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Control of ventilation in the hypercapnic skate *Raja ocellata*: I. Blood and extradural fluid

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Abstract. In order to study the role of CO_2 and acid–base status in contributing to ventilatory drive, skate were exposed to normoxic hypercapnia (PI_{CO_2} = 7.5 Torr) under conditions where the primary O_2 drive would remain unaltered. Blood O_2 transport was markedly insensitive to CO_2, with no Root effect and only a small Bohr effect. Red blood cell pHi was not preferentially regulated, and there was no evidence of RBC swelling or nucleoside triphosphate adjustment. Although there were no changes in arterial O_2 levels during hypercapnia, ventilation immediately increased 2.7-fold through large changes in stroke volume and small changes in frequency, and declined only slightly through 24–48 h. Pa_{CO_2} equilibrated rapidly with PI_{CO_2}, driving down arterial pHa, which was 65% corrected through HCO_3^- accumulation by 24 h. In contrast, the extradural fluid outside the brain equilibrated only very slowly, and was clearly not involved in the ventilatory stimulation. Increased ventilation during hypercapnia may be related to depressions in pHa.

Animal, skate; Blood, O_2 transport properties in skate; Bohr effect in skate; Cerebrospinal fluid, acid–base balance; Control of breathing, cerebrospinal fluid in skate, response to CO_2; Hypercapnia, ventilatory response in skate; pH, intracellular; Red cell, intracellular pH in skate; Ventilation, sensitivity to CO_2 in skate

It is now firmly established that O_2, and not CO_2 or internal acid–base status, sets the primary ventilatory drive in water-breathing fish, in contrast to air-breathing mammals. Circumstantial evidence points to the presence of blood-based receptors for O_2 content on the arterial side downstream from the gills, possibly in the brain. These receptors appear to function independently from the water-based O_2 receptors on the first gill arch which control heart rate (for reviews, see Shelton *et al.*, 1986; Perry and Wood, 1989).

The possible role of CO_2 or acid–base status in contributing to ventilatory control in fish remains far from clear. Early studies, which often involved unrealistically high

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P_{CO_2} exposures, have been reviewed by Dejours (1973); in general, they produced no definite conclusions. More recent investigations, using more physiological P_{CO_2} levels (< 10 Torr) have found increases in ventilatory water flow ($\dot{V}w$), though the magnitude and duration of the response varied greatly amongst species (*e.g.* Dejours, 1973; Janssen and Randall, 1975; Randall *et al.*, 1976; Truchot *et al.*, 1980; Smith and Jones, 1982; Thomas *et al.*, 1983). The explanation generally offered has been that the ventilatory response to hypercapnia is really a response to hypoxemia mediated through the O_2 receptor system, because arterial O_2 content is lowered by the Bohr and/or Root effects associated with respiratory acidosis. In support of this idea, Smith and Jones (1982) reported that a level of environmental hyperoxia sufficient to maintain arterial O_2 content eliminated the increase in ventilation caused by hypercapnia in trout.

However, other workers have shown that even during intense hyperoxia, fish still respond to moderate hypercapnia with increased ventilation (Truchot *et al.*, 1980; Thomas *et al.*, 1983). Indeed, Smith and Jones (1982) noted that hyperoxia did not eliminate the elevated ventilation associated with higher levels of P_{CO_2} (7–15 Torr). Recently, Heisler *et al.* (1988) have presented evidence that hyperoxic dogfish adjust their ventilation in response to arterial acid–base status. These observations suggest that CO_2 and/or acid–base status may exert a secondary but important controlling influence on the ventilatory drive in fish, separate from that of O_2. The goal of the present study was therefore to test this hypothesis, by exposing fish to moderate hypercapnia (PI_{CO_2} = 7.5 Torr) under conditions where the primary O_2 drive would remain unaltered.

The Atlantic big skate (*Raja ocellata*) was selected for several reasons. Firstly, we suspected that like other elasmobranchs, it would have a relatively low blood O_2 capacity, lack a Root effect, and exhibit a minimal Bohr shift (Butler and Metcalfe, 1989). Thus, the anticipated effects of P_{CO_2} on blood O_2 transport would be small. Secondly, the dorsal inhalent spiracle and discrete ventral gill slits, combined with the skate's sedentary behaviour in captivity, make it an ideal subject for ventilation measurements. Thirdly, by analogy to mammals, any direct CO_2 or acid–base sensitivity of ventilation might reside at the level of the brain or its fluids. The brain is readily accessible under a thin layer of chondocranial cartilage. The curious extra-brain fluid of elasmobranchs (extradural fluid = EDF) is particularly voluminous in the skate and easily sampled by catheter. The internal or cerebrospinal fluid (CSF) of the brain is much less abundant, but anaerobic samples can be readily obtained.

This first of two papers describes the basic respiratory properties of the blood in *Raja ocellata*, and then characterizes the ventilatory response to prolonged hypercapnia (24–48 h) under conditions where any O_2 signal remains unchanged. The mechanisms stabilizing blood O_2 transport during hypercapnia have been examined. Particular attention has been devoted to the acid–base chemistry of the arterial blood and extradural fluid to see whether either correlates with the observed ventilatory changes. The companion paper (Wood *et al.*, 1990) examines the acid–base chemistry of the cerebrospinal fluid, and the intracellular acid–base status of the brain and other tissues during an identical hypercapnic regime.

Materials and methods

Experimental animals. The experiments were conducted during July–August at the Marine Sciences Research Laboratory, Logy Bay, Newfoundland, Canada. Specimens of the big skate (*Raja ocellata* Mitchill; 0.75–5.0 kg) were caught on baited long lines in Conception Bay, Newfoundland. Hook wounds were sutured, and the fish allowed to recover for 2–6 weeks, in 40 000 L flow-through seawater tanks at 12.0 ± 1.0 °C, salinity 30 ± 1 ppt, which represented the subsequent experimental conditions. Daily feeding with capelin (*Mallotus villosus* Muller) was halted at least one day prior to surgery.

Surgical procedures. Skate (N = 11) for the ventilation series were fitted with ventilation collection funnels, extradural fluid catheters, and arterial catheters during a 60–90 min operation. Additional animals (N = 45) used in the companion study (Wood *et al.*, 1990) or as blood donors in the present study were fitted with catheters only. During the operation, anaesthesia was maintained by irrigation of the gills via the spiracles with MS-222 (Sigma; 0.05–0.125 g/L as required) in aerated seawater.

The ventral gill slits are bilaterally discrete in skate. Each animal was custom-fitted with two separate funnels (length approximately 10 cm) for the collection of ventilatory water flow, one around the gill slits on each side. The funnels were cut from latex surgical gloves, tightly sutured to the skin, and sealed with cyanoacrylate glue. A length of PE90 tubing (Clay-Adams) was secured 2.5 cm inside each funnel for sampling purposes.

