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# PHYSIOLOGICAL RESPONSE OF THE ATLANTIC COD (*GADUS MORHUA*) TO HYPOXIA AT VARIOUS ENVIRONMENTAL SALINITIES

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## Summary

Atlantic cod (*Gadus morhua* L.) acclimated to water salinities ranging from 28‰ to 7‰ were exposed to mild (8.0 kPa) or severe (4.0 kPa) hypoxic conditions for 6 h. In each experiment, respiratory, acid–base, ionic, haematological and metabolic disturbances were analyzed. During mild hypoxia, a strong hyperventilatory response was observed, resulting in a respiratory alkalosis that persisted throughout the 6-h trial. Plasma $Cl^-$ and pyruvate levels were the only other variables to display significant changes: they both increased. In more severe hypoxic conditions, although the ventilatory response was the same, a weak metabolic acidosis was superimposed. The haematological response (increased haematocrit and decreased mean cellular haemoglobin content) suggested that catecholamines were released into the blood. Both $Na^+$ and $Cl^-$ concentrations increased significantly. Metabolic perturbations occurred: plasma lactate, pyruvate and glucose concentrations increased markedly. Though lactate concentrations in liver, heart and white muscle increased, the concentrations of pyruvate, glucose and glycogen did not change significantly. Water salinity affected the amplitude of the ionic responses during hypoxia: the amplitude decreased with decreasing salinity. Irrespective of water salinity, 23 of 29 fish survived the severe hypoxic conditions. This relatively good tolerance of low water oxygenation, as compared with other marine bottom-feeders, suggests that this species may face poorly oxygenated waters in the wild. Together with temperature and salinity, water oxygen content may thus be an important variable to take into account in the study of the distribution and migration patterns of Atlantic cod.

## Introduction

Intertidal waters are characterized by wide fluctuations in salinity and temperature owing to freshwater run-off and to the action of tides, currents and waves near the coast. Much smaller fluctuations are to be expected offshore, though large-

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scale variations are found in salinity and temperature. Dunbar *et al.* (1980) and Sameoto and Herman (1990) showed that the water column is vertically stratified at all seasons in the Gulf of Saint Lawrence and on the Nova Scotia Shelf. Surprisingly, this stratification was also present for water oxygenation level. Given the low oxygen concentrations reported near the bottom in these studies, this environmental variable may influence the behaviour of marine fishes living in such waters. Studying the interactions between the physiology of the animals and the natural variations in physicochemical variables, such as dissolved oxygen, can help us to understand the distribution and small-scale migration patterns of those species. This knowledge may also be useful for the development of aquaculture, as it will allow the assessment of the extreme conditions tolerated by these species.

The present study evaluates the capacity of the Atlantic cod (*Gadus morhua* L.) to tolerate low water oxygenation levels. Various physiological mechanisms were taken into account; ventilation, respiration, osmoregulation, and acid–base and metabolic regulation. These processes were studied at two levels of hypoxia. The first level (4.0 kPa) was established close to the tolerance limit as determined in preliminary experiments. The second level (8.0 kPa) was the lower critical oxygen value, that is the minimal oxygen level allowing the fish to meet its normal energetic requirements without setting up compensatory mechanisms, (Sundnes, 1957; Saunders, 1963). As cod is known to be a euryhaline species (Odense *et al.* 1966; J.-D. Dutil, J. Munro, C. Audet and M. Besner, in preparation), we also considered the possibility of a modified capacity to tolerate hypoxia resulting from the acclimation to low water salinity. Indeed, respiration, acid–base regulation and osmoregulation in fish mainly take place through the same branchial ion exchange pathways. Combined decreases in oxygen levels and salinity may thus result in an antagonism between these physiological processes. For example, the increased respiratory surface observed during hypoxia, resulting from the recruitment of gill secondary lamellae, also results in an increased surface for ion and water movements which, in turn, may threaten the osmotic balance of the fish. Similarly, the extrusion of protons from the extracellular fluid through the gill $Na^+/H^+$ exchangers may also conflict with the hypo-osmoregulatory processes. Finally, the osmotic status of the gill epithelial cells will determine their volume and, thus, the size of the passive ion diffusion channels (McDonald *et al.* 1989). Conversely, the possible reduction of the energetic cost of osmoregulation, as external salinity approaches the isosmotic point, may allow a reallocation of the internal energy fluxes. The hypoxic experiments were therefore conducted under four different salinity conditions, 28, 21, 14 and 7 ‰.

