

Physiological impact of salinity increase at organism and red blood cell levels in the European flounder (*Platichthys flesus*)

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Abstract

Blood respiratory, acid–base, and ionic changes in response to hyperosmotic shock were studied in vivo and in vitro in the European flounder. One primary aim was to evaluate regulatory changes in red blood cell (RBC) volume and its interrelationship with blood O₂ transporting properties. An acute increase in the ambient salinity from 10 to 30 ppt caused small but significant increases in extracellular osmolality (<20 mosM kg⁻¹), [Na⁺], and [Cl⁻], which were corrected within 48 h. RBC volume was not significantly changed 3 h after the in vivo exposure to elevated salinity. A small metabolic acidosis was fully developed within 3 h, and this acidosis seemed responsible for a modest decrease in blood O₂ affinity (i.e., increased P₅₀-O₂ tension at 50% O₂ saturation). RBC organic phosphates were unchanged. In vitro elevation of whole blood extracellular osmolality by 60 mosM kg⁻¹ caused immediate RBC shrinkage. The subsequent regulatory volume increase (RVI) showed a graded dependency on blood O₂ saturation (S_{O₂}). At S_{O₂} values of 0% and 20%, there were full RBC volume recoveries within 120 min, RVI was partial at S_{O₂} values of 45% and 55%, and RVI was absent at a S_{O₂} of 100%. S_{O₂} and P₅₀ did not change significantly during RBC shrinkage and RVI. Thus, the up-concentration of cellular haemoglobin and organic phosphates in hyperosmotically shrunken RBCs had minimal influence on blood O₂ transporting properties. The degree of cell

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shrinkage and time needed for RVI were positively correlated with the magnitude of the rise in extracellular osmolality. The RVI proceeded via elevation of cellular $[\text{Na}^+]$, $[\text{Cl}^-]$, and to some extent also $[\text{K}^+]$. Cell volume regulatory mechanisms are only needed to correct minor volume disturbances *in vivo*, because changes in extracellular osmolality were limited by an efficient osmotic regulation at the epithelial interface between extracellular compartment and environment. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The European flounder (*Platichthys flesus*) is extremely euryhaline and tolerates ambient media ranging from freshwater to solutions that are more concentrated than full-strength seawater. Populations inhabiting the internal coastal waters of Denmark (e.g., Skagerak, the Belts, and the Baltic Sea) are exposed to large fluctuations in ambient salinity as result of the admixture of high-salinity water from the North Sea (via Skagerak) and low-salinity waters from rivers and the Baltic Sea. Salinities may thus vary from a few parts per thousand to full-strength seawater on a short time scale. Such changes in ambient salinity perturb internal osmolality, ionic concentrations, and cell volume in fish (Madsen, 1990; Marshall et al., 1999) and may also lead to disturbances of gas transport and acid–base regulation (Nonnotte and Truchot, 1990; Maxime et al., 1991; Talbot et al., 1992; Madsen et al., 1996). The ability to thrive in a brackish water environment with variable salinity thus demands effective and integrative control with a multitude of physiological mechanisms.

Acute exposure of fish to an increased ambient salinity normally increases the extracellular osmolality and shrinks tissue cells, which subsequently activates cellular mechanisms for regulatory volume increase (RVI) in order to restore the original cell volume. At the same time, branchial, renal, and intestinal ion transport mechanisms are involved in the correction of extracellular ion concentrations. RVI requires the activation of either $\text{K}^+/\text{Na}^+/\text{Cl}^-$ cotransport or Na^+/H^+ exchange (with accompanying $\text{Cl}^-/\text{HCO}_3^-$ exchange). The latter is often the case in teleost RBCs (Cossins and Gibson, 1997) and has also been reported in flounder (Weaver et al., 1999). The influx of Na^+ and Cl^- into the cells is accompanied by osmotically obligated water that leads to cell volume recovery (Cala, 1977; Weaver et al., 1999). The Na^+ influx following shrinkage depends on RBC oxygenation conditions, being large in deoxygenated cells and low in oxygenated cells, whereby RVI is complete in deoxygenated RBCs and practically absent in oxygenated RBCs (Weaver et al., 1999). The degree of RVI at intermediate O_2 saturations has not yet been evaluated. Another interesting aspect is the possibility that RBC volume influences the oxygen binding properties of the RBCs. Thus, RBC shrinkage will lead to increased cellular concentrations of haemoglobin and nucleoside triphosphates (NTPs, being primarily ATP and GTP), which may be predicted to decrease haemoglobin oxygen affinity (Lykkeboe and Weber, 1978; Jensen, 1990). A recent *in vitro* study on whole

blood from rainbow trout and carp, however, suggested that the influence of hyperosmotic cell shrinkage on RBC oxygen affinity is limited (Brauner et al., 2002), making it pertinent to obtain information on additional species in order to establish the generality of this finding.

Whereas mechanisms of osmoregulation and RVI are often studied, the extent to which red blood cell volume and extracellular osmolality and acid–base conditions are perturbed during *in vivo* salinity changes is less known. As part of a large research initiative dealing with intestinal ion transport mechanisms and osmoregulation in flounder in relation to salinity, we evaluated *in vivo* changes in extracellular electrolytes and acid–base status as well as red blood cell volume and gas transport properties during an increase in ambient salinity. In addition, we conducted *in vitro* experiments on whole blood to investigate RVI at different oxygenation conditions and the impact of volume changes on RBC O₂ binding.

2. Materials and methods

European flounder (*P. flesus*) weighing 323.7 ± 15.4 g were caught by a local fisherman in the coastal waters near Kerteminde, Funen, Denmark, and brought to the Aquatic Research Centre of Odense University in Kerteminde. The fish were kept in 500-l tanks in seawater with salinity of 10 ppt (obtained by adding appropriate amount of distilled water to natural seawater pumped from outside the Research Centre) and temperature of 13 °C. The water was passed through a biological filter and aerated to maintain normoxic conditions, and it was periodically replaced with fresh seawater (10 ppt). Fish were allowed to acclimate at least 4 days before experiments. Animals were not fed before and during the experiments.

