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Tolerance of chronic hypercapnia by the European eel *Anguilla anguilla*

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Summary

European eels were exposed for 6 weeks to water CO_2 partial pressures (P_{CO_2}) from ambient (approx. 0.8 mmHg), through 15±1 mmHg and 30±1 mmHg to 45±1 mmHg in water with a total hardness of 240 mg l^{-1} as $CaCO_3$, pH 8.2, at 23±1°C. Arterial plasma P_{CO_2} equilibrated at approximately 2 mmHg above water P_{CO_2} in all groups, and plasma bicarbonate accumulated up to 72 mmol l^{-1} in the group at a water P_{CO_2} of 45 mmHg. This was associated with an equimolar loss of plasma Cl^-, which declined to 71 mmol l^{-1} at the highest water P_{CO_2}. Despite this, extracellular acid–base compensation was incomplete; all hypercapnic groups tolerated chronic extracellular acidoses and reductions in arterial blood O_2 content (Ca_{O_2}), of progressive severity with increasing P_{CO_2}. All hypercapnic eels, however, regulated the intracellular pH of heart and white muscle to the same levels as normocapnic animals. Hypercapnia had no effect on such indicators of stress as plasma catecholamine or cortisol levels, plasma osmolality or standard metabolic rate. Furthermore, although Ca_{O_2} was reduced by approximately 50% at the highest P_{CO_2}, there was no effect of hypercapnia on the eels' tolerance of hypoxia, aerobic metabolic scope or sustained swimming performance. The results indicate that, at the levels tested, chronic hypercapnia was not a physiological stress for the eel, which can tolerate extracellular acidosis and extremely low Cl^- levels while compensating tissue intracellular pH, and which can meet the O_2 requirements of routine and active metabolism despite profound hypoxaemia.

Key words: hypercapnia, European eel, *Anguilla anguilla*, acid–base balance, aerobic scope, hypoxia, metabolic rate, stress, swimming performance.

Introduction

The partial pressure of CO_2 (P_{CO_2}) in surface waters is typically less than 1 mmHg (1 mmHg = approx. 0.0132 kPa), and that of fish blood is usually equilibrated at approx. 2–3 mmHg above this (Howell, 1970; Cameron and Randall, 1972; Randall and Cameron, 1973). The P_{CO_2} of freshwater may increase as a consequence of microbial metabolism and inadequate surface gas exchange. Such hypercapnic episodes cause acid–base imbalances in fish when CO_2 diffuses into the blood across the gills; a 10 mmHg increase in inspired water P_{CO_2} (Pw_{CO_2}) is adequate to reduce plasma pH by 0.4–0.5 pH units (reviewed by Heisler, 1984, 1993). The primary negative effect of this extracellular acidosis is a decline in blood O_2-carrying capacity, as a consequence of Root and Bohr effects (Heisler, 1984). Severe levels of hypercapnia may also cause a reduction in intracellular pH (pHi), and interfere with cellular metabolism through effects on the function of pH-sensitive proteins (Heisler, 1984, 1993). Teleosts can compensate for hypercapnic acidosis, and consequent effects on blood O_2 carrying capacity and pHi, by ion-exchange at the gills, accumulating bicarbonate (HCO_3^-) ions at the expense of an equimolar loss of chloride (Cl^-) (Heisler, 1984, 1993). Plasma HCO_3^- has accumulated to levels above 50 mmol l^{-1} in teleosts exposed for extended periods (days to weeks) to Pw_{CO_2} levels of up to 25 mmHg (e.g. Dimberg, 1988; Larsen and Jensen, 1997). This implies a significant loss of plasma Cl^-, and it has been suggested that in fish there may be a 'ceiling' to HCO_3^- accumulation, and consequent acidotic compensation (Heisler, 1984, 1993). Indeed, chronic exposure to Pw_{CO_2} >15 mmHg causes significant mortalities in salmonids (Larsen and Jensen, 1997; Fivelstad et al., 1998, 1999).

Steffensen and Lomholt (1990), however, measured water Pw_{CO_2} levels exceeding 30 mmHg in European eel farms using closed-cycle recirculating water systems. It is unlikely that teleosts would ever experience such severe hypercapnia in nature, and no previous investigations into the effects of chronic hypercapnia have employed a Pw_{CO_2} of above 25 mmHg and rarely for longer than 10 days (Heisler, 1984, 1993; Dimberg, 1988; Larsen and Jensen, 1997; Fivelstad et al., 1998, 1999). Unusually low plasma Cl^- concentrations have, however, been reported to occur spontaneously in the

European eel (Farrell and Lutz, 1975), which indicates that it may have an elevated capacity to accumulate plasma HCO_3^- and compensate for acidosis. Furthermore, McKenzie et al. (2002) found that the eel was extremely tolerant of hypercapnic acidosis *per se*. That is, although acute sequential 30 min exposures to water P_{CO_2} levels (Pw_{CO_2}) of 5, 10, 20, 40, 60 and 80 mmHg caused a reduction in arterial pH (pHa) from 7.9 to below 7.2 and a consequent 80% decline in arterial blood total O_2 content (Ca_{O_2}), this had no effect on cardiac output or whole animal O_2 uptake (McKenzie et al., 2002). The selective pressures that led to the evolution of these physiological adaptations are not known, but they do indicate that the eel may be particularly tolerant of chronic hypercapnic acidosis and hypoxaemia. Nonetheless, severely hypercapnic water (Steffensen and Lomholt, 1990) should represent a sub-optimal aquaculture environment and, therefore, elicit elements of a teleost stress response (Barton and Iwama, 1991; Wendelaar Bonga, 1997) in the eels. In particular, chronic hypoxaemia consequent to Bohr and Root effects has the potential to impair the regulation of O_2 delivery, and therefore aerobic metabolism, in response to changes in water O_2 supply (hypoxia) or increased tissue O_2 demand (e.g. sustained aerobic exercise). Acute exposure to a Pw_{CO_2} of 25 mmHg caused a decline in the ability of the eel to regulate metabolic rate in hypoxia (Cruz-Neto and Steffensen, 1997).