The extradural space is extremely large in this species (10–20 ml in a 1 kg skate) and easily recognized as a lightly coloured, nearly transparent triangular region near the snout. A PE50 catheter tipped with a 22 gauge needle was inserted through the anterior dorsal surface of the chondocranium into this space to allow repetitive sampling of extradural fluid (EDF) without disturbance. In order to implant a chronic arterial catheter for blood sampling, a 3–5 cm incision was made in the ventral midline, and the stomach and intestine exteriorized. The anterior mesenteric artery, near the small curvature of the stomach, was occlusively cannulated with PE50, which for smaller skate was stretched to a finer diameter. The catheter was fed up the artery as far as possible so as to sample blood from the dorsal aorta. The gastro-intestinal organs were returned to the body cavity, the catheter tied in a position which would assure blood flow, the wound dusted with tetracycline (Eastern Drug Services, Newfoundland), and the incision closed with silk suture. Both EDF and arterial catheters were filled with heparinized skate saline (100 i.u./ml sodium heparin; Sigma). The composition of this saline in g/L was: NaCl = 15.80; urea = 11.40; KCl = 0.380; $CaCl_2 \cdot 2H_2O$ = 0.558; $MgCl_2 \cdot 6H_2O$ = 0.510; $NaHCO_3$ = 0.420; Na_2HPO_4 = 0.120; KH_2PO_4 = 0.029; $MgSO_4 \cdot 7H_2O$ = 0.123. Saline pH was adjusted to 7.8. Following surgery, the animals were placed in individual experimental chambers, which were covered with black plastic, and allowed to recover in flowing normoxic, normocapnic seawater for 24–72 h.

Ventilation measurements. Two techniques were employed for measurement of ventilatory water flow ($\dot{V}w$). The direct method ($N = 5$) involved timed collection of $\dot{V}w$ using the modified Van Dam boxes designed by Cameron *et al.* (1971) for the stingray, which has a very similar morphology (dorsoventrally flattened). The skate rested on a platform in the central chamber, and was restrained by several ties around the tail. This central chamber was served by a water inflow well in excess of the animal's $\dot{V}w$. The ventilation funnels were led out through the bottom and connected via latex tubing (2 cm ID) to two separate lateral chambers, one for each funnel. Each chamber drained by a constant level overflow. A water manometer was used to set the overflow at identical heads in all three chambers. Cameron *et al.* (1971) provide a useful illustrative diagram. The sum of simultaneous timed collections (30–60 sec, in duplicate) from the two lateral chambers yielded a direct measure of $\dot{V}w$; flow from the two sides was usually the same. Visual observations of the movements of dye placed in various locations around the fish's head indicated that resting skate inhaled almost entirely through the dorsal spiracles (occasionally, very small amounts entered via the mouth) and exhaled entirely via the ventral gill slits into the funnels (see also Hughes, 1960).

An indirect method ($N = 6$) based on the Fick principle (Dejours, 1973) was also employed. Here the ventilation funnels served as mixing tubes to ensure representative PE_{O_2} measurements. Skate were placed in sealed perspex boxes tapered to their morphology (flattened, guitar-shaped) and served with an inflow of water well in excess of the $\dot{V}w$. This single inflow entered close to the head and a single outflow exited at the tail. The dead space was reduced as much as possible such that the box volume was approximately twice that of the animal. The skate was again supported on a platform, hollow at the anterior end, which prevented collapse of the ventilation funnels. Simultaneous measurements of the flow rate (X) of seawater through the box, and of P_{O_2}s at the box inflow (Pin_{O_2}), box outflow ($Pout_{O_2}$), inspired water ($P\bar{I}_{O_2}$; the mean of measurements at the entrance to each spiracle), and mixed expired water ($P\bar{E}_{O_2}$; the mean of measurements from each ventilation funnel) yielded an indirect estimate of $\dot{V}w$:

$$\dot{V}_w = \frac{Pin_{O_2} - Pout_{O_2}}{P\bar{I}_{O_2} - P\bar{E}_{O_2}} \cdot X. \quad (1)$$

While this approach avoided the restraint used in the direct method, its accuracy was limited by the requirement for 7 different measurements and the fact that any extrabranchial uptake of O_2 would cause a proportional overestimate of $\dot{V}w$.

With both methods, respiratory stroke volume ($V_{S,R}$) was calculated from $\dot{V}w$ (on a kg body weight basis) and the observed respiratory frequency (f_R) based on spiracular closings counted visually through a small window in the black plastic:

$$V_{S,R} = \frac{\dot{V}w}{f_R}. \quad (2)$$

O_2 uptake ($\dot{M}_{O_2}$) was calculated as:

$$\dot{M}_{O_2} = \dot{V}w\,(P\bar{I}_{O_2} - P\bar{E}_{O_2}) \cdot \alpha_{O_2}, \tag{3}$$

where α_{O_2} was the tabulated O_2 solubility in seawater at experimental temperature and salinity (Boutilier *et al.*, 1984). The percentage utilization of O_2 from water flowing over the gills (Uw_{O_2}) was estimated as:

$$Uw_{O_2} = \frac{(P\bar{I}_{O_2} - P\bar{E}_{O_2})}{PI_{O_2}} \times 100\%. \tag{4}$$

Experimental protocols. Ventilatory, blood and EDF responses were monitored in 11 skate (5 with direct $\dot{V}w$ methodology, 6 with indirect $\dot{V}w$ methodology) subjected to 24 h of normoxic hypercapnia. Seawater flowing to the experimental chambers percolated through a 1.0 m × 0.1 m gas exchange column which was bubbled with either air or a CO_2/air mixture from a Wösthoff 3011a-F pump (Bochum, F.R.G.), yielding PI_{CO_2} = 0.3 Torr (normocapnia) or 7.5 Torr (hypercapnia), respectively, at a constant PI_{O_2} = 155 Torr. Two control measurements, separated by about 1 h, were taken under normocapnia. Hypercapnia was then instituted, and experimental measurements taken at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h. In two fish, the hypercapnic exposure was continued until 48 h. Ventilatory measurements (PI_{CO_2}, PI_{O_2}, PE_{O_2}, $\dot{V}w$, f_R) were taken at all sample times. EDF samples (0.25 ml) and arterial blood samples (1.1 ml) were drawn at all times except 0.25 h. EDF samples were replaced with an equal volume of non-heparinized saline, the composition of which (see above) was very similar to that of EDF (table 2). In order to avoid any decline in blood O_2 capacity resulting from repetitive sampling, blood samples were replaced with arterial blood freshly drawn from the catheter of a donor skate. Blood replacements were adjusted to approximately the same hematocrit as found in the experimental fish. No problems of cross-reactivity were noted. Arterial blood samples were assayed for Pa_{CO_2}, true plasma Ca_{CO_2}, whole blood pHa, red cell lysate pH (RBC pHi), Pa_{O_2}, Ca_{O_2}, hematocrit (Hct), hemoglobin (Hb), total nucleoside triphosphates (NTP), and plasma protein, Na^+, K^+, Ca^{2+}, and Cl^- concentrations. EDF samples were analyzed for pH, C_{CO_2}, and in some cases P_{CO_2}, protein, and ions.

Blood respiratory properties. Red cell counts and the mean red cell volume measurements were performed with a Coulter Counter Channelyzer (Coulter Electronics, Hialeah, Florida), using blood sampled by catheter from 5 skates and diluted with skate saline. These, together with Hb measurements, allowed calculation of mean Hb content per red cell (MHC).

The O_2 dissociation characteristics of skate blood at 12 °C were determined on 3 separate animals sampled by catheter, using the mixing technique of Scheid and Meyer (1978). A Lex-O_2-Con Model TL (Lexington Instruments, Waltham, Massachusetts) was used to verify the O_2 contents of oxygenated and de-oxygenated pools, mixtures,

and plasma O_2. A potential drawback of the mixing technique has been noted by Wells and Weber (1983), namely a decrease in NTP levels in the de-oxygenated pool. Over the 3 h tonometry period used in the present study, NTP levels declined by ~30% in the de-oxygenated blood of the skate, comparable to the data of Wells and Weber (1983) on dogfish blood, whereas NTP levels were stable or increased slightly in the oxygenated blood. Wells and Weber (1983) concluded that these changes produced only a small change in P50 (~2 Torr). P_{CO_2} levels representative of the control (0.75 Torr) and hypercapnic conditions (7.50 Torr) were employed, yielding a ΔpH (−0.42) comparable to that observed *in vivo* upon initial exposure to hypercapnia. The resulting dissociation curves allowed calculation of the Bohr (Δlog P50/ΔpH) and Hill ("n") coefficients (at P50) for whole blood.

