VENTILATION IN *AMIA CALVA*: A COMPARISON WITH WATER-BREATHING FISH.

by

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ABSTRACT

Aspects of ventilation and ventilatory control were investigated in an air-breathing fish, *Amia calva*, to determine the extent to which *Amia* is similar to water-breathing fish.

The possibility that *Amia* uses the air-breathing organ to maintain gas-exchange during periods of aestivation was tested. During gradual air-exposure, *Amia* showed no reduction in oxygen consumption, no increase in plasma urea levels or in urea excretion. Arterial blood pH (pH_a) remained constant, and arterial plasma total carbon dioxide (T_{aCO2}) and carbon dioxide partial pressure (P_{aCO2}) increased. Arterial plasma total ammonia (T_{amm}) and NH_3 concentrations rose significantly. Exposure to elevated total ammonia concentrations in the water did not elicit an increase in urea production or air-breathing. Aquatic hypoxia without access to air did not cause a reduction in aerobic metabolism and moderate levels were fatal. These results indicate that *Amia* are incapable of aestivation, due to an inability to reduce metabolism and detoxify ammonia to urea, and die following three to five days of air-exposure. The air-breathing organ is used to maintain aerobic metabolism under aquatic conditions of hypoxia or raised temperature.

The characteristics of air-breathing and gill ventilatory responses to internal acid-base disturbances were investigated in *Amia*. Acid infusions lowered pH_a and arterial blood oxygen content (C_{aO2}), raised P_{aCO2}, and stimulated air-breathing and gill ventilation. Ammonium bicarbonate infusions did not change pH_a or C_{aO2}, raised P_{aCO2}, and did not stimulate any ventilatory responses. Acid infusions

during aquatic hyperoxia lowered pH_a and raised P_{aCO2}. Arterial blood O_2 content declined but remained above normoxic levels. There were no ventilatory responses. These results indicate that air-breathing and gill ventilation responses in *Amia* are most closely correlated with blood O_2 status, not pH_a or P_{aCO2}. Air-breathing and gill ventilation responses following acid infusion were associated with a release of catecholamines into circulation. Catecholamine infusion stimulated gill ventilation but not air-breathing in *Amia*, suggesting that endogenous catecholamine release may have mediated gill ventilatory responses to hypoxaemia. These ventilatory reflex responses to acid-base disturbance, and the correlation between gill ventilation responses and catecholamine release are similar to observations made on water-breathing fish.

Ventilatory responses to increases in T_{aCO2} and T_{amm} were investigated in rainbow trout, and compared with responses by *Amia*. In trout, infusion of $NaHCO_3$ raised pH_a and T_{aCO2}, did not change P_{aCO2} or C_{aO2}, and stimulated ventilation. Infusion of NH_4HCO_3 did not change pH_a or C_{aO2}, raised T_{aCO2}, P_{aCO2} and T_{amm}, and stimulated ventilation. Infusion of NH_4Cl lowered pH_a, raised T_{amm}, and stimulated ventilation. Infusion of HCl lowered pH_a, T_{aCO2} and C_{aO2}, and stimulated ventilation. Infusion of NaOH raised pH_a but did not stimulate ventilation until twenty minutes post-infusion. Infusion of NaCl had little or no effect on pH_a, C_{aO2}, T_{aCO2} or T_{amm}, and no effect on ventilation. These results indicate that trout show a ventilatory response to increases in T_{aCO2}, increases in T_{amm} and decreases in pH_a and C_{aO2}, but not to increases in pH_a. Following HCl and $NaHCO_3$ infusion, there was a significant increase in the level of circulating

catecholamines, indicating that the ventilatory responses to reductions in pH_a and C_{aO2} and increases in T_{aCO2} may be humorally mediated by catecholamine release. The ventilatory responses to increases in T_{amm} were not associated with a catecholamine release. Unlike trout, *Amia* do not show a ventilatory response to infusion of NH_4HCO_3, i.e. to increases in T_{aCO2} and T_{amm}.

Sites and afferent pathways for ventilatory reflex responses to blood and water O_2 status were determined in *Amia*. Air-breathing and gill ventilatory reflex responses to hypoxia, sodium cyanide (NaCN), hypoxaemia and catecholamines were investigated in intact *Amia*, and compared with responses in animals following section of branchial branches of cranial nerves IX and X, and extirpation of the pseudobranch. In intact, sham-operated animals, hypoxia stimulated an increase in air-breathing and gill ventilation. Following denervation, the air-breathing response was abolished, and the gill ventilation response significantly attenuated. In sham-operated animals, NaCN in the water flowing over the gills stimulated air-breathing and gill ventilation, and NaCN given in the dorsal aorta stimulated gill ventilation. These responses were abolished following denervation. In intact animals, HCl infusion stimulated air-breathing and gill ventilation, but following denervation, the air-breathing response was abolished. The ventilatory response to catecholamines was significantly attenuated in denervated animals as compared with shams. These results indicate that air-breathing and gill ventilation reflex responses are controlled by oxygen-sensitive receptors in the gills and pseudobranch, innervated by cranial nerves VII, IX and X. These sites and afferent pathways are similar to receptors controlling hypoxic

reflex responses in water-breathing fish. The effects of catecholamines on gill ventilation are mainly exerted via stimulation of receptors in the gills, which are separate from those controlling air-breathing. The gill ventilatory responses to hypoxia, hypoxaemia and acidosis following denervation may be mediated by central effects of circulating catecholamines, or by an extrabranchial oxygen or pH receptor.

In conclusion, *Amia* is an entirely aquatic animal with the primary ventilatory control mechanisms of water-breathing fish intact, but with the added ability to breathe air at the surface.

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LIST OF ABBREVIATIONS

ABO:	Air-breathing organ
$\dot{V}_{O2}$:	Oxygen consumption
$\dot{V}_{O2}(t)$:	Total oxygen consumption
$\dot{V}_{O2}(a)$:	Oxygen consumption by air-breathing
$\dot{V}_{CO2}$:	Carbon dioxide production
RE:	Respiratory exchange ratio
pH_a:	Arterial blood pH
T_{aCO2}:	Arterial plasma total carbon dioxide content
P_{aCO2}:	Arterial plasma carbon dioxide partial pressure
HCO_3^-:	Arterial bicarbonate
C_{aO2}:	Arterial blood oxygen content
P_{aO2}:	Arterial plasma oxygen partial pressure
P_{wO2}:	Water oxygen partial pressure
T_{amm}:	Arterial plasma total ammonia content
NH_3:	Ammonia in the un-ionised form
DA:	Dorsal aorta
P_{DA}:	Dorsal aortic blood pressure
f_h:	Heart rate
P_{op}:	Opercular pressure amplitude
f_g:	Gill ventilation rate
f_{ab}:	Air-breathing frequency

NE:	Norepinephrine
E:	Epinephrine

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GENERAL INTRODUCTION

All extant terrestrial vertebrates are considered to have evolved from freshwater piscine ancestors, which developed the ability to breathe air because it conferred on them selective advantages in hypoxic water. The evolution of air-breathing and a terrestrial lifestyle was not a single event, but all terrestrial vertebrates have evolved from the same group of aquatic vertebrates, as a result of similar selective forces (Randall, Burggren, Farrell and Haswell, 1981). The colonisation of land required more substantial changes in existing systems for gas-exchange than simply the development of an ability to breathe air. The three main respiratory gases in fish are oxygen, carbon dioxide and ammonia. There are differences between air and water as respiratory media, as listed below, and the successful colonisation of land required different adaptations to control levels of respiratory gases in the body fluids. Studying respiratory adaptations in extant air-breathing fish may well give insight into the changes in respiratory control systems associated with the colonisation of land.

Water has a low capacitance for oxygen, and so in order to meet the oxygen requirements of metabolism, water-breathing fish ventilate large volumes of water across their gills. The capacitance of water for carbon dioxide is high, and so all carbon dioxide produced by metabolism is effectively flushed out of the animal across the gills, resulting in carbon dioxide tensions in the blood that are typically very low (Dejours, 1981). The result of these differential capacitances is that the primary source of respiratory drive in water-breathing fish is oxygen, and there

appears to be little sensitivity to carbon dioxide (Smith and Jones, 1982, Shelton, Jones and Milsom, 1986).

The capacitance of water for ammonia is even higher than that for carbon dioxide, and so ninety percent of ammonia produced by protein catabolism is also effectively flushed out of the animal across the gills, and about ten percent voided in the urine (Randall and Wright, 1987; Randall, 1990). The result of this is that freshwater fish excrete the majority of their nitrogenous waste as ammonia (Randall and Wright, 1987; Randall, 1990).

The oxygen content of air is high, so animals with the capacity to breathe air do not have to ventilate to the same extent as water-breathers to meet metabolic oxygen requirements. The oxygen and carbon dioxide capacitances of air are equal, so changes in ventilation will change the amount of carbon dioxide in the body fluids, and therefore acid-base status (Rahn, 1966; Rahn and Howell, 1976; Dejours, 1981). Thus there was undoubtedly selection pressure in favour of air-breathers that were able to monitor both body fluid oxygen and carbon dioxide or pH levels, and adjust ventilation to maintain homeostasis. This is an ability that all extant vertebrate air-breathing groups possess (as reviewed by Dempsey and Forster, 1982; O'Regan and Majcherczyk, 1982; Scheid and Piiper, 1986; Shelton, Jones and Milsom, 1986; Smatresk, 1990).

The capacitance of air for ammonia is extremely low, and so ammonia, which is toxic in all vertebrates, would tend to accumulate very quickly in air-breathers. The requirements of water balance on land are such that continual water loss to remove ammonia would not be sustainable. This would exert intense selection

pressure in favour of those animals that were able to detoxify ammonia, and accumulate non-toxic wastes. Thus, all terrestrial vertebrates have the ability to detoxify all nitrogenous waste as urea or uric acid (Smith, 1961), an adaptation that is absent in most extant water-breathing, bony fish (Mommsen and Walsh, 1989).

While amphibians are known to show ventilatory sensitivity to carbon dioxide and pH (MacIntyre and Toews, 1973; Ishii, Ishii and Kusakabe, 1985; Smatresk, 1990), there is very limited information about ventilatory control in air-breathing fish, and the extent to which such control systems are similar to air-breathing or water-breathing systems. It is likely that air-breathing fish are more similar to water-breathers, because the air-breathing organ (ABO) in these animals is used to supplement oxygen uptake, and the gills are used in carbon dioxide excretion (Johansen, 1970). It is probable that ventilatory sensitivity to carbon dioxide developed when the lung became a site for carbon dioxide excretion into air, as is the case in amphibians (Randall, 1974; MacIntyre and Toews, 1973).

A capacity to detoxify ammonia as urea by the ornithine cycle used by terrestrial vertebrates is found in all elasmobranchs, but only in a number of bony fish, for example as an adaptation to survive air-exposure by a air-breathing fish (Saha and Ratha, 1986) or alkaline waters in a water breathing fish (Randall, Wood, Perry, Bergman, Maloiy, Mommsen and Wright, 1989).

As stated earlier, oxygen is the primary ventilatory stimulus in water-breathing fish (Smith and Jones, 1982; Randall, 1982; Shelton *et al.*, 1986). There is

evidence, however, that fish show a ventilatory response to increases in plasma carbon dioxide content, when associated with a plasma alkalosis (Janssen and Randall, 1975), and mammals are known to show a ventilatory response to ammonia (Wischer and Kazemi, 1974). Ventilatory responses to increases in plasma carbon dioxide and ammonia content have not been investigated in either water-breathing or air-breathing fish. Increases in ventilation might function to remove excesses of endogenously produced carbon dioxide or ammonia.

Despite considerable differences in respiratory physiology, the neuro-hormones norepinephrine and epinephrine exert effects on ventilation in all vertebrate groups. Infusion of catecholamines stimulates ventilation in several water-breathing fish (Peyraud-Waitzenegger, 1979; Aota, pers. comm.), and in mammals (Dempsey, Olson and Skatrud, 1986). Furthermore, there is evidence to suggest that catecholamines are involved in respiratory control in both water-breathing fish and in mammals, via effects on peripheral chemoreceptors and the central nervous system (Dempsey *et al.*, 1986; Randall and Taylor, 1989; Aota, Holmgren, Gallaugher and Randall, 1990). There is no information about the possible role of catecholamines in gill ventilation and air-breathing responses in air-breathing fish. Ventilatory responses to increases in blood pH, carbon dioxide content or ammonia might be mediated by circulating catecholamines.

In mammals, peripheral receptors affecting ventilation are in the carotid and aortic bodies (O'Regan and Majcherczyk, 1982). In reptiles and amphibians,

oxygen and carbon dioxide sensitive receptors have also been identified in the blood vessels leaving the heart (Ishii, Ishii and Kusakabe, 1985a,b). These areas, for amphibians, reptiles and mammals, are anatomically homologous with the gill arches of fish (Romer, 1970). Indeed oxygen sensitive receptors have been identified in the first gill arch of fish (Milsom and Brill, 1986; Burleson and Milsom, 1990). The site of peripheral receptors controlling gill ventilation and air-breathing responses in air-breathing fish are unknown, although there is evidence that they are in the gills (Smatresk, 1986; Smatresk, 1987).

Amia calva is an Actinopterygian fish, the only extant species in the sub-division Halecomorpha. It is a primitive fish, only distantly related to teleosts and other extant bony fishes; with fossil remains dating from the Jurassic (Nelson, 1984). *Amia* is piscivorous, and an active predator. It occurs in shallow, slow-moving freshwater in eastern North America, from southern Ontario and Quebec to Texas (Scott and Crossman, 1973). *Amia* provides a readily available and interesting animal model of an intermediate stage in the evolution of air-breathing and survival on land. It possesses both gills for water-breathing and a swimbladder adapted to function as an air-breathing organ. The gills have an unusual structure, to prevent collapse in air (Daxboeck, Barnard and Randall, 1981; Olson, 1981), and there are anecdotal reports (Dence, 1933; Neill, 1950) that it is capable of surviving prolonged periods of air-exposure by aestivating. This apparent ability to aestivate, and the implication that *Amia* can detoxify ammonia as urea in a manner similar to the African lungfish (Smith, 1930; Smith,

1961), has never been tested. It is also unknown whether the respiratory physiology of *Amia* is similar to purely water-breathing fish, or whether it displays some characteristics of air-breathing vertebrates.

This thesis examines the responses of *Amia calva* to gradual air-exposure, to determine whether they aestivate in a manner similar to the lungfish (Smith, 1930; Delaney, Lahiri and Fishman, 1977). The possibility that *Amia* shows ventilatory responses to changes in blood pH and carbon dioxide tension is tested, and the ventilatory responses to catecholamines assessed. Ventilatory responses to increases in plasma carbon dioxide and ammonia content are investigated in water-breathing fish (trout) and compared with responses seen in *Amia*, and a possible role for circulating catecholamines in the observed responses is discussed. The site of peripheral chemoreceptors controlling gill ventilation and air-breathing responses, and the site of catecholamine stimulation of ventilation is determined in *Amia*. The results of these experiments are discussed to assess the extent to which *Amia* is similar to water-breathing or air-breathing vertebrate groups.

GENERAL MATERIALS AND METHODS.

Experimental animals:

Amia calva were netted in Lake Erie, southern Ontario, Canada. Animals were air-freighted to U.B.C., in large, heavy duty bags containing one-third water and two-thirds 100% O_2. Mortality from transit was less than one percent. At U.B.C., *Amia* were maintained in large outdoor circular fibreglass tanks, with a constant flow of dechlorinated Vancouver tapwater (Temperature = 7 to 12°C, pH approx. 6.5). The animals were fed live goldfish, trout fry or salmon fry, usually *ad-libitum*, and at least once per week. All animals were allowed a minimum of three weeks recovery following transit, before experimentation.

All experiments were conducted at 20°C. *Amia* were placed in small plexiglass holding tanks, and the temperature raised to 20°C over a minimum of three days (usually five).

Rainbow trout were obtained from West Creek Trout Farms, Aldergrove, B.C. and maintained in large, outdoor circular fibreglass tanks with a constant flow of dechlorinated Vancouver tapwater, at a temperature of 7 to 12°C. Animals were fed trout chow daily, and starved for at least 48h prior to use in any experiments.

Cannulations:

Fish were fitted with chronic indwelling cannulae in the dorsal aorta and, when required, in the operculum, under general anaesthesia in short operations lasting 15 to 20 minutes. All fish were anaesthetised in Tricaine Methane Sulphonate

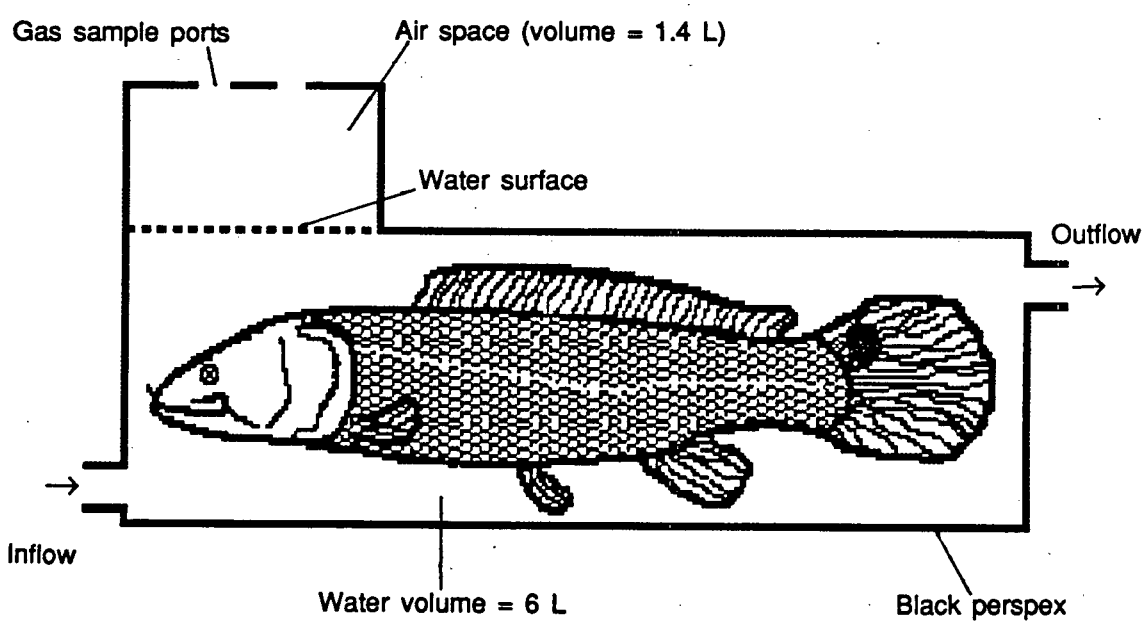
(MS222) at concentrations of 1:10,000 until ventilatory movements ceased, and then placed ventral side up on an operating table where the gills were irrigated with an MS222 solution at 1:20,000.

Dorsal aortic cannulations were performed using the technique of Soivio, Westman and Nyholm (1972). A sharpened wire was inserted into PE-50 tubing such that only the tip protruded at the end. A blind puncture was made in a caudal direction (at a 45 degree angle) in the midline of the branchial basket, between the first and second gill arches. The wire punctured the wall of the dorsal aorta, and then was used to guide the tubing into the vessel. The wire was removed, and the tubing advanced for three to five centimetres. The cannula was secured to the roof of the mouth with a suture, and led out of the roof of the mouth in front of the nares, via a flanged section of tubing (PE-200).

Opercular cannulation was performed by drilling a hole in the centre of the operculum, and feeding a flanged section of tubing (PE-200) through from the inside. A flanged cuff was then attached on the outside, to hold the cannula tightly in place.

All fish were allowed to recover from surgery for 48h in individual perspex boxes, with an ample flow of water. *Amia* were allowed to recover in boxes with an anterior air-space (fig 1), to allow air-breathing. Dorsal aortic cannulae were flushed daily with heparinised (10,000 USP units/L sodium heparin) Cortland's saline (Wolf, 1963).

Figure 1: Individual plexiglass box with anterior air-space for air-breathing by *Amia*.


Gas sample ports
Air space (volume = 1.4 L)
Water surface
Outflow
Inflow
Water volume = 6 L
Black perspex

Measurement of blood and plasma variables:

Arterial blood pH was measured using a Radiometer microelectrode (E5021) and acid-base analyser (PHM 72) thermostatted to the temperature of the fish, and calibrated with Radiometer precision phosphate buffers S1500 and S1510.

Arterial blood P_{O2} was measured on anaerobically collected blood samples using a Radiometer electrode (E5046) and acid-base analyser, thermostatted as for pH_a. The O_2 electrode was calibrated with water-saturated N_2 and air.

Arterial blood O_2 content was measured using a Radiometer electrode (E5046) calibrated with O_2-free sodium sulphite, and air-saturated water, and the method described by Tucker (1967), at the fish temperature, using 30 µl blood samples collected anaerobically in a gastight Hamilton syringe.

Plasma for T_{aCO2} determination was obtained by centrifuging (Damon/IEC) blood, immediately upon collection, in heparinised microhaematocrit capillary tubes, and withdrawing 25 µl of plasma into a gastight syringe. 25 µl of plasma was shaken for three minutes with 1 ml of 0.1M HCl and 7 ml of 100% N_2 in a 10 ml gastight syringe, to liberate all the CO_2 into the gas phase and ensure equilibration of the gas and liquid phase. At least 5.5 ml of the gas was injected via a drying filter into a 1 ml sample loop of a gas chromatograph (Carle GC100) with a Poropak Q CO_2 discriminating column. The gas chromatograph was calibrated with 10 mM T_{CO2} standards. The T_{aCO2} was calculated by integrating the signal from the gas chromatograph with a data acquisition card (Data Translation 2801) and an Olivetti M24 computer.

Plasma for T_{amm} and urea concentration ([urea]) determination was obtained by centrifuging whole blood samples in 1.5 ml micro test-tubes (Eppendorf) with a micro-centrifuge (Fisher, Model 235), within five minutes of collection. Plasma was stored on ice for a maximum of 30 minutes. Total ammonia concentration was determined colorimetrically with a UV spectrophotometer (Shimadzu UV 160), and a Sigma kit. Plasma [urea] was determined by incubating plasma with urease (Boeringher) and then analysing the samples with the Sigma ammonia kit.

Plasma samples for catecholamine analysis were collected by centrifuging whole blood in a micro-centrifuge (Fisher, Model 235), decanting plasma, and immediately freezing in liquid N_2. Catecholamine concentrations ([NE] and [E]) were determined on alumina-extracted plasma samples by high performance liquid chromatography (HPLC) with electrochemical detection, using a Waters Plasma Catecholamines reverse-phase column, Waters M460 Electrochemical Detector and Waters 510 solvent delivery pump (Waters/Millipore), as described by Woodward (1982) and Primmett, Randall, Mazeaud and Boutilier (1986), with peaks generated on a chart recorder (Soltec 1241). Catecholamine concentrations were calculated by integrating the area under peaks with Sigmascan (Jandel Scientific) and an Olivetti M24 computer, and comparing with peaks from DHBA, NE and E standards.

Measurement of water and air variables:

Water pH was measured using a Radiometer combination pH electrode (GK 2402B) and a Radiometer acid-base analyser (PHM 72). The electrode was calibrated with standard Radiometer buffer solutions at pH 7 and 4.

Water and air P_{O2} were measured as for P_{aO2}, but with large (5 to 10 ml) samples in gastight syringes.

Water T_{CO2} was measured as for T_{aCO2}, but 1 ml of water was mixed with 1 ml 0.1M HCl and 9 ml 100% N_2 in a gastight syringe. The gas-chromatograph was calibrated with 0.5mM T_{CO2} standards.

Water total ammonia concentration was measured colorimetrically with a UV spectrophotometer (Shimadzu UV 160) using a micro-modification (D.G MacDonald, pers. comm.) of the technique of Verdouw, van Echteld and Dekkers (1978). Water [urea] was measured using a micromodification (C.M. Wood, pers. comm.) of the Crocker (1973) technique.

Calculations:

Plasma P_{CO2} was calculated using the Henderson-Hasselbalch equation:

$$P_{aCO2} = \frac{T_{aCO2}}{(\alpha Co2) \,.\, (1 + \text{antilog}\,(pH - pK))}$$

Apparent pK and αCO_2 values for trout plasma were used as calculated for the correct temperature in Boutilier, Heming and Iwama (1984), for trout, and for

Amia the apparent pK and αCO_2 for gar (*Lepisosteus osseus*) plasma as calculated at 20°C in Smatresk and Cameron (1982) was used.

Plasma NH_3 ($[NH_3]$) concentration was calculated with the Henderson-Hasselbalch equation:

$$\text{Plasma } [NH_3] = \frac{T_{amm} \cdot (\text{antilog } pH_a - pK)}{1 + (\text{antilog } pH_a - pK)}$$

Plasma pK was calculated from values given for *Oncorhynchus mykiss* in Cameron and Heisler (1983).

Water P_{CO2} was calculated using the Henderson-Hasselbalch equation, and the apparent pK and αCO_2 as calculated (for the experimental temperature) in Boutilier *et al.* (1984).

Water $[NH_3]$ was calculated as for plasma $[NH_3]$, using the pK at the correct water temperature from Boutilier *et al.* (1984).

Chapter 1: Physiological responses to gradual air-exposure in *Amia*.

INTRODUCTION

There are reports in the literature that *Amia* can survive prolonged emersion by aestivating, in a manner similar to the African lungfish, *Protopterus* sp. (Smith 1961). Dence (1933) found an *Amia* living in a mud puddle, in northeastern U.S.A. (New York) and the animal quickly burrowed into the substrate when disturbed. In southeast U.S.A. (Georgia), Neill (1950) found an *Amia* in a spherical underground chamber, at some distance from a recently flooded river. The animal was apparently in good health.

To aestivate, an animal must be able to reduce water loss, avoid a toxic accumulation of wastes and rely on air-breathing for gas exchange. Avoiding desiccation requires that ventilation, and therefore oxygen uptake, be reduced, leading to a reduction in aerobic metabolism. This also allows conservation of energy stores, since feeding is impossible. Burrowing further reduces evaporative water losses. Detoxification of ammonia to urea allows the animal to store nitrogenous wastes, avoid ammonia toxicity and reduce urine volumes. Thus, if *Amia* aestivate, they must be capable of reductions in aerobic metabolism and of converting ammonia to urea.

In the present study, *Amia* were gradually air exposed over a ten day period, and various respiratory and internal variables measured to determine whether they aestivate. It is possible that emersion is not an adequate stimulus to initiate aestivation, so two further experiments were performed. Elevated aquatic ammonia levels are known to stimulate increases in urea production in the

goldfish (*Carassius auratus*) (Olson and Fromm 1971), and an increase in water borne irritants leads to an increase in air-breathing in gar (*Lepisosteus osseus*) (Smatresk 1988). If an *Amia* were trapped in a gradually evaporating mud puddle, a build up of ammonia in the water, rather than dehydration, might stimulate an increase in urea production and air-breathing. Thus, *Amia* were exposed to elevated aquatic ammonia levels, and air and water breathing and urea production monitored. In sturgeon (*Acipenser transmontanus*) aquatic hypoxia elicits a reduction in aerobic metabolism (Burggren and Randall 1979) and the same is true in trout (*Oncorhynchus mykiss*) (Boutilier *et al.* 1988). *Amia* were exposed to differing degrees of aquatic hypoxia, without access to air breathing to supplement their oxygen uptake, to determine if this resulted in a decrease in aerobic metabolism.

MATERIALS AND METHODS.

Experimental animals:

Amia calva weighing between 300 and 1000 g were maintained and temperature acclimated as described in General materials and methods.

Animal preparation:

Following three to five days at 20°C, the animals were anaesthetized in a buffered ($NaHCO_3$) tricainemethanesulphonate (MS222) solution at a concentration of 1:10,000 and transferred to an operating table, where they were maintained at a MS222 concentration of 1:20,000. Dorsal aortic cannulae (PE50) were implanted using the technique of Soivio *et al.* (1972). The fish were then left to recover for 48 hours in the plexiglass holding tanks.

Experimental protocols:

1) Control Measurements:

Animals were placed in individual black plexiglass boxes (volume = 9 l) with access to a forward air space for air-breathing (volume 1.4 l), and allowed 24 hours to recover. Following recovery, a 1 ml blood sample was removed, replaced with an equal volume of heparinised (1:1,000) Cortland's saline (Wolf 1963), and pH_a, T_{amm} and plasma urea concentration ([urea]) measured as described in General materials and methods. The forward airspace was then sealed, and the decline in P_{O2} as a result of air-breathing by the *Amia* measured over a two hour

period, following which the space was re-opened. Samples of inflow and outflow water were also analysed for P_{wO2}. The blood sampling regime was repeated at two to three day intervals. This protocol was followed for a minimum of ten days.

2) Air Exposure:

Following the post-surgical recovery period, while still in the plexiglass holding tank, 1.5 ml blood samples were collected anaerobically, in a gastight syringe (Hamilton), and replaced with an equal volume of heparinized saline. Arterial blood pH, T_{aCO2}, T_{amm} and plasma [urea] were measured, as described in general materials and methods.

The animals were then placed in black plexiglass chambers (volume approx. 120 l) containing a known volume of water (approx. 50 l) and a substrate of either washed river sand or 1/8" mesh plastic netting slung between bags of washed river sand. A control chamber containing the same substrate and water volumes, but no fish, was also prepared. The chambers were placed at a slight diagonal inclination using ramps of bagged sand, and the water circulated via an inlet at the topmost corner of the chamber lid and an outlet at the bottommost corner of the chamber. The water was circulated from experimental to control chamber, and vice-versa, using a peristaltic pump (Watson Marlowe MRHE 100 using Marprene 0.5 cm I.D. tubing). Water flow rate was maintained at approximately 12 litres per hour. The lids of the chambers could be closed to produce an airtight seal. The water in control and experimental chambers could

be circulated separately and mixed samples of both water and air in the chambers removed to monitor changes in P_{O2}.

Water volume was decreased at a rate of approximately four litres per day, from both control and experimental chambers, so that the fish were completely air exposed at ten days (exact volumes of water removed differed slightly for each fish, as a result of differing initial volumes). Water samples were taken every day, and pH, T_{amm} and [urea] measured. Every third day, a 1.5 ml blood sample was collected anaerobically, and replaced with an equal volume of heparinized saline. Arterial blood pH, T_{aCO2}, T_{amm}, and plasma [urea] were analysed. If the fish's cannula was not patent, then an attempt to remove a blood sample was made the following day. Every attempt was made to disturb the animal as little as possible during sample collection. Twenty four hours after first placing the fish in the chamber, or blood sampling, water flow between control and experimental chambers was separated and the chambers sealed. Water (a minimum of 3 x 5 ml) and air (a minimum of 3 x 10 ml) samples were removed from each chamber and analysed for P_{O2} and T_{CO2}. Based on measurements of $\dot{V}_{O2}$ under control conditions, the experimental chamber remained sealed long enough to produce an approximately 20 mmHg decline in P_{O2}, and then additional water and air samples were removed, P_{O2} measured, and the chamber unsealed.

Once the animal was completely air-exposed, water removal was stopped but blood, air and water sampling continued, as described, until the fish's death.

3) Ammonium Chloride exposure:

Fish were placed in individual black plexiglass boxes identical to those used

for control measurements. 24 hours later, a 1 ml blood sample was removed, and replaced with an equal volume of heparinized saline. Arterial blood pH, T_{amm} and plasma [urea] were measured. Daily measurements were made of inflow and outflow water P_{O2}, and of the decline in P_{O2} in the forward chamber over a two hour closure period. The flow of water through the boxes was then shut off, and water samples removed and analysed for [urea]. Two hours later, further samples were collected and the same parameter measured. Flow was then resumed.

Following the removal of control blood, air, and water samples, ammonium chloride (NH_4Cl) was pumped into a large header tank at a constant rate, where it was mixed with incoming water, so that the fish were exposed to water with an NH_4Cl concentration of 923 ±9 (mean ± S.E.) μmol/l. Water pH was 6.68 ± 0.1, water [NH_4+] was 921 μmol/l and water [NH_3] was 1.9 μmol/l. Slightly higher NH_4Cl concentrations led to over 50% mortality. The water and air sampling protocol described above was followed daily for 10 days of exposure to NH_4Cl. Every second day, the blood sampling regime was repeated.

Eight fish were put through the above protocol, but all animals no longer had patent cannulae after four days. In order to obtain blood readings from animals later in the exposure regime, fish were exposed to the NH_4Cl for three to eight days, and then chronically cannulated. Cannulation was by the same method as desribed earlier, but fish were anaesthetized in water with MS-222 and 923 ±9 mmol/l NH_4Cl, and irrigated during surgery with water at the same NH_4Cl concentration. Following a 48 hour recovery period, blood samples were withdrawn and analysed for T_{amm} and [urea].

4) Hypoxic Exposure:

Amia were placed in individual black plexiglass boxes (volume = 6 l), without access to an airspace, and with a water flow rate of approximately 500 ml/min. 24 hours later, samples of inflow and outflow water were analysed for P_{O2} and T_{CO2}, and a 1 ml blood sample withdrawn anaerobically, replaced with an equal volume of heparinized saline, and pH_a, T_{aCO2}, and T_{amm} measured immediately.

The P_{O2} of the inflow water was then reduced using a gas exchange column with nitrogen gas flowing counter-current to water flow. The water P_{O2} was reduced to one of four levels: 111±0.59 mmHg, 85.2±2.37 mmHg, 59.3±0.34 mmHg, or 30±0.22 mmHg. Following 24 hours' exposure to one of these P_{wO2} levels, samples of inflow and outflow water were analysed for P_{O2} and T_{CO2}, and a 1 ml blood sample withdrawn, replaced with saline, and the relevant variables measured.

Analytical methods:.

During air exposure, $\dot{V}_{O2}$ was calculated, given the change in P_{O2} in the boxes while sealed, the time elapsed, the water and air volumes, and the weight of the fish. Oxygen consumption was expressed as mg/kg/hr, and corrected for any background oxygen consumption as measured in the control box. Oxygen consumption and $\dot{V}_{CO2}$ were calculated in aquatic hypoxia, using the Fick principal and the values of P_{O2} and T_{CO2} for inflowing and outflowing water. Oxygen consumption under control conditions and during ammonium chloride exposure

was calculated in the same way for the water phase ($\dot{V}_{O2}(w)$). Under all these circumstances, inflow and outflow gas tensions were in steady state. $\dot{V}_{O2}$ in air ($\dot{V}_{O2}(a)$) was calculated knowing the volume of the air space, the decline in P_{O2} during closure, the closure time and the fish's weight. Total $\dot{V}_{O2}$ ($\dot{V}_{O2}(t)$) was calculated by adding water and air quantities.

Water and plasma P_{CO2} were calculated using the Henderson-Hasselbalch equation, as described in general materials and methods. Plasma bicarbonate concentration ($[HCO_3^-]$) was calculated using the following equation:

$$\text{plasma } [HCO_3^-] = T_{aCO2} - (\alpha\ CO_2\ .\ P_{aCO2})$$

Plasma and water $[NH_3]$ were calculated using the Henderson-Hasselbalch equation, as described in General materials and methods.

Urea excretion rates during ammonium chloride exposure were calculated given the initial and final water urea concentrations, the closure time, the box volume and the fishes' weight.

Statistical analysis:

All measured variables under control, air exposure and NH_4Cl exposure conditions were plotted against time, and the relationship described with a best-fit linear regression. The regression coefficient of each variable was compared with a coefficient of zero, and the regression coefficients of the control variables were compared with the regression coefficients of the same variables during air or NH_4Cl exposure, using a modification of the Student's T-test (Zar 1984). The least squares fit of the regression to the data was not improved by using second or third order regressions. The regression coefficients of T_{aCO2}, P_{aCO2} and $[HCO_3^-]$ during air exposure were compared in the same way.

