. These cardiovascular responses were not significantly dose-dependent.

Table 3-1 lists the absolute changes in a given variable during each treatment. While 2% CO_2 induced a larger increase in ventilation amplitude and frequency than seawater alone, 4% CO_2 elicited a significantly greater change in V_f, V_{amp}, H_f, V_b, and R_S than did aerated seawater. However, these incremental changes were not statistically significant in a dose-dependent manner. By way of comparison, changes in cardiorespiratory values from seawater (control) injections were no different than with injections of seawater titrated to a pH of 7.0 or 6.3.

Figure 3-1. The effects of injections (60 ml kg^{-1}) into the buccal cavity of seawater (SW; control), seawater equilibrated with 2% or 4 % CO_2 in air (2% and 4% CO_2, respectively), and seawater titrated to the complimentary pH with HCl (pH = 7.0 and 6.3, respectively), on A) ventilation amplitude (V_{AMP}; N = 9 – 11) and B) ventilation frequency (V_f; N = 8 - 11) in Atlantic salmon (*Salmo salar*). Black bars represent pre-injection (pre) values; white bars represent maximal responses obtained within 1 min post-injection. Values are means ± 1 SEM; * denotes a significant difference between pre value and maximal response ($P < 0.05$), while † denotes a significant difference between the control (seawater) value.

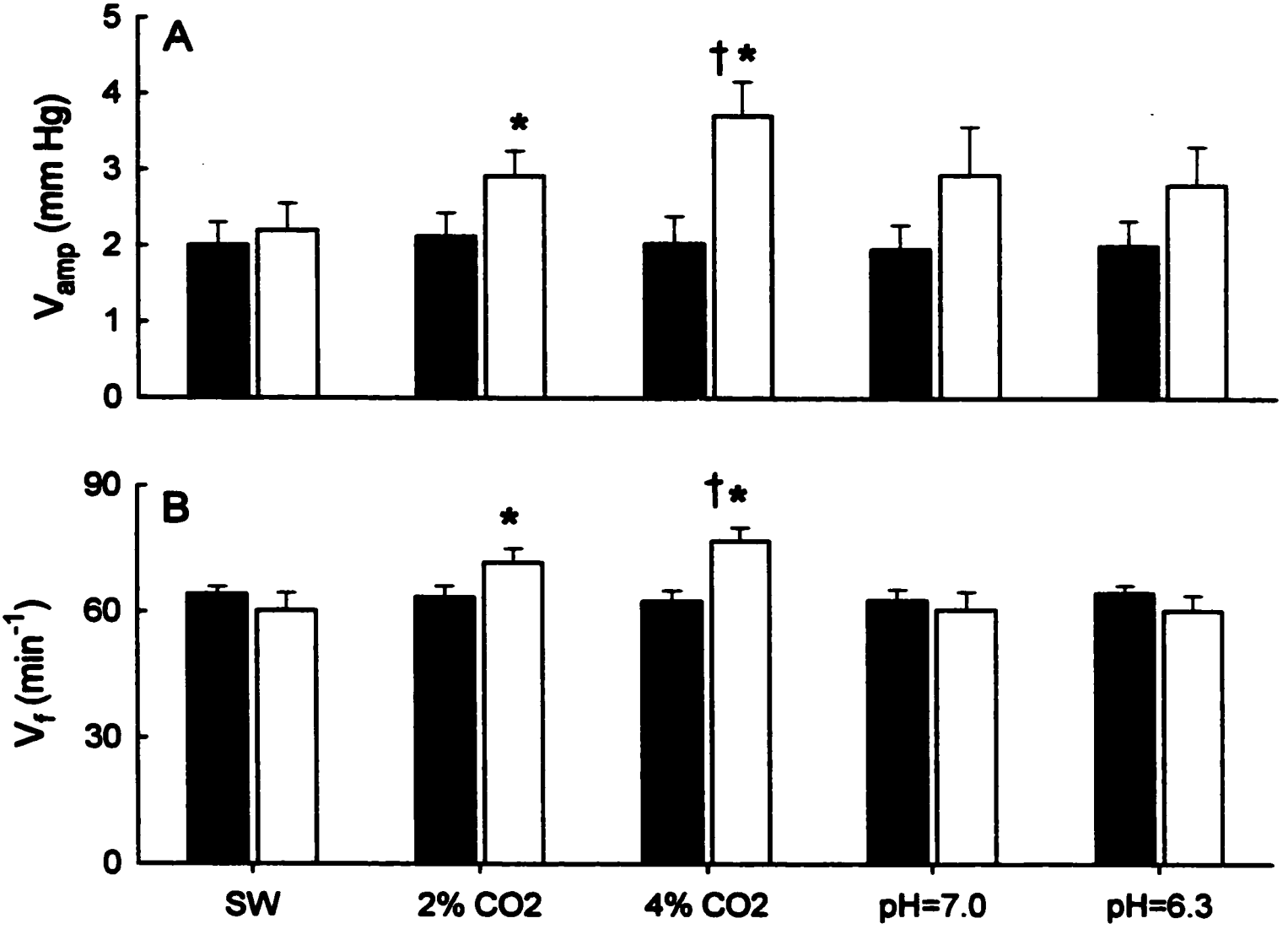
A
B
V_{amp} (mm Hg)
V_f (min^{-1})
0
1
2
3
4
5
30
60
90
*
†*
SW
2% CO2
4% CO2
pH=7.0
pH=6.3

Figure 3-2. The effects of injections (60 ml kg^{-1}) into the buccal cavity of seawater (SW; control), seawater equilibrated with 2% or 4 % CO_2 in air (2% and 4% CO_2, respectively), and seawater titrated to the complimentary pH with HCl (pH = 7.0 and 6.3, respectively), on A) heart rate (H_f; N = 9 - 11), B) cardiac output (V_b; N = 9 - 11), C) arterial blood pressure (P_A; N = 9 - 11), and D) systemic vascular resistance (R_S; N = 9 – 11), in Atlantic salmon (*Salmo salar*). Black bars represent pre-injection values; white bars represent maximal responses obtained post-injection. Values are means ± 1 SEM; * denotes a significant difference between pre value and maximal response ($P < 0.05$).

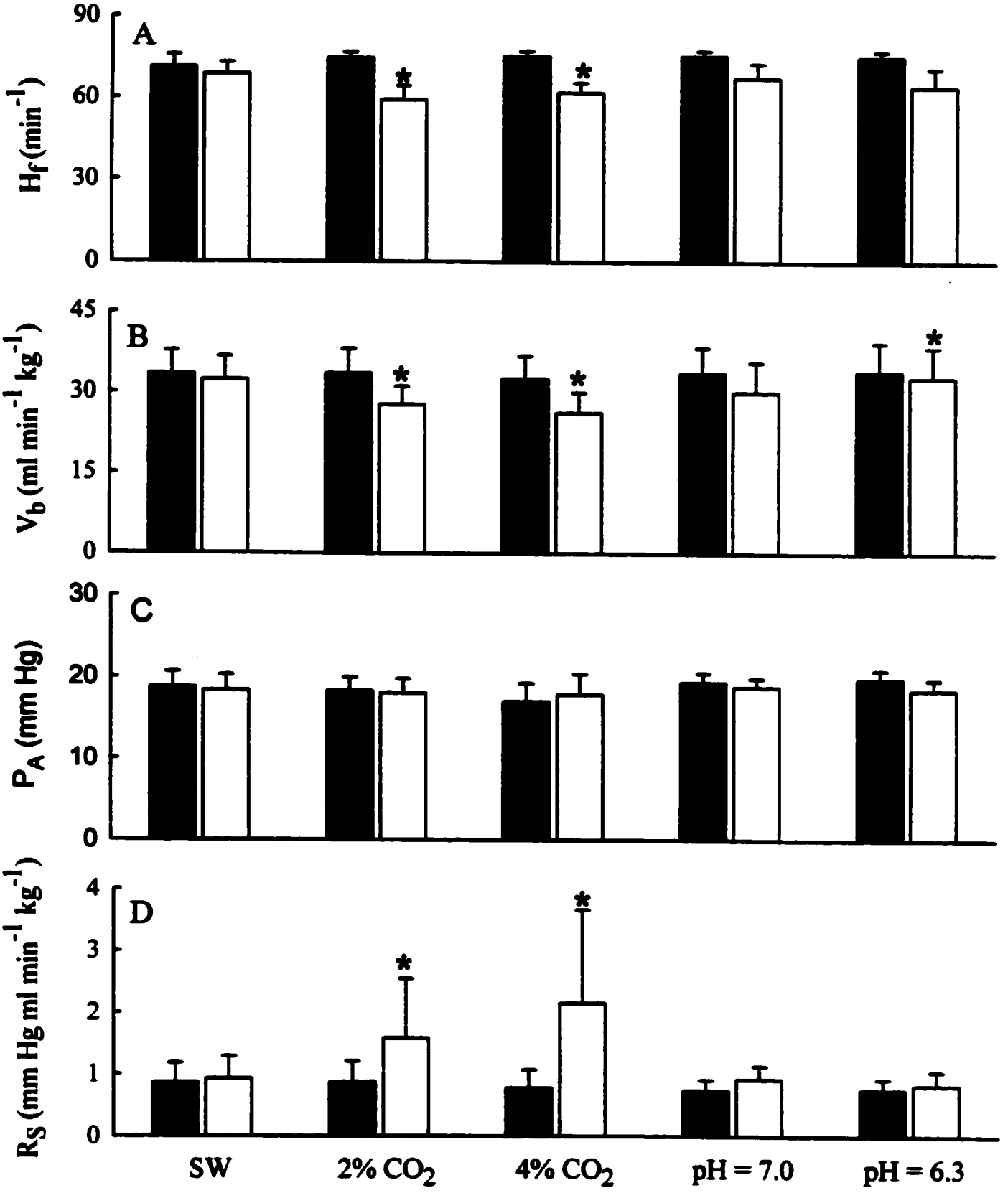
A
B
C
D
H_f (min^{-1})
V_b (ml min^{-1} kg^{-1})
P_A (mm Hg)
R_S (mm Hg ml min^{-1} kg^{-1})
90
60
30
0
45
30
15
0
20
10
4
3
2
1
*
SW
2% CO_2
4% CO_2
pH = 7.0
pH = 6.3

Table 3-1. The changes in ventilation frequency (V_f), ventilation amplitude (V_{AMP}), arterial blood pressure (P_A), heart rate (H_f), cardiac output (V_b), and systemic vascular resistance (R_S) in Atlantic salmon (*Salmo salar*) after injections (60 ml kg^{-1}) into the buccal cavity of seawater (SW; control), seawater equilibrated with 2% or 4 % CO_2 in air (2% and 4% CO_2, respectively), and seawater titrated with HCl to the complimentary pH (pH = 7.0 and 6.3, respectively). † denotes a statistically significant difference from the seawater (control) treatment; N = 8 - 11 ($P < 0.05$).

	V_f (Δ min^{-1})	V_{AMP} (Δ mm Hg)	P_A (Δ mm Hg)	H_f (Δ min^{-1})	V_b (Δ ml min^{-1} kg^{-1})	R_S (Δ mm Hg ml min^{-1} kg^{-1})
SW	-3.9 ± 3.8	0.2 ± 0.2	-0.4 ± 0.2	-2.5 ± 1.6	-1.1 ± 0.6	0.06 ± 0.05
2% CO_2	8.2 ± 1.8 †	0.8 ± 0.2 †	-0.3 ± 0.5	-13.5 ± 3.6	-5.8 ± 2.2	0.71 ± 0.63
4% CO_2	14.4 ± 1.8 †	2.3 ± 0.5 †	1.1 ± 0.9	-15.4 ± 5.5 †	-6.3 ± 1.8 †	1.38 ± 1.23 †
pH = 7.0	-2.4 ± 3.7	1.0 ± 0.7	-0.6 ± 0.7	-8.1 ± 5.7	-3.6 ± 2.6	0.18 ± 0.13
pH = 6.3	-4.2 ± 3.3	0.8 ± 0.5	-1.4 ± 0.9	-10.9 ± 7.2	-1.2 ± 0.6	0.07 ± 0.06

DISCUSSION

The results of the present study provide the strongest evidence to date that the hypercarbia-induced cardioventilatory adjustments are mediated via changes in the partial pressure of CO_2 in the inspired water rather than by changes in water pH. Furthermore, owing to the extremely acute, localized nature of the CO_2 exposure in this investigation, the data provide additional qualitative evidence supporting the growing theory of externally oriented branchial CO_2 chemoreceptors in fish.

In these experiments, the treated seawater would have a PCO_2 of either 15 mm Hg (2% CO_2) or 30 mm Hg (4% CO_2) which would mix with normocarbic inspired water within the buccal cavity before passing over the gills. Thus, although the levels of CO_2 in the injected water were high, the mixing and dilution with inspired water would likely lower the final PCO_2 substantially. Regardless, the CO_2 levels are within those encountered by many fish species as well as levels experienced in aquaculture facilities. For example, when fish are densely stocked and supplied with recirculating ground water CO_2 tensions around 20 mm Hg are not uncommon (Eddy *et al.*, 1977). Furthermore, Thomas (1983) pointed out the magnitude of PCO_2 and PO_2 fluctuations that can be seen diurnally (increases in PCO_2 of ~3 Torr and increases in PO_2 of ~350 Torr). Although, 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 $PwCO_2$'s 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.

It should be emphasized that this study is the first to directly investigate, in such a comparative manner, the relative importance of elevated ambient CO_2 versus the concomitant drop in pH observed during hypercarbia. Moreover, by administering a randomized series of treated seawater injections consisting of either hypercarbic seawater or aerated seawater titrated with HCl to the equivalent pH, these results definitively demonstrate $PwCO_2$, not water pH (pHw), is the trigger for hypercarbia-induced cardioventilatory adjustments. Although it is becoming increasingly apparent that there exists a wide variety of cardiorespiratory responses among different fish species to increases in ambient CO_2 (Randall et al., 1976; Graham et al. 1990; Perry and Gilmour, 1996; Perry et al., 1999; Reid et al., 2000; Sundin et al., 2000; McKendry and Perry, submitted), there is, to date, no evidence for a fish species which is more sensitive to pHw than $PwCO_2$. In fact, previous attempts to correlate the changes in ventilation observed in the skate (*Raja ocelatta)* during hypercarbic exposure to changes in arterial PCO_2, arterial pH, cerebrospinal fluid pH and calculated brain intracellular pH found no close correlation with any of these variables (Wood et al., 1990). The results of the present investigation suggest that, in the skate, ventilation may have been most closely correlated to water PCO_2, rather than the blood and cerebral fluid variables examined. Because elasmobranchs and teleosts respond similarly to acute hypercarbic exposure (McKendry and Perry, submitted), it now appears, for future studies, as though $PwCO_2$ would be the most closely correlated to changes in both ventilation and cardiovascular parameters.

Until quite recently, the relatively unchallenged consensus had been that ventilatory, and indeed cardiovascular, responses to hypercarbia in fish were an indirect effect of the marked Bohr and Root effects that can occur in hypercapnic fish blood. These effects can impair the blood oxygen transport capability (Hughes and Shelton, 1962; Cameron, 1971; Eddy, 1971, 1973) and were believed to be indirectly triggering the hypercarbic reflex via receptors sensitive to changes in arterial oxygen content (Smith and Jones, 1982; Perry et al., 1989). There were some difficulties, however, with the theory that the cardioventilatory response in teleosts was simply a reflection of reduced blood O_2-transport capability.

A review article by Milsom (1995) points out that goldfish blood, for example, shows Bohr and Root effects but these fish show no respiratory response to hypercarbia or acid exposure (Dejours, 1972, 1973). Thomas et al. (1983) showed that exposure of trout to moderate PCO_2 levels (1 – 7 mmHg) during intense hyperoxia, likely negating any significant hypoxaemia, resulted in large increases in ventilation volume. In fact, Perry et al. (1999) clearly demonstrated a significant dose-dependent cardioventilatory response to step-wise increases in ambient CO_2 in rainbow trout (*O. mykiss*), while convincingly disassociating these hypercarbic reflexes from any significant hypoxaemia. The investigation showed that the degree of hypercarbia used in the study did not render the blood of the trout hypoxaemic enough to evoke the observed bradycardia and systemic hypertension. Even more recently, McKendry and Perry (2001) have shown that these cardioventilatory adjustments are triggered by the external hypercarbia itself and not the concomitant internal hypercapnia.

Both 2.0 and 4.0% CO_2 elicited significant increases in ventilatory amplitude and frequency, in keeping with the growing number of studies that have shown environmental hypercarbia as a potent ventilatory stimulus in most species of teleost (Janssen and Randall, 1975; Smith and Jones, 1982; Graham et al., 1990; Kinkead and Perry, 1991; Kinkead et al., 1993). Although there appeared to be a $[H^+]$ effect on V_{AMP}, albeit statistically insignificant, this may be the result of an immediate localized acid-base disturbance over the gill or extra-branchial receptors. Although it is unknown whether changes in ventilatory amplitude during periods of elevated ambient CO_2 are partially mediated by extra-branchial chemoreceptors, Sundin et al. (2000) recently found this to be the case with O_2 chemoreceptors during hypoxia. The cardiovascular adjustments observed also agree with recent whole animal, acute hypercarbia-exposure studies (Perry et al., 1999; McKendry and Perry, 2001; McKendry and Perry, submitted). Both heart rate and cardiac output decrease while vascular resistance increases during the period of acutely elevated ambient CO_2. Most convincingly of all, normoxic seawater titrated to pH 7.0, intended to simulate the $[H^+]$ of seawater equilibrated with 2.0% CO_2 elicited no statistically significant cardioventilatory effects. Moreover, normoxic seawater titrated to pH 6.3, intended to simulate the $[H^+]$ of seawater equilibrated with 4.0% CO_2 elicited no statistically significant cardioventilatory effects, except for a small (~3%), and likely physiologically insignificant, drop in Vb.