## Materials and methods

### *Fish holding*

Experiments were carried out on adult Atlantic cod (*Gadus morhua*) of both sexes weighing 0.8–1.5 kg. Fish were captured in the Gulf of Saint Lawrence (NAFO fishing area 4T) in September 1989 at a maximum depth of 30 m. They

were transferred to 12 $m^3$ indoor tanks supplied with running filtered sea water and acclimated for 2 months. Water temperature was maintained at 5±0.5°C and salinity remained in the range 26–30‰. The artificial lighting followed the natural photoperiod. Fish were fed once or twice a week with frozen capelin and smelt (40 g per week per kilogram of fish).

Following acclimation to laboratory conditions, 20 fish were transferred directly to one of four salinities (28, 21, 14 or 7‰) and acclimated for an additional month. Experimental salinities were obtained by mixing known proportions of sea water and fresh water. During this second period of acclimation, photoperiod, water temperature and feeding rate were unchanged. Feeding was discontinued 48 h prior to surgery.

### *Surgery*

Cod were anaesthetized in a solution of ethyl-*m*-aminobenzoate (0.5 $g\,l^{-1}$; MS222). To allow blood sampling, a catheter (Clay Adams PE60) was implanted in the subclavian artery according to Thomas and Le Ruz (1982). Throughout the surgical procedure, gills were perfused with oxygenated water containing a small dose of MS222 (0.1 $g\,l^{-1}$). Following surgery, the excised area was treated with antibiotics and the catheter was filled with heparinized saline and sealed. The anaesthetic dose being light, fish resumed ventilatory activity within 5 min when ventilated with anaesthetic-free water. Fish were then transferred to a holding chamber and allowed to recover for 18 h. The catheter was passed through a hole in the chamber, allowing undisturbed blood sampling. The chamber was supplied with the same water as the acclimation tank. Before reaching the fish, the water flowed through a counter-current gas exchange column bubbled with either air, for the control condition, or nitrogen to induce hypoxia.

### *Experimental protocol*

Seven (28, 21 and 7‰ acclimated groups) or eight (14‰ acclimated group) fish were picked at random from the acclimation tanks to be exposed to severe hypoxia (4.0 kPa) and three others to mild hypoxia (8.0 kPa). Blood was sampled *via* the catheter, under control normoxic conditions and after 1, 3 and 6 h of hypoxia. Blood $P_{O_2}$ and pH were measured on 100 $\mu$l samples using a Hamilton gas-tight syringe. A second sample was withdrawn with a 1 ml heparinized syringe. Part of this sample was kept for haematocrit and haemoglobin determinations and for tonometry. The remainder was centrifuged at 5000 ***g*** for 5 min and the plasma stored at −80°C until analyzed for $Na^+$, $K^+$, $Cl^-$, lactate, pyruvate and glucose. The total blood volume sampled from each fish never exceeded 6 ml (i.e. approximately 5 % of the fish blood volume). At the end of the 6-h hypoxic period, fish were quickly killed by a blow to the head. The heart and liver and a piece of the epaxial muscle were excised, freeze-clamped and stored at −80°C until assayed. For each salinity group, control values for tissue metabolites were obtained from four (21 and 14‰ acclimated groups) or five (28 and 7‰ acclimated

groups) fish maintained under normoxic conditions but subjected to the same surgical and recovery procedures.

The 28‰ salinity experiments were performed in December 1989 and January 1990. The 21‰ experiments took place the following February and March, the 14‰ experiments were realised in March and April and the 7‰ experiments in April and May.