2.1. Surgery

Fish were anaesthetised with 0.1 g l⁻¹ MS-222 (3-aminobenzoic acid ethyl ester, Sigma) in 10 ppt seawater, and a PE50 polyethylene catheter was inserted into the caudal blood vessel (Grosell and Jensen, 2000). The catheter was filled with physiological saline containing 100 IU heparin and held in place with sutures. After surgery, the fish were placed in individual tanks containing 20–25 l of aerated seawater at 10 ppt and 13 °C. Water was renewed daily, and the fish were allowed to recover for 3 days prior to experiments.

2.2. *In vivo* experiments

These experiments evaluated the *in vivo* influences of a 10- to 30-ppt transfer on blood respiratory, acid–base, and osmoregulatory variables. Following withdrawal of a 1-ml control blood sample (time zero), the water salinity was raised from 10 to 30 ppt. The 10-ppt water was removed to just above the fish (lying quiet on the bottom), and then high-salinity seawater (obtained by adding balanced amounts of sea salt to the natural seawater) was added to achieve a final salinity of 30 ppt. Control fish were

treated similarly, except that the exchanged water maintained a salinity of 10 ppt. The exchange of water could be done without any noticeable disturbance to the fish. Subsequent 1-ml blood samples were drawn at 3, 24, and 48 h. At each blood sampling, an equivalent volume of physiological saline was injected into the fish via the catheter.

2.3. *In vitro* experiments

The *in vitro* effects of hyperosmotic conditions on blood respiratory, acid–base, and osmoregulatory parameters were determined in two series of experiments.

2.3.1. Series I

The oxygen dependence of red blood cell (RBC) regulatory volume increase (RVI) was studied by equilibrating 3-ml blood samples (taken from cannulated flounders at 10 ppt) for 1 h at 13 °C in shaking Eschweiler tonometers (Kiel, Germany). The tonometers were supplied with humidified gas mixtures containing 0.4% CO₂ and 0%, 1%, 2%, 3%, or 30% O₂. Pure N₂ was the balancing gas. The gas mixtures were made with Wösthoff gas mixing pumps (Bochum, Germany). After the removal of control (time zero) samples, microlitre samples of 1.6 M NaCl were added to elevate plasma osmolality by approximately 60 mosM kg⁻¹. Subsequent samples were taken from the tonometers at 3, 20, 40, 80, and 120 min. Blood O₂ content (C_{O₂}), pH, haemoglobin (Hb) concentration, haematocrit (Hct), and plasma osmolality were measured.

2.3.2. Series II

In this series, blood was equilibrated to 0.4% CO₂/1% O₂/98.6% N₂ and the increase in osmolality was twice (120 mosM kg⁻¹) that in Series I (addition of microlitre samples of 3 M NaCl). Samples were taken at 0, 3, 20, 40, 80, 120, 180, and 240 min. In addition to the measurements made in Series I, extracellular and intracellular ion concentrations were also measured.

2.4. Analysis

In vivo blood pH and oxygen tension (P_{O_2}) were measured immediately after sampling, using a Radiometer BMS 3 electrode assembly (Copenhagen, Denmark) thermostatted at 13 °C. Intracellular pH of RBCs was measured after twice freezing (in liquid nitrogen) and thawing of packed RBC. Blood oxygen content (C_{O₂}) was measured with the Tucker (1967) method and plasma total CO₂ content (C_T) was measured with the Cameron (1971) method. Haematocrit (Hct) values were determined by centrifugation (3 min, 12 000 rpm, 14 980×g). Blood haemoglobin (Hb) concentration was measured spectrophotometrically after converting all Hb to cyanmethaemoglobin, using an extinction coefficient of 11 mmol⁻¹ l⁻¹ cm⁻¹ at 540 nm. Mean cellular haemoglobin concentration (MCHC) was obtained from blood [Hb]/Hct. Methaemoglobin was measured according to the method of Benesch et al. (1973). Red blood cell counts (N_{RBC}) were done with a Bürker–Türk

counting chamber and a microscope, and the mean cellular volume (MCV) was obtained from $\text{Hct}/N_{\text{RBC}}$.

Plasma and erythrocytes were separated by centrifugation in weighed Eppendorf tubes. Plasma osmolality was measured with a Gonotec Osmomat 030. Plasma $[\text{Cl}^-]$ was measured with a Radiometer CMT 10 chloride titrator. Plasma sodium was measured by flame photometry (Instrumentation Laboratory 243), and plasma potassium, calcium, and magnesium were determined by atomic absorption spectrophotometry (Perkin-Elmer 2380). Packed RBCs were dried for 24 h at 90 °C, and the RBC water content was determined from the difference between wet and dry masses. The dry RBC pellet was extracted with 1 ml of 0.6 M HClO_4 . After centrifugation, the supernatant was used for the analysis of intracellular ions. The RBC ions were measured with the same apparatus as plasma ions. RBC ion contents are expressed as millimoles per kilogram of dry cell mass. The erythrocytic nucleoside triphosphate (NTP, includes both ATP and GTP) content was measured enzymatically (Sigma No. 366-UV) following deproteination of blood in 12% trichloroacetic acid. Lactate was determined after deproteination of blood with 0.6 N HClO_4 (Boehringer-Mannheim lactate dehydrogenase method).

2.5. Calculations

In vivo blood P_{CO_2} was calculated from the Henderson–Hasselbach equation, using the measured pH and C_{T} and appropriate values for $\text{p}K'$ and CO_2 solubility (Heisler, 1989). Plasma $[\text{HCO}_3^-]$ was calculated from C_{T} by subtracting dissolved CO_2 .