The mean ± S.E. was calculated for the initial ("day 0") values of the control variables, and compared with the same variables during air and NH_4Cl exposure using T-tests. $P<0.05$ was taken as the fiducial limit of significance.

For the hypoxic exposure experiment, measured variables at each level of P_{wO2} were compared using the Kruskal-Wallis test for non-parametric analysis of variance. In those cases where there was a significant ($p < 0.05$) difference amongst variables at different P_{wO2} levels, a modification of the Tukey "a posteriori" test (Zar 1984) was used to compare the control and experimental conditions.

RESULTS

Gradual air exposure on a substrate of autoclaved alluvial mud (n = 4), sterile potting soil (n = 3) or washed river sand (n = 6) did not result in any attempts to burrow by the *Amia*. During gradual air-exposure the fish created a shallow depression in the substrate by moving their body from side to side. Neither air exposure nor NH_4Cl exposure led to an increase in urea production or excretion. Air exposure and aquatic hypoxia without access to air did not elicit any reduction in $\dot{V}_{O2}$.

1) Control:

The best fit linear regression equations, R^2 and mean initial values ± S.E. of the control variables are in Table 1. Figures 2A and B show the effects of time on all variables measured. None of the regression coefficients of the control variables showed a significant difference from zero, indicating that these parameters remained constant over time.

2) Air-exposure:

All the fish survived at least 24 hours of complete air exposure, and most animals survived for three to five days. Following complete air-exposure, the

Table 1: Best-fit linear regression equations and mean initial values of respiratory and blood gas variables under control aquatic conditions.

Variable	N	n	Best-fit	R^2	R.C.S.E.	mean±SE
$\dot{V}_{O2}(t)$	6	45	y=.51x+63.04	0.01	0.72	61.5±13.4
$\dot{V}_{O2}(a)$	6	49	y=0.31x+7.27	0.05	0.20	3.9±2.8
pH_a	6	31	y=0.08x+6.91	0.06	0.06	7.70±0.03
T_{amm}	6	28	y=1.40x+312.6	.001	0.04	314.6±55.7
$[NH_3]$	6	25	y=-0.15x+5.9	0.09	-0.3	6.1±1.4
[urea]	6	27	y=.51x+121.7	0.08	-0.28	108.6±2.9

N = number of fish, n = number of observations.

R.C.S.E. = regression coefficient standard error.

Units: $\dot{V}_{O2}(t)$ and $\dot{V}_{O2}(a)$ = mg/kg/hr; T_{amm}, [NH] and [urea] = μmol/l.

Figure 2A: The relationship between $\dot{V}_{O2}(t)$, $\dot{V}_{O2}(a)$ and pH_a and Time (days) under control aquatic conditions. n = 6

Each symbol represents an individual animal.

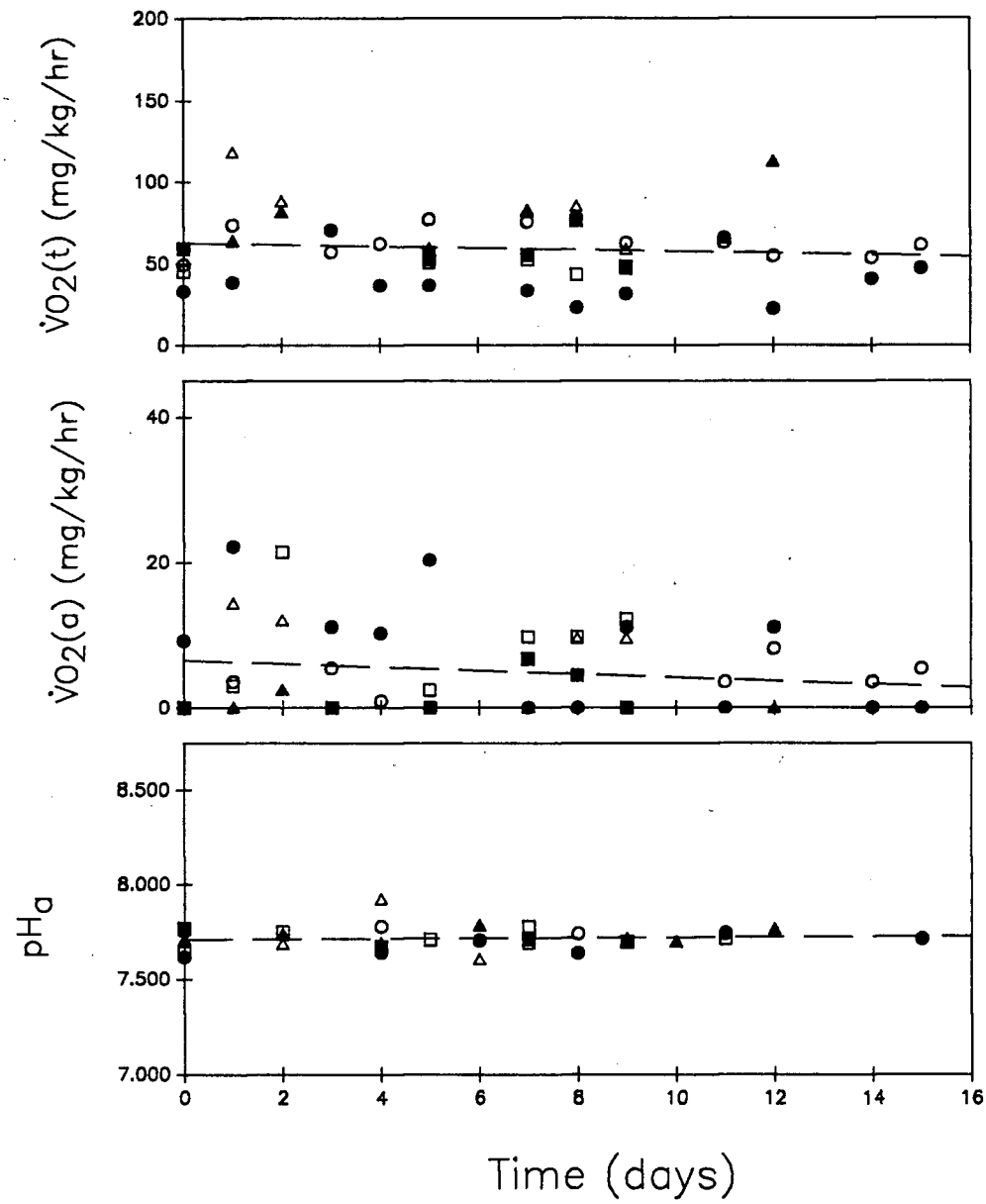
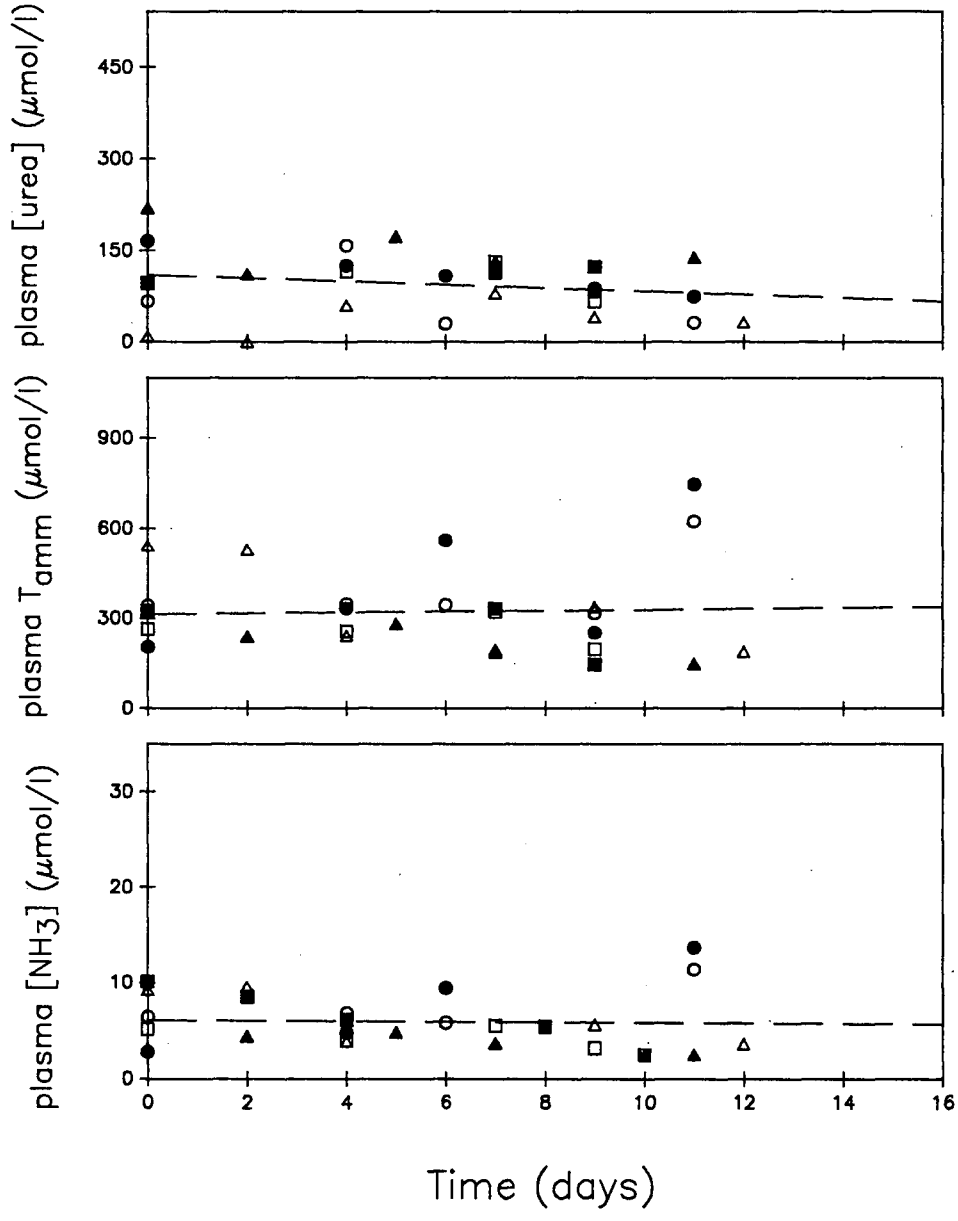

$\dot{V}O_2(t)$ (mg/kg/hr)
200
150
100
50
0
$\dot{V}O_2(a)$ (mg/kg/hr)
40
20
0
pH_a
8.500
8.000
7.500
7.000
0
2
4
6
8
10
12
14
16
Time (days)

Figure 2B: The relationship between T_{amm}, $[NH_3]$ and [urea] and Time (days) under control aquatic conditions. n = 6

Each symbol represents an individual animal.


plasma [urea] (µmol/l)
450
300
150
0
plasma T_{amm} (µmol/l)
900
600
300
0
plasma [NH_3] (µmol/l)
30
20
10
0
0
2
4
6
8
10
12
14
16
Time (days)

animals continued to make gill ventilation movements, interspersed with air-breathing behaviour. At death, most animals had a characteristically bloated appearance, suggesting over-inflation of the swimbladder.

The relationship between time (days), and measured respiratory and blood gas variables during gradual emersion can be seen in figures 3A, B and C . The best fit linear regression equations, R^2 and mean initial values ± S.E. of these variables are shown in Table 2. The mean initial values for $\dot{V}_{O2}$, pH_a, T_{amm}, and plasma $[NH_3]$ were not significantly different from control values. The mean initial value for plasma [urea] was significantly higher in the animals that subsequently underwent air exposure than in the control animals. There was no reduction in $\dot{V}_{O2}$ during gradual air-exposure, indeed, $\dot{V}_{O2}$ rose slightly as the *Amia* gradually became emersed, and the regression coefficient was significantly different from zero (fig 3A). However, the regression coefficient during gradual emersion was not significantly different from the regression coefficient derived for control measurements.

Arterial blood pH and plasma [urea] (fig 3A) did not change during gradual emersion. Arterial plasma total CO_2, P_{aCO2} and $[HCO_3^-]$ (fig 3B) all rose significantly during air exposure, especially following complete air-exposure at 10 days. The regression coefficients for T_{aCO2}, P_{aCO2} and $[HCO_3^-]$ were not significantly different from each other, all increased to the same extent. Arterial plasma total [ammonia] and plasma $[NH_3]$ both increased significantly during gradual emersion (fig 3C), especially following complete air-exposure at 10 days.

Table 2: Best-fit linear regression equations and mean initial values of respiratory and blood gas variables during gradual air-exposure.

Variable	N	n	Best-fit	R^2	R.C.S.E.	mean±SE
$\dot{V}_{O2}$	7	30	y=2.6x+63.61	0.13	1.25	77.8±9.4
pH_a	8	34	y=0.01x+7.71	0.13	3.34	7.72±0.01
T_{aCO2}	6	24	y=0.76x+12.13*	0.48	0.17	11.8±0.57
P_{aCO2}	6	24	y=0.79x+8.34*	0.32	0.25	7.76±0.39
$[HCO_3^-]$	6	24	y=0.72x+11.7*	0.48	0.16	11.40±0.55
T_{amm}	9	38	y=60.x+251.0*	0.24	13.9	383.1±59.0
$[NH_3]$	9	36	y=0.67x+4.9*	0.31	0.17	6.7±1.0
[urea]	6	25	y=1.80x+315.8	0.01	4.08	304.3±53

N = number of fish, n = number of observations.

R.C.S.E. = regression coefficient standard error

Units: $\dot{V}_{O2}$ = mg/kg/hr; T_{aCO2} and $[HCO_3^-]$ = mmol/l; P_{aCO2} = mmHg; T_{amm}, $[NH_3]$ and [urea] = μmol/l.

* = significantly different from zero and/or control regression.

Figure 3A: The relationship between $\dot{V}_{O2}$, pH_a and [urea] and Time (days) during gradual air-exposure

Each symbol represents an individual animal.

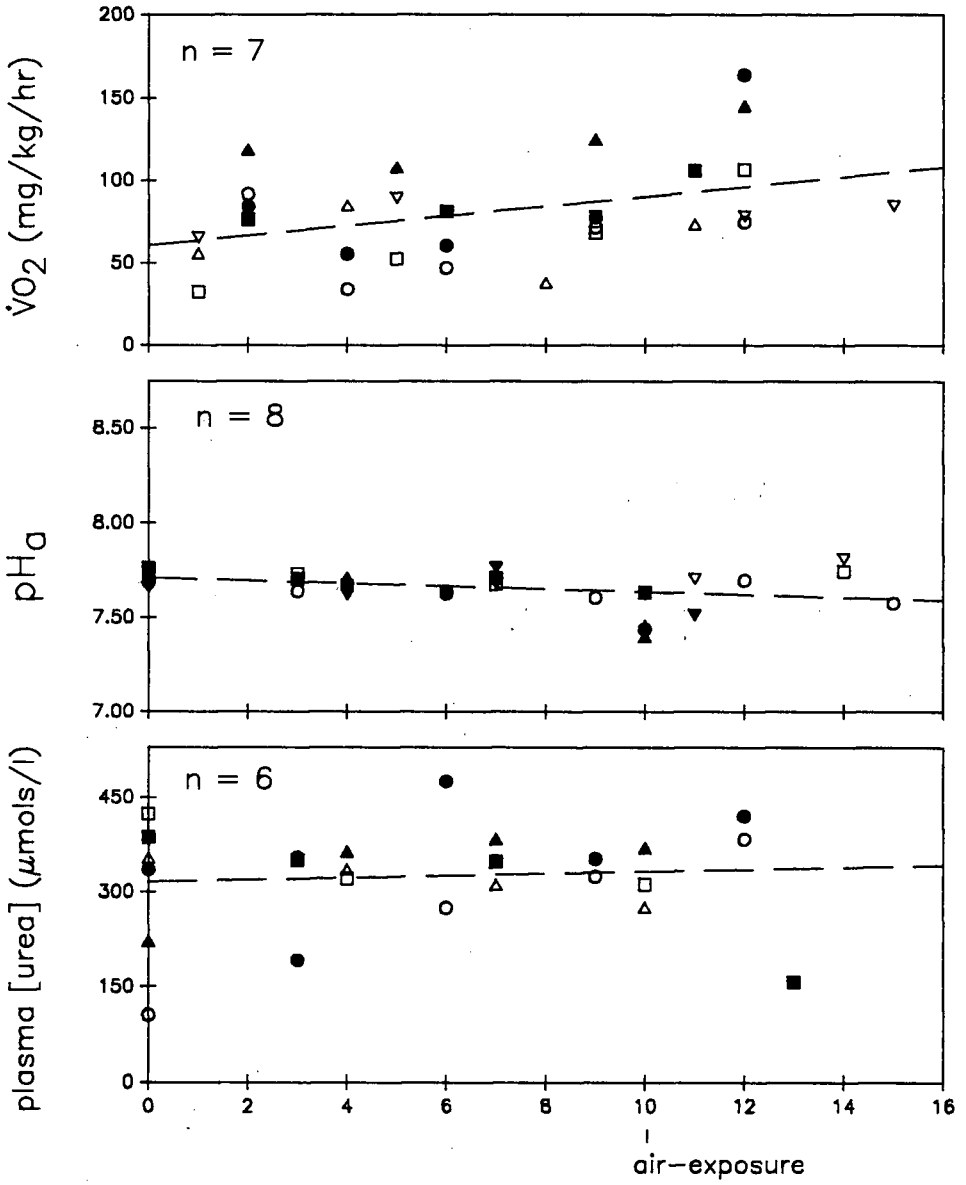

n = 7
$\dot{V}O_2$ (mg/kg/hr)
200
150
100
50
0
n = 8
pH_a
8.50
8.00
7.50
7.00
n = 6
plasma [urea] (μmols/l)
450
300
150
0
0
2
4
6
8
10
12
14
16
air–exposure
Time (days)

Figure 3B: The relationship between T_{aCO2}, $[HCO_3^-]$, and P_{aCO2} and Time (days) during gradual air-exposure

Each symbol represents an individual animal.

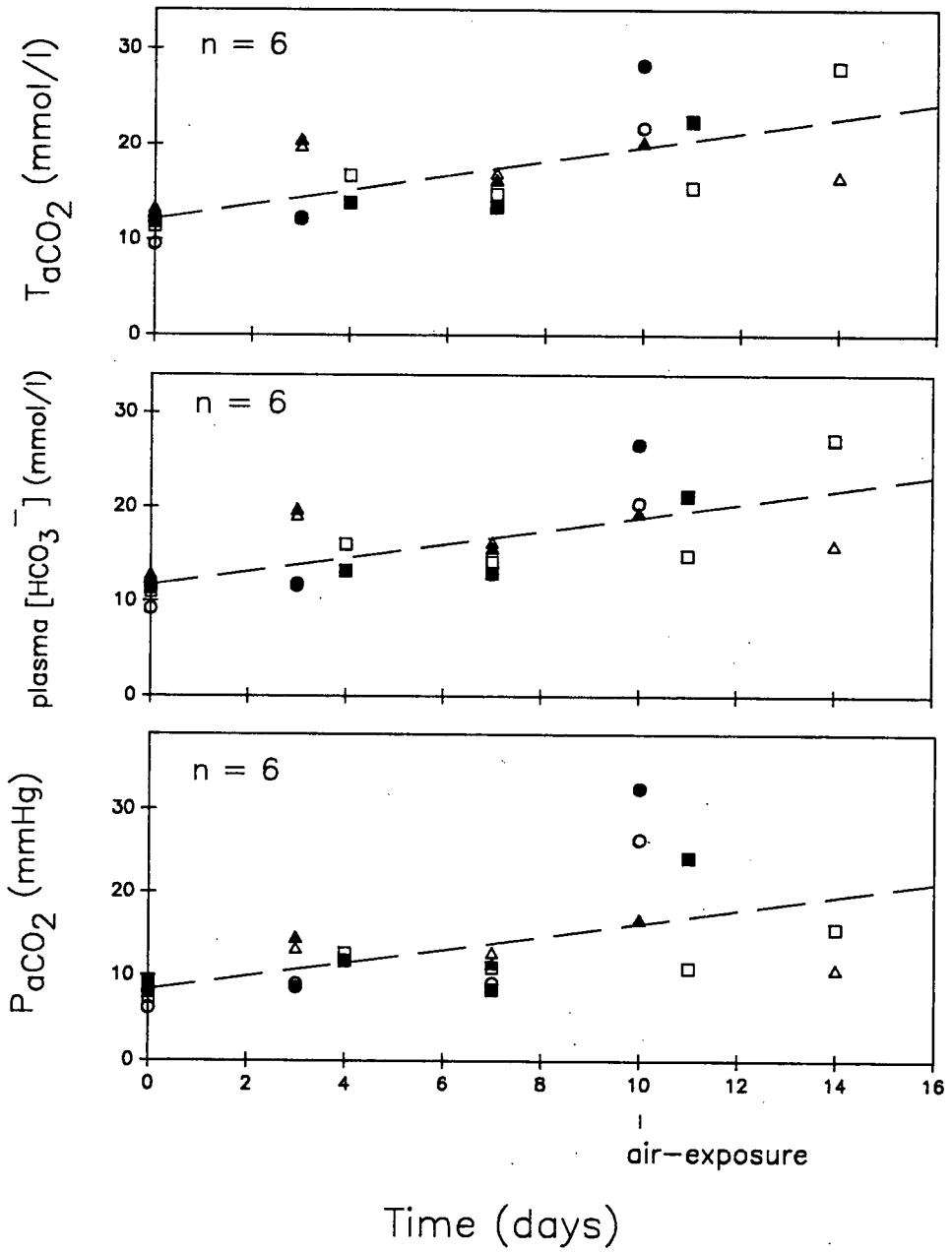
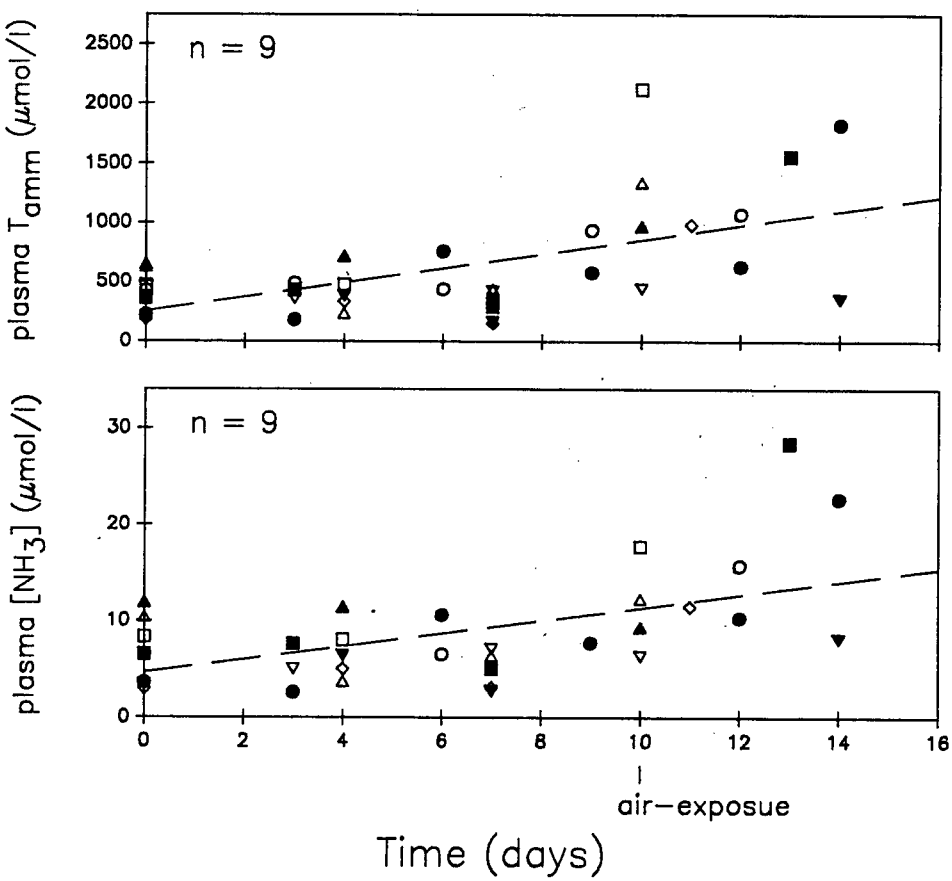

n = 6
n = 6
n = 6
T_{aCO_2} (mmol/l)
plasma $[HCO_3^-]$ (mmol/l)
P_{aCO_2} (mmHg)
0
10
20
30
0
2
4
6
8
10
12
14
16
air–exposure
Time (days)

Figure 3C: The relationship between T_{amm} and $[NH_3]$ and Time (days) during gradual air-exposure.
Each symbol represents an individual animal.


plasma T_{amm} (μmol/l)
2500
2000
1500
1000
500
0
n = 9
plasma [NH_3] (μmol/l)
30
20
10
0
n = 9
0
2
4
6
8
10
12
14
16
air–exposue
Time (days)

The relationship between time (days) and water pH, P_{CO2}, $[NH_3]$, and [urea] can be seen in figures 4A and B. The best fit linear regression equations and R^2 values are in table 3. Water pH did not change significantly during air exposure.

Water P_{CO2} rose initially, and then reached a new equilibrium between excretion of CO_2 by the fish and diffusive loss to the atmosphere. The regression coefficient for water P_{CO2} was not significantly different from zero, indicating that the CO_2 diffusion gradient between plasma and water increased significantly as the fish became emersed. Water T_{CO2} was dependent on water pH, rising as pH rose and vice-versa. Water $[NH_3]$ rose greatly during three experiments but remained constant during two, the combined data leading to a regression coefficient that indicated a significant increase. Increases in plasma $[NH_3]$ were correlated with increases in water $[NH_3]$ up to air exposure, in those individuals in which both parameters were measured simultaneously. Following air exposure, however, there was no clear relationship between water and plasma $[NH_3]$ levels. Water [urea] increased significantly during air exposure, but translation of daily water [urea] measurements into daily excretion rates yielded variable results that did not indicate a significant increase in urea excretion.

Table 3: Best-fit linear regression equations for water variables during gradual air-exposure.

Variable	N	n	Best-fit	R^2	R.C.S.E.
pH	7	78	y=0.04x+6.44	0.02	0.02
P_{CO2}	7	32	y=0.03x+7.32	0.003	0.05
$[NH_3]$	5	60	y=0.61x-1.74*	0.39	0.10
[urea]	4	48	y=3.58x+13.6*	0.36	0.70

N = number of fish, n = number of observations.

R.C.S.E. = regression coefficient standard error.

Units: P_{CO2} = mmHg, $[NH_3]$, [urea] = μmol/l.

* = significantly different from zero.

Figure 4A: The relationship between water pH and P_{CO2} and Time (days) during gradual air-exposure.

Each symbol represents an individual animal.

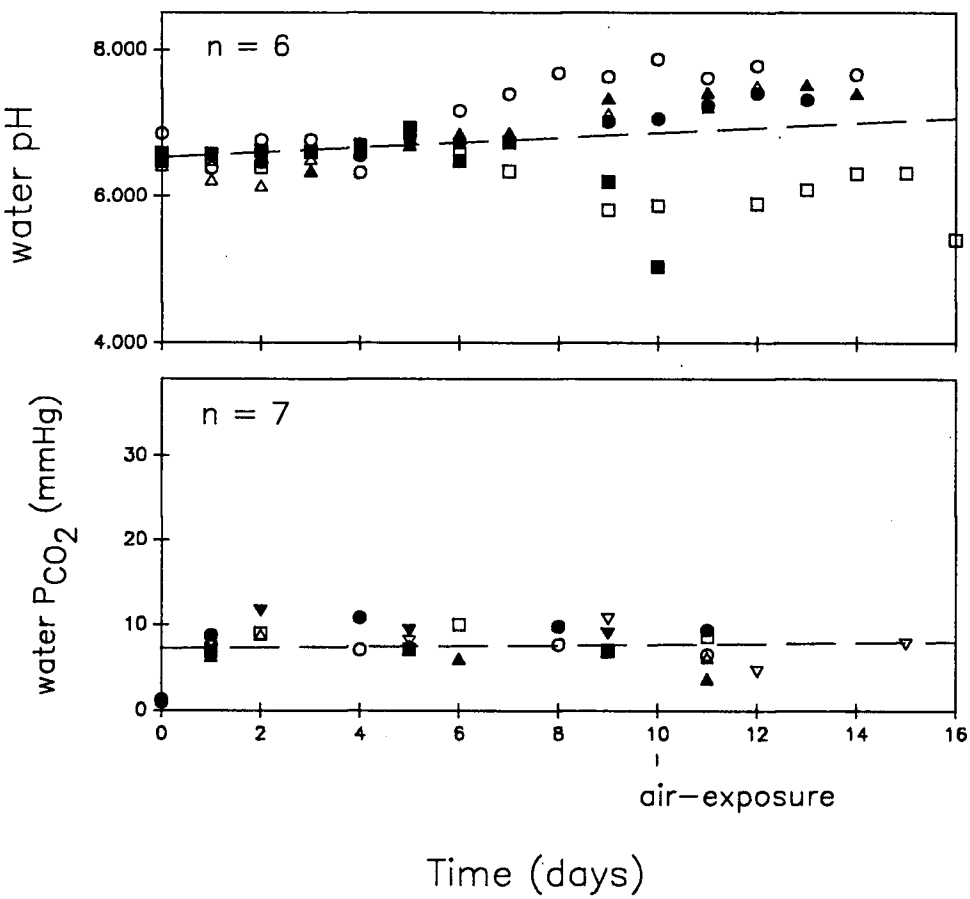
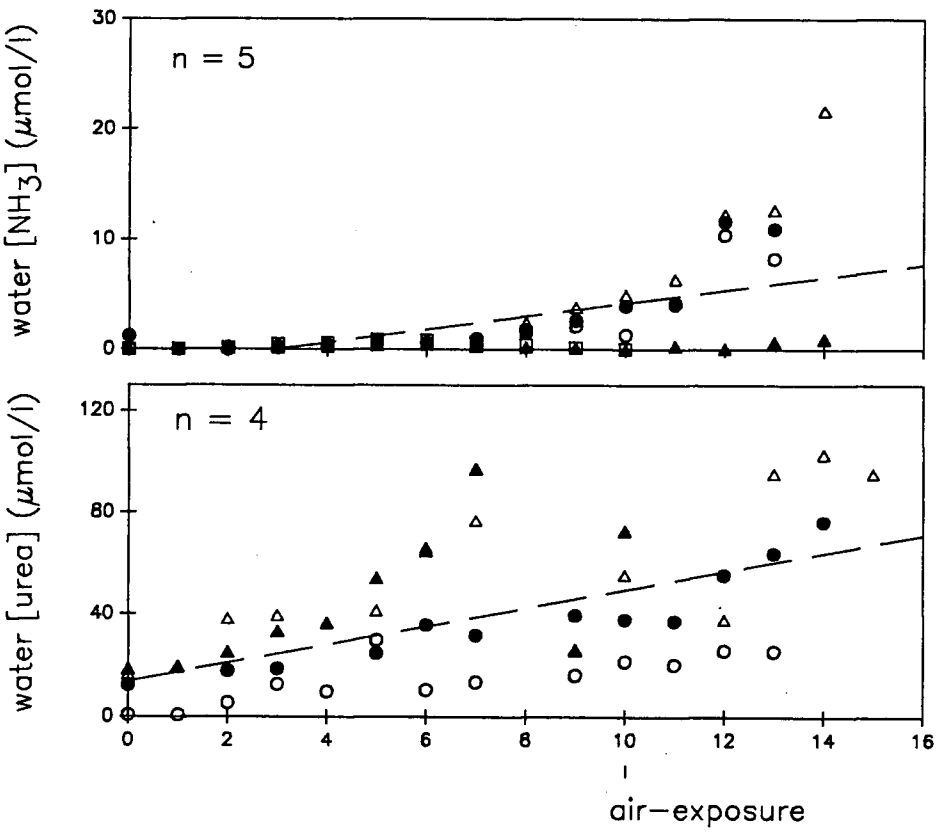

n = 6
8.000
6.000
4.000
water pH
n = 7
30
20
10
0
water P_{CO_2} (mmHg)
0
2
4
6
8
10
12
14
16
air–exposure
Time (days)

Figure 4B: The relationship between water [NH_3] and [urea] and Time (days) during gradual air-exposure.

Each symbol represents an individual animal.


n = 5
water [NH3] (µmol/l)
30
20
10
0
n = 4
water [urea] (µmol/l)
120
80
40
0
0
2
4
6
8
10
12
14
16
air–exposure
Time (days)

3) Ammonium chloride exposure:

The best fit linear regression equations, R^2 values and mean initial values ± S.E. of all measured respiratory and internal variables can be seen in Table 4. There were no significant differences between the initial values of $\dot{V}_{O2}(t)$, $\dot{V}_{O2}(a)$, T_{amm} and plasma [urea] for this experiment and the values for the same parameters under control conditions.

Total O_2 consumption, $\dot{V}_{O2}(a)$, T_{amm}, and plasma [urea] did not change during NH_4Cl exposure (fig 5A), their regression coefficients were not significantly different from zero. The regression coefficient for plasma [urea] was significantly different from that derived under control conditions, but the coefficients for $\dot{V}_{O2}(t)$, $\dot{V}_{O2}(a)$ and T_{amm} were not.

Urea excretion represented 9.9% of total nitrogen excretion under control conditions, with an excretion rate of 30.2 ± 8.0 mmol/kg/hr cf 606.6 ± 86.8 mmol/kg/hr total ammonia excretion. Urea excretion rates did not increase during NH_4Cl exposure.

4) Hypoxic exposure:

The effect of aquatic hypoxia without access to air breathing on measured respiratory and internal variables can be seen in Figure 6. At P_{wO2} = 111 mmHg, no variable showed a significant change. At P_{wO2} = 85 mmHg, $\dot{V}_{CO2}$ and R.E. both increased significantly over control values. Arterial plasma total CO_2 and P_{aCO2} both

Table 4: Best-fit linear regression equations and mean initial values of respiratory, blood gas and excretory variables during 900μmol/l NH_4Cl exposure.

Variable	N	n	Best-fit	R^2	R.C.S.E.	Mean±SE
$\dot{V}_{O2}(t)$	6	48	y=0.39x+44.47	0.01	0.57	50.46±9.95
$\dot{V}_{O2}(a)$	6	48	y=0.20x+7.10	0.01	0.25	8.27±2.88
T_{amm}	7	26	y=6.61x+340.5	0.07	4.80	310.8±38.4
[urea]	7	26	y=7.10x+112.6	0.15	3.45	108.8±17.7
Urea exc.	6	52	y=-0.74x+23.8	0.03	0.57	30.2±17.9

N = number of fish, n = number of observations.

R.C.S.E. = regression coefficient standard error.

Urea exc. = urea excretion rate.

Units: $\dot{V}_{O2}(t)$, $\dot{V}_{O2}(a)$ = mg/kg/hr; T_{amm}, [urea] = μmol/l; urea exc. = μmol/kg/hr.

Figure 5A: The relationship between $\dot{V}_{O2}(t)$ and $\dot{V}_{O2}(a)$ and Time (days) during 900μmol/l NH_4Cl exposure.

Each symbol represents an individual animal.