The non-HCO_3^- buffer characteristics of skate blood at 12 °C were determined on 7 different pools of blood sampled from 3 different skate by catheter. The pools were chosen so as to differ in Hct and Hb levels, allowing an assessment of the influence of these variables on β_{NB}, the non-HCO_3^- buffer capacity. Each pool was equilibrated at P_{CO_2}s of 1.1, 2.2, 4.3, and 7.5 Torr (in air), covering the range observed *in vivo* in the hypercapnia experiments; pH and C_{CO_2} of true plasma were measured at each P_{CO_2}. The β_{NB} values (in slykes = mmol HCO_3^-/pH unit/L) were calculated from the linear slopes ($\Delta HCO_3^-/\Delta pH$) of the resulting relationships. For each fish, the β_{NB} value of separated plasma was also determined.

In the above equilibrations, the blood was heparinized at 1000 i.u./ml (sodium heparin; Sigma). Rotating glass tonometer flasks (100 ml volume), each containing 0.5–5.0 ml of blood or plasma, were employed; the equilibration period at each P_{CO_2} was 1 h. Precision gas mixtures were provided by a Wösthoff 301a-F pump fed from analyzed cylinders. The gases were humidified at 12 °C before entering the tonometers.

Analytical techniques. Water, blood, and EDF samples were drawn into gas-tight glass syringes and held briefly on ice prior to gas measurements. Aliquots of blood were immediately fixed for Hb (20 μl into 5 ml Drabkin's reagent) and NTP (200 μl into 600 μl ice-cold 8% trichloroacetic acid, followed by immediate freezing). Whole blood samples from a few skate under control conditions were also fixed for lactate (100 μl into 200 μl ice-cold 8% perchloric acid). Plasma for ionic analysis, and a red cell pellet for RBC pHi were obtained by centrifuging 500 μl whole blood, at 9000 × *g* for 2 min. The centrifuge tube was filled entirely and sealed; measurements of plasma pH before and after centrifugation demonstrated that there was no loss of CO_2.

The O_2 content of blood (C_{O_2}) was determined with a Lex-O_2-Con Model TL, using an injection volume of 50 μl. Blood and water P_{O_2}, P_{CO_2}, blood pH, and RBC pHi (freeze-thaw lysate method of Zeidler and Kim, 1977) were determined by standard techniques, using Radiometer Copenhagen electrodes thermostatted to the experimental temperature and displayed on PHM 71 or 72 acid–base analyzers. The recommendations of Boutilier *et al.* (1984) were followed to increase the accuracy of blood and water P_{CO_2} determinations. True plasma C_{CO_2} measurements (Cameron chamber method, with an injection volume of 50 μl) were performed on plasma taken anaerobi-

cally from sealed capillary tubes centrifuged at 5000 × *g* for 4 min for hematocrit (Hct) determination. Plasma and EDF HCO_3^- were calculated from their respective P_{CO_2} and C_{CO_2} levels, using the tabulated value for α_{CO_2} at the experimental temperature in elasmobranch plasma, from Boutilier *et al.* (1984). EDF P_{CO_2} was not routinely measured but rather calculated from the measured pH and C_{CO_2} levels, using the Henderson–Hasselbalch equation.

It was found that apparent pK values for the CO_2/HCO_3^- equilibrium (pKapp), as derived from either the nomogram for dogfish plasma or the general equations provided by Boutilier *et al.* (1984), systematically overestimated P_{CO_2} relative to measured levels in skate blood and EDF. Therefore, an empirical relationship was generated, based on 48 simultaneous determinations of pH, P_{CO_2}, and C_{CO_2} *in vivo*:

$$\text{pKapp} = -0.186\,\text{pH} + 7.418 \quad (r = 0.45,\ P < 0.001). \tag{5}$$

Whole blood hemoglobin (Hb) was determined by the cyanmethemoglobin technique against mammalian hemoglobin standards (Sigma reagents) and expressed as g/100 ml whole blood. An *approximate* conversion is 1 g Hb/100 ml = 0.155 mmol Hb/L, assuming all measured hemoglobin is tetrameric and similar to mammalian Hb in molecular weight. Whole blood lactate was determined by the L-lactate dehydrogenase/NADH method, and whole blood NTP by the phosphoglycerate phosphokinase/glyceraldehyde phosphate dehydrogenase enzyme system (Sigma reagents). This method does not differentiate between the different nucleoside triphosphates, but rather provides a lumped overall value ('NTP'). The two most important compounds in fish blood are usually ATP and GTP, for which precise, specific enzymatic assays have been developed (Albers *et al.*, 1983). Nevertheless, the NTP method has been shown to provide a reliable measure of the sum of ATP plus GTP in the red cells of another elasmobranch, *Squalus acanthias* (Wells and Weber, 1983). NTP levels have been expressed as μmol per g hemoglobin (NTP/Hb), and mean hemoglobin concentration (MCHC) as g hemoglobin/ml RBC (i.e. Hb/Hct). Plasma and EDF protein levels were determined using a Goldberg refractometer (American Optical, Rochester, New York) recalibrated for the higher ionic strength of skate body fluids, and expressed as g/100 ml. Na^+, K^+, and Ca^{2+} were determined by atomic absorption (Varian-Tectron AA5) and Cl^- by coulometric titration (Radiometer CMT10).

Data have been generally reported as means ± 1 SEM (*N*), where *N* is the number of fish. The significance of differences during hypercapnia relative to control levels within a group was assessed by the paired Student's two-tailed *t*-test, using each animal as its own control. In all cases, the control value was taken as the mean of the two separate control measurements. The unpaired Student's two tailed *t*-test was used to evaluate differences between groups. Linear regression lines were fitted by the method of least squares, and the significance of Pearson's correlation coefficient was tested. A significance level of $P \leq 0.05$ was used throughout.

Results

Blood respiratory properties. A variety of respiratory properties determined on the blood of resting skate, sampled by catheter, are summarized in table 1. Skate blood was characterized by relatively low hematocrit, hemoglobin concentration (Hb), and blood O_2 capacity, and a low density of unusually large red blood cells, yielding a high mean Hb content per RBC (MHC). On an absolute basis, the concentration of plasma protein was actually higher than that of Hb. Other properties related to Hb such as NTP/Hb, mean cell Hb concentration (MCHC), and O_2 capacity per unit Hb were not unusual. The O_2 dissociation curve of the blood (*e.g.* fig. 1A) was approximately hyperbolic with a Hill coefficient (at P50) less than 2 (*i.e.* low co-operativity) and a low O_2 affinity (P50 $\simeq$ 28 Torr at P_{CO_2} = 0.75 Torr, representative of normal *in vivo* Pa_{CO_2}). The blood exhibited no Root effect and only a small Bohr shift (- 0.29) in the face of a 10-fold elevation in P_{CO_2} from 0.75 to 7.50 Torr ($\Delta pH \simeq -0.42$). From these data, it is clear that the experimental exposure to normoxic hypercapnia, which raised Pa_{CO_2} by a comparable amount (*cf.* fig. 3A), should have negligible effect on blood O_2 transport.