In the present study, physiological adaptations to chronic severe hypercapnia were examined by exposing eels to Pw_{CO_2} levels of either 0.8 mmHg (ambient), 15 mmHg, 30 mmHg or 45 mmHg for 6 weeks. The extent of acid–base compensation was assessed both with reference to control normocapnic eels, and to the acid–base disturbances elicited by acute exposure to hypercapnia (McKenzie et al., 2002), to investigate the hypothesis that a capacity to tolerate low plasma Cl^- concentrations would permit the accumulation of large quantities of plasma HCO_3^-. The hypothesis that chronic hypercapnia would elicit a stress response in the eels was investigated using plasma catecholamine and cortisol titres, plasma osmolality and standard metabolic rate as indicators (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Finally, responses to progressive hypoxia and sustained aerobic exercise performance were measured to investigate the hypothesis that hypoxaemia would impair the eels' capacity to regulate O_2 delivery and aerobic metabolism. The results reveal that the European eel is exceptionally tolerant of chronic hypercapnia, and only confirm the first of the three stated hypotheses.

Materials and methods

Experimental animals

European eels (*Anguilla anguilla* L.) were obtained from commercial suppliers in Northern Italy and transported to 'La Casella' Fluvial Hydrobiology Station, via Argine del Ballottino, 29010, Sarmato (PC), Italy, where they were maintained indoors in 1 m^2 tanks, each with a water volume of 250 l and a continuous supply of freshwater at 23±1°C derived from a closed cycle biofilter (total volume 90 m^3). The water had pH 8.2 and total hardness 240 mg l^{-1} as $CaCO_3$; precise details of its chemical composition are as reported by McKenzie et al. (2001). Two sizes of eel were studied: one group with an average live mass of approximately 300 g was used for all experiments except swimming performance, for which eels with an average live mass of approximately 100 g were used. For the large eels, 25 fish were stocked in each tank, whereas for the small eels, 100 fish were stocked per tank. The animals were exposed through skylights to a natural photoperiod and fed extruded pellets (Provimi Grower Select, Provimi Italia, Piacenza, Italy) of appropriate size at a rate of 1% wet mass day^{-1}. The eels were allowed one month's acclimation to the conditions at La Casella prior to exposure to hypercapnia.

Duplicate tanks, of each size of eel, were then exposed to one of four Pw_{CO_2} levels: 0.8±0.1 (ambient control), 15±1 mmHg, 30±1 mmHg and 45±1 mmHg. Pw_{CO_2} levels were monitored indirectly as water pH and regulated around each hypercapnic setpoint with the automated feedback system described in McKenzie et al. (2002). The eels were acclimated to hypercapnia gradually, by increasing the Pw_{CO_2} by 5 mmHg every 2–3 days. Thus, 9 days were required to reach a Pw_{CO_2} of 15 mmHg, higher levels requiring proportionally more time. The eels were then exposed to their appropriate Pw_{CO_2} for at least 6 weeks' prior to use in any experiments, and were fed at a rate of 1% body mass day^{-1} throughout the exposure period.

Surgical preparation

The large eels were anaesthetised and cannulated in the dorsal aorta and operculum, as described in McKenzie et al. (2000), in water equilibrated either with air (controls) or the appropriate air/CO_2 mixture. Following surgery, fish were transferred to individual 6 l opaque black Plexiglas chambers where they recovered for 48 h in a continuous flow of water at the appropriate Pw_{CO_2}. The two cannulae were drawn out of a small hole in the lid of the chamber so that they could be manipulated without disturbing the fish. The dorsal aortic cannula was flushed twice daily with heparinised (10 i.u. l^{-1}) Cortland's saline (Wolf, 1963).

Blood acid–base and dissolved gas status

Following 48 h recovery from surgery, a 2 ml blood sample was collected from the dorsal aortic cannula and replaced by an equal volume of Cortland's saline. Arterial blood pH (pHa) was measured with a Radiometer BMS2 capillary pH electrode (Brønshøj, Denmark) thermostatted to the same water temperature as the fish, with the signals displayed on a Radiometer PHM73 acid–base analyser. Arterial blood total CO_2 content (Ca_{CO_2}) and total O_2 content (Ca_{O_2}) were measured as described by Cameron (1971) and Tucker (1967), respectively, with appropriate Radiometer electrodes thermostatted to 37°C. Arterial plasma P_{CO_2} (Pa_{CO_2}) and arterial [HCO_3^-] were calculated from the measured values of pHa and arterial [CO_2] using the Henderson–Hasselbach equation and apparent pK and αCO_2 values for trout plasma at

23°C, calculated as described in Boutilier et al. (1984). The remaining blood was rapidly centrifuged and the decanted plasma stored in liquid nitrogen for subsequent analysis of osmolality, ion concentrations and hormone titres.

Ventilation

In all cases, ventilation was measured prior to collection of the blood sample and care was taken to minimise disturbance to the fish prior to and during measurements. The water-filled opercular cannula was attached to a differential pressure transducer (Validyne 45DF, Northridge, CA, USA) and gill ventilation rate (f_G, beats min^{-1}) and opercular pressure amplitude (P_{OP}, in Pa) were displayed on a chart recorder (Gould Windograf, Valley View, OH, USA) as described by McKenzie et al. (2000). Following manipulation of the opercular cannula, the eels were allowed at least 1 h for ventilatory activity to stabilise prior to collection of data for 30 min. To quantify ventilation, the rate was counted for three 5 min periods within each sampling interval, and P_{OP} averaged from 10 measurements of individual waveforms within that period. P_{OP} was used as an index of ventilatory effort.