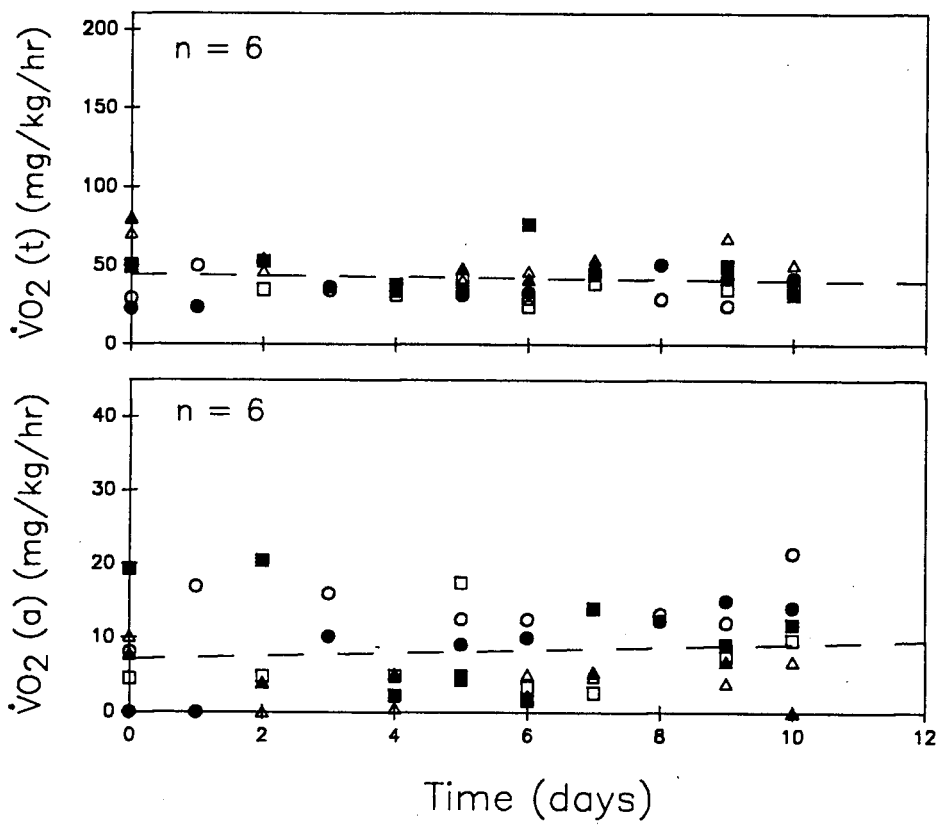

n = 6
$\dot{V}O_2$ (t) (mg/kg/hr)
200
150
100
50
0
n = 6
$\dot{V}O_2$ (a) (mg/kg/hr)
40
30
20
10
0
0
2
4
6
8
10
12
Time (days)

Figure 5B: The relationship between T_{amm}, [urea] and urea excretion and Time (days) during 900μmol/l NH_4Cl exposure.

Each symbol represents an individual animal for urea excretion.

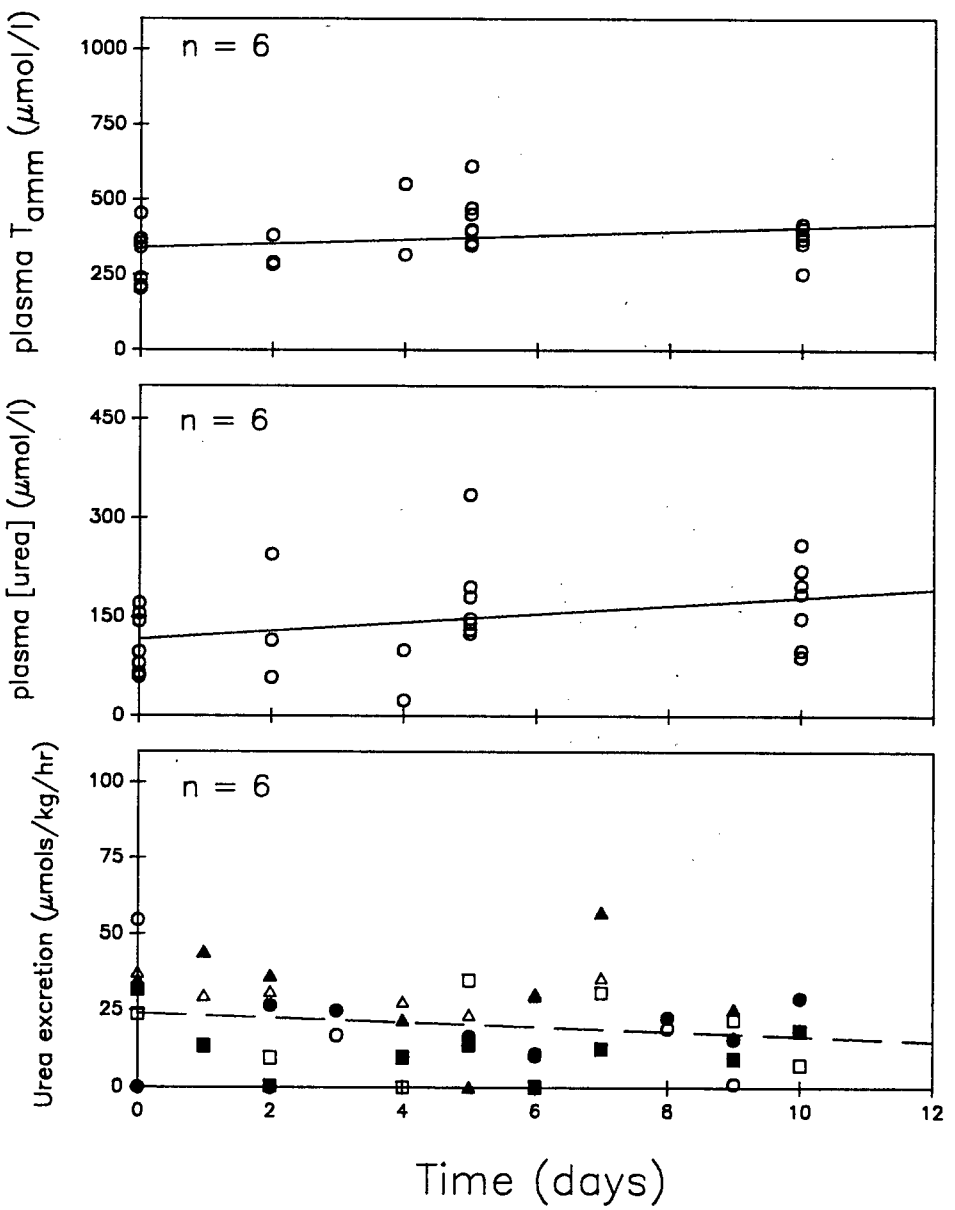
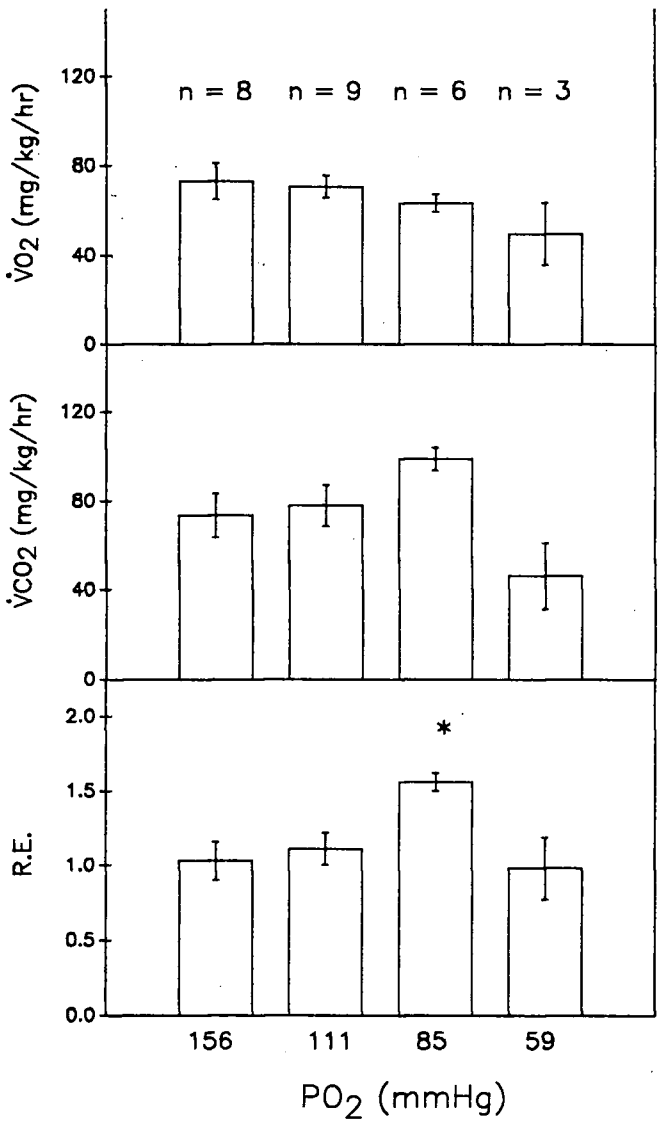
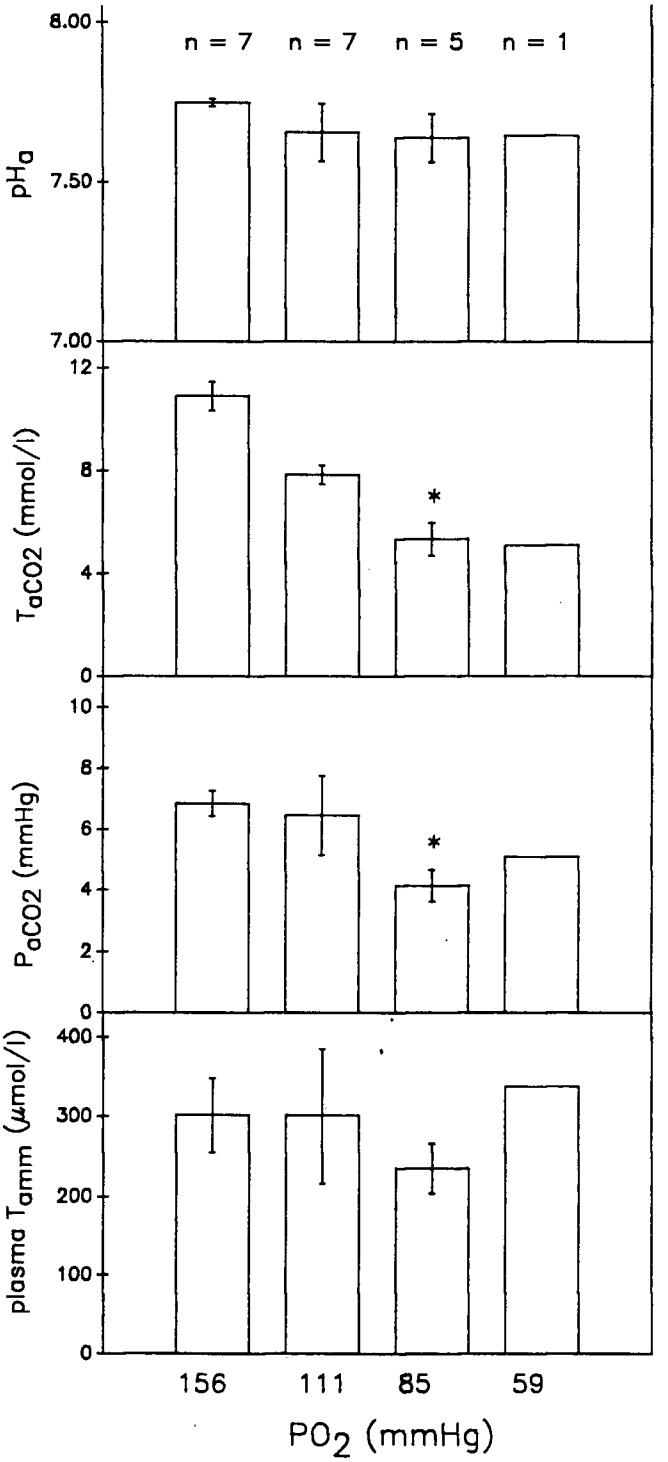

plasma T_{amm} (μmol/l)
1000
750
500
250
0
n = 6
plasma [urea] (μmol/l)
450
300
150
0
n = 6
Urea excretion (μmols/kg/hr)
100
75
50
25
0
n = 6
0
2
4
6
8
10
12
Time (days)

Figure 6: Respiratory and blood gas variables at different levels of aquatic hypoxia.

* = significantly different from control at $P<0.05$.


n = 8 n = 9 n = 6 n = 3
$\dot{V}O_2$ (mg/kg/hr)
120
80
40
0
$\dot{V}CO_2$ (mg/kg/hr)
120
80
40
0
R.E.
2.0
1.5
1.0
0.5
0.0
*
156 111 85 59
PO_2 (mmHg)


n = 7 n = 7 n = 5 n = 1
pH_a
8.00
7.50
7.00
T_{aCO2} (mmol/l)
12
8
4
0
*
P_{aCO2} (mmHg)
10
8
6
4
2
0
*
plasma T_{amm} (μmol/l)
400
300
200
100
0
156 111 85 59
PO_2 (mmHg)

dropped significantly. All other variables did not change. At P_{wO2} = 59 mmHg, only three of eight fish survived 24 hours, and blood samples were obtained from only one animal. $\dot{V}_{O2}$ dropped in all three animals, as did $\dot{V}_{CO2}$, but the differences were not significant. Respiratory exchange ratio was similar to the mean control value. Arterial blood pH was very low, as were T_{aCO2} and P_{aCO2}, as compared to the mean control value. Arterial plasma total ammonia was similar to the mean control value. At P_{wO2} = 30 mmHg, none of the fish survived more than two hours of hypoxic exposure.

DISCUSSION

Air Exposure:

A number of actinopterygian fish are known to be capable of surviving periods of water deprivation, e.g *Symbranchus marmoratus* (Bicudo and Johansen 1979) and *Lepidogalaxias salamandroides* (Pusey 1986). This capacity must involve the ability to avoid desiccation, depletion of energy stores, and toxic accumulation of wastes. No studies to date have investigated the physiological changes associated with water deprivation in the above animals, although each is able to breathe both water and air. There are, however, a number of studies of the responses to water deprivation seen in other bimodally breathing vertebrates, responses commonly described as "aestivation".

The african lungfish, *Protopterus* sp. burrows during periods of drought, surrounding itself in a mucus cocoon (Smith 1961). Various anurans, e.g. *Scaphiophus couchi* (Seymour 1973), *Bufo marinus*, (Boutilier *et al.* 1979) and *Pyxicephalus adspersus*, (Loveridge and Withers 1981) also burrow in response to drought. This reduces evaporative water loss. Oxygen consumption is reduced in all of these animals, indicating a reduction in metabolism or the use of alternative, anaerobic metabolic pathways. *Protopterus* is ammonotelic when in water, but during aestivation converts all nitrogenous waste to urea (Janssens 1964; Janssens and Cohen 1968, a,b) thereby avoiding the toxic effects of excessive ammonia accumulation in the tissues. Urea levels in the blood rise, as urine volumes decrease to conserve water (Delaney *et al.* 1977; Babikker and El Hakeem 1979). A plasma respiratory acidosis develops in aestivating *Protopterus*

(Delaney *et al.* 1977) and *Pyxicephalus adspersi* (Loveridge and Withers 1981). In *Bufo marinus*, a respiratory acidosis develops, but is gradually corrected (Boutilier *et al.* 1979). The acidosis is probably a result of impeded gas exchange across the skin whilst in a burrow, exacerbated in *Protopterus* by the increased respiratory dead space that results from breathing through a mucus tube extending to the surface of the mud (Delaney *et al.* 1974).

In the present study, northern, cold water adapted *Amia calva* did not make any of the physiological adjustments to air exposure seen in lungfish and anurans, with no evidence of a metabolic suppression or detoxification of ammonia to urea.

In teleosts, in general, 45 to 100% of total ammonia excretion is by passive diffusion of NH_3 (Randall and Wright 1987). In this study, prior to air exposure, plasma [NH_3] increases were correlated with water [NH_3] increases. Following air exposure, there were marked increases in plasma [total ammonia] and no clear correlation between water and plasma [NH_3] levels. Water [NH_3] levels continued to rise, indicating some continued ammonia excretion.

In *Amia*, under aquatic conditions, over 90% of CO_2 excretion occurs at the gills (Randall *et al.* 1981). During gradual emersion, T_{aCO2} increased, although there was no respiratory acidosis. *Amia* satisfied all their oxygen requirements in air using their respiratory swimbladder, and there was no evidence of a metabolic acidosis. The rigid, seive like structure of the gills (Daxboeck *et al.* 1981) probably allowed some continued ammonia and CO_2 excretion following air exposure, by trapping water in the pores between secondary lamellae.

Ammonium chloride exposure:

An increase in water [total ammonia] leads to an increase in urea production via uricolysis in the goldfish, *Carassius auratus*, (Olson and Fromm 1971) and via ureagenesis in the primitive air breathing fish, *Heteropneustes fossilis*, (Saha and Ratha 1987). In this study, the water [total ammonia] was over two times that used by Olson and Fromm (1971), but because of the low water pH, (6.5) the $[NH_3]$ was only 1.9 μmols/l; 28% of the mean plasma $[NH_3]$. Biological membranes are relatively impermeable to NH_4^+ (Randall and Wright 1987), so there was no significant increase in plasma [total ammonia] during NH_4Cl exposure, despite high NH_4^+ levels in the water. *Amia* relied on urea excretion to remove only 10% of nitrogenous waste under control conditions, and showed no increase in plasma urea or in urea excretion following ten days of NH_4Cl exposure. Mommsen and Walsh (1989) report that *Amia* does not have functional levels of ornithine cycle enzymes in isolated hepatocytes, unlike a number of aquatic animals that are known to aestivate (Janssens 1964; Saha and Ratha 1987).

There is some evidence that increases in water-borne irritants can cause an increase in air breathing in gar, *Lepisosteus osseus*, (Smatresk 1988). *Amia* showed no change in $\dot{V}_{O2}(t)$ or $\dot{V}_{O2}(a)$ during NH_4Cl exposure. Thus, under conditions of drought, if an *Amia* were trapped in a gradually evaporating puddle, a build up of water ammonia levels *per se* would not lead to an increase in urea production and excretion, or to increased reliance on air-breathing.

Hypoxic exposure:

Amia are facultative air breathers, and at low water temperatures, the gills are the main site of gas exchange (Johansen *et al.* 1970). In Southern Ontario, Canada, they overwinter under ice cover. As temperature rises, aerial uptake begins to predominate, but *Amia* do not die if denied access to air at 30°C under aquatic normoxia (Johansen *et al.* 1970; Randall *et al.* 1981). During acute aquatic hypoxia, *Amia* is capable of meeting all of its oxygen requirements by air-breathing (Randall *et al.* 1981). The present study showed that if denied access to air, *Amia* were not capable of sustaining oxygen delivery with the gills at relatively moderate degrees of aquatic hypoxia. At P_{wO2} = 85 mmHg, there were clear indications of gill hyperventilation, resulting in very low P_{aCO2} levels, and a significant increase in $\dot{V}_{CO2}$ and R.E. over control values. This respiratory alkalosis was presumably offset by a metabolic acidosis, as pH_a values were not significantly different from control values. At P_{wO2} = 59 mmHg there was a reduction in $\dot{V}_{O2}$, indicating an inability to sustain oxygen delivery via the gills, and there was only 50% survival after 24 hours at this level of hypoxia. Rainbow trout (*Oncorhynchus mykiss*), fish adapted to well-oxygenated fast flowing waters, are able to survive at P_{wO2} = 25 mmHg, at 15°C (Claireaux *et al.* 1988), and display a reduction in total metabolism at P_{wO2} = 80 mmHg and below (Boutilier *et al.* 1988). Sturgeon (*Acipenser transmontanus*) reduce $\dot{V}_{O2}$ in concert with a reduction in aquatic P_{O2} (Burggren and Randall 1978). *Amia* are clearly incapable of initiating a reduction in aerobic or total metabolism in response to hypoxia.

In summary, these results suggest that northern, cold adapted *Amia calva* are not able to aestivate, as they are incapable of reducing aerobic metabolism during air exposure, and are not able to detoxify their nitrogenous wastes as urea. Under the conditions of these experiments, air exposure resulted in death of *Amia.* Their respiratory swimbladder functions only to sustain aerobic metabolism under aquatic conditions of raised temperature or lowered P_{wO2}, not to aid in gas-exchange during prolonged emersion. Previous reports (Dence 1933; Neill 1950) of "aestivating" *Amia* were probably animals that had recently become air exposed, although it is possible that *Amia* from the southern areas of the species' range may be capable of aestivation.

Chapter 2: Ventilatory and Cardiovascular Responses to Blood pH, Plasma P_{CO2}, Blood O_2 content and Catecholamines in *Amia*

INTRODUCTION

The results of Chapter 1 indicate that *Amia* is an entirely aquatic animal, but with the added ability to breathe air. *Amia*, therefore, is an extant example of an intermediate stage in the evolution from water-breathing to air-breathing ventilatory control systems in vertebrates. The extent to which ventilatory responses in *Amia* are similar to those of water or air breathers is unknown.

In water-breathing fish, O_2 is the primary stimulus for ventilatory and cardiovascular reflex responses (Dejours, 1973; Randall and Jones, 1973; Smith and Jones, 1982; Randall, 1982). Apart from a modest sensitivity to P_{aCO2} and/or pH_a that has been demonstrated in hyperoxic dogfish (Heisler, Toews and Holeton, 1988), ventilatory responses by water-breathing fish to changes in plasma P_{CO2} and blood pH only occur when they are associated with reductions in blood O_2 content, via Bohr and Root effects (Smith and Jones, 1982; Perry, Kinkead, Gallaugher and Randall, 1989). Air-breathers (amphibians, reptiles, birds and mammals) exhibit direct ventilatory and cardiovascular responses to P_{aCO2} and/or pH_a, as well as blood O_2 status (Dempsey and Forster, 1982; O'Regan and Majcherczyk, 1982; Scheid and Piiper, 1986; Smatresk, 1990, for reviews). It is still unclear whether reflex responses in mammals are to P_{CO2} or pH, as there is evidence of sensitivity to both (Shams, 1985).

These differences between vertebrate groups in the reflex control of breathing are considered to be related to the differential capacitances of water and air for O_2 and CO_2 (Dejours, 1981). It is unknown when ventilatory and cardiovascular sensitivity to P_{CO2}/pH first appeared in the evolution of air-breathing. *Amia* shows

ventilatory sensitivity to O_2; increasing gill ventilation and air-breathing in aquatic hypoxia (Johansen *et al.*, 1970; Randall *et al.*, 1981) but it is unknown whether it also responds to CO_2 and/or pH.

There is recent evidence that release of circulating catecholamines (NE and E) from chromaffin tissue may mediate ventilatory responses to hypercapnia and acidosis in water-breathing fish (Perry *et al.*, 1989; Aota, Holmgren, Gallaugher and Randall, 1990). Catecholamines stimulate ventilation in some water-breathing fish (Peyraud-Waitzennegger, 1979) and air-breathing vertebrates (Dempsey, Olson and Skatrud, 1986), and they are released into the circulation in response to blood acidosis in water-breathing fish (Boutilier, Iwama and Randall, 1986; Tang and Boutilier, 1988; Perry *et al.*, 1989; Aota *et al.*, 1990). The effects of circulating catecholamines on ventilation and their potential role in ventilatory responses have not been examined in air-breathing fish.

This study compared cardiovascular and ventilatory responses, and endogenous catecholamine release, in *Amia* exposed to blood acidosis, to transient increases in plasma P_{CO2} without acidosis, and to blood acidosis when C_{aO2} was maintained above normoxic levels, to discover whether reflex responses were best correlated with P_{aCO2}, pH_a or C_{aO2}. Any associated changes in blood catecholamine levels were recorded and cardiovascular and ventilatory responses to pharmacological doses of catecholamines were assessed, under normoxia and hypoxia, to investigate their possible role in ventilatory responses to acidosis.

MATERIALS AND METHODS

Experimental Animals:

Bowfin were maintained and temperature acclimated as described in general materials and methods.

Surgical Procedures:

Animals were anaesthetized in a buffered ($NaHCO_3$) tricainemethanesulphonate (MS222) solution at a concentration of 1:10,000 and transferred to an operating table, where they were ventilated with a MS222 solution at 1:20,000. A dorsal aortic cannula (PE50, Intramedic) was implanted using the technique of Soivio, Westman and Nyholm (1972). An opercular cannula was fitted, using flared PE190 (Intramedic) passed through a small hole drilled in the operculum, secured with a cuff and sutures. The fish was allowed to recover in a black plexiglass box (volume 9 l) with access to a forward space, for airbreathing (volume 1.6 l), for 48 hours before use in an experiment. DA cannulae were flushed with heparinized Cortland's saline (Wolf, 1963) twice daily.

Cardiovascular and Ventilatory Measurements:

During experiments, dorsal aortic peak systolic blood pressure (P_{DA}, cmH_2O) and heart rate (f_h, beats/min) were measured using a Statham (P23Db) pressure transducer attached to the 20 cm, saline-filled dorsal aortic cannula. Gill ventilation frequency (f_g, beats/min) and opercular pressure amplitude (P_{op}, cm H_2O) were measured using a Statham (P23BB) pressure transducer attached to the 20 cm, water-filled opercular cannula. The output from both transducers was

displayed on a pen recorder (Gould 8188-2202-XX). Opercular pressure amplitude was used as an index of ventilatory effort. The frequency of air breathing (f_{ab}) was visible as large pressure excursions on the opercular trace, associated with changes in f_h and P_{DA} (fig 7). These air breaths were verified visually through a small hole in a screen between the experimenter and the bowfin. Ventilatory and cardiovascular variables were considered to be in steady state when they remained stable for 30 minutes.

Experimental Protocols:

Once ventilatory and cardiovascular variables were in steady-state, bowfin were exposed to the following treatments:

Series 1:

Treatment 1) 2.5 ml/kg Cortland's saline infusion into the DA, followed by one hour recovery, and then 2.5 ml/kg 0.1M hydrochloric acid (HCl) infusion, in a Cortland's saline vehicle.

Treatment 2) 2.5 ml/kg Cortland's saline infusion, followed by a one hour recovery period, and then 2.5 ml/kg 0.2M ammonium bicarbonate (NH_4HCO_3) infusion, in a Cortland's saline vehicle.

Treatment 3) One hour's exposure to aquatic hyperoxia (P_{wO2} = 643 ± 12 mmHg), created by bubbling 100% O_2 counter-current to water flow through a gas-exchange column, followed by the same infusion series as in treatment 1.

Series 2:

Treatment A) 0.5 ml/kg Cortland's saline injection, followed by a 2 hour recovery period. Then, 0.5 ml/kg 10^{-5}M epinephrine hydrochloride (Sigma) injection, in a

saline vehicle, followed by a two hour recovery period. Subsequently, a 0.5 ml/kg 10^{-5}M norepinephrine bitartrate (Sigma) injection, in a saline vehicle. Both epinephrine (E) and norepinephrine (NE) solutions were at pH 7.7. In half the animals studied, the order of epinephrine and norepinephrine injections was reversed.

Treatment B) Three animals were exposed, for two hours, to moderate aquatic hypoxia (P_{wO2} = 59±1.9 mmHg), obtained by bubbling N_2 counter-current to water flow through a gas-exchange column, and then treated to E and NE injections as described for treatment (A). No cardiovascular responses were measured in this treatment

All infusions were performed over 7 to 10 minutes, at approximately 0.3 $ml.min^{-1}$. This infusion rate avoided any struggling associated with irritant or behavioural responses. Injections in Series 2 were performed over 1 minute. All animals were used in more than one treatment, assigned randomly, with a 48 hour recovery period between each treatment.

Baseline measurements of P_{DA}, f_h, f_g and P_{op} were recorded for 10 minutes as a control, and for 30 minutes post-infusion in Series 1. In Series 2, variables were measured continuously for 1 hour post-injection. f_{ab} was measured for 30 minutes post-infusion in Series 1; for 1 hour post-injection in Series 2. At 5 minutes post-infusion (or injection), a 1 ml blood sample was withdrawn in both series of treatments.

Sample Analysis:

0.5 ml of blood was immediately centrifuged, the plasma decanted and frozen

in liquid nitrogen for subsequent analysis of plasma catecholamine levels, as described in general materials and methods. The remaining blood was analysed for pH_a, T_{aCO2}, P_{aCO2}, C_{aO2} and P_{aO2}, as described in general materials and methods.

Data analysis and statistics:

Heart rate and f_g were assessed by counting for 30 seconds within each minute, for two minutes immediately prior to intervention, and at 1, 2.5, 4, 10, 15, 20, and 30 minutes following intervention (for Series 2, also at 60 minutes). P_{DA} and P_{op} were averaged from 6 measurements taken within the same periods used for measuring f_h and f_g. Cardiovascular and ventilatory responses were normalized for each time interval as per cent change from control values. Following arc-sine transformation, responses were analysed by ANOVA. Within each treatment mean values of blood gas variables following saline infusion (injection) were compared with mean values following experimental infusions (injection) using a paired t-test. Air-breath frequency following saline and experimental infusions (injection) within each treatment was compared with a paired t-test. Mean values of blood variables during hyperoxia were compared with the same parameters during normoxia using unpaired t-tests. $P = 0.05$ was taken as the fiducial limit of significance.

RESULTS

SERIES 1

For all treatments, infusion of 2.5 ml/kg Cortland's saline had no significant effect on steady-state cardiovascular (f_h and P_{DA}), or ventilatory (f_g, P_{op} and f_{ab}) variables (figs 8, 9 and 10).

In Treatment 1, HCl infusion caused a significant increase in P_{DA}, P_{op}, and f_{ab} (figs 7 and 8, table 5). The changes in P_{op} were initiated towards the last minute of infusion, and peak response in both P_{DA} and P_{op} occurred between 1 and 5 minutes post-infusion (p.i.). Blood pressure returned to control at 30 minutes p.i., and P_{op} at 10 minutes. Air-breaths all occurred within the first ten minutes p.i., and the majority occurred within the first five minutes. There was no significant effect on f_h or f_g, although some change is visible in figure 8. These cardiovascular and ventilatory changes were associated, at five minutes p.i., with a significant decrease in pH_a, T_{aCO2} and C_{aO2}, and a significant increase in P_{aCO2}, P_{aO2} and the concentrations ([NE] and [E]) of circulating catecholamines (table 5).

In Treatment 2, NH_4HCO_3 infusion had no significant effects on P_{DA}, f_h, P_{op} f_g or f_{ab} (fig 9, table 5). At five minutes p.i. there was no significant change in pH_a, C_{aO2}, P_{aO2} or [NE] and [E] as compared with values obtained following saline infusion, but T_{aCO2} and P_{aCO2} showed a significant increase (table 5).

Table 5: Effects of HCl, NH_4HCO_3 and HCl in Hyperoxia on f_{ab}, blood gases, [NE] and [E].

	HCl		NH_4HCO_3		HCl + Hyperoxia	
	sal.	exp.	sal.	exp.	sal.	exp.
f_{ab}	0.32 ±0.36	4.34* ±1.48	0.66 ±0.46	1.00 ±0.74	0 -	0 -
pH_a	7.60 ±0.04	7.31* ±0.07	7.66 ±0.02	7.67 ±0.03	7.67 ±0.03	7.31* ±0.10
T_{aCO2}	9.47 ±0.12	8.65* ±0.19	9.58 ±0.10	12.41* ±1.31	10.15 ±0.21	9.05* ±0.15
P_{aCO2}	8.80 ±0.84	15.29* ±1.68	7.39 ±0.30	9.61* ±0.66	8.35 ±0.80	15.1* ±2.65
P_{aO2}	35 ±5	48* ±8	59 ±18	51 ±9	368+ ±29	313+ ±51
C_{aO2}	5.8 ±0.7	4.1* ±0.6	6.0 ±0.8	5.4 ±0.6	10.2+ ±0.2	8.5+* ±0.7
[NE]	13.3 ±5.5	720.0* ±240.0	14.2 ±5.2	17.4 ±7.2	57.2 ±28.0	48.0 ±12.0
[E]	9.0 ±2.2	703.1* ±194.0	21.0 ±3.3	16.2 ±2.4	54.4 ±27.8	47.1 ±12.2

Values = mean ± S.E., N = 6
* = significantly different from control; + = significantly different from normoxic control (P=0.05)
Units: f_{ab} = breaths/hr; T_{aCO2} = mmol/l; P_{aCO2} and P_{aO2} = mmHg; C_{aO2} = vol.%; [NE] and [E] = nmol/l

Figure 7: Representative traces of blood pressure and ventilation. A) An air breath (ab). B) The effects of HCl infusion during aquatic normoxia, (ab = air breath). C) The effects of HCl infusion during aquatic hyperoxia. inj.= infusion.

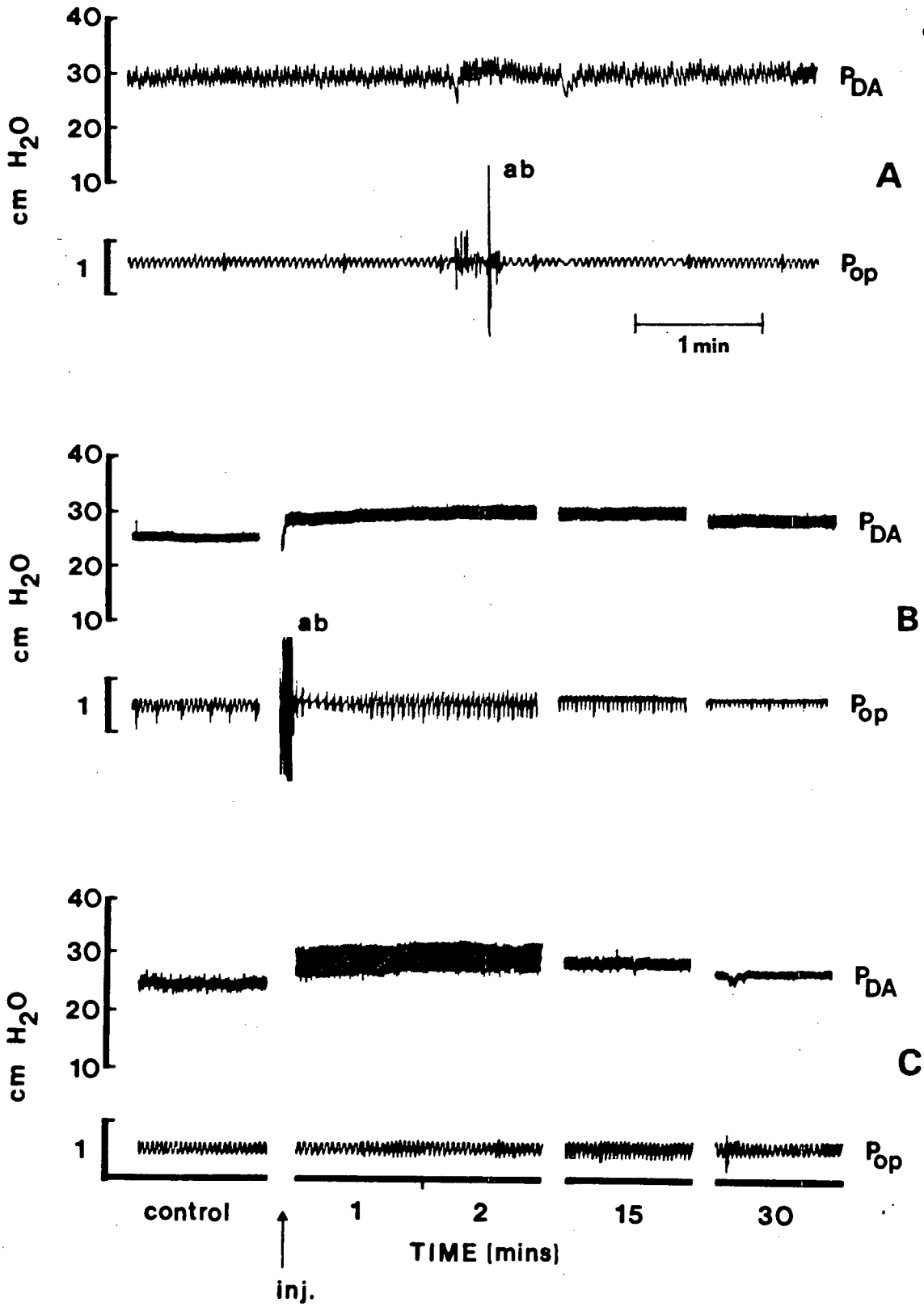

40
30
20
10
cm H2O
PDA
ab
A
1
Pop
1 min
40
30
20
10
cm H2O
PDA
B
ab
1
Pop
40
30
20
10
cm H2O
PDA
C
1
Pop
control
1
2
15
30
TIME (mins)
inj.