Haemoglobin bound oxygen (HbO_2) was calculated as $C_{\text{O}_2} - \beta_{\text{O}_2} \times P_{\text{O}_2}$, where β_{O_2} is the solubility of oxygen at 13 °C (Christoforides and Hedley-Whyte, 1969) and P_{O_2} is the prevailing O_2 tension. The in vitro addition of NaCl caused an increase in plasma osmolality by approximately 60 mosM kg^{-1} . By analogy with seawater, this would correspond to a change in salinity from 11 to 13 ppt, which would decrease O_2 solubility by 1.2%. This change in β_{O_2} is of minor importance but was taken into account in the calculations. The O_2 saturation of Hb (S_{O_2}) was calculated by dividing HbO_2 with the theoretical O_2 binding capacity (4 O_2 per Hb molecule) for the functional Hb (i.e., total Hb minus metHb). In the experiments, the RBCs showed a time-dependent change of volume, whereby it was not possible to establish full O_2 equilibrium curves at any given time (i.e., this requires long-term equilibration of steady state blood at different P_{O_2} 's). However, the determined S_{O_2} values (at known P_{O_2} values) per se give information on O_2 binding properties, and it was possible to obtain point estimates of O_2 affinity by calculating P_{50} (the P_{O_2} at 50% S_{O_2}) from S_{O_2} and P_{O_2} using the rearranged Hill equation:

$$P_{50} = 10^{(\log P_{\text{O}_2} - \log(S_{\text{O}_2}/(100 - S_{\text{O}_2}}))/n).$$

In order to obtain a proper value for the whole blood Hill coefficient n , two full O_2 equilibrium curves were constructed by equilibrating blood from control (10 ppt) flounder to variable P_{O_2} at constant P_{CO_2} (3 mmHg, equivalent to 0.4% CO_2). From

the slope of Hill plots [$\log S_{O_2}/(100-S_{O_2})$ vs. $\log P_{O_2}$], n was found to be 1.2 in both cases, and this was the value used in the calculations. P_{50} was only evaluated for S_{O_2} values between 15% and 85%, since the Hill equation is only applicable in this S_{O_2} range.

In vitro cell volume changes were evaluated from the relative change in Hct at time t compared to time zero control values: $\Delta\text{Hct} (\%) = 100 \times [\text{Hct}(t) - \text{Hct}(0)] / \text{Hct}(0)$.

2.6. Statistics

The data are presented as means \pm S.E.M. and were analysed by repeated measures analysis of variance (ANOVA) followed by the Tukey multiple comparison test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. In vivo response to increased ambient salinity

Three hours after transfer of flounder from 10 to 30 ppt seawater, the extracellular osmolality was significantly increased compared to time zero control values and to control fish (Fig. 1A). The disturbance was, however, modest (the rise in osmolality was below 20 mosM kg^{-1}) and it was corrected within the 48-h experimental period (Fig. 1A). The rising osmolality resulted mainly from increases in plasma $[\text{Cl}^-]$ and $[\text{Na}^+]$ (Fig. 1C,D). A small significant increase occurred in plasma magnesium (Fig. 1E), whereas there were no noteworthy changes in plasma calcium (Fig. 1F) and potassium (Fig. 1B).

Hyperosmotic exposure caused a small but significant change in extracellular acid–base status. Between 0 and 3 h, extracellular pH and $[\text{HCO}_3^-]$ decreased, and this metabolic acidosis dominated the acid–base change, but there was also a small decrease in P_{CO_2} , suggesting a respiratory compensation via hyperventilation (Fig. 2). Following this acute change, acid–base status did not change further up to 48 h. Control fish maintained their extracellular acid–base status within narrow limits (Fig. 2). Blood lactate was low (typically 0.1–0.2 mmol l^{-1}) in both groups throughout the experiment. The extracellular acidosis was paralleled by a small decrease in the intracellular pH of the RBCs (not illustrated).

Blood [Hb] decreased with time in both experimental and control fish, mainly as result of the repeated blood sampling (Fig. 3A). The mean cellular volume of the RBCs did not change significantly in flounder transferred to 30 ppt seawater (Fig. 3B), suggesting that RBC shrinkage caused by the extracellular hyperosmolality experienced in vivo was either limited or corrected before the first sampling time at 3 h. Blood P_{O_2} stayed constant in control fish, but increased significantly in experimental fish (Fig. 3C), supporting the abovementioned hyperventilation in fish transferred to 30 ppt seawater. Despite this increase in P_{O_2} , S_{O_2} remained unchanged (Fig. 3D). This was explained by the fact that blood P_{50} increased in flounder transferred from 10 to 30 ppt, resulting in a significant difference in P_{50} between experimental and control fish at 3 and 48 h (Fig. 3E). The erythrocytic concentration