Tissue intracellular pH

Following collection of the blood sample, the eels were injected with 1 ml of a 5 g l^{-1} solution of MS-222 in saline, which caused loss of ventilatory movements within 5 s. The animals were removed rapidly from their chamber, decapitated, and samples of white muscle and the heart 'freeze-clamped' with aluminium tongs and frozen in liquid nitrogen within 30 s of death. Tissue intracellular pH (pHi) was measured as described by Pörtner et al. (1990). The tissue was ground to a fine powder while frozen under liquid nitrogen. The powdered tissue was then defrosted in a sealed microcentrifuge (Eppendorf) tube containing metabolic inhibitor solution and, following centrifugation, the pH of the supernatant was measured with a Radiometer capillary electrode thermostatted to the temperature of the fish and attached to a Radiometer PHM 73 blood-gas analyser. Care was taken to ensure that the Eppendorf tube contained absolutely no air bubbles, as preliminary measurements revealed that defrosting and centrifugation of the samples with access to air caused highly variable tissue pH values, presumably as a consequence of the rapid loss of gaseous CO_2 from the defrosted sample's supernatant.

Plasma ion concentrations and osmolality

Plasma Cl^- concentration was measured amperometrically with a chloride titrator (American Instruments Company, USA). Plasma cations (Na^+ and Ca^{2+}) were analysed by atomic absorption spectrophotometry (Phillips PYE Unicam SP9, Cambridge, UK). Plasma osmolality was measured with an osmometer (Fiske One-Ten, Fiske Associates, Norwood, MA, USA).

Plasma catecholamines and cortisol

Catecholamines were extracted from 250 µl samples of plasma added to 2.75 ml of phosphate buffer, pH 3, with octanesulphonic acid (0.02 mg ml^{-1}) and then loaded onto a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) pre-activated by washing with methanol (3 ml) and water (6 ml). The cartridge was eluted with 5 ml of water and 5 ml of 30% methanol, pH 3. The organic phase was collected and dried under vacuum and the residue suspended in 125 µl of methanol. Recovery rates were approximately 90% on extracted standards (three determinations). The extracted samples were measured by high performance liquid chromatography (HPLC) (Waters) with a Model 510 pump (Waters) and Rheodyne 7715 injector (Rohnert Park, CA, USA). A 50 µl sample was injected onto a 5 µm Symmetry (Waters) C_{18} Shield column (250 mm × 4.6 mm i.d.) with precolumn. The eluent was composed of two solutions, A and B (87:13 mixture, v/v), where A was 100 mmol l^{-1} NaH_2PO_4, 100 mg l^{-1} EDTA, 250 mg l^{-1} octansulphonic acid, pH 3, and B was a 3:2 (v/v) solution of methanol:acetonitrile. Elution was performed in isocratic mode at a flow rate of 1.3 ml min^{-1}. A coulometric detector (Coulochem II, ESA, Chelmsford, MA, USA) was used with the following analytical conditions: Guard cell, +250 mV; Cell 1, –50 mV; Analytical cell, –200 mV (gain range 200 nA). The data were acquired and integrated by dedicated software (Waters Millenium 2010). Samples were compared against curves derived from standard solutions of noradrenaline (25.5 ng l^{-1}) and adrenaline (29.5 ng l^{-1}) (Sigma, Sigma-Aldrich Srl, Milan, Italy).

Cortisol was extracted by adding 250 µl plasma to 20 µl of glacial acetic acid, loading the resultant solution onto Ultrafree-PF filter (5000 M_r cut-off, Millipore, Bedford, MA, USA), and filtering by centrifuging at 100 ***g*** for 10 min. The filtrate was then transferred to a 10 ml test tube, and extracted twice with 4 ml of diethyl ether. The organic phases were collected, dried under vacuum, and the residue resuspended in 70 µl of methanol. Recovery was approximately 90% when measured on duplicate standard samples. Cortisol was measured by HPLC, as described in Volin (1992). A 50 µl sample of extract was injected onto a Symmetry C_{18} 250 mm×4.6 mm i.d. column with precolumn. Analyses were performed in gradient mode using two pumps (Waters Model 510) equipped with a Rheodyne 7715 injector coupled with a Model 996 photodiode-array detector (Waters). The eluents were (A) 30 mmol l^{-1} NaH_2PO_4, pH 3, and (B) acetonitrile, and the linear gradient was 30 min at 40:60 ratio of A:B, followed by 35 min at 100% A; flow-rate was 1.5 ml min^{-1}. Detection was carried out at 245 nm and the chromatograms stored in the range 200–400 nm. The data were acquired and integrated by dedicated software (Waters Millenium 2010). Samples were compared against a curve derived from a standard solution of cortisol (50 mg l^{-1}) (Sigma).

Metabolic rate and tolerance of hypoxia

Metabolic rate was measured as O_2 uptake. Automated intermittent flow-through respirometry (Steffensen, 1989) was used to measure the instantaneous O_2 uptake rates ($\dot{M}_{O_2}$, in mmol O_2 kg^{-1} h^{-1}) of individual non-instrumented eels once

Table 1. *Extracellular and intracellular acid–base status in eels exposed for at least 6 weeks to water CO_2 partial pressures (Pw_{CO_2}) of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg*

	Pw_{CO_2} (mmHg)			
	Approx. 0.8	15±1	30±1	45±1
a[CO_2] (mmol l^{-1})	13.0±1.6[a]	41.6±1.6[b]	59.8±2.6[c]	74.7±4.0[d]
pHa	7.89±0.04[a]	7.75±0.02[a,b]	7.63±0.02[b]	7.57±0.04[b]
Pa_{CO_2} (mmHg)	3.7±0.3[a]	17.5±1.3[b]	35.0±1.7[c]	47.5±2.9[d]
a[HCO_3^-] (mmol l^{-1})	12.8±1.5[a]	40.8±1.6[b]	58.2±2.6[c]	72.5±4.1[d]
White muscle pHi	7.13±0.05[a]	7.03±0.02[a]	7.03±0.05[a]	7.01±0.03[a]
Heart pHi	7.02±0.03[a]	7.10±0.03[a]	7.18±0.03[a]	7.13±0.01[a]

1 mmHg=approx. 0.01316 kPa.

a[CO_2], arterial blood total CO_2 content; pHa, arterial pH; Pa_{CO_2}, arterial CO_2 partial pressure; a[HCO_3^-], arterial bicarbonate concentration; pHi, intracellular pH.