The non-HCO_3^- buffer capacity of the blood (β_{NB}, true plasma measurements) was a linear function of Hb concentration (fig. 1B). Despite the very low levels of Hb

TABLE 1

Respiratory properties of blood of the big skate, *Raja ocellata*, at 12 °C. Means ± 1 SEM (*N*).

Hematocrit %	12.5	±	0.7	(53)
Hemoglobin (g/100 ml)	2.82	±	0.14	(43)
Plasma protein (g/100 ml)	3.86	±	0.14	(48)
Mean cell Hb concentration (MCHC-g/ml)	0.239	±	0.016	(43)
RBC concentration[a] (10^9 cells/ml)	0.20	±	0.02	(5)
Mean RBC volume[a] (μm^3)	888.8	±	55.2	(5)
Mean Hb per RBC[a] (MHC-pg/cell)	127.6	±	30.1	(5)
Blood O_2 capacity[b] (mmol/L)	1.58	±	0.14	(5)
NTP/Hb (μmol/g Hb)	13.40	±	1.66	(16)
Hemoglobin O_2 capacity (μmol/g Hb)	53.66	±	3.27	(5)
Plasma O_2 capacity[c] (mmol/L)	0.268	±	0.027	(3)
P50 at P_{CO_2} = 0.75 Torr[d] (Torr)	27.6	±	2.6	(3)
P50 at P_{CO_2} = 7.50 Torr[e] (Torr)	34.6	±	1.4	(3)
Bohr coefficient (Δlog P50/ΔpH)	- 0.29	±	0.06	(3)
Hill coefficient at P_{CO_2} = 0.75 Torr[d] (n)	1.78	±	0.12	(3)
Hill coefficient at P_{CO_2} = 7.50 Torr[e] (n)	1.99	±	0.12	(3)
Whole blood non-HCO_3^- β[f] (slykes)	11.04	±	0.26	(6)
Plasma non-HCO_3^- β (slykes)	6.58	±	0.27	(3)

[a] [Hb] = 2.41 ± 0.38 (5) g/100 ml.
[b] [Hb] = 2.56 ± 0.26 (5) g/100 ml.
[c] P_{O_2} = 158.2 ± 2.8 (3) Torr
[d] pH = 7.82 ± 0.04 (3).
[e] pH = 7.40 ± 0.01 (3).
[f] Normalized to [Hb] = 2.82 ± 0.14 g/100 ml, from equation of fig. 1.

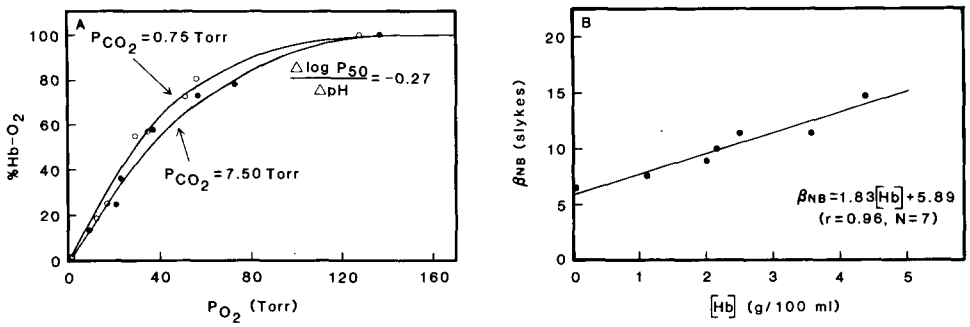

Fig. 1. (A) Typical *in vitro* O_2-dissociation curves at 12 °C of the blood of a single skate (*R. ocellata*) sampled by catheter. The two P_{CO_2} levels employed were representative of the levels present *in vivo* during control and hypercapnic exposures. Note the absence of a Root effect, and the small size of the Bohr effect. (B) The *in vitro* relationship at 12 °C between the whole blood non-HCO_3^- buffer capacity (β_{NB}) and its hemoglobin concentration ([Hb]). Blood was sampled by catheter from 3 skate and made into 7 pools of differing Hb concentrations. The linear regression relationship ($P \leq 0.01$) is given; the intercept at [Hb] = 0 represents the β_{NB} value attributable to plasma proteins.

normally present, typical blood β_{NB} values were quite high (11 slykes; table 1). This reflected both a high β_{NB} per unit Hb (1.83 slykes per g/100 ml; fig. 1B) and a high β_{NB} per unit plasma protein (1.70 slykes per g/100 ml). The latter was indicated both by the intercept (5.89 slykes) of the β_{NB} *vs* Hb regression at 0 Hb (fig. 1B), and by separate measurements of the β_{NB} of separated plasma (6.58 slykes; table 1). Thus plasma accounted for more than half of total blood non-HCO_3^- buffering (table 1). As judged from hematocrit, there was no evidence of RBC swelling during tonometry at higher P_{CO_2} and lower pH levels.

Responses to hypercapnia. There were no significant differences for any parameter, at any sample time, between the 5 skate studied using direct $\dot{V}w$ methodology and the 6 skate studied using indirect $\dot{V}w$ methodology by Fick principle. This suggests that there were no differences in the extent of disturbance with the two procedures, and that the two methods for measuring $\dot{V}w$ yielded similar estimates of all respiratory and ventilatory parameters. Therefore, the data were combined for all analyses. There were also no significant differences between the first and second control measurements, suggesting that sampling itself caused negligible disturbance.

Exposure to $P_{I_{CO_2}}$ = 7.5 Torr caused a rapid increase in ventilation which had reached its maximum (~2.7 × resting $\dot{V}w$) by 0.25 h (fig. 2A).The increased $\dot{V}w$ was maintained at this elevated level for the next 8 h, but declined slightly (~2.1 × resting $\dot{V}w$) by 24 h of hypercapnic exposure. In the 2 fish followed until 48 h, there was no further reduction in $\dot{V}w$. This increase in $\dot{V}w$ was achieved by large increases in $V_{S,R}$ (~2 × ; fig. 2B) and only small increases in f_R (~1.35 × ; fig. 2B).

The increase in $\dot{V}w$ was accompanied by a persistent fall in the Uw_{O_2} from 36% to about 20% (fig. 2C) with only a slight recovery (26%) by 24 h. There was also an initial increase in $\dot{M}_{O_2}$ at 0.25 and 0.5 h, but this declined to levels not significantly different