The results of this study suggest that the vast majority, if not all, of the hypercarbic reflex is mediated via externally oriented chemoreceptors, triggered by changes in $PwCO_2$ itself, not $[H^+]$. Similar studies will be required in numerous other fish species before $PwCO_2$ itself can be unequivocally adopted as trigger for hypercarbia-

induced cardioventilatory adjustments in fish, although at this point, at least in Atlantic salmon this appears to be the case.

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Chapter 4

Branchial CO_2 receptors and cardiorespiratory adjustments during hypercarbia in Pacific spiny dogfish (*Squalus acanthias*)

ABSTRACT

Adult Pacific spiny dogfish (*Squalus acanthias*) were exposed to acute hypercarbia (~20 min). Experiments were performed to examine the influence of hypercarbia on aspects of cardiorespiratory physiology including arterial blood pressure, systemic vascular resistance (Rs), cardiac output (Vb) and frequency (Hf), as well as ventilatory amplitude (Vamp) and frequency (Vf). Separate series of experiments using the muscarinic receptor antagonist atropine (100 nmol kg^{-1}) or complete branchial denervation were performed to investigate putative CO_2-chemoreceptive sites on the gills and their link to the autonomic nervous system and cardiorespiratory reflexes.

In untreated fish, moderate hypercarbia (water CO_2 partial pressure; $PwCO_2$ = 6.4 ± 0.1 mm Hg) elicited significant increases in Vamp (~92%) and Vf (~18%) as well as decreases in Hf (~64%), Vb (~29%) and blood pressure (~13%); Rs did not change significantly.

Prior administration of the muscarinic receptor antagonist atropine (100 nmol kg^{-1}) abolished the hypercarbia-induced ventilatory responses and virtually eliminated all CO_2-elicited cardiovascular adjustments. Although the atropinised dogfish displayed a hypercarbic bradycardia, the magnitude of the response was significantly attenuated (36 ± 6% decrease in Hf in controls *versus* 9 ± 2% decrease in atropinised fish). Denervation of the branchial branches of the IXth and Xth cranial nerves to the pseudobranch and each gill arch eliminated all cardiorespiratory responses to hypercarbia.

Thus, the results of the present study reveal the presence of gill CO_2 chemoreceptors in dogfish that are linked to numerous cardiorespiratory reflexes.

Because all cardiorespiratory responses to hypercarbia were abolished or attenuated by atropine, the CO_2 chemoreception process and/or one or more downstream elements likely involve cholinergic (muscarinic) neurotransmission.

INTRODUCTION

The cardiorespiratory responses of fish to environmental hypoxia have been studied extensively (for reviews see Randall, 1982; Shelton *et al.* 1986; Smatresk, 1990; Burleson and Smatresk, 1992; Fritsche and Nilsson, 1993; Milsom *et al.* 1999). Although there is considerable inter-specific variation (e.g. see Fritsche, 1990), teleosts typically respond to hypoxia with hyperventilation, bradycardia and hypertension (caused by increased systemic vascular resistance). Elasmobranchs also exhibit hyperventilation and bradycardia (reviewed by Butler and Metcalfe, 1988) but in contrast to most teleosts, blood pressure is decreased (Butler and Taylor, 1971; Short *et al.* 1979) during hypoxia. The mechanisms underlying the cardiorespiratory reflex responses to hypoxia are reasonably well understood and are initiated by branchial chemoreceptors of both internal and external orientation. The external receptors are thought to trigger the cardiovascular and ventilatory responses whereas the internal receptors are believed to be involved solely in mediating the ventilatory responses (Burleson and Smatresk, 1992).

Considerably less is known about the cardiorespiratory responses of fish to elevated environmental CO_2 (hypercarbia) and few studies have addressed the underlying mechanisms. Although hyperventilatory responses of fish to hypercarbia are well-documented, the precise cause and the physiological significance of this response have been debated (see Perry and Wood, 1989). Owing to the pronounced role of blood O_2 status in setting respiratory drive in fish (Randall, 1982; Smith and Jones, 1982), it has proven difficult to distinguish between the primary effects of CO_2/H^+ and the indirect

effects caused by acidosis-induced hypoxaemia (via the Root effect). In recent years, however, there have been compelling arguments for direct effects of CO_2/H^+ on ventilation (Heisler *et al.* 1988; Kinkead and Perry, 1991; Perry and Gilmour, 1996; Burleson and Smatresk, 2000) and thus current models of respiratory control in fish incorporate a specific, albeit secondary, role for CO_2/H^+ (Milsom, 1995a; 1995b).

Although evidence of hypercarbic bradycardia was presented as early as 1978 (Kent and Peirce, 1978), only recently have the cardiovascular effects of elevated CO_2 been addressed in any detail (Perry *et al.* 1999a; McKendry and Perry, 2001; Sundin *et al.* 2000; Reid *et al.* 2000). In trout, the cardiovascular responses to hypercarbia (principally bradycardia, lowered cardiac output and elevated systemic resistance) are reflexively mediated by the autonomic nervous system (Perry *et al.* 1999a). Furthermore, these hypercarbia-induced adjustments are not related to hypoxaemia (Perry *et al.* 1999a) and are likely initiated by externally oriented CO_2/H^+ receptors (McKendry and Perry, 2001). The importance of gill receptors in mediating hypercarbic bradycardia was demonstrated using a branchial denervation technique in two tropical teleosts, the Tambaqui (*Colossoma macrpomum*; Sundin, *et al.* 2000) and the Traira (*Hoplias malabaricus*; Reid *et al.* 2000).

Despite the robust ventilatory and cardiovascular responses to hypercarbia in dogfish, nothing is known about the location of the sensory receptors in this species. Because it lacks a Root effect, the dogfish is a particularly useful model species to study aspects of CO_2-induced cardiorespiratory reflexes in fish. Thus, unlike in some previous studies (Sundin *et al.* 2000; Reid *et al.* 2000) any effects of hypercarbia can be attributed

specifically to external and/or internal changes in CO_2/H^+ without any need to use hyperoxia as a tool to prevent hypoxaemia (Burleson and Smatresk, 2000).

Therefore, the present study addresses for the first time the sensitivity and location of CO_2/H^+ receptors in dogfish thus permitting a direct assessment of the effects of hypercarbia. Specifically, this study tests the hypothesis that the cardiorespiratory responses to hypercarbia in dogfish are triggered by branchial chemoreceptors. This was achieved by comparing physiological responses in intact, sham-operated and gill-denervated animals. Complete gill denervation was accomplished via surgical transection of the IXth cranial nerve (glossopharyngeal) and the branchial branches of the Xth cranial nerve (vagus). Owing to the possible role of cholinergic neurotransmission in O_2 chemoreception (Burleson and Milsom, 1995a) and the importance of increased vagal activity in mediating bradycardia, experiments also were performed in fish pre-treated with atropine, a muscarinic receptor antagonist.

MATERIALS AND METHODS

Experimental animals

Pacific spiny dogfish, *Squalus acanthias*, were angled or collected by net during trawls by local fishermen and held indoors under natural photoperiod at the Bamfield Marine Station in a 75,000 l opaque circular tank continuously supplied with fresh aerated seawater. Dogfish were fed twice weekly with herring.

Surgical procedures

Fish, weighing between 1250 g and 3460 g (experimental N = 28) were anaesthetized using benzocaine (0.1g l^{-1}ethyl-*p*-aminobenzoate, Sigma) in seawater until breathing movements stopped. The fish were then placed onto an operating table where their gills were force-ventilated with oxygenated anaesthetic solution. Bi-directional cannulation of the coeliac artery was required to establish an extracorporeal arterial blood circulation (see below). After making a ventral incision and externalising much of the viscera, the coeliac artery was cannulated in the orthograde and retrograde directions using polyethylene tubing (Clay Adams PE 50). The viscera were re-inserted, the wound sutured, and the cannulae were secured firmly to the ventral musculature. 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 arterial blood pressure (P_A) measurements, the caudal vein cannula permitted injections and/or repeated blood sampling. To allow for the recording of ventilation adjustments, a heat-flared cannula

(PE 160) was inserted into a spiracular cavity and secured using a single silk ligature and Vetbond™.

Animals were fitted with a cardiac flow (V_b) 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 conus arteriosus. A 3S or 4S ultrasonic flow probe (Transonics Systems Inc., Ithaca, NY. USA) was placed non-occlusively around the conus to enable the measurement of cardiac output (Olson *et al.* 1997). Lubricating jelly was used with the perivascular flowprobe as an acoustic couplant. The incision was sutured closed and sealed with Vetbond™; the flow probe cable was secured externally to the ventral surface of the fish using silk ligatures and Vetbond™.

Additional surgery was required for the denervation series. A small incision (~ 3 cm), beginning approximately 2 cm behind the spiracular opening was made in the caudal direction. This incision permitted access to cranial nerve IX (glossopharyngeal) and X (vagus), including all branchial branches from the vagus (X) to each gill arch. The glossopharyngeal (IX) as well as the branchial branches of the vagus (X) cranial nerve, leading to each gill arch, were delicately dissected free of surrounding connective tissue and severed with micro-scissors. In all cases the cardiac and visceral branches of the vagus (X) were left intact. All denervations were confirmed *post mortem* by autopsy. In the sham-operated group, dogfish underwent the identical procedure, without the actual denervation, i.e. the nerves were exposed but not sectioned.

All cardiovascular cannulae were filled with heparinised (50 i.u. ml^{-1} sodium heparin) saline (500 mmol l^{-1} NaCl). After surgery, fish were revived, placed into opaque flow-through acrylic boxes and left to recover for ~24 h prior to experimentation.

Experimental protocol

Measurement of water/blood respiratory variables

Inspired water was withdrawn continuously from the box via a peristaltic pump and passed over PO_2 and PCO_2 electrodes (Radiometer). O_2 and CO_2 electrodes were housed in temperature controlled cuvettes and connected to a Radiometer blood gas analyzer. Blood gases were assessed using an extracorporeal arterial blood shunt (Thomas, 1994; Perry and Gilmour, 1996) that permitted continuous and simultaneous measurements of arterial blood pH (pHa), oxygen partial pressure (PaO_2) and carbon dioxide partial pressure ($PaCO_2$). This was achieved by pumping (peristaltic pump; 0.4 ml min^{-1}) blood from the coeliac artery through pH, PCO_2 and PO_2 electrodes connected in series and returning it to the coeliac return cannula. The extracorporeal shunt contained approximately 1.0 ml of blood representing less than 3% of the total blood volume. Ventilatory and cardiovascular variables were recorded concurrently (see below).

Prior to experimentation, the extracorporeal shunt was rinsed for 20 - 30 min with a solution of ammonium heparin (540 units ml^{-1}) in saline to prevent blood from clotting in the tubing and electrode chambers. After starting the extracorporeal circulation, a period of approximately 10 - 15 min was required to achieve stable baseline recordings of pHa, $PaCO_2$ and PaO_2. All electrodes were calibrated prior to each individual experiment (Perry *et al.* 1999b).

Measurement of cardiovascular and ventilation variables

V_{AMP} was assessed by monitoring pressure changes in the spiracle associated with each breathing cycle. The spiracle catheter was filled with seawater, connected to a 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 caudal artery cannula was flushed with heparinised saline (50 units ml^{-1}) to prevent clotting and then connected to a pressure transducer (Bell and Howell) that was 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 analog signals (water and blood gases, 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 40 sec^{-1}) and a Pentium™ PC. Thus, continuous data recordings were obtained for the measured variables as well as mass-specific $\dot{V}b$, cardiac frequency (H_f; automatic rate calculation from the pulsatile $\dot{V}b$ trace), cardiac stroke volume (H_{SV}; V_b/H_f), ventilation frequency (V_f; automatic rate calculation from the raw ventilation pressure traces), V_{AMP} (the difference between maximum and minimum ventilation pressures), mean blood pressures (P_A) and systemic vascular resistance (R_S; mean $P_{DA}/\dot{V}b$).

Cardiorespiratory effects of external hypercarbia

Experiments commenced on intact, sham-operated or gill denervated fish with a 10 min recording period under conditions of normoxic normocarbia. After this 'pre'-period, the water was rendered hypercarbic for 20 min by gassing a water equilibration column with 1.5% CO_2 in air (Cameron flowmeter model GF-3/MP, Cameron Instruments Inc.), then switched back to normocarbic water. To test the efficacy of the gill denervation, 1.0 ml of sodium cyanide (0.5 mg ml^{-1}), was injected as a bolus into the mouth of fish and ventilation was monitored.

A separate group of fish were administered a bolus injection (via the caudal vein) of the muscarinic receptor antagonist atropine (100 nmol kg^{-1}) dissolved in saline (100 nmol ml^{-1}). These fish were given at least 15 min to allow the atropine to circulate and take effect before undergoing the same procedure previously described in the non-atropinized group. To determine the efficacy of the atropine derived vagal blockade of the heart, fish were administered an intravenous injection (1 ml kg^{-1}) of the potent muscarinic receptor agonist methacholine (100 nmol kg^{-1}).

Statistical analyses

Data are presented as mean values ± one standard error of the mean (SEM). For all experiments, the statistical significance of the observed effects was determined using paired t-tests, while differences between group means employed unpaired t-tests. One-way ANOVA followed, where necessary, by Dunnett's all-pairwise post hoc comparison was used across treatments in Table 4-1. 'MAX' values represent the maximal response

for a given variable over a 1 min interval during maximal hypercarbic exposure. All statistical analyses were performed using SigmaStat 2.0 commercial software package (SPSS Inc.) with a fiducial limit of 5%. All figures were plotted using the Sigmaplot 4.0 commercial graphics software package (SPSS Inc.).

RESULTS

Cardiorespiratory effects of hypercarbia

The interactive effects of the various treatments (i.e. denervation, atropine) and acute hypercarbia on water/blood gases are depicted in Table 4-1. During normocarbia, blood gases were similar in the control, sham and atropine-treated fish whereas the denervated fish displayed a significant reduction in PaO_2. The denervated fish also appeared to exhibit elevated $PaCO_2$ and reduced pHa however the differences were not statistically significant. In all treatment groups, P_WCO_2 was increased to ~6 mm Hg during hypercarbia resulting in pronounced respiratory acidosis (lowered pHa owing to increased $PaCO_2$) of more-or-less equivalent magnitude (Table 4-1). Although PaO_2 appeared to increase in all treatment groups during hypercarbia, the change was statistically significant only in the control fish.

Control and sham fish displayed marked increases in both breathing frequency (V_f) and amplitude (V_{AMP}) during hypercarbia (Figure 4-1). Denervating the gills via surgical transection of the branchial branches of the IXth and Xth cranial nerves eliminated these hyperventilatory responses (Figure 4-1). It is important to point out that denervation itself, while without affect on V_f, caused a marked reduction in V_{AMP} (Figure 4-1).

Unlike the effects on V_{AMP}, none of the measured or calculated cardiovascular variables were affected by denervation, alone (Figure 4-2). Hypercarbia elicited a statistically significant decrease in H_f and $\dot{V}b$ within the untreated and sham-operated

groups whereas these variables remained unchanged in the denervated group (Figure 4-2A & B). Although arterial blood pressure (P_A) decreased (~9%) in the control group, there was no significant effect of hypercarbia on P_A in the sham-operated or denervated dogfish (Figure 4-2C). Furthermore, despite fractional increases in systemic vascular resistance (R_S) in control fish, only the sham-operated group demonstrated a significant increase in R_S during hypercarbia (Figure 4-2D).

Figure 4-3 clearly shows the inhibitory effect of the muscarinic receptor antagonist atropine (100 nmol kg^{-1}) on a variety of cardiorespiratory variables in dogfish experiencing an acute bout of hypercarbia ($PwCO_2$ = 5.94 ± 0.28 mm Hg). Atropine eliminated the hypercarbia-induced ventilatory response (Figure 4-3A & B), and virtually abolished all aforementioned cardiovascular adjustments (Figure 4-3C – F). Although the atropinised dogfish still displayed a significant bradycardia, the magnitude of the responses was greatly diminished. For example, the decrease in H_f of 35.9 ± 5.6% for the control group was significantly larger than the decrease H_f of 8.5 ± 2.0% for the atropine treated group.

Effectiveness of the denervation in blocking O_2 chemoreceptor-mediated responses

The effectiveness of the branchial denervation was assessed by comparing the ventilatory responses of fish to the O_2 chemoreceptor stimulant sodium cyanide. The control and sham-treated fish exhibited marked hyperventilatory responses to cyanide (Figure 4-4). The cyanide-induced increase in V_f was eliminated by branchial denervation and although V_{AMP} still increased significantly, the magnitude of the

response was drastically diminished. Interestingly, the hyperventilatory responses to external cyanide were also eliminated in the atropine-treated fish (Figure 4-4).

External injection of cyanide caused a significant bradycardia (20% decrease in H_f from 14.6 ± 1.2 to 11.7 ± 0.5 min^{-1}) and lowering of P_A (15% decrease from 13.4 ± 1.7 to 11.4 ± 1.8 mm Hg) without affecting $\dot{V}b$ or R_S (data not shown). The cyanide-induced bradycardia and hypotension were eliminated in denervated or atropinised fish (data not shown).

Efficacy of atropine in establishing cardiac muscarinic blockade

Figure 4-5 illustrates the efficacy of atropine in achieving complete muscarinic receptor blockade. First, atropine, itself, caused an approximate doubling of H_f. Second, the decrease in H_f (~55%) after an intravenous injection of the muscarinic receptor agonist methacholine was eliminated in the atropinised fish.

Figure 4-1. The effects of external hypercarbia ($PwCO_2$: control, 6.38 ± 0.13 mm Hg; sham, 6.58 ± 0.44 mm Hg; denervated, 5.99 ± 0.54 mm Hg) on A) ventilation frequency (V_f; N = 5 - 8) and B) ventilation amplitude (V_{AMP}; N = 4 – 8). Black bars represent pre-hypercarbic (i.e. normocarbia) values; white bars represent maximal responses obtained during hypercarbia. Control fish were untreated, sham fish underwent the identical procedure as the denervated fish except for the actual transection of cranial nerves IX and X and denervated dogfish underwent surgical transection of all branchial branches of the IX^{th} and X^{th} cranial nerves. Values are means ± 1 SEM; * denotes a significant difference between normocarbia and hypercarbia ($P < 0.05$).