Values are means ± 1 S.E.M. of six determinations, except for blood acid–base variables, where N=7 at Pw_{CO_2} of 30 mmHg and N=5 at Pw_{CO_2} of 45 mmHg.

A common superscript indicates no significant difference between groups (P>0.05).

every 10 min, using a system described in detail by McKenzie et al. (2000). Eels were placed in a respirometer chamber and allowed to recover overnight in water at the appropriate P_{CO_2}. $\dot{M}_{O_2}$ was then measured for 24 h, and routine metabolic rate (RMR) calculated as the average rate measured over the entire 24 h period. Standard metabolic rate (SMR), the amount of O_2 required for minimum maintenance metabolism, was estimated as the average of the six lowest measured values of $\dot{M}_{O_2}$ for each individual animal within the 24 h period, thus representing, a cumulative time of 1 h (Cruz-Neto and Steffensen, 1997).

Hypoxia tolerance in the eels was assessed as the critical water P_{O_2} level (Pw_{O_2}) at which the eels were no longer able to maintain SMR during progressive hypoxia (McKenzie et al., 2000). Eels from each experimental group were exposed to gradual progressive hypoxia, with Pw_{O_2} reduced from saturation (Pw_{O_2}=*circa* 150 mmHg) to 120, 100, 80, 60, 40, 30 and 20 mmHg every 20 min (two complete 10 min measurement cycles of the automated system), and the critical Pw_{O_2} for maintenance of SMR then calculated as described by McKenzie et al. (2000).

Sustained aerobic exercise performance

Exercise performance and associated respirometry were measured on small eels (mean live mass approximately 100 g) with the automated Brett-type swim-tunnel respirometer described by McKenzie et al. (2001). Eels were placed in the respirometer and trained to swim at a water speed equivalent to 0.5 body lengths per second ($BL\ s^{-1}$) for at least 12 h (overnight). The following day, the eels were exposed to progressive increments in swimming speed, of 0.25 $BL\ s^{-1}$ every 40 min, until exhaustion. Measurements of $\dot{M}_{O_2}$ were collected at each swimming speed, and used to calculate the theoretical rate of O_2 uptake of a stationary resting fish (Brett, 1958). This notional value, termed 'immobile metabolic rate' (IMR), was used to derive aerobic scope and the net aerobic metabolic cost of swimming at each speed (Beamish, 1978), as described in detail by McKenzie et al. (2001). Maximum sustainable (critical) swimming speed (U_{crit}) was calculated (in $BL\ s^{-1}$) as described by Brett (1964).

Statistical analyses

Comparisons amongst groups for any given variable were made by one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* tests to establish significant differences amongst groups. Statistical significance was attributed at a 95% limit of confidence (P<0.05).

Results

Exposure to chronic hypercapnia elicited profound physiological responses in the eels, but did not cause any mortalities.

Extracellular and intracellular acid–base compensation

All of the hypercapnic groups exhibited a marked increase in a[CO_2], progressively from 13 mmol l^{-1} in control animals to almost 75 mmol l^{-1} in animals exposed to 45 mmHg Pw_{CO_2} (Table 1). This was associated with a progressive decline in pHa (Table 1). Calculation of Pa_{CO_2} revealed that it was equilibrated at approximately 2 mmHg above Pw_{CO_2} in each group, while plasma bicarbonate concentrations were elevated in all hypercapnic groups, to above 70 mmol l^{-1} in the group at a Pw_{CO_2} of 45 mmHg (Table 1). As can be seen in the pH/bicarbonate (Davenport) diagram (Fig. 1), the decline in pHa during hypercapnia did not parallel the non-bicarbonate buffer line calculated by Hyde et al. (1987) for plasma of the closely related American eel *Anguilla rostrata*, but deviated significantly above it. Fig. 1 also carries data reported by McKenzie et al. (2002), describing the effects on acid–base status of acute exposure to hypercapnia, consisting of sequential exposure at 30 min intervals to Pw_{CO_2} levels of 5, 10, 20, 40, 60 and 80. Comparison with the data from the present study reveals that the animals in chronic hypercapnia

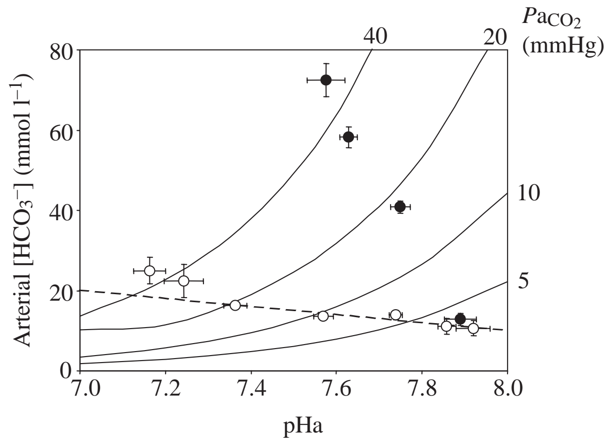

Fig. 1. A pH–HCO_3^- Davenport diagram describing (black symbols) the blood acid–base status of eels exposed for at least 6 weeks to water CO_2 partial pressures (Pw$_{CO_2}$) of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg (1 mmHg=approx. 0.01316 kPa). Pa$_{CO_2}$ levels are equilibrated at approximately 2 mmHg above Pw$_{CO_2}$ in each group (see text). The diagram also carries data (white symbols) replotted from McKenzie et al. (2002) describing blood acid–base status following acute sequential 30 min exposures to Pw$_{CO_2}$ levels of 5, 10, 20, 40, 60 and 80 mmHg. The dashed line indicates the *in vitro* whole blood non-HCO_3^- buffer line for *A. rostrata* provided by Hyde et al. (1987). Values are means ± S.E.M., N=5–7 determinations.