### *Analytical techniques*

Ventilation frequency was obtained by visual observation and using a stopwatch. Water $P_{O_2}$ was measured continuously using an Orion 97–08 oxygen electrode connected to an Orion ion analyzer EA940+. Blood $P_{O_2}$ and pH were measured in triplicate with a Radiometer BMS3-Mk2 blood microsystem, connected to a Radiometer PHM-72 acid–base analyzer. Blood $P_{CO_2}$ was determined by the Astrup method (Astrup, 1956) using a thermostatted tonometer and humidified gas mixtures of known $P_{CO_2}$. Bicarbonate concentration was calculated using the Henderson–Hasselbalch equation and the appropriate dissociation constant and solubility coefficient (Boutilier *et al.* 1985). Blood haematocrit values were obtained after centrifugation in heparinized microhaematocrit tubes at 5000 ***g*** for 5 min. Blood haemoglobin concentration was determined by the cyanmethaemoglobin method (Sigma procedure no. 525). Mean cellular haemoglobin content (MCHC) was calculated as (haemoglobin concentration/haematocrit)×100. Plasma $Na^+$, $K^+$ and $Cl^-$ concentrations were measured using a Ciba–Corning 644 $Na^+K^+Cl^-$ analyzer. Plasma pyruvate, lactate and glucose were analyzed enzymatically at 340 nm (Sigma procedures nos 726-UV, 826-UV and 16-UV, respectively). Tissues were homogenized under ice-cold conditions in chilled 6% perchloric acid. Part of the homogenate was saved for glycogen determination (according to Keppler and Decker, 1974), and the rest was centrifuged at 10 000 ***g*** for 10 min. The supernatant was then assayed for pyruvate, lactate and glucose using the procedures described above.

### *Statistical analysis*

Plasma variables were analyzed as follows. Normoxic data were first compared using SAS procedure GLM (SAS Institute, 1985) to verify that there was no difference between fish submitted to the two hypoxic treatments (factor HL) and to detect differences attributable to the salinity of the acclimation medium (factor SAL). The data were then treated in two different ways.

When no differences occurred in normoxia between salinities, the GLM procedure was used to perform unbalanced multivariate analyses of variance for two-way designs, i.e. SAL and HL. Since several measurements were made on each individual, they were considered as repeated measures (REPEATED option). Pairwise comparisons were made to determine which measurement differed from the normoxic value (CONTRAST option). Interaction terms were also considered. When no significant interaction between salinity and level of hypoxia (SAL×HL), time and salinity (TIME×SAL) or time and level of hypoxia

(TIME×HL) was found, the observations for one factor were pooled to assess the second factor. Otherwise, levels of one factor were compared for each level of the second factor separately using one-way designs. The data from the two levels of hypoxia were never pooled.

For those variables between which significant differences were found during normoxia, the same statistical analysis was performed, but a nested design was used, i.e. the effect of hypoxia was tested within each salinity treatments. When the data from those variables that showed heterogeneity between salinities in normoxia had to be pooled, they were standardized (mean values observed during normoxia were subtracted).

Tissues metabolite data were also analyzed for the effect of salinity during normoxia. They were tested in the same way as the plasma variables but they were not treated as repeated measures. Pairwise tests were used to compare hypoxic to normoxic values for each level of hypoxia and to compare values between hypoxic treatments at the end of the hypoxic trials. When data for those variables that showed heterogeneity between salinities during normoxia had to be pooled, they were also standardized (mean values observed during normoxia were subtracted).

As six fish died between the third and the fourth hour in the deep hypoxia trials (two for each of the 28, 21 and 14 ‰ salinity groups), dead and surviving fish were compared at $t$=180 min. As no significant differences were observed ($P$>0.05), data from the dead and surviving fish were pooled at $t$=0, 60 and 180. In the statistical analysis and the figures, $N$ values are as follows. During mild hypoxia, $N$=3 at all time for each salinity treatment. During deep hypoxia, salinity 28 and 21 ‰, $t$=0, 60 and 180 min; $N$=7. At $t$=360 min; $N$=5. Salinity 14 ‰, at $t$=0, 60 and 180 min; $N$=8. At $t$=360 min; $N$=6. Salinity 7 ‰; $N$=7 at all sampling time. Thus, $N$ refers to the number of samples included in the analysis and not to the number of fish examined.

## Results

### *Ventilation and blood gases*

Respiratory responses are summarized in Table 1. Under normoxic conditions, ventilation frequency was not affected by water salinity ($P$>0.05), but arterial blood $P_{O_2}$ and $P_{CO_2}$ were ($P \leq 0.02$ and $P \leq 0.001$, respectively). $Pa_{O_2}$ was lower in the fish acclimated to 21 and 14 ‰ than in those acclimated to 28 and 7 ‰ salinity. $Pa_{CO_2}$, in contrast, increased steadily with decreasing salinity.