Figure 8: Mean % change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following HCl infusion. n = 6.

C = control, shaded bar represents infusion period.

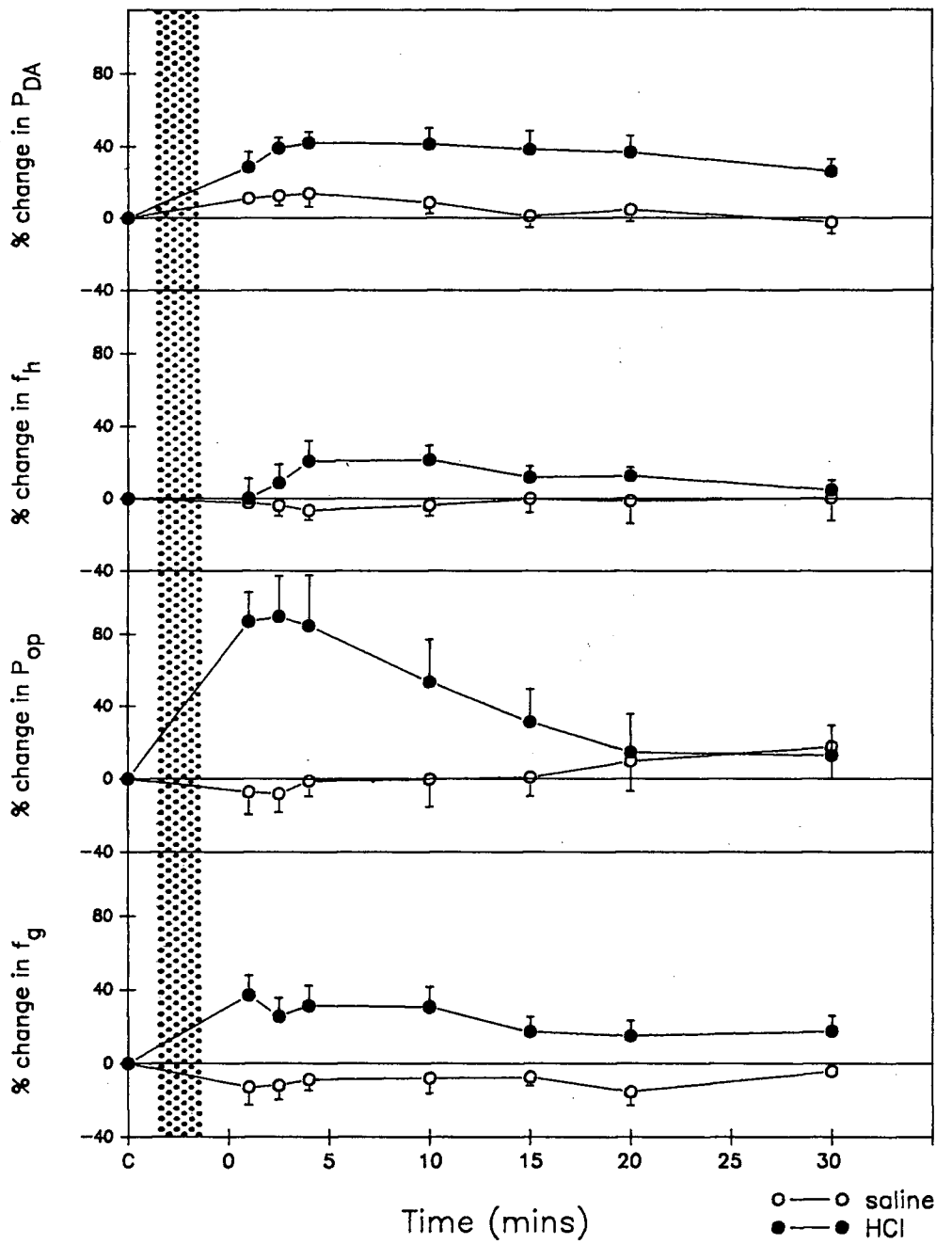
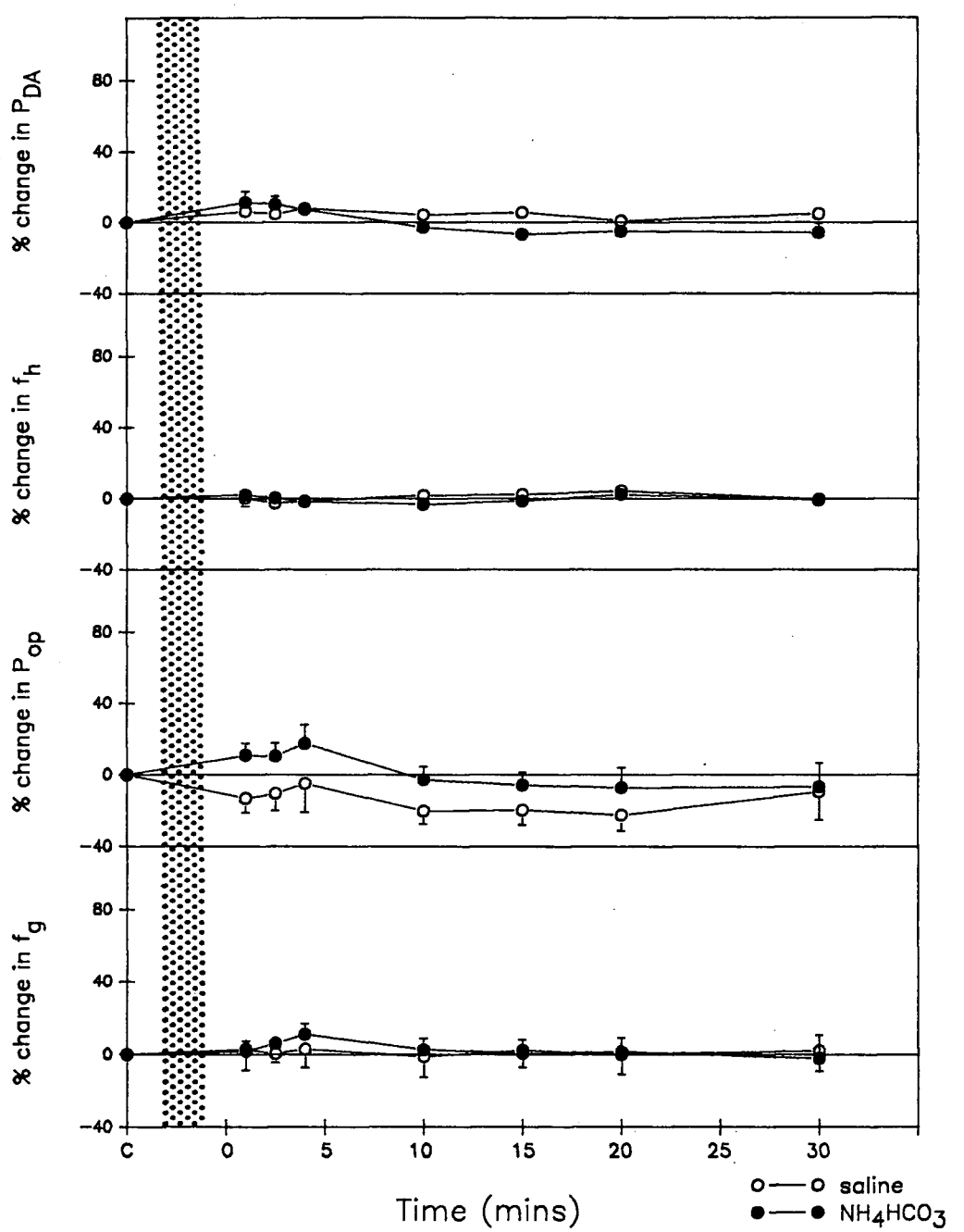

% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
80
40
0
-40
C
0
5
10
15
20
25
30
Time (mins)
o—o saline
●—● HCl

Figure 9: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following NH_4HCO_3 infusion. n = 6.

C = control, shaded bar represents infusion period.


% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
80
40
0
-40
C
0
5
10
15
20
25
30
Time (mins)
o—o saline
●—● NH_4HCO_3

In Treatment 3, aquatic hyperoxia caused a significant increase in P_{aO2} and C_{aO2}, as compared with normoxic conditions (Table 5). There was no reduction in gill ventilation (f_g or P_{op}) as compared with fish in normoxia, but there was no air breathing (table 5). HCl infusion during hyperoxia effected no significant changes in cardiovascular or ventilatory variables, except P_{DA}, which was increased immediately p.i., and remained elevated until 20 minutes (Figures 7 and 10). There is no evidence of the response profile for P_{op} and f_g visible following acid infusion in normoxia. At five minutes p.i, there was a significant drop in pH_a, C_{aO2}, and T_{aCO2}, and a significant increase in P_{aCO2} . Blood O_2 partial pressure, [NE] and [E] did not change (table 5). Arterial blood O_2 content was still significantly higher than normoxic values five minutes following HCl infusion (table 5).

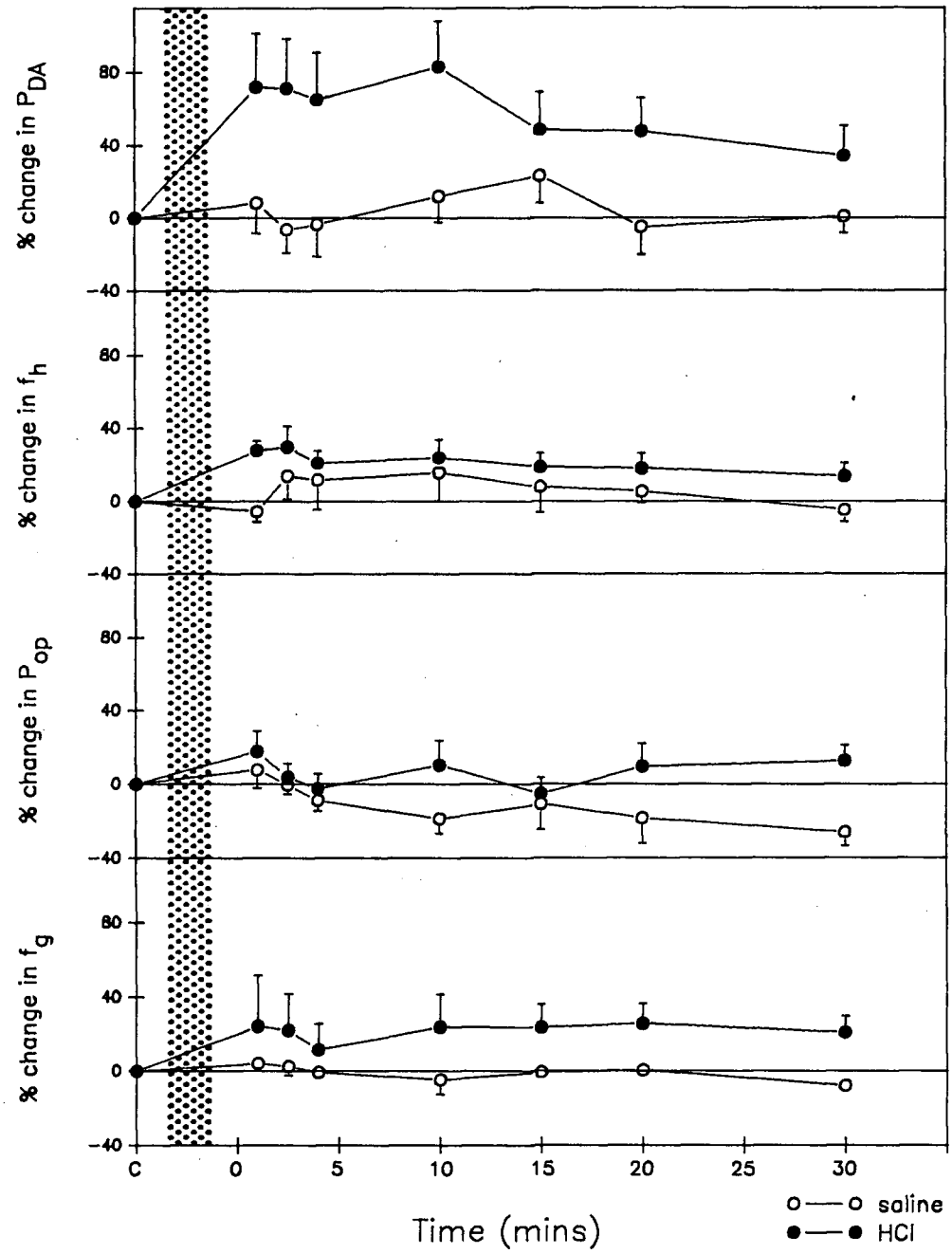
SERIES 2:

Saline injection resulted in no change in steady-state values of P_{DA}, f_h, P_{op}, f_g (figs 11 and 12) or f_{ab} (table 6).

Treatment A) Resting plasma [NE] and [E] measured in this study are an order of magnitude higher than those reported for water breathing fish (Perry *et al.* 1989),

Figure 10: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following HCl infusion during hyperoxia. n = 6

C = control, shaded bar represents infusion period.


% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
80
40
0
-40
C
0
5
10
15
20
25
30
Time (mins)
o—o saline
●—● HCl

and injection of NE and E stimulated a large endogenous release, with both [NE] and [E] increasing after either NE or E infusion (table 6). Norepinephrine injection stimulated significant cardiovascular and gill ventilatory responses; P_{DA}, P_{op} and f_g all increased (fig. 11), although f_h did not. P_{DA} increased immediately, and remained significantly elevated until 4 mins p.i., P_{op} increased significantly at 2 mins p.i. and returned to control levels at 10 mins. f_g increased at 2.5 minutes p.i., and returned to control at 10 minutes. There was no stimulation of air breathing (table 6). There was no significant change in pH_a, but a significant increase in P_{aO2} and C_{aO2} (table 6). Following epinephrine injection, f_h, P_{DA} and P_{op} all increased significantly, but f_g and f_{ab} did not change (fig. 11, table 6). P_{DA} rose immediately, and remained elevated until 15 mins post-injection, and f_h increased at 2 mins and returned to control levels at 15 mins post-injection. P_{op} increased at 2 mins and returned to control levels at 15 mins post-injection. Epinephrine effected no significant change in pH_a or P_{aO2}, but significantly increased C_{aO2} (table 6). Epinephrine appeared to stimulate cardiovascular variables more than norepinephrine, which had a greater effect on ventilation, but these differences were not significant when compared at each time interval with a t-test. Catecholamine infusions at doses of 1 ml/kg 10^{-4} M or 10^{-3} M, during aquatic normoxia, did not stimulate air breathing.

Treatment B) During moderate hypoxia, there was a significant increase in pre-injection f_{ab}, but no change in gill ventilatory variables or blood gases, as compared with normoxia (table 6). Opercular pressure amplitude and f_g were not

Table 6: Effects of NE and E on f_{ab} and blood gases in normoxia and hypoxia, and on [NE] and [E] in normoxia.

	Saline	NE	E
Normoxic f_{ab}	0.32±0.36	0.32±0.36	0
Hypoxic f_{ab}	6.67±1.33+	6.00±0+	6.00±2.00+
Normoxic pH_a	7.65±0.03	7.68±0.01	7.67±0.03
Hypoxic pH_a	7.73±0.07	7.70±0.05	7.77±0.06
Normoxic P_{aO2}	43±8	72±13*	53±7
Hypoxic P_{aO2}	41±7	48±8	38±7
Normoxic C_{aO2}	5.8±0.4	7.2±0.5*	6.8±0.5*
Hypoxic C_{aO2}	5.8±1.1	7.1±1.0*	5.8±0.2
Normoxic [NE]	21.1±5.0	564.8±90.0*	416.2±183*
Normoxic [E]	11.1±3.3	231.0±88.1*	1079±160*

Values = mean ± S.E.; N = 6 in normoxia; 3 in hypoxia.
* = significantly different from saline, + = significantly different from normoxia (P=0.05)
Units: f_{ab} = breaths/hr; P_{aO2} = mmHg; C_{aO2} = vol.%; [NE] and [E] = nmol/l.

Figure 11: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following NE or E injection during normoxia. n = 6.

C = control, shaded bar represents infusion period.

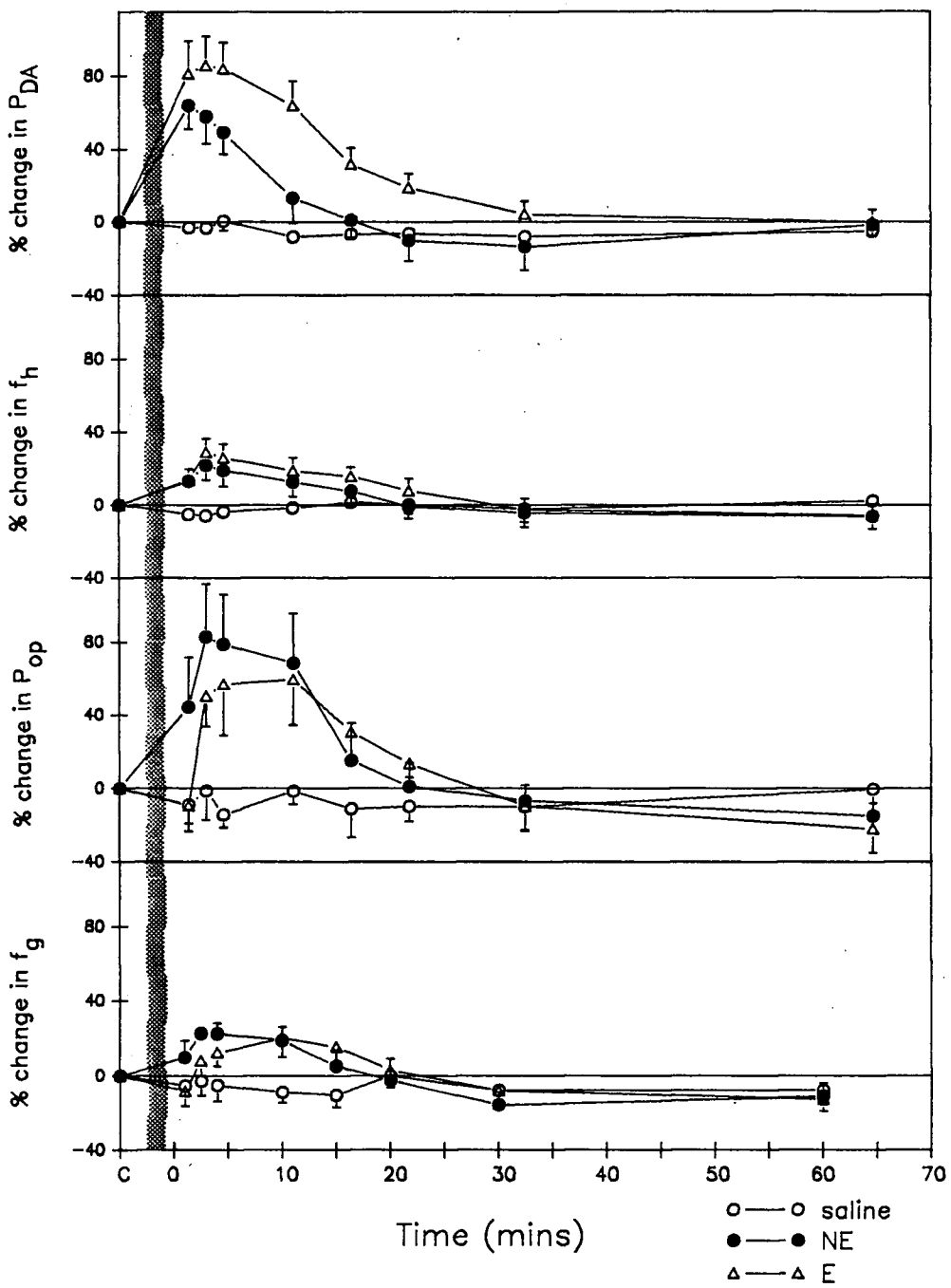
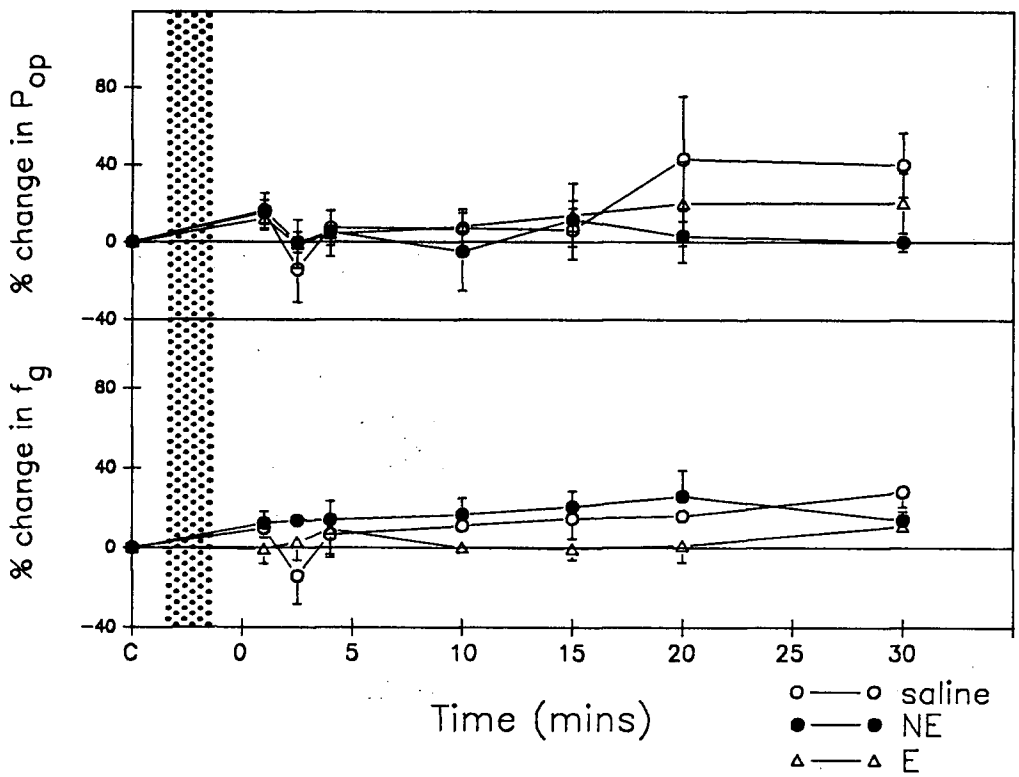

% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
80
40
0
-40
C
0
10
20
30
40
50
60
70
Time (mins)
saline
NE
E

Figure 12: Mean per cent change (± S.E.) in P_{op} and f_g following NE or E infusion during moderate hypoxia. n = 3

C = control, shaded bar represents infusion period.


% change in P_{op}
% change in f_g
80
40
0
-40
C
0
5
10
15
20
25
30
Time (mins)
saline
NE
E

significantly higher than normoxic levels, but this may reflect the small number of fish studied, and the fact that the same fish were not measured under both conditions. NE and E had no significant effect on P_{op} and f_g (fig 12) and did not increase f_{ab} (table 6). Norepinephrine effected a significant increase in C_{aO2}, but there were no other significant effects on blood gases.

DISCUSSION

SERIES 1.

Cardiovascular and ventilatory responses seen in treatments 1, 2 and 3 have been correlated with P_{aCO2}, pH_a and C_{aO2} levels measured at five minutes p.i., to assess the possible role of each of these blood gas variables in the reflex responses observed. Possible mechanisms behind observed responses will be discussed.

The relationship between P_{aCO2} and cardiovascular and ventilatory responses:

Acid infusion in normoxia stimulated P_{DA}, P_{op} and f_{ab}, and was associated with a significant increase in P_{aCO2}. Ammonium bicarbonate infusion, however, caused a significant increase in P_{aCO2} (table 5) that was not associated with any significant changes in f_h, P_{DA}, P_{op}, f_g or f_{ab}. Acid infusion in hyperoxia also increased P_{aCO2}, but there was no stimulation of ventilatory variables. These results indicate that *Amia* do not show cardiovascular or ventilatory sensitivity to an increase in P_{aCO2}.

The relationship between pH_a and cardiovascular and ventilatory responses:

Acid infusion in normoxia was associated with a significant reduction in pH_a, and significant increases in P_{DA}, P_{op} and f_{ab}. During hyperoxia, acid infusion resulted in a significant reduction in pH_a that was associated with an increase in P_{DA}, but no ventilatory responses. This suggests that a reduction in pH_a *per se* causes increases in blood pressure, but not gill ventilation or airbreathing.

The relationship between C_{aO2} and cardiovascular and ventilatory responses:

Acid infusion in normoxia was associated with a significant reduction in C_{aO2}, and elicited increases in P_{DA}, P_{op} and f_{ab}. Acid infusion in hyperoxia caused a significant decrease in C_{aO2}, but at 5 minutes p.i. C_{aO2} was still significantly higher than control, normoxic, levels. There were no ventilatory responses in hyperoxia. This suggests that both gill ventilation and air-breathing are stimulated by a reduction in C_{aO2} below normoxic levels in *Amia*.

These results indicate that the primary stimulus for ventilatory responses to internal acid-base disturbances in *Amia* is hypoxaemia, as is the case for water-breathing fish, and that *Amia* do not show a ventilatory response to P_{aCO2}. If there is a ventilatory response to pH_a, then it is inhibited by hyperoxia. The cardiovascular responses are interesting, as they indicate that there is an effect of a reduction in pH_a on blood pressure. It is unknown how a reduction in pH_a might effect increases in P_{DA}, although thromboxane release associated with acid infusion stimulates increases in blood pressure in mammals (Shams, Peskar and Scheid, 1988).

There is evidence for water-breathing fish that ventilatory and cardiovascular responses to changes in external and internal O_2 status are neurally mediated, by O_2-sensitive chemoreceptors in the gills (Milsom and Brill, 1986; Burleson, 1986; Burleson and Smatresk, 1986; Burleson and Milsom, 1986). There is similar evidence in lungfish (*P. aethiopicus*) (Lahiri, Szidon and Fishman, 1970) and in both spotted gar (*Lepisosteus oculatus*) and longnose gar (*L. osseus*) (Smatresk, 1986; Smatresk, 1987). The site of putative internal receptors in *Amia* is

unknown.

In longnose gar, internally oriented receptors appear to set the level of hypoxic drive, and external receptors influence the balance between gill ventilation and air-breathing, with central integration of the external and internal afferent input (Smatresk, Burleson and Azizi, 1986). In gar, internal hypoxia will stimulate both air-breathing and gill ventilation responses (Smatresk *et al.*, 1986). In this study, both air-breathing and gill ventilation were stimulated by a reduction in C_{aO2}, indicating that *Amia* are more similar to gar than to *Ancistrus*, where air-breathing responses are only influenced by external hypoxia (Graham and Baird, 1982). It is unknown whether the gill ventilation and air-breathing are stimulated by the same peripheral receptors in *Amia*.

In water-breathing fish, there is evidence that ventilatory responses to internal acidosis may be humorally mediated. It is known that internal acidosis in normoxic water-breathing fish is associated with a ventilatory increase and release of NE and E into the circulation (Boutilier *et al.*, 1986; Tang and Boutilier, 1988) and that the catecholamine release is in response to a reduction in C_{aO2} (Perry *et al.*, 1989; Aota *et al.*, 1990). Both NE and E stimulate ventilation in water-breathing fish (Peyraud-Waitzennegger, 1979), and Aota *et al.*, (1990) demonstrated that the ventilatory response and catecholamine release following acid infusion are abolished by hyperoxia, and that the β-adrenergic receptor blocker propranolol abolishes the ventilatory response but not the catecholamine release. This indicates that NE and E might be responsible for stimulating the ventilatory response to acid infusion in water-breathing fish.

In the present study, increases in blood [NE] and [E] only occurred in treatment 1, when there was a reduction in C_{aO2} below normoxic levels, and an increase in P_{op} and f_{ab}. Thus the correlation between catecholamine levels and ventilatory responses seen in water-breathing fish holds true in *Amia* also. This suggests that NE and E might be responsible for mediating the observed increases in P_{op} and f_{ab}. Further evidence for this possibility will be gained by looking at the ventilatory responses to NE and E infusion in *Amia.*

SERIES 2:

Infusion of NE and E had pronounced effects on cardiovascular and gill ventilatory variables during normoxia, but not during moderate hypoxia. There was no stimulation of airbreathing in either treatment. The large endogenous release seen following NE and E injection in normoxia is similar to that seen in the American eel, *Anguilla rostrata* (Epple and Nibbio, 1985).

Catecholamines are known to stimulate ventilation in water-breathing fish (Peyraud-Waitzennegger, 1979), but the mechanism by which this stimulation occurs is unknown. Catecholamines can cross the blood:brain barrier in fish (Nekvasil and Olson, 1986), suggesting that the increases in P_{op} and f_g following NE and E may be a centrally mediated effect. In air-breathers (mammals), catecholamines affect ventilation at both central and peripheral sites (Dempsey *et al.*, 1986).

It is conceivable that the lack of an air-breathing response to catecholamines during normoxia was the result of a gating effect. In *Amia,* a change in ventilatory pattern, with increased emphasis on air-breathing, occurs in moderate

hypoxia (Johansen *et al.*, 1970; Randall *et al.*, 1981; table 6). Catecholamines may exert effects on ventilation at a site that will stimulate either gill ventilation or air-breathing, depending on the prevailing level of hypoxic drive. In normoxia, the emphasis was on gill ventilation, so catecholamine infusion increased P_{op} and f_g. If the lack of an air-breathing response to NE and E in normoxia was the result of a gating mechanism, then one might expect pharmacological doses of NE and E to stimulate increases in f_{ab} during moderate hypoxia (f_{ab} still has scope for increase in moderate hypoxia). This was not the case, indicating that NE and E exerted effects on a structure responsible for stimulating gill ventilation alone. It is unknown why NE and E did not stimulate P_{op} and f_g in hypoxia.

Thus, the results of treatments (A) and (B) indicate that if endogenous catecholamines do mediate ventilatory responses to internal acidosis in *Amia*, then they only mediate gill ventilatory responses. It is clear, however, that their release is an adaptive response, as catecholamine infusion increased C_{aO2}, indicating that endogenous release would ameliorate the effects of acid infusion on blood O_2-carrying capacity. Catecholamines are known to have this effect during acidosis in water-breathing fish (Perry and Kinkead, 1989).

In conclusion, it appears that in *Amia*, air-breathing and gill ventilatory responses to a reduction in pH_a only occur if there is an associated reduction in C_{aO2}. Arterial blood pH may have a direct effect on P_{DA}. Catecholamine infusion stimulates gill ventilation but not air-breathing. Increases in endogenous catecholamines may mediate gill ventilatory responses to a reduction in C_{aO2}, and

catecholamine release probably ameliorates the effects of acidosis on C_{aO2}

Chapter 3: Ventilatory and Cardiovascular Responses to Increases in Plasma Total CO_2 and Total Ammonia in Rainbow Trout and *Amia.*

INTRODUCTION

In water-breathing fish, blood and water O_2 status is the primary stimulus for ventilatory responses (Smith and Jones, 1982; Randall, 1982; Shelton *et al.*, 1986), and the results of Chapter 2 indicate that this is also true for *Amia*. There is evidence, however, that some water-breathing fish may exhibit a ventilatory response to increases in T_{aCO2} (Janssen and Randall, 1975), and mammals are known to show a ventilatory response to increases in T_{amm} (Wischer and Kazemi, 1974).

Janssen and Randall (1975) showed that infusion of sodium bicarbonate ($NaHCO_3$) stimulated ventilation in rainbow trout. This ventilatory response could be an effect of increases in T_{aCO2}, P_{aCO2} or HCO_3^-. $NaHCO_3$ infusion also caused a significant increase in pH_a (Janssen and Randall, 1975). Thus, the ventilatory response may have been to changes in other blood variables associated with an alkalosis. For example ammonia, the major end-product of protein catabolism, dissociates into ionised (NH_4^+) and un-ionised (NH_3) states when in solution. The degree of dissociation in plasma is dependent on blood pH, with NH_3 levels increasing as pH increases. The NH_3 form is freely permeable to cell membranes. Increases in blood pH might lead to changes in ammonia distribution between tissue compartments, with a gradient from alkalotic extracellular compartments to intracellular compartments at lower pH, e.g. the brain. Ammonia is known to stimulate ventilation via a central, intracellular effect in mammals (Wischer and Kazemi, 1974), but ventilatory responses to ammonia have not been examined in fish. Thus there is evidence to suggest that water-breathing fish may show

ventilatory responses to all three major respiratory gases, O_2, CO_2 and ammonia.

It is possible that the ventilatory response to increases in blood T_{aCO2} or T_{amm} is mediated by a release of catecholamines into circulation. Exogenous catecholamine infusion is known to stimulate ventilation in eels, *Anguilla anguilla* (Peyraud-Waitzennegger, 1979), and in rainbow trout (Aota, pers. comm.). Catecholamines are released in response to stress in fish (Nakano and Tomlinson, 1967; Perry at al., 1989), and may mediate the ventilatory response to hypoxaemia (Aota *et al.*, 1990).

Cardiovascular changes associated with ventilatory responses to increases in T_{aCO2} and T_{amm} have not been described in fish.

The present experiment was designed to determine whether water breathing fish (rainbow trout) show ventilatory and cardiovascular responses to increased T_{aCO2} and T_{amm}, and to compare responses with those seen in *Amia*. For trout, an initial experiment controls for the effects on ventilation of changes in pH_a, produced by sodium hydroxide or hydrochloric acid infusion into the dorsal aorta. This is followed by an investigation of the effects on ventilation of changes in T_{aCO2} and T_{amm}, produced by sodium bicarbonate, ammonium bicarbonate and ammonium chloride infusion. The possibility that ventilatory responses to increased T_{aCO2} and T_{amm} are stimulated by a reduction in C_{aO2} or increases in the levels of circulating catecholamines is investigated. Ventilatory and cardiovascular responses to increases in T_{aCO2} and T_{amm} are described in *Amia*, and the results compared with those seen in trout.

MATERIALS AND METHODS.

Experimental animals:

Rainbow trout, *Oncorhynchus mykiss*, weighing between 240 and 360 g, from the Sun Valley Trout Farm (Mission, B.C.), were maintained in large outdoor tanks, with a constant flow of dechlorinated Vancouver tapwater. Fish were fed regularly with trout chow. Water temperature was 8 to 12°C.

Amia (500 to 1100 g) were maintained and temperature acclimated as described in general materials and methods.

