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THE ROLE OF CIRCULATING CATECHOLAMINES IN THE REGULATION OF BREATHING IN TELEOSTS

BY

RICHARD KINKEAD

A THESIS SUBMITTED TO THE UNIVERSITY OF OTTAWA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

The observations that intra-arterial infusions of catecholamines can modify ventilation in the European eel (*Anguilla anguilla L.*), and that conditions which elicit hyperventilation are often associated with elevated plasma catecholamine levels, suggested that these hormones might play a role in the control of ventilation in fish. This thesis attempts to elucidate the role of epinephrine and/or norepinephrine in the regulation of ventilation in teleosts while also considering the effects of other potential ventilatory modulators such as blood respiratory/acid-base status and blood pressure.

Two basic approaches were utilized. First, the ventilatory responses to various external respiratory challenges were quantified and compared with those in fish pre-treated with adrenoceptor antagonists. Second, the effects of experimental elevation of circulating catecholamines on gill ventilation volume ($\dot{V}w$) were assessed. These experiments were performed under different environmental conditions that provoked a wide range of ventilatory responses and respiratory/acid-base disturbances, but that were not severe enough to stimulate mobilization of endogenous catecholamines.

It was observed that during hypoxia, pre-treatment of fish with either α- or β-adrenoceptor antagonists did not affect the ventilatory response of rainbow trout (*Oncorhyncus mykiss*) or Atlantic cod (*Gadus morhua*), regardless of the degree of hypoxia used and the corresponding effects on circulating catecholamine levels. Furthermore, since increases in $\dot{V}w$ were observed under mild hypoxia (partial pressure of O_2 in water (PwO_2) $\geq$ 72 Torr) or hypercapnia (partial pressure of CO_2 in water ($PwCO_2$) $\leq$ 4.5 Torr) it is concluded that elevation of circulating catecholamines is not a prerequisite for hyperventilatory responses to these stimuli.

Pre-treatment of trout with a β-adrenoceptor antagonist (propranolol) prior to exposure to external hypercapnia ($PwCO_2$ = 5.5 ± 0.2 Torr) prevented a sustained hyperventilation despite the absence of significantly elevated catecholamines in the circulation. This revealed that catecholamines of non-humoral origin are involved in the hyperventilatory response to hypercapnia.

It is unlikely, at least in trout, that catecholamines play a stimulatory role in the regulation of ventilation. This statement is derived from the observation that intra-arterial infusion of physiologically relevant doses of catecholamines induced transient depressions of $\dot{V}w$ regardless of the presence or absence of external ventilatory stimulants. The effects were unrelated to the concomitant changes in blood pressure.

Monitoring of blood respiratory and acid-base parameters suggested that respiratory acidosis may play a role in the control of ventilation in this species since external hypercapnia prevented the hypoventilatory response normally associated with hyperoxia. Furthermore, the rapid variations of $\dot{V}w$ observed during elevations or small depressions of PwO_2 suggested that the corresponding ventilatory responses were initiated by PwO_2 directly or by PaO_2 which were the only respiratory/acid-base variables altered under these conditions.

ABREGE

Les observations que l'infusion intra-artérielle de catécholamines peut modifier la ventilation chez l'anguille européenne (*Anguilla anguilla*) et que les conditions qui suscitent l'hyperventilation sont souvent associées à des niveaux élevés de catécholamines plasmiques, ont suggéré que ces hormones pourraient jouer un rôle dans le contrôle de la ventilation chez les poissons. Cette Thèse a tenté d'éclaircir le rôle de l'épinephrine et/ou de la norépinephrine dans la régulation de la ventilation chez les téléostéens tout en considérant les effets d'autres modulateurs potentiels de la ventilation tels que le statut acido-basique/respiratoire sanguin et la pression sanguine.

Deux approches de base furent utilisées. Premièrement, les réponses ventilatoires à des défis respiratoires externes ont été quantifiées et comparées à celles de poissons pré-traités avec des antagonistes d'adrénocepteurs. Deuxièmement, les effets d'élévations expérimentales des catécholamines circulantes sur le volume de ventilation branchial ($\dot{V}w$) furent évalués. Ces expériences ont été effectuées sous différentes conditions environnementales créant ainsi un large éventail de réponses ventilatoires et de perturbations respiratoires/acido-basiques, qui n'étaient cependant pas suffisamment sévères pour stimuler la mobilisation des catécholamines endogènes.

Il a été observé que pendant l'hypoxie, le pré-traitement des poissons avec soit un antagoniste d'adrénocepteurs α ou β n'a pas affecté la réponse ventilatoire de la truite arc-en-ciel (*Oncorhyncus mykiss*) ou de la morue de l'Atlantique (*Gadus morhua*) et ce, indépendamment du degré d'hypoxie utilisé (pression partielle d'O_2 dans l'eau (PwO_2) $\geq$ 72 Torr) ou d'hypercapnie (pression partielle de CO_2 dans l'eau ($PwCO_2$ $\leq$ 4.5 Torr). Il est conclu que l'élévation des catécholamines circulantes n'est pas un prérequis à la réponse

hyperventilatoire à ces stimuli.

Le pré-traitement de la truite avec un antagoniste d'adrénocepteur β (propranolol) préalablement à l'exposition à l'hypercapnie externe ($PwCO_2$ = 5.5 ± 0.2 Torr) a empêché une hyperventilation maintenue malgré l'absence d'une élévation significative des catécholamines dans la circulation. Ceci a révélé que les catécholamines d'origine non-humorale sont impliquées dans la réponse ventilatoire à l'hypercapnie.

Il est peu probable, du moins chez la truite, que les catécholamines agissent comme stimulant dans la régulation de la ventilation. Cette affirmation dérive de l'observation que l'infusion intra-artérielle de doses physiologiquement appropriées de catécholamines a provoqué des dépressions transitoires du $\dot{V}w$ et ce, indépendamment de la présence ou l'absence de stimulants ventilatoires externes. Ces effets n'étaient pas reliés aux fluctuations concomitantes de pression sanguine.

Les mesures des paramètres respiratoires et acido-basiques suggèrent que l'acidose respiratoire peut jouer un rôle dans le contrôle de la ventilation chez cette espèce puisque l'hypercapnie externe a empêché la réponse hypoventilatoire normalement associée à l'hyperoxie. De plus, les variations rapides du $\dot{V}w$ observées lors d'élévations ou dépressions de la PwO_2 ont suggéré que les réponses ventilatoires correspondantes étaient initiées par la PwO_2 directement ou par la PaO_2 qui étaient les seules variables respiratoires/acido-basiques modifiées sous ces conditions.

ABBREVIATIONS

C_{CO2}	Total carbon dioxide content
C_{O2}	Total oxygen content
CO_2	Carbon dioxide
EPI	Epinephrine
f_V	Ventilation frequency
Hct	Haematocrit
Hb	Haemoglobin
$\dot{M}CO_2$	Carbon dioxide excretion
$\dot{M}O_2$	Oxygen uptake
NE	Norepinephrine
O_2	Oxygen
P_{da}	Dorsal aortic blood pressure
pHa	Arterial blood pH
P_{va}	Ventral aortic blood pressure
PO_2	Partial pressure of oxygen
PCO_2	Partial pressure of carbon dioxide
RBC	Red blood cell
V_{sv}	Ventilation stroke volume
$\dot{V}w$	Gill ventilation volume

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CHAPTER 1

GENERAL INTRODUCTION

The environment

Depending on whether they live in an aquatic or terrestrial environment, animals have developed and exploited particular aptitudes with respect to breathing. This fundamental physiological function, which will simply be defined as the extraction of oxygen from the surrounding environment along with the excretion of carbon dioxide, is performed using strategies that have been shaped by the physical and chemical properties of the environment.

At a given altitude, the gaseous composition of air is remarkable for its homogeneity throughout the terrestrial environment. This contrasts with the aquatic milieu which can experience important daily fluctuations. It is known that ambient factors such as temperature, salinity and carbonation, affect the capacitances of O_2 and CO_2 in water. Furthermore, respiration of aquatic organisms may have an enormous influence in determining O_2 and CO_2 concentrations of water because the diffusion coefficients of these gases in water are low (approximately 8500 times less than air) and the convection currents are less in water than in air.

For example, the PwO_2 of air equilibrated water at sea level is ~155 Torr; but the O_2 consumption by living organisms can cause PwO_2 to drop almost to zero. On the other hand, O_2 production by photosynthesis may raise PwO_2 to values well over 300 Torr (Dejours, 1973), while $PwCO_2$ can fluctuate daily between 0.2 and 3.0 Torr in intertidal rock pools and even reach values of 40 Torr in tropical ponds (Truchot, 1987). Since adequate gas transfer is an important physiological function, this indicates that water breathing animals must be able to detect and adjust to such changes within their environment. The present thesis is focused on the modifications of water convection over the gill, or gill ventilation volume ($\dot{V}w$), which is only one mechanism by which fish can maintain efficient gas transfer in the face of

environmental fluctuations.

Particularities of water breathers

The respiratory system of fish is characterized by its counter-current organisation such that the blood in the gill flows in a direction opposite to the unidirectional and continuous movement of water. Consequently $\dot{M}O_2$, along with $\dot{M}CO_2$, will be accomplished effectively as long as fish ventilate at rates that will meet the water convection requirements to maintain a partial pressure gradient sufficient to facilitate gas diffusion from water to blood or vice versa. An important characteristic of water is its capacitance for O_2 which is approximately 30 times less than air. Thus, O_2 is more likely to be a limiting factor for water breathers. It is understandable, therefore, that oxygen is a key factor around which all ventilatory adjustments should be accomplished in fish.

This is precisely what the work of Smith and Jones (1982) confirmed. Their study suggested that many of the conditions acknowledged as ventilatory stimulants (e.g. hypoxia, anaemia or hypercapnia) directly or indirectly depress the oxygen status of blood, the primary intermediate in the gas exchange function. More precisely, Ca_{O2} was thought to be the common factor that influences $\dot{V}w$ under these experimental conditions. That study, as well as others, reinforce the consensus that unlike mammalian ventilation, which is mainly driven by the blood acid-base status, teleosts adjust ventilation according to their internal oxygen status (see reviews by Jones and Milsom, 1982; Shelton *et al.* 1986).

In teleosts, the structures responsible for the detection of O_2 fluctuations, termed chemoreceptors, were initially localized on the first pair of branchial arches (Daxboeck and Holeton, 1978; Smith and Jones, 1978), regions considered to be the phylogenetic precursor

of the mammalian carotid and aortic bodies (Heymans and Neils, 1958). Until recently however, the link between the stimulus and the ventilatory response was obscure. It was shown that in the Channel catfish (*Ictalurus punctatus*) internal O_2-sensitive chemoreceptors, which are thought to be located in or just downstream from the gills, will reflexively increase $\dot{V}w$ when they are stimulated by hypoxemia (Burleson and Smatresk, 1990).

Factors influencing blood respiratory status

It is only when Ca_{O2} is depressed to a critical value that hormonal factors will come into play to help $\dot{V}w$ compensate for this detrimental condition. Figure 1 presents a model which summarizes the series of physiological events that may contribute to the regulation of Ca_{O2}. Indeed, it has been shown that hypoxemia, or more precisely depression of Ca_{O2}, is the proximate stimulus for catecholamine (norepinephrine and/or epinephrine) mobilisation from the chromaffin tissue of the head kidney (Perry *et al.* 1989) an organ homologous to the mammalian adrenal medulla. Once released into the blood stream, catecholamines will stimulate a variety of tissues within the organism and will exert their action to alleviate the initial stimulus.

The blood pressure increase mediated by catecholamines will promote lamellar recruitment; i.e., perfusion of the more distal gill lamellae. This will cause a more uniform blood distribution within the lamellae as well as increasing the diffusive conductance for gas transfer (Gdiff) (for review, see Randall and Daxboeck, 1984). All these factors will tend to facilitate gas transfer by increasing the functional surface area and thus, increase PaO_2. If the fish, however, is simultaneously experiencing environmental or metabolic disturbances that promote acidemia, the previously described strategies deployed to enhance gas transfer will

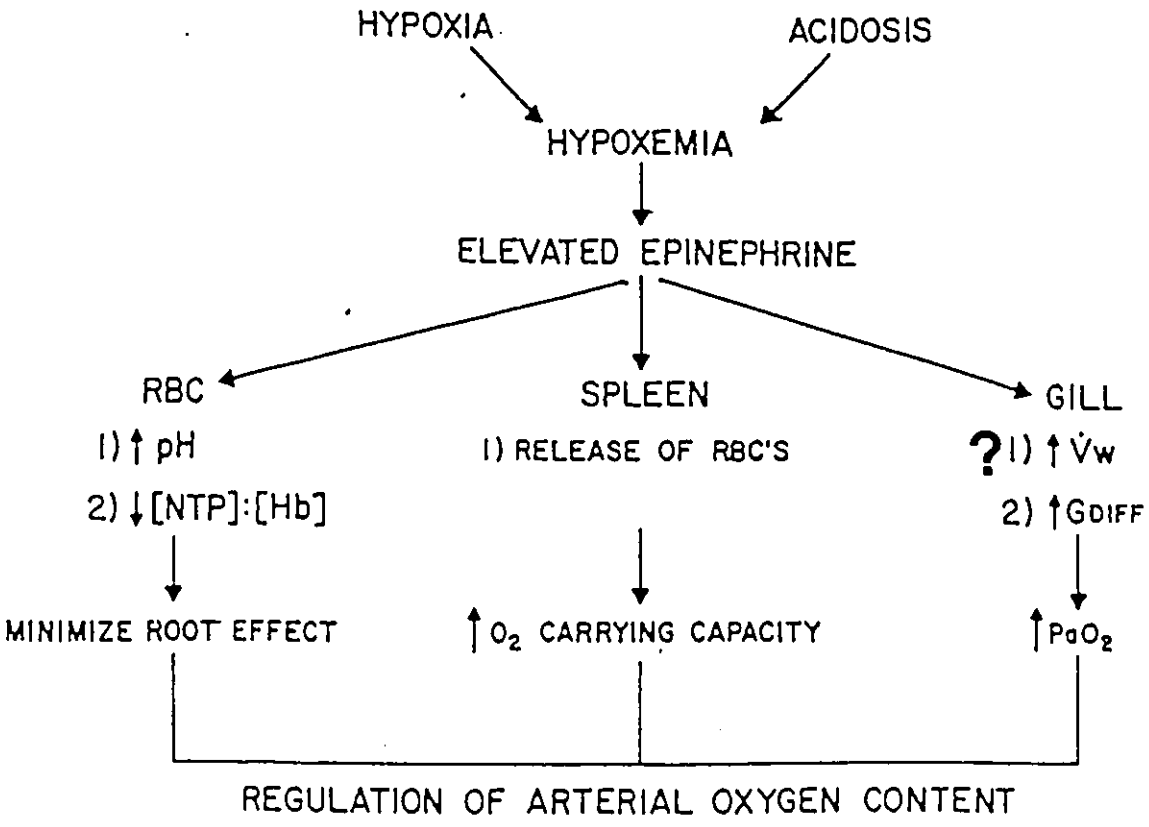
not be effective unless RBC pH is regulated.

Indeed, acidification of the RBC impairs the binding affinity of Hb for O_2, a phenomenon termed the Bohr effect. Oxygen binding will therefore be impaired, causing PaO_2 to remain elevated although Hb is not O_2-saturated. This will consequently affect O_2 uptake at the gill due to the reduced partial pressure gradient. In addition, when Hb is contained within a low pH environment, its total capacity for binding O_2 is diminished, further contributing to the hypoxemia. This phenomenon is known as the Root effect (Root, 1931).

Many fish species are capable of preferentially regulating RBC intracellular pH (pHi). The results of *in vivo* as well as *in vitro* experiments revealed that RBC pHi regulation involves a Na^+/H^+ antiporter which is activated upon catecholamine binding with β-adrenoceptors located on the membrane of the erythrocytes (for review, see Nikinmaa and Tufts, 1989). Activation of the Na^+/H^+ antiporter will cause an increase of intracellular osmolarity which will passively instigate swelling of the RBC. This phenomenon will be beneficial to the O_2-carrying capacity owing to dilution of the intracellular concentrations of nucleoside triphosphates (NTP) which are known to be negative allosteric modifiers of O_2 Hb affinity (Wood *et al.* 1975).

The importance of this adrenergic mechanism in the regulation of Ca_{O2} was confirmed experimentally by Vermette and Perry (1988*a*). These workers demonstrated that rainbow trout (*Oncorhyncus mykiss*) pre-treated with the β-adrenoceptor antagonist, propranolol, displayed a significant reduction of CaO_2 during acute hypercapnia.

Figure 1. Model summarizing the series of physiological events regulating arterial O_2 content after mobilization of epinephrine and norepinephrine into the blood stream. ? indicates the question that is addressed in the present study. Gdiff: Gill diffusive conductance for gas; [NTP]: nucleoside triphosphate concentration. This figure is adapted from Perry and Wood (1989).


HYPOXIA
ACIDOSIS
HYPOXEMIA
ELEVATED EPINEPHRINE
RBC
1) ↑ pH
2) ↓[NTP]:[Hb]
MINIMIZE ROOT EFFECT
SPLEEN
1) RELEASE OF RBC'S
↑ O_2 CARRYING CAPACITY
GILL
? 1) ↑ $\dot{V}_W$
2) ↑ G_{DIFF}
↑ P_aO_2
REGULATION OF ARTERIAL OXYGEN CONTENT

Another important adrenergic response that contributes to the compensation of hypoxemia by increasing the O_2-carrying capacity of the blood is the recruitment of RBC's from the spleen. *In vitro* studies have showed that α-adrenergic stimulation can cause contraction of the spleen (Nilsson and Grove, 1974) and thus elevate the Hct. These observations have been confirmed by the results of *in vivo* studies which demonstrated that after exhaustive exercise or hypoxia (Yamamoto *et al.* 1985), or catecholamine infusion (Vermette and Perry, 1988*b*; Perry and Kinkead, 1989), blood Hb concentration was elevated. The fact that stimulation of α-adrenoceptors is an important mechanism in the spleen response to hypoxemia was confirmed by the abolishment of Ca_{O2} regulation *via* increase in Hct and blood Hb concentration by pre-treating fish with the α-adrenoceptor antagonist phentolamine. Furthermore, the demonstration that adrenergic stimulation cannot induce an increase in blood Hb concentration in splenectomized trout led Perry and Kinkead (1989) to conclude that contraction of the spleen is the dominant response causing arterial blood O_2-carrying capacity to increase during surges of plasma catecholamines.

Putative ventilatory modulators

In view of more recent studies, it is possible that the rationale which suggests that variation of Ca_{O2} is the predominant factor affecting $\dot{V}w$ omits several important aspects of the physiology of the animal to be an adequate "model" for the control of ventilation. There follows a brief description of other factors that might be important modulators in piscine ventilation, along with the reason(s) why they should be considered as such.

The identity and the location of the receptors involved in the O_2 drive for ventilation are presently the subjects of intense investigation. On the other hand, the CO_2/H^+ sensing

structures involved in the control of breathing in fish have received little attention. The results of the investigations that have argued in favour of their existence as well as a role for CO_2/H^+ in the regulation of $\dot{V}w$ are discussed below.

The hyperventilatory response to moderate hypercapnia (below 9 Torr) generally has been attributed to the acidosis-induced hypoxemia (*via* the Bohr and Root effects), rather than depression of whole blood pH, or to elevation of $PaCO_2$, *per se* (Smith and Jones, 1982; for reviews see Jones and Milsom, 1982; Shelton *et al.* 1986; Perry and Wood, 1989). Recent work performed on elasmobranchs, however, demonstrated that during conditions of reduced oxygen-related respiratory drive (hyperoxia), variations of acid-base parameters are important modifiers of $\dot{V}w$ (*Scyliorhynus stellaris*; Heisler *et al.* 1988). Similar conclusions were drawn from experiments performed on the Atlantic big skate (*Raja ocellata*). When exposed to moderate hypercapnia, a condition that did not affect the blood O_2 status of these fish, a hyperventilatory response that appeared to be linked to the respiratory acidosis was observed (Wood *et al.* 1990). These authors proposed that a structure sensitive to arterial pH variations, rather than $PaCO_2$, could likely account for such responses. It is possible therefore, that in trout, the relationship between blood acid-base status and $\dot{V}w$ has been underestimated.

In mammals, it is believed that chemoreceptors and baroreceptors act in concert to achieve homeostasis (Heistad *et al.* 1975; for review see Daly, 1986). Despite the fact that these two types of receptors interact to regulate heart rate in rainbow trout (*Oncorhyncus mykiss*) (Wood and Shelton, 1980), the possibility of a relationship between the respiratory and cardiovascular systems in fish has received little attention. The only study which has addressed this question was performed on the European eel (*Anguilla anguilla*) in which mechanical increases in blood pressure by 50 to 100% caused hyperventilation (Soulier *et al.*

1988); this in contrast to the well-established inverse relationship between blood pressure and ventilation reported for mammals. Thus, these observations suggest that the effect of hypertension on the ventilatory responses to diverse stimuli might have been previously underestimated.

Involvement of catecholamines in the control of breathing in teleosts

As was discussed earlier, depression of Ca_{O2} is a known stimulus for catecholamine release in rainbow trout (Perry *et al.* 1989). Conditions which provoke increases in $\dot{V}w$ are often associated with the release of catecholamines into the circulation (e.g. hypoxia; Boutilier *et al.* 1988; anaemia; Iwama *et al.* 1987; hypercapnia; Perry *et al.* 1989; Perry and Kinkead, 1989; exercise; Milligan and Wood, 1987; intra-vascular acid-infusion; Aota *et al.* 1990). This "coincidence", combined with the knowledge that i) NE can modify the response of peripheral O_2-sensitive chemoreceptors (O'Reagan and Majcherczyk, 1982; Milsom and Sadig, 1983) and ii) circulating catecholamines are powerful ventilatory stimulants in the eel (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980), led to the supposition that a direct relationship exists between hyperventilation and elevated circulating catecholamine levels.

The role of circulating catecholamines in the regulation of breathing in fish has been a topic of extensive debate and investigation in recent years (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980; Aota *et al.* 1990; Playle *et al.* 1990). These discussions can be summarized by describing the different premises that are now prevailing.

I. Based on the observation that i) intra-arterial infusion of selective adrenoceptor agonists can induce an increase or a decrease of opercular movement depending on the seasonal dominance of the adrenoceptors (β- in summer = hyperventilation; α- in winter =

hypoventilation) and that ii) catecholamines can easily cross the blood-brain barrier in eel (Peyraud-Waitzenegger *et al.* 1979), Peyraud-Waitzenegger *et al.* (1980) postulated that circulating catecholamines could modulate $\dot{V}w$ by directly affecting the respiratory control centres in the central nervous system. Nevertheless, the fact that the eel can hyperventilate during hypoxia without releasing these amines into the blood stream lessens their apparent physiological importance in the control of $\dot{V}w$ (Peyraud-Waitzenegger and Soulier, 1989)

II. Aota *et al.* (1990) suggested that the stimulation of β-adrenoceptors by circulating catecholamines during hypoxia is required to maintain stimulation of breathing when the afferent signal from chemoreceptors becomes fainter during severe hypoxic conditions (M. Burleson, personal communication). This hypothesis arose from the observation that the β-adrenoceptor antagonist, propranolol, prevented the hyperventilatory response to severe hypoxia (24-60 Torr), a condition that promoted a metabolic acidosis along with an increase in plasma catecholamine concentration.

III. In addition, Aota *et al.* (1990) reported that propranolol abolished the hyperventilation, otherwise encountered, during intra-arterial acid infusion. This led these authors to conclude that catecholamines play a role in the reflexive response to elevated CO_2/H^+ in the blood, possibly by interacting with the putative CO_2/H^+ receptor that they report as being situated in the central nervous system. On the other hand, it has been postulated that catecholamines can stimulate $\dot{V}w$ indirectly by promoting retention of CO_2 (Wood and Perry, 1985), owing to inhibition of HCO_3^- dehydration through the RBC (for review see Perry and Wood, 1989).

There exists, however, one study that does not support a stimulatory role for catecholamines on $\dot{V}w$ (Playle *et al.* 1990). Indeed, these authors reported that intra-arterial infusion of catecholamines in normoxic trout caused a transient hypoventilation. Thus, as far

as a potential stimulatory role of these hormones in the regulation of breathing in fish is concerned, no clear conclusion can be drawn as yet and this area of physiology certainly warrants further research.

Hypothesis

It was with this knowledge in mind that the working hypothesis of the present thesis was formulated:

Circulating catecholamines play a stimulatory role in the regulation of ventilation in teleosts.

It must be emphasized that, because of the controversy surrounding this particular topic along with the disbelief of certain researchers that ventilatory responses could be solely mediated by humoral mechanisms (Perry and Wood, 1989), the hypothesis can be formulated in a negative or affirmative fashion, depending on the school of thought that is favoured. Nevertheless, the primary objective of this research was to investigate the role of these amines in the ventilatory responses of fish exposed to different environmental stimuli.

Experimental approach

This work was performed using two different strategies that can summarily be described as follows. First, the ventilatory responses of fish to different environmental stimuli were quantified. These responses were then compared, in terms of magnitude and pattern, to those measured in other groups of fish that were pre-treated with pharmacological agents

to selectively prevent the interaction of endogenous catecholamines with adrenoceptors.

The second approach was to stimulate the potential loci and/or variables previously described as being putatively involved in the ventilatory responses. The endogenous levels of catecholamines were experimentally elevated by means of intra-arterial infusion of EPI, NE or both, under a variety of external conditions while $\dot{V}w$ was monitored. In addition, many variables that were previously described as potential ventilatory modulators, namely blood acid-base/respiratory status as well as blood pressure, were quantified in order to assess their relative importance in the regulation of breathing in fish.

CHAPTER 2

GENERAL METHODS

Experimental animals

Rainbow trout (*Oncorhyncus mykiss*) of either sex were obtained from Thistle Springs (Ashton, Ontario) for the studies of Chapters 3 and 5 or Linwood Acres (Campbellcroft, Ontario) for the study of Chapter 6. Fish were maintained indoors in large fibreglass tanks that were supplied with flowing, dechlorinated and vigorously aerated city of Ottawa tapwater ($[Na^+]$= 0.12 mmol·l^{-1}; $[Cl^-]$= 0.15 mmol·l^{-1}; $[Ca^{2+}]$= 0.35-0.40 mmol·l^{-1}; $[K^+]$= 0.03 mmol·l^{-1}; pH= 7.5-8.0; photoperiod 12h light, 12h dark). The temperature of the holding and experimental water varied seasonally and is specified in each Chapter. Fish were fed daily with floating commercial trout pellets (Purina trout chow). Food was withheld 48 h before the experiments commenced.

Animal preparation

In all experimental groups, trout were anaesthetized in a 1:10000 (W/V) solution of ethyl-*m*-aminobenzoate (MS 222; Sigma Chemical Company; adjusted to pH 7.5 with $NaHCO_3$) and then placed onto an operating table which allowed continuous irrigation of the gills with anaesthetic solution. An indwelling cannula was implanted into the dorsal aorta (Soivio *et al.* 1975) using flexible polyethylene tubing (Clay-Adams PE 50; internal diameter = 0.580 mm, outer diameter = 0.965 mm) to permit measurements of P_{da}, periodic blood sampling and drug injection, when required. A piece of heat-flared flexible PE 50 was implanted inside the buccal cavity (e.g. Davie *et al.* 1982) to provide a measure of breathing frequency. The fish was then fitted with a latex mask sutured around the mouth and attached to the divider of an opaque and aerated van Dam box (Cameron and Davis, 1970). This

installation separates the box into two compartments. When the water level is uniform in the box, the movement of water from the cephalic to the caudal compartment reflects the ventilatory water flow. This causes an overflow in the caudal compartment which can be monitored thereby allowing a direct measurement of $\dot{V}w$ (Fig 2).

Each fish was allowed to recover from anaesthesia and surgery 48 h before experiments commenced. During the first 24 h, recuperation was facilitated by a slight pressure head in the cephalic compartment (~1 cm). The pressure differential between the cephalic and caudal compartment was left at zero during the final 24 h of recovery. The dorsal aortic cannula was flushed at least once daily with 0.2 ml of heparinized (10 units·ml^{-1} ammonium heparin; Sigma) Cortland saline (Wolf, 1963).