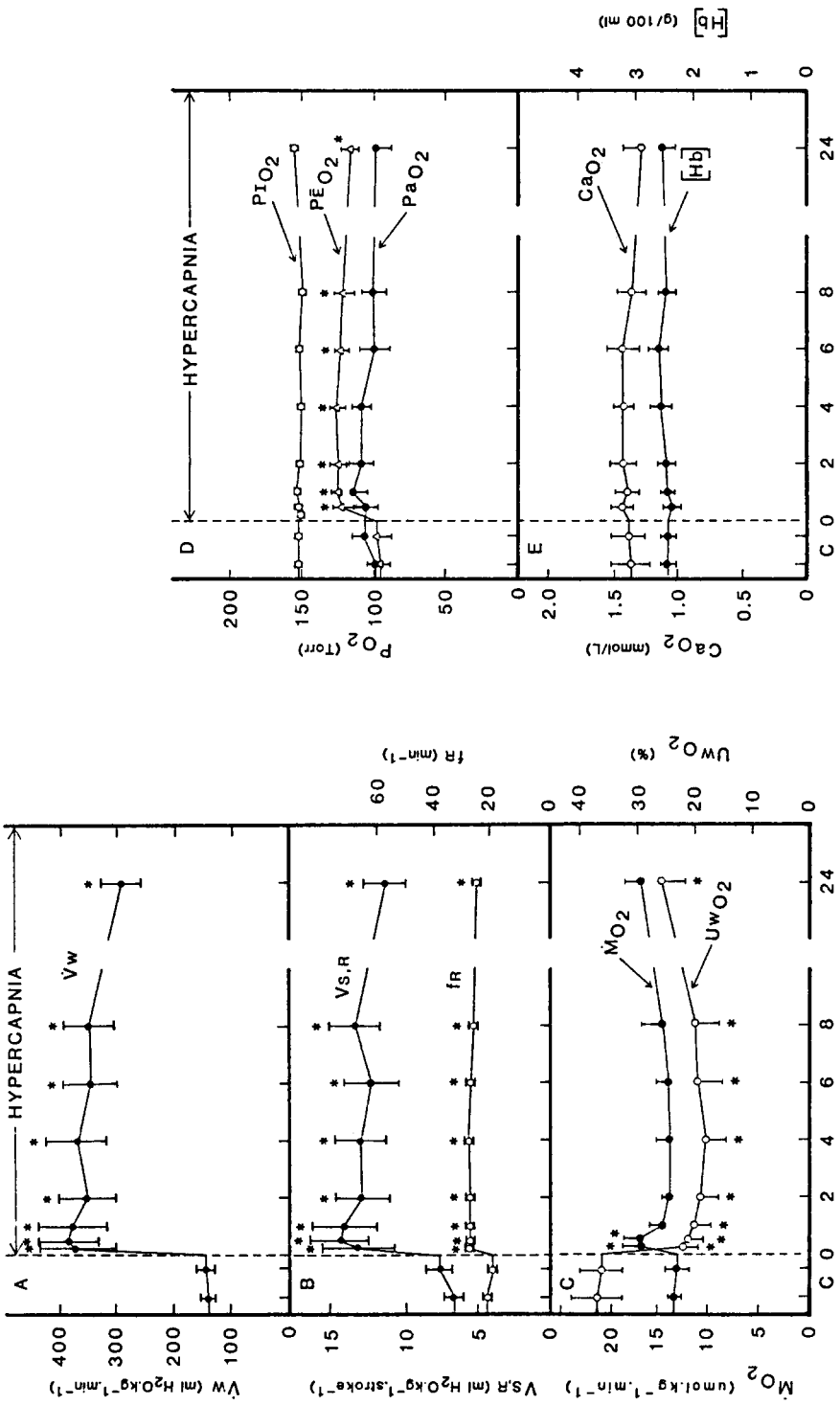

Fig. 2. The effects in *R. ocellata* of exposure to normoxic hypercapnia at 12 °C on (A) ventilation volume ($\dot{V}w$); (B) respiratory stroke volume ($V_{S,R}$) and frequency (f_R); (C) O_2 consumption rate ($\dot{M}_{O_2}$) and utilization (Uw_{O_2}) from water flowing over the gills; (D) inspired (PI_{O_2}), mean expired (PE_{O_2}), and arterial (Pa_{O_2}) O_2 tensions; and (E) arterial blood O_2 (Ca_{O_2}) and hemoglobin ([Hb]) concentrations. The two normocapnic control measurements (at PI_{CO_2} = 0.3 Torr, PI_{O_2} = 155 Torr) are designated as C. Exposure to hypercapnia (PI_{CO_2} = 7.5 Torr, PI_{O_2} = 155 Torr) was instituted at time 0. Values are means ± 1 SEM (N = 8–11). Asterisks indicate experimental means significantly different ($P \leq 0.05$) from the mean of the two normocapnic control measurements.

from control for the remainder of the exposure (fig. 2C). Note that PE_{O_2} was slightly lower than Pa_{O_2} under control conditions (fig. 2D), indicative of the efficient multicapillary exchange system in elasmobranch gills. However, this effect was lost during hypercapnia as PE_{O_2} rose above Pa_{O_2} and Uw_{O_2} fell with increased $\dot{V}w$.

PI_{O_2} and Pa_{O_2} values remained unchanged throughout the exposure (fig. 2D). More importantly, Ca_{O_2} remained constant (fig. 2E) in the face of the 10-fold elevation of P_{CO_2}, in accord with the lack of Root effect and minimal Bohr shift described earlier for the blood O_2 dissociation curve (fig. 1A). Blood Hb was also maintained at a constant level (fig. 2E) throughout the experiment by virtue of red cell replacement from donor animals, so O_2 saturation of the Hb was not altered. Thus, there was no evidence that the hyperventilation during hypercapnia was related to a change in the O_2 drive.

Exposure to hypercapnia caused a profound disturbance of internal acid-base

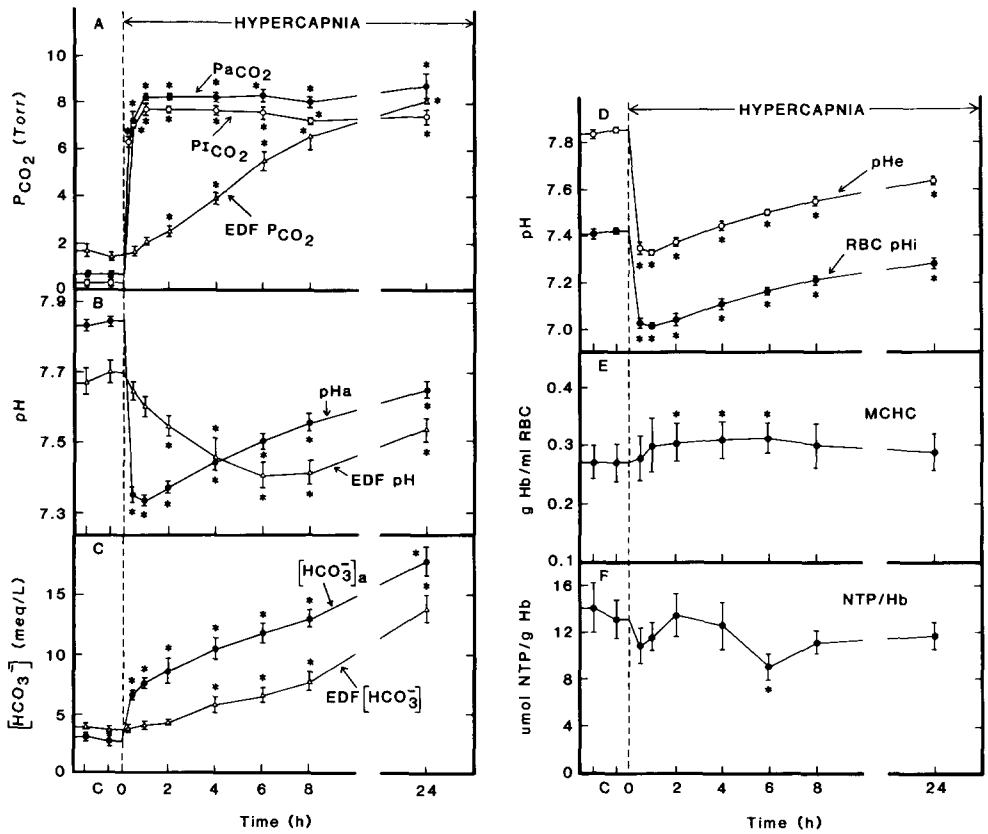

Fig. 3. The effects in *R. ocellata* of exposure to normoxic hypercapnia on (A) inspired (PI_{CO_2}), arterial (Pa_{CO_2}), and extradural fluid (EDF P_{CO_2}) CO_2 tensions; (B) arterial plasma (pHa) and EDF pH; (C) arterial plasma ($[HCO_3^-]a$) and EDF bicarbonate concentrations, (D) arterial plasma extracellular (pHe = pHa) and red blood cell intracellular pH (RBC pHi); (E) mean red blood cell hemoglobin concentration (MCHC); and (F) the nucleoside triphosphate to hemoglobin ratio (NTP/Hb). Means ± 1 SEM (N = 8–11). See legend of fig. 2 for other details.