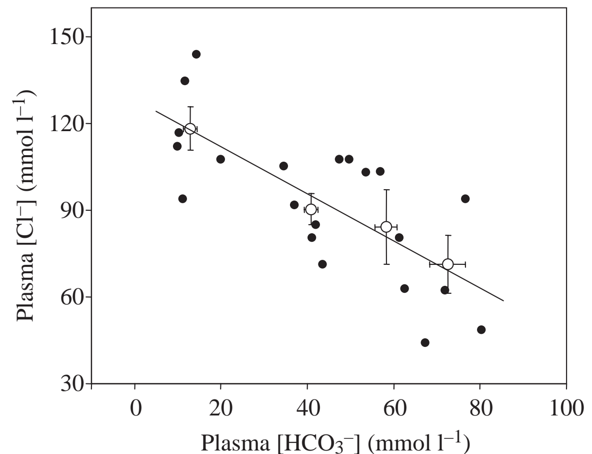

Fig. 2. The relationship between plasma concentrations of chloride and bicarbonate in eels exposed for at least 6 weeks to water CO_2 partial pressures (Pw$_{CO_2}$) of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg (1 mmHg=approx. 0.01316 kPa). The black symbols indicate individual data points, the white symbols denote the mean values for each Pw$_{CO_2}$. The regression line for the individual data points is described by the linear relationship [HCO_3^-]=–0.817[Cl^-]+128 (r^2=0.536, N=24), with concentrations in mmol l^{-1}.

exhibited a profound accumulation of plasma bicarbonate and a consequent compensation of pHa for any given Pa$_{CO_2}$. The compensation of pHa was, however, only partial and animals at a Pw$_{CO_2}$ of 30 and 45 mmHg were suffering from chronic extracellular acidosis (Table 1). All of the hypercapnic groups, however, regulated the intracellular pH of their white muscle and heart to levels unchanged from those of normocapnic animals (Table 1).

Plasma ion concentrations

As shown in Fig. 2, there was a highly significant negative linear relationship between plasma bicarbonate and plasma chloride (P<0.0001), with an almost equimolar loss of plasma chloride for each bicarbonate ion accumulated. However, plasma Cl^- concentrations were only significantly lower than the controls at a Pw$_{CO_2}$ of 45 mmHg, due to wide individual variations (Farrell and Lutz, 1975) within all groups. There were no significant effects of chronic hypercapnia on plasma Na^+ or Ca^{2+} concentrations (Table 2).

Blood O_2 content and ventilatory responses

The increased Pa$_{CO_2}$ in the hypercapnic groups, and the accompanying extracellular acidosis, was linked to a progressive reduction in Ca$_{O_2}$, presumably as a result of Root and Bohr effects on haemoglobin-O_2 binding. The hypoxaemia was significant in animals at 30 and 45 mmHg Pw$_{CO_2}$ (Table 3). Fig. 3 compares the percentage reduction in Ca$_{O_2}$ in eels from the present study with data replotted from McKenzie et al. (2002), describing the effects of acute hypercapnia. The comparison reveals that during chronic exposure the eels had a less severe hypoxaemia at any given Pa$_{CO_2}$. However, Fig. 3 also shows that the relationship between pHa and Ca$_{O_2}$ was the same in eels exposed to chronic hypercapnia (this study) and in those exposed to acute hypercapnia as reported by McKenzie et al. (2002). Thus, the increased Ca$_{O_2}$ at any given Pw$_{CO_2}$ in chronic *versus* acute exposure was only a result of compensation of pHa, not modifications of haemoglobin-O_2 affinity. Despite the chronic hypoxaemia at a Pw$_{CO_2}$ of 30 or 45 mmHg, however, there were no significant effects on either ventilatory frequency or effort, where this latter was measured as P_{OP} (Table 3).

Stress indicators

Exposure to hypercapnia had no effect on plasma

Table 2. *Plasma ion concentrations in eels exposed for at least 6 weeks to water CO_2 partial pressures* (Pw$_{CO_2}$) *of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg*

Pw$_{CO_2}$ (mmHg)	[Cl^-] (mequiv l^{-1})	[Na^+] (mequiv l^{-1})	[Ca^{2+}] (mequiv l^{-1})
Approx. 0.8	118±7^{a}	140±7^{a}	6.9±0.5^{a}
15±1	90±5^{a,b}	116±5^{a}	7.1±2.5^{a}
30±1	84±13^{a,b}	120±8^{a}	6.6±0.4^{a}
45±1	71±10^{b}	116±7^{a}	9.2±1.0^{a}

1 mmHg equals=0.01316 kPa.

Values are mean ± 1 S.E.M., N=5–7 determinations.

A common superscript indicates no significant difference between groups (P>0.05).

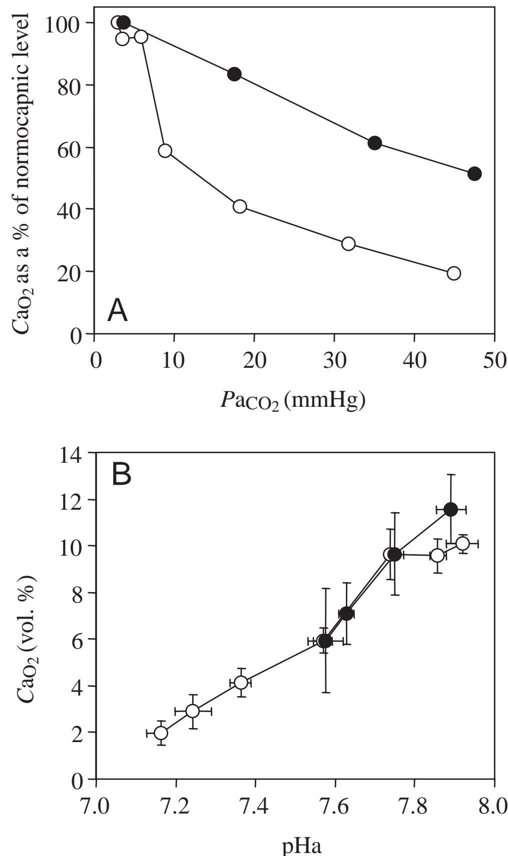

Fig. 3. (A) The effects of increasing arterial CO_2 partial pressures Pa_{CO_2} on arterial total O_2 content (Ca_{O_2}), expressed as the percentage change relative to normocapnia, in eels exposed either to chronic (black symbols) or acute (white symbols) hypercapnia. (B) The relationship between arterial blood pH (pHa) and Ca_{O_2} in eels exposed to either chronic (black symbols) or acute (white symbols) hypercapnia. Chronic hypercapnia implies at least 6 weeks exposure to water CO_2 partial pressures (Pw_{CO_2}) of approx. 2mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg; acute hypercapnia implies sequential 30 min exposures to a Pw_{CO_2} of 5, 10, 20, 40, 60 and 80 mmHg (1 mmHg=approx. 0.01316 kPa). Data for acute hypercapnia are replotted from McKenzie et al. (2002). Values are means ± S.E.M., N=5–7 determinations.