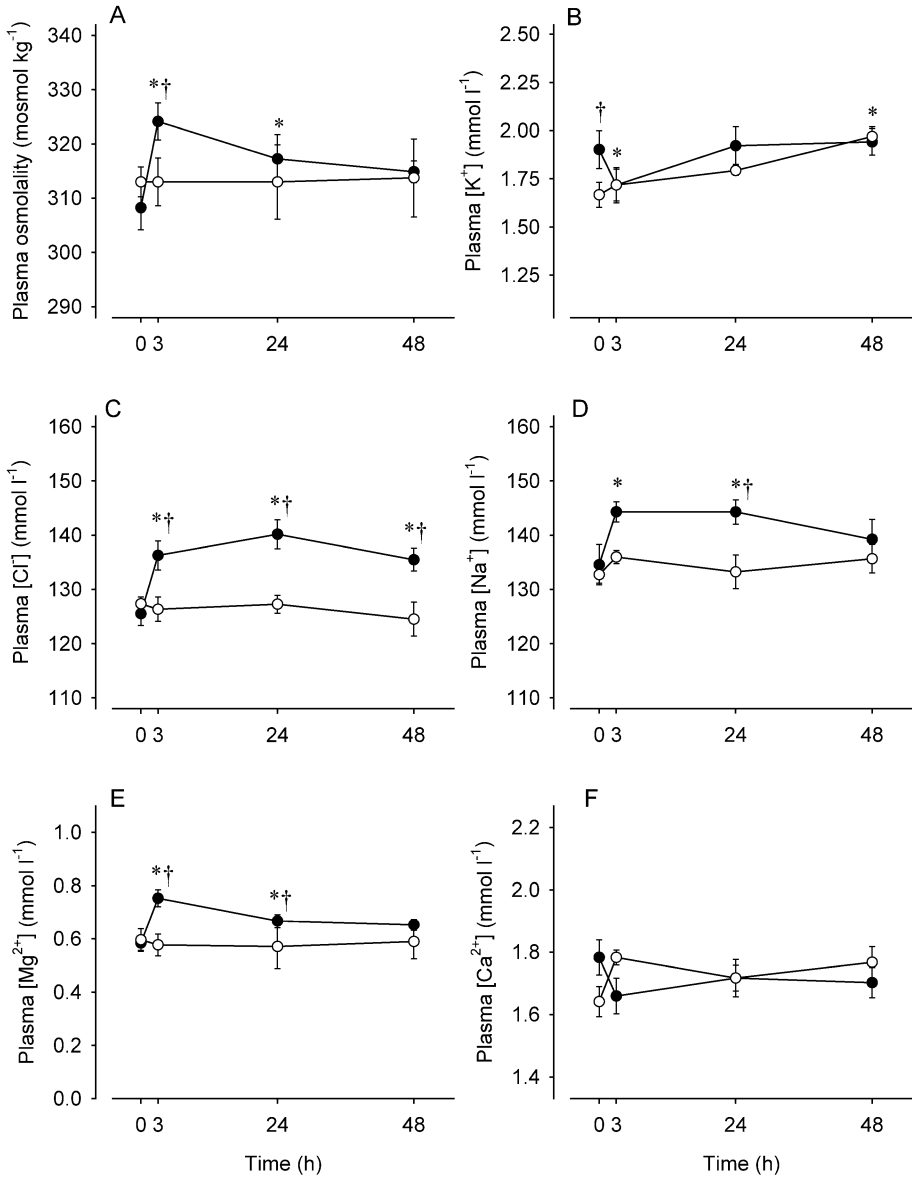


Fig. 1. Changes in plasma osmolality and concentrations of K⁺, Cl⁻, Na⁺, Mg, and Ca in flounder transferred from seawater with salinity of 10 ppt to seawater with salinity of 30 ppt (●, n=8) and in flounder kept in 10 ppt seawater (○, controls, n=4) (means ± S.E.M.). *Significant (P < 0.05) difference from the time zero control value within each group. †Significant (P < 0.05) difference between experimental and control fish at any given time.

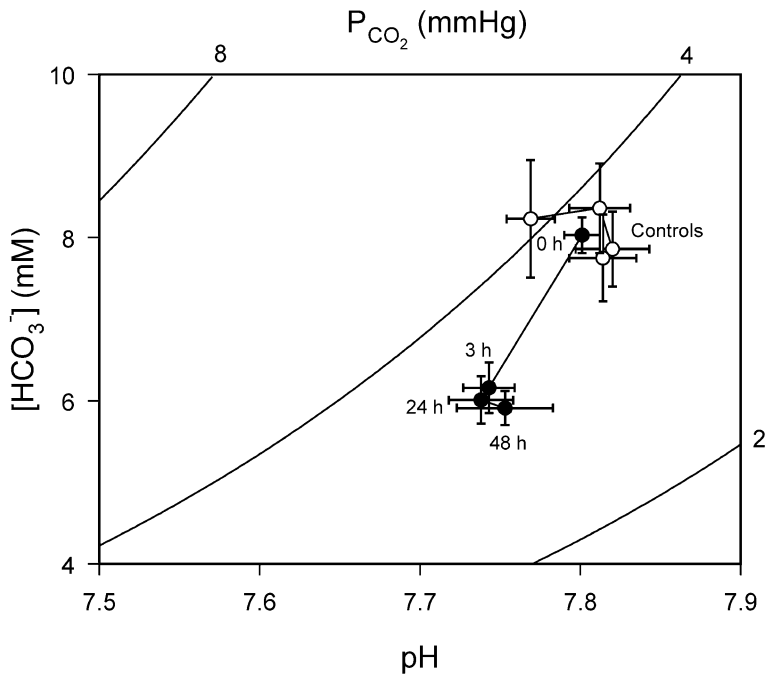


Fig. 2. Plasma $[\text{HCO}_3^-]$ vs. pH diagram with P_{CO_2} isopleths and the in vivo changes in extracellular acid–base status of flounder transferred from 10 to 30 ppt (●, $n=8$) and flounder kept at 10 ppt (○, controls, $n=4$) (means \pm S.E.M.).

of NTP was approximately 10 mmol l^{-1} RBC and did not change significantly with time (Fig. 3F).

3.2. In vitro response to hyperosmolality

The extracellular osmolality of whole blood equilibrated to different P_{O_2} values at constant P_{CO_2} (3 mmHg) was elevated by circa 60 mosM kg^{-1} via addition of NaCl (Fig. 4A). This caused an immediate shrinkage of the RBCs, as reflected by significant decreases in ΔHct (Fig. 4C). The extent of shrinkage at 3 min was similar at the different P_{O_2} levels, but the subsequent volume recovery was strongly dependent on the oxygen conditions. In the absence of oxygen, there was full volume recovery (ΔHct returning to zero) within 120 min, whereas there were no RVI at the highest O_2 level (30% O_2) that corresponded to a S_{O_2} of 100% (Fig. 4B,C). At intermediate O_2 levels, there was a graded transition between these two extremes. At 1% O_2 (corresponding to a S_{O_2} of 20%), RVI appeared equally efficient as in the absence of oxygen, whereas at 2% and 3% O_2 (corresponding to S_{O_2} values of approximately 45% and 55%, respectively), volume recovery was partial within 120 min (Fig. 4C).