chemistry. Pa_{CO_2} (only 0.75 Torr under control conditions) increased 10-fold in concert with PI_{CO_2} to approximately 8 Torr by 0.5 h of hypercapnia (fig. 3A). This re-established the normal Pa_{CO_2}–PI_{CO_2} gradient of 0.5 Torr. The gradient gradually widened to about 1.3 Torr by 24 h. Arterial pHa fell by 0.5 units (fig. 3B) and plasma HCO_3^- more than doubled to 6.6 meq/L (fig. 3C) within 0.5 h of exposure to hypercapnia. By 2 h, active compensation had started with a progressive accumulation of HCO_3^- and increase in pHa. Compensation of the pH change reached 40% at 8 h, and 65% at 24 h, by which time plasma HCO_3^- had risen to 18 meq/L. The 2 skate followed until 48 h exhibited a small further rise in Pa_{CO_2}, but no further pHa compensation, suggesting that the maximum was achieved by 24 h.

Despite the marked changes in plasma acid–base status during hypercapnia, major plasma electrolytes (Na^+, Cl^-, K^+, Ca^{2+}) were relatively stable (table 2). There were no significant changes at any time up to 8 h, whereas at 24 h, both Na^+ and Cl^- were elevated relative to control levels. There was no detectable change at any time in the 'strong ion difference' (SID = $Na^+ + K^+ + Ca^{2+} - Cl^-$) which stayed at approximately 35 meq/L. Plasma protein concentration (data not shown) also remained unchanged at about 3.7 g/100 ml throughout the exposure to hypercapnia.

In contrast to the rapid changes in the blood, the acid–base status of the extradural fluid responded slowly. EDF P_{CO_2} and EDF pH did not change significantly until 2 h (fig. 3A,B). Thereafter, EDF P_{CO_2} increased linearly with time, reaching the level of Pa_{CO_2} only after 24 h. EDF pHa continued to decline until 6 h and then rose very

TABLE 2

Plasma and extradural fluid electrolyte concentrations under control conditions and after 8 and 24 h of exposure to normoxic hypercapnia in the big skate, *Raja ocellata*, at 12 °C. Means ± 1 SEM (N = 7–11).

	Control	Hypercapnia	
	0 h[a]	8 h	24 h
Plasma			
Na^+ (meq/L)	270 ± 4	275 ± 7	283 ± 5[b]
Cl^- (meq/L)	246 ± 5	250 ± 3	260 ± 4[b]
K^+ (meq/L)	4.2 ± 0.3	4.2 ± 0.4	4.3 ± 0.4
Ca^{++} (meq/L)	8.5 ± 0.2	8.9 ± 0.2	8.1 ± 0.3
Extradural Fluid			
Na^+ (meq/L)	268 ± 8	–	282 ± 14
Cl^- (meq/L)	260 ± 5	–	268 ± 6
K^+ (meq/L)	3.6 ± 0.3	–	4.1 ± 0.6
Ca^{++} (meq/L)	7.3 ± 0.3	–	7.7 ± 0.6

[a] There were no significant differences in any plasma electrolyte relative to control levels at 0.5, 1, 2, 4 or 6 h hypercapnia (data not shown).

[b] Significantly different $P < 0.05$) from corresponding control value.

gradually in concert with the slow rise in EDF HCO_3^- concentration (fig. 3C). As a result of this slow pattern of change, the absolute decrease in EDF pH was much less than in pHa (fig. 3b). Compensation was about 50% complete by 24 h, but continued beyond this time, in contrast to the situation in arterial blood. In the 2 skate followed until 48 h, EDF HCO_3^- had risen to almost equal that in arterial plasma, and pH compensation had reached about 65%. Clearly, there was no relationship between EDF P_{CO_2} or EDF pH and $\dot{V}w$ (fig. 2A).

Major electrolytes in the extradural fluid were measured only under control conditions and after 24 h of hypercapnia (table 2). There were no significant changes. Note that the ionic composition of extradural fluid was very similar to that of plasma, but the SID was lower ($\sim$20 *vs* $\sim$35 meq/L), probably reflecting the much lower EDF protein concentration (1.80 ± 0.08 g/100 ml, $N = 37$) relative to that in plasma (3.86 ± 0.14 g/100 ml, $N = 48$, table 1).

Blood O_2 content remained constant during hypercapnia (fig. 2E); therefore, several possible modulators of Hb O_2 binding were monitored. RBC pHi fell by $\sim$0.4 units with the onset of hypercapnia, in the face of a slightly larger decrease in pHe (fig. 3D). There appeared to be no preferential regulation of pHi, which thereafter gradually recovered in parallel to the recovery of pHe. Both remained significantly depressed at 24 h. MCHC was elevated significantly by about 15% at 2, 4, and 6 h of hypercapnia (fig. 3E). This suggests that a small shrinkage of the red cells occurred, rather than the swelling normally associated with hypercapnia. Similarly there was no evidence of RBC swelling with hypercapnia during tonometry *in vitro*. The NTP/Hb ratio remained unchanged, apart from an unexplained decrease at 6 h (fig. 3F). These same basic trends (depressed RBC pHi without preferential regulation; unaltered or slightly increased MCHC; unchanged NTP/Hb) were seen in skate sampled only once, terminally (Wood *et al.*, 1990). Thus, they cannot be attributed to repetitive blood sampling or red cell replacement.

Discussion

Blood respiratory properties. The blood of *Raja ocellata* (table 1; fig: 1A,B) had a low O_2 capacity and affinity, high non-HCO_3^--buffer capacity (β_{NB}), and was relatively insensitive to CO_2 and acidity with respect to O_2 transport. These properties were fairly typical of other elasmobranchs which have been investigated in comparable detail (*cf.* Butler and Metcalfe, 1989), such as the Pacific dogfish (*Squalus suckleyi*; Lenfant and Johansen, 1966), the Atlantic spiny dogfish (*Squalus acanthias*; Wells and Weber, 1983; Weber *et al.*, 1983), and an Atlantic skate termed *Raia oscillata* by Dill *et al.* (1932). While this name is not listed as a synonym for *Raja ocellata*, we suspect that in fact they may be one and the same species. Indeed, the data reported by Dill *et al.* (1932) for P50, Root effect (absent), Bohr coefficient, Hill coefficient, RBC concentration, and most notably the very high β_{NB} attributable to plasma protein, were all remarkably close to the present observations (table 1, fig. 1A,B). Hematocrits, blood O_2 capacities, and β_{NB}

values attributable to RBCs were all about 50% higher in '*Raia oscillata*', but this could reflect hemoconcentration due to sampling stress (unanaesthetized, restrained venipuncture) in contrast to the cannulation sampling used in the present study.