Analytical procedures

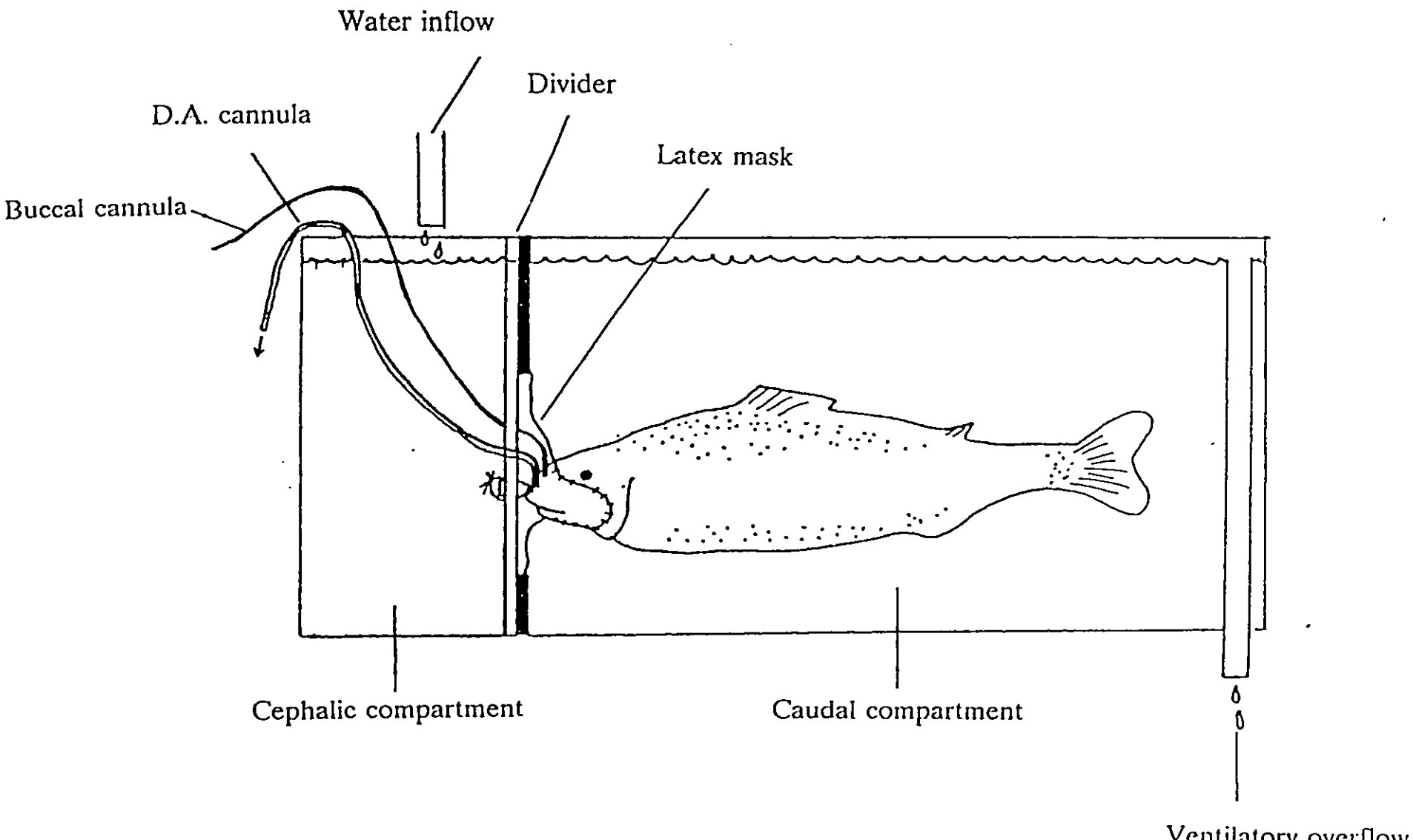
Ventilation frequency (f_V) was monitored by connecting the buccal cavity catheter to a pressure transducer (Bell and Howell, type 4-327-I). Changes in buccal cavity pressure associated with inspiration and expiration were recorded over 1 min intervals at each measuring period on a chart recorder (Lafayette Instrument Co., model 7610). Gill ventilation volume ($\dot{V}w$) was determined simultaneously by collecting and weighing the water exiting the overflow standpipe of the caudal compartment of the van Dam box. Ventilatory stroke volume (V_{sv}) was calculated by dividing $\dot{V}w$ by f_V. Dorsal aortic blood pressure (P_{da}) was monitored concurrently with the ventilatory measurements by connecting the dorsal aortic cannula to a second pressure transducer.

Arterial blood pH were determined with a micro-capillary pH electrode (Radiometer G299A); water and blood PO_2 was measured using a Radiometer PO_2 electrode (E 5047-0)

and water PCO_2 was measured using a Radiometer PCO_2 electrode (E 5037-0). The pH, PCO_2 and PO_2 electrodes were maintained at ambient water temperature and utilized in conjunction with Radiometer PHM-71 acid-base analyzers and BMS3 Mk2 blood micro-systems or thermostated chambers. The PO_2 and PCO_2 electrodes were calibrated with air saturated water and pre-analyzed gas mixtures (Air Products Inc.), respectively, while the pH electrode was calibrated with precision buffers (Radiometer). Plasma Ca_{O2} was measured on 100 µl samples according to the method of Tucker (1967) using a Radiometer PO_2 electrode in a sealed chamber. Total carbon dioxide content (Ca_{CO2}) was determined on 100 µl plasma samples using a total CO_2 analyzer (Corning model 905).

Plasma EPI and NE levels were determined on alumina-extracted plasma samples with high performance liquid chromatography (HPLC) using the basic method of Woodward (1982) in conjunction with an electrochemical detector (Waters, model 460) equipped with a glassy carbon electrode (operating voltage = +0.6 V). The HPLC consisted of a reverse phase Ultratech 5 ODS column and a Waters HPLC pump (model 510 equipped with pulse dampeners; part # 98060 and 25561). 80 µl of the extracted sample was injected *via* a Waters U6K injector. The mobile phase was a commercial catecholamine eluting reagent (Waters, part # 040521). Data integration was performed by a Waters 740 Data module.

Figure 2. A schematic diagram of the van Dam technique utilized to measure $\dot{V}w$ in fish. The dorsal aortic (D.A.) cannulae was utilized to withdraw blood samples, inject and/or infuse drugs and measure P_{da}, whereas the buccal cannula was used to monitor f_V.


Water inflow
Divider
D.A. cannula
Latex mask
Buccal cannula
Cephalic compartment
Caudal compartment
Ventilatory overflow

CHAPTER 3

AN INVESTIGATION OF THE ROLE OF CIRCULATING CATECHOLAMINES IN THE REGULATION OF VENTILATION DURING ACUTE MODERATE HYPOXIA IN RAINBOW TROUT (*Oncorhyncus mykiss*)

INTRODUCTION

Teleost fish demonstrate a close relationship between oxygen requirements and gill ventilation. Consequently, ventilatory adjustments occur when the oxygen tension of the environment (PwO_2) fluctuates. Smith and Jones (1982) demonstrated that many of the conditions acknowledged as ventilatory stimulants (e.g. hypoxia, anaemia or hypercapnia) directly or indirectly depress Ca_{O2}. Thus, Ca_{O2} appears to be a common factor influencing $\dot{V}w$ under these experimental conditions. As was discussed in Chapter 1, it is unclear, however, whether ventilatory changes during hypoxemia are mediated directly by oxygen-sensitive chemoreceptors or indirectly by intervention of circulating catecholamines.

Since it is conceivable that elevation of circulating catecholamines during hypoxemia may contribute to the well-established hyperventilatory response, the present study assessed the potential independent effects of altered blood oxygen status and circulating catecholamines in the ventilatory responses of rainbow trout to acute modification of PwO_2. Environmental hypoxia, normoxia and hyperoxia were utilized to generate a range of ventilatory responses. The involvement of circulating catecholamines in the hyperventilatory response to moderate hypoxia was determined by monitoring $\dot{V}w$ after selective adrenoceptor blockade and by assessing the effects of intra-arterial infusion of catecholamines.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhyncus mykiss*) of either sex, weighing between 129 and 318 g (mean mass = 220 ± 5.5 g (standard error of the mean; S.E.M.); experimental N = 48) were obtained from Thistle Springs Trout Farm (Ashton, Ont). The temperature of the holding and experimental water varied between 10°C and 19°C (June to August).

Experimental protocol

1. Experimental conditions

Series I: Ventilatory adjustments to different external oxygen tensions.

The conditions of external normoxia (PwO_2 ~ 155 Torr, N = 7), hyperoxia (PwO_2 = 643 ± 32 Torr, N = 6) or moderate hypoxia (PwO_2 = 72 ± 6.0 Torr, N = 5) were achieved by gassing the cephalic compartment of the van Dam box as well as a counter-current gas exchange column (which supplied water to the cephalic compartment), with air, oxygen, or nitrogen, respectively. New steady state water PO_2 (PwO_2) was achieved within 2 min. All gases were supplied by Air Products Inc. (Ottawa, Ont.). A water sample (10 ml) was withdrawn from the cephalic compartment at 10 min intervals during all experiments to determine PwO_2.

The hypoxic fish served as a control group for the fish treated with α- or β-

adrenoceptor antagonists. Therefore, these animals were sham-injected *via* the dorsal aortic cannula with 0.4 ml of saline (pH = 7.8) 2 h before the initiation of hypoxia.

Separate groups of fish were pre-injected with either α- or β-adrenoceptor antagonists prior to being exposed to acute moderate external hypoxia. The first group (PwO_2 = 72 ± 5.8 Torr, N = 6) was treated with the β-adrenoceptor antagonist, propranolol, (DL-propranolol; Sigma Chemical Company) 2 h before the onset of moderate hypoxia to ensure adequate antagonistic activity (Nilsson, 1983). The drug was dissolved in Cortland saline immediately before use, adjusted to a final pH of 7.8 and injected into the dorsal aortic cannula (approximately 0.2 ml) at a dose of 2 mg · kg body weight^{-1}. Previous experiments in our laboratory (e.g. Vermette and Perry, 1988*a, b*; Wright *et al.* 1989) have demonstrated that identical doses of propranolol can abolish or diminish β-adrenergic responses in trout (e.g. rbc alkalinization) for up to 8 h after injection. The cannula was flushed with an additional 0.2 ml of saline after each injection to ensure complete delivery of the antagonist to the circulation. An identical protocol was repeated on a second group (PwO_2 = 80 ± 6.2 Torr, N = 5) using the α-adrenoceptor antagonist, phentolamine (Rogitine; obtained from Ciba-Geigy). The effectiveness of the α-antagonistic action with this protocol was demonstrated previously in an other study (Perry *et al.* 1989).

Series II: Ventilatory responses to catecholamine infusions.

After the two "Pre" measurements of the ventilatory and cardiovascular parameters (see below), the dorsal aorta cannula was infused with physiological saline at a rate of 0.6 ml·h^{-1} for 60 min using a syringe pump (Sage; model 352). Then, a blood sample (0 min) was withdrawn *via* a 3-way valve in the infusion tubing. In one group (N = 6), each fish was

given a bolus injection of 0.3 ml of 2 x 10^{-5} mol·l^{-1}L-epinephrine. (bitartrate salt, Sigma chemical Company) dissolved in physiological saline (final pH = 7.8). The cannula was flushed with 0.2 ml of saline and was then attached immediately to the pump which dispensed a solution of 2 x 10^{-5} mol·l^{-1} L-epinephrine at the same rate (0.6 ml·h^{-1}). All syringes and tubing were opaque to impede oxidation of catecholamines. The fish were infused 30 min before a second blood sample (30 min) was taken. Recovery was effected by infusing the fish with physiological saline for 30 min. The experiment was terminated by withdrawing a final (recovery) blood sample. The same procedure was also performed on another group of fish (N = 7) using norepinephrine bitartrate (arterenol, Sigma Chemical Company) or physiological saline in the control group (N = 6).

2. Blood sampling

Three blood samples of 750 µl each were taken from each fish. An initial sample was withdrawn from the dorsal aortic cannula before the onset of the experiment (0 min), a second one after 30 min of exposure to the experimental condition (30 min) and a final sample 30 min after cessation of the experiment (recovery).

After each blood sample, an equivalent volume of heparinized saline (10 units·ml^{-1}) was injected into the fish to partially restore blood volume. This procedure was always followed by a recording of $\dot{V}w$, f_V and P_{da} to determine if these were affected by blood sampling. The arterial blood was analyzed immediately after sampling to determine PaO_2, pHa, Ca_{CO2}, and Ca_{O2} as described in Chapter 2 (analytical procedures). $PaCO_2$ was calculated from the measured Ca_{CO2} and pHa values using the Henderson-Hasselbalch equation. Remaining blood was centrifuged and the plasma (~250 µl) was combined with

10 µl of 5 $mMol \cdot l^{-1}$ sodium bisulphite and 20 µl of heparin (2500 $units \cdot ml^{-1}$) before being stored at -70°C for subsequent determination of catecholamine levels.

3. Ventilatory and cardiovascular measurements

In series I, ventilatory and cardiovascular parameters of each fish were monitored in the following sequence: two pre-experimental measurements (Pre 1 and Pre 2) were taken 10 min apart. Another measurement was taken 10 min later (0 min), just prior to withdrawal of the first blood sample. The animal was then exposed to the particular experimental condition (normoxia, hyperoxia, or moderate hypoxia) for 30 min. At the end of that period a second blood sample was taken. During the experiment, the ventilatory and cardiovascular parameters were monitored every 10 min (see below). The fish was then allowed to recover under normoxic conditions (PwO_2 = ~155 Torr) for 30 min before a final blood sample was taken.

In series II, in addition to the ones described for series I, ventilatory and cardiovascular measurements were performed after 2, 5, 15, and 30 min of catecholamine or saline-infusion and after 30 min recovery (saline-infusion).

Statistical analysis

The results have been statistically analyzed using paired or unpaired Student's t-test (two-tailed) for differences between means; 5% was taken as the fiducial limit of significance.

RESULTS

I. Ventilatory responses to different environmental oxygen tensions

Moderate hypoxia elicited an approximate 2.5-fold increase in $\dot{V}w$ during the 30 min experimental interval (Fig 3). This response was due solely to an increase of V_{sv} because f_V was unaffected (Table 1). After 30 min of hypoxia, PaO_2 and Ca_{O2} were decreased significantly whereas $PaCO_2$ and pHa were unchanged (Table 2). Plasma catecholamine levels were unaltered. PaO_2 and $\dot{V}w$ returned to pre-hypoxic values after 30 min of recovery but Ca_{O2} remained depressed slightly (Fig 3, Table 2). Hyperoxia caused an approximate 3-fold decrease in $\dot{V}w$ (Fig 3) that was due entirely to reduced ventilatory stroke volume (Table 1). PaO_2 was elevated while Ca_{O2} remained constant (Table 2). $\dot{V}w$ and PaO_2 returned to pre-experimental levels after cessation of the treatment. Hyperoxia did not alter pHa, $PaCO_2$ or catecholamine levels (Tables 1 and 2). No measured variable changed significantly under normoxic (control) conditions (Fig 3, Tables 1 and 2).

Changes in $\dot{V}w$ during variation of PwO_2 were correlated with changes in both PaO_2 (Fig 4A) and Ca_{O2} (Fig 4B). It is difficult to distinguish independent effects of PwO_2, PaO_2 and Ca_{O2} on $\dot{V}w$ because they co-vary. During hyperoxia, however, Ca_{O2} remained constant despite a large increase in PO_2 (arterial or water, Fig 5). It would appear therefore, that the hypoventilatory response to hyperoxia was mediated solely by changes in PO_2 (Fig 5). Pre-treatment of fish with α or β-adrenoceptor antagonists did not prevent, hinder or modify the hyperventilatory responses normally observed during hypoxia (Fig 6). Fish pre-treated with phentolamine displayed a significant increase of epinephrine and norepinephrine levels during exposure to hypoxia (Table 1) yet $\dot{V}w$ was unaltered. Catecholamine levels also increased in

fish pre-treated with propranolol although the changes were not statistically significant due to the variability of the data (Table 1). Ca_{O2} was depressed to the greatest extent in hypoxic fish pre-treated with adrenoceptor antagonists (Table 2). Catecholamine levels were unaltered after 30 min of hypoxia in fish pre-treated with saline (controls; Table 1) and yet, the fish were hyperventilating.

II. Ventilatory responses to infusion of catecholamines.

After 30 min of intra-arterial catecholamine infusion, the circulating levels of EPI and NE were elevated to 164 nM and 206 nM, respectively (Table 1). Epinephrine infusion caused a depression of $\dot{V}w$ which was mediated by a decrease in stroke volume (Table 3). The hypoventilatory response was transient and apparent only at the 2 min measurement (Fig 7, Table 3). In contrast, NE infusion elicited a hypoventilatory response that persisted throughout the infusion period (Fig 5). Interestingly, of all the different stimuli utilized to modulate $\dot{V}w$, norepinephrine infusion was the only experimental condition in which $\dot{V}w$ was altered by simultaneous modification of stroke volume and breathing frequency (Table 3). This type of response was observed during the initial 5 min of infusion. In both cases, catecholamine infusion was accompanied by an increase of dorsal aortic pressure during the entire infusion period (Table 3). All the measured changes caused by catecholamine infusion were reversed upon cessation of the treatment. Infusion with saline (sham) did not affect $\dot{V}w$ or any other variable monitored during the experiment (Fig 7, Table 3).

Figure 3. Ventilatory responses of rainbow trout to external hyperoxia (■——■; N = 6), hypoxia (♦——♦; N = 5) or sustained normoxia (●——●; N = 7). * indicates a value significantly different from its respective pre-experimental measurement (time = 0 min). The differences in resting ventilation volume among experimental groups are due to the seasonal fluctuation of ambient water temperature (see materials and methods, Chapter 3).

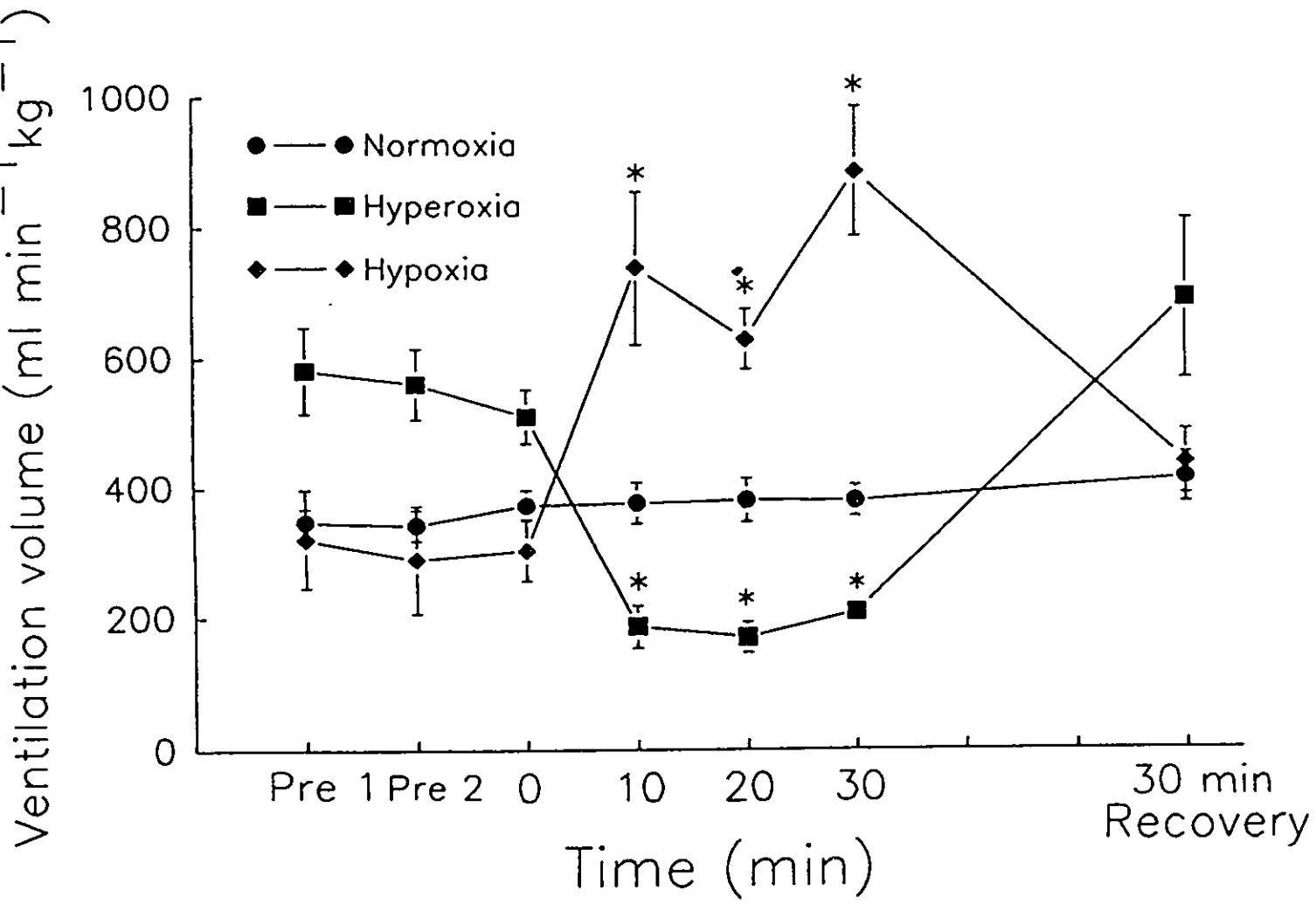

Ventilation volume (ml min^{-1}kg^{-1})
1000
800
600
400
200
0
Normoxia
Hyperoxia
Hypoxia
*
Pre 1
Pre 2
0
10
20
30
30 min Recovery
Time (min)

Figure 4. The relationships between the changes in ventilation volume (Δ ventilation volume; $\dot{V}w$) and the changes in A) arterial oxygen tension (Δ PaO_2) or B) arterial oxygen content (Δ CaO_2) during hypoxia, hyperoxia or sustained normoxia. Each data point represents the difference between the 30 and 0 min values. The linear regression for the relationship between Δ $\dot{V}w$ and CaO_2 is Δ $\dot{V}w$ = -115 ml $min^{-1} \cdot kg^{-1}$/ml $O_2 \cdot 100$ ml blood x Δ CaO_2 + 17.7 ml min^{-1} kg^{-1}; r = 0.77. The curvilinear relationship between Δ $\dot{V}w$ and Δ PaO_2 was fitted by eye.

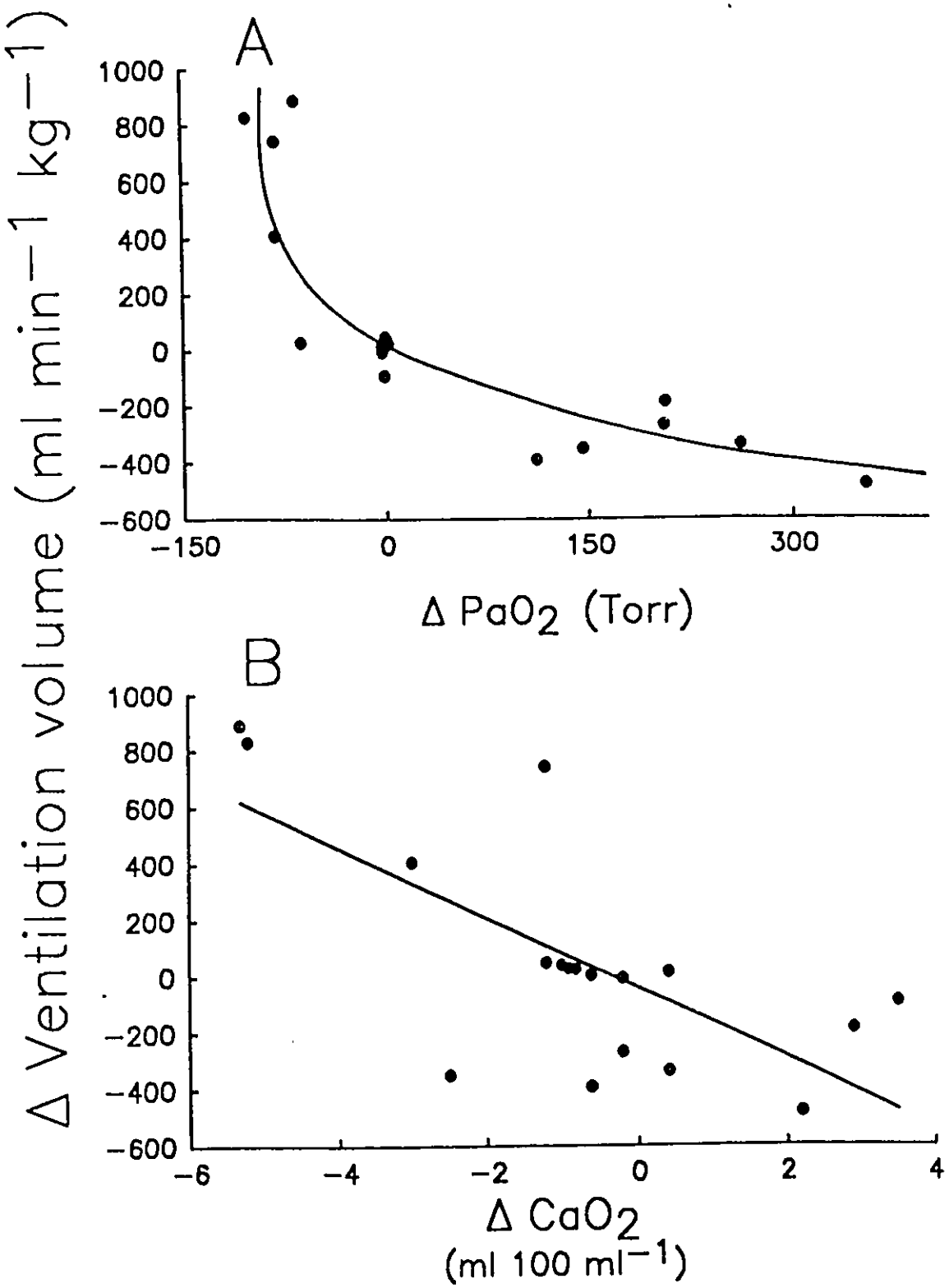

A
B
Δ Ventilation volume (ml min−1 kg−1)
1000
800
600
400
200
0
−200
−400
−600
−150
0
150
300
Δ PaO2 (Torr)
−6
−4
−2
0
2
4
Δ CaO2
(ml 100 ml−1)

Figure 5. Changes in ventilation volume (Δ ventilation volume) during hypoxia, hyperoxia or sustained normoxia expressed either as a function of changes in arterial oxygen tension (Δ PaO_2; ●——●) or changes in arterial oxygen content (Δ CaO_2; ■----■). Each data point is the mean variation between the 30 and 0 min measurements ± 1 S.E.M.

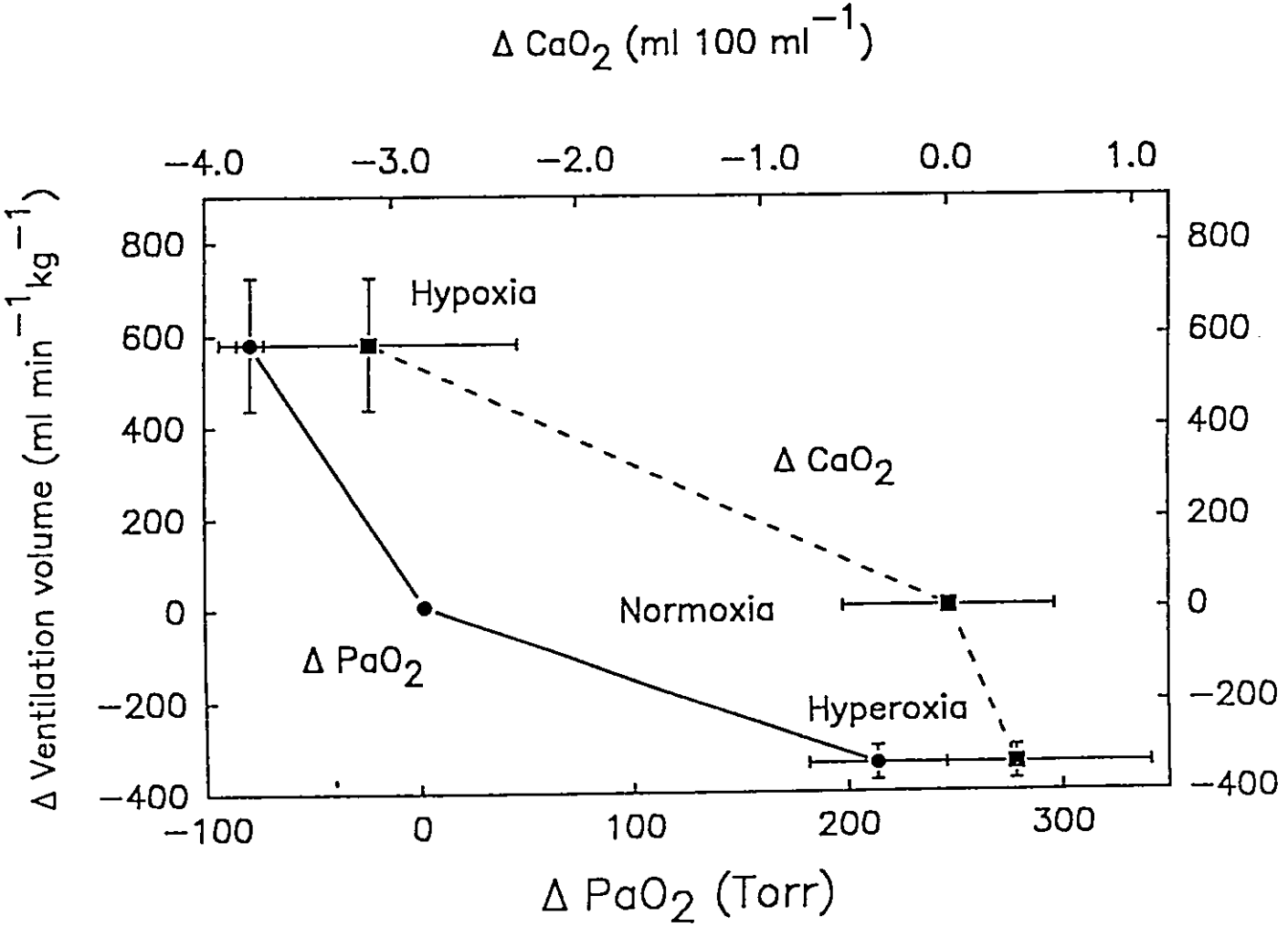

Δ CaO2 (ml 100 ml−1)
−4.0
−3.0
−2.0
−1.0
0.0
1.0
800
600
400
200
0
−200
−400
Δ Ventilation volume (ml min−1 kg−1)
Hypoxia
Δ CaO2
Normoxia
Δ PaO2
Hyperoxia
−100
0
100
200
300
Δ PaO2 (Torr)

Figure 6. Ventilatory responses of rainbow trout to moderate hypoxia after pre-treatment with saline (♦——♦; N = 5, control group), α- (▽——▽; N = 5) or β- (◇——◇; N = 6) adrenoceptor antagonists. * indicates a value significantly different from the pre-experimental measurement (time = 0 min). Since the increases in $\dot{V}w$ are significant for all groups during hypoxia (10, 20, and 30 min), only one * is shown per measurement for clarity.