noradrenaline, adrenaline or cortisol levels, which were low in all groups (Table 4). There were no differences in plasma osmolality amongst the four experimental groups, the evidence of increased osmolality at a Pw_{CO_2} of 30 mmHg and 45 mmHg was not significant (Table 4). Furthermore, as can be seen in Table 4, there were no significant effects of hypercapnia on RMR or SMR.

Regulation of aerobic metabolism during hypoxia and sustained aerobic exercise

In all groups, exposure to hypoxia was associated with a decline towards and beyond SMR as hypoxia deepened (data not shown), but there was no effect of hypercapnia on the critical water P_{O_2} for maintenance of SMR (Table 5), indicating that all groups were equally tolerant of hypoxia.

Table 3. *Arterial O_2 content and ventilatory activity in eels exposed for at least 6 weeks to water CO_2 partial pressures (Pw_{CO_2}) of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg*

Pw_{CO_2} (mmHg)	Ca_{O_2} (vol.%)	f_G (beats min^{-1})	P_{OP} (Pa)
Approx. 0.8	11.6±1.5[a]	34±4[a]	23±9[a]
15±1	9.6±1.7[a,b]	32±1[a]	19±4[a]
30±1	7.1±1.3[b]	29±3[a]	32±1[a]
45±1	5.9±2.2[b]	39±6[a]	32±7[a]

1 mmHg=approx. 0.01316 kPa.

Ca_{O_2}, arterial total O_2 content; f_G, gill ventilation frequency; P_{OP}, opercular pressure amplitude.

Values are mean ± 1 S.E.M., N=5–7 determinations.

A common superscript indicates no significant difference between groups (P>0.05).

There were also no significant effects of hypercapnia on any aspect of exercise metabolism or performance. The eels from all groups showed an exponential increase in O_2 consumption with increased swimming speed, Fig. 4 shows this exponential relationship between swimming speed and total O_2 uptake in the control group and the group exposed to the highest P_{CO_2}, at 45 mmHg, and also the power relationship between swimming speed and net cost of swimming in these same two groups. These exponential and power relationships were essentially indistinguishable amongst all the four groups, and maximum $\dot{M}_{O_2}$, aerobic scope and U_{crit} were not statistically different (Table 5).

Discussion

The exceptional tolerance of chronic hypercapnia exhibited by the European eel is greater than has been described in any other teleost to date (Heisler, 1993; Larsen and Jensen, 1997) and is another example of the remarkable capacity for physiological adaptation that characterises the genus *Anguilla* (Tesch, 1977; van Waarde, 1983; Hyde et al., 1987). Some elements of the hypercapnia tolerance observed in the present study may have been a consequence of the hard water employed; Larsen and Jensen (1997) found that increased water [HCO_3^-] improved the ability of rainbow trout *Oncorhynchus mykiss* to accumulate plasma HCO_3^- and tolerate hypercapnia, but that the best acid–base compensation was observed in trout exposed in water containing both high HCO_3^- and high Ca^{2+} concentrations. The recirculating water in European eel farms is rich in sodium bicarbonate, which is added to maintain water pH (Steffensen and Lomholt, 1990), so it would be interesting to study the responses of eels to hypercapnia in softwater.

The accumulation of plasma HCO_3^- to levels above 70 mmol l^{-1} by the eels exposed to a Pw_{CO_2} of 45 mmHg is the highest yet reported in a teleost (Börjeson, 1977; Jensen and Weber, 1982; Dimberg, 1988; Larsen and Jensen, 1997)

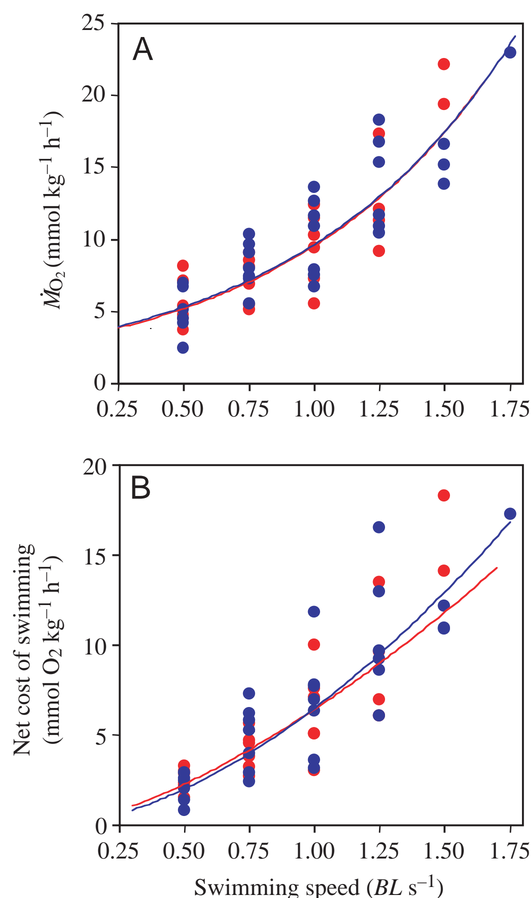

Fig. 4. (A) The relationship between swimming speed and O_2 uptake, and (B) between swimming speed and net cost of swimming (as O_2 uptake; see text for details), in normocapnic control eels (red symbols and regression line) and in eels exposed for at least 6 weeks to a water CO_2 partial pressure of 45±1 mmHg (blue symbols and regression line). In A, for control eels the relationship was described by the exponential equation $y=2.85e^{1.20x}$ ($r^2=0.699$, $N=23$ observations on six animals), whereas for eels exposed to hypercapnia the equation was $y=2.94e^{1.19x}$ ($r^2=0.714$, $N=29$ observations on seven animals). In B, for control eels the relationship was described by the power equation $y=6.45x^{1.50}$ ($r^2=0.755$, $N=23$ observations on six animals), whereas for eels exposed to hypercapnia the relationship was described by the equation $y=6.51x^{1.70}$ ($r^2=0.739$, $N=29$ observations on 7 animals).