As in *Squalus suckleyi* (Lenfant and Johansen, 1966), the plasma contributed more than the red cells to whole blood β_{NB} (table 1, fig. 1B). This differs from the situation in teleost fish and higher vertebrates, and may correlate with the absence of a Root effect, and negligible Bohr and Haldane effects in elasmobranchs in general (Lenfant and Johansen, 1966; Albers and Pleschka, 1967; Wells and Weber, 1983; Weber *et al.*, 1983; Butler and Metcalfe, 1989). A simple, unifying explanation for all these properties would be a low buffer capacity of the hemoglobin in elasmobranchs. Unfortunately, this is not the case, as illustrated by the present data (1.83 slykes per g Hb/100 ml; fig. 1B) and that (1.87 slykes per g Hb/100 ml) of Albers and Pleschka (1967) on the larger-spotted dogfish (*Scyliorhinus stellaris*). These values are comparable to figures in mammals and generally higher than those in teleost fish. A more complex explanation related to the specific chemistry of elasmobranch hemoglobin may be involved. Unusual properties of the hemoglobin may further correlate with the absence of RBC pHi regulation (fig. 3D), the lack of RBC swelling (fig. 3E), and the general stability of NTP/Hb (fig. 3F) in the skate during hypercapnia, all of which differ from the situation in teleost fish (Perry and Wood, 1989). If intracellular acidosis has little effect on hemoglobin O_2 transport, then there is presumably no need for such protective adjustments.

Respiratory parameters under control conditions. Relative to a variety of other elasmobranchs summarized by Butler and Metcalfe (1989), *R. ocellata* exhibited much lower values of $\dot{V}w$, $\dot{M}_{O_2}$, and f_R (fig. 2). The low $\dot{M}_{O_2}$ values were comparable to those reported by Hughes (1978) for uncannulated specimens of the torpedo (*Torpedo marmorata*), an inactive skate-like elasmobranch. Arterial pH values were normal for the experimental temperature, but both Pa_{CO_2} and plasma HCO_3^- levels were markedly lower (fig. 3A,B,C) and Pa_{O_2} values (fig. 2D) generally higher than in other elasmobranchs. This high efficiency of gas exchange, low resting metabolism and ventilation, and low blood lactate level (< 0.4 mequiv/L in all 5 fish tested) indicated that the skate were in truly resting condition and not adversely affected by the experimental procedures, thus underlining the suitability of this species for research.

Blood acid–base responses to hypercapnia. Correction of arterial acid–base status (fig. 3B,C) during hypercapnia in *R. ocellata* was somewhat slower and less complete than seen in the extensive studies of Heisler and co-workers on *Scyliorhinus stellaris* (reviewed by Heisler, 1989). Fig. 4A displays the plasma acid–base data of the skate in the form of a pH-HCO_3^- diagram, together with the appropriate non-HCO_3^- buffer line (β_{NB} from the equation of fig. 1B). This display demonstrates that during the first 2 h of hypercapnia, plasma HCO_3^- accumulation in the skate was less than predicted from passive non-HCO_3^- buffering by the blood. This could reflect an initial loss of HCO_3^- equivalents (= gain of acidic equivalents) to the external seawater or intra-

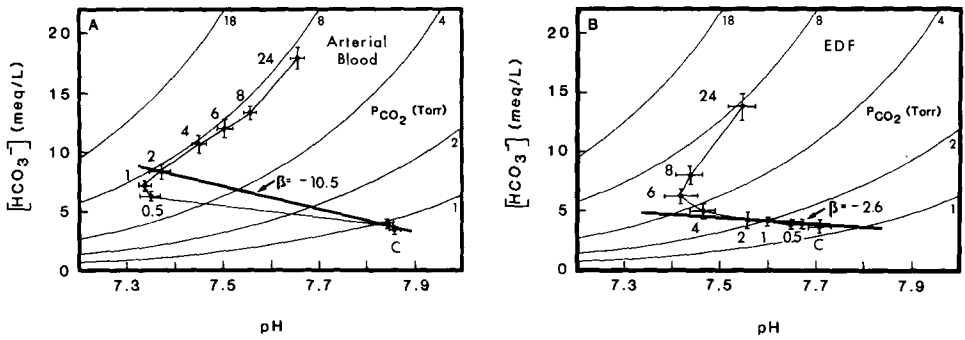

Fig. 4. A graphical representation in *R. ocellata* of the simultaneous changes in pH, HCO_3^-, and P_{CO_2} levels in (A) arterial blood plasma; and (B) extradural fluid during 24 h exposure to normoxic hypercapnia. In (A), the non-HCO_3^- buffer relationship (heavy line) was plotted using the β_{NB} value appropriate for the mean measured Hb concentration (from fig. 1B); in (B), the relationship was plotted using the β_{NB} value appropriate for the mean measured EDF protein concentration (from fig. 1 of Wood *et al.*, 1990). Mean ± 1 SEM (N = 8–11). See legend of fig. 2 for other details.

cellular compartment, and/or a metabolic acid accumulation of unknown origin. Whatever the cause, the phenomenon explained the slower compensation in *R. ocellata* relative to *S. stellaris*. Thereafter, the pattern was similar to that in the dogfish. Similar mechanisms of acidic equivalent exchange between the extracellular compartment, external seawater and intracellular compartment were likely responsible for this later stage of partial acid–base compensation in the two species (*cf.* Heisler, 1989). There was no evidence of preferential RBC pHi adjustment, relative to pHe, during hypercapnia (fig. 3D). This contrasts with the situation in teleosts where catecholamine-mediated increases in RBC pHi are commonly seen (Perry and Wood, 1989). As discussed earlier, the insensitivity of hemoglobin O_2 transport to acidosis in skate presumably renders this response unnecessary. The precise relationships between extracellular and intracellular pH adjustments in other tissues are discussed in the subsequent paper (Wood *et al.*, 1990).

The lack of decrease in plasma Cl^- (table 2) and lack of increase in SID, accompanying the observed increase in plasma HCO_3^- during hypercapnic compensation (fig. 3C) was surprising, but agrees with the observations of Randall *et al.* (1976) on *Scyliorhinus stellaris*. This contrasts with the commonly reported pattern in both freshwater and marine teleosts (*e.g.* Toews *et al.*, 1983). A possible explanation may the unusually high buffer capacity of plasma protein in elasmobranchs; electrical neutrality may be achieved largely through a reduction in protein negative charge during acidosis, rendering plasma SID changes so small as to be undetectable.

Another unusual feature of the present data was the gradual 2–3-fold widening of the Pa_{CO_2}–PI_{CO_2} gradient during environmental hypercapnia (fig. 3A). Both P_{CO_2} values were measured directly, rather than calculated, and an identical pattern was observed in independent terminal measurements where repetitive sampling and blood replacement were not employed (Wood *et al.*, 1990). Thus the phenomenon was not an

experimental artifact. While this response has not been widely reported in previous investigations on environmental hypercapnia (*e.g.* Toews *et al.*, 1983; Heisler, 1989), several studies have noted a similar trend of smaller magnitude (Janssen and Randall, 1975; Randall *et al.*, 1976; Truchot *et al.*, 1980). The response may reflect a combination of the reduced CO_2 capacitance coefficient of the water at high P_{CO_2} (Truchot *et al.*, 1980), plus the breakdown of efficient multicapillary or countercurrent gas exchange accompanying elevated $\dot{V}w$ (fig. 2A).