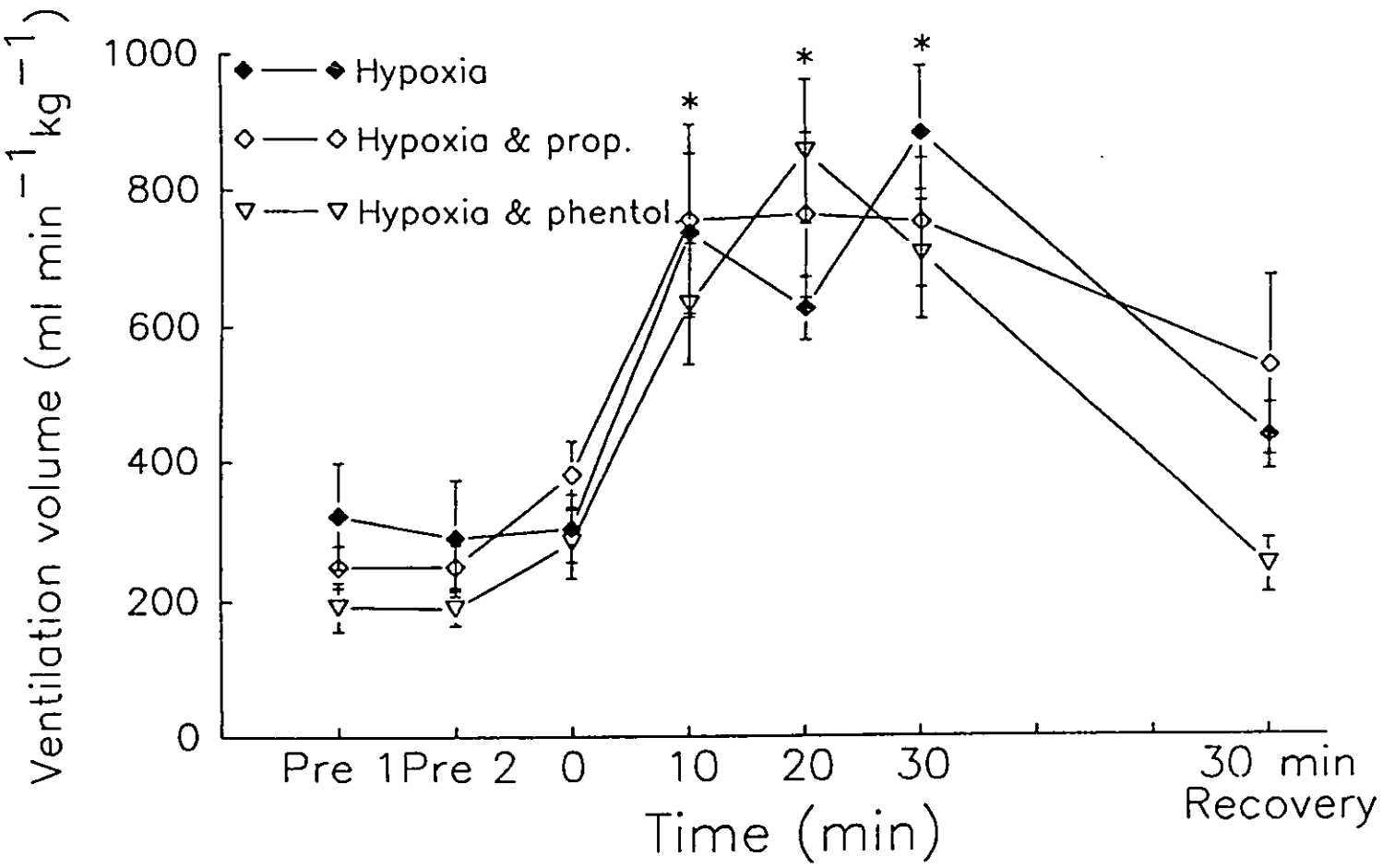

Ventilation volume (ml min^{-1}kg^{-1})
1000
800
600
400
200
0
Hypoxia
Hypoxia & prop.
Hypoxia & phentol
*
*
*
Pre 1
Pre 2
0
10
20
30
30 min Recovery
Time (min)

Figure 7. Ventilatory responses of rainbow trout to intra-arterial infusion of epinephrine (▲——▲; N = 6), norepinephrine (■——■; N = 7) or saline (●——●; N = 6, control group). * indicates a value significantly different from the pre-experimental measurement (time = 0 min).

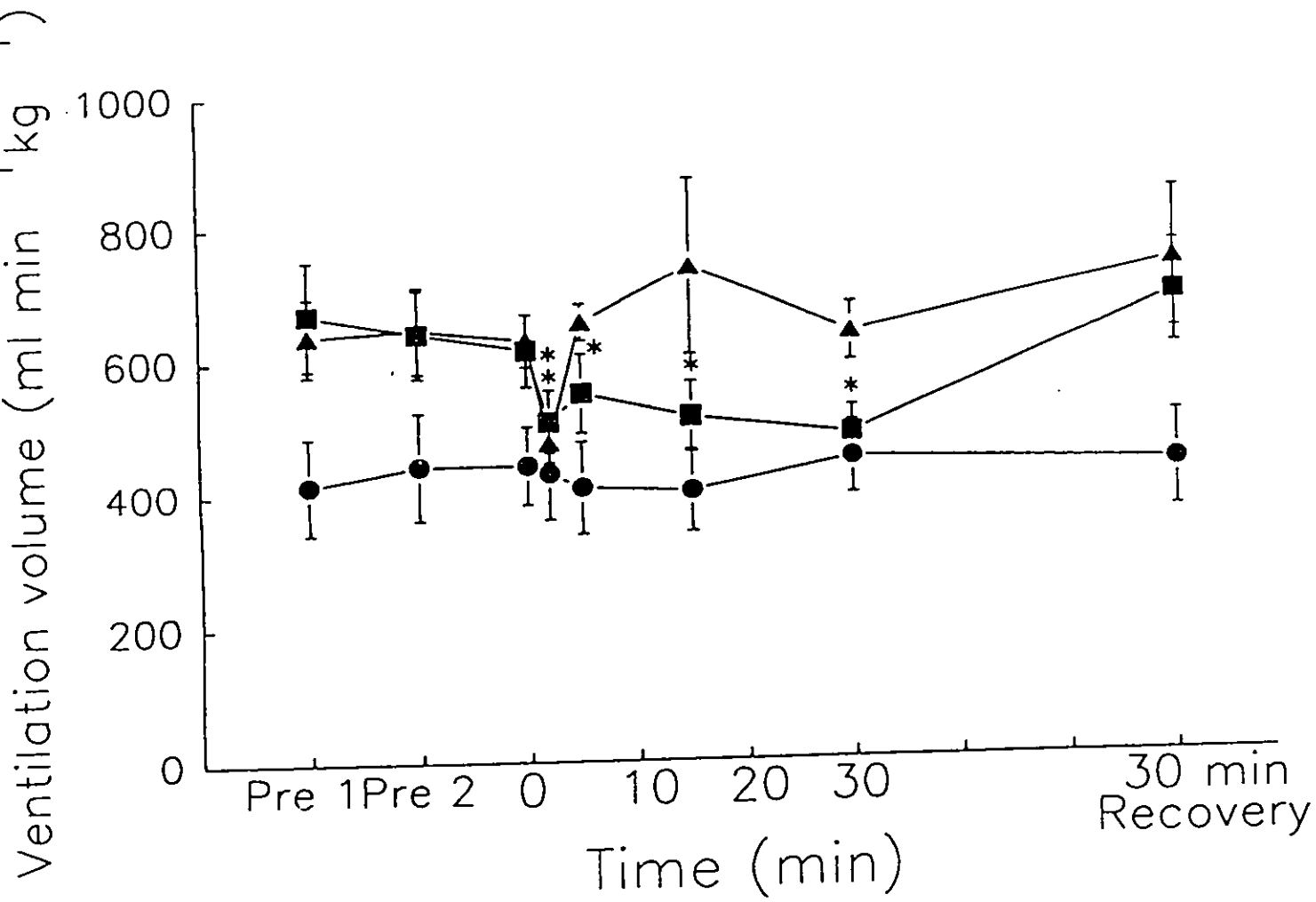

Ventilation volume (ml min^{-1}kg^{-1})
1000
800
600
400
200
0
Pre 1
Pre 2
0
10
20
30
30 min
Recovery
Time (min)

Table 1: Plasma catecholamine levels and ventilatory variables monitored under varied external oxygen tensions. Data are shown as means 1 ± standard error of the mean (S.E.M.). Numbers of experimental fish are indicated in parentheses.
* indicates a value significantly different from the 0 min measurement.

	Time (min)	Normoxia (N = 7)	Hyperoxia (N = 6)	Hypoxia (N = 5)	Hypoxia + propranolol (N = 6)	Hypoxia + phentolamine (N = 5)
EPI (nM)	0	0.76 ± 0.13	1.01 ± 0.30	1.76 ± 0.65	1.08 ± 0.57	2.74 ± 1.11
	30	0.72 ± 0.14	1.07 ± 0.40	1.94 ± 0.92	4.7 ± 4.0	12.12 ± 3.70*
	rec.	0.61 ± 0.05	1.11 ± 0.30	1.79 ± 0.80	2.32 ± 1.0	2.21 ± 0.48
NE (nM)	0	0.96 ± 0.37	1.05 ± 0.09	0.97 ± 0.29	0.92 ± 0.35	1.20 ± 0.19
	30	1.49 ± 0.38	1.13 ± 0.08	1.18 ± 0.34	6.38 ± 5.47	5.22 ± 1.57*
	rec.	0.89 ± 0.18	0.99 ± 0.12	0.95 ± 0.32	2.26 ± 1.27	1.14 ± 0.23
Ventilation frequency (min^{-1})	0	81 ± 2	90 ± 2	71 ± 3	76 ± 3	76 ± 3
	30	83 ± 3	85 ± 2	77 ± 3	76 ± 3	74 ± 2
	rec.	81 ± 2	95 ± 3	70 ± 3	74 ± 5	76 ± 3
Stroke volume ($ml \cdot kg^{-1}$)	0	4.5 ± 0.4	5.6 ± 0.4	4.3 ± 0.7	5.0 ± 0.5	4.0 ± 0.8
	30	4.5 ± 0.5	2.0 ± 0.3*	12.0 ± 1.6*	10.1 ± 1.2*	10.0 ± 1.2*
	rec.	5.0 ± 0.6	7.1 ± 1.1	6.2 ± 0.6	7.3 ± 1.6	3.4 ± 0.8

Table 2: Blood respiratory and acid base variables monitored under varied external oxygen tensions. Data are shown as means 1 ± standard error of the mean (S.E.M.). Numbers of experimental fish are indicated in parentheses.
* indicates a value significantly different from the 0 min measurement.

	Time (min)	Normoxia (N = 7)	Hyperoxia (N = 6)	Hypoxia (N = 5)	Hypoxia + propranolol (N = 6)	Hypoxia + phentolamine (N = 5)
PaO_2 (Torr)	0	120 ± 10.8	120 ± 10	112 ± 8.4	112 ± 6.9	119 ± 9.1
	30	129 ± 8.7	334 ± 40*	34 ± 7.7*	25 ± 2.4*	26 ± 5.4*
	rec.	135 ± 6.0	166 ± 20	113 ± 11.3	115 ± 5.2	129 ± 5.5
CaO_2 ($ml \cdot 100\ ml^{-1}$)	0	7.4 ± 0.6	8.0 ± 0.7	6.7 ± 0.5	6.6 ± 0.6	5.2 ± 0.4
	30	7.4 ± 0.9	8.3 ± 0.3	3.6 ± 0.6*	2.3 ± 0.3*	2.5 ± 0.4*
	rec.	7.0 ± 0.8	6.4 ± 0.4	5.7 ± 0.3*	4.6 ± 0.4*	5.0 ± 0.4
$PaCO_2$ (Torr)	0	1.43 ± 0.09	1.16 ± 0.18	1.17 ± 0.13	1.40 ± 0.16	1.22 ± 0.04
	30	1.36 ± 0.11	1.99 ± 0.46	0.98 ± 0.07	1.21 ± 0.18	1.21 ± 0.04
	rec.	1.39 ± 0.09	1.29 ± 0.23	1.35 ± 0.19	1.90 ± 0.25*	1.31 ± 0.08
pHa	0	7.71 ± 0.04	7.89 ± 0.02	7.99 ± 0.05	7.79 ± 0.03	7.95 ± 0.02
	30	7.74 ± 0.03	7.78 ± 0.07	8.03 ± 0.05	7.81 ± 0.04	7.98 ± 0.06
	rec.	7.74 ± 0.03	7.91 ± 0.05	7.88 ± 0.07	7.67 ±0.03*	7.95 ± 0.02

Table 3:Plasma catecholamine levels, cardiovascular, and ventilatory variables monitored during infusion of catecholamines. * indicates values significantly different from the measurement made at 0 min.

	Time (min)	Saline infusion (N = 5)	Adrenaline infusion (N = 6)	Noradrenaline infusion (N = 7)
	0	4.1 ± 3.2	3.2 ± 2.5	0.4 ± 0.1
Epinephrine	30	4.7 ± 3.6	164 ± 73*	1.4 ± 0.3
(nM)	recovery	11.8 ± 6.3	1.6 ± 0.7	0.7 ± 0.2
	0	3.0 ± 2.3	5.5 ± 4.6	1.1 ± 0.4
Norepinephrine	30	3.8 ± 2.2	5.8 ± 4.6	206 ± 26*
(nM)	recovery	5.9 ± 4.9	4.7 ± 3.4	2.3 ± 0.7
	pre 1	86 ± 5.3	99 ± 3.8	110 ± 5.0
	pre 2	87 ± 4.3	99 ± 4.3	110 ± 4.9
Ventilation	0	89 ± 2.9	102 ± 2.4	110 ± 4.4
frequency	2	89 ± 3.1	104 ± 2.7	106 ± 4.5*
(min^{-1})	5	90 ± 3.1	100 ± 2.7	103 ± 3.7*
	15	92 ± 3.2	100 ± 3.5	107 ± 2.9
	30	93 ± 2.4	101 ± 2.9	109 ± 3.5
	recovery	93 ± 2.7	106 ± 4.3	110 ± 6.3
	pre 1	4.7 ± 0.7	6.5 ± 0.8	6.0 ± 0.6
	pre 2	5.1 ± 0.8	6.7 ± 0.9	5.8 ± 0.5
Stroke	0	5.0 ± 0.8	6.2 ± 0.4	5.5 ± 0.4
volume	2	4.7 ± 0.6	4.6 ± 0.3*	4.9 ± 0.4*
($ml \cdot kg^{-1}$)	5	4.4 ± 0.6	6.6 ± 0.3	5.3 ± 0.5
	15	4.9 ± 0.7	6.3 ± 0.5	4.8 ± 0.5*
	30	4.6 ± 0.6	6.3 ± 0.4	4.5 ± 0.3*
	recovery	4.6 ± 0.7	7.2 ± 0.4	6.1 ± 0.4
	pre 1	34 ± 2.9	28 ± 3.4	32 ± 1.9
	pre 2	33 ± 3.5	28 ± 3.5	31 ± 1.7
Dorsal	0	32 ± 2.1	32 ± 2.6	32 ± 1.2
aortic	2	35 ± 2.4	89 ± 5.4*	61 ± 3.8*
pressure	5	33 ± 2.6	70 ± 1.3*	55 ± 4.0*
(cm H_2O)	15	33 ± 3.0	56 ± 3.0*	51 ± 4.3*
	30	31 ± 3.8	68 ± 4.2*	47 ± 3.6*
	recovery	28 ± 2.1	39 ± 8.7	28 ± 1.3*

hypoxia and hyperoxia, respectively, confirm the results reported in numerous studies on a variety of teleost species (Dejours, 1973; Dejours *et al.* 1977; Randall and Jones, 1973; Wood and Jackson, 1980; Wilkes *et al.* 1981; Smith and Jones, 1982; Peyraud-Waitzenegger and Soulier, 1989; see review by Shelton *et al.* 1986). During acute moderate hypoxia, fish did not develop metabolic acidosis unlike trout exposed to more severe or chronic hypoxia (e.g. Thomas and Hughes, 1982; Tetens and Lykkeboe, 1985; Boutilier *et al.* 1988). Furthermore, moderate hypoxia did not induce catecholamine mobilization which is known to occur during acute deep hypoxia (Fievet *et al.* 1987; Tetens and Christensen, 1987) or long-term moderate hypoxia (Boutilier *et al.* 1988).

Perry *et al.* (1989) demonstrated that hypoxemia is a specific stimulus causing the release of catecholamines from chromaffin tissue in trout but also suggested that acidosis might potentiate this response. The absence of blood acidosis in the present study may explain the lack of catecholamine release even though arterial oxygen content was reduced by approximately 50%. It is conceivable that the blood sampling protocol employed did not permit the detection of transient surges of catecholamines.

It is clear, however, that after 30 min of hypoxia, two potential ventilatory stimulants, elevated catecholamines (Peyraud-Waitzenegger *et al.* 1980) and blood acidosis (Heisler *et al.* 1988), were not contributing to the observed hyperventilatory response. Thus, it would appear that depression of PaO_2 and/or Ca_{O2} were exclusively modulating $\dot{V}w$ during acute moderate hypoxia. This affirmation is supported by the fact that in Channel catfish (*Ictalurus punctatus*) internal O_2-sensitive chemoreceptors, which are thought to be located in or just downstream from the gills, will reflexively increase $\dot{V}w$ when they are stimulated by hypoxemia (Burleson and Smatresk, 1990). The possibility that PwO_2 contributed to this response however, cannot be neglected since in that same study it was shown that externally oriented

chemoreceptors monitor the PO_2 of the inspired water and elicit bradycardia and hyperventilation when stimulated by aquatic hypoxia.

The study of Smith and Jones (1982) suggested that $\dot{V}w$ in trout is directly related to Ca_{O2} during hypercapnia, hypoxia and hyperoxic hypercapnia; a conclusion derived from a linear relationship between Ca_{O2} and $\dot{V}w$ (see review by Randall, 1982). In the present study, a similar correlation between $\dot{V}w$ and Ca_{O2} was observed (Fig 4B). Indeed, the results of a variety of studies (Eclancher and Dejours, 1975; Holeton, 1977; Smith and Jones, 1982; Smatresk, 1986) indicate the presence of peripheral chemoreceptors that are receptive to changes in Ca_{O2} or oxygen delivery rate, rather than to PaO_2 itself. During hyperoxia, however, PaO_2 was elevated markedly without significantly affecting Ca_{O2}. Thus, it is suggested that during environmental hyperoxia, a change in PaO_2 (or PwO_2) rather than Ca_{O2}, is the stimulus promoting hypoventilation.

The fact that fish displayed a two-fold increase of $\dot{V}w$ without any concomitant increase of circulating catecholamines demonstrates that elevated circulating catecholamines are not a prerequisite for hyperventilation during moderate hypoxia. Furthermore, in certain instances (phentolamine-treated fish, Table 1) circulating catecholamine levels did increase during hypoxia, yet the ventilatory response was unaffected. In addition, it was confirmed that potential transient (undetected) surges of catecholamines were not modulating ventilation because selective α- or β- adrenoceptor blockade was without effect on ventilation during hypoxia. These results differ from predictions based on the study of Peyraud-Waitzenegger (1979). In that study, it was shown that stimulation of β-adrenoceptors caused hyperventilation whereas stimulation of α-adrenoceptors caused hypoventilation in the European eel (*Anguilla anguilla*). If a similar system was operative in trout, hypoxic fish pre-treated with phentolamine would display a more pronounced hyperventilation whereas fish

pre-treated with propranolol might be expected to display a diminished hyperventilatory response. Clearly, this was not the case as illustrated in Fig 6.

Hypoxia or norepinephrine increase afferent discharge of the chemoreceptors of the carotid bodies of the rabbit, a response that can be diminished by propranolol (Milsom and Sadig, 1983). Furthermore, these authors showed that there is a direct relationship between the degree of hypoxia to which the animal is exposed and sensitivity of the receptor to circulating norepinephrine levels. If the situation is similar in trout, fish pre-treated with propranolol should display a hindered hyperventilatory response during hypoxia. A possible explanation for this discrepancy is that the degree of hypoxia achieved in the present study was not severe enough to allow propranolol to impair chemoreceptor discharge and therefore the ventilatory response.

In their study, Aota *et al.* (1990) suggested that circulating catecholamines play a role in the hyperventilatory response to hypoxia. This conclusion was based on the fact that a similar dose of propranolol, reduced the hyperventilatory response to hypoxia (PwO_2 ranging from 24 to 60 Torr) in *Oncorhyncus mykiss*. Based on the observation that in trout, chemoreceptor activity is depressed when they are exposed to external O_2 tensions below 50 Torr (M. Burleson, personal communication), Aota *et al.* (1990) suggested that catecholamines could augment the ventilatory response to hypoxia as a last resort mechanism to compensate for the reduced response of the chemoreceptors to the hypoxic stimulus.

The hypoventilatory responses observed during catecholamine infusion also do not support a role for these hormones in stimulating $\dot{V}w$ in trout and differ from similar experiments on eel (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980) in which $\dot{V}w$ was stimulated in summer. The measured levels of catecholamines after 30 min of infusion were comparable to peak values reported after exhaustive exercise (e.g. Tang and Boutilier, 1988; Milligan *et al.* 1989) and estimated levels in the studies of Peyraud-Waitzenegger and co-workers. The physiological significance of the hyperventilatory response of eel to catecholamines is unclear because it was demonstrated recently that severely hypoxic eels (PwO_2 = 40 Torr) hyperventilate in the absence of elevated plasma catecholamine levels (Peyraud-Waitzenegger and Soulier, 1989).

The mechanism(s) of the hypoventilatory responses caused by catecholamine infusion in trout is unclear, as is the reason(s) for the differences in the duration of the responses caused by norepinephrine (persistent) or epinephrine (transient). It has been suggested, however, that only norepinephrine can cross the blood-brain barrier in trout (Nekvasil and Olson, 1986). Interestingly, trout infused with norepinephrine were the only treatment group to display modified breathing frequency, an effect which is well described in mammals (Eldridge and Millhorn, 1981). Thus, it is conceivable that norepinephrine can modulate $\dot{V}w$ by acting directly upon the respiratory centres situated in the CNS. It is unlikely that the initial response observed during epinephrine infusion may reflect the large increase in arterial blood pressure which, if as in mammals, would have stimulated the baroreceptors to cause a temporary hypoventilation. As it is demonstrated in Chapters 4 and 6, $\dot{V}w$ is not sensitive to elevations of blood pressure. Finally, it must be stressed that the hypoventilatory responses to catecholamines were observed in normoxic trout and therefore may not resemble the ventilatory responses to similar levels of catecholamines during hypoxia. In summary, the

results of the present study do not support a role for circulating catecholamines in stimulating ventilation in moderately hypoxic rainbow trout. The decreases in PwO_2, PaO_2 and/or Ca_{O2} appear to be the dominant factors promoting hyperventilation during moderate hypoxia whereas elevated PaO_2 (and/or PwO_2) is likely to be the key factor causing hypoventilation during hyperoxia.

CHAPTER 4

THE ROLE OF CIRCULATING CATECHOLAMINES IN THE VENTILATORY AND HYPERTENSIVE RESPONSE TO HYPOXIA IN THE ATLANTIC COD (*Gadus morhua*)

INTRODUCTION

It is suggested that in fish, catecholamine mobilization may be an important factor promoting hyperventilation when the animal experiences hypoxemia (see Chapter 1). A role for circulating catecholamines in promoting hyperventilation was further established by Aota *et al.* (1990). In that study, it was shown that during severe hypoxia (PwO_2 = 24-60 Torr) or intra-arterial acid infusion, β-adrenoceptor blockade hindered the usual hyperventilatory responses. In Chapter 3, the use of a similar protocol did not significantly modify the ventilatory response to moderate hypoxia (PwO_2 = 72 Torr), an experimental condition that did not elicit catecholamine release into the blood. The significantly different levels of hypoxia utilized in the two studies (Aota *et al.* 1990; present study, Chapter 3) with corresponding dissimilar effects on blood respiratory/acid-base status and plasma catecholamine concentrations may explain the discrepancies between the reported results. Thus it is not possible, given our present state of knowledge, to generalize on the role of circulating catecholamines in the ventilatory responses of fish to hypoxia.

The first objective of this study, therefore, was to determine if catecholamines, whether of humoral or neural origin, are involved in the ventilatory response of Atlantic cod (*Gadus morhua*) to pure internal and/or external hypoxia. This was accomplished by slowly exposing the fish to hypoxia so that catecholamine mobilization occurred in the absence of metabolic acidosis (a potential ventilatory stimulant).

The Atlantic cod displays a pronounced hypertension when exposed to hypoxia (Fritsche and Nilsson, 1989; 1990). Thus, this animal is an excellent experimental model to study the potential relationship between the cardiovascular and respiratory systems in fish. To test the hypothesis that acute changes in blood pressure can influence breathing during

hypoxia (see Chapter 1 for further details), $\dot{V}w$ was monitored when the hypertensive response was either delayed, or abolished, pharmacologically.

MATERIALS AND METHODS

The experiments reported in this Chapter were performed at the Zoological Institute of the University of Göteborg, Sweden. This explains the differences of the methods/techniques utilized in comparison to those described in Chapter 2 (general methods).

Experimental animals

Atlantic cod, *Gadus morhua*, of either sex weighing between 358 and 1276 g (mean mass = 690 ± 26 g) were captured off the west coast of Sweden. Fish were maintained indoors in large fibreglass tanks that were supplied with aerated recirculating sea water. The temperature of holding and experimental water was 10°C. Fish were not fed, and were used within 1 week of capture. Photoperiod was maintained at 12 h light/12 h dark. The experiments were performed from September to December.

Animal preparation

Fish were anaesthetized by immersion in sea water containing a 1:10000 (W/V) solution of ethyl-*m*-aminobenzoate (MS 222; Sigma) and then transferred to an operating table. During surgery, the gills were continuously irrigated with aerated sea water containing anaesthetic (1:20000, W/V) that was maintained at 10°C. In all fish, a polyethylene cannula (Clay-Adams PE 50, tipped with Clay-Adams PE 90) was inserted occlusively into the afferent branchial artery of the third gill arch. This enabled periodic withdrawal of pre-branchial (termed venous) blood. A second similar cannula was inserted occlusively into the efferent

branchial artery of the same gill arch for periodic withdrawal of post-branchial (termed arterial) blood. All cannulae were pre-filled with heparinized (50 units·ml^{-1} ammonium heparin) 0.9% (W/V) NaCl (saline) and secured to the skin with sutures after implantation. At least once a day, both cannulae were flushed with approximately 0.4 ml of heparinized NaCl solution to ensure their patency.

A custom-made plastic probe support was fixed on the snout of the fish with surgical silk (2-0) and then a latex membrane was sutured around the mouth and the support (Fig 8). Fish were allowed to recover from anaesthesia and surgery for 48 h before the experiments commenced. Prior to experimentation (24h), an electromagnetic flow probe (i.d. = 16 mm; calibrated by measuring circulation of sea water at known flow rates) was carefully mounted onto the support.

Experimental protocol

1. Experimental conditions

Series I: Ventilatory and blood pressure adjustments to hypoxia

Hypoxia (final PwO_2 = 46 Torr) was achieved by recirculating a static volume (~30 l) of 10°C sea water, gassed appropriately with N_2, to the experimental box. This method, owing to the relatively large volume of the boxes, resulted in gradual depression of the oxygen tension in the inspired water (PwO_2) such that the final PwO_2 was reached only after 25 min. PwO_2 was monitored continuously via polyethylene tubing that was inserted into the flow probe (see Fig 8). The recirculation system was flushed and renewed frequently to prevent

accumulation of waste substances.

Series II: Effects of phentolamine, sotalol or bretylium on the ventilatory and blood pressure adjustments to hypoxia.

Phentolamine metasulphonate (2 $mg \cdot kg^{-1}$ body weight, conc. = [2 $mg \cdot ml^{-1}$]), an α-adrenoceptor antagonist, was slowly (2-5 min) injected into the post-branchial cannulae of the first group of fish (N = 7) 30 min prior to the onset of the experiment. The cannulae were then flushed with approximately 0.2 ml of saline to ensure complete delivery of the drug. An identical protocol was repeated on a second group of fish (N = 7) using the β-adrenoceptor antagonist, sotalol hydrochloride (2.7 $mg \cdot kg^{-1}$ body weight, conc = [2.7 $mg \cdot ml^{-1}$]). In a third group (N = 7), bretylium tosylate (Wellcome Foundation Ltd; 10 $mg \cdot kg^{-1}$ body weight, conc. = [10 $mg \cdot ml^{-1}$]), a drug that prevents catecholamine release from peripheral adrenergic neurones (Smith *et al.* 1985; Fritsche and Nilsson, 1990), was slowly injected (5-10 min) *via* the afferent branchial artery 24 h prior to experimentation during which time the side effects of bretylium dissipated (Smith *et al.* 1985; Fritsche and Nilsson, 1990).

Since the volumes of drugs injected were similar to the daily flushing of the cannulae, the fish exposed to normoxia (PwO_2 = 155 Torr, N = 6) or hypoxia alone (N = 8) served as control groups.

2. Blood sampling

Three pairs of blood samples [arterial (pre-branchial) and venous (post-branchial)] of 750 µl each were taken from all fish. An initial sample (pre) was withdrawn before the onset

of the experimental recording, a second one after 30 min of exposure to the experimental condition (30 min) and a final sample was taken 60 min after cessation of the experiment (recovery).

After each blood sample, an equivalent volume of RBC's was reinjected into the animal. These RBC's originated from the blood of donor fish. A polyethylene cannulae was chronically implanted into the third afferent branchial artery of the donor fish at least 24 h before withdrawal of blood (see above). The donor blood was centrifuged (500 x G, 10 min) and resuspended to the same Hct in 0.9% saline. The washing procedure was repeated twice to ensure low levels of catecholamines in the suspension; the values in the reinjected blood were not significantly different from the resting values normally seen in cod ([NE] = 3.1 ± 1.3 nM; [EPI] = 1.9 ± 0.8 nM). No fish displayed any obvious signs of distress that might be linked to this "transfusion" procedure.

The arterial and venous blood samples were analyzed immediately after sampling to determine PaO_2, PvO_2, pHa, pHv and Ca_{O2}, Cv_{O2} as described in Chapter 2 (analytical procedures). Remaining blood was centrifuged and the plasma (~250 µl) was combined with 20 µl of 0.2 M EGTA/ 0.2 M glutathione solution before being stored at -70°C for subsequent determination of catecholamines levels.

3. Ventilatory and blood pressure measurements.

Gill ventilation volume was quantified by measuring the flow of water through the electromagnetic flow probe (Fig 8) which was connected to a flow meter (Biotronex, BLI). The signal of the flow meter was recorded on a Grass polygraph recorder (model 7 or 79). To obtain zero water flow for calibration of the system after completion of each experiment,

a piece of plastic was placed in front of the flow probe to prevent any water movement. Ventilation frequency (f_V) was recorded from the biphasic $\dot{V}w$ signal, triggering a 7P44 tachograph preamplifier in the same polygraph.