and was linked to the ability of the European eel to tolerate unusually low plasma Cl^- levels (Farrell and Lutz, 1975), as demonstrated by the inverse approximately 1:1 relationship between plasma [HCO_3^-] and plasma [Cl^-]. A similar equimolar relationship has been described in rainbow trout exposed to hypercapnia (Larsen and Jensen, 1997). The HCO_3^- accumulation allowed the eels to compensate for the acute acidosis that occurs upon initial exposure to hypercapnia (McKenzie et al., 2002). It seems unusual, however, that the eels did not completely compensate for the chronic extracellular acidosis at a $P\mathrm{w}_{CO_2}$ of 15 or 30 mmHg if they were capable of the profound accumulation of HCO_3^- observed at a $P\mathrm{w}_{CO_2}$ of 45 mmHg. That is, the eels tolerated some degree of extracellular acidosis at all levels of hypercapnia. One possible explanation for the incomplete regulation of pHa may be that the eels regulated extracellular pH only as far as was required to ensure regulation of pHi. Although it is well established that teleosts regulate pHi in hypercapnia (Heisler, 1984, 1993), the present study may have been the most extreme conditions for which such regulation has been demonstrated.

Given the ability of the eel to regulate pHi during hypercapnia, the primary physiological imbalances appear to have been the chronic extracellular acidosis, exceptionally low plasma chloride levels and hypoxaemia. McKenzie et al. (2002) found that an 80% reduction in $C\mathrm{a}_{O_2}$ elicited by acute hypercapnia exposure had no effect on routine metabolic rate in the eel, so the reduction in $C\mathrm{a}_{O_2}$ levels of approximately 50% observed at a $P\mathrm{w}_{CO_2}$ of 45 mmHg in the present study would not, therefore, be expected to limit the ability of the eels

Table 4. *Plasma concentrations of stress hormones, plasma osmolality and metabolic rate in eels exposed for at least 6 weeks to water CO_2 partial pressures ($P\mathrm{w}_{CO_2}$) of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg*

	$P\mathrm{w}_{CO_2}$ (mmHg)			
	Approx. 0.8	15±1	30±1	45±1
Adrenaline (nmol l^{-1})	3.11±0.31	3.42±0.33	3.11±0.62	3.92±0.31
Noradrenaline (nmol l^{-1})	5.31±0.90	5.72±1.10	7.32±1.22	6.90±1.11
Cortisol (nmol l^{-1})	22.30±3.72	33.61±6.33	27.62±7.82	27.80±10.51
Osm (mosmol kg^{-1})	292±6	296±4	309±12	300±4
RMR (mmol O_2 kg^{-1} h^{-1})	–	0.94±0.37	1.16±0.37	1.07±0.18
SMR (mmol O_2 kg^{-1} h^{-1})	0.85±0.10	0.61±0.13	0.74±0.31	0.80±0.24

1 mmHg=approx. 0.01316 kPa.

Osm, osmolality; RMR, routine metabolic rate; SMR, standard metabolic rate (please see text for details).

Values are means ± 1 S.E.M.

For plasma hormones and osmolality, N=5–7 determinations.

For metabolic rate, N=8 in all cases.

There were no significant differences amongst groups for any variable (P>0.05).

Table 5. *Tolerance of hypoxia, and selected metabolic and performance traits for sustained aerobic exercise, in eels exposed for at least 6 weeks to water CO_2 partial pressures ($P_{W_{CO_2}}$) of approx. 0.8 mmHg (ambient control), 15±1 mmHg, 30±1 mmHg or 45±1 mmHg*

	$P_{W_{CO_2}}$ (mmHg)			
	Approx. 0.8	15±1	30±1	45±1
$P_{W_{O_2crit}}$ (mmHg)	31±14	24±11	32±10	27±12
IMR (mmol O_2 kg^{-1} h^{-1})	–	3.05±0.557	3.75±0.34	3.36±0.58
AMR (mmol O_2 kg^{-1} h^{-1})	14.70±2.29	16.81±3.35	16.64±2.40	15.25±2.37
Aerobic scope (AMR/IMR)	4.80±0.37	6.07±1.15	4.66±1.00	5.44±1.26
U_{crit} (BL s^{-1})	1.42±0.09	1.39±0.10	1.43±0.09	1.45±0.13

1 mmHg=approx. 0.01316 kPa.

$P_{W_{O_2crit}}$, critical hypoxic water O_2 partial pressure required for maintenance of standard metabolic rate; IMR, immobile metabolic rate; AMR, active metabolic rate; U_{crit}, maximum sustainable aerobic swimming speed (please see text for details); *BL*, body length.

Values are means ±1 S.E.M., *N*=8 in all cases.

There were no significant differences amongst groups for any variable.

to meet their routine O_2 requirements. Indeed, there were no significant effects of chronic hypercapnia on RMR or SMR, and this would explain the absence of any hyperventilation in the hypercapnic groups. However, the $P_{a_{CO_2}}$, pHa and $C_{a_{O_2}}$ levels measured in the hypercapnic groups in the present study elicited a significant hyperventilation when they were created by acute exposure (McKenzie et al., 2002), revealing compensation of the ventilatory response during chronic exposure (Larsen and Jensen, 1997).