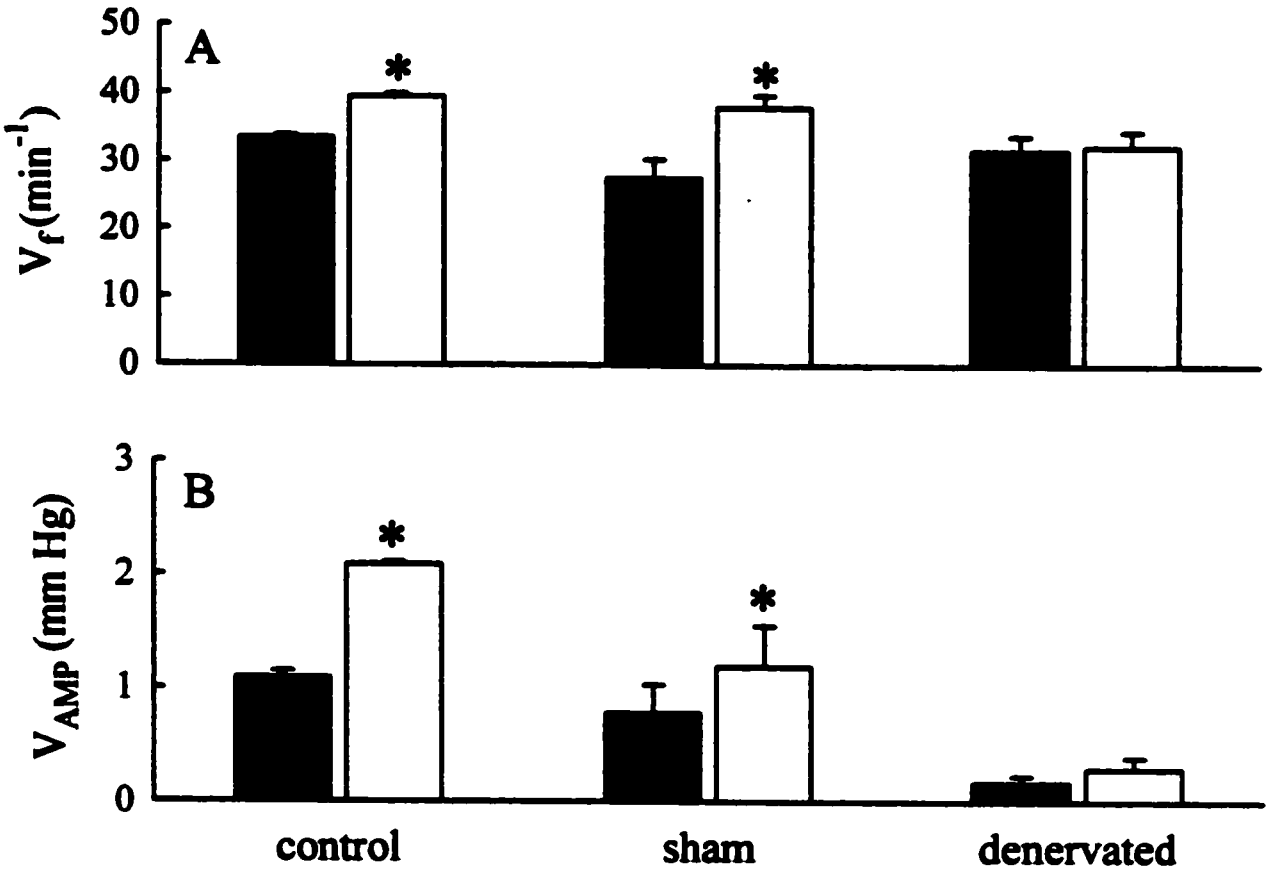
A
B
V_f(min^{-1})
V_{AMP} (mm Hg)
50
40
30
20
10
0
3
2
1
0
*
control
sham
denervated

Figure 4-2. The effects of external hypercarbia ($PwCO_2$: control, 6.38 ± 0.13 mm Hg; sham 6.58 ± 0.44 mm Hg; denervated, 5.99 ± 0.54 mm Hg) on cardiovascular variables in the Pacific spiny dogfish (*Squalus acanthias*) including A) heart rate (H_f; N = 5 – 9), B) cardiac output (V_b; N = 5 - 10), C) arterial blood pressure (P_A; N = 5 - 9), and D) systemic vascular resistance (R_S; N = 5 - 9). Black bars represent pre-hypercarbic (i.e. normocarbia) values; white bars represent maximal responses obtained during hypercarbia. Control fish were untreated, sham fish underwent the identical procedure as the denervated fish except for the actual transection of cranial nerves IX and X and denervated dogfish underwent surgical transection of all branchial branches of the IX^{th} and X^{th} cranial nerves. Values are means ± 1 SEM; * denotes a significant difference between normocarbia and hypercarbia ($P < 0.05$).

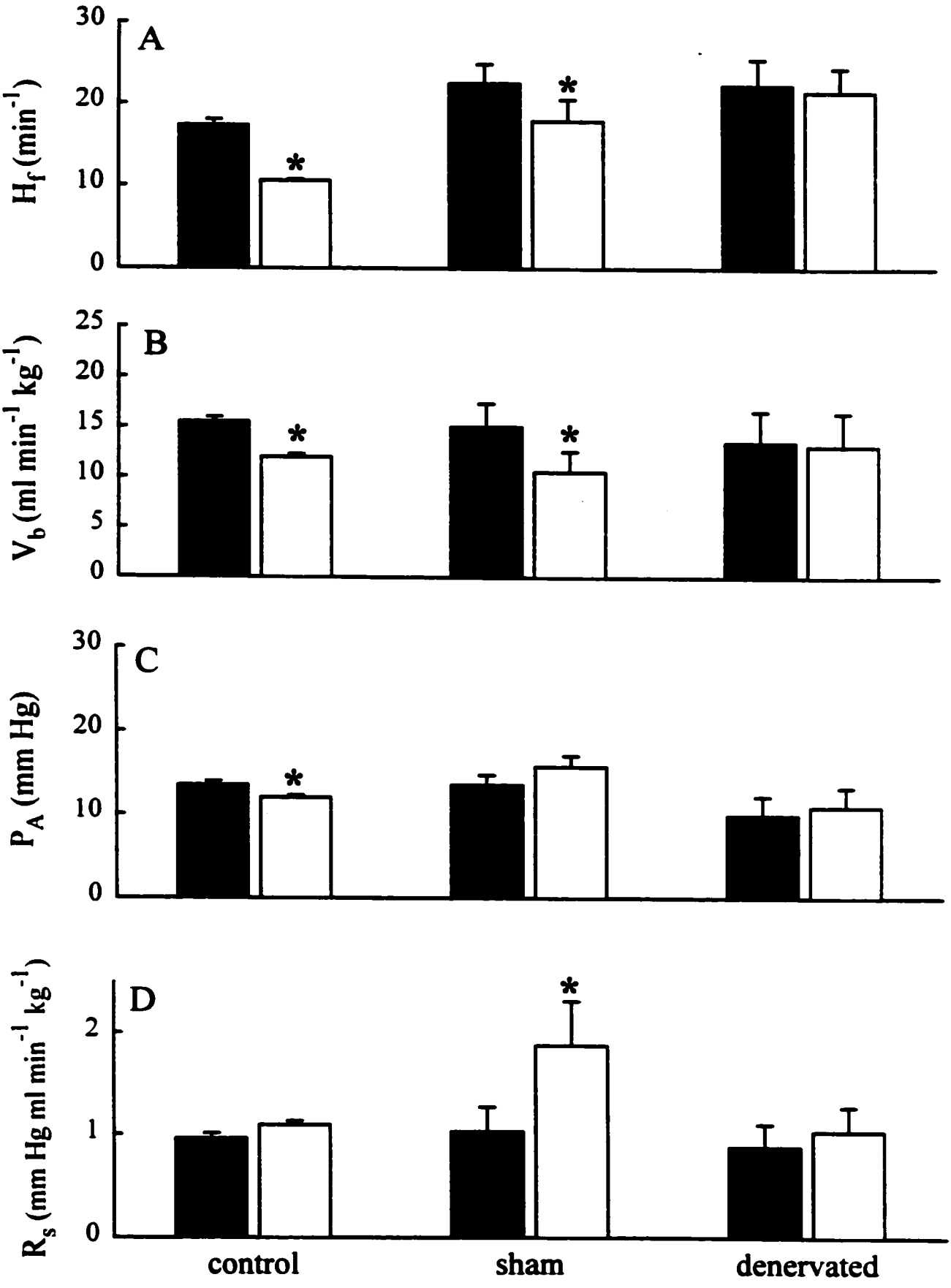
A
B
C
D
H_f (min^{-1})
V_b (ml min^{-1} kg^{-1})
P_A (mm Hg)
R_s (mm Hg ml min^{-1} kg^{-1})
30
20
10
0
25
15
5
2
1
*
control
sham
denervated

Figure 4-3. The effects of external hypercarbia ($PwCO_2 = 5.94 \pm 0.28$) on cardiovascular variables in atropine treated (100 nmol kg^{-1}) Pacific spiny dogfish (*Squalus acanthias*) including A) ventilation frequency (V_f; N = 7) and B) ventilation amplitude (V_{AMP}; N = 7), C) heart rate (H_f; N = 7), D) cardiac output (V_b; N = 7), E) arterial blood pressure (P_A; N = 7), and F) systemic vascular resistance (R_S; N = 7). Black bars represent pre-hypercarbic (i.e. normocarbia) values; white bars represent maximal responses obtained during hypercarbia. Values are means ± 1 SEM; * denotes a significant difference between normocarbia and hypercarbia ($P < 0.05$).

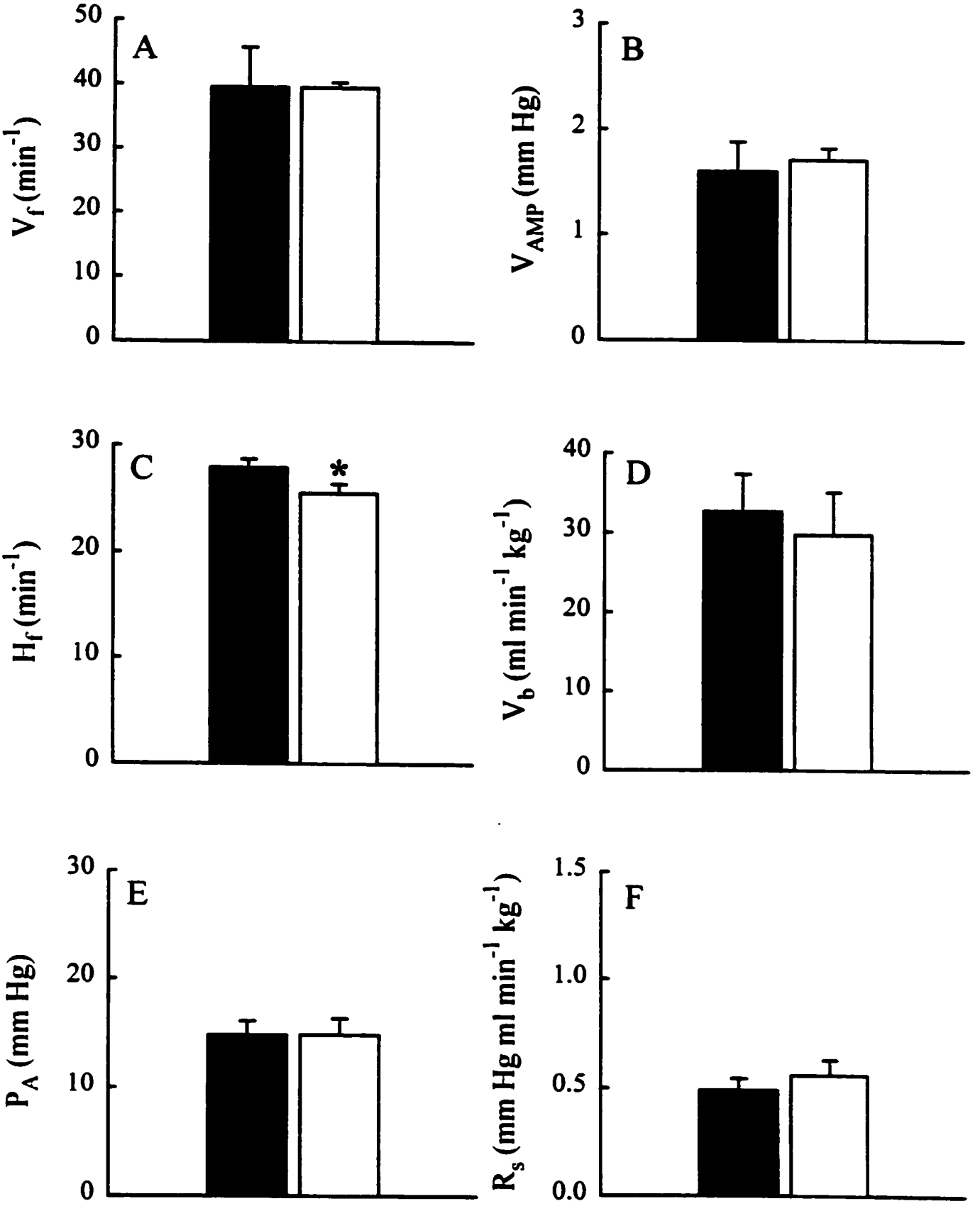
A
B
C
D
E
F
V_f (min^{-1})
V_{AMP} (mm Hg)
H_f (min^{-1})
V_b (ml min^{-1} kg^{-1})
P_A (mm Hg)
R_s (mm Hg ml min^{-1} kg^{-1})
*
0
10
20
30
40
50
1
2
3
0.0
0.5
1.0
1.5

Figure 4-4. The effects of a bolus injection into the mouth of 0.5 ml of sodium cyanide (0.5 mg ml^{-1}) on (A) ventilation amplitude (V_{AMP}) and (B) ventilation frequency (V_f) in control, sham, denervated or atropinised dogfish (*Squalus acanthias*). Black bars represent pre-cyanide values; white bars represent maximal responses obtained after cyanide injection. Values are means ± 1 SEM; * denotes a significant difference from pre-cyanide value ($P < 0.05$).

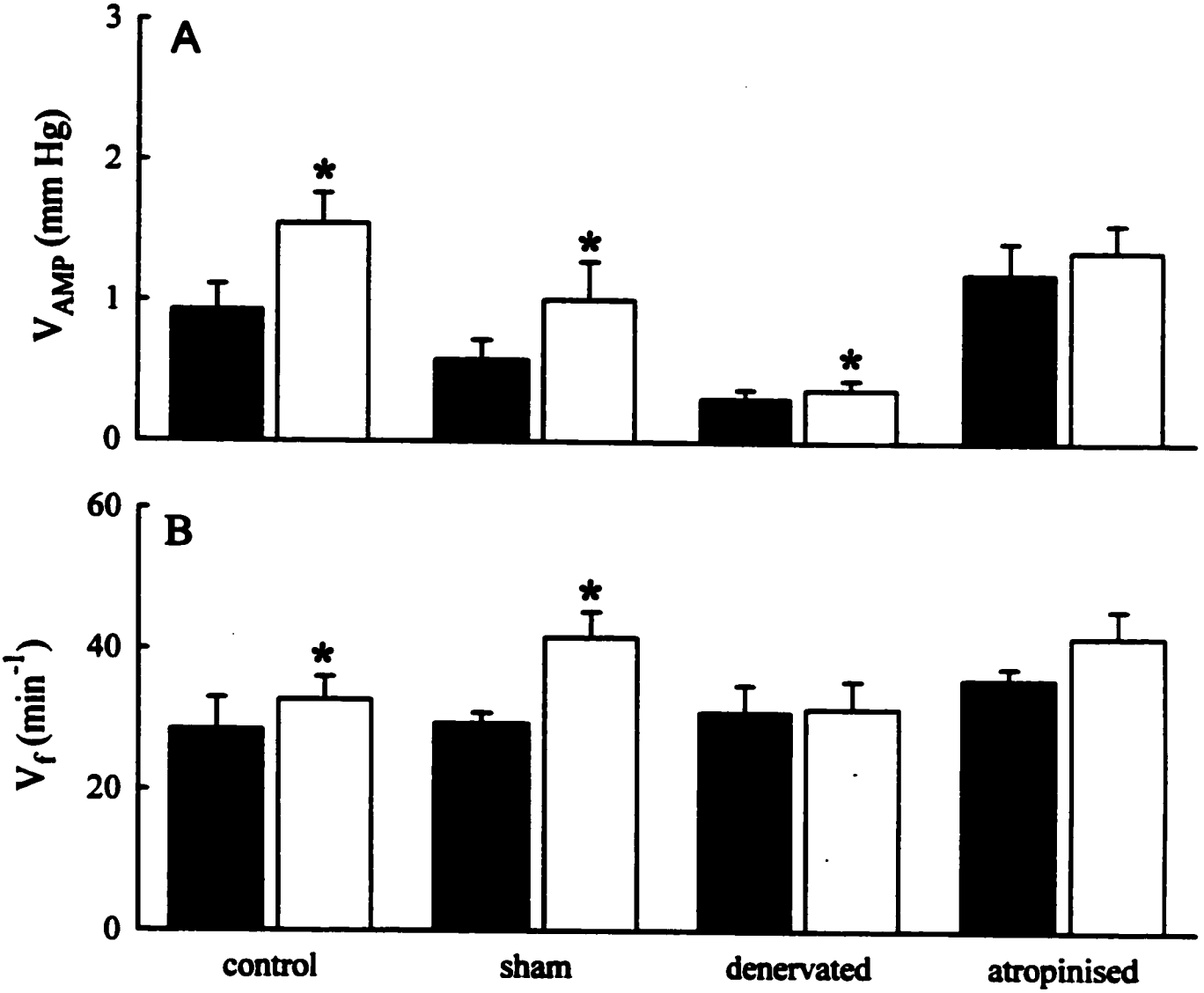
A
B
V_{AMP} (mm Hg)
V_f (min^{-1})
3
2
1
0
60
40
20
0
*
control
sham
denervated
atropinised

Figure 4-5. The effectiveness of the muscarinic receptor antagonist atropine (100 nmol kg^{-1}) in blocking a methacholine-induced (100 nmol kg^{-1}) reduction in heart rate (H_f) in Pacific spiny dogfish (*Squalus acanthias*). Black bars represent pre-methacholine (i.e. resting) values; white bars represent maximal responses obtained following intravenous injection of methacholine. Values are means ± 1 SEM; * denotes a significant difference between 'resting' H_f and post-methacholine H_f, ($P < 0.05$).