Animal Preparation:

Two different surgical procedures were followed to prepare trout for ventilatory measurements, but all *Amia* were treated as in procedure 2:

Procedure 1.

Trout were anaesthetized in a buffered ($NaHCO_2$) MS-222 solution at a concentration of 1:10,000, and transferred to an operating table where they were maintained at an MS-222 concentration of 1:20,000. Dorsal aortic cannulae (PE 50) were implanted using the technique of Soivio, Westman and Nyholm (1972). A latex mask was sutured below the eyes and in front of the opercula, and attached to the divider in a Van Dam box. In this way, all water flow from anterior to posterior chambers was via the mouth and gills (Cameron and Davis, 1970). The water level was adjusted so that the fish had a positive pressure head

across the gills, and the animal allowed 48 hrs to recover. The DA cannula was flushed twice daily with heparinised (1:1,000) Cortland's saline (Wolf, 1963).

Procedure 2.

Trout or *Amia* were anaesthetised and fitted with a dorsal aortic cannula as described above. An opercular cannula was fitted, using flared PE 190 tubing passed through a small hole drilled in the operculum, secured with a cuff and sutures. Trout recovered from surgery in individual black plexiglass boxes, and *Amia* in individual plexiglass boxes with access to a forward airspace, to allow air-breathing, for 48 hrs. The DA cannula was flushed as described above.

Ventilatory and cardiovascular measurements:

Procedure 1.

One hour before the experiments, water level was adjusted in the Van Dam box, so that the trout had to ventilate actively, and any water overflow from the posterior chamber was a result of ventilation. Ventilation volume ($\dot{V}g$) was measured by collecting the overflow for two one minute periods. No cardiovascular measurements were made in fish treated by this procedure.

Procedure 2.

Following recovery, the water-filled opercular cannula was attached to a presssure transducer (Statham P23BB). This allowed gill ventilation rate (f_g, beats/min), and opercular pressure amplitude (P_{op}, cm H_2O) to be recorded and

displayed on a brush recorder (Gould 8188-2202-XX). P_{op} was used as an index of stroke volume. In *Amia*, air-breaths were visible as large pressure excursions in opercular pressure amplitude, and were verified visually via a small hole in a screen separating experimenter and fish. In Series 3 and 4 (see below) the saline-filled DA cannula was attached to a pressure transducer (Statham P23dB). This allowed heart rate (f_h, beats/min) and dorsal aortic blood pressure (P_{DA}, cm H_2O) to be recorded and displayed on the same recorder as used for ventilatory recordings.

Protocol:

Trout were used in three series of infusions.

Series 1:

Trout surgically prepared by procedure (1) were exposed to the following treatments:

1) 5 ml/kg Cortland's saline infusion.

2) 5 ml/kg 0.05 M sodium hydroxide (NaOH) infusion, in a Cortland's saline vehicle.

3) 5 ml/kg 0.05 M hydrochloric acid (HCl) infusion, in a Cortland's saline vehicle.

Series 2:

Trout prepared by procedure (2) were exposed to the following treatments:

1) 5 ml/kg 0.2 M sodium chloride (NaCl) infusion, in a Cortland's saline vehicle.

2) 5 ml/kg 0.2 M sodium bicarbonate ($NaHCO_3$) infusion, in a Cortland's saline vehicle.

3) 5 ml/kg 0.2 M ammonium bicarbonate (NH_4HCO_3) infusion, in a Cortland's saline vehicle.

4) 5 ml/kg 0.2 M ammonium chloride (NH_4Cl) infusion, in a Cortland's saline vehicle.

Series 3:

Trout prepared by procedure 2 were exposed to NaCl, $NaHCO_3$, NH_4HCO_3 and HCl infusions as described above, and ventilatory and cardiovascular parameters measured.

Amia were used in the following treatments:

Series 4:

1) 5 ml/kg 0.2 M NaCl infusion, in a Cortland's saline vehicle.

2) 5 ml/kg 0.2 M NH_4HCO_3 infusion, in a Cortland's saline vehicle.

All infusions were performed over 7 to 10 minutes, at 0.6 ml/min. At this infusion rate, there were no signs of irritant or behavioural responses.

Ventilation was measured for a control period before each infusion, and then for 60 minutes following infusion in Series 1 and 2, and for 30 minutes in series 3 and 4. Heart rate and P_{DA} were measured for a control period before infusion

and for 30 minutes post-infusion in Series 3 and 4. Immediately prior to each infusion, a 1.4 ml blood sample was collected, and replaced with an equal volume of heparinised saline. At five minutes post-infusion (p.i), another 1.4 ml blood sample was collected, and replaced with an equal volume of heparinised saline. In Series 1, 2 and 4, 500 µl of blood were immediately centrifuged, the plasma decanted and frozen in liquid nitrogen for subsequent analysis of plasma [NE] and [E], as described in general materials and methods. In Series 1 and 2, the remainder of the sample was analysed for pH_a, T_{aCO2} and T_{amm}, as described in general materials and methods. Arterial plasma P_{aCO2} and NH_3 concentrations ($[NH_3]$) were calculated from T_{aCO2} and T_{amm}, respectively, as described in general materials and methods. In Series 3 and 4, blood was analysed for pH_a, C_{aO2} and P_{aO2}, as described in general materials and methods.

Data Analysis and Statistics:

In Series 1, ventilation was measured directly, for two minutes pre-infusion and at designated intervals up to 60 minutes post-infusion . In Series 2 to 4, f_g was calculated for designated time intervals by counting 30 seconds in each minute, up to 30 minutes. P_{op} was averaged from six measurements taken within the same period as used to calculate f_g. In Series 3 and 4, f_h was measured by counting 30 seconds in each minute, at the same intervals counted for f_g. Peak systolic P_{DA} was averaged from six measurements taken within the same period as f_h. In all treatments, individual ventilatory and cardiovascular responses were normalised as % change from control (pre-infusion) values. Following arc-sine transformation,

the significance of responses was assessed by ANOVA. In one case ($NaHCO_3$ infusion in Series 3), the variance of the transformed ventilatory and cardiovascular measurements within each time interval was heterogenous, and so a non-parametric, Kruskal-Wallis analysis by ranks was used. In all treatments, blood and plasma parameters were compared before and after infusion using a paired T-test. $P < 0.05$ was taken as the confidence level of probability.

RESULTS

Series 1:

Saline (control) infusion resulted in no significant changes in $\dot{V}g$ (fig 13). NaOH and HCl infusions both caused significant changes in $\dot{V}g$ (fig 13). All three infusions resulted in significant changes in blood gases (table 7).

Following saline infusion, there was a small but significant drop in pH_a and increase in T_{aCO2} and P_{aCO2}. Plasma T_{amm} and $[NH_3]$ did not change (table 7) and neither did [NE] and [E] (table 9).

At 5 minutes following NaOH infusion, there was no significant change in ventilation, pH_a was significantly higher than control values, and P_{aCO2} was significantly depressed. Arterial plasma T_{CO2}, T_{amm}, and $[NH_3]$ did not change (table 7). The level of circulating catecholamines did not change (table 9). At 20 minutes p.i., there was a significant increase in ventilation that was sustained until 45 minutes post-injection (fig 13).

HCl infusion produced a different response. At the end of infusion, there was an increase in $\dot{V}g$, which remained significantly elevated until 12 minutes post-infusion (fig 13). This increase in ventilation was associated, at 5 minutes p.i, with a significant drop in pH_a and T_{aCO2}. Arterial plasma P_{CO2} increased significantly, but T_{amm} and $[NH_3]$ did not change (table 7). Plasma [NE] and [E] increased significantly (table 9).

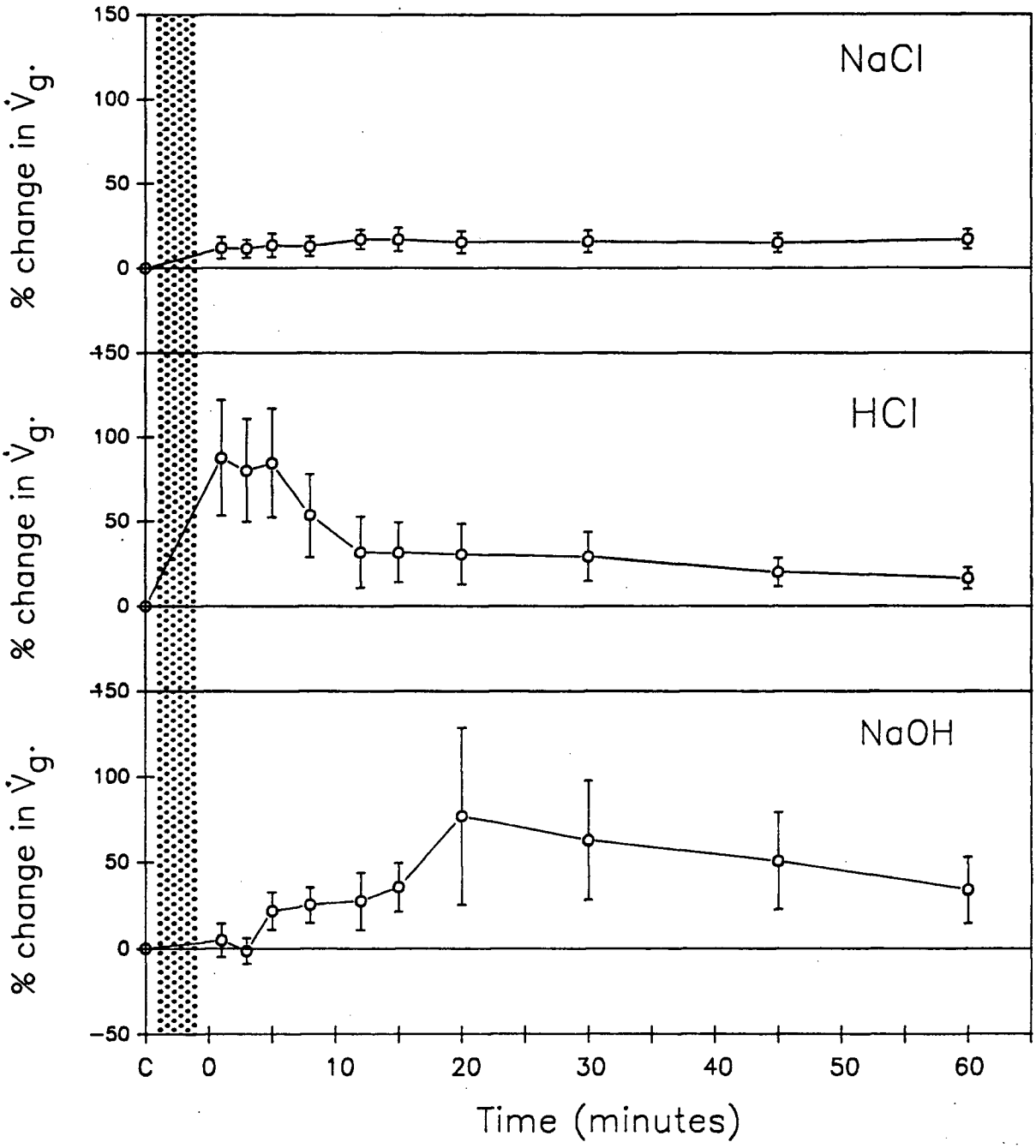
Table 7: Blood Gas Measurements for Series 1 and 2.

	pH_a	T_{aCO2} (mmol)	P_{aCO2} (mmHg)	T_{amm} (μmol)	$[NH_3]$ (μmol)
Series 1					
Pre-Saline	7.86 ±0.02	10.64 ±0.89	3.8 0.2	97.1 ±16.6	1.5 ±0.2
Post-Saline	7.81* ±0.03	11.13* ±0.97	4.5* ±0.1	108.6 ±26.9	1.5 ±0.3
Pre-NaOH	7.80 ±0.01	8.82 ±0.3	3.7 ±0.1	56.7 ±9.1	0.8 ±0.1
Post-NaOH	8.06* ±0.08	9.94 ±0.68	2.2* ±0.4	69.5 ±10.6	2.2 ±0.8
Pre-HCl	7.75 ±0.02	8.84 ±0.59	4.3 ±0.3	66.0 ±6.2	0.8 ±0.1
Post-HCl	7.60* ±0.03	7.34* ±0.49	5.0 ±0.5	65.3 ±3.0	0.6 ±0.1
Series 2					
Pre-NaCl	7.91 ±0.06	9.64 ±0.57	2.7 ±0.2	183.5 ±24.3	2.7 ±1.0
Post-NaCl	7.84* ±0.06	10.21* ±0.56	3.3* ±0.3	233.0 ±27.3	2.9 ±0.9
Pre-$NaHCO_3$	8.03 ±0.04	10.04 ±0.84	2.1 ±0.1	204.7 ±42.3	3.9 ±1.4
Post-$NaHCO_3$	8.16* ±.03	15.66* ±0.93	2.3 ±0.2	205.3 ±39.7	5.3 ±1.4
Pre-NH_4HCO_3	7.85 ±0.04	10.06 ±0.19	3.2 ±0.2	205.2 ±35.1	2.3 ±0.2
Post-NH_4HCO_3	7.85 ±0.03	11.69* ±0.22	3.7* ±0.3	1219.3* ±94.2	14.7* ±1.9
Pre-NH_4Cl	7.86 ±0.03	10.36 ±0.20	3.2 ±0.2	138.0 ±25.3	1.6 ±0.2

Post-NH_4Cl	7.52* ±0.05	8.44* ±0.14	5.7* ±0.5	2685.2* ±543.0	14.3* ±2.0

All values = mean ± S.E.
* = significantly different from pre-injection ($P<0.05$).

Figure 13: Mean per cent change (± S.E.) in $\dot{V}g$ following NaCl, NaOH or HCl infusion. n = 6. C = control, shaded bar represents infusion period.


NaCl
HCl
NaOH
% change in $\dot{V}_g$
% change in $\dot{V}_g$
% change in $\dot{V}_g$
150
100
50
0
-50
C
0
10
20
30
40
50
60
Time (minutes)

Series 2:

NaCl infusion did not result in any significant changes in ventilation (fig 14). Plasma [total ammonia] and [NH_3] did not change, pH_a showed a small but significant decrease, and T_{aCO2} and P_{aCO2} a small but consistent increase (table 7). Catecholamine levels did not change significantly (table 9).

$NaHCO_3$, NH_4HCO_3 and NH_4Cl all caused significant increases in ventilation (fig 14), and significant changes in blood and plasma variables (table 7). Representative traces of ventilatory responses to NaCl, $NaHCO_3$ and NH_4HCO_3 infusion are presented in fig 15.

$NaHCO_3$ infusion increased both P_{op} and f_g. P_{op} returned to control values within 12 minutes p.i. and f_g within 8 minutes p.i. (figs. 14 and 15). These ventilatory responses were associated, at 5 minutes p.i., with a significant increase in pH_a and T_{aCO2}. Arterial plasma P_{CO2}, T_{amm} and [NH_3] did not change (table 7). The level of circulating catecholamines increased significantly (table 9).

NH_4HCO_3 infusion resulted in an immediate increase in P_{op}, which remained significantly elevated until 20 minutes post-infusion. Gill ventilation frequency, however, did not change (figs 14 and 15). At 5 minutes p.i., there was a significant increase in T_{aCO2}, P_{aCO2}, T_{amm} and [NH_3], but no change in pH_a (table 7). Neither [NE] nor [E] increased significantly (table 9).

NH_4Cl infusion stimulated both P_{op} and f_g. Opercular pressure amplitude increased immediately, and remained elevated until 3 minutes p.i., but f_g did not

Figure 14: Mean per cent change (± S.E.) in P_{op} and f_g following $NaHCO_3$, NH_4HCO_3, HCl and NaCl infusion. n = 6.

C = control, shaded bar represents infusion period

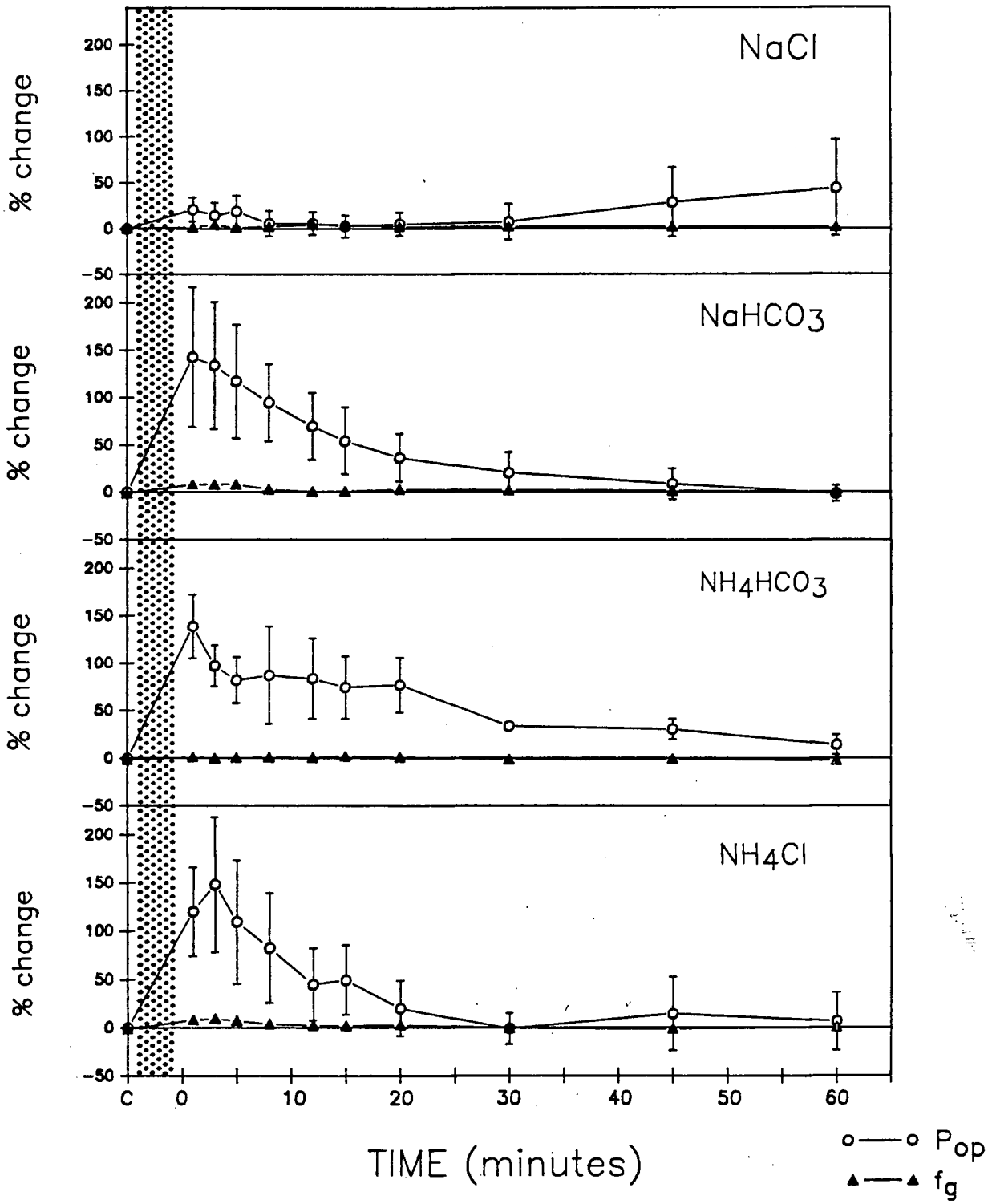
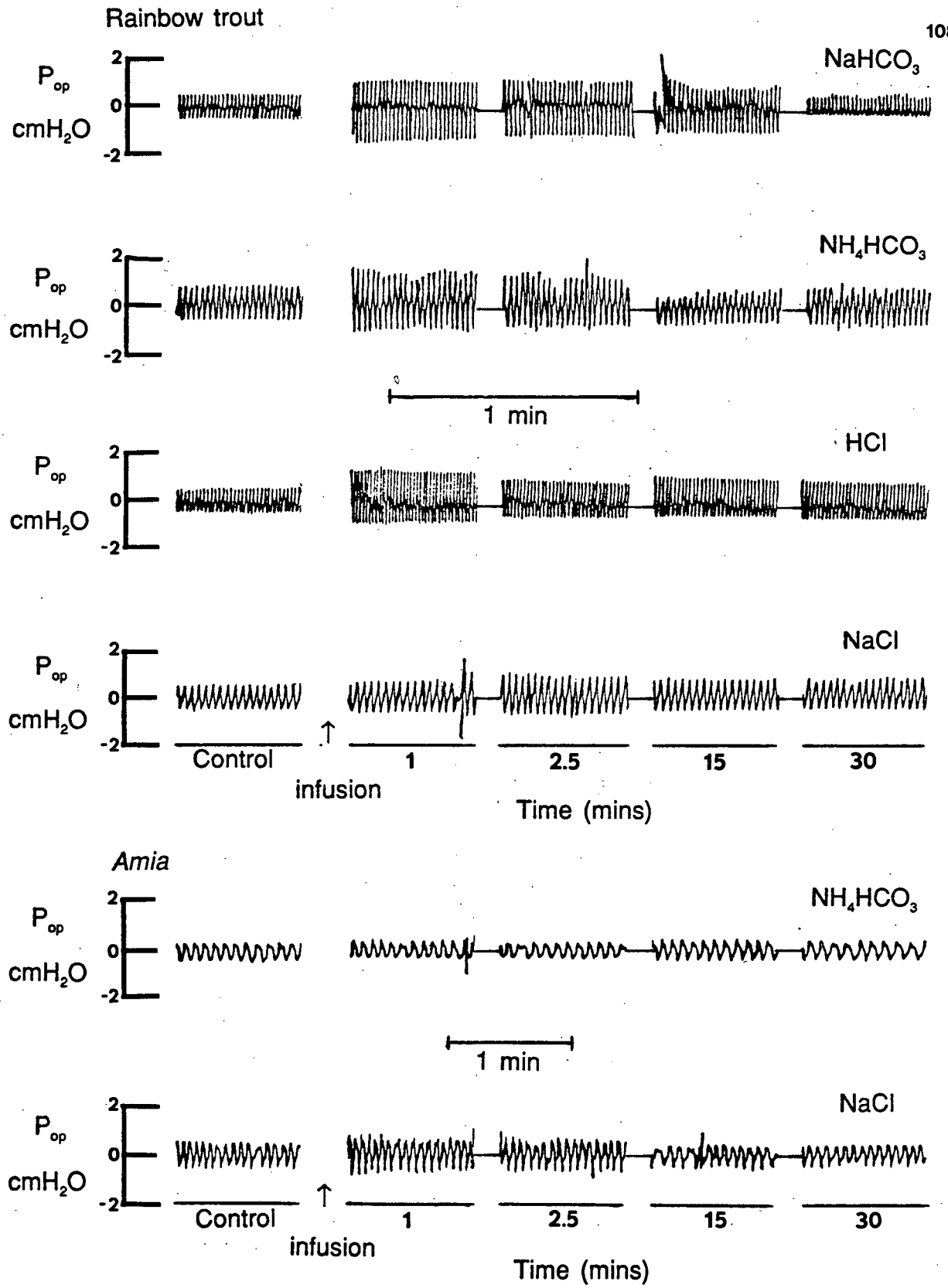

NaCl
NaHCO3
NH4HCO3
NH4Cl
% change
% change
% change
% change
200
150
100
50
0
-50
C
0
10
20
30
40
50
60
TIME (minutes)
o—o Pop
▲—▲ fg

Figure 15: Representative traces of ventilatory responses to $NaHCO_3$, NH_4HCO_3, HCl and NaCl in rainbow trout, and NH_4HCO_3 and NaCl in *Amia*.


Rainbow trout
P_{op}
cmH_2O
2
0
-2
$NaHCO_3$
NH_4HCO_3
1 min
HCl
NaCl
Control
infusion
1
2.5
15
30
Time (mins)
Amia
NH_4HCO_3
1 min
NaCl
Control
infusion
1
2.5
15
30
Time (mins)

increase until 3 minutes p.i., and returned to control levels at 8 minutes post-infusion (fig 14). These ventilatory changes were associated with a significant decrease in pH_a and T_{aCO2}, and an increase in P_{aCO2}, T_{amm}, and $[NH_3]$ (table 7). There was no significant increase in [NE] and [E], but resting levels were unusually high in all animals in this treatment, and the data were not included in Table 9.

Series 3:

NaCl infusion had no significant effect on either ventilatory variables or f_h, but caused a significant increase in P_{DA} (fig 16). NaCl caused no significant changes in pH_a, P_{aO2} or C_{aO2} (table 8), or [NE] and [E] (table 9). $NaHCO_3$, NH_4HCO_3 and HCl stimulated both cardiovascular and ventilatory variables (figs 17, 18 and 19). A representative trace of the effects of HCl infusion on ventilation is presented in fig 15.

$NaHCO_3$ infusion resulted in a significant increase in P_{op}, f_g and P_{DA} and f_h (fig 17). In two fish, ventilatory increases were up to 500 per cent of pre-injection values. These responses were associated with a significant increase in pH_a, a significant decrease in P_{aO2}, but C_{aO2} did not change (table 8).

NH_4HCO_3 infusion elicited a significant increase in P_{op}, f_g, P_{DA} and f_h (fig 18). There were no significant changes in pH_a, C_{aO2} or P_{aO2} (table 8).

HCl infusion elicited a significant increase in P_{op}, f_g and P_{DA}, but there was no significant change in f_h (fig 19). At 5 minutes p.i., these ventilatory responses

Table 8: Blood Gas Measurements for Series 3 and 4

	pH_a	C_{aO2} (vol.%)	P_{aO2} (mmHg)
Series 3 : Trout			
Pre-NaCl	7.77 ±0.02	7.5 ±0.6	147 ±8
Post-NaCl	7.75 ±0.02	6.5 ±0.3	142 ±4
Pre-$NaHCO_3$	7.76 ±0.03	7.4 ±0.8	135 ±6
Post-$NaHCO_3$	8.74 ±0.10*	6.4 ±0.9	80 ±11*
Pre-NH_4HCO_3	7.85 ±0.01	7.6 ±0.6	143 ±8
Post-NH_4HCO_3	7.81 ±0.02	7.5 ±0.7	145 ±3
Pre-HCl	7.77 ±0.02	6.6 ±0.8	140 ±7
Post-HCl	7.51 ±0.05*	5.5 ±0.8*	196 ±27
Series 4: *Amia*			
Pre-NaCl	7.68 ±0.03	6.7 ±1.2	48 ±9
Post-NaCl	7.69 ±0.03	5.4 ±1.2*	42 ±6
Pre-NH_4HCO_3	7.72 ±0.01	6.4 ±0.6	30 ±6
Post-NH_4HCO_3	7.63 ±0.03*	5.4 ±1.1	27 ±5

All values = mean ± S.E.; n = 6 in Series 3, n = 5 in Series 4.
* = significantly different from pre-infusion ($P<0.05$).

Table 9: Plasma [NE] and [E]

	[NE]		[E]	
Trout	**Pre**	**Post**	**Pre**	**Post**
NaOH	11.7±4.72	3.19±0.62	1.72±0.56	1.10±0.29
HCl	1.27±0.48	6.17±0.71*	3.67±0.70	32.4±11.0*
$NaHCO_3$	3.85±1.12	22.8±6.65*	4.05±1.36	61.3±21.3*
NH_4HCO	4.17±0.80	6.96±1.38	12.7±3.47	39.6±17.2
NaCl	3.60±0.42	3.32±0.57	10.7±3.27	11.4±2.01
Saline	10.7±3.23	8.03±1.87	5.41±1.57	3.43±1.48
Amia				
NH_4HCO	18.4±1.94	31.7±6.99	16.3±3.65	27.9±7.32
NaCl	13.4±4.57	23.1±8.01	7.64±2.20	23.4±13.3

Units = nM

All values = mean ± S.E., * = significantly different from pre-injection.

Figure 16: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following NaCl infusion in rainbow trout. n = 6.

C = control, shaded bar represents infusion period.

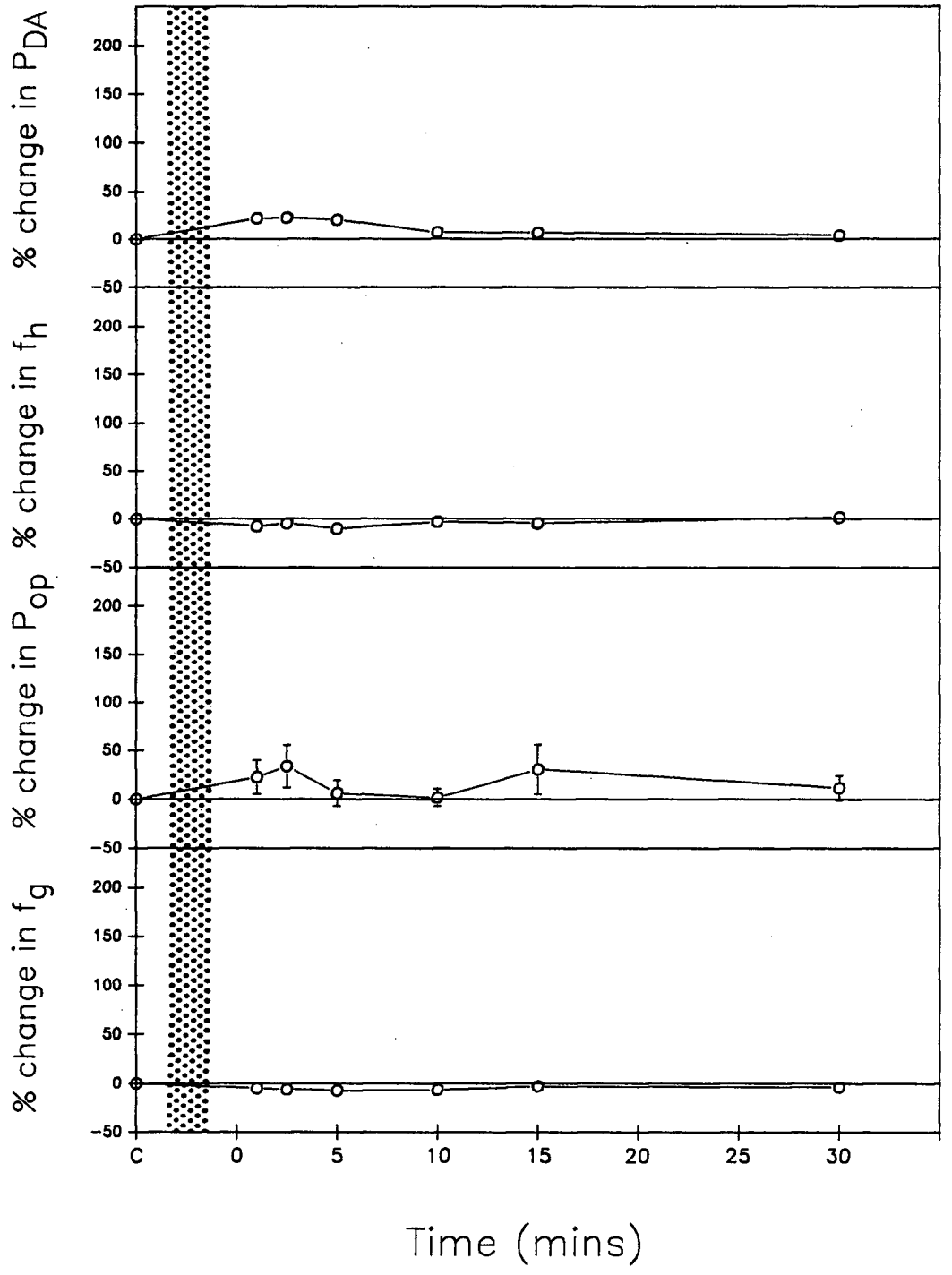

% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
200
150
100
50
0
−50
C
0
5
10
15
20
25
30
Time (mins)

Figure 17: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following $NaHCO_3$ infusion in rainbow trout. n = 6. C = control, shaded bar represents infusion period.

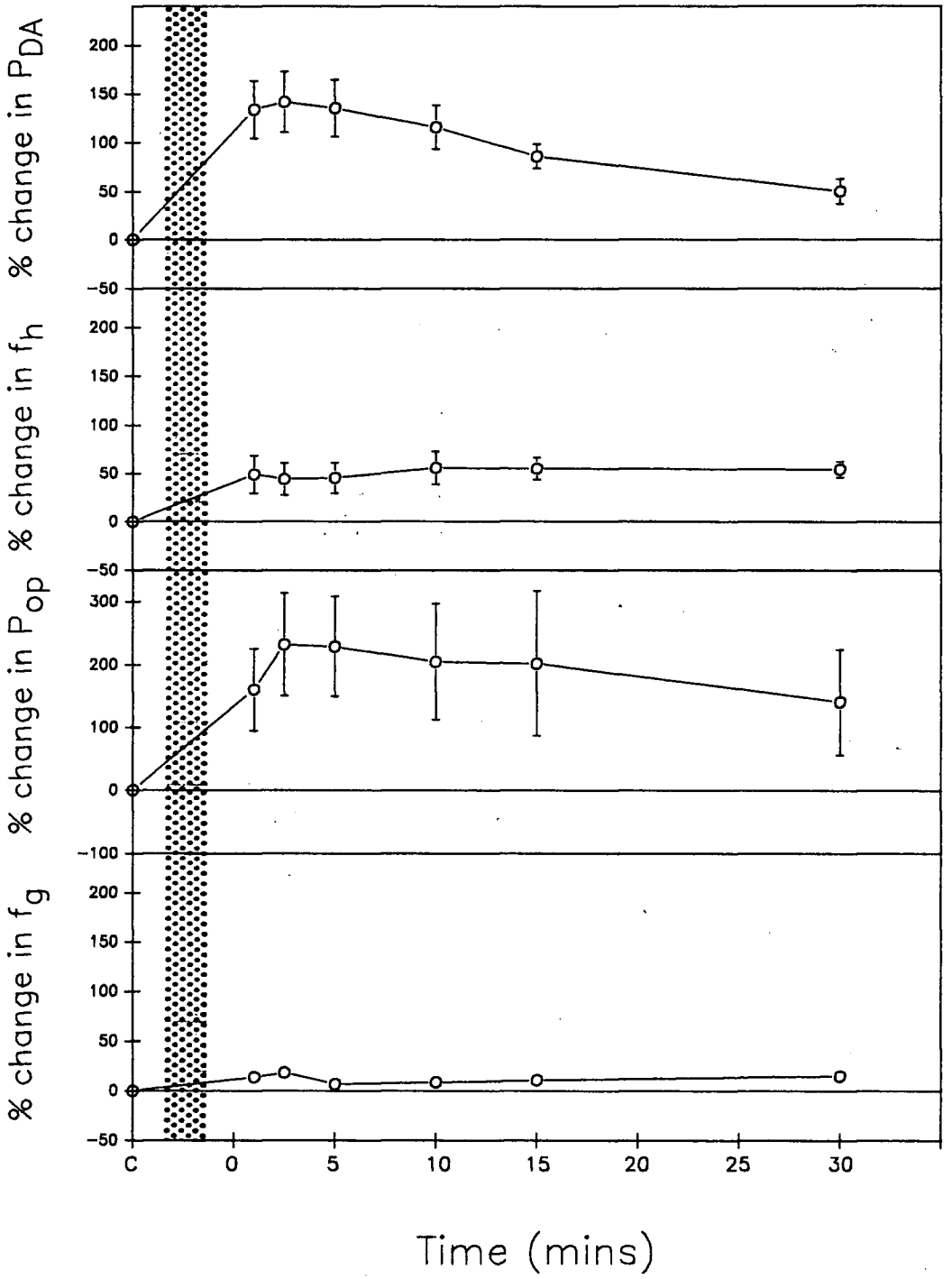

% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
200
150
100
50
0
-50
300
-100
C
0
5
10
15
20
25
30
Time (mins)

Figure 18: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following NH_4HCO_3 infusion in rainbow trout. n = 6

C = control, shaded bar represents infusion period.