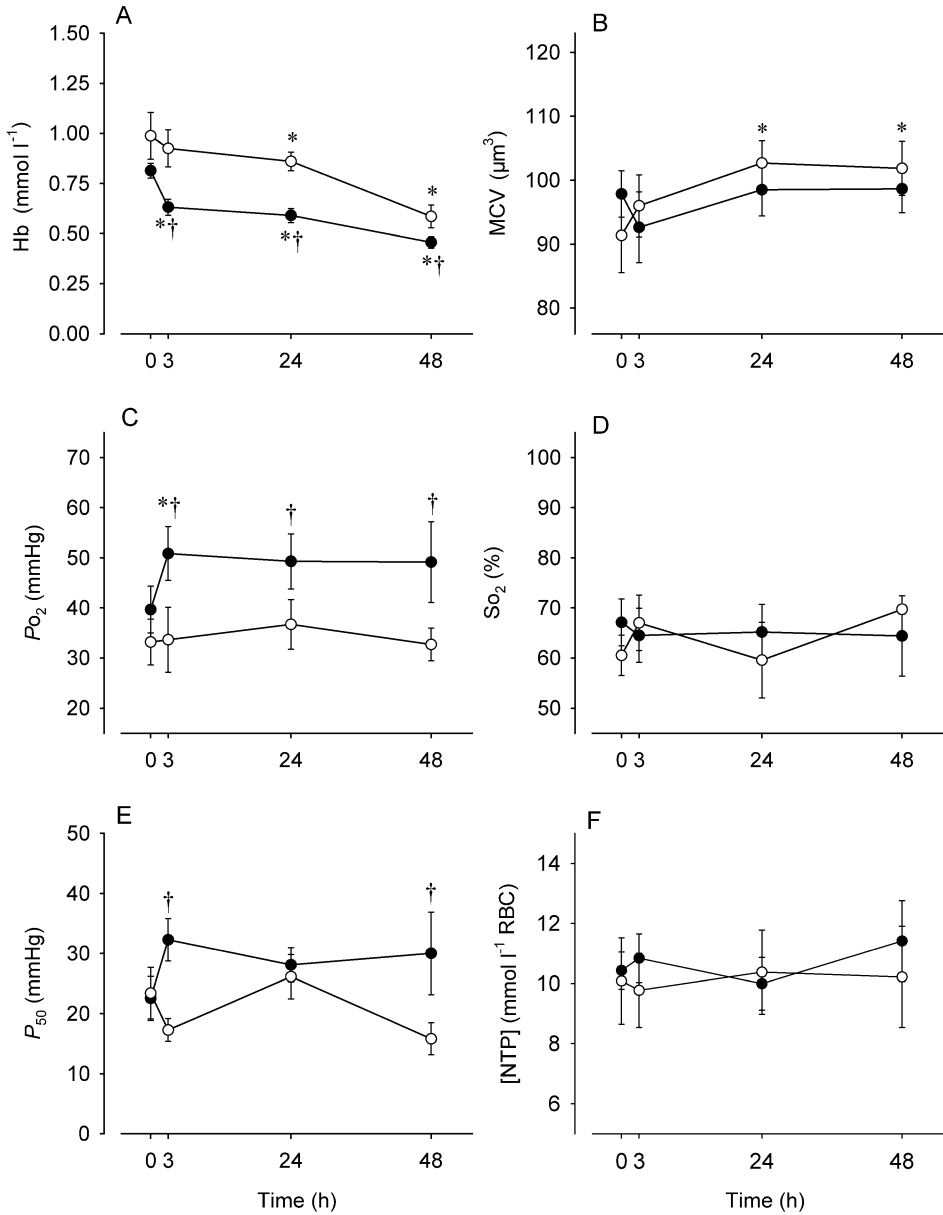


Fig. 3. Changes in tetrameric haemoglobin (Hb) concentration, erythrocyte mean cellular volume (MCV), oxygen tension (P_{O_2}), oxygen saturation (S_{O_2}), oxygen affinity (P_{50}), and erythrocyte nucleoside triphosphate (NTP) concentration in blood of flounder transferred from 10 to 30 ppt (●, $n=8$) and in flounder kept at 10 ppt (○, controls, $n=4$) (means±S.E.M.). See legend to Fig. 1 for further details.