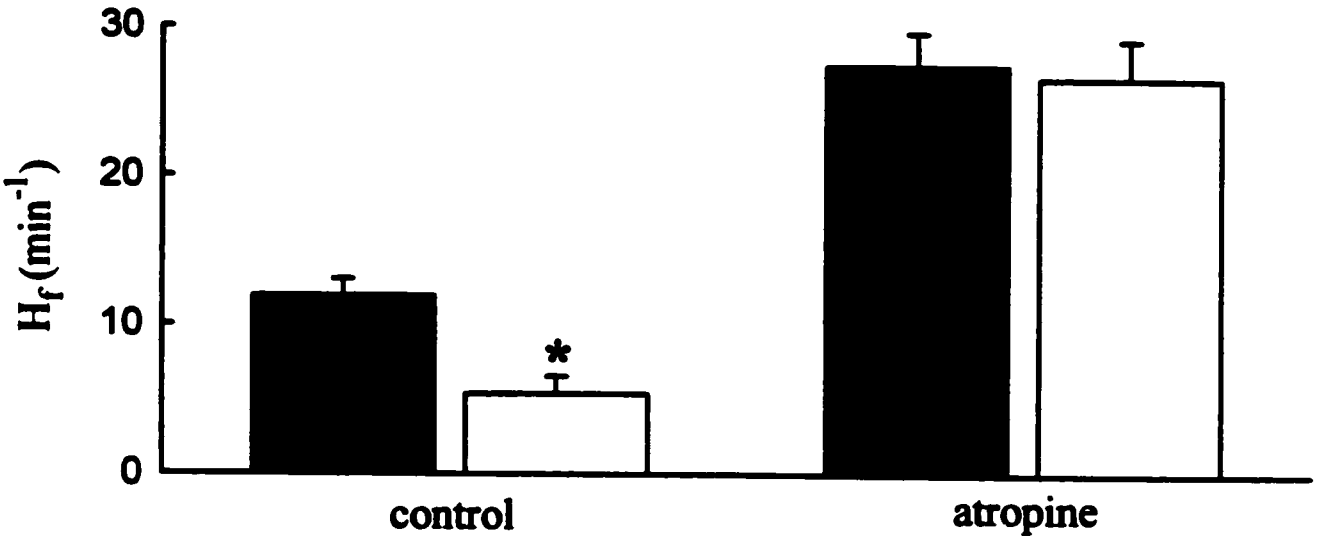
30
20
10
0
H_f (min^{-1})
*
control
atropine

Table 4-1. The effects of hypercarbia on water/blood gases in untreated (control; N = 7 - 9), atropinised (atropine; N = 5 – 7; 100 nmol kg^{-1}), sham-operated (sham; N = 4 - 7), and denervated (i.e. branchial branches of the IX^{th} and X^{th} cranial nerves were surgically transected; N = 4 - 5) Pacific spiny dogfish (*Squalus acanthias*). * denotes a statistically significant difference between the normocarbic and hypercarbic value within each treatment; † denotes a significant difference between the designated treatment and all other treatments at a given $PwCO_2$ ($P < 0.05$).

	Normocarbia				Hypercarbia			
	control	atropine	sham	denervated	control	atropine	sham	denervated
PwO_2 (mm Hg)	147.0 ± 1.0	137.7 ± 1.7	146.7 ± 3.3	141.2 ± 2.5	147.3 ± 0.9	139.3 ± 0.9	147.6 ± 2.4	142.9 ± 2.7
$PwCO_2$ (mm Hg)	0.74 ± 0.45	0.62 ± 0.06	0.66 ± 0.21	0.63 ± 0.09	6.38 ± 0.13*	5.94 ± 0.28*	6.68 ± 0.34*	5.99 ± 0.54*
PaO_2 (mm Hg)	120.9 ± 2.2	117.8 ± 6.8	112.7 ± 8.6	52.9 ± 17.3†	128.8 ± 2.0*	125.0 ± 9.1	115.1 ± 11.0	84.8 ± 19.1
$PaCO_2$ (mm Hg)	1.20 ± 0.10	2.21 ± 0.20	1.93 ± 0.48	3.46 ± 0.38	6.83 ± 0.24*	6.29 ± 0.57*	6.87 ± 0.29*	6.61 ± 0.86*
pHa	7.71 ± 0.04	7.59 ± 0.03	7.92 ± 0.31	7.48 ± 0.04	7.41 ± 0.04*	7.33 ± 0.03*	7.60 ± 0.36*	7.29 ± 0.04*

DISCUSSION

Although previous studies have addressed aspects of ventilatory control during hypercarbia in elasmobranchs (Randall *et al.* 1976; Heisler *et al.* 1988; Graham *et al.* 1990; Perry and Gilmour, 1996), this is the first study to examine in detail both the ventilatory and cardiovascular responses to hypercarbia and also the first to attempt to localise the receptors that mediate the cardiorespiratory responses. Lacking a Root effect, elasmobranchs are ideal for such studies because unlike in teleosts (Burleson and Smatresk, 2000; Sundin *et al.* 2000; Reid *et al.* 2000), the results are not confounded by indirect effects of CO_2-induced hypoxaemia. Two approaches were used in this study to assess cardiorespiratory control during hypercarbia. First, complete branchial denervation was used as a tool to eliminate sensory input from the gill arches and the pharynx and second, atropine was used to block muscarinic receptors so as to provide insight into the involvement of cholinergic nervous transmission.

Critique of the methods

Because of the diffuse branchial location of oxygen chemoreceptors in elasmobranchs (Taylor, 1992), denervation of all gill arches is required to eliminate the reflex hypoxic bradycardia (Butler *et al.* 1977). Assuming a similar distribution of the presumptive CO_2/H^+ receptors, we also chose to denervate all gill arches by bilateral sectioning of cranial nerves IX and X. As previously demonstrated for *Scyliorhinus canicula* (Butler *et al.* 1977), this led to a reduction in Pa_{O_2} (from 121 to 53 mm Hg) that

was probably related to the markedly reduced breathing amplitude. Although not statistically significant, the trend toward respiratory acidosis in denervated dogfish is also consistent with impaired gas transfer. However, the absence of a Root effect in dogfish coupled with its relatively high affinity haemoglobin (P_{50} = 13.5 - 17.3 mm Hg in *S. acanthias*; Butler and Metcalfe, 1988) and small Bohr effect (Butler and Metcalfe, 1988), suggests that denervation had only minor effects on overall blood O_2 transport. Moreover, aside from V_{AMP} and PaO_2, none of the other measured cardiorespiratory variables were significantly affected by denervation. The marked reduction in V_{AMP} after denervation suggests that the scope to increase amplitude during hypercarbia may have been compromised in these fish. Thus, the effects of denervation on the V_{AMP} response to hypercarbia must be interpreted with caution. Fortunately, similar concerns need not apply to the remaining cardiorespiratory variables that were examined.

The deleterious effects of gill denervation on gas transfer are not confined to dogfish but have also been reported in the bowfin *Amia calva* (McKenzie *et al.* 1991) and the channel catfish *Ictalurus punctatus* (Burleson and Smatresk, 2000). Most studies, however, employing gill denervation as a tool to localise chemoreceptors in fish have not measured arterial blood gases (e.g. Sundin *et al.* 2000; Reid *et al.* 2000). It seems likely, however, that impaired gas transfer is a universal response to complete gill denervation.

The effects of hypercarbia in intact dogfish

As previously documented (Perry and Gilmour, 1996), spiny dogfish exhibited a pronounced hyperventilatory response during hypercarbia consisting of increases in both V_f and V_{AMP}. As reported for a variety of elasmobranch (Randall *et al.* 1976; Graham *et*

al. 1990; Perry and Gilmour, 1996) and teleost (Thomas, 1983; Perry and Gilmour, 1996; Reid *et al.* 2000) species, the increase in V_{AMP} (92%) was considerably greater than the increase in V_f (18%). Comparing all the fish species that have been examined, however, no clear pattern of ventilatory response emerges. Indeed, some species display a predominant (channel catfish; Burleson and Smatresk, 2000) or exclusive (tambaqui; Sundin *et al.* 2000) V_{AMP} response to hypercarbia.

Few studies have examined the cardiovascular effects of hypercarbia in fish and to our knowledge this is only the second investigation to report the effects in dogfish or any elasmobranch species. Moreover, the single previous study on dogfish (Kent and Peirce, 1978) was performed on anaesthetised, restrained and force-ventilated animals. With so few data, it is difficult to construct a generic model describing the cardiovascular effects of CO_2 in fish. In rainbow trout *(Oncorhynchus mykiss)*, hypercarbia elicits a reflex bradycardia and an elevation of arterial blood pressure (Perry *et al.* 1999a). The hypertensive response to hypercarbia in trout reflects a profound increase in systemic vascular resistance that is caused by vasoconstriction following sympathetic activation of vascular smooth muscle α-adrenoreceptors (Perry *et al.* 1999a). This reflex vasoconstriction serves to counteract the specific dilatory effects of elevated CO_2 on the systemic vasculature (McKendry and Perry, 2001). The receptors mediating the reflex cardiovascular responses to hypercarbia in trout appear to be externally oriented because increases in $PaCO_2$, alone, are without effect on cardiac frequency or systemic resistance (McKendry and Perry, 2001). Results obtained using other species have yielded conflicting results. For example, while two neotropical teleost species (tambaqui and traira) both exhibit CO_2-evoked bradycardia, the tambaqui (*Colossoma macropomum)*

responds with hypertension (Sundin *et al.*, 2000) whereas the traira (*Hoplias malabaricus*) exhibits mild hypotension (Reid *et al.*, 2000); in neither study was systemic resistance determined. Finally, although channel catfish (*Ictalurus punctatus*) display a pronounced hyperventilatory response to hypercarbia, blood pressure and cardiac frequency are unaffected (Burleson and Smatresk, 2000).

In the present study, dogfish displayed a bradycardia, reduced cardiac output and lowered arterial (post-branchial) blood pressure in response to acute hypercarbia. Because systemic vascular resistance was unaltered or increased (sham group), the hypotension likely was caused by the lowering of cardiac output although based on the study of Kent and Peirce (1978), the possibility of increased gill vascular resistance contributing to the overall hypotension cannot be excluded. The hypotensive response of dogfish to moderate hypercarbia (P_wCO_2 = 6.4 mm Hg) in the present study resembles the slight (8%) decrease in P_A in this species during exposure to much higher levels of $PwCO_2$ (~37.5 mm Hg; Kent and Peirce, 1978). Interestingly, elasmobranchs, unlike most teleosts, also exhibit a hypotensive response during exposure to environmental hypoxia (Satchell, 1961; Butler and Taylor, 1971). Thus, the cardiovascular (and indeed the ventilatory) responses of dogfish to hypercarbia are more-or-less identical to those previously reported for hypoxia. This raises the question as to whether the physiological responses to hypercarbia and hypoxia are mediated by a single class of receptor or different classes of receptors linked to similar efferent pathways (see below).

CO_2 versus H^+ receptors

In the present study, no attempt was made to discern between CO_2 and H^+ receptors. During hypercarbia (P_wCO_2 = 6 – 6.5 mm Hg), seawater pH was undoubtedly lowered. Although this likely constituted a small but significant reduction in pH, any specific impact of H^+ on the cardiorespiratory responses is unlikely. For example, we have demonstrated previously (J.E. McKendry and S.F. Perry; unpublished data) that bolus injections of seawater equilibrated with 2% (15 mm Hg) or 4% (30 mm Hg) CO_2 into the mouth of dogfish or Atlantic salmon (*Salmo salar*) caused pronounced cardiorespiratory responses (increased V_f, V_{AMP}, decreased H_f). These responses were not observed when aerated seawater was titrated to equivalent pH's (7.0 and 6.3, respectively) with HCl. Similarly, Reid *et al.* (2000) demonstrated that the cardiorespiratory responses to hypercarbia in traira were not mimicked by external injection of HCl.

Cardiorespiratory responses to hypercarbia in denervated dogfish

Denervation of all gill arches (via transection of cranial nerves IX and X) eliminated all cardiorespiratory responses to hypercarbia. These results clearly demonstrate the existence of branchial chemoreceptors in dogfish that are stimulated by elevated CO_2 and/or H^+ as well as providing evidence for the absence of central CO_2/H^+ chemoreceptors. Whether the receptors are oriented externally and monitor inflowing water and/or are oriented internally and monitor the blood cannot be distinguished from the present experimental design. However, we (J.E. McKendry and S.F. Perry,

unpublished data) have recently demonstrated that injections into the caudal vein of dogfish of saline equilibrated with 4% CO_2 (PCO_2 = 30 mm Hg) did not elicit any cardiorespiratory responses (unlike external injections of seawater gassed with 4% CO_2 into the buccal cavity; see above). These data would argue in favour of exclusively externally oriented CO_2 chemoreceptors.

Because all gill arches were denervated in the present experiments, we cannot comment on the relative distribution of the branchial CO_2 receptors in dogfish. However, if distributed in a similar manner as O_2 chemoreceptors in elasmobranchs, they are likely to be diffusely distributed amongst all gill arches. On the other hand, unlike the additional location of O_2 chemoreceptors within the buccal cavity of dogfish (Butler *et al.* 1977), the CO_2 chemoreceptors appear to be confined to the gills (i.e. all cardiorespiratory responses were prevented despite continuing innervation of the orobranchial cavity via cranial nerves V and VII).

Because the data on cardiorespiratory responses to hypercarbia in teleosts are sparce, it is not yet possible to formulate a general model for any fish group. Based on the heterogeneity in the existing data, however, construction of a generic fish model seems unlikely. For example, in tambaqui, the receptors mediating reflex hypercarbic bradycardia are localised to the first gill arch (Sundin *et al.* 2000) whereas in traira, the receptors mediating the bradycardia are found within all the gill arches (Reid *et al.* 2000). Clearly, further studies on a variety of species are required to determine whether the first gill arch (as appears to be the case for O_2 chemoreception in teleosts – see Fritsche and Nilsson, 1993 for review) is the predominant site of CO_2 chemoreception.

In tetrapod vertebrates, peripheral chemoreceptors are responsive to both O_2 and CO_2 (Iturriaga, 1993). In fish, however, it is unclear as to whether there is a single population of branchial receptors that are responsive to lowered O_2 and elevated CO_2 or rather two distinct populations of CO_2 and O_2 chemoreceptors. In dogfish, there is evidence for and against a single population of receptors. In support of a single receptor population, the cardiorespiratory responses to hypercarbia (increased V_f, V_{AMP}, decreased H_f, P_A) are similar to the responses observed during hypoxia. In opposition, and perhaps more importantly, external injections of cyanide (a potent activator of O_2 chemoreceptors), while eliciting bradycardia and hypotension, did not elicit the marked reduction in cardiac output that accompanies hypercarbia.

Cardiorespiratory responses to hypercarbia in atropinised dogfish

The marked attenuation of the hypercarbic bradycardia and ensuing reduction in cardiac output and blood pressure in atropinised fish was not surprising because increased vagal tone is the key contributor to reflex bradycardia in dogfish (Butler and Metcalfe, 1988). However, because the reflex bradycardia was not totally abolished by atropine, elevated blood PCO_2, in itself, may contribute slightly to the hypercarbic bradycardia. The more interesting result was the blockade of the hyperventilatory responses during hypercarbia in the atropine-treated fish. Previous studies have demonstrated that O_2 chemoreception in fish (Burleson and Milsom, 1995a) and mammals (Fitzgerald, 2000; Fitzgerald *et al.* 2000) involves an important cholinergic element. According to the "cholinergic hypothesis" of O_2 chemotransduction for the mammalian carotid body (see Fitzgerald, 2000 for review), acetylcholine (Ach) is released from the O_2-sensing glomus

cells and interacts with post-synaptic cholinergic receptors on neigbouring afferent neurons as well as autoreceptors on the glomus cells, themselves. There is evidence for the presence of both nicotinic and muscarinic receptors at both sites (Fitzgerald, 2000; Fitzgerald *et al.* 2000; Shirahata *et al.* 2000) although the relative contribution of these receptor sub-types to the overall chemotransduction process is unclear. It is generally accepted, however (see Fitzgerald, 2000), that the nicotinic and M_1 muscarinic receptors are excitatory while the M_2 muscarinic receptors are inhibitory.

The absence of any ventilatory responses to hypercarbia after atropine treatment suggests a critical involvement of muscarinic receptors in the chemosensory response of dogfish to CO_2. This finding is consistent with the results of earlier studies demonstrating that atropine blocks Ach- or cyanide-evoked increases in trout gill O_2 chemoreceptor activity *in situ* (Burleson and Milsom, 1995a) and that injection of muscarine markedly stimulates breathing in trout *in vivo* (Burleson and Milsom, 1995b). Although the focus of the present study was on CO_2 rather than O_2 chemoreception, the observation that atropine eliminated the hyperventilatory responses to external cyanide suggests a crucial role for muscarinic receptors in the response of dogfish to both hypercarbia and hypoxia.