Except in the 28 ‰ acclimated group, induction of hypoxia resulted in an increase in ventilation frequency and a decrease in both $Pa_{O_2}$ and $Pa_{CO_2}$. However, all three variables reached a new steady state within an hour and thereafter remained unchanged. Therefore, measurements made after 1, 3 and 6 h were averaged (Table 1).

During hypoxia, the overall increase in ventilation frequency was the same for both hypoxic treatments, and water salinity had no impact on this response ($P$>0.05). The effect of time was therefore analyzed after pooling all salinity

Table 1. *Ventilatory and respiratory responses of cod to deep (4.0 kPa) and mild (8.0 kPa) hypoxia at various water salinities*

| | Salinity 28‰ | | | Salinity 21‰ | | | Salinity 14‰ | | | Salinity 7‰ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normoxia | Mild hypoxia | Deep hypoxia | Normoxia | Mild hypoxia | Deep hypoxia | Normoxia | Mild hypoxia | Deep hypoxia | Normoxia | Mild hypoxia | Deep hypoxia |
| $N$ | 10 | 9 | 19 | 10 | 9 | 19 | 11 | 9 | 22 | 10 | 9 | 21 |
| $Pw_{O_2}$ (kPa) | 20.1±0.3 | 8.3±0.4 | 3.8±0.1 | 20.9±0.1 | 9.2±0.3 | 3.8±0.1 | 20.6±0.1 | 7.9±0.2 | 3.7±0.1 | 20.8±0.1 | 7.7±0.1 | 3.6±0.1 |
| Ventilation frequency (beats $min^{-1}$) | 18.5±1.0 | 19.0±0.8 | 23.7±0.8 | 21.7±0.9 | 26.0±1.0 | 22.6±1.2 | 18.0±1.0 | 26.0±0.6 | 23.8±1.3 | 18.8±1.0 | 26.4±1.3 | 25.7±0.6 |
| $Pa_{O_2}$ (kPa) | 10.2±1.3 | 4.8±0.5 | 2.1±0.2 | 7.6±0.3 | 3.5±0.3 | 1.6±0.1 | 7.6±0.7 | 4.0±0.2 | 1.7±0.1 | 10.5±0.9 | 5.1±0.4 | 2.1±0.1 |
| $Pa_{CO_2}$ (Pa) | 200.0±53.3 | 133.3±13.2 | 40.0±12.3 | 253.3±13.3 | 120.0±22.7 | 80.0±15.5 | 333.3±26.5 | 240.0±26.7 | 93.3±13.8 | 346.6±9.9 | 266.6±14.2 | 186.6±14.5 |

For each salinity group, as no differences between samplings were observed during the hypoxic period, measurements made at $t$=60, 180 and 360 min have been pooled.

See text for more details.

Values are mean±S.E.M.

treatments and was found to be highly significant ($P \leq 0.01$). This response over time was also influenced by the salinity of the acclimation medium (TIME×SAL, $P=0.01$), at least for the relatively smaller hyperventilatory response observed at 28‰ during moderate hypoxia and at 21‰ salinity during severe hypoxia. The differences in the time courses of the ventilatory response between salinity treatments were significant after 1 h of hypoxia ($P=0.01$).

The overall $Pa_{O_2}$ and $Pa_{CO_2}$ values measured in these experiments were influenced by salinity ($P=0.02$ and $P \leq 0.01$, respectively) and by the intensity of hypoxia ($P \leq 0.01$). Again, the duration of exposure to hypoxia determined the blood gas values (TIME; $P \leq 0.01$), but in both cases this response over time was also influenced by the intensity of the hypoxic treatment (TIME×HL; $Pa_{O_2}$, $P=0.01$ and $Pa_{CO_2}$, $P=0.03$). The effect of salinity on blood gases during hypoxia resulted solely from differences already present during normoxia (TIME×SAL, $P>0.05$). Therefore, we calculated the differences between hypoxic and normoxic values for each fish and pooled the data from all salinities (see *Statistical analysis* for details). The time course of changes in $Pa_{O_2}$ and $Pa_{CO_2}$ differed significantly between hypoxic treatments after 1 h of hypoxia ($P \leq 0.05$).

### *Blood acid–base status*

Whole-blood bicarbonate concentration ($[HCO_3^-]$) was significantly affected by salinity ($P \leq 0.01$) during normoxia (Fig. 1). For this reason, $[HCO_3^-]$ data were transformed to give differences between normoxic and hypoxic values. No effect of salinity was found for arterial blood pH (pHa) ($P>0.05$).