The circulating levels of the catecholamines adrenaline and noradrenaline increase in teleost fishes immediately in response to a variety of physical and environmental stresses that require enhanced O_2 transport, such as exhaustive exercise, hypoxia or hypercapnia (Randall and Perry, 1992). It is perhaps somewhat surprising, therefore, that the hypercapnic eels did not show a chronic elevation of plasma catecholamines, despite their extracellular acidosis and hypoxaemia. Catecholamines are, however, an indicator of acute rather than chronic stress in fish (Randall and Perry, 1992), and members of the genus *Anguilla* do not show a pronounced catecholamine release when compared with salmonids (Gilmour, 1998). An elevation of plasma cortisol is the most widely used primary indicator of chronic sublethal physiological stress in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). The absence of any increase in plasma cortisol is an indication, therefore, that hypercapnia at the levels tested was not stressful to the eel. In many freshwater teleosts, reductions in plasma osmolality and increases in metabolic rate can occur as a secondary consequence of an underlying endocrine response (Wendelaar Bonga, 1997). The absence of any variations in osmolality or SMR in the eels is, therefore, consistent with the absence of an endocrine response, and is further evidence that chronic hypercapnia did not present a sublethal stress. The fact that hypercapnia did not cause a decline in SMR indicates that there was no anaesthetic effect of CO_2 (Bernier and Randall, 1998). Conversely, the absence of any increase in SMR, which represents the minimum costs of organismal maintenance, may indicate that energetic costs for acid–base and ion regulation are low in the eel, as they appear to be in other teleosts (Morgan and Iwama, 1999).

It was unexpected that the ability of eels exposed to hypercapnia to regulate aerobic metabolism in hypoxia was equal to the normocapnic controls, despite significant hypoxaemia in animals at $P_{W_{CO_2}}$ levels of 30 mmHg and 45 mmHg. A previous study found that acute hypercapnia exposure decreased tolerance of hypoxia in the eel (Cruz-Neto and Steffensen, 1997), and this difference from the results of the present study may be linked to the severe effects of acute exposure on blood O_2 content (McKenzie et al., 2002) that were ameliorated in chronic hypercapnia by the compensation of acid–base status. The differences in hypoxia tolerance may also have been a consequence of the effects of water chemistry on the regulation of acid–base balance (Larsen and Jensen, 1997). Cruz-Neto and Steffensen (1997) studied eels in water with a hardness of 150 mg l^{-1} as $CaCO_3$, compared with the hardness of 240 mg l^{-1} as $CaCO_3$ in the present study. Nonetheless, the ability of the eels in the present study to regulate their aerobic metabolism in hypoxia, despite quite profound hypoxaemia, demonstrates that their cardiovascular and ventilatory systems possess an exceptional capacity to meet the O_2 demands of their tissues (McKenzie et al., 2002).

In salmonids, sustained aerobic swimming performance is impaired by elevated water CO_2 levels (Dahlberg et al., 1968), and their maximum performance appears to be closely matched to the capacity of their cardiovascular system for O_2 convection, as reductions in $C_{a_{O_2}}$ cause reductions in aerobic scope, AMR and U_{crit} (Jones, 1971; Brauner et al., 1993; Gallaugher et al., 1995, 2001). Contrary to expectations, chronic hypercapnia did not have any negative impact on these performance traits in the eel and, despite a 50% reduction in $C_{a_{O_2}}$, the animals adapted to a $P_{W_{CO_2}}$ of 45 mmHg exhibited the same fivefold increase in $\dot{M}_{O_2}$ and achieved the same AMR as observed in normocapnia. The fact that eels in normocapnia did not perform better than those adapted to a $P_{W_{CO_2}}$ of

45 mmHg, despite having twice the Ca_{O_2}, leads to the conclusion that the capacity for O_2 convection in the eel is not closely matched to maximum tissue O_2 demands during exercise, but can exceed them. Although it must be presumed that sufficiently extreme reductions in Ca_{O_2} will eventually limit O_2 convection and exercise performance, the present results may indicate that, unlike in salmonids, aerobic scope and AMR in the eel can be determined by the maximum capacity of the respiring tissues to use O_2 and perform work. Compensation of heart pHi may have been important in preserving cardiac performance and O_2 convection during exercise, although the eel heart possesses an exceptional intrinsic tolerance of both hypercapnic acidosis (McKenzie et al., 2002) and hypoxia (Davie et al., 1992). Compensation of pHi in the working muscles may have ensured their maximum performance and oxygen consumption, and would explain the absence of any effects of hypercapnia on the net aerobic metabolic costs, the energetic efficiency (Beamish, 1978), of swimming. Sustained aerobic exercise performance has long been considered a valid means of revealing underlying sublethal stresses in fish (Brett, 1958; Randall and Brauner, 1991), so these results are yet further evidence that the eel was not stressed by such severe chronic hypercapnia.

In conclusion, the results indicate that the eel's exceptional tolerance of chronic hypercapnia is due to an ability to withstand chronic extracellular acidosis, hypoxaemia and extremely low Cl^- levels, an ability to regulate pHi independently of extracellular acid–base status, and a remarkable capacity to meet the O_2 demands of routine and active metabolism, despite hypoxaemia. The life history of the eel must have provided the selective pressures that led to the evolution of these particular physiological adaptations. One ecological trait that may be particularly relevant is the habit of eels to make excursions into air, reportedly to escape poor water quality or to migrate through damp vegetation to colonise new water bodies (Tesch, 1977). Such air-exposure inhibits O_2 uptake and CO_2 excretion, causing acidosis and hypoxaemia (Hyde et al., 1987), which can be expected to be exacerbated if the animals were also exercising. This, then, might select for the observed ability to tolerate acidosis, and also for an ability to meet the O_2 demands of routine and active metabolism when O_2 supply is limited – a profound reserve capacity for O_2 convection by the cardiovascular system. The habit of the eel to live in confined spaces when in water (Tesch, 1977), where O_2 supply may be limited, may also have selected for a reserve capacity for O_2 convection. It is less easy to speculate about which selective pressures might have led to the eel's ability to withstand extremely low plasma Cl^- levels (Farrell and Lutz, 1975), and the unusual physiological adaptations that allow the eel to tolerate chronic severe hypercapnia are all interesting topics for further study.

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