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Chapter 5

INTER-SPECIFIC VARIATION IN THE CARDIO-RESPIRATORY RESPONSES OF FISHES TO ENVIRONMENTAL CARBON DIOXIDE

ABSTRACT

The goal of this study was to assess, in a comparative manner, the homogeneity of the cardiovascular and ventilatory responses amongst fish to environmental hypercarbia. The experiments were performed *in vivo* using two marine teleosts, Atlantic salmon (*Salmo salar*) and Pacific sanddab (*Citharychthus sordidus*); two freshwater teleosts, brown bullhead (*Ictalurus nebulosus*) and American eel (*Anguilla rostrata*); as well as one marine elasmobranch, the Pacific spiny dogfish (*Squalus acanthias*). These results are compared with data previously obtained from rainbow trout (*Oncorhynchus mykiss*; and Perry, 2001).

Fish were exposed acutely (approximately 20 min) to elevated levels of environmental CO_2 ranging from 6.1 ± 0.4 mm Hg (brown bullhead) to 8.1 ± 0.2 mm Hg (Pacific sanddab). All species compared in this study displayed an increase in ventilation frequency (increases of 17 - 36%), except the American eel which did not increase ventilation frequency or amplitude. Ventilation amplitude did, however, increase significantly in the Atlantic salmon, dogfish and sanddab.

In terms of cardiovascular responses, rainbow trout and Atlantic salmon both displayed an elevated arterial blood pressure (47 and 36%, respectively) and significant increases in systemic vascular resistance (80 and 16% respectively). In contrast, dogfish experienced a significant decrease (13%) in arterial blood pressure during the hypercarbic period. The effect on cardiac frequency also varied among the species studied. While heart rate did not change during hypercarbia in the American eel or brown bullhead, it decreased significantly in the rainbow trout (12%), Atlantic salmon (22%), dogfish (33%)

and sanddab (67%). The results of this investigation reveal that there is large inter-specific variation in the cardiovascular and ventilatory responses of fishes to environmental hypercarbia.

INTRODUCTION

It is well known that fish exhibit pronounced physiological responses, including hyperventilation and catecholamines release, when exposed to environmental hypercarbia (see review by Perry and Wood, 1989). Until recently, it was generally accepted that the physiological responses to hypercarbia are not directly attributable to changes in PCO_2/pH but instead are an indirect effect of the marked Bohr and Root effects that occur during hypercapnic acidosis. It has been argued that these cardiorespiratory effects reflect an impairment in blood O_2 transport capability (e.g. Randall, 1982; Smith and Jones, 1982) that is believed to initiate physiological responses via receptors sensitive to changes in arterial blood O_2 content (Janssen and Randall, 1975; Smith and Jones, 1982; Perry *et al.* 1989). However, an analysis of prior studies performed on elasmobranchs (that lack a Root effect) clearly reveals a specific involvement of CO_2/pH in the modulation of ventilation (Heisler *et al.*, 1988; Graham *et al.*, 1990; Wood *et al.*, 1990; Perry and Gilmour, 1996). Within the teleosts, there also is emerging evidence that CO_2/pH can exert a specific effect on ventilation (Kinkead and Perry, 1991; Burleson and Smatresk, 2000; reviewed by Perry and Wood, 1989). More recently, CO_2/pH has been identified as a specific regulator of cardiovascular function in several teleosts (Perry *et al.*, 1999; Reid *et al.*, 2000; Sundin *et al.*, 2000). The specific effects of CO_2/pH on cardio-respiratory function in fish are mediated, at least in part, by branchial chemoreceptors (Burleson and Smatresk, 2000; Reid *et al.*, 2000; Sundin *et al.*, 2000) that are probably externally oriented (McKendry and Perry, 2001)

CO_2/H^+ chemoreception and its effects have been studied in invertebrates (Erlichman *et al.*, 1994; Erlichman and Leiter, 1997 a & b), amphibians (Coates et al., 1998), reptiles (Furilla, 1991), birds (Burger et al., 1976; Osborne et al., 1977; Furilla and Bernstein, 1995), and mammals (Cross et al., 1990; Thomas et al., 1999). Although fish represent the largest group of vertebrates and possibly a 'pre-terrestrial' evolutionary perspective with respect to CO_2/H^+ chemoreception they have garnered remarkably little attention.

Within animals, there exists a wide variation in responsiveness to CO_2 (Boggs and Kilgore, 1983; Kilgore *et al.*, 1985). For example, burrowing owls like burrowing mammals, encounter elevated CO_2 concentrations in their natural environment and seemingly have reduced their reliance on a compensatory hyperventilation. Although most investigations in fish have centered on the salmonids, it seems likely that the vast diversity of aquatic microenvironments has led to marked variability in hypercarbia responsiveness similar to that observed in birds and mammals.

Therefore, to investigate the variability among fish species with respect to their CO_2/H^+ chemoreceptive abilities and their cardiovascular and ventilatory responses to CO_2, a variety of species from differing aquatic habitats have been exposed to parallel treatments of hypercarbia. The species examined were two marine teleosts, Atlantic salmon (*Salmo salar*) and Pacific sanddab (*Citharychthus sordidus*); two freshwater teleosts, brown bullhead (*Ictalurus nebulosus*) and American eel (*Anguilla rostrata*); as well as one marine elasmobranch, the Pacific spiny dogfish (*Squalus acanthias*). The present results are compared with recent data collected from rainbow trout (*Oncorhynchus mykiss*; McKendry and Perry, 2001).

MATERIALS AND METHODS

Experimental animals

Brown bullhead, *Ictalurus nebulosus*, and American eel, *Anguilla rostrata* of either sex were obtained from a commercial fishery on the St. Lawrence River (Cornwall, Ontario) and were transported in oxygenated water to the University of Ottawa. Both species were maintained on a 12L:12D photoperiod in 1300 l fiberglass tanks supplied with flowing, aerated and dechlorinated City of Ottawa tap water (14°C). They were fed using commercial pelleted diets.

Atlantic salmon, *Salmo salar*, of either sex were obtained from a fish farm (Vancouver Island, British Columbia) and were transported in oxygenated water to Bamfield Marine Station where they were housed outdoors in fiberglass tanks supplied with fresh flowing full strength seawater (12° C); they were fed daily with commercial salmonid pellets. Pacific sanddab, *Citharychthus sordidus*, and spiny dogfish, *Squalus acanthias*, were angled or collected by net during trawls by local fishermen and held indoors under natural photoperiod at the Bamfield Marine Station in 200 l open-air holding tanks and a 75,000 l opaque circular tank, respectively. Both tanks sizes were supplied with a continuous flow of aerated seawater. Dogfish were fed twice weekly with herring; sanddab were not fed.

Surgical procedures

Atlantic salmon, weighing between 300 and 575 g (experimental N = 11) were anaesthetized using benzocaine (0.1 g l^{-1}ethyl-m-aminobenzoate, Sigma) in seawater 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 (Sovio *et al.*, 1975; Olson *et al.* 1997).

In order to record ventilation frequency and amplitude 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™.

Pacific sanddab, weighing between 180 and 350 g (experimental N = 10), and Pacific spiny dogfish, weighing between 1250 and 3460 g (experimental N = 10) were anaesthetized as above. To permit measurement of P_A, a lateral incision was made in the caudal peduncle immediately below the lateral line to expose, separate from the surrounding tissue, and cannulate (PE 50) the caudal artery in the anterograde direction (Axelsson and Fritsche, 1994). To allow for the recording of ventilation adjustments, a heat-flared cannula (PE 160) was threaded through either a puncture in the upward-facing operculum (sanddab) or inserted into the spiracular cavities (dogfish) and secured using a single silk ligature and Vetbond™.

American eels weighing between 350 and 950 g (experimental N = 8) were immersed in an anaesthetic solution of benzocaine (1.6g l^{-1}ethyl-m-aminobenzoate) in dechlorinated tap water for approximately 10 min and placed on a dissection tray without

gill irrigation. To permit measurement of P_A, a lateral incision was made immediately below and parallel to the lateral line approximately 5 cm behind the pectoral fin in order to expose the pneumogastric artery and dissect it free from the overlying tissue. A cannula (PE 10) was then occlusively inserted into the pneumogastric artery and advanced into the dorsal aorta. Heat-flared ventilation cannula (PE 160) were threaded through the opercular opening, into the buccal cavity and secured to the outer body wall with silk ligatures.

Brown bullhead weighing between 275 and 450 g (experimental N = 8) were immersed in an oxygenated anaesthetic solution of benzocaine (1.6g l^{-1}ethyl-m-aminobenzoate) in dechlorinated tap water for approximately 5-10 min and placed on an operating table where their gills were force-ventilated with the same oxygenated anaesthetic solution. To permit measurement of P_A, a polyethylene cannula (PE 50) was surgically implanted into the caudal artery using the same procedure as for the dogfish and sanddab. Heat-flared ventilation cannula (PE 160) were threaded through the opercular opening, into the buccal cavity and secured to the outer body wall with silk ligatures.

With the exception of the pacific sanddab, each species was fitted with cardiac flow (Vb) 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 or conus arteriosus (dogfish). A 3S or 4S ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY. USA) was placed non-occlusively around the bulbus/conus to enable the measurement of cardiac output (Olson et al., 1997). The incision was sutured closed and sealed with Vetbond™ (3M Animal Care Products, MN),

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 appropriate heparinized (50 i.u. ml^{-1} sodium heparin) saline [dogfish, 500 mmol l^{-1} NaCl; seawater teleosts, 0.9% NaCl; freshwater teleosts, Cortland saline (Wolf, 1963)]. After surgery, fish were revived and placed in opaque flow-through acrylic boxes and left to recover for ~24 h prior to experimentation.

Experimental protocol

Measurement of water respiratory variables

A pump-driven loop continuously withdrew inspired water from the box directly in front of the fish and passed it over PO_2 and PCO_2 electrodes (Radiometer) linked to data acquisition system. O_2 and CO_2 electrodes were housed in temperature controlled cuvettes and connected to a Radiometer blood gas analyzer. All electrodes were calibrated prior to each individual experiment (Perry et al. 1999).

Measurement of cardiovascular and ventilation variables

The arterial and ventilation cannulae were connected to UFI model 1050BP (UFI, Morro Bay, CA) pressure transducers that were pre-calibrated against a static column of water. Mean arterial blood pressure was calculated as: (systolic pressure + diastolic pressure)/2. Cardiac output was determined by connecting the 3S or 4S ultrasonic flow probe to a Transonic T106 small animal blood flow meter. Analog signals were converted to digital data and collected at 40 samples sec^{-1} using a computer linked to a

data-acquisition system (Biopac System Inc., Goleta, CA) using AcqKnowledge 3.0 software. Recordings began once all parameters had stabilized.

Cardiovascular effects of hypercarbia

Experiments commenced with a 10 min recording period under conditions of normoxic normocarbia. After this 'pre'-period, the water was rendered hypercarbic for 20 min by gassing a water equilibration column with 1.5% CO_2 in air (Cameron flowmeter model GF-3/MP, Cameron Instruments Inc.), then switched back to normocarbic water. Systemic vascular resistance (R_S) was calculated using the formula: $R_S = P_A/Vb$. Systemic resistance was not calculated for the Pacific sanddab because it was not possible to successfully implant a cardiac flow probe into these relatively small flat fish.

Statistical analyses

Data are presented as mean values ± one standard error of the mean (SEM). For all experiments, the statistical significance of the observed effects for each species was determined using paired t-tests. All recordings were analyzed by taking mean values over 1 min periods. All statistical analyses were performed using SigmaStat 2.0 commercial software package (SPSS Inc.) with a fiducial limit of 5%. All figures were plotted using the Sigmaplot 4.0 commercial graphics software package (SPSS Inc.).

RESULTS

It should be noted that in each species-specific series, $PwCO_2$ increased significantly, while PwO_2 remained unchanged. The only exception was a slight (~3.5%), but statistically significant rise in PwO_2 with the Pacific sanddab.

Cardiovascular effects of environmental hypercarbia

Rainbow trout demonstrated a hypertensive response when exposed to an acute increase in water PCO_2 ($PwCO_2$; maximum $PwCO_2 = 6.1 \pm 0.3$ mm Hg), P_A increased significantly (47%), R_S increased 80% and H_f was decreased by 12% (Figures 5-1 to 5-3). P_A also increased significantly (30 %) in Atlantic salmon but decreased (13%) in the dogfish (Fig 5-1). Atlantic salmon displayed a significant increase in R_S (16%), while brown bullhead and American eel exhibited no change (Figure 5-2). Similar to the rainbow trout, *S. salar*, *C. sordidus* and *S. acanthias* displayed a significant bradycardia during their bouts of increased $PwCO_2$ exposure (Figure 5-3). The level of environmental hypercarbia (Table 5-1) used in this study had no effect on P_A, R_S or H_f in *I. nebulosus* and *A. rostrata* (Figures 5-1 to 5-3).

Figure 5-4 illustrates three distinct cardiovascular responses to environmental hypercarbia. *C. sordidus* (Figure 5-4A) displayed a remarkable bradycardia with little effect on arterial blood pressure. This particular trace shows a slight increase in mean P_A that was associated with a decrease in H_f of ~60%. The increase in P_A bandwidth is likely indicative of a compensatory elevation in stroke volume although stroke volume could not be measured in this species. The rainbow trout (Figure 5-4B) reacted somewhat differently to a similar exposure to acute hypercapnic acidosis. There was an

increase in stroke volume, although not as pronounced as in the flatfish, but the bradycardia was less pronounced. The substantial increase in P_A (~10 - 15 mm Hg, Figure 5-1) presumably resulted from the increase in R_S (Figure 5-2). Finally, the brown bullhead (Figure 5-4C), like the American eel, displayed no cardiovascular adjustments when subjected to acute environmental hypercarbia. Although this particular example appears to show a substantial decrease in blood pressure pulsatility during hypercarbia this was not the case in most bullhead observed.

Respiratory effects of environmental hypercarbia

Exposing *S. salar*, *C. sordidus*, and *S. acanthias* to an acute increase in $PwCO_2$ (6.3 ± 0.4 mm Hg, 8.1 ± 0.2 mm Hg, 6.4 ± 0.1 mm Hg respectively; Table 5-1) elicited increases in Vf of 36%, 27%, and 18% respectively (Figure 5-5). These same three species also experienced significant increases in V_{AMP} of 91 – 186% (Figure 5-6). *I. nebulosus* (max $PwCO_2$ = 6.1 ± 0.4) demonstrated a 17% increase in Vf without any statistically significant increase in V_{AMP}, while *A. rostrata* (max $PwCO_2$ = 6.4 ± 0.2) showed no effect of environmental hypercarbia on V_{AMP} or Vf (Figures 5-5 & 5-6).

Figure 5-7 contains representative ventilation traces from *C. sordidus*, *S. salar* and *A. rostrata* (A, B and C, respectively). Although *C. sordidus* and *S. salar* displayed different resting ventilation amplitudes and frequencies, both were increased significantly by similar relative magnitudes in response to similar increases in $PwCO_2$. *A. rostrata*, on the other hand, was unaffected by levels of environmental hypercarbia used in this study and demonstrated no ventilatory adjustment in during periods of acute increases in $PwCO_2$.

Figure 5-1. The effects of hypercarbia on arterial blood pressure (P_A) in two marine teleosts (Atlantic salmon, N= 7; Pacific sanddab, n = 7), three freshwater teleosts (rainbow trout, N = 9; brown bullhead, N = 8; American eel, N = 8), and one marine elasmobranch (spiny dogfish, N = 9). * denotes a statistically significant difference between normocarbia (filled bars) and maximal hypercarbia (unfilled bars) for each species via paired t-test ($P < 0.05$). The data for rainbow trout were reported previously in McKendry and Perry (2001).

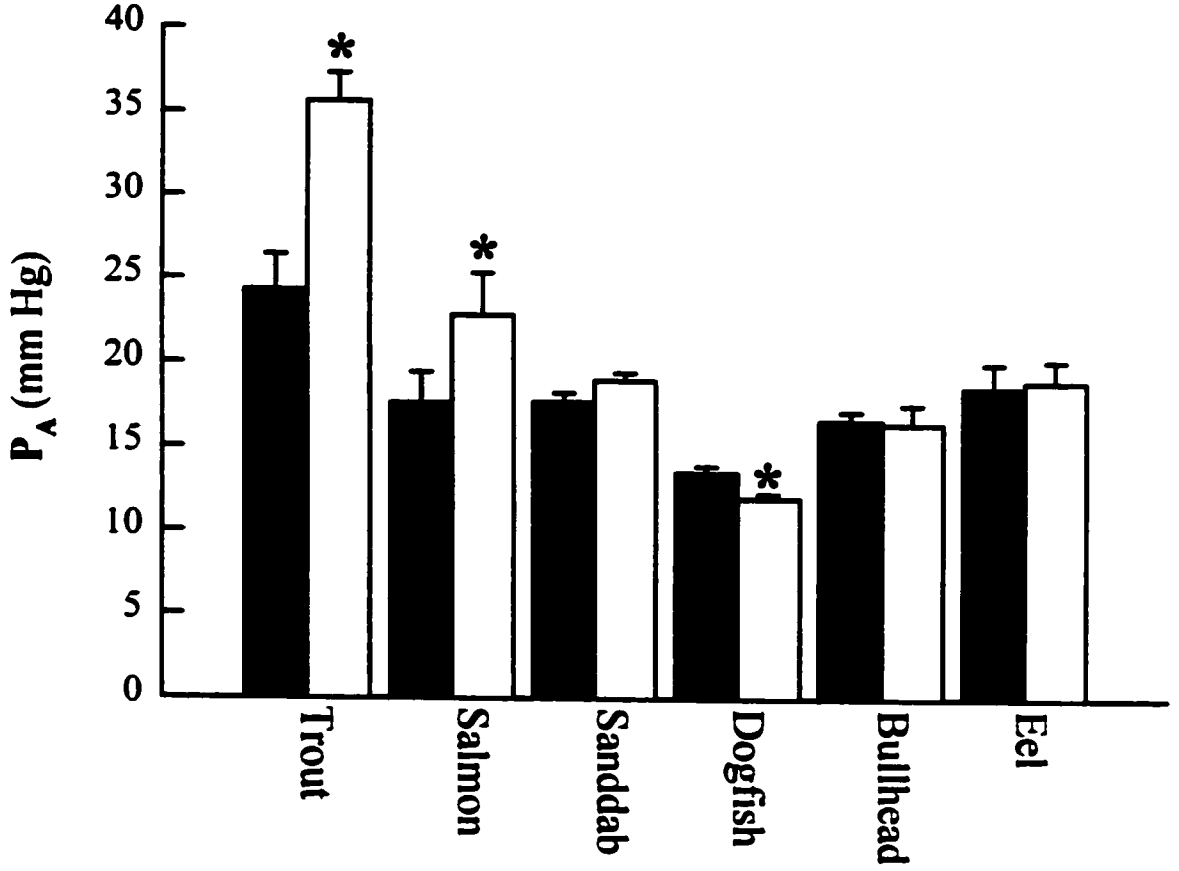
40
35
30
25
20
15
10
5
0
P_A (mm Hg)
*
*
*
Trout
Salmon
Sanddab
Dogfish
Bullhead
Eel

Figure 5-2. The effects of hypercarbia on systemic vascular resistance (R_S) in a marine teleost (Atlantic salmon, N = 6), three freshwater teleosts (rainbow trout, N = 9; brown bullhead; N = 8; American eel, N = 8), and a marine elasmobranch (spiny dogfish, N = 9). * denotes a statistically significant difference between normocarbia (filled bars) and maximal hypercarbia (open bars) for each species via paired t-test ($P<0.05$). The data for rainbow trout were reported previously in McKendry and Perry (2001).