The validity of this seldom used method of measuring $\dot{V}w$ was assessed by comparing the blood respiratory status (O_2 tension and content) in arterial and venous blood, plasma catecholamines levels as well as breathing frequency of 8 fish before and after installation of the flow probe apparatus.

Ventral aortic blood pressure was measured from the afferent branchial artery cannulae which was filled with heparinized saline and attached to a Statham P23 pressure transducer that was connected to a Grass polygraph. The transducer was calibrated against a static column of water.

In addition to the polygraph recording, the ventilatory parameters and P_{va} were sampled by a microcomputer (IBM PPC) during the experiments. The software used (AD/DATA; P. Thorén, Department of Physiology, University of Göteborg, Sweden) allowed continuous sampling every 5^{th} sec. Samples were pooled and mean values created for 1 min periods.

The data acquisition was initiated first under normoxic condition (PwO_2 ~150 Torr for 10 min) 5-10 min after the first pair of blood samples (Pre) was taken to ensure that the resting values of the parameters of interest were not affected by the sampling procedure. Then, the hypoxia [normoxia (PwO_2 ~150 Torr) in the control group] was initiated for 30 min. At the end of that period, a second pair of blood samples (30 min) was taken and flow of normoxic water was re-commenced. The data acquisition was continued for another 60 min. At the end of that recovery period the computer sampling was stopped and the final pair of blood samples (recovery) was taken.

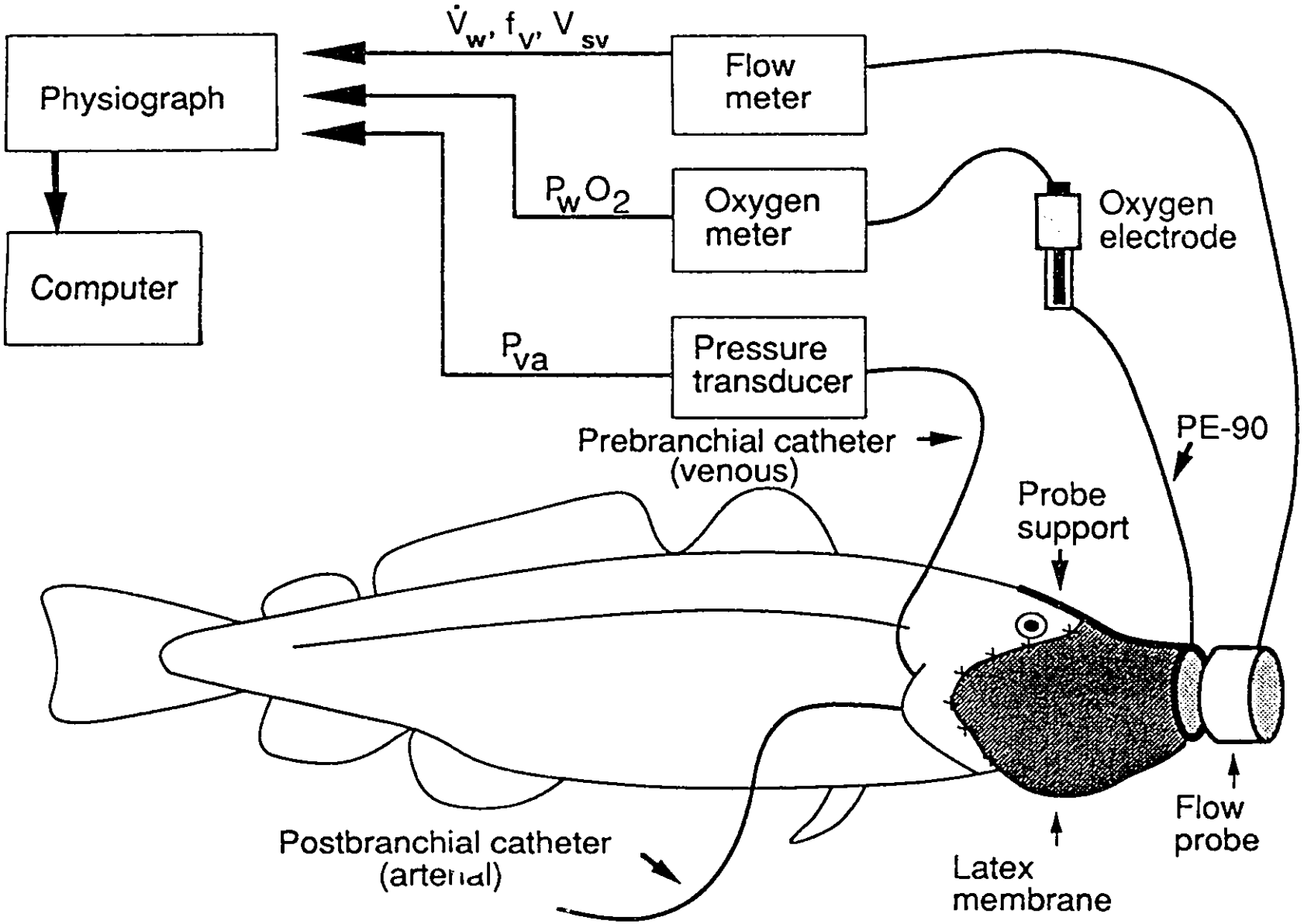
Analytical procedures

PwO_2 was sample ¹ from a piece of PE 90 cannulae that was mounted onto the flow probe and connected to the electrode chamber in such a way that gravity allowed continuous flow of the inspired water. The PwO_2 measurements were recorded on the polygraph. Plasma EPI and NE levels were determined on alumina-extracted plasma samples with high performance liquid chromatography (HPLC) in conjunction with electrochemical detection using the method described by Fritsche and Nilsson (1990).

Statistical analysis

Variability of the data is indicated by ± 1 S.E.M. Results have been statistically analyzed by two way analysis of variance followed by a two-tailed Dunnett's t-test for multiple comparisons to a control group (level of significance = 0.05) which, in this case, was the respective 0 min value of each group.

Figure 8. A schematic diagram of the experimental set up for the flow mask along with the data acquisition system for ventilatory and cardiovascular parameters.


Physiograph
Computer
$\dot{V}_w$, f_V, V_{sv}
Flow meter
P_wO_2
Oxygen meter
Oxygen electrode
P_{va}
Pressure transducer
Prebranchial catheter (venous)
PE-90
Probe support
Postbranchial catheter (arterial)
Latex membrane
Flow probe

RESULTS

The results of the initial validation experiments demonstrated that none of the variables assessed (f_V, O_2 tension and content along with plasma [catecholamines]) were affected by the installation of the flow probe apparatus. The data are not shown here for brevity.

I: Ventilatory and hypertensive responses to hypoxia.

Depression of PwO_2 caused a rapid increase in $\dot{V}w$ that plateaued after 10 min (PwO_2 ~ 59 Torr, Fig 9). During the initial 10 min of hypoxia, the hyperventilatory response was caused solely by an increase of f_V (Fig 9). When hypoxia became more severe, however, ($PwO_2 \leq 53$ Torr), a significant increase in V_{sv} was observed (Fig 11) while the increase in f_V was of lesser importance (Fig 10).

The pattern and magnitude of the P_{va} response to hypoxia were similar to results previously reported for cod (Fritsche and Nilsson, 1989; 1990). Figure 12 illustrates that P_{va} increased rapidly from a value of 41 cm H_2O to a peak of 82 cm H_2O within 5 min of the onset of hypoxia.

Figure 13 is a representative original recording of selected ventilatory and cardiovascular variables from a single fish which illustrates the sensitivity of cod to hypoxia. Note that a depression of PwO_2 from only 155 to 110 Torr was sufficient to provoke a pronounced hyperventilation, which was mediated largely by an increase in f_V; an increase in P_{va} was also observed simultaneously. It is also evident from this figure that the changes in $\dot{V}w$, f_V and P_{va} were initiated well before the final PwO_2 of 110 Torr was reached.

After 30 min of exposure to hypoxia, plasma concentrations of both catecholamines (NE and EPI) were elevated in venous blood (Table 4). No differences were observed in the catecholamines concentrations between plasma from pre- (arterial) or post-branchial (venous) blood. In both arterial and venous blood, oxygen tension and content were lowered while pH was elevated (Table 5).

Blood acid-base/respiratory variables, plasma catecholamines levels along with P_{va}, $\dot{V}w$, V_{sv} and f_V returned to their pre-experimental levels one hour after cessation of the experiment. Note that in the normoxic control group, none of the measured variables were significantly affected by the blood sampling and replacement protocol (Tables 4 and 5, Figs 8, 9, 10, 11 and 12).

II: Ventilatory and hypertensive responses to hypoxia during selective peripheral or nervous adrenergic blockade.

In all treatment groups, the ventilatory response to hypoxia was similar to the hypoxic control group (Fig 9 A, B and C) with the exception of the phentolamine treated group in which the $\dot{V}w$ increase was mediated solely by changes V_{sv} (Figs 10A and 11A). In addition, when expressed on a percent change basis, it is clear that none of the treatments affected the peak ventilatory response to hypoxia (Fig. 14). This type of normalized comparison was made since the resting $\dot{V}w$ in the various groups varied, although analysis of variance demonstrated that the differences were not statistically significant.

An increase in plasma NE and/or EPI levels was observed in all treatment groups during hypoxia (Table 4). Blood oxygen status was depressed and pHa and/or pHv elevated in a similar manner as the hypoxic control group except in the phentolamine-treated group

which did not exhibit the typical alkalosis or a significant reduction of oxygen content.

The blood pressure response of phentolamine-treated fish paralleled the one of the normoxic control group since no increase was observed during the hypoxic period (Fig 12 A). Bretylium treatment, on the other hand, only delayed the hypoxia-induced hypertension since P_{va} remained at the pre-hypoxic level for at least 5 min, until PwO_2 decreased below 96 Torr. The blood pressure response in the sotalol-treated group did not differ from the control group (Fig 12 B).

Sotalol treatment appeared to impair recovery from hypoxia because V_{sv} remained slightly elevated 60 min after return to normoxia (Fig 11 B). Furthermore, plasma NE concentration remained elevated while PvO_2 did not return to pre-hypoxic levels (Tables 4 and 5).

Figure 9. The effect of exposure to progressive hypoxia for 30 min on gill ventilation volume followed by the recovery upon return to normoxia. Panel A shows the response of the hypoxic (O---O) and normoxic (▵---▵) control groups as well as the one of another group pre-treatment with the α-adrenoceptor antagonist phentolamine (♦——♦). Panels B and C shows the responses of groups pre-treated with the β-adrenoceptor antagonist sotalol (●——●) or the adrenergic nerve release inhibitor bretylium (▲——▲) respectively. In each panel, the response of the normoxic control group was transposed to enable comparison. The average PwO_2 measured throughout the experiment is indicated on the x-axis of panel A. * indicates a value statistically different from the 0 min value (Pre hypoxic).

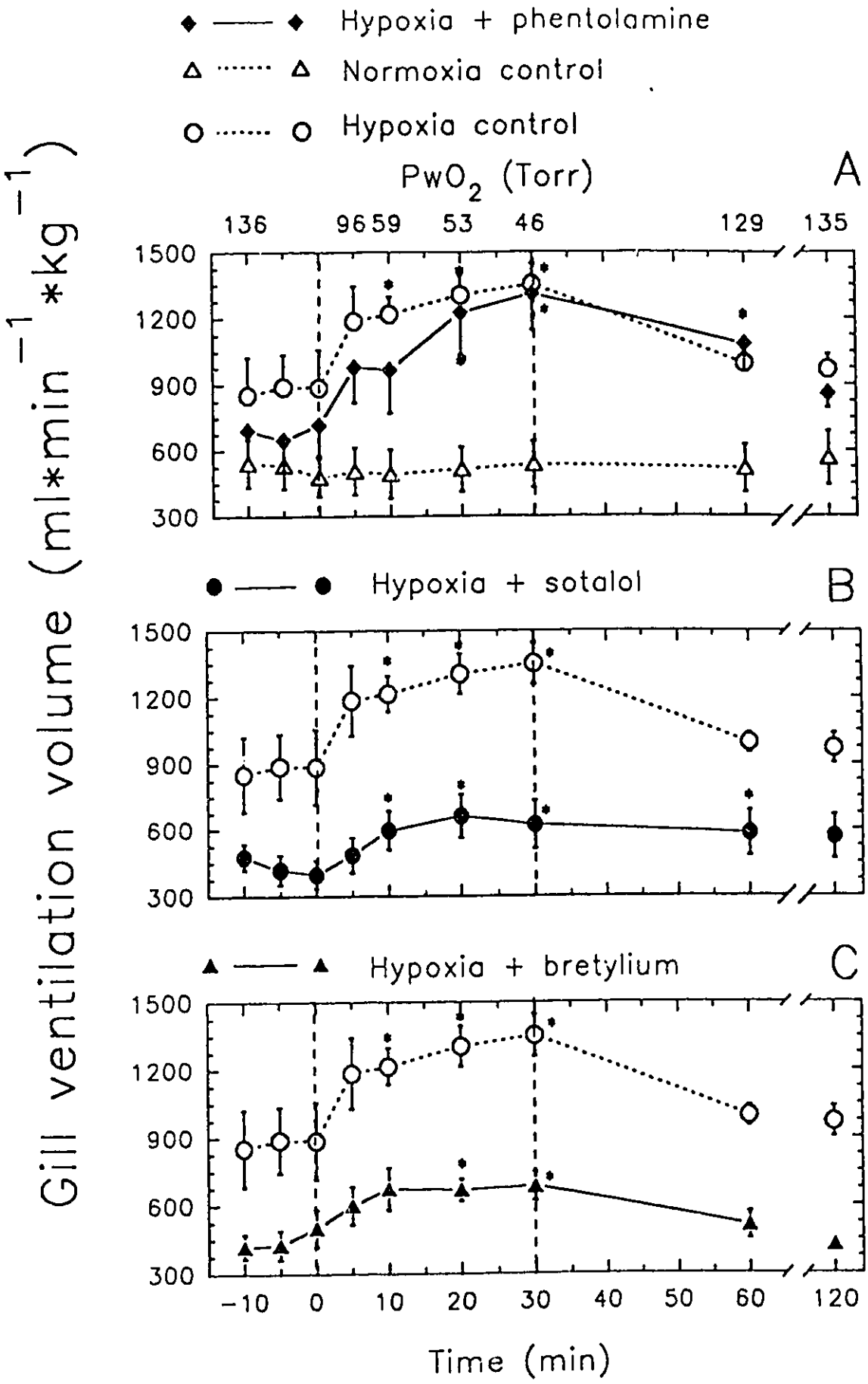

Hypoxia + phentolamine
Normoxia control
Hypoxia control
PwO2 (Torr)
A
136
96
59
53
46
129
135
1500
1200
900
600
300
Hypoxia + sotalol
B
Hypoxia + bretylium
C
−10
0
10
20
30
40
50
60
120
Time (min)
Gill ventilation volume (ml*min−1*kg−1)

Figure 10. The effect of exposure to progressive hypoxia for 30 min on ventilation frequency followed by the recovery upon return to normoxia. Further details of this figure are given in figure legend 9.

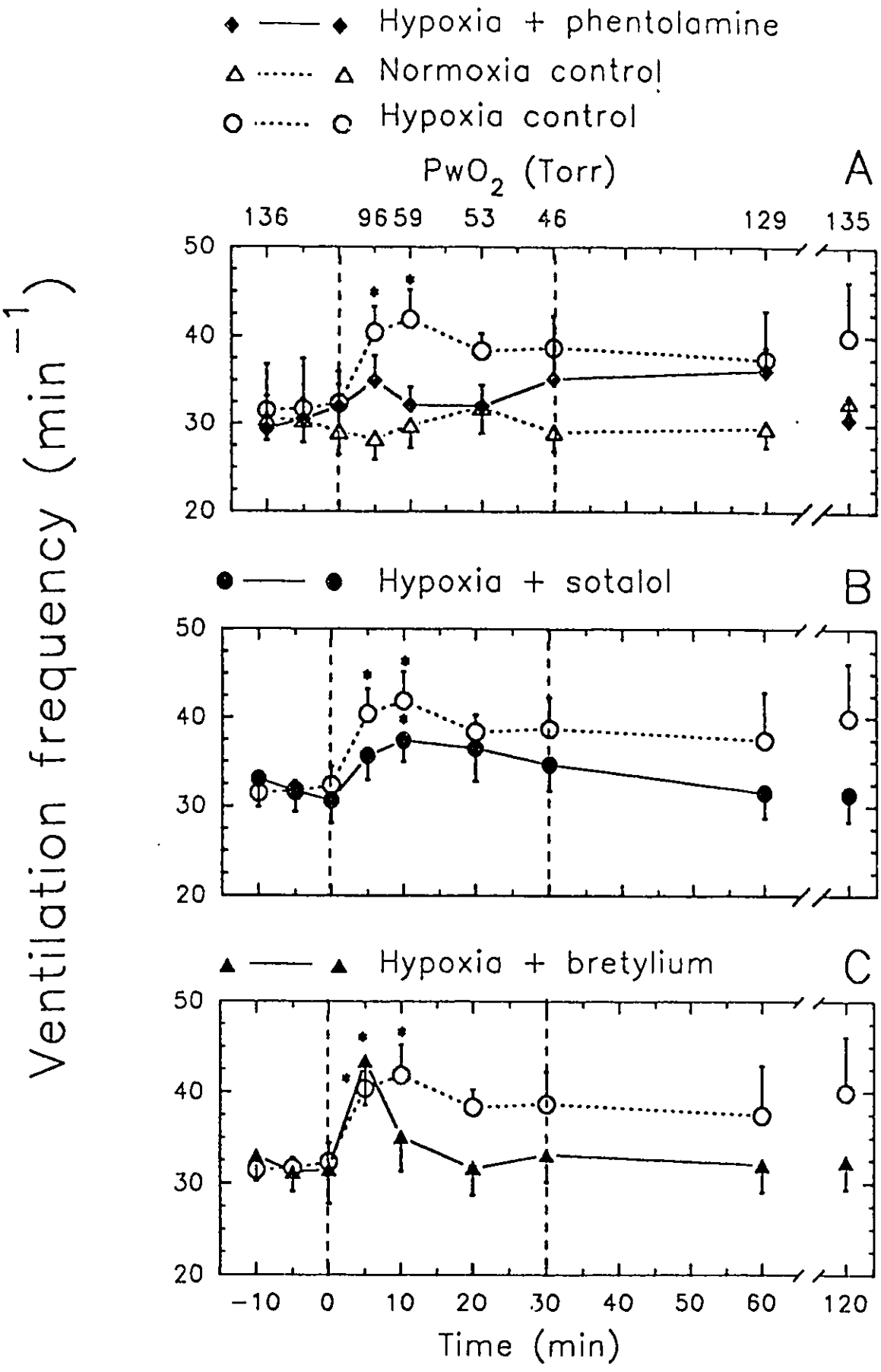

Hypoxia + phentolamine
Normoxia control
Hypoxia control
PwO2 (Torr)
A
136
96 59
53
46
129
135
50
40
30
20
Hypoxia + sotalol
B
Hypoxia + bretylium
C
−10
0
10
20
30
40
50
60
120
Time (min)
Ventilation frequency (min−1)

Figure 11. The effect of exposure to progressive hypoxia for 30 min on ventilation stroke volume followed by the recovery upon return to normoxia. Further details of this figure are given in figure legend 9.

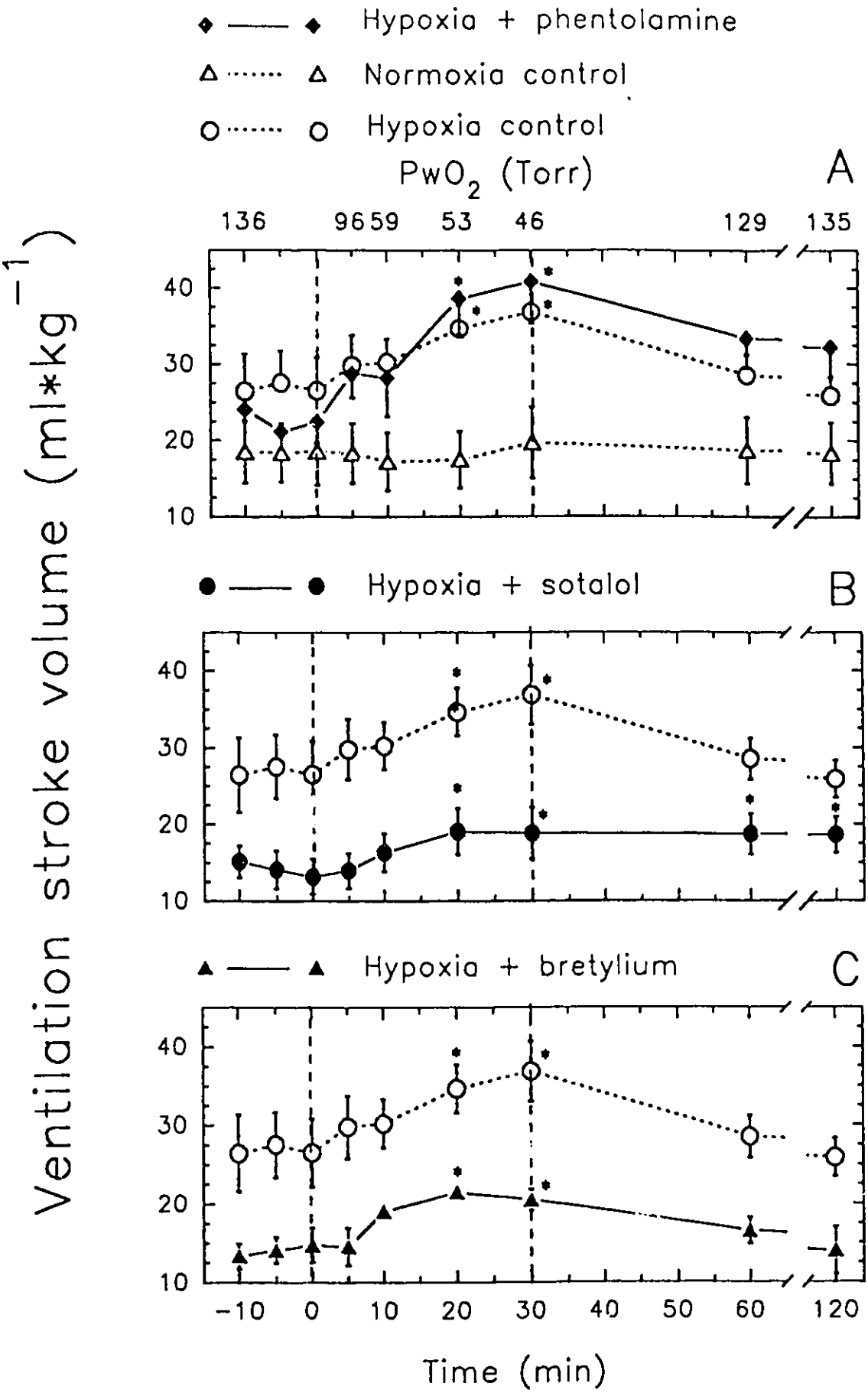

Hypoxia + phentolamine
Normoxia control
Hypoxia control
PwO2 (Torr)
A
136
96
59
53
46
129
135
40
30
20
10
Hypoxia + sotalol
B
Hypoxia + bretylium
C
Ventilation stroke volume (ml*kg-1)
-10
0
10
20
30
40
50
60
120
Time (min)

Figure 12. The effect of exposure to progressive hypoxia for 30 min on ventral aortic blood pressure followed by the recovery upon return to normoxia. Further details of this figure are given in figure legend 9.

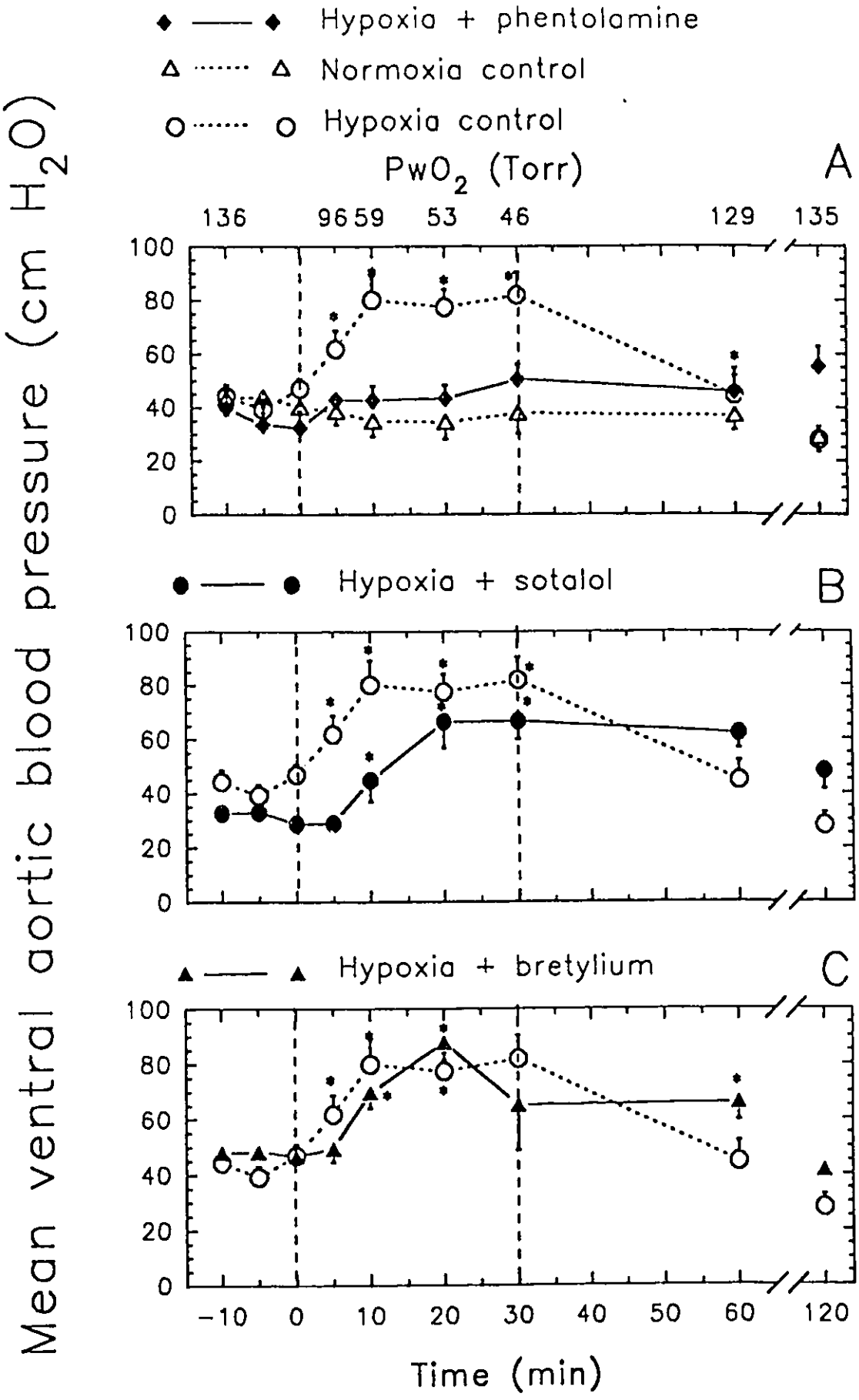

Hypoxia + phentolamine
Normoxia control
Hypoxia control
PwO2 (Torr)
A
136
96 59
53
46
129
135
Hypoxia + sotalol
B
Hypoxia + bretylium
C
Mean ventral aortic blood pressure (cm H2O)
Time (min)
100
80
60
40
20
0
-10
0
10
20
30
40
50
60
120

Figure 13. Original recording displaying the ventilatory ($\dot{V}w$ and f_v) and blood pressure responses (P_{va}) of a single fish to mild hypoxia.

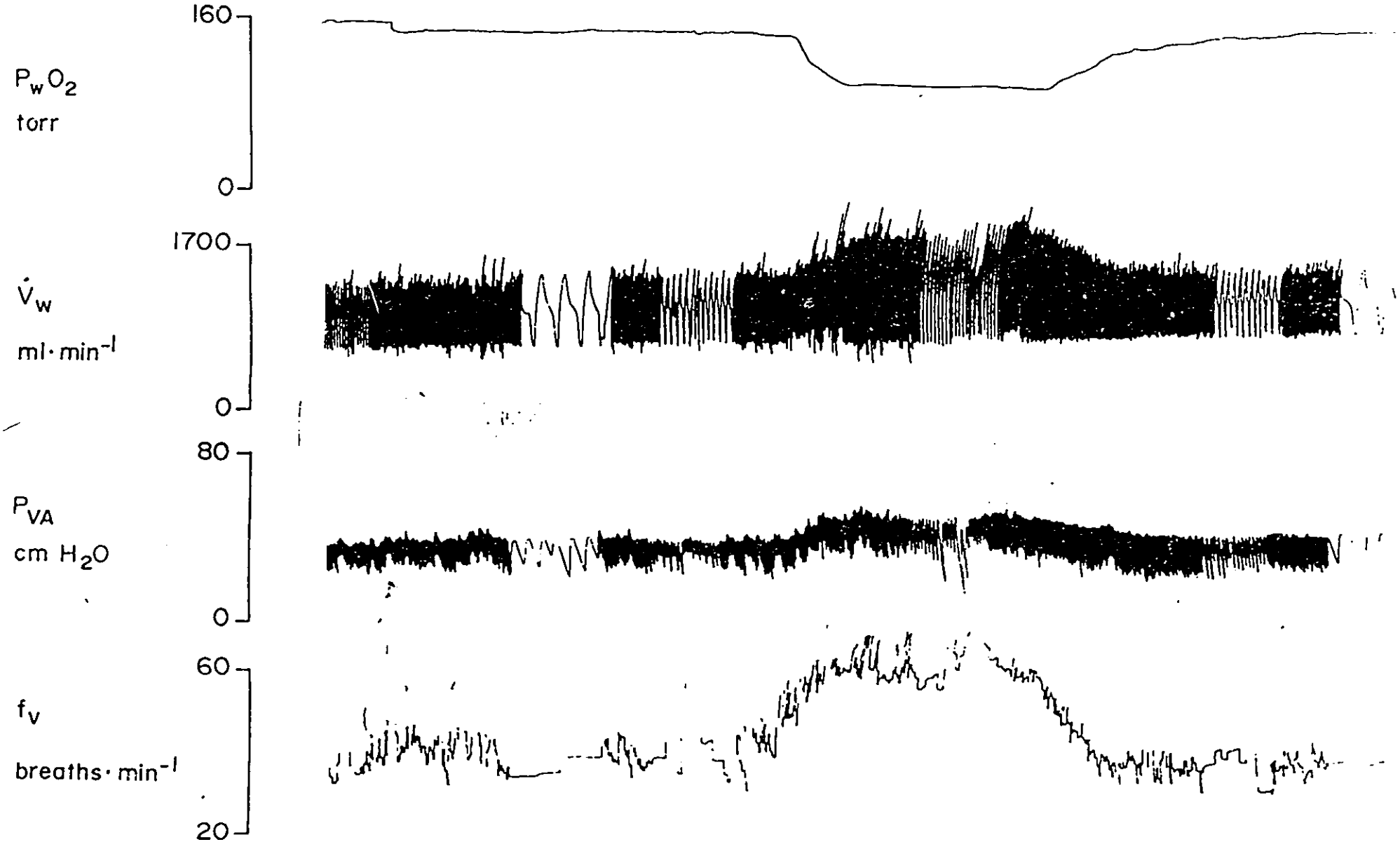

160
P_wO_2
torr
0
1700
$\dot{V}_w$
ml·min^{-1}
0
80
P_{VA}
cm H_2O
0
60
f_v
breaths·min^{-1}
20
2 min

Figure 14. Peak ventilatory responses to the experimental conditions of the 5 groups expressed in proportion to the resting $\dot{V}w$. * indicates a value significantly different from the hypoxic control group.