Hypoxia resulted in a significant decrease in plasma $[HCO_3^-]$, this decrease being linked both to the level of hypoxia and to the water salinity ($P \leq 0.01$). The duration of exposure influenced the bicarbonate level ($P \leq 0.01$) and the time course of the $[HCO_3^-]$ response was found to be linked to the level of hypoxia (TIME×HL, $P=0.02$). In contrast, since there was no interaction between water salinity and the bicarbonate response over time (TIME×SAL, $P>0.05$), the overall salinity effect measured during hypoxia can be accounted for by the differences observed under control conditions. The differences in the response over time between hypoxic treatments was found to be significant after 1 h.

Arterial blood pH values were found to be dependent upon hypoxia level ($P=0.02$) but not upon water salinity ($P>0.05$), so results for all salinities were pooled. Time was also found to influence pHa values ($P \leq 0.01$). Moreover, there was a TIME×HL interaction ($P=0.03$). Indeed, while alkalosis was maintained throughout the experiment during mild hypoxia (significant at $t=60$ and 180 min), a relative extracellular acidosis developed during the last half of the deep hypoxic trials. Though this acidosis was never significant compared to control conditions, the difference in arterial blood pH between the two experimental treatments was found to be significant at $t=360$ min.

### *Haematological variables*

During normoxia, haematocrit (Hct), haemoglobin concentration ([Hb]) and

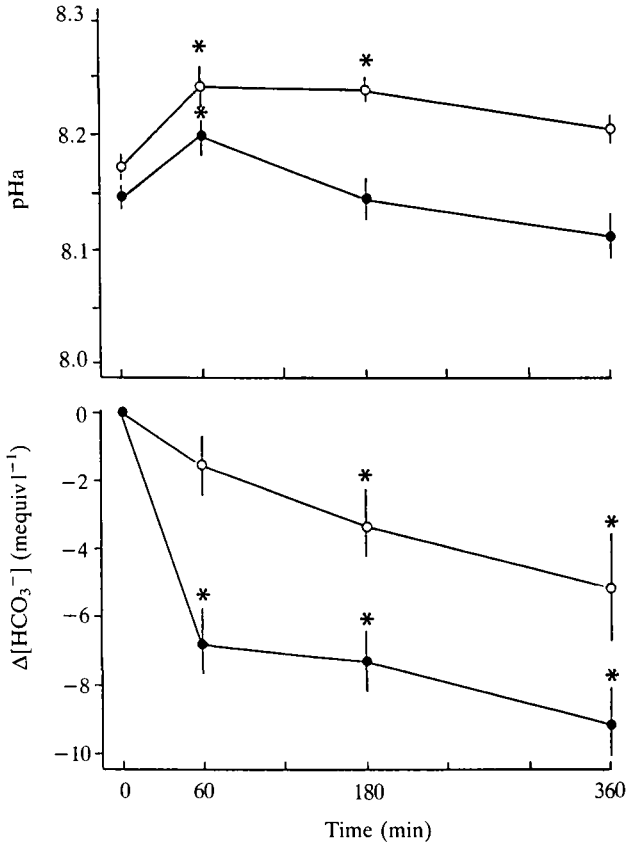


Fig. 1. Acid–base response of cod acclimated to various water salinities and exposed to 4.0 kPa (filled symbols) and 8.0 kPa (open symbols) hypoxia. As salinity had no effect on the response to hypoxia, salinity treatments were pooled (see *Statistical analysis* for more details). * significantly different from the normoxic level ($P \leq 0.05$).

mean cellular haemoglobin content (MCHC) were not significantly affected by the salinity of the acclimation medium ($P>0.05$). MCHC was the only variable to display significant differences in the response to hypoxia that were related to the salinity of the acclimation medium ($P=0.02$) (Fig. 2). None of the other variables was affected by salinity (SAL $P>0.05$; SAL×HL $P>0.05$). In contrast, all three variables changed with time (Hct; $P \leq 0.01$, [Hb]; $P \leq 0.01$, MCHC; $P \leq 0.05$). Only Hct and MCHC displayed a positive TIME×HL interaction ($P \leq 0.01$), suggesting that the response over time was affected by the level of hypoxia. These differences were significant at $t=60$ min for Hct and at $t=180$ min for MCHC. No TIME×SAL interaction occurred for any of the three variables ($P>0.05$).