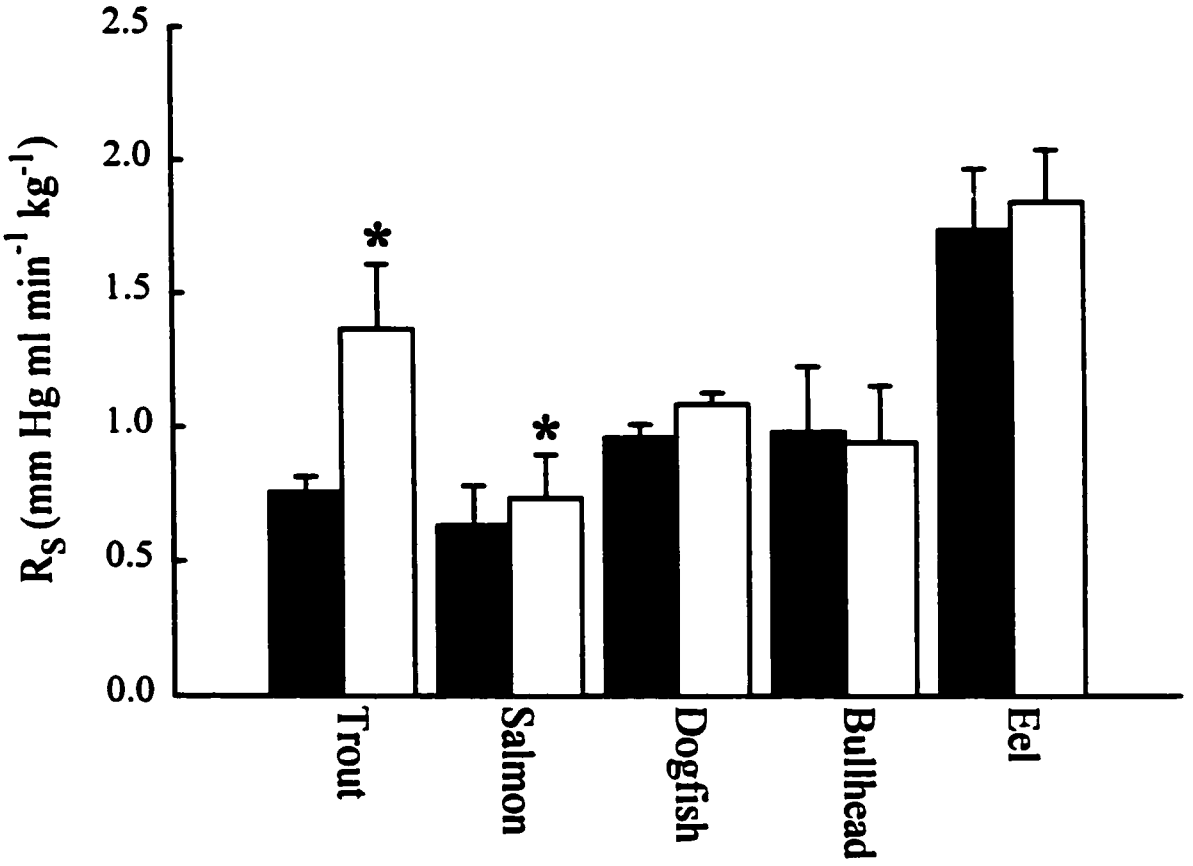
2.5
2.0
1.5
1.0
0.5
0.0
R_S (mm Hg ml min^{-1} kg^{-1})
*
*
Trout
Salmon
Dogfish
Bullhead
Eel

Figure 5-3. The effects of hypercarbia on heart rate (H_f) in two marine teleosts (Atlantic salmon, N = 10; Pacific sanddab, N = 7), three freshwater teleosts (rainbow trout, N = 8; brown bullhead, N = 8; American eel; N = 8), and a marine elasmobranch (spiny dogfish, N = 9). * denotes a statistically significant difference between normocarbia (filled bars) and maximal hypercarbia (unfilled bars) for each species via paired t-test($P < 0.05$). The data for rainbow trout were reported previously in McKendry and Perry (2001).

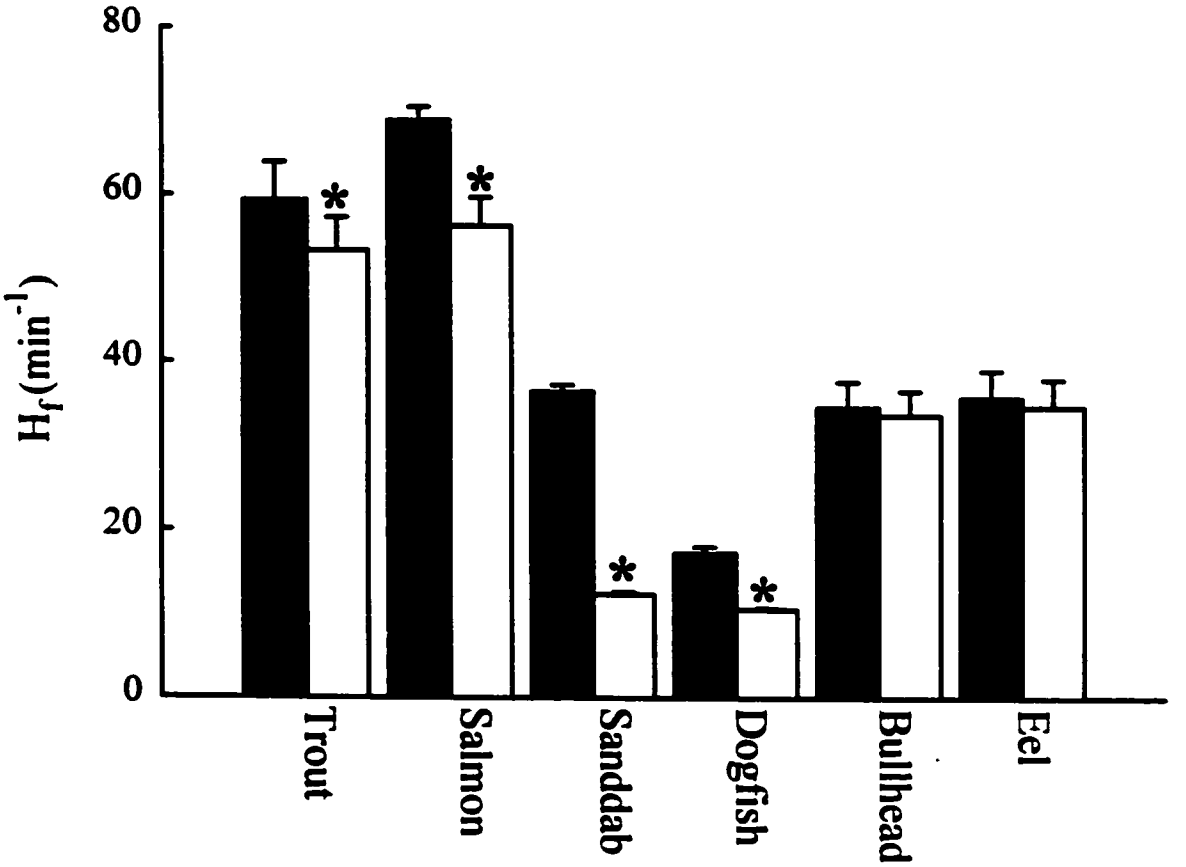
80
60
40
20
0
$H_f(min^{-1})$
*
*
*
*
Trout
Salmon
Sanddab
Dogfish
Bullhead
Eel

Figure 5-4. Representative blood pressure traces from the Pacific sanddab (A), rainbow trout (B), and brown bullhead (C) with highlighted segments from before, during and after the hypercarbic period (shaded in A).

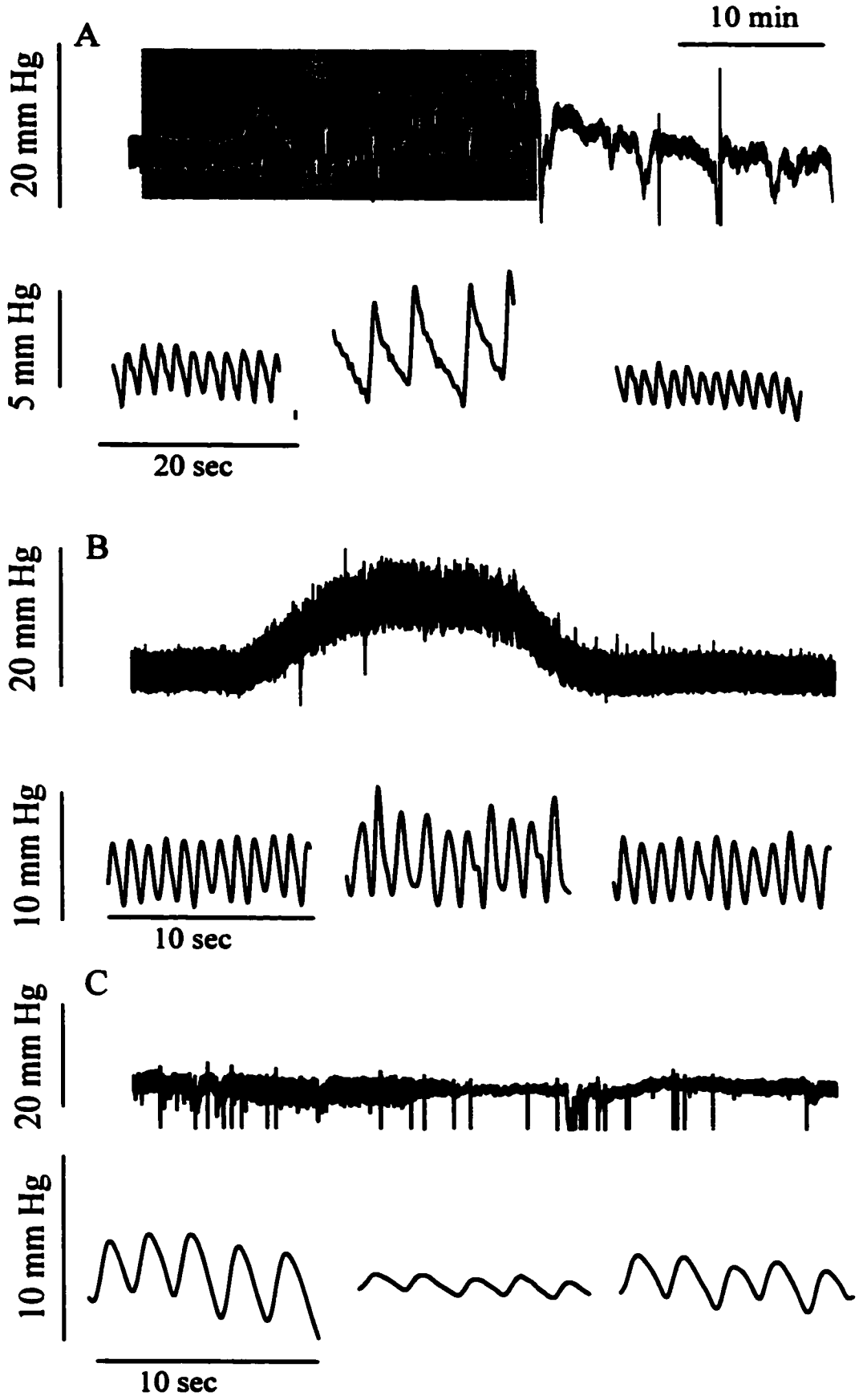
A
10 min
20 mm Hg
5 mm Hg
20 sec
B
20 mm Hg
10 mm Hg
10 sec
C
20 mm Hg
10 mm Hg
10 sec

Figure 5-5. The effects of hypercarbia on ventilation frequency (V_f) in two marine teleosts (Atlantic salmon, N = 11; Pacific sanddab, N = 10), two freshwater teleosts (brown bullhead, N = 8; American eel, N = 8), and a marine elasmobranch (spiny dogfish, N = 8). * denotes a statistically significant difference between normocarbia (filled bars) and maximal hypercarbia (unfilled bars) for each species ($P < 0.05$).

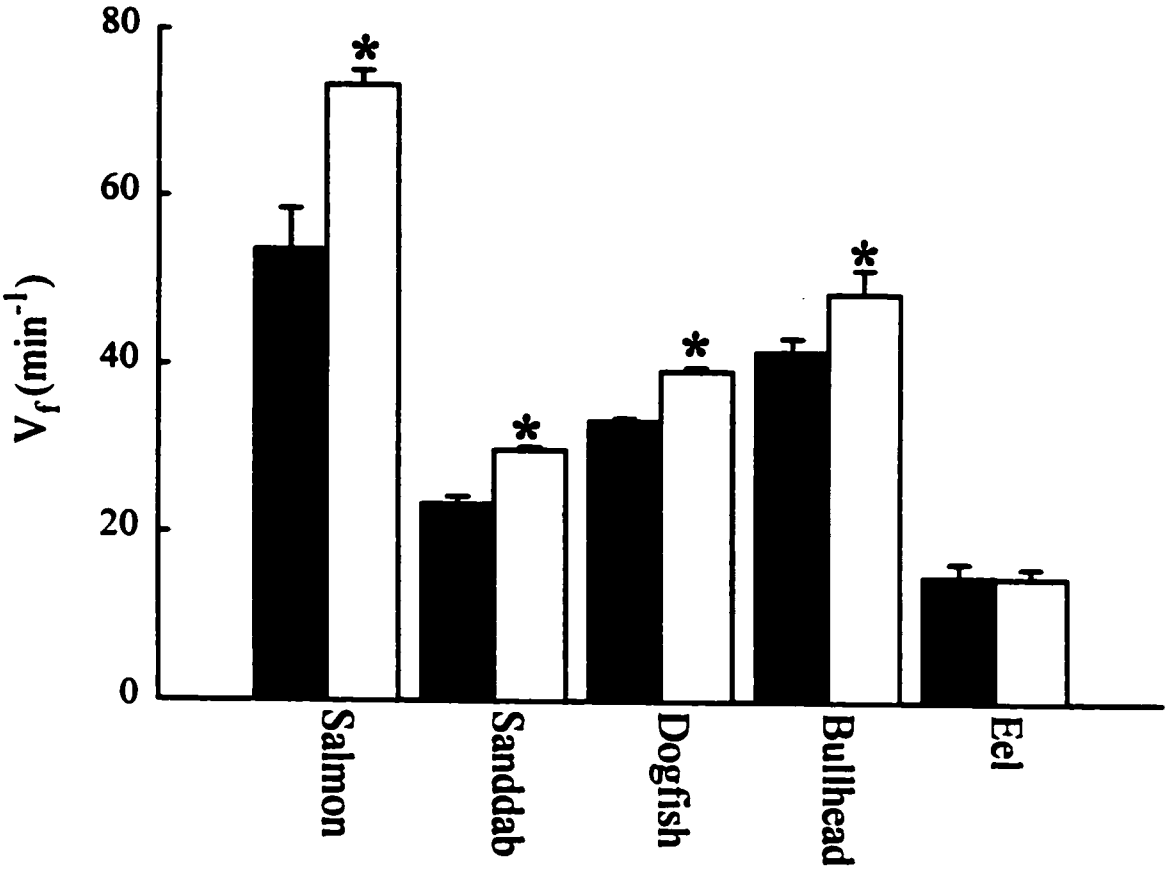
80
60
40
20
0
$V_f(min^{-1})$
*
*
*
*
Salmon
Sanddab
Dogfish
Bullhead
Eel

Figure 5-6. The effects of hypercarbia on ventilation amplitude (V_{AMP}) in two marine teleosts (Atlantic salmon, N = 11; Pacific sanddab, N = 10), two freshwater teleosts (brown bullhead, N = 7; American eel, N = 8), and a marine elasmobranch (spiny dogfish, N = 8). * denotes a statistically significant difference between normocarbia (filled bars) and maximal hypercarbia (unfilled bars) for each species ($P < 0.05$).