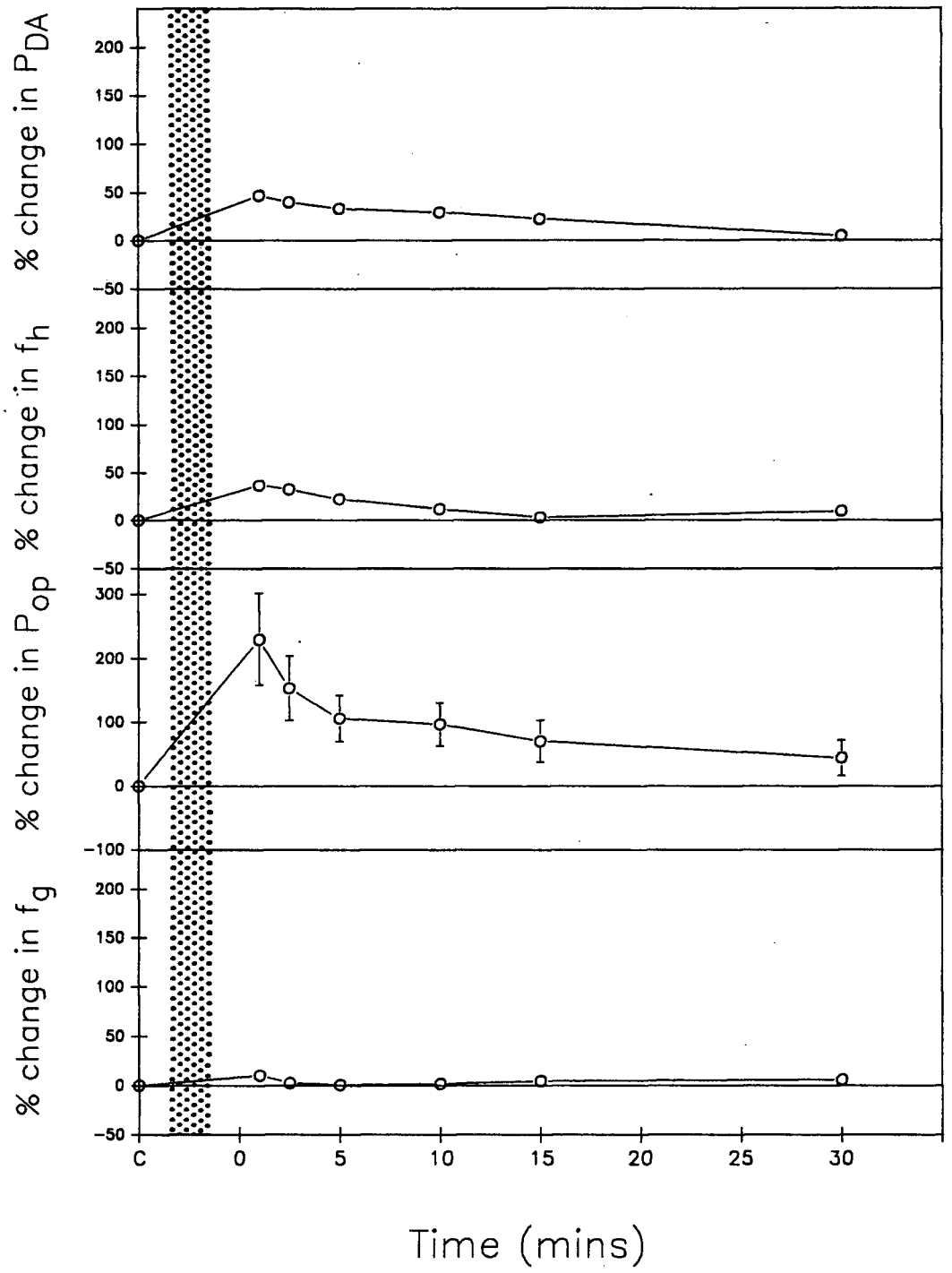
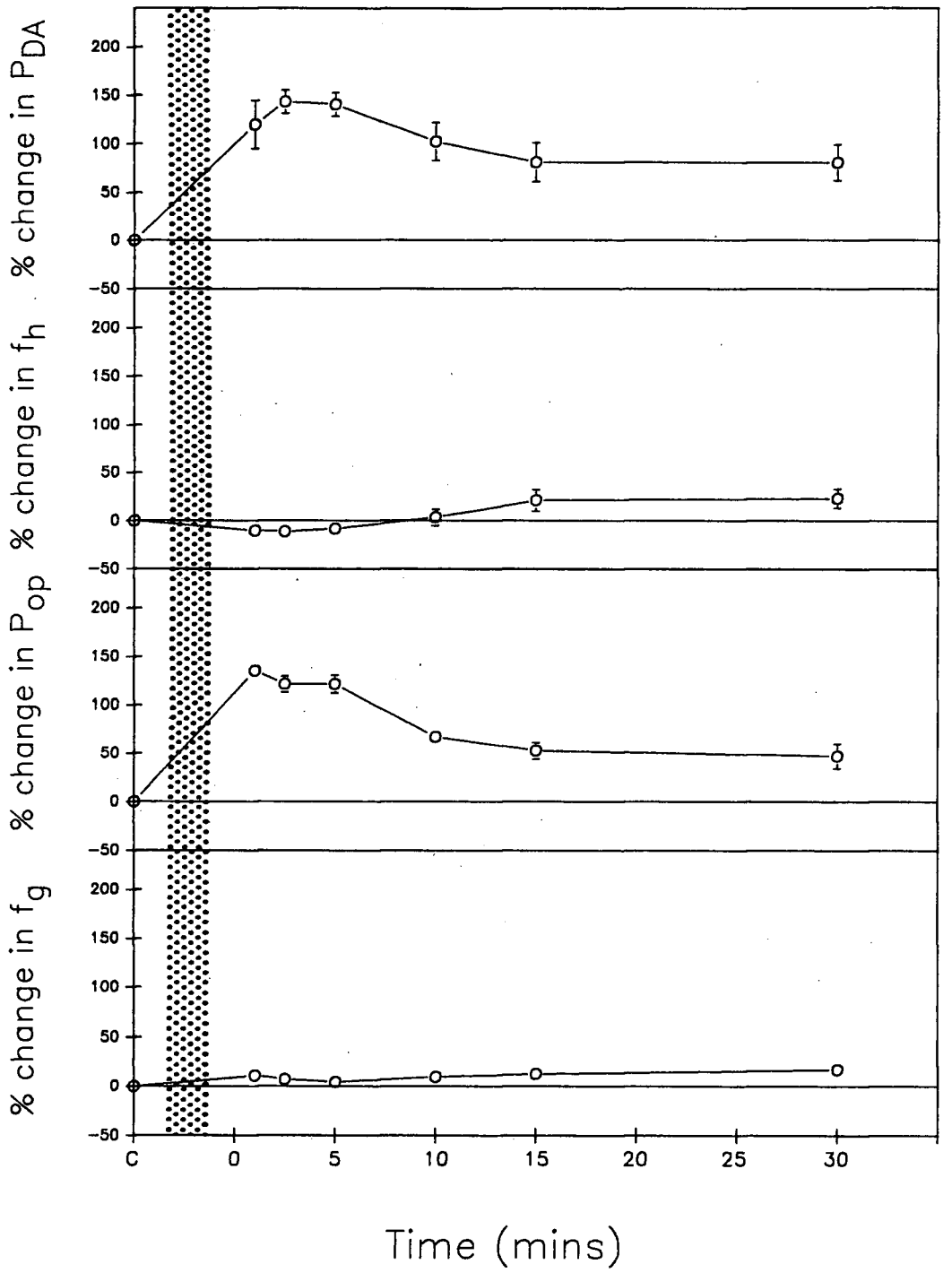

% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
200
150
100
50
0
-50
300
-100
C
0
5
10
15
20
25
30
Time (mins)

Figure 19: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following HCl infusion in rainbow trout. n = 6

C = control, shaded bar represents infusion period.


% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
200
150
100
50
0
-50
C
0
5
10
15
20
25
30
Time (mins)

were associated with a significant reduction in pH_a and C_{aO2}, but no significant change in P_{aO2} (table 8).

Series 4:

Gill ventilation frequency under control conditions was significantly lower in *Amia* compared with trout. Opercular pressure amplitude, arterial blood pressure and heart rate were not significantly different in the two species. Blood gases differed between the two species. *Amia* had a significantly lower blood pH and P_{aO2}. Arterial blood O_2 content was not significantly different (table 8). These differences are probably related to the difference in water temperature (10°C in trout vs 20°C in *Amia*), and possibly to the different ventilatory strategies and activity levels of the two species. *Amia* had resting catecholamine levels in the plasma about one order of magnitude higher than those in trout.

NaCl infusion resulted in a small but significant increase in P_{op}, but no significant changes in f_g, P_{DA} or f_h (fig 20). Air-breathing frequency did not change, remaining at 0.66 ±0.33 (mean ± S.E.) breaths/hr. At 5 minutes p.i., there was a significant reduction in C_{aO2}, but no changes in pH_a or P_{aO2} (table 8). Circulating [NE] and [E] did not change significantly (table 9). A representative trace of the effects of NaCl infusion in *Amia* is shown in fig 15.

NH_4HCO_3 infusion had no significant effects on P_{op}, f_g (fig 21) or f_{ab}, but significantly increased P_{DA} and f_h. At 5 minutes p.i., there was a small but significant reduction in pH_a, but no changes in C_{aO2} or P_{aO2} (table 8). Circulating catecholamine levels did not change (table 9).

Figure 20: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following NaCl infusion in *Amia*. n = 5.

C = control, shaded bar represents infusion period.

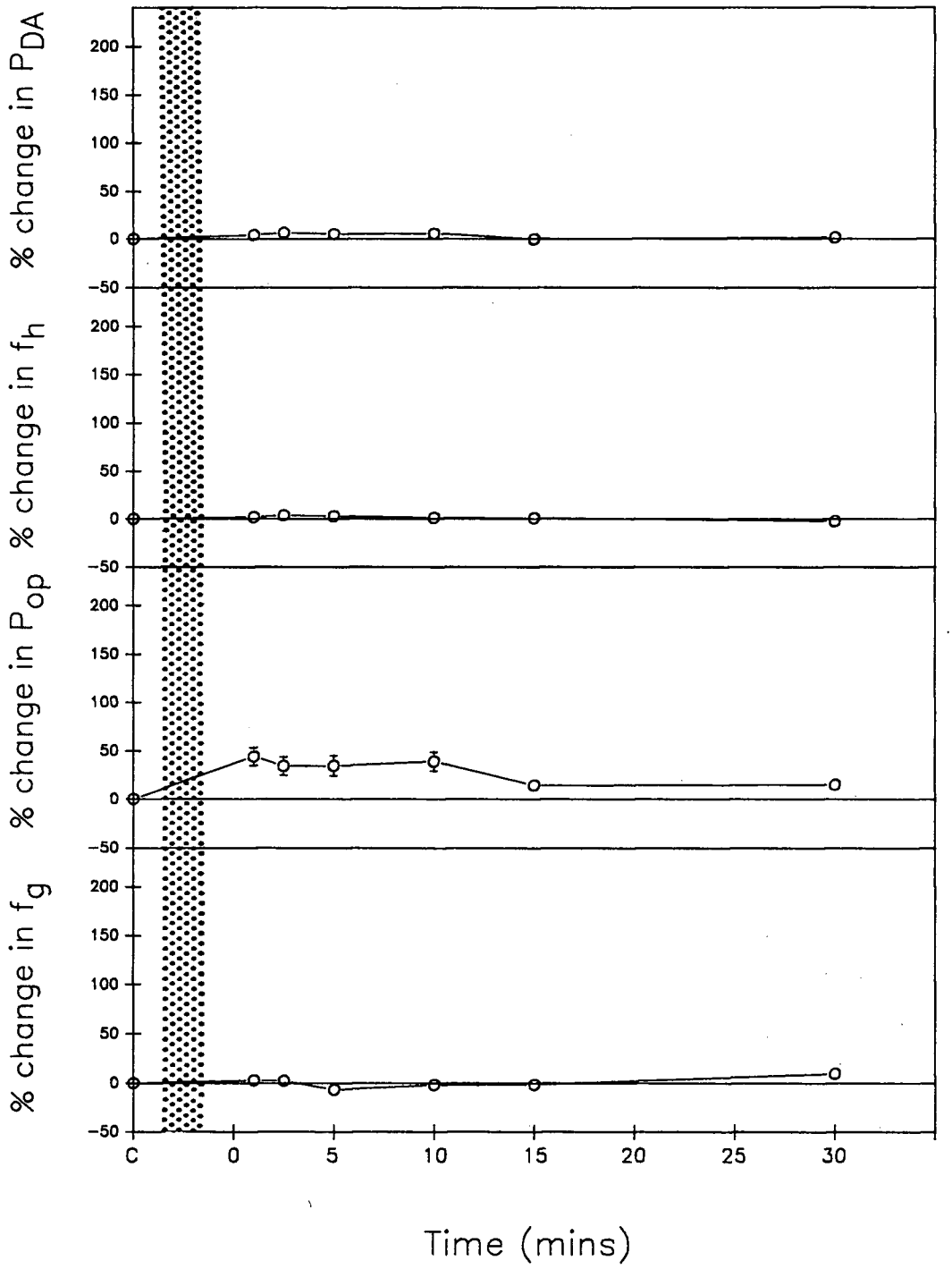
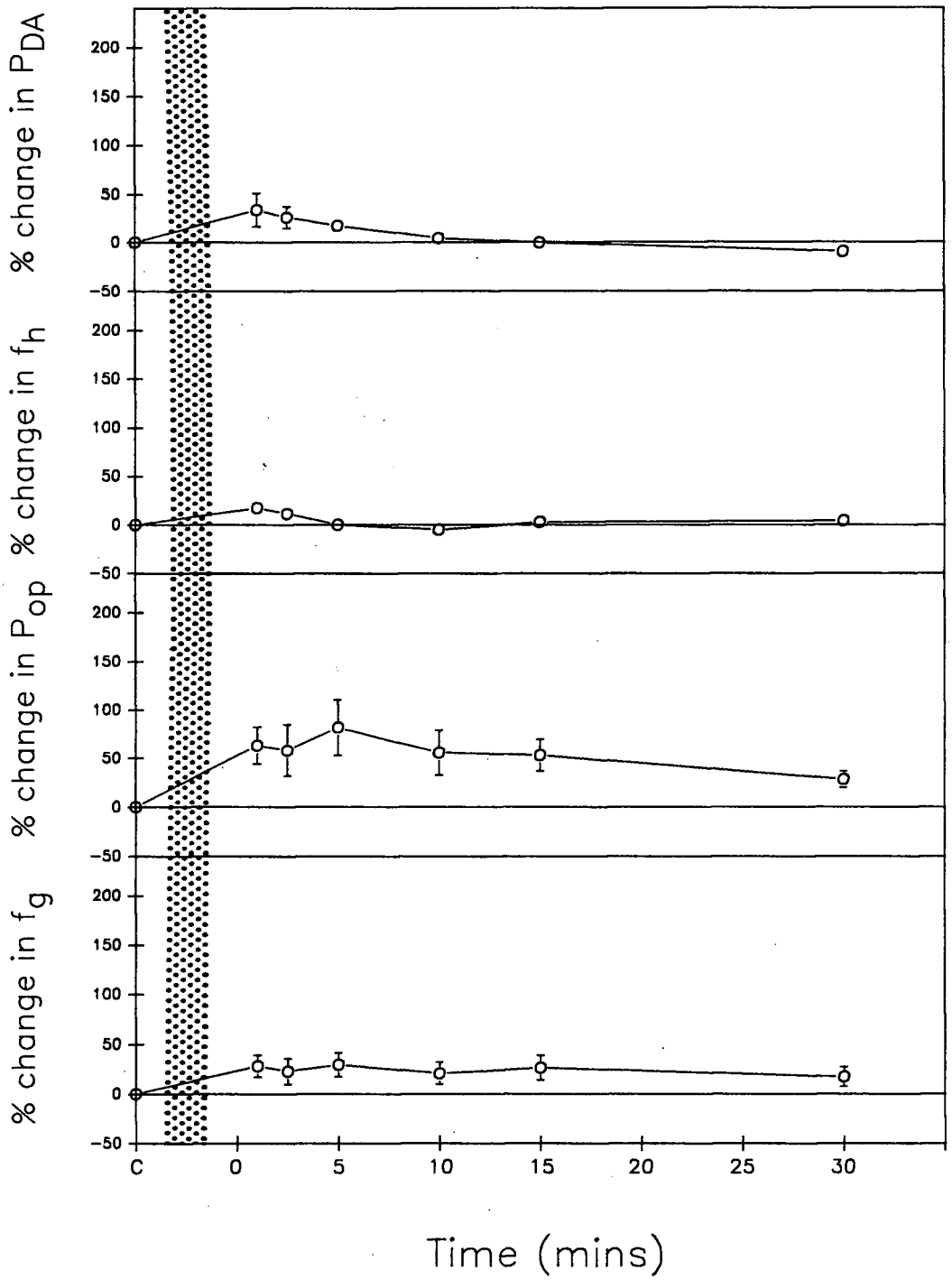

% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
200
150
100
50
0
-50
C
0
5
10
15
20
25
30
Time (mins)

Figure 21: Mean per cent change (± S.E.) in P_{DA}, f_h, P_{op} and f_g following NH_4HCO_3 infusion in *Amia*. n = 5

C = control, shaded bar represents infusion period.


% change in P_{DA}
% change in f_h
% change in P_{op}
% change in f_g
200
150
100
50
0
−50
C
0
5
10
15
20
25
30
Time (mins)

DISCUSSION

The blood gas measurements in Series 1 and 2 allow analysis of the relative roles of pH_a, T_{aCO2} and T_{amm} in the ventilatory responses observed for trout.

The relationship between pH_a and ventilation:

Following NaOH infusion there was a significant increase in blood pH, but this was not directly associated with any immediate increase in $\dot{V}g$. This indicates that increases in pH_a are not a direct ventilatory stimulant, and thus the increase in ventilation observed following $NaHCO_3$ infusion (Janssen and Randall, 1975; fig 14) was not a result of the associated increase in pH_a. It is interesting that infusions of NaOH at doses 1.5 or 2 times those reported here do not stimulate ventilation in a pattern similar to that following $NaHCO_3$ infusion, but immediately lead to convulsions and death (unpublished observations). The significant increase in ventilation seen 20 minutes following NaOH infusion is difficult to interpret, as no associated blood gas measurements were collected.

Decreases in pH_a resulting from infusion of HCl or NH_4Cl are directly associated with ventilatory responses, a result similar to that observed by Aota *et al.* (1990).

The relationship between T_{amm}, T_{aCO2} and ventilation:

Infusions of NH_4Cl and NH_4HCO_3 stimulated ventilation significantly, and were associated with increases in both T_{amm} and $[NH_3]$. The decrease in pH_a following NH_4Cl infusion might have been responsible for the ventilatory increase seen, but

following NH_4HCO_3 infusion, there was no change in pH_a.

Sodium bicarbonate infusion, however, also stimulated ventilation but was not associated with any significant increase in plasma T_{amm} or $[NH_3]$. This indicates that the response was stimulated by the measured increase in T_{aCO2}. The ventilatory response occurred in the absence of a significant increase in P_{aCO2}, indicating that it was a result of increases in plasma HCO_3^- concentration ($[HCO_3^-]$). The ventilatory response to NH_4HCO_3 may also have been a result of increases in $[HCO_3^-]$, in addition to the increase in T_{amm}. The ventilatory responses to NH_4Cl, NH_4HCO_3 and $NaHCO_3$ were not a result of changes in plasma osmolarity following infusion, because infusion of NaCl did not have any effects on ventilation.

These results indicate that water-breathing fish show a ventilatory response to increases in T_{aCO2} and T_{amm}. The evidence indicates that increases in $[HCO_3^-]$ were the cause of the ventilatory response seen following $NaHCO_3$ infusion. Ventilatory responses to NH_4Cl and NH_4HCO_3 infusions are evidence of ventilatory sensitivity to ammonia, with a reduction in pH_a (NH_4Cl infusion) or increases in $[HCO_3^-]$ (NH_4HCO_3 infusion) probably contributing to the response.

Possible mechanisms behind ventilatory responses:

As stated in the Introduction, the primary source of ventilatory drive in water-breathing fish is water and blood O_2 status. Ventilatory responses to blood O_2 status in fish appear to be more closely correlated with changes in blood O_2

content than with O_2 partial pressure (Dejours, 1981; Smith and Jones, 1982; Randall, 1982).

It is conceivable that all the ventilatory responses observed in Series 1 and 2 were a result of a reduction in C_{aO2}. The results of Series 3 indicate, however, that this was not the case. Following HCl infusion, C_{aO2} declined significantly, as a result of the effects of a reduction in pH_a on blood O_2 carrying capacity, via Bohr and Root effects. The observed ventilatory increase, therefore, was most likely stimulated by the reduction in C_{aO2}; results similar to those of Smith and Jones (1982) and Aota *et al.* (1990), for rainbow trout. By contrast, the ventilatory responses to $NaHCO_3$ and NH_4HCO_3 infusion were not associated with a significant reduction in C_{aO2}. Thus, the effect of HCO_3^- and T_{amm} on ventilation do not appear to be mediated by changes in C_{aO2}. Following $NaHCO_3$ infusion, there was a significant reduction in P_{aO2}, which probably contributed to the observed ventilatory response. It is unknown why $NaHCO_3$ infusion caused a reduction in P_{aO2}. NH_4HCO_3 infusion, however, did not effect any changes in blood O_2 status.

Cardiovascular responses observed in Series 3 also indicate that there is a difference in the characteristics of the response to HCl and the responses to $NaHCO_3$ and NH_4HCO_3. HCl has no effect on f_h, whereas $NaHCO_3$ and NH_4HCO_3 infusion both result in a tachycardia.

In fish, catecholamines (NE and E) are released into circulation during stress (Nakano and Tomlinson, 1967; Perry *et al.*, 1989). Catecholamines stimulate ventilation in the eel, *A. anguilla* (Peyraud-Waitzennegger, 1979), and in trout

(Aota, pers. comm.). There is evidence that the ventilatory responses to internal acidosis are mediated by catecholamines in trout (Aota *et al.*, 1990). In the present experiment, there was a significant increase in the levels of circulating NE and E associated with the ventilatory responses to HCl and $NaHCO_3$. This suggests that the ventilatory responses to increases in $[HCO_3^-]$ may have resulted from release of catecholamines, which then stimulated peripheral or central sites involved in ventilatory control.

Catecholamines are known to increase P_{DA} and f_h in rainbow trout (Wood and Shelton, 1980), which might explain the cardiovascular responses observed in this study. Increased acidity has a negative inotropic and chronotropic effect on the isolated trout heart, that is offset by the effects of epinephrine (Farrell, MacLeod and Chancey, 1986). Thus, following acid infusion, catecholamine release may have ameliorated the negative chronotropic effects of an acidosis, resulting in no change in f_h. In the absence of an acidosis, catecholamines release may have stimulated heart rate, producing the tachycardia seen following $NaHCO_3$ infusion. Following NH_4HCO_3 infusion, however, there was a tachycardia that was not associated with a significant increase in [NE] and [E].

It is unknown whether the ventilatory and cardiovascular responses to $NaHCO_3$ actually represent direct evidence of sensitivity to $[HCO_3^-]$ in trout. It is possible that the infusions stimulated catecholamine release as a generalised stress response and the catecholamines then stimulated an increase in ventilation and cardiovascular variables as a secondary effect. The proximate reason for catecholamine release following HCO_3^- loading is unknown, although release may

have been stimulated by the reduction in P_{aO2}. In mammals, a reduction in P_{aO2} will stimulate catecholamine release from the adrenal medulla (Nishijima, Breslow, Raff and Traystman, 1989).

The ventilatory responses to NH_4Cl and NH_4HCO_3 occurred in the absence of a catecholamine release or (in the case of NH_4HCO_3) of any change in blood O_2 status, indicating that trout show a direct ventilatory response to ammonia, possibly similar to that described in mammals (Wischer and Kazemi 1974).

It is conceivable that the increase in ventilation and heart rate seen following $NaHCO_3$ and NH_4HCO_3 infusion functioned to aid in returning T_{aCO2} or T_{amm} to normal, by increasing passive diffusion of CO_2 and NH_3 from blood to water at the gills. Iwama, Boutilier, Heming and Randall (1987) showed that changes in gill water flow had virtually no effect on P_{aCO2}, and the factor limiting CO_2 excretion appears to be HCO_3^- dehydration by the red blood cell (Perry, Davie, Daxboeck and Randall, 1982; Randall, 1990). However, Iwama *et al.* (1987) did not determine whether CO_2 diffusion might be limiting when T_{aCO2} was significantly elevated above control values. At the dose used in this study, immediately following $NaHCO_3$ and NH_4HCO_3 infusion, T_{aCO2} would have approximately doubled and T_{amm} increased sevenfold, such that CO_2 and NH_3 excretion may have been transiently limited by gill water and blood flow. It is unknown whether trout would normally experience such large increases in T_{aCO2}, so it is unknown whether observed ventilatory and cardiovascular responses are of any functional significance. There is evidence, however, that plasma T_{amm} increases following feeding in sockeye salmon (*Oncorhynchus nerka*) (Brett and

Zala, 1975).

In *Amia*, unlike trout, there was no ventilatory response to changes in HCO_3^- or T_{amm}, but a ventilatory response to NaCl. The ventilatory response to 2 M NaCl is presumably a result of the measured decrease in C_{aO2}, and occurred in the absence of a significant increase in [NE] and [E]. *Amia* did show an increase in P_{DA} and f_h in response to NH_4HCO_3 infusion, similar to that seen in trout, which is presumably a response to increases in T_{amm}.

In conclusion, water-breathing fish (trout) show a ventilatory response to increases in plasma T_{aCO2} and T_{amm}. There is evidence that the ventilatory and cardiovascular response to T_{aCO2} is mediated by catecholamines, and the ventilatory response to T_{amm} may be similar to that seen in mammals. *Amia* do not demonstrate a ventilatory response to either T_{aCO2} or T_{amm}, but show a cardiovascular response to T_{amm} similar to that of trout.

Chapter 4: The Effects of Branchial Denervation and Pseudobranch Ablation on Cardiovascular and Ventilatory Responses in *Amia*

INTRODUCTION

Amia increases gill ventilation and air-breathing in response to aquatic hypoxia (Johanson, Hansen and Lenfant, 1970; Randall, *et al.* 1981), and as discussed in chapter 2, air-breathing and gill ventilatory responses to internal acid-base disturbances appear to be more closely correlated with C_{aO2} than with pH_a. Thus, *Amia* is similar to water breathing fish, where the primary stimulus modality for ventilatory and cardiovascular reflexes is O_2 (Randall and Jones, 1973; Dejours, 1973; Smith and Jones, 1982; Randall, 1982). The putative sites of O_2 sensitive receptors responsible for hypoxic and hypoxaemic reflexes in *Amia* (and other air-breathing fish) remain controversial. In teleosts, recent evidence suggests that reflex responses to hypoxia are mediated by O_2-sensitive chemoreceptors in the gills and pseudobranch, innervated by cranial nerves VII, IX and X. Recordings of nerve activity sensitive to both internal and external O_2 levels have been obtained from the vagus nerve to the first gill arch of tuna (Milsom and Brill, 1986) and the glossopharyngeal nerve to the first gill arch of trout (Burleson and Milsom, 1990). Section of the IXth and Xth cranial nerves to the first gill arch abolishes hypoxic bradycardia in salmonids (Smith and Jones, 1978; Smith and Davie, 1984) and cod (Fritsche and Nilsson, 1989).

Various studies employing section of gill nerves failed, however, completely to abolish ventilatory responses to hypoxia in water breathing fish (Shelton, 1959; Hughes and Shelton, 1962; Saunders and Sutterlin, 1971; Butler, Taylor and Short, 1977) and in one air-breathing fish, the gar, *Lepisosteus osseus* (Smatresk, 1987). This may be because, as suggested by Smatresk (1990), in each

of these studies, the pseudobranch and/or nerves serving one particular gill arch were left intact. Complete branchial denervation abolishes ventilatory responses to hypoxia and NaCN in anaesthetized channel catfish. In the catfish, O_2 sensitive receptors appear to be located diffusely throughout the gills, and even one branchial nerve left intact unilaterally is sufficient to stimulate a ventilatory response (Burleson, 1986).

Recent evidence suggests that circulating catecholamines (NE and E) might be responsible for mediating ventilatory responses to hypoxia and hypoxaemia in dogfish and trout, possibly via a direct effect on central respiratory motoneurons (Randall and Taylor, 1988; Taylor and Randall, 1989; Aota *et al.*, 1990). There is evidence to suggest that catecholamines might also play a role in gill ventilatory responses to hypoxaemia in *Amia* (see chapter 2).

This study employs total branchial denervation (branchial branches of IX and X) and pseudobranch ablation to assess the role of sensory input from the gills and pseudobranch in cardiovascular, air-breathing and ventilatory responses to hypoxia, NaCN, catecholamines and acidaemia in *Amia.* A conscious animal preparation was used to avoid the effects of anaesthesia, which may compromise cardiovascular and ventilatory reflex responses.

MATERIALS AND METHODS

Amia weighing 400 to 1000 g were maintained and temperature acclimated as described in general material and methods.

Animal Preparation:

Animals were anaesthetized in a tricainemethanesulphonate (MS-222) solution at a concentration of 1:10,000, buffered with sodium bicarbonate. Following transfer to an operating table, fish were artificially ventilated with a buffered MS-222 solution at 1:20,000, bubbled with O_2 to help maintain P_{aO2}. A dorsal aortic (DA) cannula (PE 50, Intramedic) was implanted using the technique of Soivio, Westman and Nyholm (1972). A buccal cannula was fitted using flared PE50 (Intramedic) passed through a small hole drilled in the roof of the mouth, and secured with a cuff and sutures. An opercular cannula was fitted, using flared PE190 (Intramedic) passed through a small hole drilled in the operculum, secured with a cuff and sutures. The operculum was reflected forward, and a small (1 to 1.5 cm) incision made in the epithelium dorsal and posterior to the fourth gill arch. This allowed access to the branchial branches of cranial nerve X, serving all four gill arches. These were gently dissected free of connective tissue and sectioned with iris scissors, care being taken not to damage gill vasculature, musculature or cardiac and visceral branches of X. The incision was closed with absorbable Vicryl coated polyglactin sutures (Ethicon 2.0). Cranial nerve IX, serving the first gill arch, was exposed by making a small (0.3 to 0.5 cm)

incision, at the base of the gill filaments, where the arch meets the roof of the opercular cavity, and dissecting the nerve free of connective tissue. The nerve was then sectioned with iris scissors. The pseudobranch in bowfin is glandular and situated in the roof of the mouth just anterior to the first gill arch. It was exposed via a small (0.5 cm) incision and removed by cautery. The same procedure was followed on the other side, so that denervation and pseudobranch ablation were bilateral. Surgery took between 30 and 40 minutes and nerve section was confirmed post-mortem, by dissection. Sham-operated animals (shams) had their gill nerves exposed as described, but not sectioned. The fish were transferred to individual, darkened plexiglass chambers (water volume 9 l), ventilated with water until ventilatory movements resumed and then allowed to recover for 48 hr with a continuous flow of aerated water (approx. 900 mls/kg/min) through the chamber. The anterior end of the chamber had an air-space (volume 1.6 l), to allow air-breathing.

Protocol:

Following recovery, $\dot{V}_{O2}$ was measured in both denervated and sham-operated fish. Samples of inflow and outflow water were analysed for P_{O2}, as described in General materials and methods, and water $\dot{V}_{O2}$ ($\dot{V}_{O2}(w)$) calculated, knowing the fish's weight and the water flow rate. To measure air-breathing $\dot{V}_{O2}$ ($\dot{V}_{O2}(a)$), the decline in P_{O2} in the anterior air-breathing chamber when sealed for a two to three hour period was recorded, and $\dot{V}_{O2}(a)$ calculated knowing the volume of the air-space and the fish's weight.

Subsequently, the water-filled opercular cannula was attached to a pressure transducer (Statham P23BB), allowing measurement of ventilation rate (f_g, beats/min) and opercular pressure amplitude (P_{op}, cm H_2O). The saline-filled DA cannula was attached to a pressure transducer (Statham P23Db), for measurement of heart rate (f_h, beats/min) and DA blood pressure (P_{DA}, cm H_2O). The output from both transducers was displayed on a pen recorder (Gould 8188-2202-XX). Air breathing frequency (f_{ab}, breaths.hr) was visible as large pressure excursions on the opercular trace, associated with changes in f_h and P_{DA}. Air breaths were all verified visually through a small hole in a screen separating experimenter and fish. When required, 700 µl blood samples were withdrawn anaerobically from the DA cannula, via a three-way stopcock at the pressure transducer. Blood samples were replaced with an equal volume of heparinised saline. 500 µl of blood was immediately centrifuged, the plasma decanted and frozen in liquid nitrogen for subsequent analysis of plasma catecholamine levels, as described in general materials and methods. Blood pH (pH_a), P_{O2} (P_{aO2}) and O_2 content (C_{aO2}) were all measured as described in general materials and methods.

Ventilatory and cardiovascular responses to a variety of different stimuli were measured in shams and denervates. Experiments on any one fish were performed over a two day period, with overnight recovery between days. No experiments were initiated until ventilatory and cardiovascular parameters had remained stable for 30 minutes. Animals were exposed to the following treatments, assigned randomly:

Treatment 1: Using a three way valve at the inflow of the plexiglass chamber, animals were exposed to hypoxic water (P_{wO2} = 47.3 ± 1.2 mmHg) obtained by bubbling N_2 counter-current to water flowing through a stripping column. Fish were exposed for 15 minutes, and then normoxic flow was resumed. Blood samples were collected immediately before, and after 5 minutes exposure. Animals were then allowed a minimum of two hours recovery.

Treatment 2: 300 µl heparinised saline control and 300µg NaCN (dissolved in 300 µl saline) delivered as a bolus injection via the DA cannula.

Treatment 3: 1 ml saline control and 1mg NaCN (in 1 ml saline) given as a bolus injection into the buccal cavity via the buccal cannula.

Treatment 4: 0.5 ml/kg saline control, 0.5 ml/kg 10^{-5} M norepinephrine hydrochloride (Sigma), in saline, and 0.5 ml/kg 10^{-5} M epinephrine bitartrate (Sigma), in saline, infused via the DA cannula.

In all treatments, control and experimental injections or infusions were performed in random order. Animals were given a minimum of 30 min to recover between injections in treatments 2, 3 and 4.

Upon completion of this protocol, denervates were given a 2.5 ml/kg control, saline infusion followed by 1 hr recovery and a 2.5 ml/kg 0.1 M hydrochloric acid (HCl) infusion, in a saline vehicle. Infusions were performed as described in

general materials and methods. Blood samples were withdrawn immediately before and 5 min after each infusion.