EDF acid–base responses to hypercapnia. Relative to arterial blood, the acid–base status of extradural fluid changed very slowly during hypercapnia (fig. 3A-C). Display of these data on a pH-HCO_3^--diagram (fig. 4B), together with the appropriate EDF non-HCO_3^--buffer value from Wood *et al.* (1990), demonstrates that until about 4 h, these changes were entirely passive. Thereafter, there was a gradual active accumulation of HCO_3^-. There was no evidence of a compounding metabolic acidosis at any time, in contrast with the pattern in blood (fig. 4A). While a few scattered observations from the early literature have been summarized by Maren (1967), there have been no previous systematic measurements of the acid–base or ionic status of EDF during an acid–base disturbance. The present data show that CO_2 penetrates very slowly into the EDF, and that the ensuing HCO_3^- accumulation is correspondingly delayed. Therefore, these findings support the commonly held view that EDF is simply a slowly formed, slowly exchanging exudate of the blood plasma (Maren, 1967; Fenstermacher and Patlak, 1977). Furthermore, our observations of much faster acid–base changes in CSF and brain tissue, and differential radiolabel entry into EDF *vs* CSF and brain tissue (Wood *et al.*, 1990), demonstrate that EDF does not exchange CO_2, HCO_3^-, or other substances at a significant rate with the brain. The dural membranes must serve as an effective barrier. The complete lack of correlation between EDF acid–base status and ventilatory changes further indicates that the EDF is not involved in the control of ventilation. Apart from perhaps serving as a hydrostatic cushion for the brain, the function of this curious fluid remains unknown.

The action of CO_2 in ventilatory control. The increase in ventilation occurring during normoxic hypercapnia ($P_{I_{CO_2}}$ = 7.5 Torr) in *R. ocellata* (fig. 2A) was substantial ($\sim 2.7\times$), prolonged (>48 h), and generally similar in overall pattern to that described by Janssen and Randall (1975) and Thomas *et al.* (1983) in the rainbow trout, *Salmo gairdneri*. These responses were very different from the smaller ($\sim 1.7\times$) and transitory (<2 h) elevation in ventilation observed by Randall *et al.* (1976) in the dogfish, *S. stellaris*. The reason for this difference is unknown, though the lower level of hypercapnia ($P_{I_{CO_2}}$ = 5 Torr) used in the dogfish study could be a factor. Janssen and Randall (1975) and later Smith and Jones (1982) interpreted the response of *S. gairdneri* as an effect of increased Pa_{CO_2} acting directly and/or indirectly through secondary hypoxemia, and discounted any direct role for pHa. Nevertheless, the time course of ventilatory disturbance and subsequent correction in the trout appeared well correlated with the time course of pHa changes.

In the skate, hypoxemia is not the explanation, for Ca_{O_2}, Pa_{O_2}, PI_{O_2}, and PE_{O_2} all stayed constant or increased during hypercapnia (fig. 2). Pa_{CO_2} and pHa are possible, though by no means exclusive, candidates for the proximate stimulus of increased ventilation during environmental hypercapnia. Both changed markedly in concert with the elevation in $\dot{V}w$ (figs. 2A, 3A,B) and remained disturbed throughout the entire period during which $\dot{V}w$ was elevated. Interpretation is complicated by the fact that Pa_{CO_2} and pHa are not entirely independent variables. Nevertheless, there are several reasons for arguing that pHa is the more important stimulus.

First, there was a small reduction in $\dot{V}w$ (fig. 2A) accompanying the partial correction of pHa by 24 h (fig. 3B), despite the fact that Pa_{CO_2} exhibited a further slight increase at this time (fig. 3A). Second, in preliminary experiments on two skate subjected to exhaustive exercise (C.M. Wood, M.S. Graham, J.D. Turner, unpublished results), Pa_{CO_2} rose only slightly, yet post-exercise increases in ventilation and depressions in pHa were comparable to those occurring during hypercapnia (figs. 2A, 3B). Ca_{O_2} was unaffected. The time course of ventilatory recovery coincided with that of pHa correction. Third, on an individual fish basis, the correlation between the change in pHa and change in $\dot{V}w$ during hypercapnia was more striking than inspection of the mean data in figs. 2A and 3B might indicate. In 9 of the 11 skate subjected to hypercapnia, there were significant relationships (r = 0.60-0.99) between ΔpHa and relative $\dot{V}w$, with slopes ranging from 1.8 to 8.3 × $\dot{V}w$/pH unit. The mean overall relationship (slope = 3.4 × $\dot{V}w$/pH unit) is shown in fig. 5. Fortuitously, the two skate exhibiting greatest and

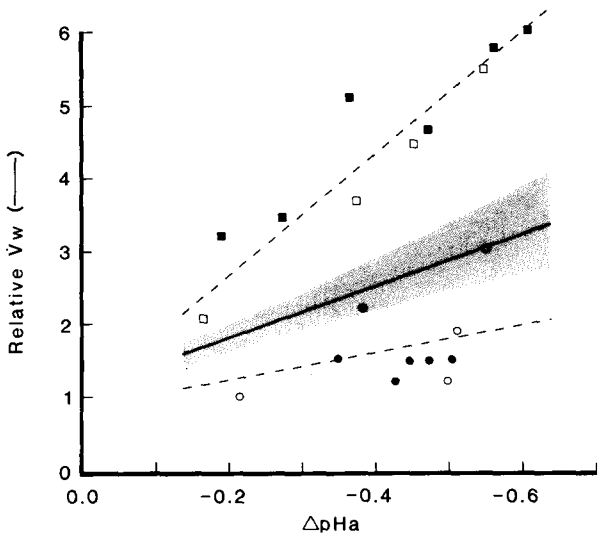

Fig 5. The relationship between relative ventilation volume (Y; control $\dot{V}w$ = 1.0) and the change in arterial pH (ΔpHa, X) from control levels in *R. ocellata* exposed to normoxic hypercapnia. The mean overall relationship in 9 skate (all exposure times) is shown by the solid line with ± 1 SEM indicated by the shaded area: Y = −3.44 (±0.69) X + 1.15 (±0.10) ($P \leq 0.001$). Individual relationships for 2 skate (squares, circles) are also shown by means of dashed lines, with closed symbols representing points recorded during hypercapnia, and open symbols representing points recorded during recovery from exhaustive exercise.

least slope were those subjected to both the hypercapnia and exercise experiments; their individual relationships are also shown in fig. 5. Note that points obtained after exercise (when Pa_{CO_2} was not greatly elevated) followed the same relationships as those during hypercapnia, adding further strength to the argument.

This is not the first study to argue that depressions in pHa may serve as a ventilatory stimulus in elasmobranchs. In *S. stellaris* subjected to hyperoxia-induced hypercapnia, Heisler *et al.* (1988) related changes in ventilation to changes in arterial acid–base, particularly pHa. In a more detailed analysis of these responses based on additional unpublished experiments from his laboratory, Heisler (1989) concluded that $\dot{V}w$ changes were specifically related to pHa, and not to Pa_{CO_2} or HCO_3^-. The present study is in agreement with this conclusion, and provides additional evidence from completely different experimental conditions (environmental hypercapnia, post-exercise recovery). A common feature of all this evidence is that it was obtained under circumstances where O_2 uptake and delivery were not compromised. Thus the primary O_2 drive was unaltered, allowing demonstration of a pHa-related drive. Therefore, a direct acid–base sensitivity could have been present before the evolutionary transition from water- to air-breathing, in contrast to common belief. Heisler (1989) suggested that this secondary respiratory drive may be provided by 'pH-sensitive structures in, or closely related to, the arterial bloodstream'. The acid-base status of the cerebrospinal fluid and brain tissue itself are also certainly important in this regard in higher vertebrates (Shelton *et al.*, 1986); these possibilities are examined by Wood *et al.* (1990).

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