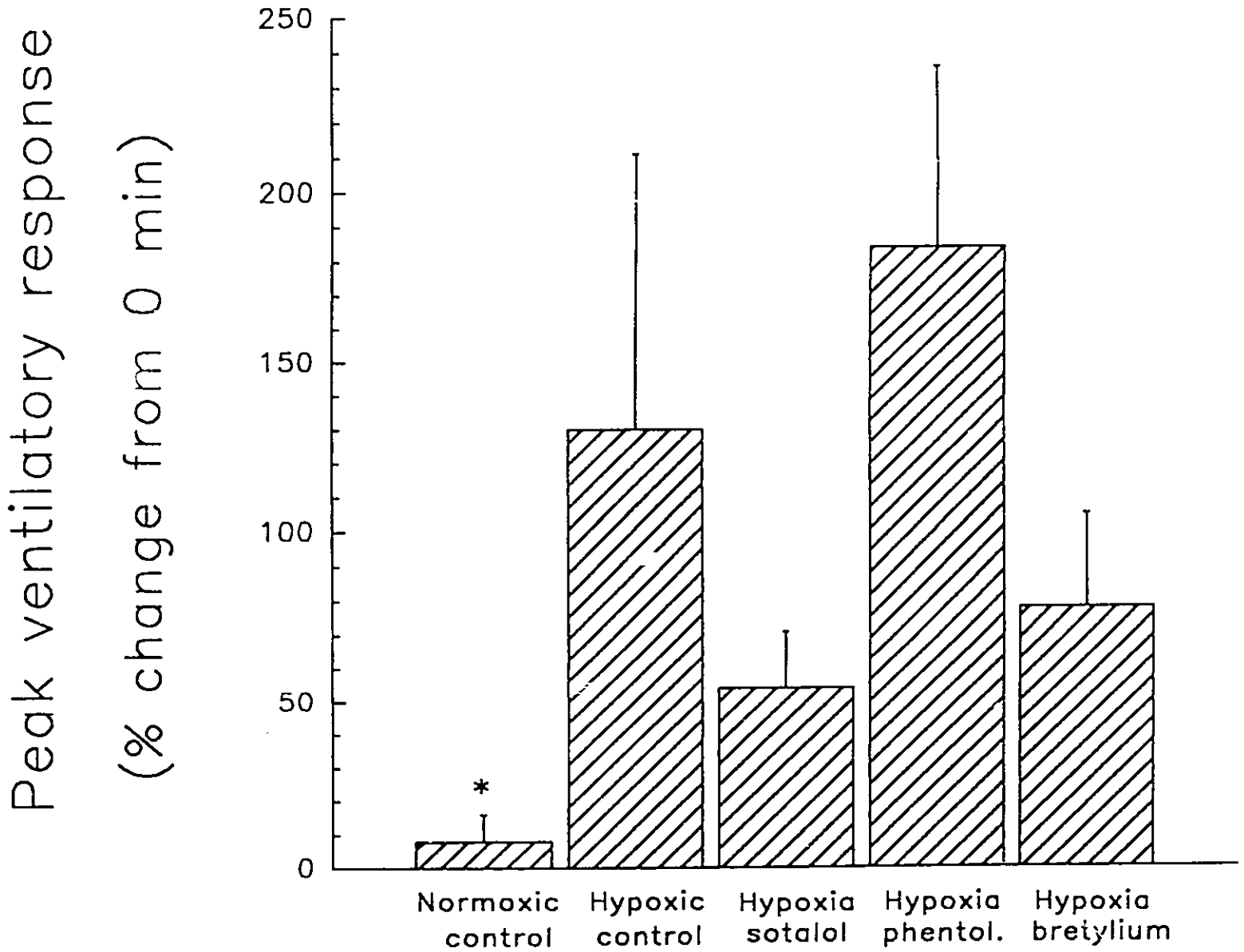

Peak ventilatory response
(% change from 0 min)
250
200
150
100
50
0
*
Normoxic control
Hypoxic control
Hypoxia sotalol
Hypoxia phentol.
Hypoxia bretylium

Table 4: Plasma catecholamine [epinephrine (EPI) and norepinephrine (NE)] levels monitored before and after 30 min of exposure to hypoxia as well as after 60 min of recovery. Data are shown as means ± 1 standard error of the mean which is indicated in parentheses. * indicates a value significantly different from the 0 min measurement.

		NORMOXIA CONTROL [N = 7]		HYPOXIA CONTROL [N = 8]		HYPOXIA SOTALOL [N = 7]		HYPOXIA PHENTOLAMINE [N = 7]		HYPOXIA BRETYLIUM [N = 7]	
		ART	VEN	ART	VEN	ART	VEN	ART	VEN	ART	VEN
PRE (0 MIN)	NE	1.8 (0.8)	2.4 (1.6)	4.1 (2.1)	4.0 (1.4)	0.8 (0.3)	1.0 (0.4)	3.5 (1.0)	3.7 (1.0)	2.8 (1.4)	0.6 (0.3)
	EPI	1.3 (0.3)	3.4 (1.4)	3.5 (1.4)	2.8 (0.7)	0.7 (0.2)	0.9 (0.2)	2.8 (1.4)	1.5 (0.4)	2.5 (1.1)	2.5 (0.2)
30 MIN	NE	0.6 (0.2)	4.4 (2.4)	11.0 (5.4)	11.0* (2.8)	4.9 (2.4)	3.3 (1.6)	11.8* (2.6)	22.8* (9.4)	17.5* (5.2)	6.2* (1.4)
	EPI	1.2 (0.2)	3.6 (2.0)	9.4 (5.6)	8.4* (3.6)	3.6* (1.2)	4.0* (1.1)	16.9* (4.8)	17.4* (8.2)	19.3* (5.5)	14.5* (3.1)
RECOVERY	NE	2.4 (1.6)	4.9 (2.2)	4.5 (2.0)	9.6 (4.0)	6.4* (3.1)	2.6 (1.0)	5.5 (2.3)	8.3 (2.0)	7.6 (3.6)	3.7 (1.8)
	EPI	3.3 (1.6)	2.9 (1.1)	2.2 (0.8)	2.4 (0.6)	2.6 (0.7)	1.9 (0.4)	2.7 (1.6)	4.5 (1.4)	3.2 (1.1)	2.3 (0.9)

Table 5: Blood respiratory and acid-base variables monitored before and after 30 min of exposure to hypoxia as well as after 60 min of recovery. Data are shown as means ± 1 standard error of the mean which is indicated in parentheses. * indicates a value significantly different from the 0 min measurement.

		NORMOXIA CONTROL [N = 7]		HYPOXIA CONTROL [N = 8]		HYPOXIA SOTALOL [N = 7]		HYPOXIA PHENTOLAMINE [N = 7]		HYPOXIA BRETYLIUM [N = 7]	
		ART	VEN	ART	VEN	ART	VEN	ART	VEN	ART	VEN
PO_2 (Torr)	PRE	66 (5.2)	34 (4.4)	84 (9.7)	39 (3.2)	64 (6.2)	35 (6.8)	92 (10.4)	45 (5.7)	57 (5.9)	31 (4.2)
	30 MIN	61 (5.0)	36 (3.7)	17* (0.9)	16* (1.6)	17* (2.7)	12* (1.8)	42* (5.7)	27* (3.5)	18* (1.3)	10* (0.8)
	REC	69 (5.8)	37 (2.8)	82 (4.9)	37 (3.0)	73 (7.3)	24* (2.7)	96 (16.3)	43 (4.2)	69 (8.1)	24 (2.9)
O_2 CONTENT (ml/dl)	PRE	6.7 (0.8)	3.9 (0.7)	5.9 (0.4)	3.5 (0.4)	4.6 (0.8)	3.1 (0.8)	4.1 (1.0)	2.5 (0.7)	4.2 (0.3)	2.9 (0.7)
	30 MIN	6.0 (1.0)	3.7 (0.8)	2.9* (0.3)	1.5* (0.2)	1.4* (0.4)	0.7* (0.2)	2.4 (0.3)	1.6 (0.3)	1.8* (0.2)	0.6 (0.2)
	REC	6.4 (1.1)	3.1 (0.5)	5.5 (0.3)	3.2 (0.4)	5.6 (0.5)	2.3 (0.5)	3.0 (0.6)	2.4 (0.5)	5.6 (0.6)	3.5 (0.8)
pHe	PRE	7.91 (0.02)	7.92 (0.02)	7.90 (0.004)	7.94 (0.01)	7.80 (0.02)	7.85 (0.03)	7.89 (0.01)	7.90 (0.02)	7.82 (0.03)	7.78 (0.06)
	30 MIN	7.96 (0.004)	7.95 (0.01)	8.06* (0.04)	8.05* (0.03)	7.89* (0.02)	7.84 (0.05)	7.94 (0.03)	7.84 (0.08)	7.90* (0.04)	7.93* (0.03)
	REC	7.94 (0.004)	7.93 (0.01)	7.93 (0.02)	7.96 (0.01)	7.81 (0.03)	7.85 (0.02)	7.89 (0.02)	7.92 (0.02)	7.86 (0.03)	7.88 (0.03)

DISCUSSION

This study is the first to describe the ventilatory response of the Atlantic cod (*Gadus morhua*) to external hypoxia. This quantification was performed using an electromagnetic flow probe apparatus. The results of the preliminary experiments clearly demonstrated the validity of this method to measure $\dot{V}w$ since it did not impair ventilation, gas transfer or cause higher concentrations of plasma catecholamines. In addition to the basic characterisation of the ventilatory response of cod to hypoxia, it was showed that i) circulating catecholamines, whether of neural or humoral origin, do not contribute to the hyperventilation during hypoxia and ii) under those conditions the acute changes of blood pressure do not influence breathing.

I. Characterisation of the ventilatory response of cod to hypoxia.

Cod displayed a breathing response to progressive hypoxia that can essentially be separated into two distinct phases. First, the rapid response to slight or moderate depressions in PwO_2 consisted primarily of a pronounced increase in f_V (PwO_2 > 53 Torr). The conclusions of several studies performed on teleosts argue in favour of O_2 chemoreceptors situated on the first pair of gill arches and facing the inspired water (Smith and Jones, 1978; Daxboeck and Holeton, 1978; Fritsche and Nilsson, 1990). The present results suggest that, in a fashion similar to the channel catfish (Burleson and Smatresk, 1990), the increase in f_v was initiated by these chemoreceptors. It is very doubtful that the internal O_2 content was affected by the initially mild hypoxia, given the nature of Hb-O_2 binding. Since hyperventilatory responses were observed at PwO_2 as high as 110 Torr, it is unlikely that

"internally oriented" chemoreceptors responding to Ca_{O2} variations are involved in this response. However, owing to the close relationship between water and arterial PO_2, the potential involvement of "internally oriented" chemoreceptors that responded to PaO_2 fluctuations cannot be excluded.

The second phase consisted of the increasing involvement of V_{sv} in the hyperventilation (Fig 11). This component became evident when hypoxia became more severe ($PwO_2 \leq 53$ Torr) and was more likely to have an effect on the blood O_2 content. This interesting coincidence does not necessarily reflect the progressive stimulation of internal O_2 receptors. It is possible that, by analogy with elasmobranchs, this modification of the breathing pattern was due to a breath by breath regulation of rate and amplitude mediated by mechanoreceptors, also situated on gill arches, to ensure proper ventilation-perfusion matching at the gas exchange surface (Satchell and Way, 1962; for review see Jones and Milsom, 1982). It appears, however, that α-adrenoceptor blockade can modify this pattern since no significant elevation of f_V in the ventilatory response to hypoxia was observed in the phentolamine treated group. The precise cause(s) of this modification are difficult to determine with the present data. Nevertheless, since the magnitude of the $\dot{V}w$ response of this particular group was unaltered, a possible explanation might again involve the relationship between f_V and V_{sv}.

II: Implication of catecholamines in the ventilatory response to hypoxia.

During hypoxia, cod did not develop metabolic acidosis unlike fish exposed to more severe or prolonged hypoxia (Thomas and Hughes, 1982; Tetens and Lykkeboe, 1985; Boutilier *et al.* 1988). Therefore, the present protocol enabled us to focus our study on two

potential ventilatory stimulants namely hypoxia (internal and/or external) and elevated plasma catecholamines.

The results of previous studies, which argued in favour of a stimulatory role for catecholamines on $\dot{V}w$ in fish (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980; Aota *et al.* 1990) justified the initial predictions of the present study. That is, pre-treatment with selective adrenoceptor antagonists could modify the ventilatory response to hypoxia. More precisely, since stimulation of β-adrenoceptors causes hyperventilation, while stimulation of α-adrenoceptors provokes hypoventilation in the eel (Peyraud-Waitzenegger, 1979), it was expected that fish pre-treated with phentolamine would display a significantly greater hyperventilation when catecholamines are released during hypoxia. Conversely, also based on the results of Peyraud-Waitzenegger (1979) and Aota *et al.* (1990), a diminished hyperventilatory response was anticipated in fish pre-treated with sotalol. Since neither adrenoceptor-antagonist impaired significantly the hyperventilatory responses to hypoxia, despite the increase of plasma catecholamine levels, the present data obviously differs from predictions based on the two aforementioned studies. On the other hand, these results support the study presented in Chapter 3. By using a similar pharmacological approach, no effects of selective adrenoceptor-blockade on the hyperventilatory response to moderate hypoxia (PwO_2 = 72 Torr) in rainbow trout were reported.

The discrepancies that exist between the present results and the ones reported for the eel (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980) may reflect important inter-species differences. These differences could be based on the sensitivity of the response to the circulating levels of catecholamines. That is, since Peyraud-Waitzenegger *et al.* (1980) suggested that catecholamines act on the central nervous system, it is possible that in those fish, the effects observed on ventilation following intra-arterial injections of catecholamines

reflect a highly sensitive system coupled with a blood-brain barrier permeable to catecholamines (Peyraud-Waitzenegger *et al.* 1979). This explanation is supported by observations made in rainbow trout where intra-arterial infusions of physiological doses of catecholamines only transiently depress ventilation under normoxic conditions (Playle *et al.* 1990; present study, Chapter 3). Furthermore, preliminary work performed on cod indicate that the response of this species to intra-arterial catecholamine infusion are similar to the ones reported in trout (R. Fritsche, unpublished observations). The differences that may exist between the eel and other species in terms of the sensitivity of the ventilatory response to catecholamines are difficult to assess since Chapters 3 and 6 of this study are the only one that reports measured values rather than estimates of circulating catecholamine levels at the end of the infusion.

Since the results of the work described in this Chapter are similar to the ones of Chapter 3, they obviously differ from the ones of Aota *et al.* (1990). Accordingly, the explanation of the discrepancies discussed in Chapter 3 also apply to the present case and, for the sake of brevity will not be reiterated.

The findings that cod can hyperventilate in the absence of peripheral α- or β-adrenergic function, support the conclusion that circulating catecholamines do not play a role in the stimulation of ventilation in rainbow trout (present study, Chapter 3) and the dogfish (Metcalfe and Butler, 1988) during hypoxia. Even in the European eel, where catecholamine injections were shown to cause hyperventilation (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980), a physiological role for catecholamines on $\dot{V}w$ is unclear. In fact, it was demonstrated recently that during severe hypoxia (PwO_2 = 40 Torr) European eels hyperventilate in the absence of elevated plasma catecholamine levels (Peyraud-Waitzenegger and Soulier, 1989). Hence, of all the factors previously mentioned as being potential

stimulants (see Chapter 1), only depression of the oxygen status in the water and/or in the blood, could account for the aforementioned ventilatory responses since blood pH was not lowered.

The circulating levels of catecholamines were measured in pre- and post-branchial blood since in trout, it has been reported that the gill has the capacity of influencing the general adrenergic activity through extraction and metabolism of circulating catecholamines (Nekvasil and Olson, 1986*a*). It is apparent from the present results that a different situation exists in cod as no consistent trend could be detected between pre- and post-branchial catecholamine levels. This suggests that in cod, the contribution of the gills in the regulation of plasma catecholamine concentrations *via* gill tissue uptake and/or metabolism is absent or relatively minor compared to the *in vitro* results obtained with perfused trout gills (Nekvasil and Olson, 1986*b*). It should be noted that discrepancies between results obtained from *in vitro* perfused preparations and whole animals have been reported on numerous occasions (see review by Perry and Farrell, 1989).

III: The relationship between hypertension and ventilation during hypoxia.

The observation that bretylium delayed the initial hypertension supports the hypothesis that the initial rapid increase of P_{va} during hypoxia, owing to an increase in systemic vascular resistance, is under adrenergic nervous control (Smith *et al.* 1985; Fritsche and Nilsson, 1990). The increase observed after ~10 min of hypoxia reflects the time at which plasma levels of catecholamines become elevated in cod (Perry *et al.* 1990) and thus can account for the elevation of P_{va} in the bretylium-treated fish. This was confirmed by the absence of any increase of P_{va} when the α-component of the adrenergic response was totally

abolished by phentolamine. Soulier *et al.* (1988) reported that in the European eel, a blood pressure increase of at least 50% was required to elicit significant effects on $\dot{V}w$. When control cod were subjected to hypoxia, the P_{va} increases observed (~100%) were well above this suggested threshold. Since neither bretylium nor phentolamine significantly altered the blood pressure response, affected $\dot{V}w$ during hypoxia, the hypothesis that hypertension can influence the breathing response to hypoxia in cod is not supported by the present data.

CHAPTER 5

THE EFFECTS OF CHANGES IN BLOOD RESPIRATORY STATUS AND PLASMA CATECHOLAMINE LEVELS IN THE REGULATION OF VENTILATION DURING ACUTE HYPERCAPNIA IN RAINBOW TROUT (*Oncorhynchus mykiss*)

INTRODUCTION

The two preceding Chapters demonstrated that circulating catecholamines did not mediate the regulation of the increases in $\dot{V}w$ during hypoxia. These findings differed from those of Aota *et al.* (1990) who utilized a similar protocol to assess the role of circulating catecholamines in the ventilatory responses to hypoxia and intra-arterial acid infusion in trout. These workers concluded that under those conditions, β-adrenergic stimulation is required to evoke a hyperventilation. Aside from the differences in the levels of hypoxia utilized with corresponding dissimilar effects on blood respiratory status, both experimental protocols used by Aota *et al.* (1990) induced acidemia. Since acidemia was not observed in the studies reported in Chapters 3 or 4, this may explain the discrepancies between the two studies. Thus, it is possible that i) elevated CO_2/H^+ in the blood is required for catecholamines to exert their action or ii) that circulating catecholamines play a role in the regulation of ventilatory responses associated specifically with elevated CO_2/H^+.

In the present Chapter, the role of circulating catecholamines in the regulation of $\dot{V}w$ was assessed by pre-treating fish with the selective β-adrenoceptor antagonist, propranolol, prior to exposure to physiologically relevant levels of external hypercapnia. In addition to the fact that the potential involvement of circulating catecholamines in the ventilatory response to hypercapnia has not been investigated previously, this experimental condition was chosen for its ability to stimulate ventilation as well as catecholamine mobilization while concomitantly inducing respiratory acidosis.

The elevation of $\dot{V}w$ observed during exposure to elevated environmental PCO_2 is well documented (Dejours, 1973; Janssen and Randall, 1975; Smith and Jones, 1982; Thomas *et al.* 1983, for review see Perry and Wood, 1989). Chapter 2 described in detail why it may

be incorrect to attribute the hyperventilatory response to external hypercapnia to acidosis-induced hypoxemia and to minimize, if not ignore, the potential effects of respiratory acidosis. Because Ca_{O2} and circulating catecholamine levels can also be influenced by environmental hypercapnia, hyperoxic hypercapnia was used as a tool to study the specific effects of respiratory acidosis on $\dot{V}w$ by preventing the usual reduction of Ca_{O2} and elevation of the circulating levels of catecholamines (Perry *et al.* 1989).

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex, weighing between 147 and 364 g [mean mass = 226 ± 9 g (standard error of the mean; S.E.M.)] were obtained from Thistle Springs Trout Farm (Ashton, Ontario). The temperature of the holding and experimental water varied between 13°C and 19°C (May to August; mean temperature = 15.6 ± 0.3°C).

Experimental protocol

1. Experimental conditions

Series I: Ventilatory adjustments to external hypercapnia.

The condition of external normocapnia or hypercapnia was achieved by gassing the cephalic chamber of the van Dam box, as well as a counter-current gas exchange column (which supplied water to the cephalic chamber of the box), either with air ($PwCO_2$ ~ 0.3 Torr, N = 7) or 1.0% CO_2 in air ($PwCO_2$ = 5.9 ± 0.9 Torr, N = 6). All gases were supplied by Air Products Inc. (Ottawa, Ont.). A water sample (~10 ml) was withdrawn from the cephalic compartment at 10 min intervals during all experiments to determine $PwCO_2$. Fish exposed to the 1% CO_2 in air mixture served as a control group for the fish treated with β-adrenoceptor antagonists (Series II). Therefore, these animals were sham-injected *via* the dorsal aortic cannula with 0.4 ml of saline (pH = 7.8) 2 h before the initiation of hypercapnia.

Series II: Effect of β-adrenoceptor blockade on the ventilatory adjustments to normoxic hypercapnia.

Fish were injected with the β-adrenoceptor antagonist propranolol (DL-propranolol; Sigma Chemical Company) 2 h prior to being exposed to acute external hypercapnia ($PwCO_2$ = 5.5 ± 0.2 Torr, N = 6) to ensure adequate antagonistic activity (Nilsson, 1983). Previous experiments in our laboratory (e.g. Vermette and Perry, 1988*a*, *b*; Wright *et al.* 1989) have demonstrated that identical doses of propranolol can abolish or diminish β-adrenergic responses in trout (e.g. RBC alkalinization) for up to 8 h after injection. The drug was dissolved in Cortland saline immediately before use (final pH drug solution = 7.8) and injected (approximately 0.2 ml) into the dorsal aortic cannula of fish at a dose of 2 $mg \cdot kg^{-1}$ body weight. The cannula was flushed with an additional 0.2 ml of saline after each injection to ensure complete delivery of propranolol to the circulation.

Series III: Ventilatory adjustments to external hyperoxic hypercapnia

The same protocol as for series I was utilized to achieve the conditions of hyperoxic normocapnia (PwO_2 = 697 ± 23 Torr, $PwCO_2$ ~ 0.3 Torr, N = 7) or hyperoxic hypercapnia (PwO_2 = 680 ± 45 Torr, $PwCO_2$ = 5.1 ± 0.4 Torr, N = 7) using either pure O_2 or a gas mixture of 1% CO_2 in O_2, respectively.

2. Blood sampling

Three blood samples of 750 µl each were taken from all fish. An initial sample was withdrawn from the dorsal aortic cannula before the onset of the experiment (0 min), a second one after 30 min of exposure to the experimental condition (30 min) and a final sample was taken 30 min after cessation of the experiment (recovery).

After each blood sample, an equivalent volume of heparinized (10 units·ml^{-1}) saline was injected into the fish to restore blood volume. This procedure always was followed by a recording of ventilatory variables ($\dot{V}w$ and f_V) to see if they were affected by blood sampling. Arterial blood was analyzed immediately after sampling to determine PaO_2, pHa, Ca_{CO2}, and Ca_{O2} using the procedure described in Chapter 2 (Analytical procedures). $PaCO_2$ was calculated from the measured plasma Ca_{CO2} and pHa values using the Henderson-Hasselbalch equation. Remaining blood was centrifuged and the plasma (~250 µl) was combined with 10 µl of 5 mmol·l^{-1} sodium bisulphite and 20 µl of heparin (2500 units·ml^{-1}) before being stored at -70°C for subsequent determination of catecholamine levels.

3. Ventilatory measurements

Ventilatory variables of each fish were monitored in the following sequence: two pre-experimental measurements (Pre 1 and Pre 2) were taken 10 min apart. Another measurement was taken 10 min later (0 min), just prior to withdrawal of the first blood sample. The animal then was exposed to the particular experimental condition (normoxic normocapnia, normoxic hypercapnia, hyperoxic normocapnia or hyperoxic hypercapnia) for 30 min, at which time a second blood sample was withdrawn. During that period of time, the

ventilatory and cardiovascular variables were monitored every 10 min. The fish then was allowed to recover under normoxic conditions (PwO_2 = ~155 Torr) for 30 min before a final blood sample was taken.

Statistical analysis

Variability of the data is indicated by ± 1 S.E.M. Results have been statistically analyzed by two way analysis of variance followed by a two-tailed Dunnett's *t*-test for multiple comparisons to a control group (level of significance = 0.05) which, in this case, was the 0 min value of each group.

RESULTS

Since ambient water temperature affects resting $\dot{V}w$ values in trout (Randall and Cameron, 1973), notable differences were unavoidable between the different experimental groups owing to the varying water temperatures (13°-19°C). Thus, for clarity the data are presented as absolute changes of ventilation volume. These values were obtained by subtracting the 0 min value from those measured at the different time points.

I: Ventilatory adjustments to external hypercapnia.

After 30 min of exposure to hypercapnia (1% CO_2 in air), $\dot{V}w$ increased 1.4-fold (Fig 15; resting $\dot{V}w$ = 524 ± 69 $ml \cdot min^{-1} \cdot kg^{-1}$). As predicted, a depression of Ca_{O2} accompanied the respiratory acidosis while PaO_2 was unaltered (Fig 16). An increase of circulating catecholamines was detected after 30 min of hypercapnia (1% CO_2 in air) (Fig 17). Gill ventilation volume, as well as most of the variables monitored, returned to their pre-experimental levels upon cessation of the experiment (Figs 15, 16 and 17). Ca_{O2}, however, was the exception since it remained depressed (Fig 16). The resting $\dot{V}w$ of the normoxic (control) group was 359 ± 25 $ml \cdot min^{-1} \cdot kg^{-1}$.

II: Ventilatory responses to normoxic hypercapnia during β-adrenoceptor blockade.

Fish pre-treated with propranolol were unable to sustain the increase in $\dot{V}w$ normally observed throughout the 30 min of hypercapnia. The only significant increase in $\dot{V}w$ was recorded after 10 min of exposure to 1% CO_2 in air; at the 30 min measurement $\dot{V}w$ had

returned to the pre-experimental level (Fig 18; resting $\dot{V}w = 445 \pm 44$ $ml \cdot min^{-1} \cdot kg^{-1}$). None of the measured blood respiratory variables returned to their pre-experimental values after 30 min of cessation of the treatment (Fig 16).

III: Ventilatory responses to external hyperoxic hypercapnia.

Exposure to hyperoxic normocapnia elicited a 3.2-fold decrease in $\dot{V}w$ after 30 min (Fig 15; resting $\dot{V}w = 508 \pm 43$ $ml \cdot min^{-1} \cdot kg^{-1}$). PaO_2 was elevated while Ca_{O2} remained constant (Fig 16). $\dot{V}w$ and PaO_2 returned to their respective pre-experimental levels upon cessation of the treatment. Exposure to hyperoxic normocapnia did not affect blood acid-base status; after 30 min, pHa and $PaCO_2$ were not significantly different from the pre-experimental values (Fig 16). Hyperoxic hypercapnia caused an increase of $PaCO_2$ and a reduction of pHa (Fig 16), typical of respiratory acidosis. In these fish, $\dot{V}w$ was unaltered (Fig 15; resting $\dot{V}w = 256 \pm 61$ $ml \cdot min^{-1} \cdot kg^{-1}$). Also note that no significant changes in plasma catecholamine levels were detected after 30 min of exposure to hyperoxic hypercapnia (Fig 17). After 30 min of recovery from this treatment, $PaCO_2$ remained elevated while Ca_{O2} was depressed (Fig 16).

Figure 15. Variation of ventilation volume of rainbow trout during exposure to normoxic normocapnia (O—O, control group), hypercapnia (■—■, 1% CO_2 in air), hyperoxic hypercapnia (♦—♦, 1% CO_2 in O_2) and hyperoxic normocapnia (□—□, hyperoxia). The 30 min experimental period is situated between the two vertical dotted lines. Data are shown as group averages ± SEM; * indicates statistical difference from the 0 min value of the same group.