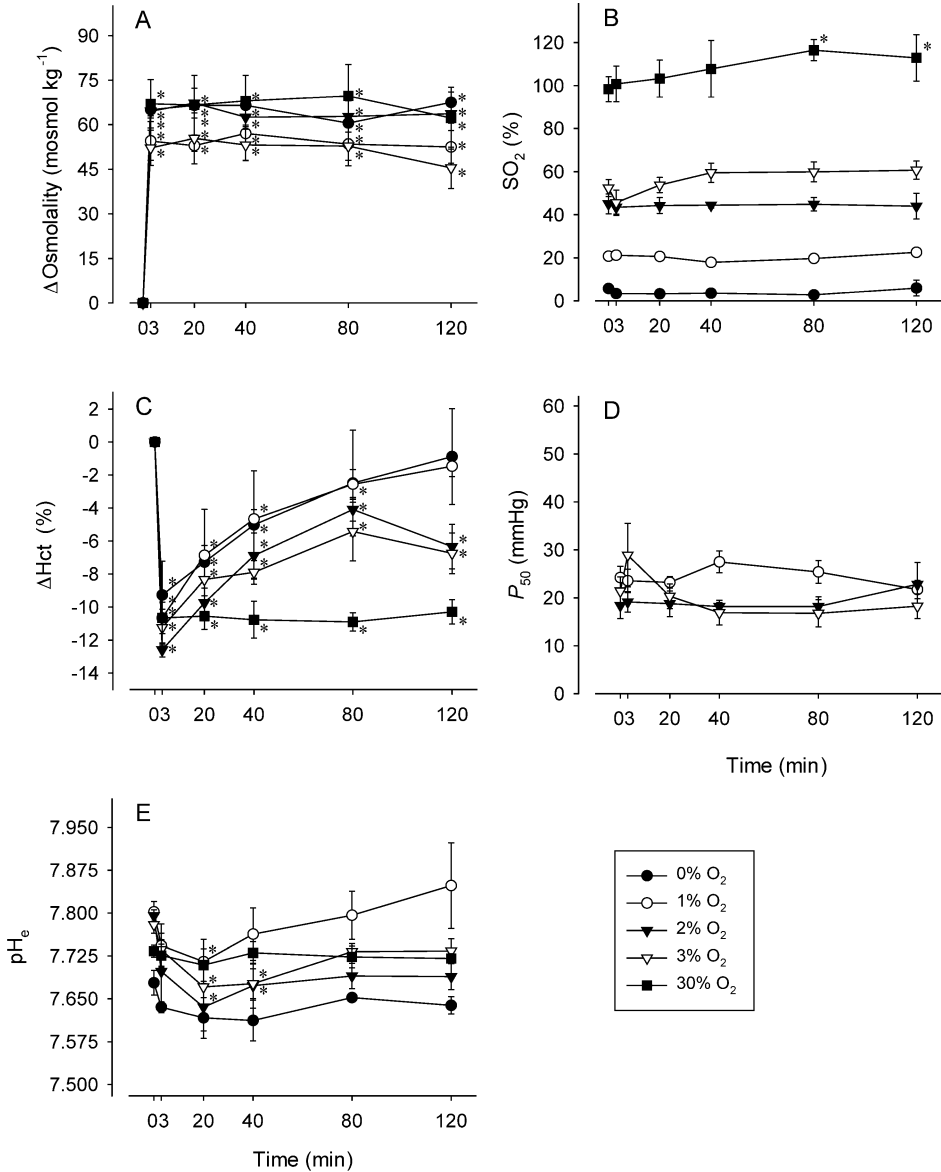


Fig. 4. Changes in osmolality, oxygen saturation (SO₂), red blood cell volume (Δ Hct), oxygen affinity (P₅₀), and extracellular pH in whole blood of flounder after addition of NaCl (time zero) at different oxygen conditions (see insert for explanation of symbols) (means \pm S.E.M., n=4–5). * Significant (P < 0.05) difference from the treatment-specific time zero values.

S_{O_2} stayed practically constant with time at the different P_{O_2} levels and, consequently, P_{50} did not change significantly (Fig. 4D). Thus, there appeared to be no significant change in oxygen affinity as result of the RBC shrinkage and subsequent volume recovery during RVI.

Following hyperosmotic shrinkage of the RBCs, plasma pH decreased for the first 20–40 min, after which pH recovery was observed (Fig. 4E).

The influence of a larger rise in osmolality (ΔosM of 120 mosM kg^{-1}) was investigated at 1% O_2 and a P_{CO_2} of 3 mmHg. This caused a slightly larger RBC shrinkage at 3 min (ΔHct of -15.4%), which was succeeded by RVI that failed to be fully completed within 250 min (Fig. 5B). The RBC shrinkage caused a significant rise in MCHC followed by subsequent recovery as RVI progressed (Fig. 5C). The change in plasma pH resembled that observed in Series I, but the initial deflection from time zero control values was

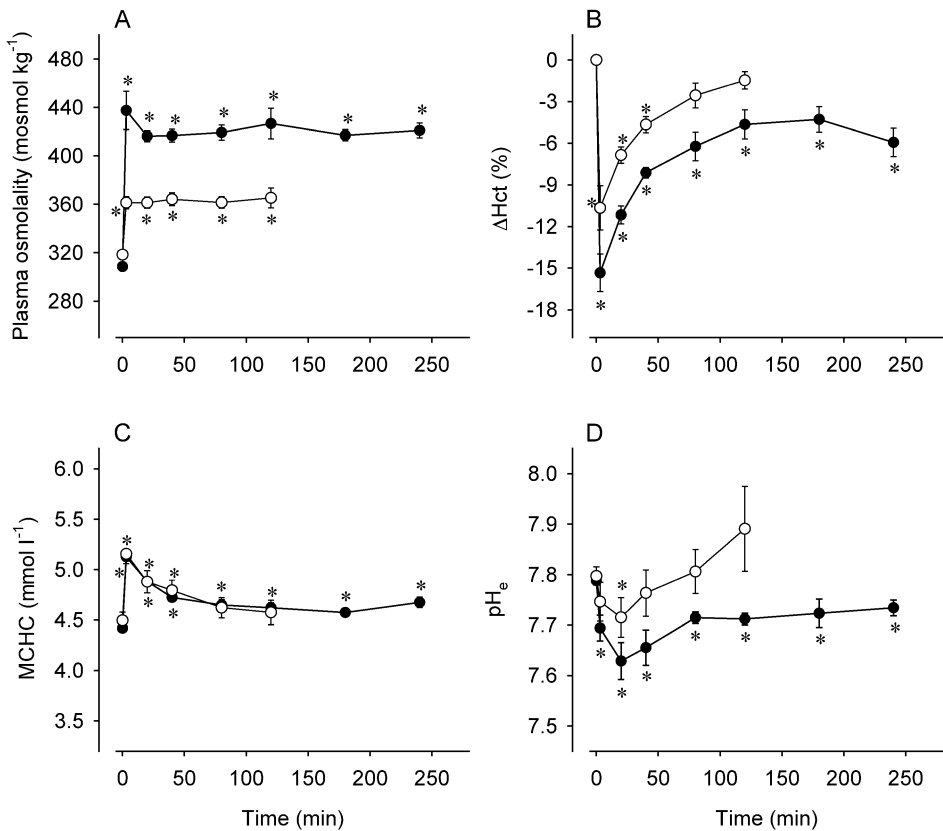


Fig. 5. Changes in plasma osmolality, red blood cell volume (ΔHct), mean cellular haemoglobin concentration (MCHC), and extracellular pH in flounder whole blood following an increase in extracellular osmolality of 120 mosM kg^{-1} (●, $n=4$). The blood was equilibrated to 1% $O_2/0.4\%$ $CO_2/98.6\%$ N_2 . The data are compared with corresponding data from a smaller increase in osmolality (○, taken from Fig. 4) under the same blood gas conditions. * Significant ($P < 0.05$) difference from the treatment-specific time zero values.