### *Plasma ions responses*

Under control conditions, plasma sodium and chloride concentrations ($[Na^+]$, $[Cl^-]$) were significantly different between salinity groups ($P \leq 0.001$ and $P \leq 0.03$,

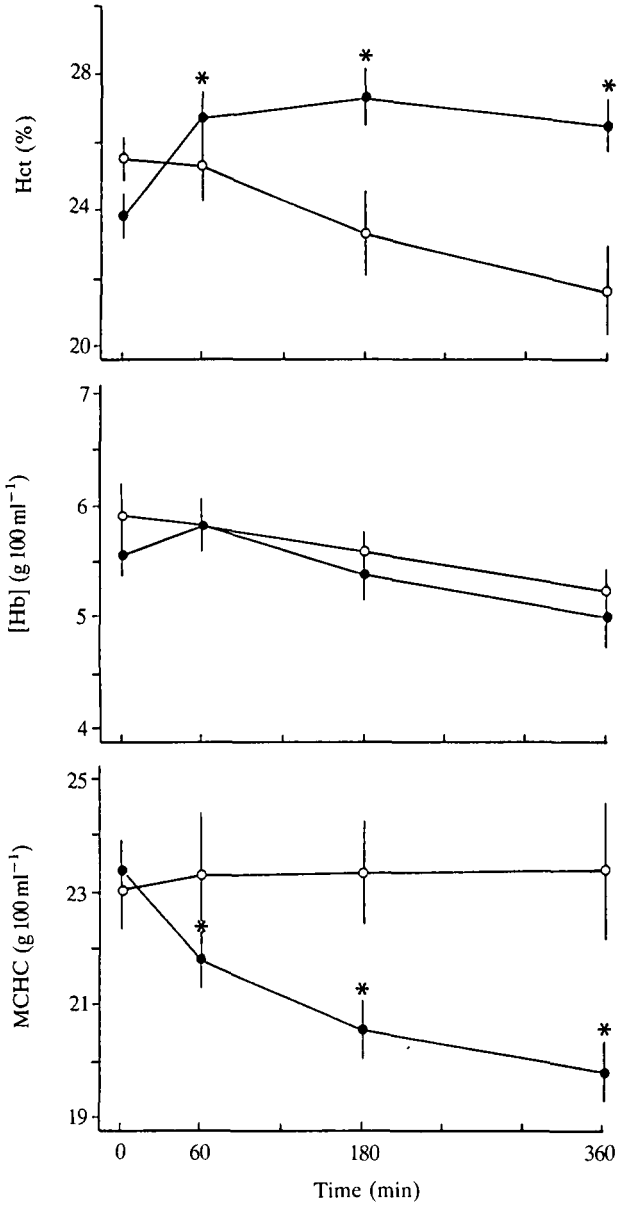


Fig. 2. Haematological response of cod acclimated to various water salinities and exposed to 4.0 kPa (filled symbols) and 8.0 kPa (open symbols) hypoxia. As salinity had no effect, salinity treatments have been pooled (see *Statistical analysis* for more details). * significantly different from the normoxic level ($P \leq 0.05$).

respectively), but this was not the case for plasma potassium ([$K^+$]; $P>0.05$, Figs 3, 4 and 5). Following the induction of hypoxia, plasma sodium and chloride concentrations were significantly influenced by water salinity ($P \leq 0.01$). More-