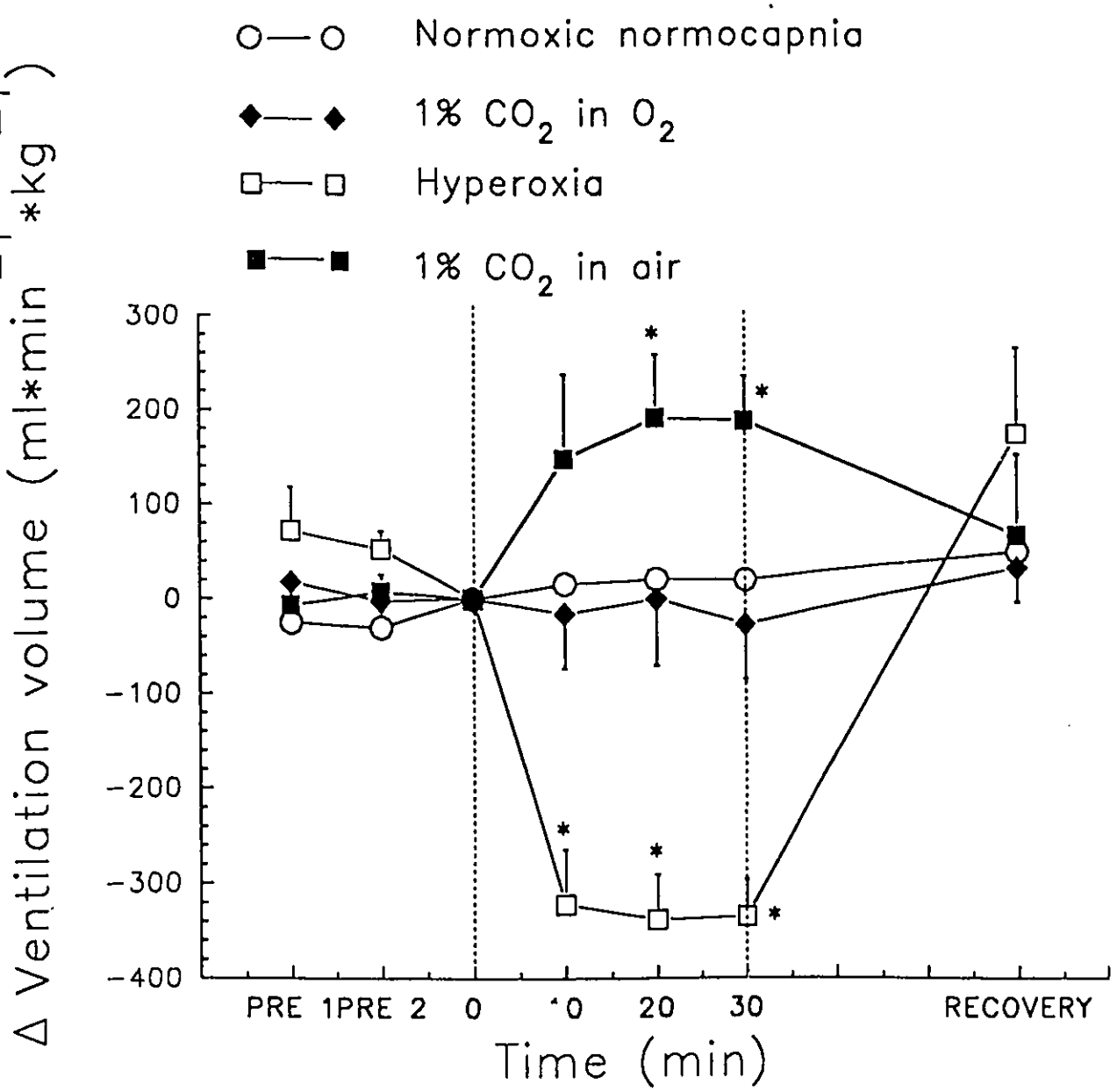

Normoxic normocapnia
1% CO_2 in O_2
Hyperoxia
1% CO_2 in air
Δ Ventilation volume (ml*min^{-1}*kg^{-1})
300
200
100
0
-100
-200
-300
-400
PRE 1
PRE 2
0
10
20
30
RECOVERY
Time (min)

Figure 16. The effects of normoxic normocapnia (control group), normoxic hypercapnia in sham-injected (1% CO_2 in air) and propranolol pre-treated fish (1% CO_2 in air + prop.) along with hyperoxia and hyperoxic hypercapnia (1% CO_2 in O_2) on selected blood acid-base and respiratory variables including A) arterial CO_2 tension ($PaCO_2$), B) arterial blood pH (pHa), C) arterial O_2 tension (PaO_2) and D) total arterial O_2 content (CaO_2). Measurements were taken before (0 min, hatched boxes) and after 30 min of exposure to the experimental condition (30 min, closed boxes). A terminal sample (recovery, open boxes) was taken 30 min after cessation of the experiment. Data are shown as group averages ± SEM; * indicates statistical difference from the 0 min value of the same group.

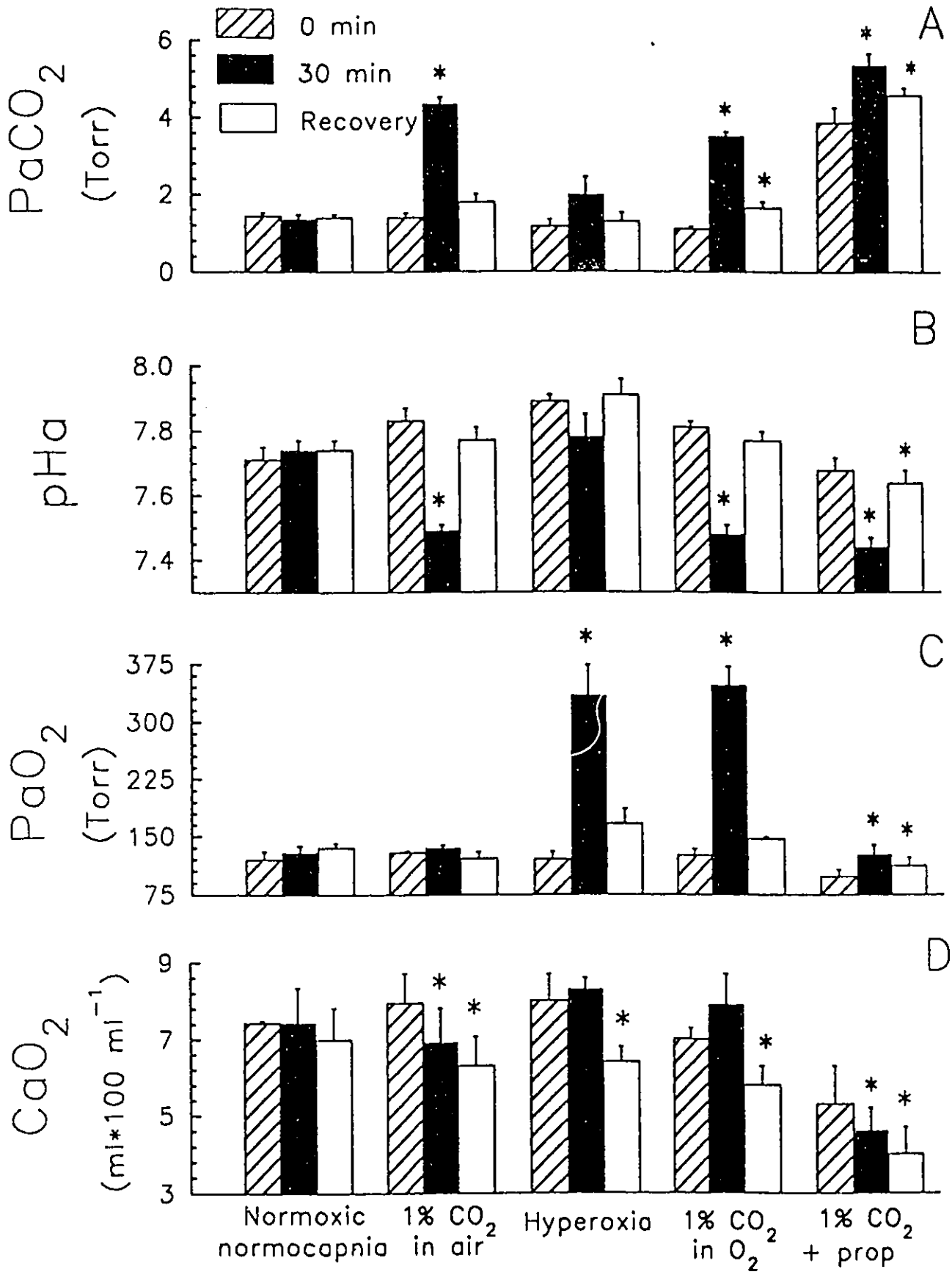

A
0 min
30 min
Recovery
$PaCO_2$ (Torr)
6
4
2
0
B
pHa
8.0
7.8
7.6
7.4
C
PaO_2 (Torr)
375
300
225
150
75
D
CaO_2 (ml*100 ml^{-1})
9
7
5
3
Normoxic normocapnia
1% CO_2 in air
Hyperoxia
1% CO_2 in O_2
1% CO_2 + prop

Figure 17. Circulating epinephrine (panel A) and norepinephrine (panel B) levels before (0 min; hatched box) and after 30 min of exposure to the experimental condition (30 min; filled box). The value of the sample taken 30 min after cessation of the experimental treatment is also shown (recovery, open box). The data (group average ± SEM) are displayed as plasma concentration (nM). * indicates statistical difference from the 0 min value.

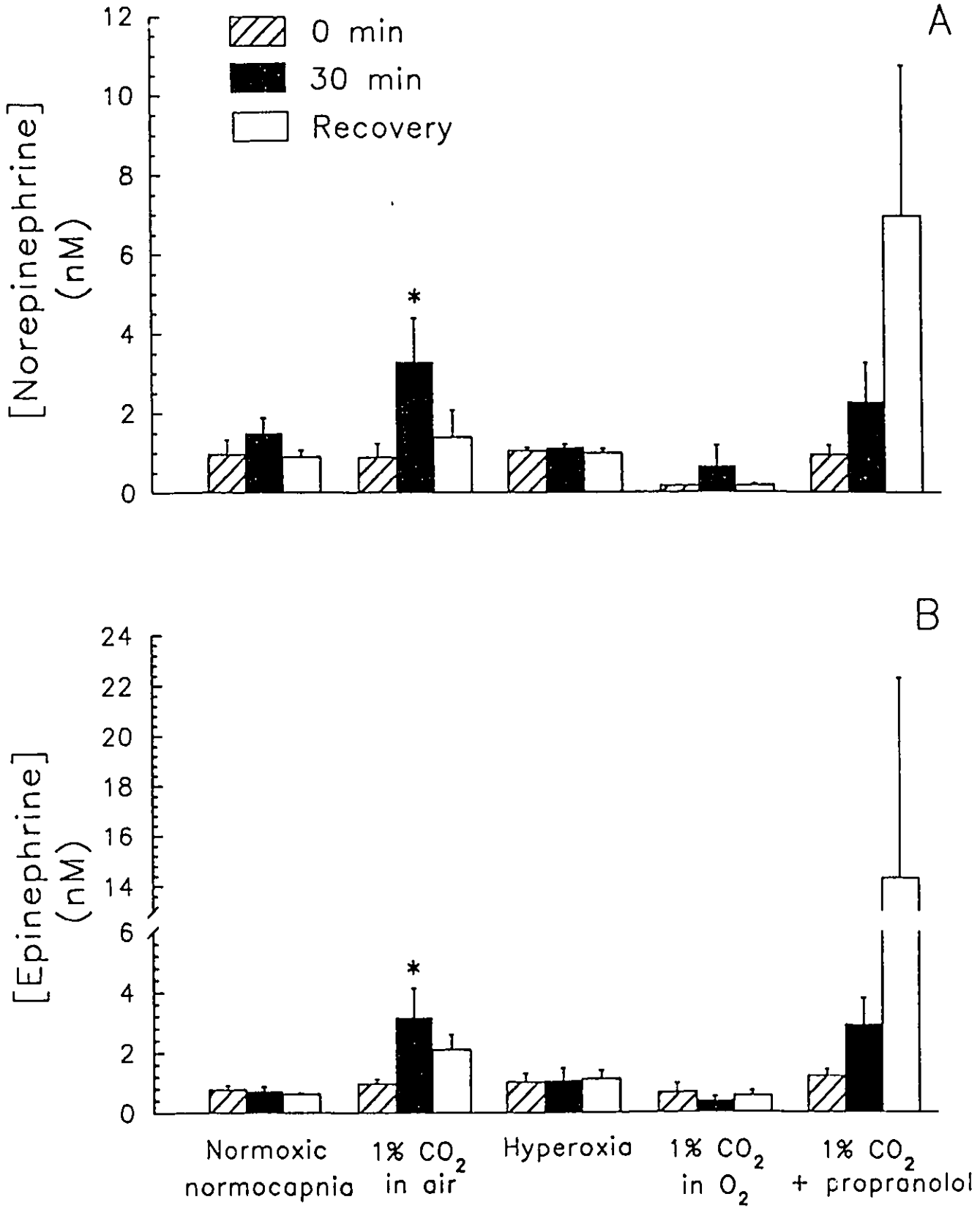
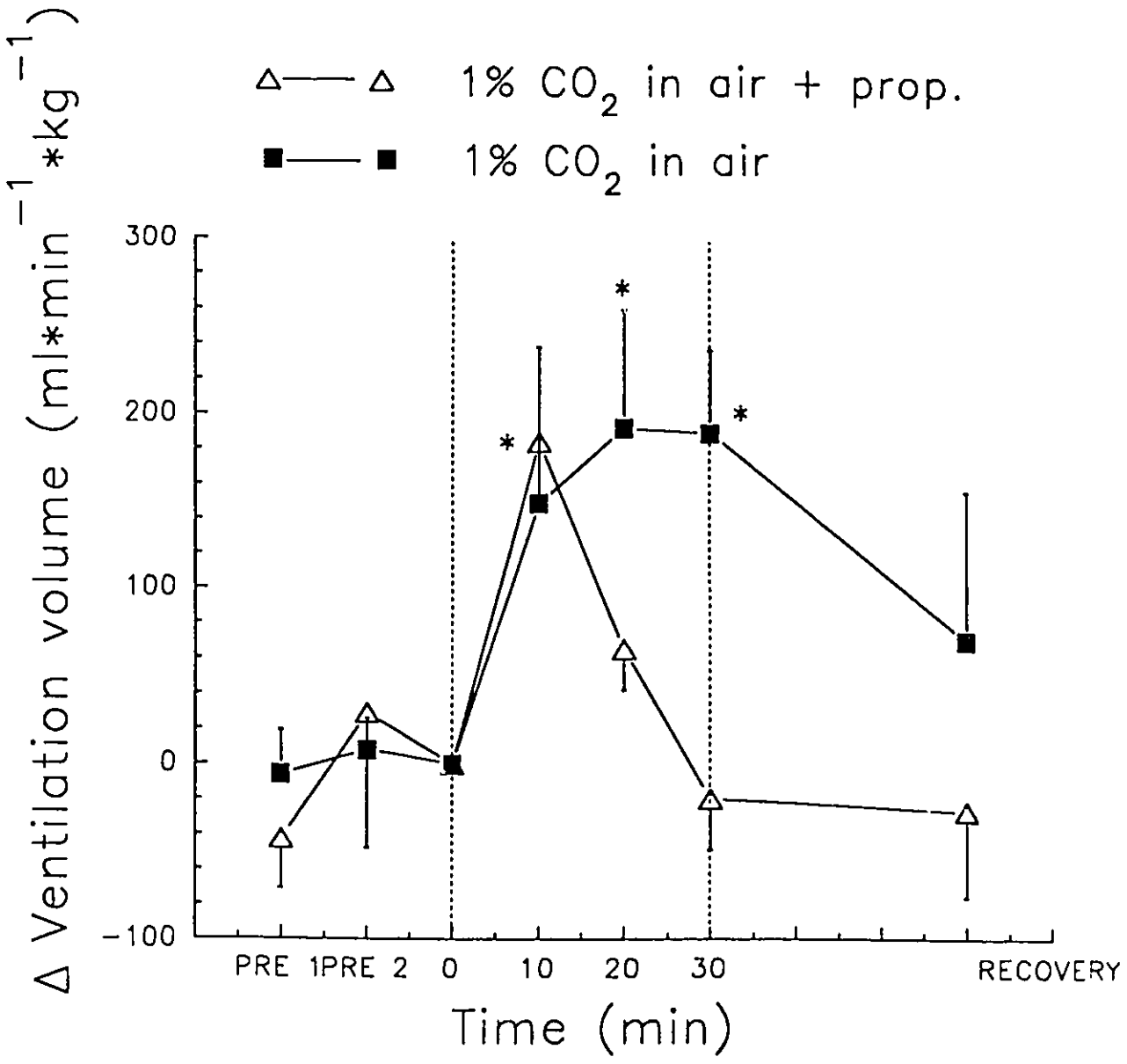

A
0 min
30 min
Recovery
[Norepinephrine] (nM)
12
10
8
6
4
2
0
*
B
[Epinephrine] (nM)
24
22
20
18
16
14
6
4
2
0
*
Normoxic normocapnia
1% CO_2 in air
Hyperoxia
1% CO_2 in O_2
1% CO_2 + propranolol

Figure 18. Variation of gill ventilation volume of rainbow trout during exposure to hypercapnia following pre-treatment with a β-adrenoceptor antagonist (▵——▵, 1% CO_2 in air + prop.). In order to facilitate comparison, the data from the sham (■——■, 1% CO_2 in air) were transposed from Fig 1. The 30 min experimental period is situated between the two vertical dotted lines. Data are shown as group averages ± SEM; * indicates statistical difference from the 0 min value.


1% CO_2 in air + prop.
1% CO_2 in air
Δ Ventilation volume (ml*min^{-1}*kg^{-1})
300
200
100
0
−100
*
*
*
PRE
1PRE
2
0
10
20
30
RECOVERY
Time (min)

DISCUSSION

I: Effect of changes in blood respiratory status

By exposing fish to hyperoxic hypercapnia, the depression of Ca_{O2} and elevation of circulating catecholamines usually associated with respiratory acidosis were prevented. The fact that fish displayed no significant change in $\dot{V}w$ under those particular conditions indicates that respiratory acidosis, alone, can stimulate $\dot{V}w$ in trout because the depressant effects of hyperoxia on ventilation were nullified by hypercapnia. Owing to their obligatory relationship, it is impossible, in the present study, to separate the potential independent effects of $PaCO_2$ (and/or $PwCO_2$) and pHa on $\dot{V}w$. Therefore, this discussion is limited to the integrated effects of respiratory acidosis. Although respiratory acidosis can stimulate $\dot{V}w$ during periods of reduced O_2 respiratory drive (e.g. hyperoxia; Heisler *et al.* 1988; hypercapnia; Graham *et al.* 1990; Wood *et al.* 1990; hyperoxic hypercapnia; present study, Chapters 5 and 6), its contribution to the stimulation of $\dot{V}w$ in rainbow trout exposed to normoxic hypercapnia is less certain because other ventilatory stimulants such as hypoxemia and, potentially, elevated catecholamine levels in the blood are often present simultaneously. Nevertheless, the results demonstrate the potential of respiratory acidosis to act as a ventilatory stimulant during hypercapnia in trout.

It is not possible, with the present data, to provide a detailed explanation for the specific effects of respiratory acidosis on $\dot{V}w$. By analogy to studies performed on elasmobranchs (Wood *et al.* 1990; Graham *et al.* 1990), it is possible that hypercapnia depresses the pH of the cerebrospinal fluid. This would act directly on the regions of the central nervous system involved in the control of $\dot{V}w$.

Our results differ from previous studies that have utilized hyperoxic hypercapnia as a tool to assess the regulation of $\dot{V}w$ in fishes (Dejours, 1973; Smith and Jones (1982). In both studies it was shown that $\dot{V}w$ in hyperoxic trout was unaffected by moderate external hypercapnia ($PwCO_2 \sim 7.5$ Torr). Those results, in addition to observations that hyperoxic trout hypoventilate despite the associated respiratory acidosis (Dejours, 1973; Truchot *et al.* 1980; Wood and Jackson, 1980; Heisler *et al.* 1988) has led to a consensus that respiratory acidosis is not a potent ventilatory stimulant if O_2 is abundant in the blood (Dejours, 1973) and that $\dot{V}w$ is governed principally by blood/water O_2 status rather than blood acid-base status. While the importance of O_2 in the control of $\dot{V}w$ is not being questioned, it is apparent from the present results as well as other studies (Thomas *et al.* 1983; Heisler *et al.* 1988; Graham *et al.* 1990; Wood *et al.* 1990), that blood acid-base status may play a more important role than previously thought (see review by Perry and Wood, 1989). The reasons for the dissimilarities between the conclusions of the aforementioned studies that investigated the control of $\dot{V}w$ under hyperoxic and/or hyperoxic hypercapnic conditions are unclear and difficult to establish due to the different experimental protocols utilized. In certain cases, fish were exposed sequentially to different stimuli, which induced blood respiratory/acid-base disturbances (Smith and Jones, 1982; Thomas *et al.* 1983) and potentially elicited catecholamine mobilisation prior to exposure to hyperoxia.

The hypoventilation during hyperoxia, which is in good accordance with prior studies (Piiper and Shuman, 1967; Randall *et al.* 1976; Wood and Jackson, 1980; Thomas *et al.* 1983; present study, Chapter 3), illustrates the relationship that exists between ventilatory convection requirement for oxygen ($\dot{V}w/\dot{M}O_2$) and PwO_2 (Dejours *et al.* 1977). However, the fact that no significant respiratory acidosis was detected in this group after 30 min of exposure to hyperoxia despite the hypoventilation is unusual and difficult to explain. It is unlikely,

however, that this observation is attributable to fish being in a "non-resting" state before the onset of the experiment. Indeed, the pre-experimental (0 min) plasma catecholamine levels (Boutilier *et al.* 1986; Tetens and Christensen, 1987; Perry *et al.* 1989; Thomas *et al.* 1990; present study, Chapters 3, 4 and 6) and blood pH values (e.g. Randall and Cameron, 1973; Aota *et al.* 1990) of all the groups were in good accordance with the values normally reported for trout at these temperatures.

Although the experimental protocol utilized in this chapter did not allow an evaluation of the potential contribution of Ca_{O2} to the hyperventilation during hypercapnia, the results of previous studies (Holeton, 1977; Smith and Jones, 1982) clearly demonstrated that depression of Ca_{O2} is a potent ventilatory stimulant. It is interesting, therefore, that after 30 min of recovery from hypercapnia and/or hyperoxia $\dot{V}w$ had returned to "resting" levels in all the groups, despite a significant reduction of Ca_{O2}. This may indicate that trout make ventilatory adjustments as a function of the changes in Ca_{O2}, rather than to the absolute values. This explanation is in accordance with the correlation between ΔCa_{O2} and $\Delta \dot{V}w$ reported in chapter 3.

II: Effects of circulating catecholamines.

The ventilatory responses depicted in Fig 18 demonstrate that the β-component of an adrenergic response is required to sustain the hyperventilatory response to acute hypercapnia since, in contrast to the sham treated group, the $\dot{V}w$ values of the propranolol treated fish recorded after 20 and 30 min of hypercapnia were not statistically different from the hypercapnic values. Note that during this experiment, fish did not hyperventilate despite the presence of strong ventilatory stimulants (depressed Ca_{O2}, elevated $PaCO_2/H^+$; Fig 16).

It is informative that plasma catecholamine levels did not significantly increase in hypercapnic fish pre-treated with propranolol (Fig 17). This observation was specific to this group and unusual because propranolol is not known to impair catecholamine mobilization (Perry *et al.* 1989). On the other hand, propranolol is known to depress resting PaO_2 and Ca_{O2} (Wright *et al.* 1989; Tetens and Christensen, 1987) in a fashion that could explain the values reported in Fig 16. The fact that a significant increase in PaO_2 was observed in the propranolol treated group after 30 min of exposure to hypercapnia may reflect the lower initial PaO_2 which was "corrected" by the hyperventilation.

It is difficult to accept that the impairment of hyperventilation caused by propranolol was due to β-adrenoceptor antagonism of catecholamines of humoral origin since plasma catecholamines did not increase. Nor can it be attributable to the absence of elevated catecholamine levels *per se*, since mild hypercapnia ($PwCO_2$ = 4.5 Torr) can elicit sustained hyperventilation without mobilizing catecholamines (Chapter 6). Instead, one must postulate that propranolol prevented the action of catecholamines released from nerve terminals either peripherally and/or centrally. It would be interesting to know whether propranolol crosses the blood-brain-barrier in trout in order to speculate further on the site of action of this drug. Regardless, there is no evidence to implicate catecholamines of humoral origin in the hyperventilatory response to hypercapnia.

CHAPTER 6

THE EFFECTS OF INTRA-ARTERIAL INFUSION OF CATECHOLAMINES ON VENTILATION IN RAINBOW TROUT (*Oncorhyncus mykiss*) EXPOSED TO EXTERNAL HYPOXIA OR HYPERCAPNIA

INTRODUCTION

Much of the evidence both for, and against, a role for circulating catecholamines in modulating ventilation is based on direct or indirect measurements of gill ventilation during intra-vascular administration of catecholamines (for further details, see Chapter 1). All previous studies (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980; Playle *et al.* 1990; present study, Chapter 3) that have assessed the effects of exogenous catecholamines on $\dot{V}w$ were performed on resting fish under normoxic conditions. Endogenous catecholamine mobilization, however, almost always occurs when $\dot{V}w$ is elevated and when blood respiratory/acid-base status is altered. It is possible, therefore, that the protocols of the aforementioned studies did not adequately assess the potential role of elevated circulating catecholamines in the regulation of $\dot{V}w$. Indeed, it is well documented that both hypoxemia and acidosis have permissive effects on adrenergic stimulation of RBC Na^+/H^+ exchange (Borgese *et al.* 1987; Motais *et al.* 1987; Nikinmaa *et al.* 1987) and therefore could also coincidentally affect potential adrenergic effects on $\dot{V}w$.

In the present study, therefore, a novel experimental protocol was used in which fish were first exposed to a ventilatory stimulant (hypoxia or hypercapnia) that did not induce an elevation of plasma catecholamine levels, but which was ultimately achieved experimentally by intra-arterial infusion of catecholamines.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhyncus mykiss*) of either sex, weighing between 150 and 523 g (mean mass = 303 ± 13 g; experimental N = 36) were obtained from Linwood Acres Trout Farms (Campbellcroft, Ontario). The temperature of the holding and experimental water varied between 10°C and 14°C (May to July, mean temperature = 10.9 ± 0.4°C).

Experimental protocol

1. Experimental conditions

Series I: The effects of catecholamine infusion on the ventilatory response to different environmental O_2 tensions

At least one hour prior to experimentation, the dorsal aortic cannula was infused with Cortland saline at a rate of 0.6 $ml \cdot h^{-1}$ using a syringe pump (Sage; model 352). After that period (I) the conditions of external normoxia (PwO_2 = 151 ± 2 Torr, N = 6) or hyperoxia (PwO_2 = 640 ± 19 Torr, N = 6) were initiated by gassing the cephalic compartment of the van Dam box as well as a counter-current gas exchange column (which supplied water to the cephalic compartment), with air or oxygen, respectively. Hypoxia (PwO_2 = 90 ± 3 Torr, N = 6) was achieved by gassing the gas exchange column with nitrogen while the cephalic compartment of the box was gassed with a mixture of 40% nitrogen in air originating from

a Wösthoff gas mixing pump (Model M301-A/F). New steady-state PwO_2 was attained within 10 min. All gases were supplied by Air Products Inc. (Ottawa, Ont.). PwO_2 of the cephalic compartment was continuously monitored throughout the experiment by using a Radiometer PO_2 electrode (housed in a thermostated cuvette).

During the first 30 min of exposure to one of these experimental conditions, fish were infused with Cortland saline (period II). At the end of period II, each fish was given a bolus injection (0.3 ml) of a catecholamine mixture containing 2×10^{-5} mol·l^{-1} L-EPI and 5×10^{-6} NE (both bitartrate salt, Sigma Chemical Company) dissolved in Cortland saline (final pH = 7.8). The cannula was flushed with 0.2 ml of saline and then attached to the syringe pump which dispensed the same solution at the rate of 0.6 ml·h^{-1} for 30 min while the experimental condition (normoxia, hyperoxia or hypoxia) was maintained (period III). All syringes and tubing were opaque to impede oxidation of catecholamines. At the end of period III, the fish was allowed to recover under normoxic conditions ($PwO_2 \sim 155$ Torr) while being infused with saline for 30 min (period IV).

Series II: The effects of catecholamine infusion on the ventilatory response to external hypercapnia under normoxic or hyperoxic conditions

To achieve normoxic hypercapnia ($PwO_2 \sim 155$ Torr, $PwCO_2 = 4.5 \pm 0.4$ Torr, N = 6) the counter-current gas exchange column, which supplied the experimental boxes, was bubbled with a gas mixture of 1% CO_2 in air originating from a Wösthoff gas mixing pump at a rate that compensated for the equilibration efficiency of the column. Thus, the inflowing $PwCO_2$ was equal to the one of the cephalic compartment which was bubbled with a commercial mixture of 0.6% CO_2 in air. Hyperoxic hypercapnia ($PwO_2 = 510 \pm 40$ Torr,

$PwCO_2$ = 4.8 ± 0.6 Torr, N = 6) was achieved in a similar fashion by substituting air for O_2 in the gas mixtures.

2. Blood sampling

Four blood samples (750 µl each) were taken from each fish *via* a 3-way valve in the infusion tubing. An initial sample was withdrawn from the dorsal aortic cannula before the onset of the experiment (period I, time = 0 min), a second sample at the end of period II, a third one at the end of period III, and a final sample at the end of the recovery period (IV).

After each sample, an equivalent volume of a homologous RBC suspension (originating from a donor fish) was reinjected into the animal. The dorsal aorta of the donor fish was cannulated (Soivio *et al.* 1975) and after a recuperation period of at least 24 h, a blood sample (~2.5 ml) was slowly withdrawn. The blood was then centrifuged (500 x g, 5 min) and rinsed twice with Cortland saline to eliminate plasma catecholamines before the RBC's were resuspended to the initial haematocrit with saline (in the suspension: [NE] = 0.53 ± 0.12 nM; [EPI] = 1.11 ± 0.32 nM, N = 11).

The arterial blood was analyzed immediately after sampling to determine PaO_2, pHa and Ca_{O2}. Remaining blood was centrifuged and the plasma (~250 µl) was combined with 10 µl of heparin (2500 units·ml^{-1}) and immediately frozen in liquid nitrogen. The plasma samples were then stored at -70°C for subsequent determination of catecholamine levels within one week.

In addition, samples of approximately 80 µl each were taken after 2, 5, 10, 15 and 20 min of exposure to the experimental condition (period II) as well as after 32, 35, 40, 45 and

50 min of exposure to experimental condition (period III). After each sample, an equivalent volume of heparinized saline (10 units·ml^{-1}) was injected into the fish to restore blood volume. These small samples were analyzed immediately to determine pHa. It must be mentioned that before withdrawing blood during the catecholamine infusion (period III), the catheter was flushed with approximately 0.3 ml of saline to avoid sample contamination.