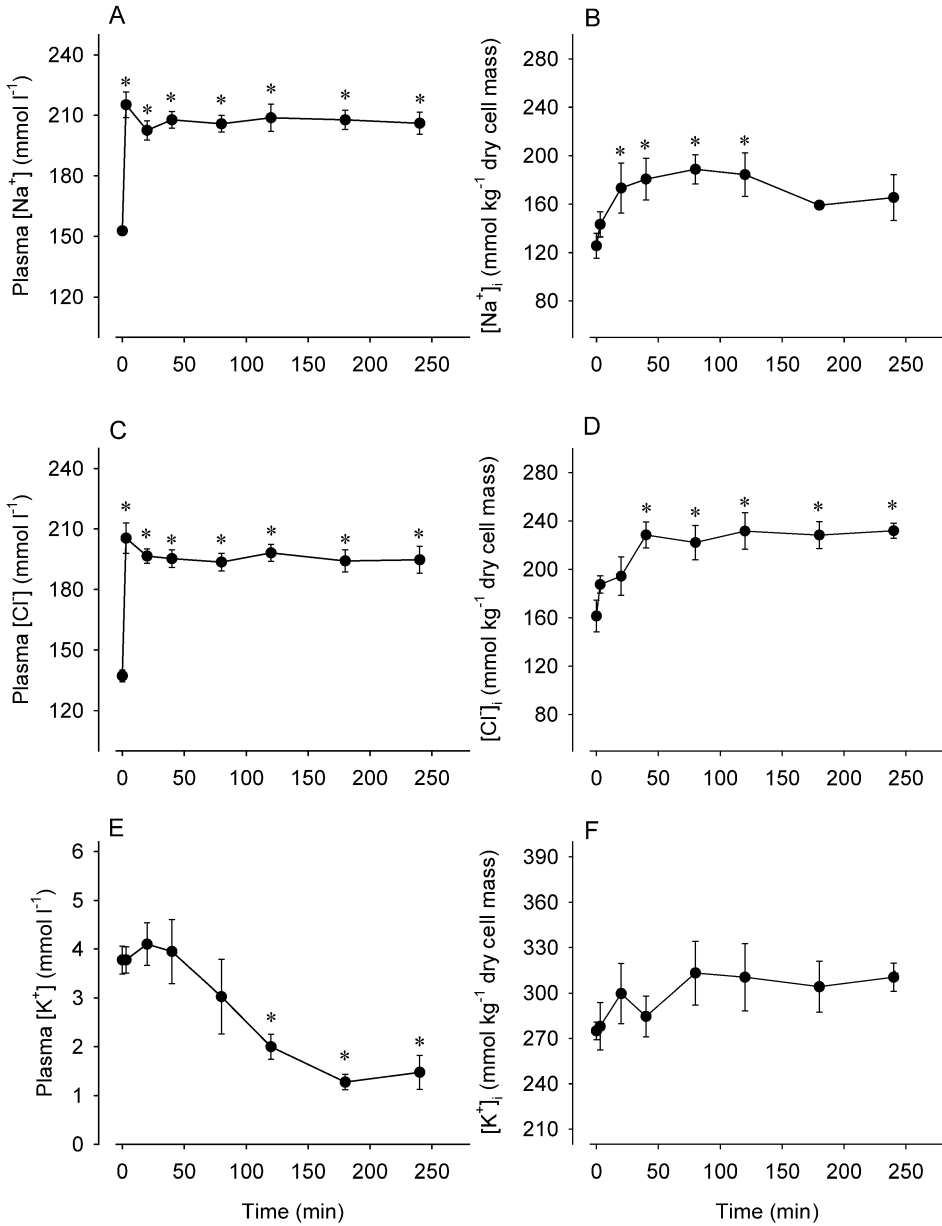


Fig. 6. Extracellular (A, C, E) and intracellular (B, D, F) concentrations of Na⁺, Cl⁻, and K⁺ in flounder whole blood subjected to an increase in extracellular osmolality of 120 mosM kg⁻¹ at time zero. Means±S.E.M. (n=4). * Significant (P<0.05) difference from time zero values.

somewhat larger (Fig. 5D). The addition of NaCl caused large increases in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ at 3 min that were succeeded by minor decreases (Fig. 6A,C). Plasma $[\text{K}^+]$ was initially unaffected but later showed a large significant decrease (Fig. 6E). In the course of RVI, the intracellular $[\text{Na}^+]$ and $[\text{Cl}^-]$ increased (Fig. 6B,D). $[\text{Cl}^-]_i$ reached a new steady level after some 50–100 min, which was kept up to 250 min. $[\text{Na}^+]_i$ showed a minor decrease at later stages of the experiment. This coincided with a small increase in intracellular $[\text{K}^+]$ (Fig. 6F) and the decrease in extracellular $[\text{K}^+]$ (Fig. 6E).

4. Discussion

4.1. *In vivo* impact of rising salinity

A change in ambient salinity from 10 to 30 ppt is a challenge that flounders in the internal coastal waters of Denmark would have to deal with naturally. The change induced an only small and transient *in vivo* rise in plasma osmolality and ion concentrations (Fig. 1). A relative stability of plasma ions upon salinity changes has also been reported in Atlantic populations of flounder (Motais et al., 1966). This reflects an efficient osmoregulation at the interface between the extracellular compartment and the environment (i.e., gill and intestinal epithelia), whereby internal ionic deflections are limited and quickly corrected. The present flounders were able to maintain plasma osmolality within narrow limits at ambient salinities ranging from 10 to 45 ppt after a few days of acclimation (R.W. Wilson et al., unpublished data). Although the rise in plasma osmolality of about 20 mosM kg^{-1} is smaller than observed in many other fish species transferred from freshwater to brackish water or seawater (e.g., Madsen, 1990; Madsen et al., 1996; Marshall et al., 1999), it might nevertheless be expected to shrink body cells. The RBC volume was, however, not significantly perturbed (Fig. 3). It appears that the potential for fast and efficient RVI that was demonstrated *in vitro* would have corrected any initial RBC shrinkage before the *in vivo* sampling time at 3 h. In other fish, transfer to increased salinity causes longer lasting shrinkage of RBCs and other cells (Madsen et al., 1996).