Data analysis and statistics:

Ventilatory and cardiovascular responses were analysed for a control period and at designated intervals following each experimental intervention. Ventilation and heart rate were counted for 30 seconds in each minute, and P_{op} and P_{DA} averaged from six measurements within that period; for two minutes control and at 1, 2.5, 5, 10 and 15 min following intervention. For acid infusion, measurements were also taken at 30 min. Mean air-breathing frequency was calculated for the 15 min period following intervention. When a blood sample was withdrawn, cardiovascular measurements were made at 4 min post-infusion. Cardiovascular and gill ventilatory responses were normalised as per cent change and, following arcsine transformation, compared at each time interval with an ANOVA, and compared "*a posteriori*" with the averaged control value. In some cases, gill ventilatory variables were compared between control and peak response using a paired t-test on normalised, transformed values. Normalised responses were used for graphical display. Control and experimental blood measurements within a treatment were compared with a paired t-test. Air-breathing frequency was compared betwen control and experimental injections or infusions using paired t-tests, and between shams and denervates using unpaired t-tests.

RESULTS.

At 20°C, in aquatic normoxia, sham-treated *Amia* obtained 0.1 per cent of their total O_2 uptake by air-breathing. Denervated animals had a significantly reduced O_2 consumption rate (30 % lower than shams), and there was no O_2 uptake by air-breathing (table 10). Mean control values for P_{DA}, f_h, P_{op} and f_g are in table 10. There was no significant difference between denervates and shams for these variables. During normoxia, f_{ab} was very low in the shams, usually zero, and only one denervate airbreathed, on two occasions (table 11). In normoxia, denervates showed no differences in pH_a and C_{aO2} as compared with shams, but P_{aO2} was significantly lower (table 12).

Effects of aquatic hypoxia:

In shams, aquatic hypoxia elicited significant cardiovascular and ventilatory responses (fig 22). A gradually developing bradycardia was evident, with f_h significantly reduced following 15 min exposure. Gill ventilation increased, with significant changes in P_{op} and f_g at five minutes that were sustained until the end of hypoxic exposure. Air-breathing frequency increased significantly (table 11), with most of the airbreaths occurring in the first five minutes of hypoxic exposure. Arterial blood pH and C_{aO2} were maintained during hypoxia, with no change from pre-exposure values at five minutes post-exposure, but P_{aO2} decreased significantly (table 12).

In denervates, the response to aquatic hypoxia was different (fig 22).

Table 10: Normoxic $\dot{V}_{O2}$, pH_a, f_h, P_{op} and f_g.

	Shams	Denervates
$\dot{V}_{O2}(t)$	52.8±4.9	36.7±3.3 *
$\dot{V}_{O2}(a)$	0.06±0.02	0*
$\dot{V}_{O2}(w)$	52.7±4.9	36.6±3.3 *
P_{DA}	28.8±0.8	29.8±0.5
f_h	30.0±0.8	25.6±0.3
P_{op}	0.66±0.05	1.56±0.08
f_g	12.2±0.6	10.7±0.4

Values are means ± S.E., * = significantly different (P=0.05)
N = 6 for sham $\dot{V}_{O2}$; N = 7 for denervate $\dot{V}_{O2}$
Cardiovascular and ventilatory variables = mean of 48 measurements on 6 shams and 64 measurements on 7 denervates.
Units: $mgO_2/kg/hr$ for $\dot{V}_{O2}$; cm H_2O for P_{DA} and P_{op}, beats/min for f_h and f_g.

Table 11: Airbreathing frequency (breaths/hr).

	Shams	Denervates	Partial Denervates
Hypoxia	5.1±1.5	0 +	-
External saline	0	0.7±0.7	0
External NaCN	1.33±0.9	0.7±0.7	4.0±1.3 *
Internal saline	0.7±0.7	0	-
Internal NaCN	0	0	-
NE infusion	0	0	-
E infusion	0	0	-
Saline infusion	-	0	-
Acid infusion	-	0	-

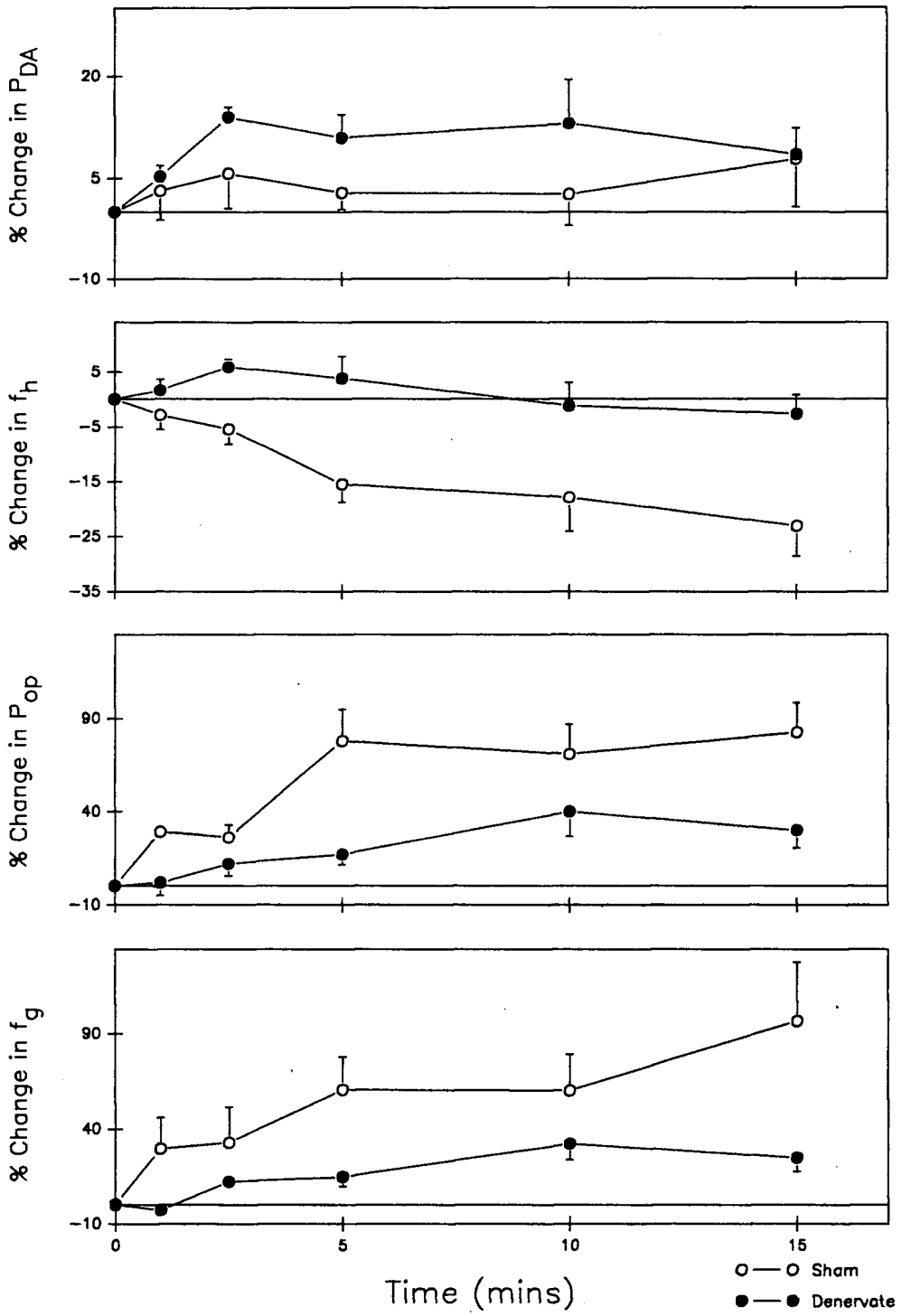
Values = mean ± S.E. + = significantly different from sham hypoxia (P=0.05); * = significantly different (P=0.05) from saline injection.
N = 6 for shams, 7 for denervates, 7 for partial denervates.

Table 12: Arterial blood gases:

	pH_a	P_{aO2}	C_{aO2}
Sham Normoxia	7.72 ±0.02	46.7 ±12.0	4.45 ±0.76
Sham Hypoxia	7.73 ±0.03	27.8* ±4.8	3.07 ±0.35
Denervate Normoxia	7.75 ±0.02	16.5+ ±1.6	3.80 ±0.68
Denervate Hypoxia	7.72 ±0.02	11.3* ±1.6	1.74* ±0.34
Denervate Pre-Saline Infusion	7.69 ±0.03	16.5 ±1.2	3.24 ±0.54
Denervate Post-Saline Infusion	7.68 ±0.03	17.2 ±1.3	3.48 ±0.60
Denervate Pre-Acid Infusion	7.68 ±0.02	17.0 ±1.8	3.25 ±0.64
Denervate Post-Acid Infusion	7.38* ±0.06	35.3* ±7.5	2.27* ±0.63

Values = mean ± S.E. * = significantly different from normoxic or pre-infusion; + = significantly different from sham normoxic.
N = 6 for both shams and denervates
Units: P_{aO2} = mmHg; C_{aO2} = vol.%.

Figure 22: The effects of aquatic hypoxia exposure on P_{DA}, f_h, P_{op} and f_g in sham-operated and denervated *Amia*. n = 6


% Change in P_{DA}
20
5
-10
% Change in f_h
5
-5
-15
-25
-35
% Change in P_{op}
90
40
-10
% Change in f_g
90
40
-10
0
5
10
15
Time (mins)
Sham
Denervate

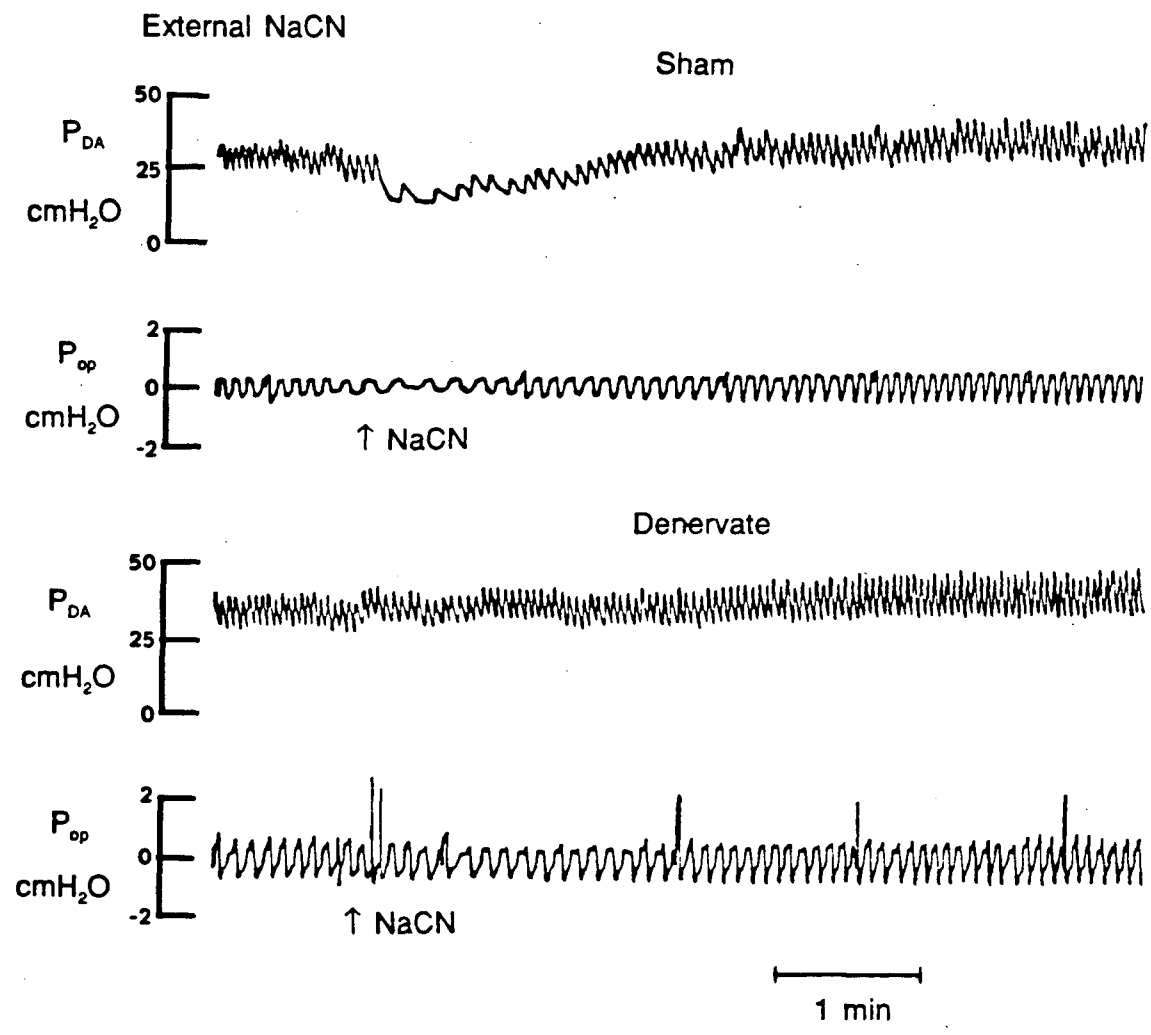
The bradycardia response was abolished. Gill ventilation responses were attenuated, with no increase until ten minutes of exposure. There was then a sustained increase in f_g and a transient increase in P_{op} that was no longer evident at 15 minutes. There was no change in f_{ab} during hypoxia, air-breathing responses were abolished (table 11). At five minutes exposure, pH_a was not changed from pre-exposure values, but C_{aO2} and P_{aO2} were significantly reduced. In animals with cranial nerve IX to the pseudobranch sectioned, air-breathing responses to hypoxia still occurred, and pseudobranch ablation was required to abolish the air-breathing response.

Effects of NaCN:

Representative traces of the ventilatory and cardiovascular responses to NaCN in shams and denervates are presented in figure 23. In shams, bolus injection of NaCN into the buccal cavity (fig 24) elicited a transient bradycardia and a transient increase in P_{op} at that time. P_{DA} and f_g did not change significantly from control. In two out of six fish, external NaCN immediately stimulated an airbreath (table 11). It is of interest to note that partial denervates (i.e. animals with a branchial branch of IX or X and/or the pseudobranch intact) showed an immediate and significant increase in f_{ab} following external NaCN (table 11). External saline control injections had no effect on any variable (fig 24).

In denervates (fig 25), all cardiovascular and ventilatory responses were abolished, with no change in any variable over time. One animal took an

Figure 23: Representative traces of cardiovascular and gill ventilatory responses to internal and external NaCN in shams and denervates.


External NaCN
Sham
50
25
0
P_{DA}
cmH_2O
2
0
-2
P_{op}
cmH_2O
↑ NaCN
Denervate
50
25
0
P_{DA}
cmH_2O
2
0
-2
P_{op}
cmH_2O
↑ NaCN
1 min

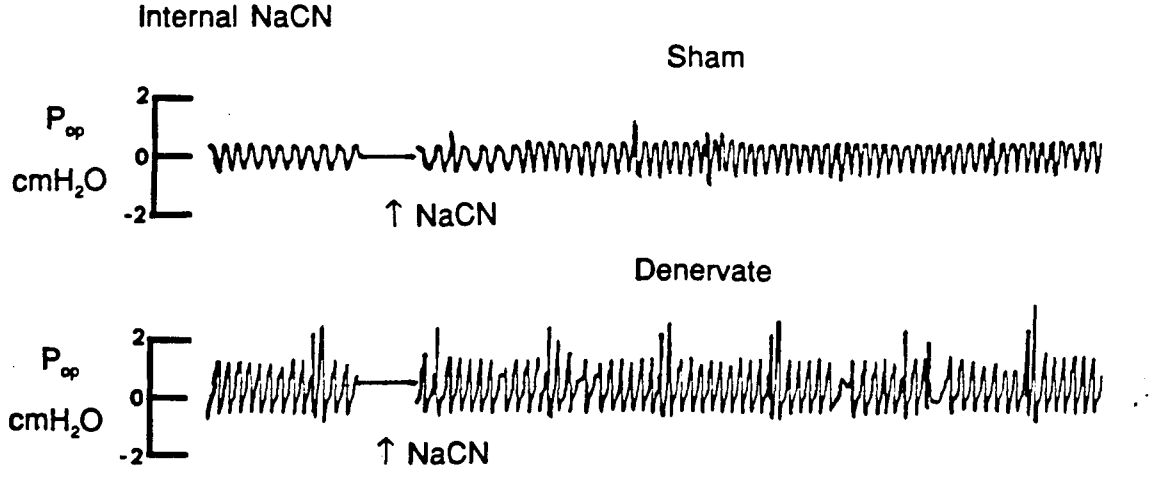

Internal NaCN
Sham
2
0
-2
P_{op}
cmH_2O
↑ NaCN
Denervate
2
0
-2
P_{op}
cmH_2O
↑ NaCN

Figure 24: The effects of externally applied NaCN on P_{DA}, f_h, P_{op} and f_g in sham operated animals. n = 6.

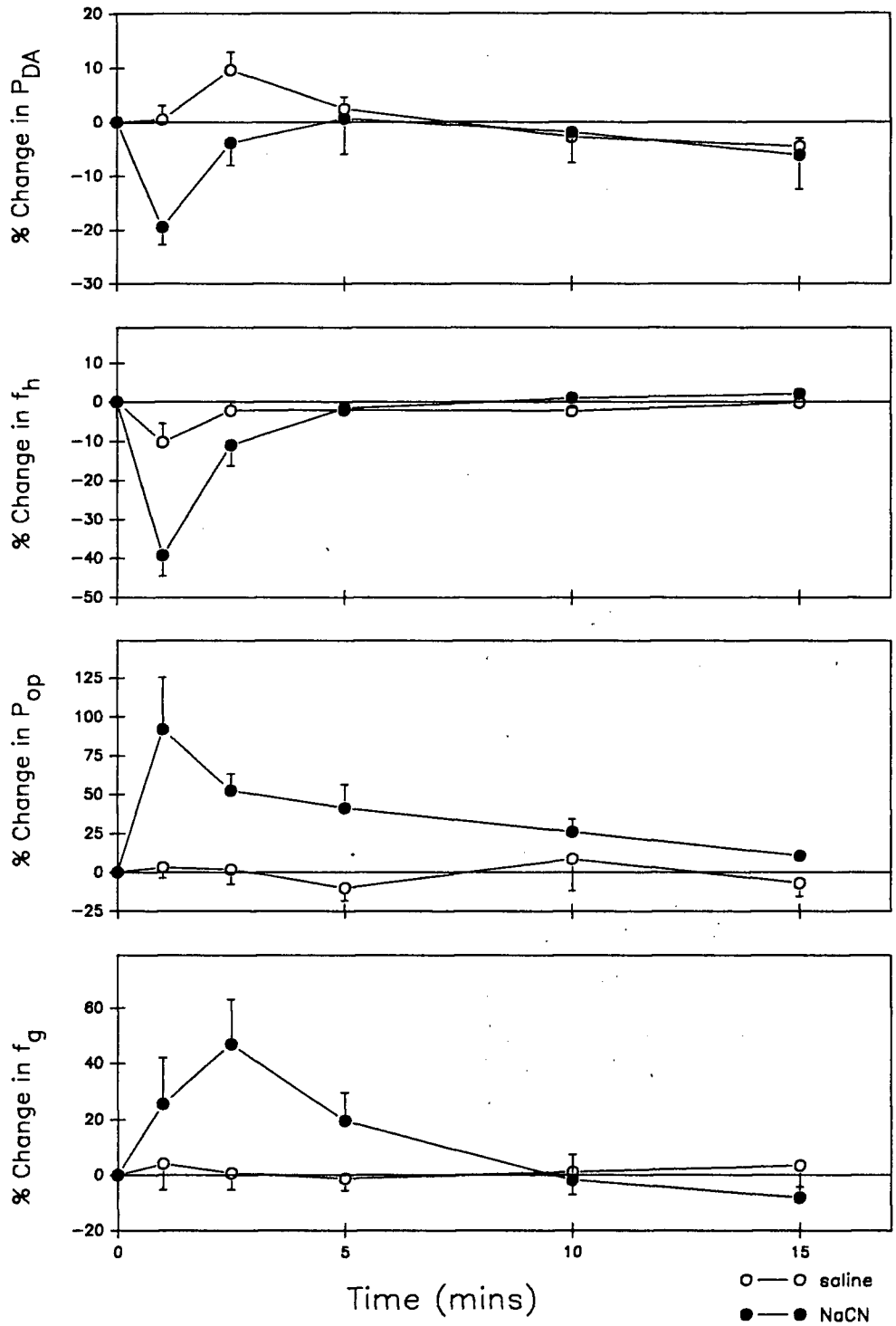
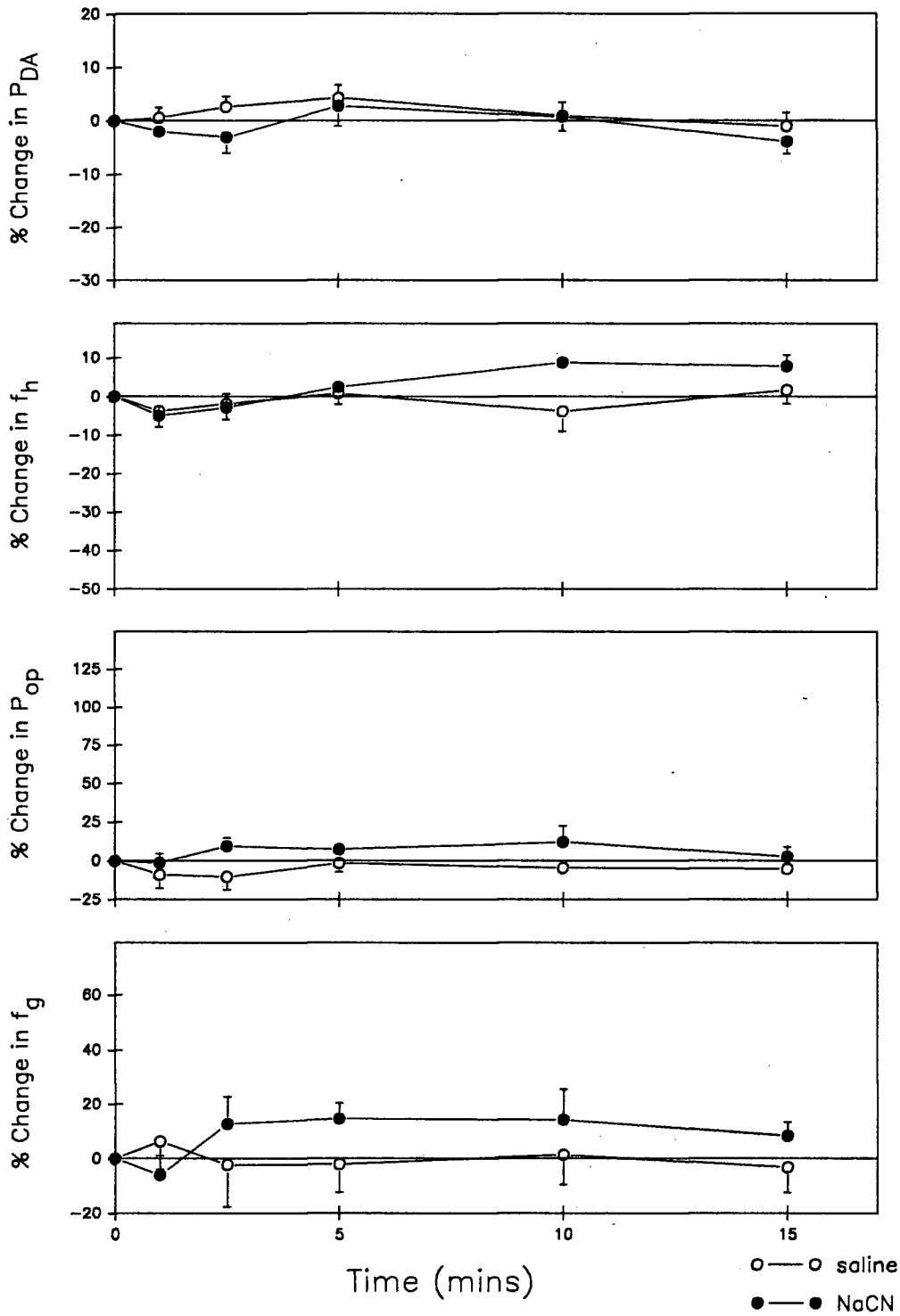

% Change in P_{DA}
20
10
0
−10
−20
−30
% Change in f_h
10
0
−10
−20
−30
−40
−50
% Change in P_{op}
125
100
75
50
25
0
−25
% Change in f_g
60
40
20
0
−20
0
5
10
15
Time (mins)
o—o saline
●—● NaCN

Figure 25: The effects of externally applied NaCN on P_{DA}, f_h, P_{op} and f_g in denervated animals. n = 7


% Change in P_{DA}
% Change in f_h
% Change in P_{op}
% Change in f_g
Time (mins)
saline
NaCN

airbreath in response to both external saline and NaCN, in the latter case, at 13 minutes post-injection (table 11). Saline bolus into the buccal cavity had no effect on any variable (fig 25). Figures 24 and 25 are drawn to the same scale, to allow comparison of responses by shams and denervates.

Bolus injection of NaCN into the DA of shams had no significant effect on P_{DA} and f_h, but significantly stimulated P_{op} and f_g (fig 26). P_{op} increased transiently at 2.5 minutes post-injection, and f_g was elevated at 1 and 2.5 minutes. Figure 26 shows some evidence of cardiovascular responses, although they were not significant. Internal NaCN had no effect on f_{ab} (table 11), and a saline bolus into the DA had no effect on any variable (fig 26).

In denervates (fig 27) the ventilatory responses to internal NaCN were abolished, with no significant changes in P_{op} or f_g. Cardiovascular variables showed a similar trend to those of shams, but the changes over time were not statistically significant. There was no air-breathing response to internal NaCN in the denervates. Saline bolus had no effect on any variable (fig 27).

Effects of Catecholamines:

In shams, NE infusion (fig 28) significantly increased P_{DA}, f_h and P_{op}. There was no significant effect on f_g, and no stimulation of f_{ab}. Blood pressure showed a transient increase at 2.5 minutes, and f_h was elevated at 2.5, 5 and 10 minutes following infusion. Opercular pressure amplitude was significantly elevated at 2.5 minutes post-infusion, and remained elevated throughout the remainder of the

Figure 26: The effects of NaCN given in the DA on P_{DA}, f_h, P_{op} and f_g in sham operated animals. n = 6

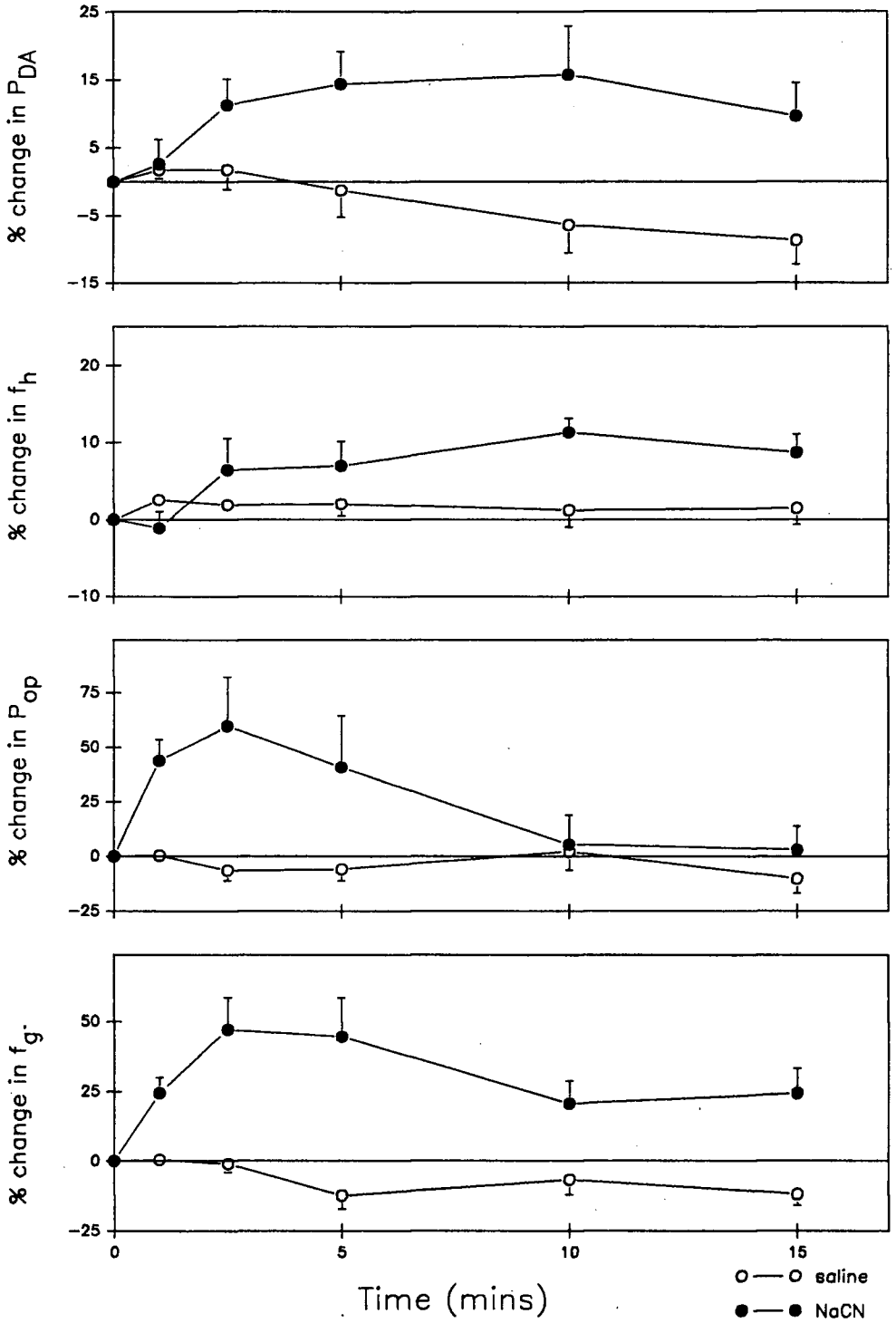
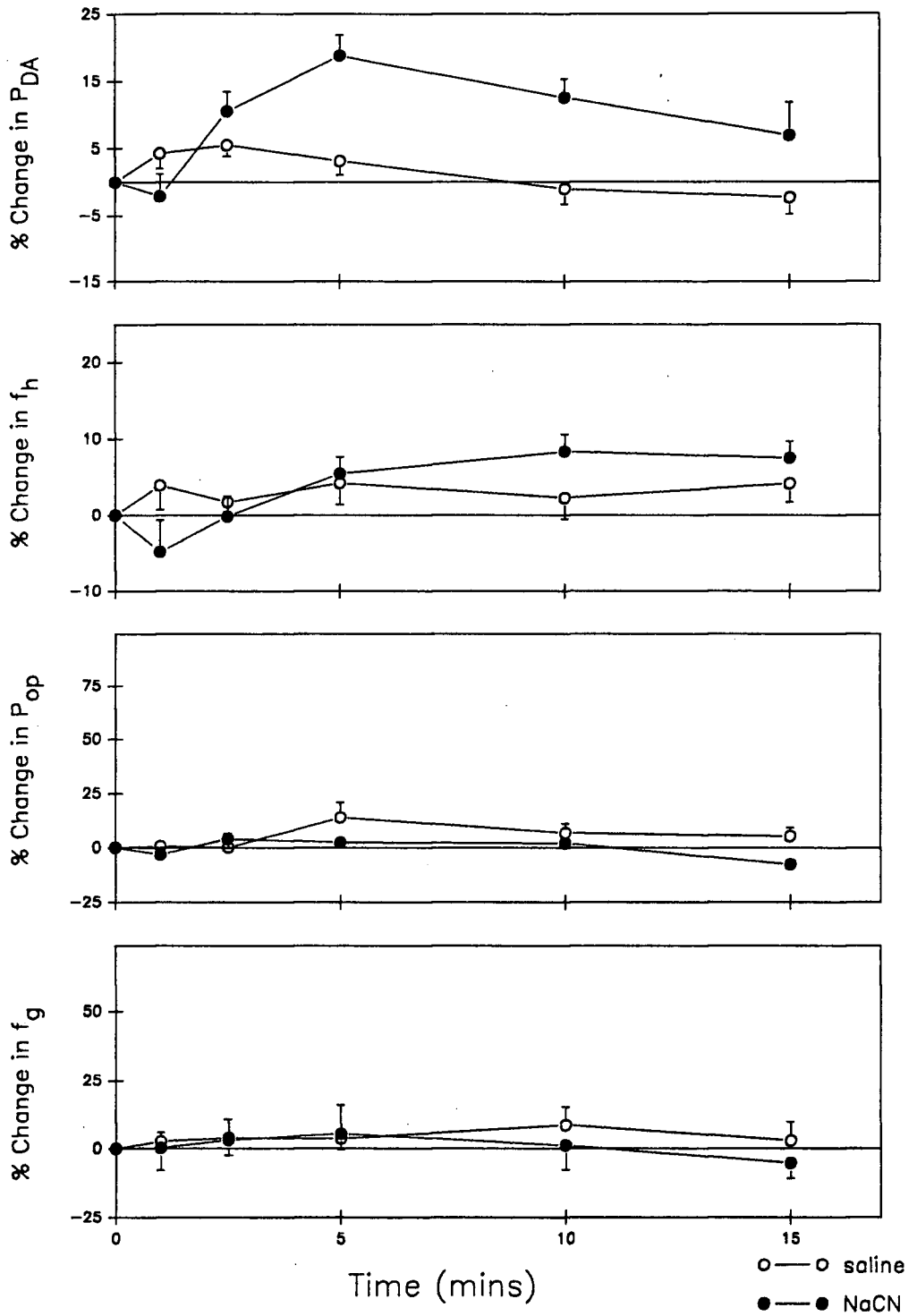

% change in P_{DA}
25
15
5
−5
−15
% change in f_h
20
10
0
−10
% change in P_{op}
75
50
25
0
−25
% change in f_g
50
25
0
−25
0
5
10
15
Time (mins)
saline
NaCN

Figure 27: The effects of NaCN given in the DA on P_{DA}, f_h, P_{op} and f_g in denervated animals. n = 7


% Change in P_{DA}
25
15
5
−5
−15
% Change in f_h
20
10
0
−10
% Change in P_{op}
75
50
25
0
−25
% Change in f_g
50
25
0
−25
0
5
10
15
Time (mins)
o—o saline
●—● NaCN

measurement period. E infusion had similar effects to NE on cardiovascular variables, significantly increasing P_{DA} from 1 minute post-infusion until the end of the measurement period, but only transiently stimulating f_h, at 2.5 minutes. There was no statistically significant stimulation of P_{op} or f_g by E, as measured by ANOVA. However, a paired t-test showed a significant increase in mean P_{op} and f_g at 2.5 minutes post-infusion. There was no stimulation of f_{ab} (table 11). Control saline infusion had no effect on P_{DA}, f_h, P_{op}, f_g or f_{ab} (fig 28).

In denervated fish (fig 29), NE had effects on P_{DA} and f_h very similar to those seen in shams, but there was no statistically significant effect on P_{op} or f_g, when measured by ANOVA. E also stimulated P_{DA} and f_h in a manner similar to the sham response. There was no significant effect on P_{op} following E infusion, but f_g increased at 2.5, 5 and 10 minutes post-infusion, when measured by ANOVA. Whilst NE and E showed no statistically significant P_{op} response as measured by ANOVA, a comparison of denervate mean control P_{op} and f_g with mean P_{op} and f_g at 2.5 minutes post-infusion, by paired t-test, shows a significant increase in both ventilatory parameters at 2.5 minutes, which is evidence of a physiologicaly significant response. Saline control infusion had no effect on any measured variable (fig 29).