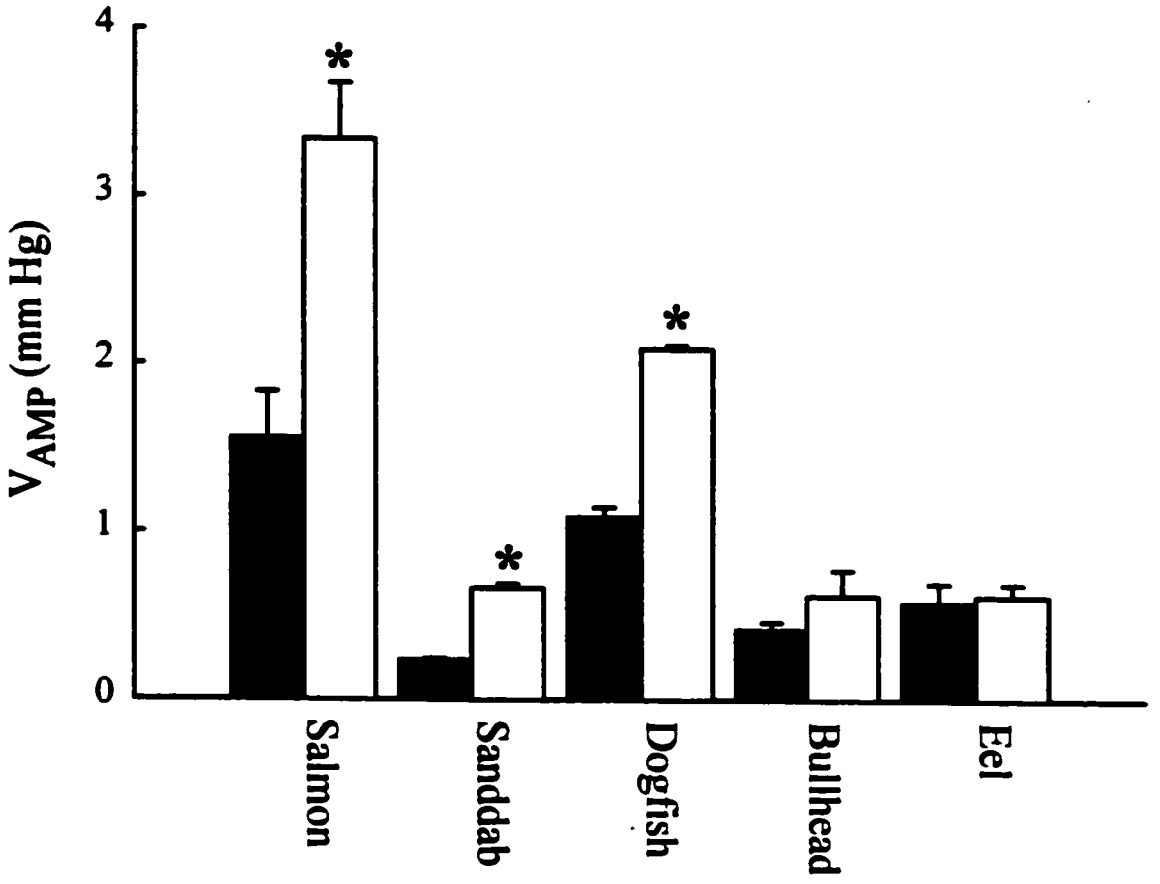
4
3
2
1
0
V_{AMP} (mm Hg)
*
*
*
Salmon
Sanddab
Dogfish
Bullhead
Eel

Figure 5-7. Representative ventilation traces from the Pacific sanddab (A), Atlantic salmon (B), and American eel (C) with highlighted segments from before, during and after the hypercarbic period (shaded boxes).

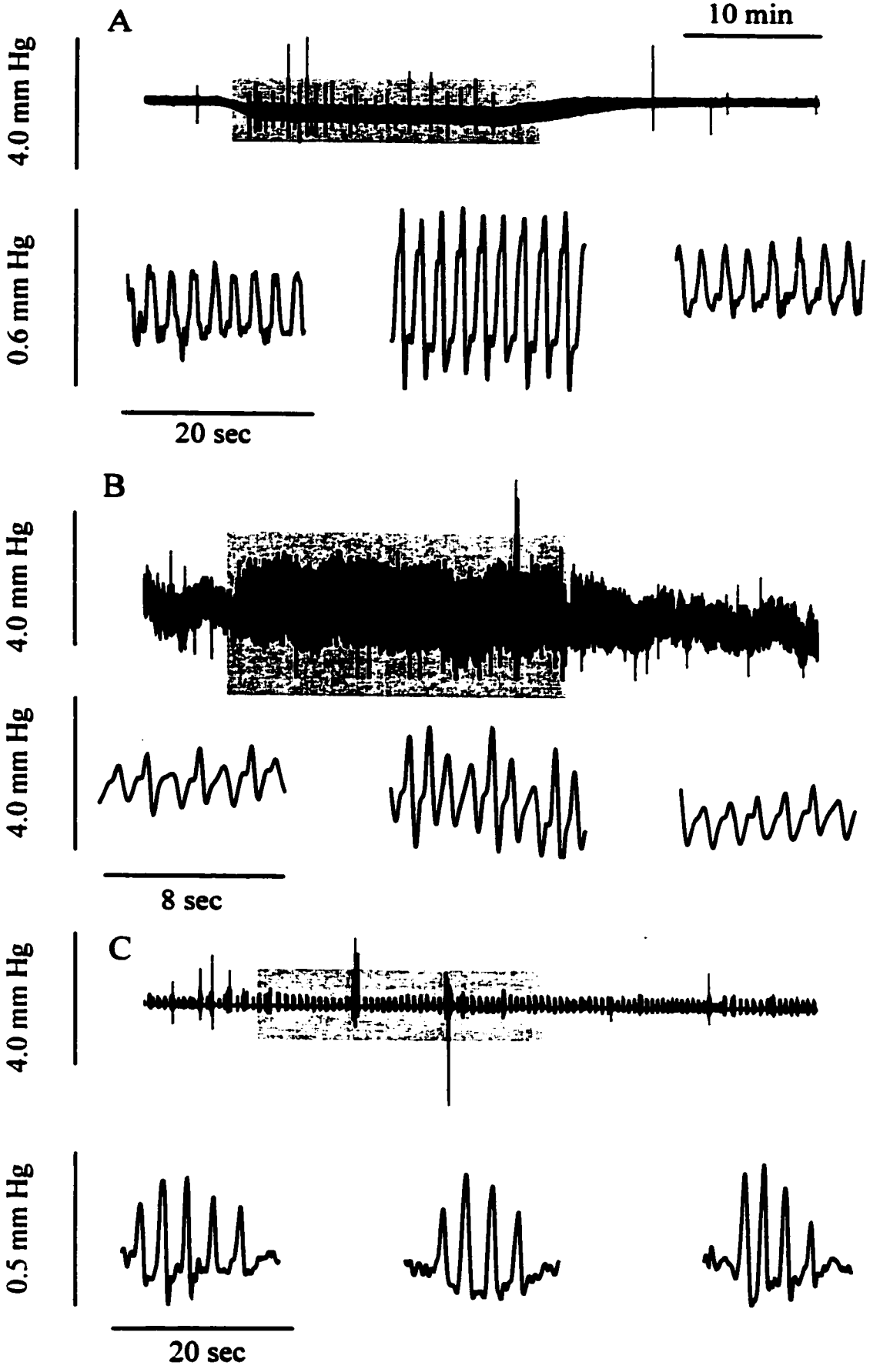
A
10 min
4.0 mm Hg
0.6 mm Hg
20 sec
B
4.0 mm Hg
4.0 mm Hg
8 sec
C
4.0 mm Hg
0.5 mm Hg
20 sec

Table 5-1. The effects hypercarbia on water gases in rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), Pacific sanddab (*Citharichthys sordidus*), brown bullhead (*Ictalurus nebulosus*), American eel (*Anguilla rostrata*), and Pacific spiny dogfish (*Squalus acanthias*). * denotes a statistically significant difference between 'PRE' (i.e. pre-hypercarbia) and 'MAX' (i.e. maximum hypercarbia) ($P < 0.05$); $N = 7 - 11$.

	$PwCO_2$ (mm Hg)		PwO_2 (mm Hg)	
	PRE	MAX	PRE	MAX
†Rainbow trout	0.6 ± 0.1	6.1 ± 0.3*	153.7 ± 2.1	152.5 ± 1.6
Atlantic salmon	0.6 ± 0.1	6.3 ± 0.4*	144.8 ± 3.9	149.0 ± 2.0
Pacific sanddab	0.8 ± 0.02	8.1 ± 0.2*	140.6 ± 0.7	145.6 ± 0.5*
Brown bullhead	1.3 ± 0.1	6.1 ± 0.4*	157.4 ± 2.7	158.0 ± 1.3
American eel	1.2 ± 0.1	6.3 ± 0.2*	161.9 ± 2.8	161.4 ± 1.9
Spiny dogfish	0.7 ± 0.1	6.4 ± 0.1*	146.6 ± 1.6	146.4 ± 0.9

†The data for rainbow trout were reported previously in McKendry and Perry (2001).

DISCUSSION

The present study demonstrates that there exists a large degree of variability among fish species with respect to their cardiovascular and ventilatory responses to environmental hypercarbia. Furthermore, this investigation raises a cautionary flag with respect to extrapolating results of future studies in piscine CO_2/H^+ chemoreception onto fish species other than the ones investigated.

The effects of hypercarbia on ventilation and blood acid-base balance are well documented in fish (e.g. Janssen and Randall, 1975; Thomas and Le Ruz, 1982; Thomas, 1983; Perry et al., 1987). Only recently, however, have the effects of hypercarbia on cardiovascular physiology been investigated in any teleost (Perry *et al.*, 1999; McKendry and Perry, 2001). These studies definitively showed an α-adrenoreceptor mediated, hypercarbia-induced systemic vasoconstriction *in vivo* (Perry *et al.*, 1999). Additionally, the hypertensive cardiovascular adjustments appear to be triggered by environmental hypercarbia and not the ensuing rise in internal CO_2 (McKendry and Perry, 2001). Previously, such hypercarbia-induced effects had been attributed to a hypoxemia arising indirectly from Root and Bohr effects. Together, these studies demonstrated that hypercarbia itself was responsible for increased blood pressure and systemic vascular resistance in rainbow trout, *Oncorhynchus mykiss*.

However, it remained unclear whether the cardio-respiratory adjustments observed in *O. mykiss* in response to environmental hypercarbia were representative of all fish species. Within other taxonomic groups there certainly exists a variety of responses to environmental hypercarbia. For example, burrowing mammals and birds have evolved

an elevated carbon dioxide threshold to allow for the long periods of time they spend in the CO_2-rich environment of their burrows (Birchard et al., 1984; Boggs et al., 1984). In order to facilitate future extrapolation onto other fish species, the basic cardio-respiratory response of hypercarbia was studied in five other species; two freshwater teleosts, two marine teleosts, and one marine elasmobranch.

Cardiovascular effects of environmental hypercarbia

The data in Table 5-1 and Figures 5-1 to 5-3 for *O. mykiss* (from McKendry and Perry, 2001) are included in this study for comparison, especially, with the related saltwater salmonid *S. salar*. Interestingly, both species exhibited an increase in P_A and R_S as well as a significant bradycardia during the hypercarbic period. The increases in P_A and R_S, in conjunction with the drop in H_f observed in both salmonid species concurs with the findings of Perry et al. (1999), in which similar changes were reported in *O. mykiss* in response to $PwCO_2 \approx 7.5$ mm Hg.

By far, the greatest hypercarbia-induced bradycardia was observed in *C. sordidus* (~67%). Although fitting these flatfish with a traditional cardiac flow-probe was not feasible, thereby negating the possibility of calculating R_S, it seems highly probable that this species also experienced a substantial increase in systemic vascular resistance because P_A was maintained despite the huge drop in H_f (indicative of decreased cardiac output). Although there was a compensatory increase in cardiac stroke volume, it is unlikely that this response could maintain cardiac output during such a severe bradycardia.

In contrast, *S. acanthias* was the only species studied which demonstrated a slight, but significant decrease in P_A during acute exposure to elevated $PwCO_2$. Kent and Peirce (1978) also observed a slight (8%) decrease in P_A in dogfish during exposure to much higher levels of $PwCO_2$ (~37.5 mm Hg). Although it is tempting to extrapolate and deem this hypotensive response representative of all elasmobranchs, more species would have to be studied over a wider range of $PwCO_2$ before such a conclusion could be drawn.

The variability in environmental hypercarbia-induced cardiovascular adjustments seems to mirror the cardiovascular changes in similar species subjected to hypoxia. Certainly, there exists sizeable variability in teleost responsiveness to hypoxia (Fritsche and Nilsson, 1993). For example, upon a rapidly induced hypoxia (PwO_2 = 30 - 40 mm Hg) three marine teleosts displayed very different responses (Fritsche, 1990). The shorthorn sculpin, *Myoxocephalus scorpius*, demonstrated the characteristic hypoxic bradycardia with no change in ventral aortic pressure (P_{VA}); the eel-pout, *Zoarces viviparus*, experienced a bradycardia with a decrease in P_{VA}; while the five bearded rockling, *Ciliata mustela*, developed no bradycardia but did increase P_{VA} significantly (Fritsche, 1990). Many teleost species are quick to develop a hypoxia-induced bradycardia, e.g. tench, *Tinca tinca* (Randall and Shelton, 1963); bluegill, *Lepomis macrochirus* (Marvin and Burton, 1973); spangled perch, *Leiopotherapon unicolor* (Gehrke and Fielder, 1988). However, other species require severe hypoxic conditions before responding with a significant bradycardia, eg. brown bullhead, *Ictalurus nebulosus* (Marvin and Burton, 1973); sea raven, *Hemitripterus americanus* (Saunders and Sutterlin, 1971). Similarly, the effect of hypoxia on arterial blood pressure has also been investigated in several fish species. Generally, during hypoxia, freshwater and marine

teleosts respond with an elevated blood pressure (Wood and Shelton, 1980; Fritsche and Nilsson, 1989). At least one exception has already been discovered; the intertidal rockpool fish, *G. cobitis*, reduces dorsal aortic pressure during hypoxia (Berschick, et al. 1987).

The similarities in variance of hypercarbia and hypoxia-induced cardiovascular adjustments apply to elasmobranchs as well as teleosts. In the present study, the elasmobranch, *S. acanthias*, was the only species that demonstrated a significant decrease in P_A during hypercarbia (see also Kent and Peirce, 1978). Interestingly, elasmobranchs have consistently shown a hypoxia-derived drop in blood pressure (Satchell, 1961; Butler and Taylor, 1971).

Clearly, the heterogeneity of the cardiovascular responses to environmental hypercarbia, as with hypoxia, is species-specific; likely determining not only the triggering threshold, but also the directionality of the response.

Respiratory effects of environmental hypercarbia

The ventilatory responses to environmental hypercarbia proved almost as diverse as the cardiovascular responses. Although the most common respiratory effect of environmental hypercarbia was an increase in ventilation amplitude (V_{AMP}) and frequency (Vf), as seen in *S. salar*, *S. acanthias* and *C. sordidus*, several other patterns of responses were observed. For example, at the level of environmental hypercarbia used in this study (6.1 ± 0.4 mm Hg), *I. nebulosus* displayed a significantly elevated Vf with no change in V_{AMP}. This result is consistent with the recent data obtained from the related channel catfish (*I. punctatus*) in which only Vf was increased during hyperoxic

hypercapnia (Burleson and Smatresk, 2000). In *A. rostrata*, V_{AMP} and Vf were both unaffected by hypercarbia. Perhaps coincidentally, *A. rostrata* was also the only episodic breather included in this study. Although the degree of episodicity varied between individuals, it may have contributed to their apparent high threshold for, or insensitivity to, increases in P_WCO_2. Previous studies on rainbow trout (Smith and Jones, 1982; Thomas and Le Ruz, 1982; Thomas *et al.*, 1983; Gilmour and Perry, 1994; Gilmour and Perry, 1996; Perry *et al.*, 1999) have demonstrated that an increase in V_{AMP} is the most consistent response to hypercarbia. The magnitude of the increases (12 – 317%), however, varied enormously, due, at least in part to differing $PwCO_2$ levels used in the various studies. Only half of the aforementioned studies observed a significant increase in ventilation frequency and the extent of these changes were minor (17 - 43%) in comparison to the changes in V_{AMP}. However, a change to V_{AMP} is not the predominant ventilatory response in all species responding to environmental hypercarbia. Recently, Sundin *et al.* (2000) observed a 45% increase in ventilation frequency in the Tambaqui, *C. macroponum*, during elevated $PwCO_2$, with no significant change in V_{AMP}. Similarly, in the present study, the brown bullhead demonstrated only a 17% increase in Vf with no significant change in V_{AMP} (see also Burleson and Smatresk, 2000). Even among species demonstrating a significant ventilatory adjustment during the hypercarbic period there are substantial differences in the magnitude of the response. For instance, *C. sordidus* ($PwCO_2$ max = 8.1 ± 0.2 mm Hg) displayed an increase in V_{AMP} of approximately 186%, while *S. acanthias* ($PwCO_2$ max = 6.4 ± 0.1 mm Hg) realized an increase of approximately 90%.

The apparent heterogeneity among species with respect to the variability in responsiveness to hypercarbia may be explained, at least in part, by a number of factors, such as: (i) lifestyle, i.e. metabolism/'aerobic-demand', (ii) differing CO_2/H^+ thresholds, and/or (iii) absence/inactivity of CO_2/H^+ chemoreceptive ability. Although a definitive explanation of the reasons for, or the physiological significance of, the variation in the hypercarbia-induced cardiorespiratory adjustments is beyond the scope of this paper, the presence and effectiveness of CO_2/H^+ chemosensory abilities in any given species are likely due to a combination of some or all of these factors.