Transfer from 10 to 30 ppt seawater changed the extracellular acid–base status towards a metabolic acidosis with a small overlapping compensatory respiratory alkalosis (decrease in P_{CO_2} due to hyperventilation). The magnitude and type of the acid–base change was rather similar to that observed in flounder transferred from freshwater to full-strength seawater (Nonnotte and Truchot, 1990), but the pH decrease was smaller than observed in some salmonids transferred from freshwater to brackish water or full-strength seawater (Maxime et al., 1991; Madsen et al., 1996). The metabolic acidosis was not due to lactic acid and must be ascribed to a decrease in the strong ion difference as in other fish exposed to ambient hyperosmolality (Maxime et al., 1991; Madsen et al., 1996).

The extracellular acidosis was paralleled by a small decrease in red blood pH, which via the Bohr effect should decrease oxygen affinity. Indeed, with the lack of a significant RBC shrinkage and the absence of changes in the erythrocytic NTP concentration, the P_{50} increase actually observed (Fig. 3) most likely can be ascribed to the blood acidosis. The

decrease in oxygen affinity resembles that reported in rainbow trout, where P_{50} was increased from 20 to 25 mmHg at 24 h after transfer to seawater (Maxime et al., 1991).

Blood P_{O_2} increased following exposure to increased ambient salinity (Fig. 3). This contrasts with the situation in salmonids, where exposure to hyperosmolality decreases arterial P_{O_2} , possibly as a result of a decreased branchial O_2 conductance associated with dehydration-linked shrinkage of the branchial epithelium (Bath and Eddy, 1979; Talbot et al., 1992; Madsen et al., 1996). In flounder, cell volume regulation in gill epithelial cells may be efficient and rapid (as it is in the RBCs), avoiding a severe disturbance of the gill gas conductance and allowing blood P_{O_2} to increase (and P_{CO_2} to decrease) via hyper-ventilation.

4.2. RBC volume regulation and O_2 binding properties in vitro

The RVI following RBC shrinkage was complete within 120 min in the absence of oxygen, whereas it was absent in fully oxygenated blood (Fig. 4). This corroborates the results of Weaver et al. (1999), but we additionally demonstrate that there is a graded transition between these two extremes at intermediate oxygen saturations (Fig. 4). An oxygen saturation of zero would never occur in the living fish, but at a S_{O_2} of 20% (which may occur in capillaries of some tissues and in exercising fish) RVI is equally efficient as in the absence of oxygen and RVI is also effective around 50% saturation, which is a typical venous S_{O_2} in resting fish (Fig. 4). One may accordingly speculate that RVI takes place while the blood is in the venous system, which constitutes the largest portion by volume of the circulation. Alternatively, the fluctuation between high and intermediate S_{O_2} in the circulation may be equally efficient as a permanent low or intermediate S_{O_2} . A more potent RVI response at low than at high P_{O_2}/S_{O_2} also applies to carp and rainbow trout RBCs (Brauner et al., 2002) and may accordingly be a general phenomenon in fish.

Examination of the mechanisms of RVI was not a major issue in the present study. However, we observed a graded increase in the RBC Na^+ and Cl^- contents during RVI (Fig. 6), which is consistent with data on winter flounder (Cala, 1977) and with the activation of Na^+ influx (Weaver et al., 1999) and Cl^- influx, which drags osmotically obligated water into the RBCs to increase their volume. Flounder RBCs possess a Na^+/H^+ exchanger that can be activated by hypertonic shrinkage and by catecholamines (Thoroed et al., 1995; Weaver et al., 1999). Stimulation of Na^+/H^+ exchange with accompanying Cl^-/HCO_3^- exchange would therefore explain the increased intracellular $[Na^+]$ and $[Cl^-]$ during RVI (Weaver et al., 1999). The minor decrease in intracellular $[Na^+]$ at later stages of RVI, which coincided with a small increase in intracellular $[K^+]$ and a decrease in extracellular $[K^+]$ (Fig. 6), suggests that part of the Na^+ taken up leave the RBCs in exchange for K^+ via the Na^+/K^+ pump. An alternative explanation of the intracellular rises in Na^+ , Cl^- , and K^+ would be the additional involvement of a $Na^+/K^+/2Cl^-$ cotransport mechanism.

Although a considerable shrinkage of the RBCs was induced in vitro, we observed no changes in S_{O_2} and P_{50} following the shrinkage or during the subsequent RVI (Fig. 4). Shrinkage of the RBCs will lead to increases in the cellular concentrations of haemoglobin and NTPs at a constant $[NTP]/[Hb]$ ratio. Experiments with carp haemoglobin suggest that

this up-concentration causes a major increase in P_{50} , notably when the Hb concentration approaches and exceeds the normal MCHC (Lykkeboe and Weber, 1978). The absence of changes in oxygen affinity in the shrunken RBCs in vitro is therefore at first sight surprising, but it supports a recent study on rainbow trout and carp, where hyperosmotic RBC shrinkage was found to have only a marginal influence on RBC O₂ binding (Brauner et al., 2002). Thus, it must be concluded that hyperosmotic shrinkage of fish RBCs has minimal impact on blood O₂ binding properties.

An elevation of osmolality by 60 mosM kg⁻¹ caused an approximately 11% decrease in RBC volume (Fig. 4), whereas an elevation by 120 mosM kg⁻¹ decreased RBC volume by 15.5% (Fig. 5). From the data of Weaver et al. (1999), it can be inferred that a 160-mosM kg⁻¹ rise in osmolality decreases RBC volume by 23% (their Fig. 4), whereas a 430-mosM kg⁻¹ increase decreases RBC volume by 22% (their Fig. 1). Thus, while there is a progressive increase in the degree of shrinkage with increasing osmolality there appears to be an upper limit to this phenomenon. Extrapolation from our data suggests that, in flounder, the less than 20 mosM kg⁻¹ increase in plasma osmolality observed in vivo should cause only a 3% shrinkage of the RBCs. This reinforces the abovementioned contention that the major handling of hyperosmotic challenge in the flounder takes place at the interface between extracellular compartment and environment, and that internal volume regulatory mechanisms need only correct minor volume disturbances in these remarkably euryhaline fish.

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