3. Ventilatory and blood pressure measurements

Gill ventilation volume ($\dot{V}w$), f_V and P_{da} were monitored in the following sequence: Two pre-experimental measurements (Pre 1 and Pre 2) were taken 10 min apart (period I). Another measurement was taken 10 min later (0 min), just prior to withdrawal of the first blood sample (750 µl). The animal was then exposed to the particular experimental condition (normoxia, hyperoxia or hypoxia) for 30 min (period II). During that period, measurements were made just before each 80 µl blood sample, i.e. at 2, 5, 10, 15, 20 min as well as before the second 750 µl sample. The same protocol was then repeated during the 30 min period of catecholamine infusion (i.e. at 32, 35, 40, 45, 50 and 60 min; period III). The final measurements were performed at the conclusion of the 30 min recovery period (90 min; period IV).

Statistical analysis

All data are presented as means ± 1 SEM. The results have been statistically analyzed using a factorial analysis of variance followed by Fishers LSD multiple comparison test; 5% was taken as the fiducial limit of significance, except where otherwise specified.

RESULTS

I: The effects of catecholamine infusion on the ventilatory response to different environmental O_2 tensions

Hypoxia elicited an approximate 2.3-fold increase (resting $\dot{V}w$ during period I = 261 ± 52 $ml \cdot min^{-1} \cdot kg^{-1}$) in $\dot{V}w$ during the first 30 min of exposure (Fig 19, period II). This treatment caused a significant depression of PaO_2 which was not accompanied by a significant reduction of Ca_{O2} (Fig 19). Despite an initial upward trend, no statistically significant changes in pHa were observed (Fig 19). The circulating levels of EPI and NE were unchanged after 30 min of hypoxia (Fig 19). P_{da} did not increase during this period (Table 6).

The injection, followed by the infusion, of catecholamines caused an approximate 1.3-fold decrease in $\dot{V}w$ (compared with the 30 min value) that persisted for 5 min before beginning to return to the pre-injection level. Simultaneously, pHa fell by 0.26 units but returned to pre-injection levels within 30 min. An increase in P_{da} was observed throughout period III, an observation that pertains to all the experimental groups (Table 6). Plasma EPI and NE levels were significantly elevated at the termination of period III. Both PaO_2 and Ca_{O2} were unaffected by the 30 min infusion of catecholamine. At the end of period IV, all measured variables had returned to pre-hypoxic levels except for P_{da} (Table 6) which remained elevated.

Hyperoxia (resting $\dot{V}w$ during period I = 280 ± 17 $ml \cdot min^{-1} \cdot kg^{-1}$) caused an approximate 3.9-fold decrease in $\dot{V}w$ during period II which was due to depression of both V_{sv} and f_V (fig 20). Interestingly, this is the only experimental treatment of this study that

caused a change in f_V in addition to changes in V_{sv}. Arterial blood pH decreased rapidly after the onset of hyperoxia (0.23 units at 30 min), probably owing to the hypoventilation and consequent retention of CO_2. Both PaO_2 and Ca_{O2} were elevated at the end of period II. Plasma catecholamine levels as well as P_{da} were unchanged during this period (Fig 20 and Table 6, respectively).

As in the hypoxic group, the catecholamine treatment during period III caused a significant depression of pHa (0.15 units) (Fig 20) and elevation of P_{da} (Table 6). On the other hand, $\dot{V}w$ was unaffected by catecholamine infusion during hyperoxia (Fig 20). Again, plasma catecholamine levels were elevated after 30 min of infusion (period III); it is worth noting here that the increase of NE in this group was the smallest of the entire study. At the end of period IV, all the variables had returned to pre-experimental levels.

The results obtained in the control group (normoxia; resting $\dot{V}w$ during period I = 251 ± 25 $ml \cdot min^{-1} \cdot kg^{-1}$) validate the protocol used in the present study. Indeed, none of the variables of interest were affected during periods I and II. The catecholamine infusion procedure induced the smallest acidosis (0.13 pH units) and did not significantly alter the blood O_2 status. Plasma catecholamine levels were elevated in the 60 min blood sample (Fig 21). As in the hypoxic group, P_{da} was the only variable that did not return to pre-experimental levels after 30 min of recovery (period IV).

II: The effects of catecholamine infusion on the ventilatory response to external hypercapnia under normoxic or hyperoxic conditions

The elevation of PCO_2 in the inspired water caused a marked depression of pHa (0.23 units) and an approximate 1.5-fold increase in $\dot{V}w$ (resting $\dot{V}w$ during period I = 490 ± 27

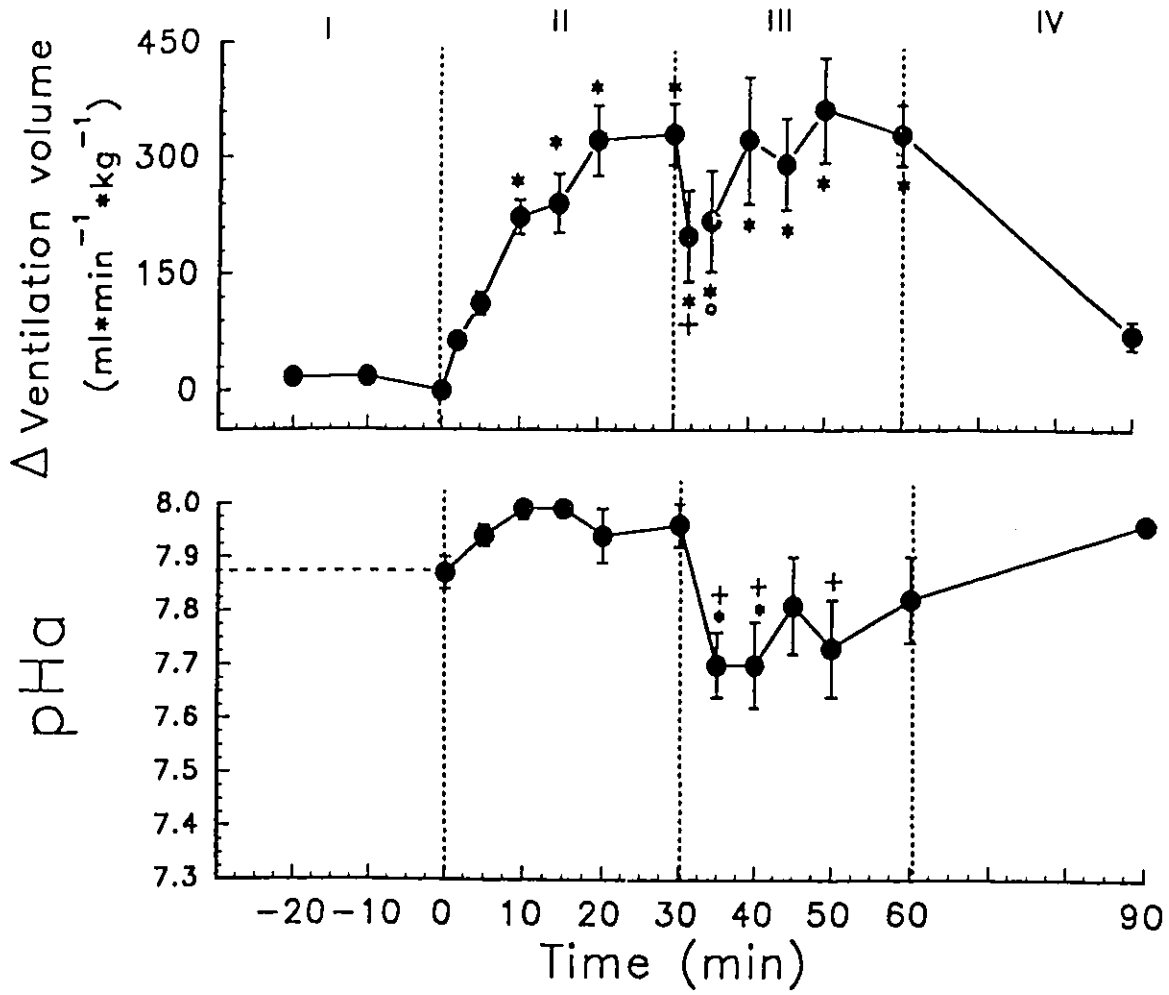
$ml \cdot min^{-1} \cdot kg^{-1}$). Arterial O_2 content and PaO_2 were unaffected by this degree of hypercapnia (Fig 22). Hypercapnia was the only treatment to cause an increase of P_{da} prior to infusion of catecholamines (Table 6). Since plasma catecholamines were not significantly increased at the end of that period (Fig 22), this hypertension is likely to be of nervous origin.

The infusion of catecholamines during hypercapnia caused $\dot{V}w$ to return to values that were statistically equal to the pre-experimental (period I) measurements. This depression of the hypercapnia-induced hyperventilation (which, however, was not different from the 30 min value) occurred concurrently with the catecholamine-mediated reduction of pHa (0.21 units). At the end of period III, both EPI and NE levels were elevated, while the blood O_2 status was unaltered (Fig 22).

Exposure of fish to hyperoxic hypercapnia evoked a severe acidosis (0.34) units, but did not alter plasma catecholamine levels, Ca_{O2} or $\dot{V}w$ (Fig 23). Gill ventilation volume (resting $\dot{V}w$ during period I = 446 ± 40 $ml \cdot min^{-1} \cdot kg^{-1}$) remained constant during catecholamine infusion despite what was later found to be the largest experimentally-induced elevation of plasma EPI in this study (the elevation of NE was not different from any other group). The consequent depression of pHa, however, was not the largest (0.19 units). Again, this treatment (period III) had no effect on the blood O_2 status.

In this series, all the variables had returned to pre-experimental values at the end of period IV (Fig 23, Table 6).

Figure 19. The effects of saline infusion during normoxia (PwO_2 = 151 ± 2 Torr; period I), followed by saline infusion during hypoxia (PwO_2 = 90 ± 3 Torr; period II), catecholamine infusion during hypoxia (PwO_2 = 90 ± 3 Torr; period III) and recovery with saline infusion under normoxia (PwO_2 = 151 ± 2 Torr; period IV) on $\dot{V}w$ (top panel) and pHa (second panel). Also shown are PaO_2 and Ca_{O2} content values (cross hatched bars and open bars, respectively, bottom left histogram) along with the plasma NE (cross hatched bars) and EPI (open bars) concentrations (bottom right histogram) at the end of each experimental period. * indicates a value significantly different ($p < 0.05$) from the 0 min value; o indicates a value significantly different from the 0 min value ($p < 0.10$); + indicates a value significantly different ($p < 0.05$) from the 30 min value.


I
II
III
IV
Δ Ventilation volume
(ml*min⁻¹*kg⁻¹)
450
300
150
0
pHa
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
-20 -10 0 10 20 30 40 50 60 90
Time (min)

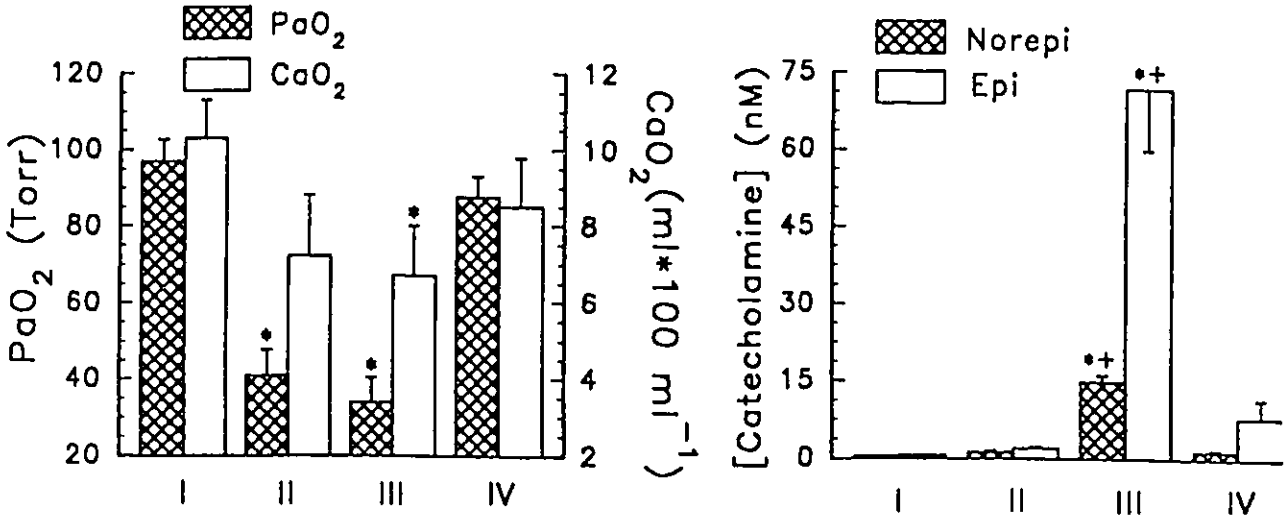
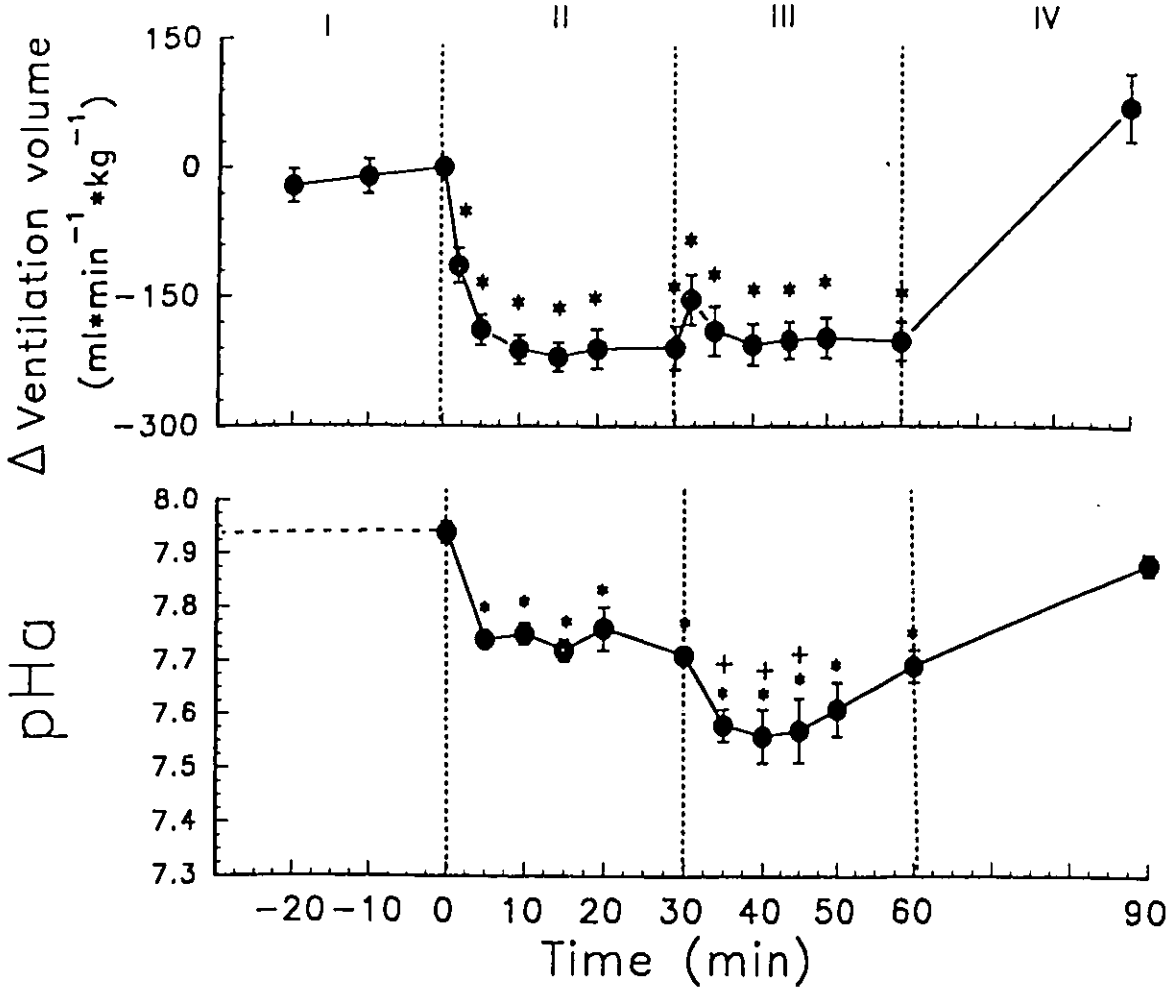

PaO2
CaO2
PaO2 (Torr)
CaO2 (ml*100 ml⁻¹)
120
100
80
60
40
20
12
10
8
6
4
2
I
II
III
IV
Norepi
Epi
[Catecholamine] (nM)
75
60
45
30
15
0

Figure 20. The effects of saline infusion during normoxia (PwO_2 = 151 ± 2 Torr; period I), followed by saline infusion during hyperoxia (PwO_2 = 640 ± 19 Torr; period II), catecholamine infusion during hyperoxia (PwO_2 = 640 ± 19 Torr; period III) and recovery with saline infusion under normoxia (PwO_2 = 151 ± 2 Torr; period IV) on $\dot{V}w$ (top panel) and pHa (second panel). See Fig legend 19 for further details.


I
II
III
IV
Δ Ventilation volume
(ml*min⁻¹*kg⁻¹)
150
0
−150
−300
pHa
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
−20 −10 0 10 20 30 40 50 60 90
Time (min)

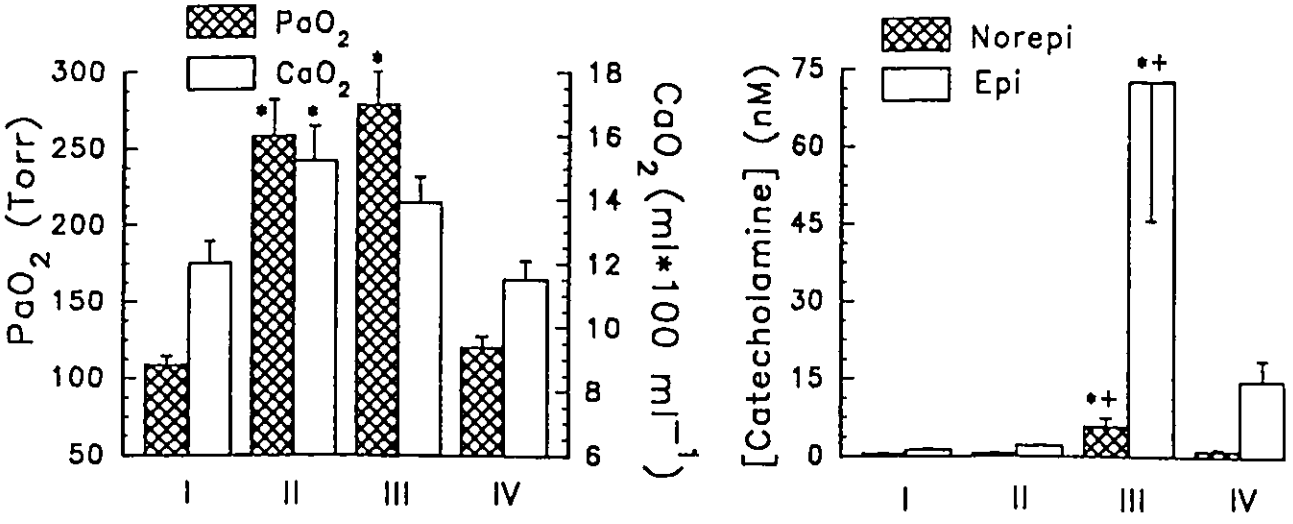
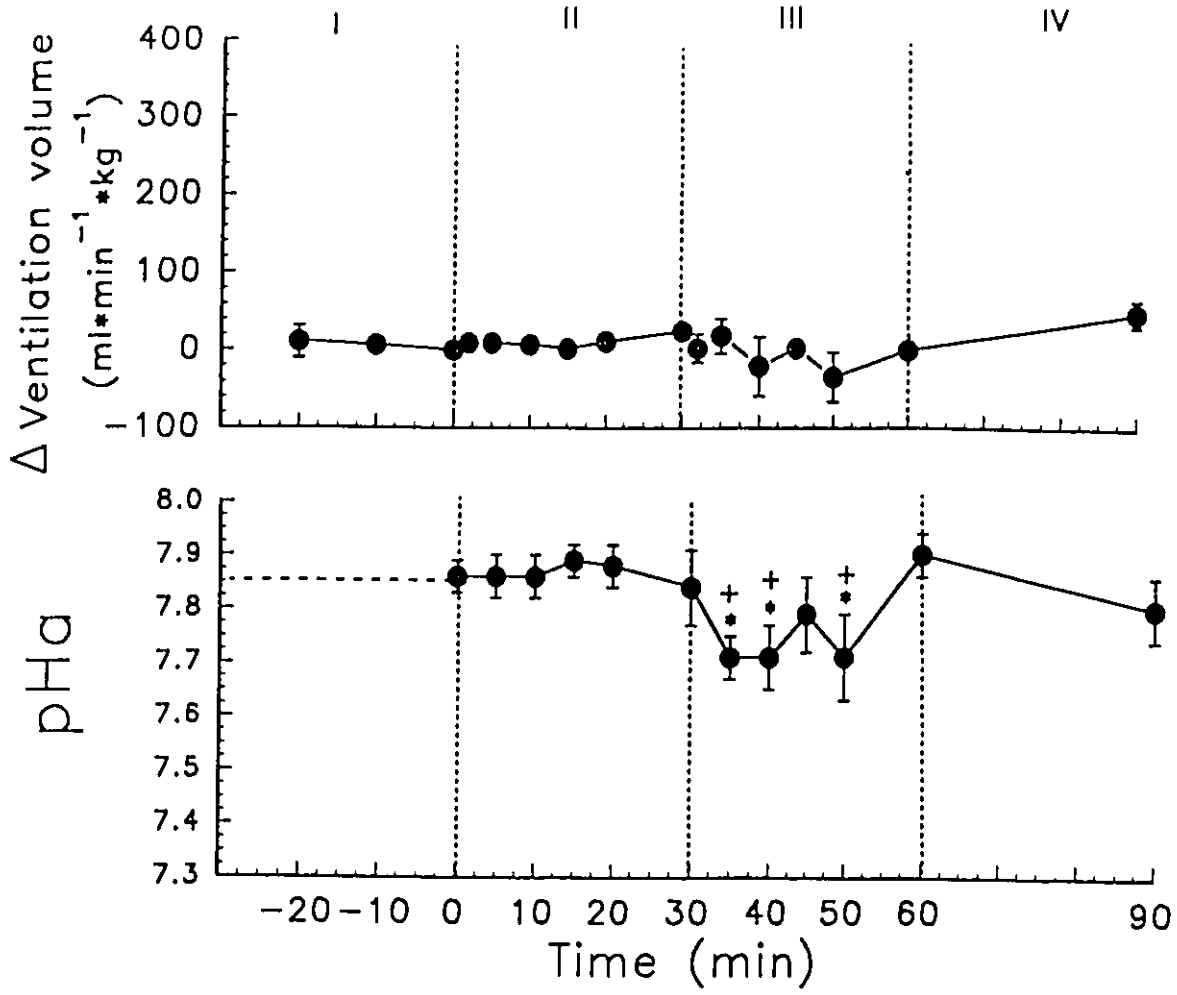
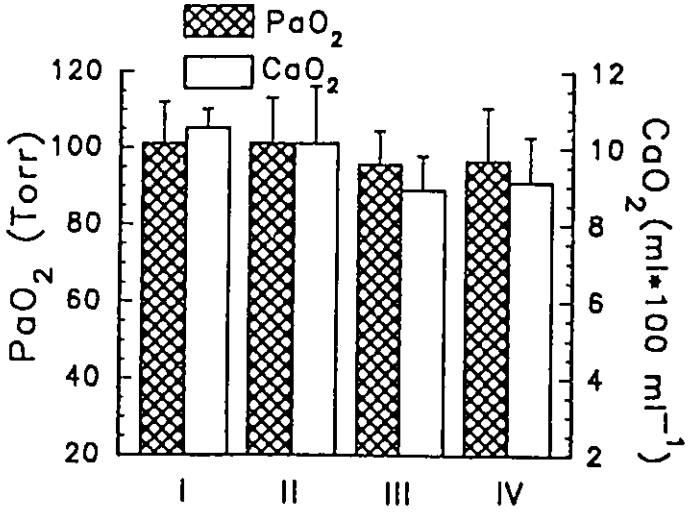

PaO2
CaO2
PaO2 (Torr)
300
250
200
150
100
50
CaO2 (ml*100 ml⁻¹)
18
16
14
12
10
8
6
I
II
III
IV
Norepi
Epi
[Catecholamine] (nM)
75
60
45
30
15
0

Figure 21. The effects of saline infusion under normoxic condition (PwO_2 = 151 ± 2 Torr; periods I, II, III and IV), followed by a second period of saline infusion under normoxia (period II), catecholamine mixture infusion during normoxia (period III) and recovery with saline infusion under normoxia (period IV) on $\dot{V}w$ (top panel) and pHa (second panel). See Fig legend 19 for further details.


I
II
III
IV
Δ Ventilation volume
(ml*min⁻¹*kg⁻¹)
400
300
200
100
0
−100
pHa
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
−20 −10 0 10 20 30 40 50 60 90
Time (min)


PaO2
CaO2
PaO2 (Torr)
CaO2 (ml*100 ml⁻¹)
120
100
80
60
40
20
12
10
8
6
4
2
I
II
III
IV

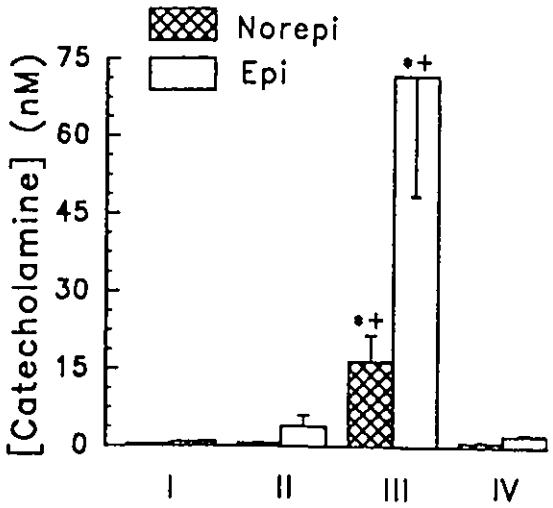
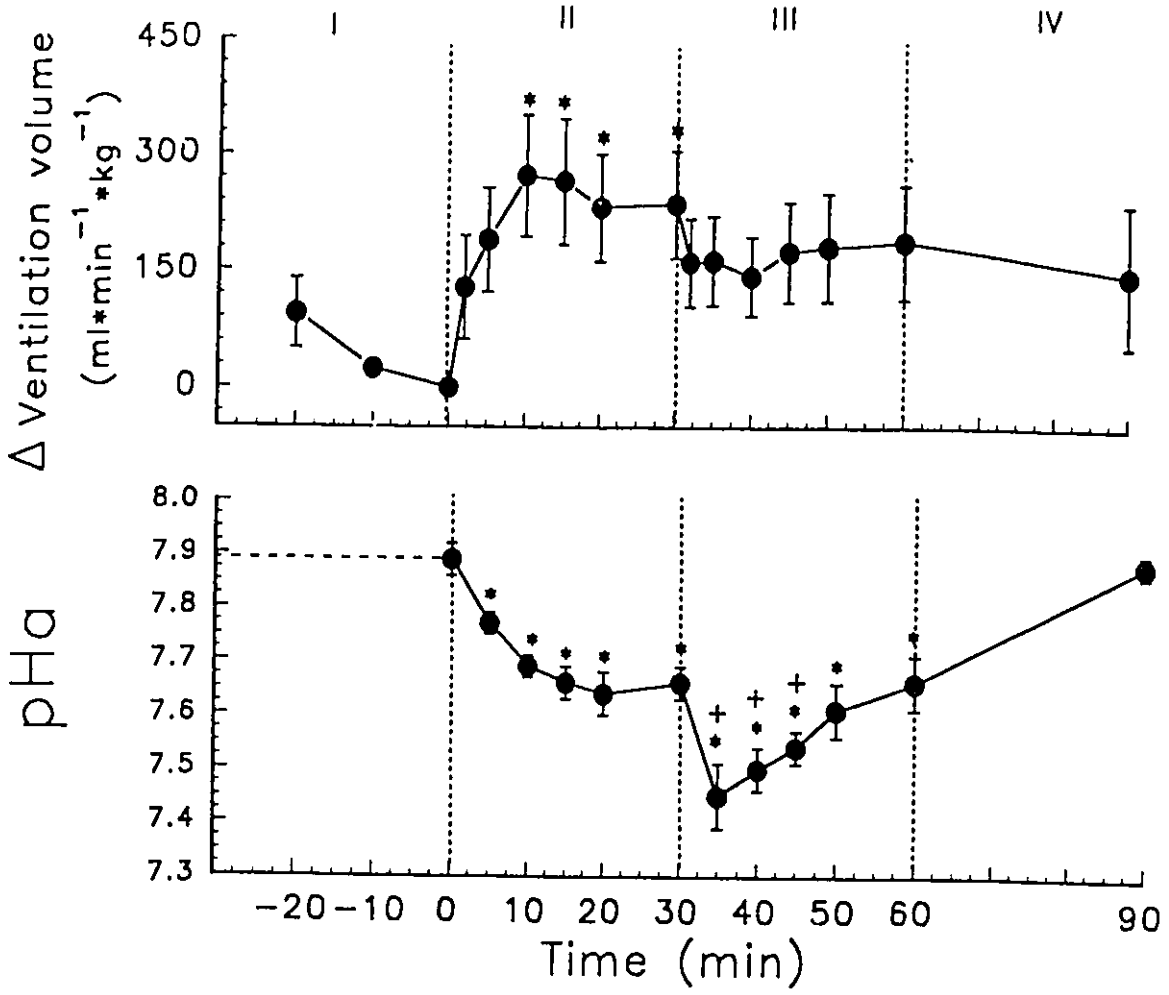
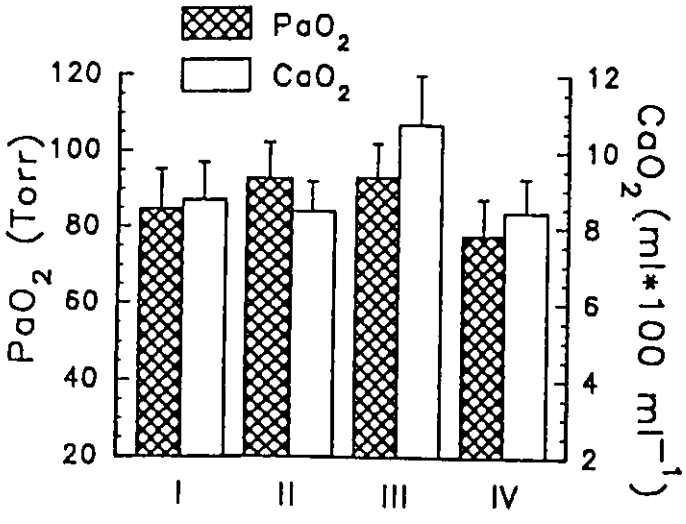

Norepi
Epi
[Catecholamine] (nM)
75
60
45
30
15
0
I
II
III
IV

Figure 22. The effects of saline infusion during normoxia (PwO_2 = 151 ± 2 Torr; period I), followed by saline infusion during hypercapnia ($PwCO_2$ = 4.5 ± 0.4 Torr; period II), catecholamine mixture infusion during hypercapnia ($PwCO_2$ = 4.5 ± 0.4 Torr; period III) and recovery with saline infusion under normoxia (PwO_2 = 151 ± 2 Torr; period IV) on $\dot{V}w$ (top panel) and pHa (second panel). See Fig legend 19 for further details.