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Chapter 6

GENERAL DISCUSSION

GENERAL DISCUSSION

Decades worth of previous studies indirectly concluded, almost without exception, that the cardioventilatory adjustments observed during periods of elevated ambient CO_2 were attributable to the impaired blood oxygen transport capability (Hughes and Shelton, 1962; Cameron, 1971; Eddy, 1971, 1973, Smith and Jones, 1982; Perry and Wood, 1989). It was believed that due to the significant Bohr and Root effects observed in the blood of most fish species during bouts of hypercapnia that acute exposure to elevated $PwCO_2$ elicited a large enough hypercarbia-induced hypoxaemia to explain, at least the increases in V_{amp} and V_f, and probably the cardiovascular adjustments as well. Although this theory was becoming increasingly questionable (Dejours, 1972, 1973; Heisler *et al.*, 1988; Perry and Wood, 1989; Wood *et al.*, 1990; Graham *et al.*, 1990; Kinkead and Perry, 1991; Perry and Gilmour, 1996), it remained the general consensus until Perry *et al.* (1999) were able to clearly differentiate between CO_2/H^+-specific effects and the hypercarbia-induced lowering of blood oxygen content during acutely elevated ambient CO_2.

Although Perry *et al.* (1999) observed a significant dose-dependent cardioventilatory response to step-wise increases in ambient CO_2 in rainbow trout (*O. mykiss*), these hypercarbic reflexes were not associated from any physiologically significant hypoxaemia. The investigation showed that the degree of hypercarbia used in the study did not render the blood of the trout hypoxaemic enough to evoke the observed bradycardia and systemic hypertension. This study represented the first definitive

separation of hypercarbia-specific cardioventilatory effects from those typically attributed to hypoxaemia. Although these findings went a long way towards dispelling the dogma that increases in ventilation, bradycardia as well as systemic hypertension were the result of hypercapnia-induced hypoxeamia, it opened the door to many new questions. For example, could the observed hypertension be explained, at least in part, by a direct effect of CO_2 on the systemic vasculature? Are the cardioventilatory adjustments mediated via increases in internal or external CO_2? Are the hypercarbia-induced effects a directly linked to the elevated CO_2 or the concomitant drops in pH? Is there a link between these putative CO_2/H^+ chemoreceptors and the autonomic nervous system, as indicated by Perry et al. (1999)? Finally, were the effects of hypercarbia, seen in rainbow trout, indicative of a universal piscine hypercarbic reflex?

Thus, the present study aimed to answer these questions and hopefully open similar doors to future investigations.

Internal versus external CO_2

In order to properly address the issue of internal versus external CO_2 (hypercapnia vs. hypercarbia) we began *in situ*, by investigating the effect of CO_2/H^+ directly on the systemic vasculature of rainbow trout in the absence of neuronal and humoral tone. As explained in chapter 2, the three levels of CO_2/H^+ examined in this study (0.6, 1.0 and 2.0%) did not elicit any vasoconstrictory effects in preparations devoid of neuronal or humoral tone. In fact, using adrenergically pre-contracted (10^{-4} M Adr) trunks, a clear vasodilatory effect of CO_2/H^+ was observed. It was determined that the net increase in systemic resistance that occurs during hypercarbia *in vivo* must reflect the opposing

influences of direct CO_2/H^+-induced vasodilation and adrenergic (neuronal and/or humoral) vasoconstriction (Perry *et al.*, 1999).

The results presented in chapter 2 confirm that *in vivo*, not only does hypercarbia induce significant increases in arterial blood pressure and systemic resistance in conjunction with a significant bradycardia in rainbow trout, but also that that the cardiovascular effects are attributable to hypercarbia, not internal hypercapnia. Both carbonic anhydrase inhibited fish and hyperoxia-exposed fish established new, stable elevated levels of arterial CO_2 ($PaCO_2$ increased from 1.9 ± 0.2 to 8.9 ± 0.7 mm Hg and from 3.2 ± 0.6 to 7.8 ± 1.1 mm Hg, respectively). In both cases, despite elevated levels of endogenous CO_2, there was no bradycardia or increase in P_{DA} or R_S. Clearly, these results support the involvement of externally directed receptors that likely reside within the gill (Burleson and Smatresk, 2000). The results of this investigation provided the strongest support to date for the involvement of external CO_2/H^+ chemoreceptors linking the cardiovascular and autonomic nervous systems.

CO_2 versus pH

Once it had been clearly established that hypercarbia, not hypercapnia, was responsible for triggering the characteristic cardioventilatory response in rainbow trout observed during periods of elevated ambient CO_2 (Perry et al., 1999; chap.2 - McKendry and Perry, 2001), we could then focus on differentiating between CO_2-specific effects and those attributable to changes in [H+]. Although the original intent was to perform these experiments, for comparisons sake, on both rainbow trout and Atlantic salmon, due

to time constraints only the salmon arm of the trial was completed and it is those results which are presented.

This study (chap. 3) is the first to directly investigate, in such a comparative manner, the relative importance of elevated ambient CO_2 versus the concomitant drop in pH observed during hypercarbia. Moreover, these findings definitively demonstrate, via external injections of treated seawater, that $PwCO_2$, not water pH (pHw), is the trigger for hypercarbia-induced cardioventilatory adjustments.

While 2% CO_2 induced a larger increase in ventilatory amplitude and frequency than seawater alone, 4% CO_2 elicited a significantly greater change in V_f, V_{amp}, H_f, V_b, and R_S than did aerated seawater. Neither aerated seawater of pH 7.0 or 6.3 caused any of the cardioventilatory variables to differ significantly from seawater (control) injections.

A recent study by Reid et al. (2000) which found that, with the exception of a decrease in heart rate, acid injections (internal and external) in the tambaqui (*H. malabaricus*) did not mimic the cardioventilatory responses observed during hypercarbia, suggesting that changes in $PwCO_2$ are more important than changes in pHw.

Neuronal Link

Using the Pacific spiny dogfish (*Squalus acanthias*) we investigated the putative link between the aforementioned externally oriented CO_2 branchial chemoreceptors and the autonomic nervous system, specifically, the effects of the muscarinic antagonist atropine on the hypercarbia-induced cardiorespiratory adjustments. Additionally, in *S. acanthias*, experiments were performed following branchial denervation, via surgical transection of the IXth cranial nerve (glossopharangeal) and the branchial branches of the

Xth (vagus). As discussed in chapter 4, the dogfish is a particularly useful model species to study aspects of CO_2-induced cardiorespiratory reflexes in fish due the absence of the confounding Root effect observed in most teleosts, thereby negating the need to prevent hypoxaemia in order to study the effects of external and/or internal changes in CO_2/H^+.

In the present study (chapter 4), dogfish displayed a bradycardia, reduced cardiac output and lowered arterial blood pressure, as well as significant increases in ventilation amplitude and frequency in response to acute hypercarbia.

The marked attenuation of the hypercarbic bradycardia and ensuing reduction in cardiac output and blood pressure in atropinised fish was not surprising because increased vagal tone is the key contributor to reflex bradycardia in dogfish (Butler and Metcalfe, 1988). However, the absence of any ventilatory responses to hypercarbia after atropine treatment suggests a critical involvement of muscarinic receptors in the chemosensory response of dogfish to CO_2. This finding is consistent with the results of earlier studies demonstrating that atropine blocks Ach- or cyanide-evoked increases in trout gill O_2 chemoreceptor activity *in situ* (Burleson and Milsom, 1995a) and that injection of muscarine markedly stimulates breathing in trout *in vivo* (Burleson and Milsom, 1995b). These findings suggest a crucial role for muscarinic receptors in the response of dogfish to both hypercarbia and hypoxia.

Denervation of all gill arches (via transection of cranial nerves IX and X) eliminated all cardiorespiratory responses to hypercarbia. These results clearly demonstrate the existence of branchial chemoreceptors in dogfish that are stimulated by elevated CO_2 and/or H^+ as well as providing evidence for the absence of central CO_2/H^+ chemoreceptors. Due to the scarcity of the data on cardiorespiratory responses to

hypercarbia in teleosts, it is not yet possible to formulate a general model for any fish group.

Other recent branchial dennervation studies have indicated the existence of separate populations of O_2 and CO_2 chemoreceptors, however distribution and mutual exclusivity of receptor populations vary widely among the species tested to date. For example, in the neotropical fish, the traira (*Hoplias malabaricus*), Reid *et al.* (2000) showed that the hypercarbia-induced increases in both breathing frequency and amplitude arose from receptors with a similar distribution that elicited the hypoxic ventilatory response. The traira also exhibited a bradycardia and mild hypotension during exposure to environmental hypercarbia (Reid *et al.*, 2000) and the receptors involved in mediating the hypoxic bradycardia were located exclusively on the first gill arch, while those responsible for the hypercarbic bradycardia were found in other gill arches as well. In addition, the same authors (Reid *et al.*, 2000) demonstrate that extra-branchial receptors may also be involved in controlling the increase in ventilation amplitude during hypercarbia.

It has also been recently suggested that tambaqui (*Colossoma macroponum*) may have separate populations of O_2-sensitive and CO_2-sensitive receptors (Sundin et al., 2000), although this is based on quantitative, not qualitative, evidence. However, it remains unclear as to whether there is a single population of branchial receptors that are responsive to lowered O_2 and elevated CO_2 or rather two distinct populations of CO_2 and O_2 chemoreceptors. In dogfish, there is evidence for and against a single population of receptors. In support of a single receptor population, the cardiorespiratory responses to hypercarbia (increased V_f, V_{AMP}, decreased H_f, P_A) are similar to the responses observed

during hypoxia. In opposition, and perhaps more importantly, external injections of cyanide (a potent activator of O_2 chemoreceptors), while eliciting bradycardia and hypotension, did not elicit the marked reduction in cardiac output that accompanies hypercarbia.

Homogeneity of the piscine hypercarbic reflex

Within other taxonomic groups there certainly exists a variety of responses to environmental hypercarbia. For example, burrowing mammals and birds have evolved an elevated carbon dioxide threshold to allow for the long periods of time they spend in the CO_2-rich environment of their burrows (Birchard et al., 1984; Boggs et al., 1984).

The final study presented in this thesis demonstrates that there exists a large degree of variability among fish species with respect to their CO_2 - chemoreceptive abilities and their cardiovascular and ventilatory responses to environmental hypercarbia. In fact, responsiveness to acute moderate hypercarbia varied from the American eel, which displayed no cardioventilatory adjustments, to the Pacific sanddab, which experienced, for example, a bradycardia of approximately 67% and an increase in V_{amp} of ~186%. Interestingly, the most dichotomous response was the effect of hypercarbia on blood pressure; dogfish experienced a significant decrease in P_A (~13%), while Atlantic salmon and rainbow trout increased (30 and 47 %, respectively). Clearly, the heterogeneity of the cardiovascular responses to environmental hypercarbia is species-specific; likely determining not only the triggering threshold, but also the directionality of the response.

Justification for species studied

Certainly rainbow trout have become the most commonly studied species in fish physiology largely due to their availability, cost, background/comparative knowledge base, surgical compliance, increasing aquacultural importance and our ability to successfully house this species on-site. Atlantic salmon were the next species selected, because of their taxonomic proximity to the freshwater rainbow trout, they offered representation by a closely related marine salmonid. The American eel and brown bullhead were selected in order to study freshwater teleosts that are not closely related to the rainbow trout and which lead a more sedentary lifestyle, not to mention their availability, relatively close to the University of Ottawa. The final teleost species, the Pacific sanddab, was selected because of its sedentary lifestyle, offering a point of comparison with the highly aerobic Atlantic salmon and their availability at Bamfield Marine Station. Finally, Pacific spiny dogfish were selected as the representative elasmobranch due to their availability on the West Coast of Canada and the existing sizeable database of studies surrounding this species.

Physiological significance

Although this thesis sought to characterize the nature of the CO_2 response and the mechanism by which these species sense changes in $PwCO_2$ the investigation stopped short of exploring the physiological significance of the hypercarbic reflex. Despite the apparent heterogeneity of the hypercarbia-induced cardioventilatory response, typically those species that do respond experience a significant bradycardia in conjunction with an increase in systemic resistance as well as a rise in ventilatory frequency and/or amplitude.

Interestingly, cardiac output seems to be maintained, presumably via a compensatory increase in stroke volume, as heart rate drops. This combination of cardiac adjustments would undoubtedly lead to increased pulsatility, which in turn would increase lamellar recruitment and residency time for blood in the gill, both of these effects would facilitate gas exchange during the apparent respiratory stress associated with hypercarbia. Furthermore, not only is it energetically beneficial to compensate for a drop heart rate with an increase in stroke volume but the increased residency time of blood in the heart allows the heart to consume more oxygen, thereby raising the diffusion gradients as the blood enters the gill.

The increase in systemic vascular resistance and the ensuing rise in arterial blood pressure also contribute to lamellar recruitment and distension. Since critical lamellar opening pressure is greater than critical closing pressure once the lamellae are being perfused they tend to stay 'open', barring any sudden drop in blood pressure. This situation certainly facilitates gas transfer. Additionally, increasing systemic resistance would lead to a slight thickening of the arteriole wall, since the vessel is contracted, and may hinder the rapid diffusion of CO_2 out of the blood stream and into the tissues.

The ventilatory response is perhaps the most easily understood since hyperventilation has been shown to lower $PaCO_2$ by approximately 0.5 mmHg. Even small fluctuations in $PaCO_2$ are known to contribute significantly to changes in pH and this ventilatory response may simply be a preventative measure, essentially safe-guarding the acid-base status of the blood.

Finally, because the partial pressure of CO_2 in water is so much lower than O_2, small changes in $PwCO_2$ are much more easily detected than small changes is PwO_2.

Since hypercarbia is nearly always associated with hypoxia, by monitoring small, more easily detectable, fluctuations in $PwCO_2$ the fish may simply be using this as an 'early warning system' for upcoming hypoxic conditions.

Of course, all of the aforementioned physiological explanations for the observed hypercarbic response remain speculative and further studies are required.

Future direction

The studies contained within this thesis have focused on cardioventilatory effects of acute hypercarbia, however it is unclear whether or not cardiorespiratory variables in these fish would eventually return to prehypercarbic levels despite continuously elevated ambient CO_2. Early studies suggest that the stimulating effects of low levels of hypercarbia are transient in most fish (Milsom, 1995). With chronic exposure to hypercarbia, ventilation volume increases initially but starts to decline after a few hours, returning to roughly prehypercarbic levels after about 24 h (Saunders, 1962; Janssen and Randall, 1975). How universal, both in magnitude and time course, is this acclimation to hypercarbia? Do all cardiovascular variables also return to baseline values? If so, could chronically-hypercarbic fish be returned to normocarbia in order to study the effect of acute hypocarbia? Perhaps these experiments should start by focusing on species that inhabit waters with unusually high PCO_2 since they may be more sensitive to hypocarbia. By simulating hypocarbia it would be possible to determine whether or not these externally oriented CO_2 chemoreceptors were tonically active. Does lowering the ambient level of CO_2 trigger a tachycardia and decreases in P_A and R_S?

Clearly, more species need to be examined. Currently there are no obvious trends among the fish species observed which would allow us to extrapolate the magnitude or directionality of the hypercarbia-induced cardioventilatory reflex onto other species. It has been suggested that studies of the distribution of cardioventilatory chemoreceptor populations (Sundin et al., 2000) as well as the magnitude and/or directionality of these hypercarbia-induced effects must be completed for a variety of species, adapted to different habitats. By increasing the number and diversity of species examined we may better be able to correlate the observed effects with factors such as evolution, phylogeny, metabolism, lifestyle, morphology, or habitat.

Lastly, immunohistochemistry might be useful in decisively differentiating between distinct populations of O_2 and CO_2 chemoreceptors, as well as better defining, at least anatomically, their neuronal links to the autonomic nervous system. In mammals, hypoxia and hypercapnia result in altered activity of respiratory neurons, although it is unclear which neuro-anatomical pathways and neurotransmitters are responsible for the processing of afferent input from different groups of chemoreceptors (Teppema et al., 1997). Much like the current situation with fish, in the rat exclusive localization and identification of specific CO_2 sensors has proven elusive, despite general agreement on the existence of superficial respiratory chemosensitive regions within specific postions of the brainstem (See review by Schlaefke, 1981). In mammals, a widely accepted tool used to map multisynaptic neuronal pathways is Fos immunohistochemistry (Sagar et al. 1988; Bullit, 1990). The expression of the proto-oncogene *c-fos* is considered to be a marker of activation of individual neurons after synaptic activation and Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Sheng and Greenberg, 1990; Morgan and Curran, 1986; Morgan

and Curran, 1991). The protein product of *c-fos*, the nuclear protein Fos, is rapidly and transiently induced, remains in the cell nucleus at elevated concentrations for hours, and can be detected immunohistochemically with antibodies. This technique has been used, with some success (Larnicol et al., 1994; Teppema et al., 1994, 1997), in mammals to differentiate between neuro-anatomical pathways associated with O_2 and CO_2 chemosensitivity. Perhaps a possibility in the not too distant future of fish physiology...

Conclusions

This series of investigations has confirmed that hypercarbia elicits a bradycardia along with a significant increase in P_A and R_S in rainbow trout (*O. mykiss*). Moreover, this thesis definitively demonstrates that the observed cardioventilatory adjustments are mediated via externally oriented receptors, that are more sensitive to changes in CO_2 than pH in salmon. In the Pacific spiny dogfish, denervation of all gill arches (via transection of cranial nerves IX and X) eliminated all cardiorespiratory responses to hypercarbia. Moreover, despite a significant attenuation of the hypercarbic bradycardia and ensuing reduction in cardiac output and blood pressure in atropinised dogfish, the absence of any ventilatory responses to hypercarbia after atropine treatment suggests a critical involvement of muscarinic receptors in the chemosensory response of *S. acanthias* to CO_2. Finally, due to the large degree of heterogeneity among species, with respect to the magnitude and directionality of their cardioventilatory hypercarbic response, many more species will need to be studied before any realistic attempt can be made to formulate model for any fish group.

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