Effects of acid infusion in denervates:

HCl infusion (fig 30) had a marked effect on P_{DA}, which rose immediately and was still significantly elevated at 30 minutes post-infusion, but f_h did not change significantly. Gill ventilatory variables showed a large increase; P_{op} was

Figure 28: The effects of NE and E infusion on P_{DA}, f_h, P_{op} and f_g in sham operated animals. n = 6 C = control, the shaded bar represents the infusion period.

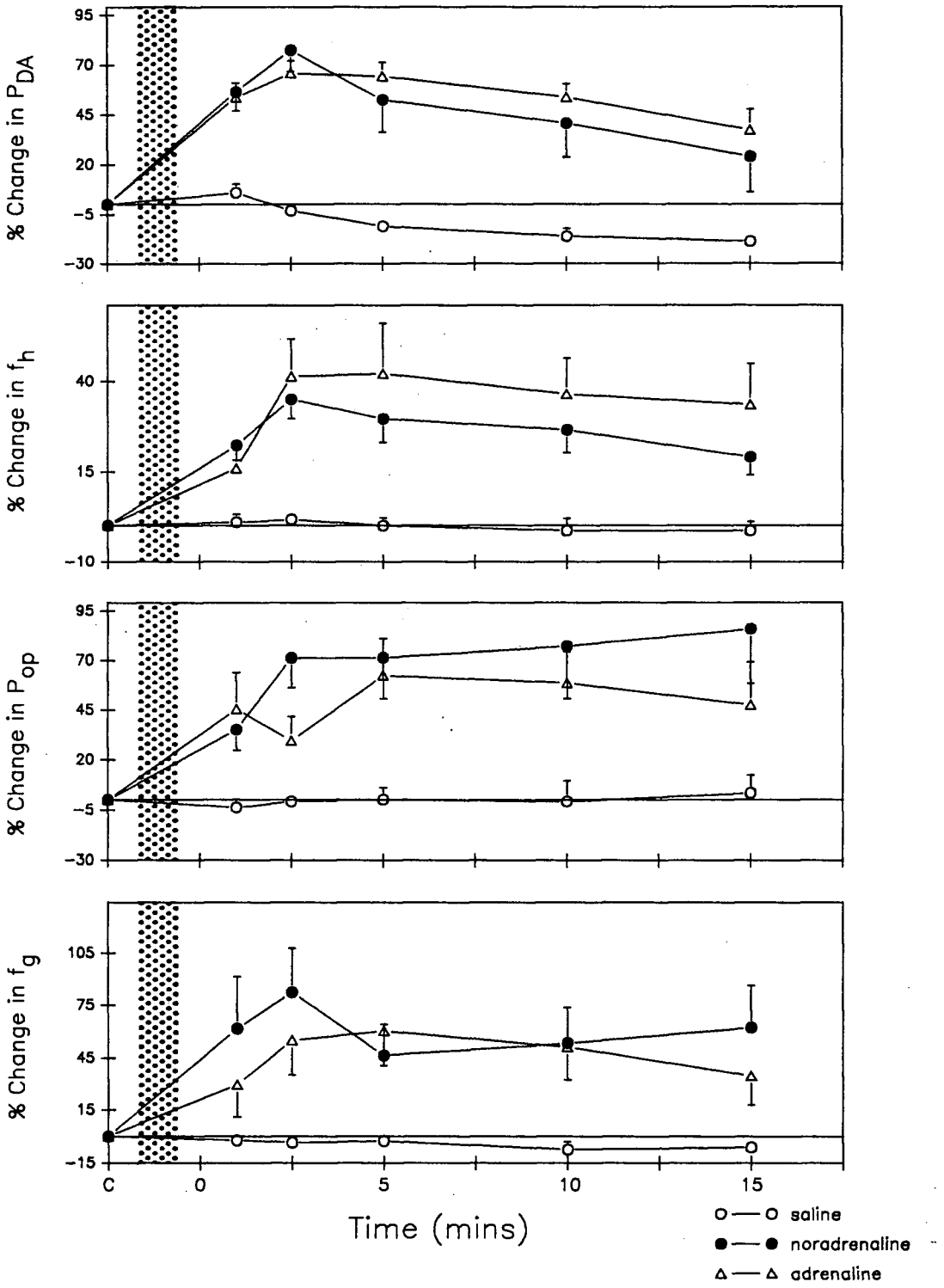
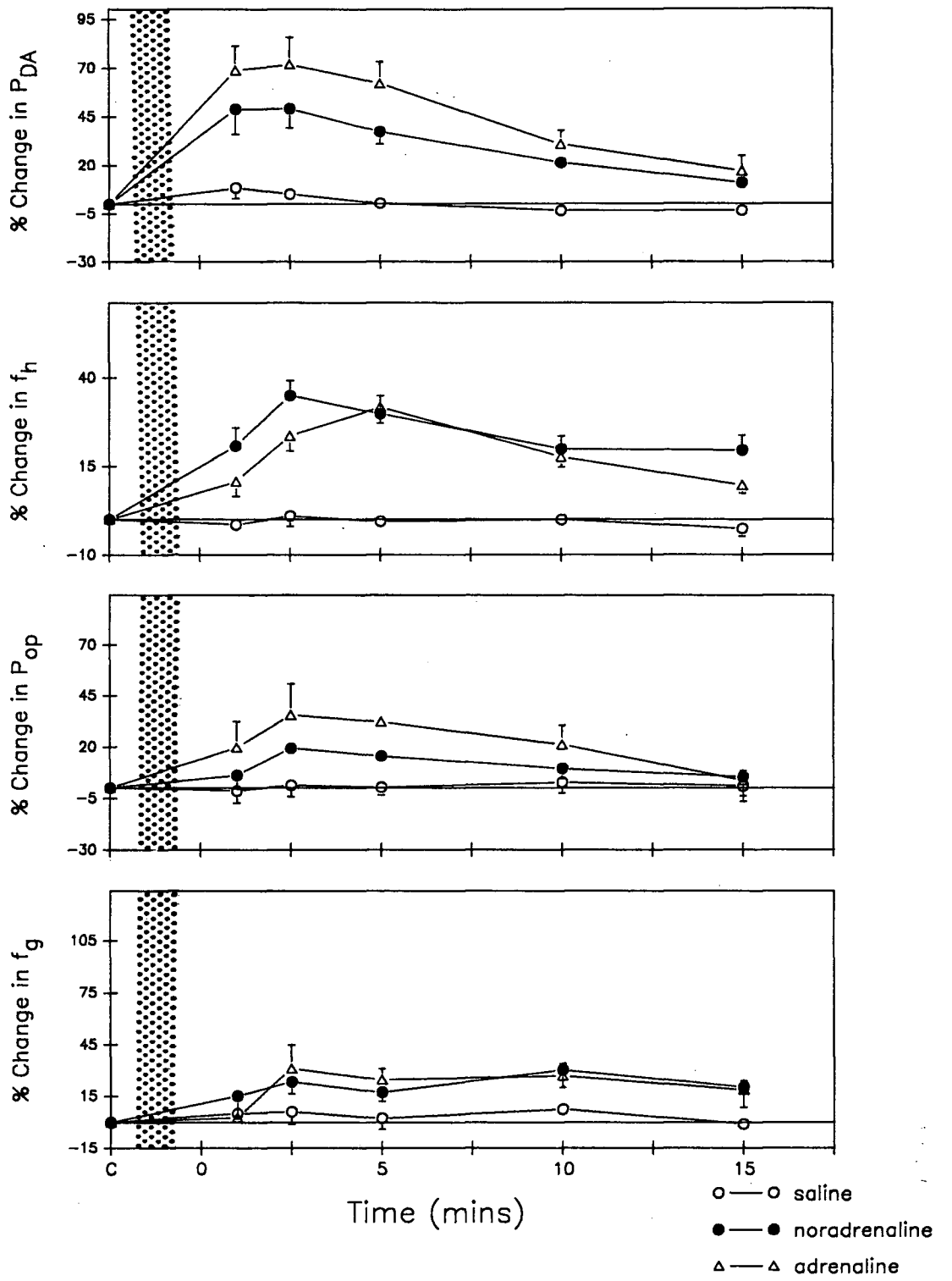

% Change in P_{DA}
95
70
45
20
-5
-30
% Change in f_h
40
15
-10
% Change in P_{op}
95
70
45
20
-5
-30
% Change in f_g
105
75
45
15
-15
C
0
5
10
15
Time (mins)
o—o saline
●—● noradrenaline
△—△ adrenaline

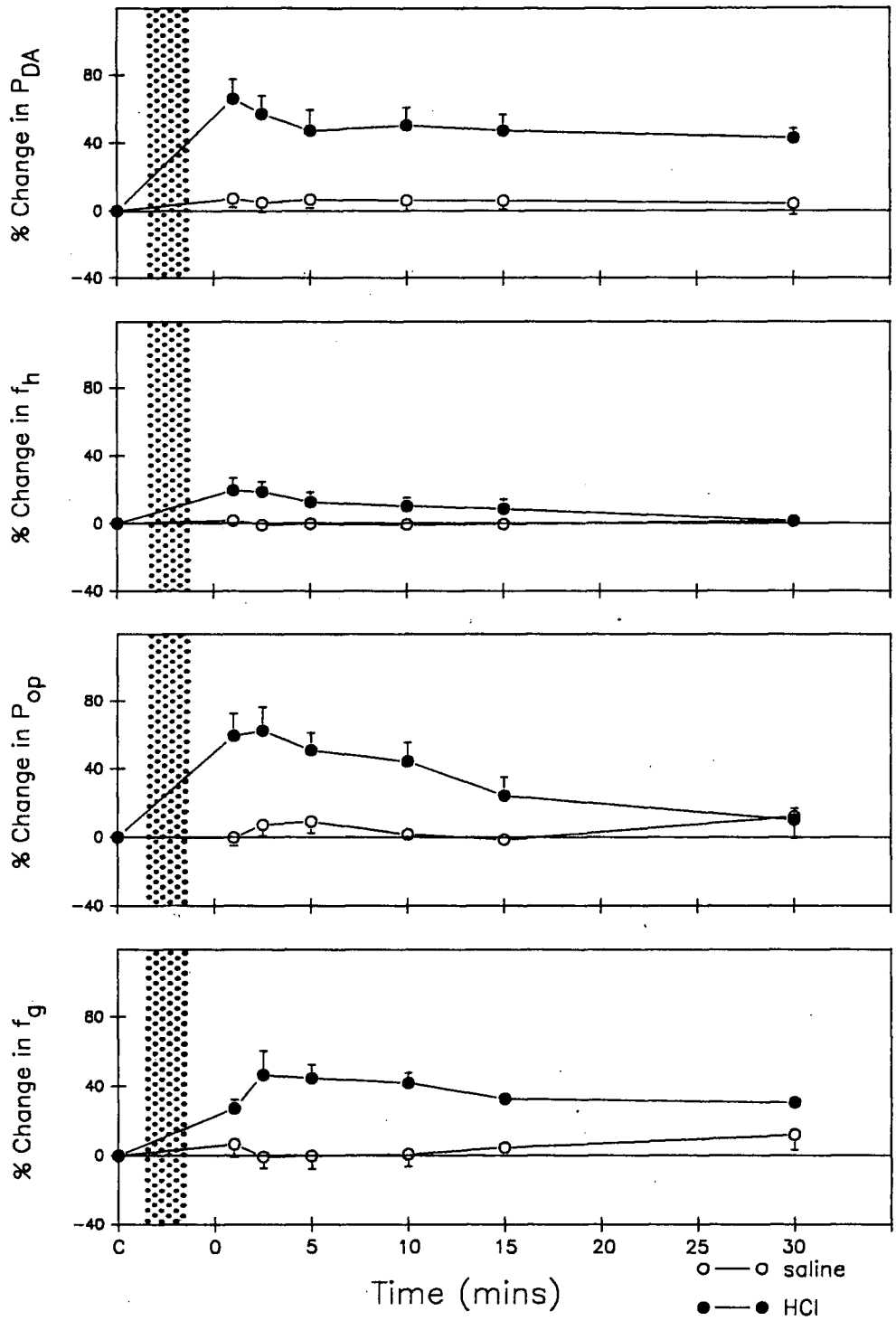
Figure 29: The effects of NE and E infusion on P_{DA}, f_h, P_{op} and f_g in denervated animals. n = 6
C = control, the shaded bar represents the infusion period.


% Change in P_{DA}
95
70
45
20
-5
-30
% Change in f_h
40
15
-10
% Change in P_{op}
70
45
20
-5
-30
% Change in f_g
105
75
45
15
-15
C
0
5
10
15
Time (mins)
saline
noradrenaline
adrenaline

significantly elevated between 1 and 10 minutes post-infusion, and f_g increased at 2.5 minutes and remained so until 30 minutes post-infusion. There was no stimulation of air-breathing (table 11). At five minutes post-infusion, pH_a and C_{aO2} were significantly depressed, and P_{aO2} significantly elevated, as compared with pre-infusion values (table 12). Control saline infusion (fig 30) had no effect on cardiovascular or ventilatory variables, and pH_a, C_{aO2} and P_{aO2} (table 12) did not change as compared with pre-infusion values.

Figure 30: The effects of acid and saline infusion on P_{DA}, f_h, P_{op} and f_g in denervated animals. n = 6

C = control, the shaded bar represents the infusion period.


% Change in P_{DA}
% Change in f_h
% Change in P_{op}
% Change in f_g
80
40
0
-40
C
0
5
10
15
20
25
30
Time (mins)
saline
HCl

DISCUSSION

The present study indicates that, in *Amia*, section of all branchial branches of cranial nerves IX and X, and extirpation of the pseudobranch, has marked effects on ventilatory and cardiovascular reflex responses to aquatic hypoxia, NaCN, catecholamine and acid infusion.

Resting cardiovascular, ventilatory and blood-gas variables:

Following gill nerve section, a reduction or abolition of afferent information about water and blood O_2 levels, and abolition of all efferent vascular and postural motor control of the gill arches, probably combined to result in the measured reduction in $\dot{V}_{O2}$ seen in the denervates as compared with sham-operated fish (shams). Denervation did not change any resting cardiovascular or gill ventilatory variables significantly. Sham-treated *Amia* did not airbreathe as much during aquatic normoxia as noted in chapter 1, or by Johansen *et al.* (1970). Denervated animals did not airbreathe at all, except for one individual that did so on two occasions within one hour. It is unlikely that denervation would abolish all air-breathing behaviours, as afferent activity from stretch receptors in the swimbladder (Milsom and Jones, 1985) may stimulate air-breathing under appropriate circumstances (branches of cranial nerve X to the swimbladder were intact), as is seen in gar (Smatresk and Azizi, 1989). The reduction in P_{aO2} in normoxic denervates, as compared with shams, indicates a possible ventilation:perfusion mismatch, for the reasons stated above, but the fact that denervates had similar C_{aO2} values to shams indicates that they were not hypoxaemic, and a normal pH_a argues against a significant degree of lactic acid accumulation or anaerobic

metabolism.

Effects of denervation on cardiovascular responses:

Abolition of the f_h response to hypoxia following gill denervation indicates that reflex bradycardia in *Amia* is controlled by receptors in the gills, as is the case in teleosts, i.e. salmonids (Smith and Jones, 1978, Smith and Davie, 1984), cod (Fritsche and Nilsson, 1989) and channel catfish (Burleson and Smatresk, 1990), where bradycardia is abolished by section of cranial nerves IX and X to the gills. In elasmobranchs cranial nerves V and VII (innervating the bucco-pharynx) also require sectioning (Butler, Taylor and Short, 1977).

In shams, stimulation of a bradycardia by external NaCN, but not by internal NaCN supports previous studies that indicate that O_2 receptors mediating bradycardia are externally oriented (Saunders and Sutterlin, 1971; Smatresk *et al.*, 1986; Burleson and Smatresk, 1990). The bradycardia was more immediate than that seen during hypoxia, because NaCN represents a transient, supra-maximal stimulus. These responses are similar to the responses of channel catfish to NaCN (Burleson and Smatresk, 1990), but are in contrast to the responses of gar, where heart rate is either not changed by external or internal NaCN (Smatresk, 1986), or internal NaCN produces a bradycardia (Smatresk, Burleson and Azizi, 1986), although dosages were much higher in this study. Abolition of all responses to NaCN following denervation confirms that the externally oriented O_2 receptors are in the gills, innervated by cranial nerves IX and X.

In shams and denervates, effects of catecholamines on both P_{DA} and f_h are

probably a result of the direct effects of catecholamines on the myocardium and peripheral vasculature in fish (Wood and Shelton, 1980; Farrell, 1983; Farrell, MacLeod and Chancey, 1986).

Large increases in P_{DA} and f_h following acid infusion in denervated fish indicates that these responses are not mediated by O_2 chemoreceptors in the gills. The response is unlikely to be the result of catechoalmine release, because in intact *Amia* increases in P_{DA} occur in response to acid infusion during hyperoxia (see chapter 2), when there is no catecholamine release, indicating the possiblity of a direct pH effect. It is possible that the increase in P_{DA} is a result of thromboxane or prostaglandin mediated effects, as acid infusion stimulates their release from erythrocytes in the cat, leading to increases in blood pressure (Shams, Peskar and Scheid, 1988)

Effects of denervation on ventilatory responses:

Increases in P_{op}, f_g and f_{ab} following hypoxic exposure in shams are similar to the response of most air-breathing fish (Smatresk, 1988). The shams did not show the hypoxic depression of gill ventilation noted by Johansen *et al.*, (1970), in *Amia*, and by Smatresk and Cameron (1982), in gar. The lack of any air-breathing by denervates during hypoxic exposure is evidence that O_2 receptors stimulating this behaviour are located in the gills or pseudobranch. This is similar to the lungfish, *Protopterus aethiopicus* (Lahiri, Szidon and Fishman, 1970) and to gar (Smatresk, 1987), where partial gill denervation attenuates the air-breathing response to hypoxia. The fact that the air-breathing responses were abolished by complete branchial denervation and pseudobranch ablation, but not by branchial

denervation and section of cranial nerve IX to the pseudobranch, indicates that cranial nerve VII to the pseudobranch must carry information adequate to stimulate air-breathing. The attenuated gill ventilatory response to hypoxia seen in this study, following complete denervation, may indicate the presence of an extrabranchial receptor (Bamford, 1974; Jones and Milsom, 1982). It is also possible, however, that the response is mediated centrally by circulating catecholamines, as suggested by Aota *et al.* (1990), for trout. Catecholamines stimulate gill ventilation in *Amia* but have no effect on air-breathing (see chapter 2), which would explain the absence of an air-breathing response in hypoxic denervates.

In shams, NaCN given into the buccal cavity increased P_{op}, but had no significant effect on f_g or f_{ab}. This response is unlike that of gar (Smatresk, 1986), where external NaCN stimulated air-breathing but had no significant effect on gill ventilation. The lack of a significant air-breathing response in *Amia* might be considered evidence that air-breathing is not controlled by O_2 chemo-receptors, except that denervation abolishes the response. It is possible, however, that information from both internally and externally oriented receptor groups is integrated to produce a final air-breathing pattern. This is known to be the case in gar (Smatresk *et al.*, 1986), where internal receptors set the level of hypoxic drive, and external receptors set the balance of air-breathing vs gill ventilation. In this study, during hypoxia, both internal and external chemoreceptors are stimulated, leading to air-breathing. External NaCN only stimulates externally oriented receptors, and thus only sometimes stimulates air-breathing. In

incomplete denervates (i.e. animals with a branchial nerve and/or pseudobranch intact) external NaCN consistently stimulated air-breathing. Partial denervation may affect the balance of information from both groups, and lead to more frequent airbreaths.

Injections of NaCN into the DA of shams elicited a similar ventilatory response to that seen in gar (Smatresk, 1986), where internal NaCN does not stimulate air-breathing, but only gill ventilation. This is unlike the response of lungfish, where internally administered NaCN stimulates air-breathing (Lahiri *et al.*, 1970). It is unknown whether internal and external NaCN injections stimulate the same or different groups of receptors in *Amia*, although two groups, oriented internally and externally, exist in gar (Smatresk *et al.*, 1986).

Complete denervation abolished all ventilatory responses to external and internal NaCN clearly indicating that the receptors responsible for mediating these reflexes are situated in the gills and pseudobranch, innervated by cranial nerves VII, IX and X. The abolition of all ventilatory responses to external and internal NaCN indicates that the P_{op} and f_g responses seen in hypoxic denervates are not mediated by an O_2 chemoreceptor on the gills, and, indeed, that following gil denervation and pseudobranch ablation, *Amia* does not exhibit any O_2 sensitivity.

Infusion of NE into shams produced ventilatory effects similar to those seen in chapter 2 for intact fish, with increases in P_{op} and f_g, but no change in f_{ab}. Following denervation, the ventilatory response to NE was no longer statistically significant, as measured by ANOVA, indicating that the P_{op} and f_g responses to NE seen in *Amia*, and the ventilatory responses seen in the eel (Peyraud-

Waitzenegger, 1979) may be mediated to a large extent by receptors in the gills. The stimulation of f_g by E in denervates but not in shams is difficult to explain, but clearly indicates that E stimulates ventilation via an extra-branchial pathway. It should be noted that there is evidence of a P_{op} response to NE and E in denervates, which may be physiologically significant, if not statistically so. At 2.5 minutes post-infusion of NE and E, mean denervate P_{op} and f_g were significantly higher than control, pre-infusion mean values (this was not the case for external and internal NaCN). The absolute magnitudes of mean P_{op} and f_g responses are also similar to those seen in hypoxic denervates. Thus, a possible role for catecholamines in mediating hypoxic gill ventilatory responses via a direct central effect, as postulated for trout (Aota *et al.*, 1990) and dogfish (Taylor and Randall, 1989) may also occur in *Amia*.

In denervates, the pattern of gill ventilatory responses and changes in blood gases following acid infusion was identical to that seen in intact animals (chapter 2), except that intact fish also showed a significant increase in f_{ab}. If the responses were mediated by a receptor sensitive to C_{aO2} (as suggested in chapter 2), then it is unusual that the receptor did not respond to NaCN, or stimulate air-breathing. It seems unlikely that the P_{op} and f_g responses are mediated by catecholamines, because the magnitude of the response to acid infusion was much greater than that seen following infusion of pharmacological levels of NE and E in denervates, unless there is a synergistic action between acid and catecholamines. It is possible that the responses are mediated by a pH receptor similar to that of air breathing vertebrates. In that case, input from such a receptor must be

integrated with (and subordinate to) information from O_2 receptors, because the response to acid infusion in intact animals was abolished by aquatic hyperoxia (chapter 2). Furthermore, the hypothetical receptor must only stimulate gill ventilation, as the air-breathing response to acid infusion was abolished in the denervates.

Summary:

Denervation of the gills and extirpation of the pseudobranch abolishes cardiovascular responses to hypoxia and NaCN, but not to catecholamine or acid infusion. All air-breathing responses to experimental intervention were abolished by denervation, as were gill ventilatory responses to NaCN. Denervates exhibited attenuated gill ventilatory responses to hypoxia and catecholamines, and a gill ventilatory response to acid infusion similar to that of intact animals. The ventilatory responses seen in denervated animals may be mediated by an extrabranchial O_2 receptor not sensitive to NaCN, by circulating catecholamines, or by a pH sensitive receptor.

GENERAL DISCUSSION

This thesis has examined various aspects of the respiratory physiology of *Amia*. The overall results demonstrate that respiratory control in *Amia* is essentially similar to that of water-breathing fish, with the added capacity to breathe air.

The results of the air-exposure experiment clearly indicate that *Amia* does not have the physiological capacities necessary to aestivate, being unable to detoxify ammonia as urea and reduce metabolism. The swimbladder functions only to sustain aerobic metabolism under purely aquatic conditions. *Amia* has an unusual gill structure (Daxboeck, Barnard and Randall, 1981; Olson, 1981), whereby the secondary lamellae of adjacent gill filaments are fused, to form a lattice arrangement. It has been suggested (Daxboeck *et al.*, 1981) that this is an adaptation to survive air-exposure. Blood perfusing the ABO in *Amia* first traverses the gills and during air-exposure, collapse of the gills would occlude blood flow to the organ (and all systemic vascular beds). This collapse can be prevented by the lattice arrangement, and this might also allow the gills some role in gas-exchange in air. The lack of any reduction in V_{O2} following air-exposure does suggest that the ABO was still receiving an adequate blood supply, but clearly the unusual gill structure alone is not adequate to sustain long-term air-exposure. The inability of *Amia* to survive even moderate hypoxia without access to air suggests that the existence of the ABO allows the fish to avoid reductions in aerobic metabolism, such that there was no selection pressure towards survival by anaerobic mechanisms.

The ventilatory sensitivity to reductions in C_{aO2} demonstrated in *Amia* is similar to that demonstrated in water-breathing fish. This C_{aO2} sensitivity suggests that *Amia* has internally oriented oxygen chemoreceptors functionally similar to those in the aortic body of mammals. Mammals have two discrete groups of arterial chemoreceptors, the carotid body, and a diffuse area of chemosensitivity in the aortic arch, the "aortic body" (Eyzaguirre, Fitzgerald, Lahiri and Zapata, 1986). All arterial chemoreceptors are sensitive to the P_{O2} at the receptor site. The carotid body has a very high, auto-regulated blood supply, such that the P_{O2} of the receptor tissue is most closely correlated with plasma P_{O2} (P_{aO2}). The aortic body does not have a high auto-regulated blood supply, and so the P_{O2} at the receptor tissue is affected by changes in blood O_2 delivery, which is the product of blood flow and C_{aO2}. Thus, aortic receptors respond vigorously to reduced blood flow, anaemia and carboxyhaemoglobinaemia, whereas carotid receptors do not (Lahiri, 1980; Lahiri, Mulligan, Nishino, Mokashi and Davies, 1981).

Indeed, as suggested by Smatresk (1990), it is only with the development of the highly specialised mammalian carotid body (or its avian analog) that a receptor sensitive primarily to P_{aO2} appears in vertebrate evolution, a receptor that is functionally equivalent to the externally oriented chemoreceptors of fish. There is no evidence for a structure similar to the carotid body in fish, and O_2 chemosensitivity appears to be diffusely organised throughout the gills and buccal cavity (Butler *et al.*, 1977; Burleson and Smatresk 1986; Smatresk, 1990, Chapter 4). It is possible that the periodic breathing behaviour of bimodally breathing

fish, and various other ectothermic vertebrate groups (Shelton *et al.*, 1986) occurs because these animals monitor blood oxygen delivery. This would explain the apparent lack of a direct correlation between P_{aO2} or P_{aCO2} and breathing in these animals (Shelton *et al.*, 1986).

Amphibian, reptile, avian and mammalian peripheral chemoreceptors all show sensitivity to P_{aCO2} and pH_a (Ishii and Ishii, 1985 a,b; Piiper and Scheid, 1986; Eyzaguirre, Fitzgerald, Lahiri and Zapata, 1986). In mammals, the response is blunted but not abolished by hyperoxia (Eyzaguirre *et al.*, 1986). The abolition of all ventilatory responses by hyperoxia in *Amia* suggests that these fish have different peripheral chemoreceptors, or that the P_{aCO2} and pH_a response is entirely abolished by hyperoxia.

Catecholamine infusion stimulates ventilation in both water-breathers and air-breathers (Peyraud-Waitzennegger, 1979; Dempsey *et al.*, 1986). The fact that in *Amia* exogenous catecholamines stimulate only gill ventilation and not air-breathing suggests that there are differences in the characteristics of air-breathing in *Amia*, as compared with terrestrial air-breathers. In mammals, catecholamines stimulate ventilation largely by stimulating peripheral chemoreceptors (Dempsey *et al.*, 1986; Eyzaguirre *et al.*, 1986). If this is the case in *Amia* also, then the receptors controlling gill ventilation may be similar pharmacologically to the peripheral chemoreceptors of terrestrial vertebrates, and those controlling air-breathing are probably separate and pharmacologically different. This is interesting, and warrants further study.

There is evidence that the effects of catecholamines are exerted centrally. In mammals, catecholamines are largely inhibitory when applied centrally, and do not cross the blood:brain barrier *in vivo* (Dempsey *et al.*, 1986). In fish, however, catecholamines cross the blood:brain barrier (Nekvasil and Olson, 1986), and when applied to respiratory neurones in the medulla stimulate ventilation (Taylor and Randall, 1990). If the gill ventilatory response to catecholamines in *Amia* is centrally evoked, then it suggests that air-breathing responses may be controlled by a different area of the brain. The fact that catecholamines do not stimulate air-breathing under moderate hypoxia, when there is a change in ventilatory pattern with an increased emphasis on air-breathing, indicates that the effects are exerted in an area not responsible for controlling air-breathing.

The endogenous release of catecholamines seen following catecholamine infusion in *Amia* also occurs in the American eel, *Anguilla rostrata* (Epple and Nibbio, 1985). Catecholamines are released from chromaffin tissue, which in actinopterygian fish is located in the posterior cardinal veins, just upstream of the heart (Nilsson, 1983). Catecholamine release in fish usually requires stimulation by the autonomic nerve supply to the chromaffin tissue (Nilsson, 1984). However, catecholamine-stimulated endogenous catecholamine release in the American eel does not require the presence of a brain or spinal cord (Hathaway, Brinn and Epple, 1989), suggesting that release can be effected by humoral influences in fish. In mammals, stimulation of peripheral chemoreceptors effects a reflex release of catecholamines from the adrenal medulla (Ungar and Phillips,

1983), but catecholamine release also occurs in the isolated adrenal gland in response to hypoxaemia (Nishijima, Breslow, Raff and Traystman, 1989). This suggests that the catecholamine release elicited by hypoxaemia in water-breathing fish (Perry *et al.*, 1989) and *Amia* may not require nervous stimulation. If catecholamine release in response to hypoxaemia in *Amia* is not a nervous reflex dependent on afferent information from chemoreceptors, but mediated humorally, then such a release may subsequently stimulate ventilation by a central effect, as postulated by Aota *et al.* (1990).

It is unusual that *Amia* do not show the ventilatory response to increases in T_{aCO2} or T_{amm} seen in trout. This may be because the dose used was not high enough, or because *Amia* are not as prone to exhibiting stress responses as trout. The ventilatory responses by trout to $NaHCO_3$ occurred in the absence of any significant change in C_{aO2}, but was associated with an increase in the level of circulating catecholamines. The stimulus for catecholamine release could be a neurally mediated "stress" response, or possibly a direct effect of HCO_3^- on chromaffin tissue. The results do indicate, however, that in water-breathing fish catecholamines may be able to stimulate ventilation in the absence of a reduction in C_{aO2}. It is interesting that $NaHCO_3$ caused a significantly higher release of NE than did HCl infusion. The ventilatory response to ammonia in mammals may be an evolutionary remnant of a response to remove excesses of endogenous ammonia in piscine ancestors.

During aquatic hypoxia, *Amia* did not show the inhibition of gill ventilation or magnitude of air-breathing increase reported by Johansen *et al.* (1970) and reported by Smatresk *et al.* (1986) for gar. *Amia* does not show the vigorous air-breathing response to externally applied NaCN seen in gar (Smatresk, 1986), and require much higher doses of external and internal NaCN to elicit a ventilatory response. This indicates that *Amia* are less sensitive to water and blood O_2 status, and may mean that *Amia* have different control mechanisms for air-breathing than those postulated for gar (Smatresk *et al.*, 1986). Gar have reduced gills compared with water-breathing fish (Smatresk and Cameron, 1982a) whereas *Amia* have gills similar in size to water-breathing fish (Daxboeck *et al.*, 1981), which suggests that *Amia* do not rely on air-breathing to the same extent as gar.

The evolution of air-breathing presumably required extensive re-organisation of the nervous system of fish. It has been suggested that lungfish have two separate central rhythm generators, one for gill ventilation and the other for air-breathing (Fishman, Galante and Pack, 1989). Other authors suggest that air-breathing in actinopterygian fish is a re-organisation of coughing and suction-feeding movements; requires relatively little neural re-organisation (Liem, 1980; Smatresk, 1990), and is critically dependent on afferent feedback (Shelton *et al.*, 1986; Smatresk, 1990). In *Amia*, following gill deafferentation, air-breathing did not occur (except in one case) in normoxia, and responses to hypoxia, NaCN and hypoxaemia were abolished, indicating that air-breathing rhythmicity and responses are dependent to a large extent on afferent information from the gills, and probably also the swimbladder.

In *Amia*, the pseudobranch contains receptors that elicit air-breathing responses, with the information carried in cranial nerve VII, because section of cranial nerve IX to the pseudobranch was not sufficient to abolish air-breathing responses to hypoxia or external NaCN, but extirpation abolished the responses. It would be interesting to determine whether pseudobranch ablation alone would abolish all air-breathing responses, since the pseudobranch in *Amia* is unusual and glandular, with different vascular relationships than that of teleosts (Allis, 1897).

Hedrick and Jones (1990) report that *Amia* show two different air-breath types, and suggest that one is driven by stretch-receptor input, and the other by a chemo-reflex. This suggests that the individual *Amia* that air-breathed following branchial denervation may have done so in response to stretch-receptor input, as the responses were not associated with experimental intervention.

The significant attenuation of gill ventilatory responses to catecholamines following gill denervation suggests that, similar to the case in mammals (Dempsey *et al.*, 1986; Eyzaguirre *et al.*, 1986), much of the ventilatory response to catecholamines is mediated peripherally. The remaining response is presumably evoked centrally. This evidence further indicates that peripheral receptors stimulating gill ventilation and air-breathing are pharmacologically and spatially separate, and that the air-breathing response is not integrated at the level of the gill ventilatory rhythm generator. In denervated animals, the most pronounced ventilatory response was an increase in ventilation frequency following epinephrine infusion, whereas catecholamines exert their major effect on opercular pressure in intact animals. The ventilatory response to hypoxia in denervates was

largely a result of an increase in opercular pressure, suggesting that there may be differences in the responses to epinephrine and hypoxia in the absence of afferent information from the gills. It is difficult to explain why epinephrine had a more pronounced effect on ventilation following denervation, since norepinephrine crosses the blood:brain barrier more freely in water-breathing fish (Nekvasil and Olson, 1986).

The vigorous gill ventilatory response to acid infusion following gill denervation suggests that *Amia* may have a central pH-sensitive receptor similar to that of mammals, but subordinate to oxygen reflex responses. Indeed, the attenuated gill ventilatory response to hypoxia may have been a result of stimulation of this receptor, since the onset of the ventilatory response occurred five minutes following blood sample collection. Interestingly, perfusion of the medullary region of the cranial space in *Amia* with mock cerebrospinal fluid at various pH levels does not stimulate gill ventilation (Hedrick, Burleson, Jones and Milsom, unpublished data). This indicates that the pH response seen in the denervates may not be central in origin, or that the mock CSF used by Hedrick *et al.* did not communicate with the sites responsible for the pH_a response.

In conclusion, *Amia calva* appears to be an aquatic animal with no ability to survive prolonged air-exposure. The ABO functions to maintain aerobic metabolism and blood oxygen delivery during aquatic hypoxia. Intact *Amia* do not exhibit a ventilatory response to pH_a and P_{aCO2}. *Amia* do not show the ventilatory sensitivity to increases in T_{aCO2} and T_{amm} seen in water-breathing fish.

Air-breathing and gill ventilation are stimulated by hypoxia and hypoxaemia, and receptors controlling the responses are in the gills. *Amia* retains the ventilatory control systems of water-breathing fish intact, and air-breathing is not stimulated by the same receptors in the gills, or fully integrated centrally. In the absence of information about blood or water oxygen status, *Amia* may show a ventilatory response to blood pH.

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