I
II
III
IV
Δ Ventilation volume
(ml*min⁻¹*kg⁻¹)
450
300
150
0
pHa
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
−20 −10 0 10 20 30 40 50 60 90
Time (min)


PaO2
CaO2
PaO2 (Torr)
CaO2 (ml*100 ml⁻¹)
120
100
80
60
40
20
12
10
8
6
4
2
I
II
III
IV

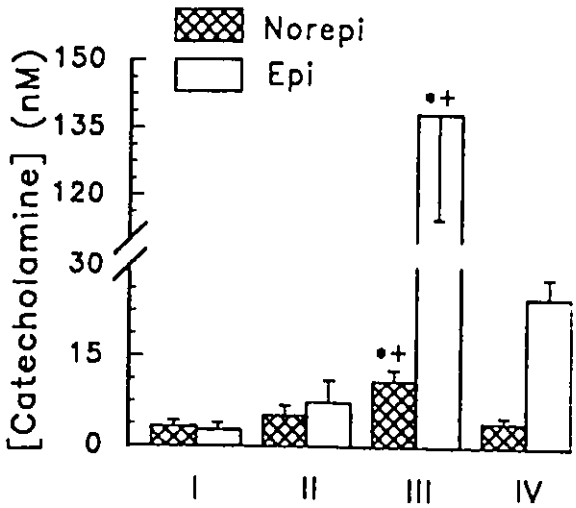
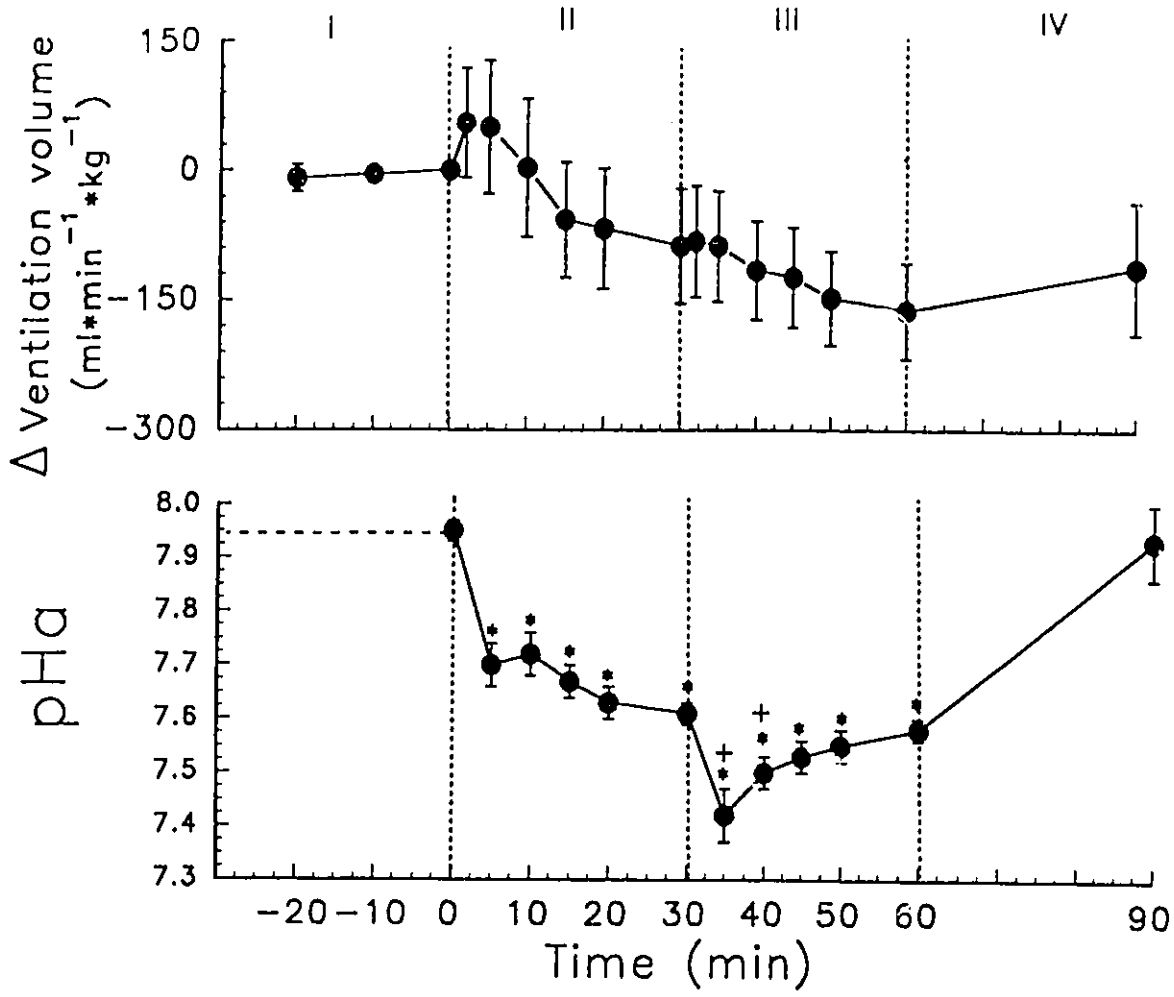

Norepi
Epi
[Catecholamine] (nM)
150
135
120
30
15
0
I
II
III
IV

Figure 23. The effects of saline infusion during normoxia (PwO_2 = 151 ± 2 Torr; period I), followed by saline infusion during hyperoxic hypercapnia (PwO_2 = 510 ± 40 Torr, $PwCO_2$ = 4.8 ± 0.6 Torr; period II), catecholamine mixture infusion during hyperoxic hypercapnia (PwO_2 = 510 ± 40 Torr, $PwCO_2$ = 4.8 ± 0.6 Torr; period III) and recovery with saline infusion under normoxic condition (PwO_2 = 151 ± 2 Torr; period IV) on $\dot{V}w$ (top panel) and pHa (second panel). See Fig legend 19 for further details.


I
II
III
IV
Δ Ventilation volume
(ml*min⁻¹*kg⁻¹)
150
0
−150
−300
pHa
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
−20 −10 0 10 20 30 40 50 60 90
Time (min)

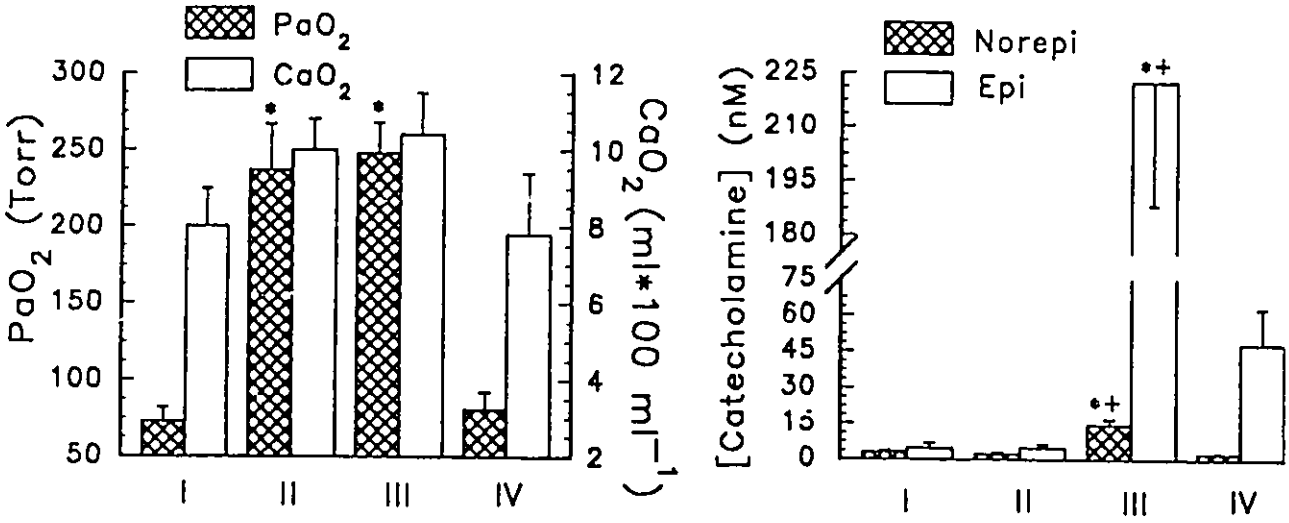

PaO2
CaO2
PaO2 (Torr)
300
250
200
150
100
50
CaO2 (ml*100 ml⁻¹)
12
10
8
6
4
2
I
II
III
IV
Norepi
Epi
[Catecholamine] (nM)
225
210
195
180
75
60
45
30
15
0

Table 6: The effects of catecholamine infusion on dorsal aortic blood pressure (P_{da}, expressed in cm of H_2O) of rainbow trout exposed to different environmental oxygen and/or carbon dioxide tensions. Values shown are means ± 1 standard error (in parentheses). * indicates a value significantly different from the 0 min value; + indicates a values significantly different from the 30 min value.

Experimental period	I Saline infusion		II Saline infusion							III Catecholamine infusion						IV Saline infusion
Time (min)	-20	-10	0	2	5	10	15	20	30	32	35	40	45	50	60	90
Normoxia	38 (2)	39 (2)	39 (2)	40 (2)	38 (2)	38 (2)	38 (2)	37 (1)	37 (2)	60*+ (2)	55*+ (2)	50*+ (3)	54*+ (6)	51*+ (4)	48 (3)	51* (3)
Hypoxia	34 (1)	34 (1)	34 (2)	34 (2)	35 (2)	36 (2)	35 (2)	38 (2)	34 (2)	60*+ (4)	53*+ (2)	53*+ (2)	56*+ (4)	56*+ (6)	48*+ (3)	41* (3)
Hyperoxia	35 (1)	35 (1)	35 (1)	32 (1)	32 (1)	32 (2)	32 (2)	34 (2)	37 (3)	62*+ (4)	53*+ (2)	58*+ (2)	57*+ (2)	53*+ (2)	50*+ (3)	40 (3)
Hypercapnia	34 (3)	32 (3)	30 (2)	29 (2)	31 (2)	34 (3)	36 (4)	41* (3)	44* (4)	59*+ (3)	52* (3)	56*+ (2)	55*+ (2)	55*+ (2)	47* (3)	33 (2)
Hyperoxic hypercapnia	35 (2)	34 (2)	34 (2)	31 (2)	34 (3)	40 (5)	40 (5)	41 (4)	39 (4)	54*+ (2)	50*+ (2)	56*+ (3)	58*+ (2)	55*+ (2)	50*+ (3)	34 (2)

DISCUSSION

The protocol used in the present study was developed to mimic stress-related elevation of endogenous circulating catecholamine levels and was modified from procedures described by Perry and Vermette (1987). That study was the first to utilize continuous intra-vascular infusion of catecholamines. This technique offers several advantages over a single rapid intra-vascular injection which, until that time, was the method used to experimentally elevate circulating catecholamine levels. Owing to the numerous mechanisms involved in catecholamine metabolism (Nekvasil and Olson, 1986*a*, *b*; Coletti and Olson, 1988; for reviews see Mazeaud and Mazeaud, 1981; Nilsson, 1983), circulating catecholamine levels rapidly return to normal following a bolus injection. Thus, intra-arterial injection (to rapidly increase circulating levels) followed by infusion (to sustain circulating levels) probably allows a more accurate assessment of the effects of catecholamine mobilisation during stress since under physiological conditions, circulating levels of these amines can remain elevated for extended periods of time if the disturbance is maintained (e.g. hypoxia; Thomas *et al.* 1990).

Several modifications were made to the protocol of Perry and Vermette (1987) to more closely mimic several aspects of the humoral adrenergic response normally encountered in fish. In addition to elevating plasma catecholamine concentrations to physiologically relevant levels, the combination of both amines in the injection and infusion solutions reproduced the natural EPI/NE ratio often reported in fish following exposure to challenging conditions (exercise, e.g. Milligan and Wood, 1987; Tang and Boutilier, 1988; hypoxia, e.g. Fievet *et al.* 1990; Perry *et al.* 1990; Thomas *et al.* 1990; anaemia, e.g. Perry *et al.* 1989). Furthermore, the experimental elevation of circulating catecholamines occurred when fish were simultaneously experiencing acid-base/respiratory disturbances and adjusting $\dot{V}w$

correspondingly. This method, therefore, also reproduced the physiological conditions which may have potentiating and/or permissive effects on adrenergic control systems in fish (for review see Nilsson, 1984).

The results clearly demonstrate the effectiveness of this infusion procedure. In addition to the final catecholamine levels reported after period III for each group, the increase of P_{da} and decrease of pHa associated with the infusion of catecholamines indicate that the circulating levels achieved were not only in the physiological range, but also sufficient to elicit adrenergic effects. Specifically, the elevation of P_{da} reflects α-adrenoceptor mediated vasoconstriction of the systemic vasculature (Wood and Shelton, 1980; Fritsche and Nilsson, 1990; for review see Nilsson, 1983) while the rapid reduction of pHa, followed by gradual recovery, is a reliable indicator of β-adrenoceptor mediated stimulation of RBC Na^+/H^+ exchange (Fievet *et al.* 1987).

The ventilatory responses to hypoxia and hypercapnia observed before and after catecholamine infusion clearly do not support a stimulatory role for these hormones in the regulation of ventilation in trout. First, the present study confirmed that fish can hyperventilate (e.g. during hypoxia or hypercapnia) in the absence of elevated catecholamines in the circulation. Other studies also have demonstrated that elevated plasma catecholamine levels is not a prerequisite for hyperventilation (e.g. Peyraud-Waitzenegger and Soulier, 1989; present study, Chapters 3 and 4). Secondly, the injection, followed by infusion, of a catecholamine mixture provoked a depression of the hyperventilatory response in the face of prominent ventilatory stimulants, namely hypoxia (Fig 19) or hypercapnia (Fig 22). It is difficult, with the present data, to give a clear explanation to these interesting observations since the only measured blood respiratory/acid-base variable that was affected by the catecholamine infusion was pHa (Figs 19 and 22). Based on the knowledge that acidosis is

a specific ventilatory stimulant in fish (Thomas *et al.* 1983; Heisler *et al.* 1988; Wood *et al.* 1990; for review see Perry and Wood, 1989) a depression of the hyperventilation was certainly not predicted. It is conceivable that the transient hypoventilation was a consequence of one or both catecholamines crossing the blood-brain barrier (NE is known to cross the blood-brain barrier in trout; Nekvasil and Olson, 1986*a*) and exerting depressant effects on the higher centres involved in the control of breathing. Since similar elevation of catecholamines and depression of pHa were observed in the control and hyperoxic groups, yet $\dot{V}w$ was unaltered, it is likely that the initial blood/water respiratory status and resulting initial $\dot{V}w$ acted to mediate the ventilatory response to elevated circulating catecholamine levels. It is informative that the absence of an adrenergic $\dot{V}w$ response in the normoxic group differed from the transient hypoventilation reported in other studies performed on trout (Playle *et al.* 1990; present study, Chapter 3). The higher circulating catecholamine levels ([NE] = 206 ± 26 nM; [EPI] = 164 ± 73 nM, respectively) reported in Chapter 3 may explain these differences. Since Playle *et al.* (1990) only report estimated values, no significant comparison can be made.

The adrenergic hypoventilation observed during hyperoxia or hypercapnia was probably not mediated by baroreceptors as is known to occur in hypertensive mammals (Heistad *et al.* 1975; for review see Daly, 1986). The fact that $\dot{V}w$ remained unchanged in the control (normoxic), hyperoxic and hyperoxic hypercapnic groups despite similar hypertensive responses to catecholamines infusion, along with the observation that hypertension does not influence $\dot{V}w$ during hypoxia (present study, Chapter 4), strongly suggests that the explanation resides in other mechanisms.

Since it is known that the O_2 status of the fish is integral in the regulation of ventilation (for review see Shelton *et al.* 1986), it is possible that the catecholamine-mediated depressions of breathing observed during hypoxia and hypercapnia were due to brief

elevations of the blood O_2-carrying capacity. The data (Figs 19 and 22), however, do not support this explanation, and therefore, can only remain as speculation based on theoretical knowledge and experimental evidence that Ca_{O2} can be increased by elevation of circulating catecholamines (Perry and Kinkead, 1989). Further experiments using a more refined method capable of detecting possible subtle or transient changes in Ca_{O2}, such as an extracorporeal circulation (Thomas and Le Ruz, 1982), are required to adequately test this idea.

The results of the normoxic, hyperoxic and hyperoxic hypercapnic groups showed that catecholamine infusion affected $\dot{V}w$ only when fish were facing a respiratory challenge (i.e. hypoxia or hypercapnia). This response occurred independently of the blood acid-base status of the fish prior to infusion since pHa of the normoxic and hypoxic groups were normal at the end of period II, whereas significant acidemia occurred in the other groups, yet only the hypoxic or hypercapnic fish hypoventilated. Further similar comparisons between groups indicate also that a depressed blood O_2-status at the end of period II was not a prerequisite for catecholamines to exert a depressant action on $\dot{V}w$. On the other hand, the data do suggest that an initial hyperventilatory state is required for these doses of catecholamines to evoke hypoventilation.

Hyperoxia and hyperoxic hypercapnia caused respiratory acidosis. The experimental elevation of catecholamines in these groups further increased the acidosis presumably *via* activation of the RBC Na^+/H^+ antiporter (Fievet *et al.* 1987). Because elevation of CO_2/H^+ in the blood can stimulate breathing in the absence of an O_2-related respiratory drive in elasmobranchs (Heisler *et al.* 1988; Graham *et al.* 1990; Wood *et al.* 1990; see review by Perry and Wood, 1989) and trout (Thomas *et al.* 1983; Kinkead and Perry, unpublished observations) an increase in $\dot{V}w$ was predicted during period III of these two groups, yet $\dot{V}w$ was unaltered. The absence of a net ventilatory effect at this time may reflect the opposing

effects of two phenomena; specific hypoventilatory effects of elevated plasma catecholamine levels *versus* stimulatory effects of the catecholamine-induced acidosis.

The present data obviously differ from results of several previous studies employing catecholamine injections, which argued in favour of a stimulatory role for catecholamines on $\dot{V}w$ in fish (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980; Aota *et al.* 1990). The discrepancies that exist between the present results (hypoventilation) and the ones reported for the eel in summer (hyperventilation: Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980) may reflect important inter-specific differences. These dissimilarities could arise from differences in the sensitivity of the response to the circulating levels of catecholamines. That is, since Peyraud-Waitzenegger *et al.* (1980) suggested that catecholamines act on the central nervous system of the European eel (*Anguilla anguilla*), it is possible that in those fish, the effects observed on ventilation following intra-arterial injections of catecholamines reflect a highly sensitive system coupled with a blood-brain barrier permeable to catecholamines (Peyraud-Waitzenegger *et al.* 1979). The differences that may exist between eels and other species in terms of the sensitivity of the ventilatory response to catecholamines are difficult to assess since the present study (Chapters 3 and 6) is the only one to report measured values rather than estimates of circulating catecholamine levels after infusion or injection of catecholamines. In addition, owing to insufficient details, any comparison with the results (reported as unpublished observations) of Aota *et al.* (1990) would be meaningless.

The results of this study do not support a stimulatory role for circulating catecholamines in the regulation of ventilation in trout during moderate environmental disturbances. First, it was shown that fish can hyperventilate during hypoxia or hypercapnia in the absence of any significant changes in the circulating levels of these amines and, second,

that experimental elevation of catecholamines in the circulation tend to diminish the hyperventilatory response to these ventilatory stimulants, independently of the blood acid-base/respiratory status of the animal. In the absence of more precise information on the blood parameters, as well as other adrenergic-sensitive systems, the mechanisms underlying this important observation remain speculative. It would appear, therefore, that if circulating catecholamines are to play a stimulatory role in the regulation of $\dot{V}w$ in trout, it must be under conditions much more severe than those utilized in this and other (e.g. Playle *et al.* 1990; present study, Chapter 3) studies as demonstrated and discussed by Aota *et al.* (1990).

CHAPTER 7

GENERAL DISCUSSION

This thesis reinforces the fact that fish react to environmental fluctuations by reflexively making $\dot{V}w$ adjustments appropriate to the gas exchange function. Indeed, the $\dot{V}w$ responses to the different external challenges occurred rapidly and probably contributed to the fish's ability to endure, as well as recover, from the corresponding disturbances.

The present study focused on the factors involved in the regulation of these ventilatory responses to environmental perturbations; and, more specifically, was aimed at elucidating the role of circulating catecholamines as potential "stimulants" of ventilation. Clearly, the results reported here refute the hypothesis that "Circulating catecholamines play a stimulatory role in the regulation of ventilation in teleosts."

Independently of the experimental approach utilized, either pre-treatment of fish with adrenoceptor antagonists or exogenous elevation of circulating catecholamines, the data obtained certainly argue against a stimulatory role for these hormones.

Chapters 3, 4 and 6, showed that during hypoxia or hypercapnia, it is possible for fish to hyperventilate without any change in the circulating EPI and/or NE levels. This indicates that catecholamine mobilization, from the chromaffin tissue, is not requisite for this response. Furthermore, if catecholamines did stimulate the $\dot{V}w$ response to external respiratory challenges, one would predict that pre-treatment of fish with adrenoceptor antagonists would impair, or at least modify, the corresponding $\dot{V}w$ increase. Aside from the notable exception (which will be discussed below) observed under hypercapnic conditions (Chapter 5), none of the antagonists utilized affected the $\dot{V}w$ responses to these stimuli. On the other hand, the experimental elevation of circulating catecholamines by intra-arterial catecholamine infusion revealed that these amines can modulate $\dot{V}w$, but in a direction opposite to the original prediction (Chapter 1); they induced hypoventilation (Chapters 3 and 6). In both studies (Chapters 3 and 6), catecholamines had a very transient influence on $\dot{V}w$ which occurred

immediately after the catecholamines were elevated. Owing to the lack of synchrony of catecholamine mobilization between fish during hypoxia, it is not surprising that a pharmacological approach involving adrenoceptor antagonists did not permit detection of the rather subtle effects that catecholamines were demonstrated to have on $\dot{V}w$ (Chapters 3 and 6).

The prevention of catecholamine elevation, by means of pharmacological or surgical treatment, was another approach tentatively utilized to study the nature of the relationship between circulating catecholamines and ventilation. Indeed, to observe the ventilatory response of fish that cannot elevate plasma catecholamine levels in the face of strong respiratory challenge, would have brought important complementary information. Metcalfe and Butler (1989) used α-methyl tyrosine in dogfish (*Scyliorhinus canicula*) to inhibit the activity of tyrosine hydroxylase, the enzyme that catalyses the hydroxylation of L-tyrosine to L-DOPA (3,4 dihydroxyphenylalanine). Since the hydroxylation of tyrosine is also the rate limiting step in catecholamine biosynthesis in fish (Jönsson and Nilsson, 1983), this treatment was reported to significantly reduce catecholamine release in this species during hypoxia (Metcalfe and Butler, 1989). This drug, however, was ineffective in rainbow trout (data not shown). In addition, surgical sectioning of the first four cranial nerves to the head kidney of cod was also attempted. The idea was inspired from the work of Wahlqvist and Nilsson (1980) along with Butler *et al.* (1989) who demonstrated that bilateral sectioning of the nerves to the head kidney significantly reduced the amount of catecholamines secreted by this organ during air exposure and exercise, respectively. The results (not shown) showed that this surgery was too invasive for the present study as sham-operated fish, in which the nerves were localized but not sectioned, could not hyperventilate normally when exposed to hypoxia. Furthermore, it was demonstrated that stimulation of sympathetic nerves to the head kidney

is not the only mechanism initiating catecholamine mobilization during hypoxia (Perry *et al.* 1990). As a consequence, this experimental approach was rejected.

The use of adrenoceptor antagonists, however, revealed that catecholamines of non-humoral origin are required for a sustained hyperventilatory response during external hypercapnia (Chapter 5). The apparent opposing effects of catecholamines, depending on their origin (humoral *versus* non-humoral) and consequently different sites of actions, are difficult to explain. Nevertheless, these results do not conflict with the conclusions drawn in other Chapters, as they do not support a stimulatory role for circulating catecholamines. It would appear, however, that this possible role for non-humoral catecholamines, is specifically related to the ventilatory responses of fish experiencing acidemia since impairment of the hyperventilation by propranolol was never reported in fish where $\dot{V}w$ was stimulated solely by a depressed water and/or blood O_2 status (Chapters 3 and 4). Acidemia, which was a condition induced by all the experimental protocols used by Aota *et al.* (1990), may be another important factor at the basis of the discrepancies between that study and the ones reported in Chapters 3, 4 and 6.

As circulating catecholamines have a depressant effect on ventilation, which is not always persistent, their role in the regulation of ventilation in fish appears to be rather limited. Thus, it is difficult to make a clear statement about the adaptive significance of this response. It should be mentioned, however, that because of the high energetic cost associated with pumping a highly viscous medium such as water, it is always advantageous for fish to minimize $\dot{V}w$ as long as the gas exchange function is performed adequately. As was discussed in Chapter 6, the adrenergic responses that cause these depressions of $\dot{V}w$ are unknown. However, because of the link that exists between blood O_2-status and $\dot{V}w$, the adrenergic regulation of Ca_{O2} may, at least in part, contribute to this phenomena under

conditions of respiratory challenge.

The diverse experimental protocols used in this thesis allowed the manipulation of different variables which may act as modulators of ventilation in fish. The simultaneous monitoring of ventilation in the presence (sham-treatment) or absence (phentolamine treatment) of the hypertensive response to hypoxia in cod (Chapter 4) suggested that changes in blood pressure do not significantly affect $\dot{V}w$ in fish. This was further supported by the observation that $\dot{V}w$ remained unaltered during normoxia in trout, despite an elevation of blood pressure stimulated by catecholamine infusion (Chapter 6). These observations highlight an important difference between fish and mammals with regards to the relationship between ventilatory and cardiovascular function. Regardless of the underlying explanations, the present data does not indicate that changes in blood pressure is an important modulator of $\dot{V}w$ in fish.

Throughout this thesis, O_2-status, whether of blood or water, emerged as being a key factor in the regulation of ventilation. Until recently, Ca_{O2} was regarded as the variable subjected to constant monitoring, which could trigger ventilatory adjustments when it deflected from its set point (see reviews by Randall, 1982; Shelton *et al.* 1986). There is, however, growing evidence arguing in favour of chemoreceptors involved in the control of ventilation that would be facing the external milieu (Burleson and Smatresk, 1990) and responding to PwO_2 fluctuations. The data obtained in Chapters 3 and 4 bring indirect support to the study of Burleson and Smatresk (1990). Indeed, it was observed that during hyperoxia, $\dot{V}w$ was depressed despite the absence of significant changes in Ca_{O2}, a response mediated solely by increases of PaO_2 and/or PwO_2 (Chapter 3). In addition, the techniques used in Chapter 4 permitted the observation of the dynamics of the hyperventilatory response to hypoxia in cod. The fact that f_V increased quickly after a small depression of PwO_2

suggests that this response is triggered directly by PwO_2 or the resulting decrease in PaO_2 since under those conditions, Ca_{O2} and plasma catecholamine levels were likely unaltered. Thus, in the light of the present results, the fundamental importance of Ca_{O2} in the regulation of ventilation in fishes should be reassessed.

Another potential ventilatory modulator that was evaluated in the present study was elevated CO_2/H^+. The results reported in chapters 5 and 6 clearly suggest that the ventilation of rainbow trout is sensitive to fluctuations of CO_2 in the blood or in the environment. The data which support this statement are the observations reported in Chapters 5 and 6, which depicted the abolishment of the hypoventilatory response to hyperoxia by external hypercapnia. As was discussed at the end of Chapter 6, the importance of CO_2/H^+ to the hyperventilatory response to external hypercapnia under normoxic conditions is difficult to evaluate since respiratory acidosis is also known to affect Ca_{O2}. The results reported in Chapter 6 suggest, however, that the hyperventilatory response to external hypercapnia under normoxic condition may have been triggered solely by the elevation of CO_2/H^+ since the blood O_2 status was not affected. According to the literature review of Jones and Milsom (1982), this response may, at least in part, be driven by chemoreceptors facing the external media. This may explain why trout did not exhibit ventilatory changes when catecholamines provoked elevations of CO_2/H^+ in the blood (Chapter 6). This lack of response, does not necessarily contradict the CO_2 retention hypothesis proposed by Perry and Wood (1989; see Chapter 1 for further details) since we cannot ignore the changes in $\dot{V}w$ that might be masked simultaneously by the depressant effects of elevated catecholamines (Chapters 3 and 6). Clearly, the respective contributions of internal *versus* external hypercapnia on the drive of $\dot{V}w$ is an area of physiology that warrants further research.

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