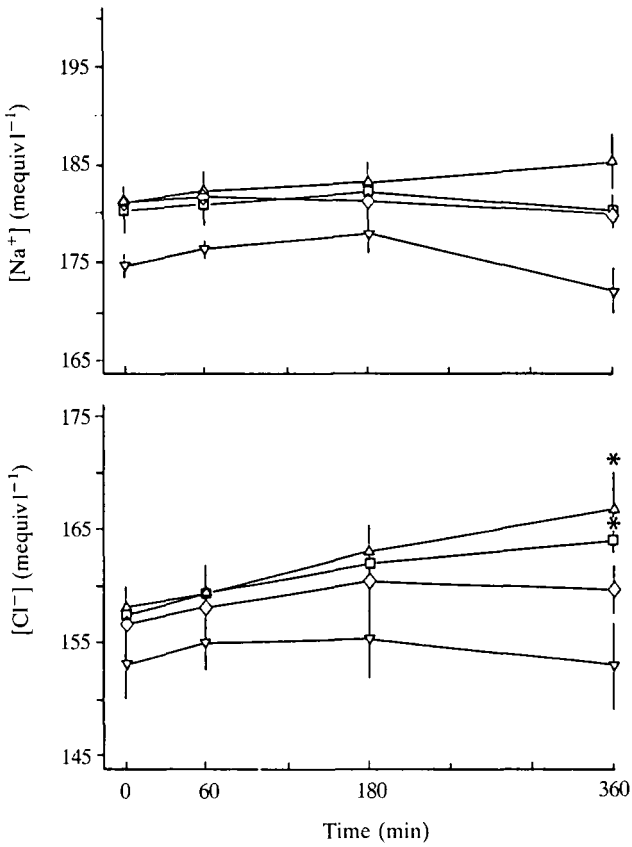


Fig. 3. Plasma sodium and chloride levels of cod acclimated to various water salinities and exposed to 8.0 kPa hypoxia. As the salinity of the acclimation medium had an impact on both the control levels and the response to hypoxia, salinity groups were analyzed individually (see *Statistical analysis* for more details): 28‰, □; 21‰, △; 14‰, ◇; 7‰, ▽. * significantly different from the normoxic level ($P \leq 0.05$).

over, plasma sodium concentration was also dependent upon the level of hypoxia ($P \leq 0.05$). Both $[Na^+]$ and $[Cl^-]$ were significantly affected by the duration of exposure to hypoxia ($P \leq 0.01$) and showed positive TIME×SAL interactions ($P \leq 0.01$). The responses over time were therefore analyzed independently for each salinity group. Only $[Na^+]$ showed a significant TIME×HL interaction ($P \leq 0.01$). The differences in the $[Na^+]$ responses over time between hypoxia treatments were found to be significant at $t=360$ min, and the effect of salinity was significant at $t=360$ min for $[Na^+]$ and at $t=180$ min for $[Cl^-]$. Plasma $[K^+]$ values (Fig. 5) were not linked to salinity or time ($P>0.05$). However, a TIME×HL interaction was found ($P=0.01$). The differences in the response over time between hypoxia treatments was significant at $t=60$ min ($P=0.05$). Strong ion difference (SID, Fig. 5) was calculated as $[Na^+]+[K^+]-[Cl^-]$. SID values were dependent upon the level of hypoxia ($P=0.02$), but were not linked to the salinity

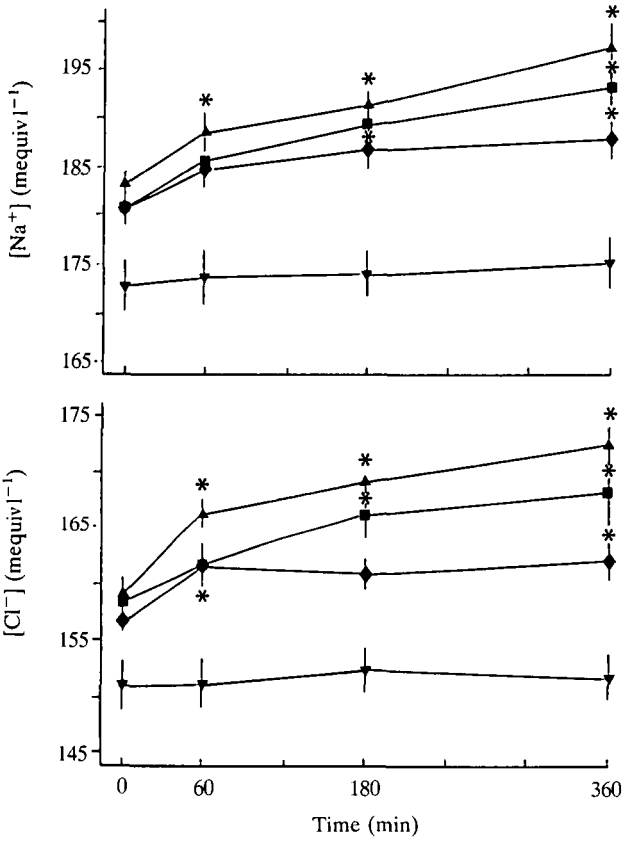


Fig. 4. Plasma sodium and chloride concentrations of cod acclimated to various water salinities and exposed to 4.0 kPa hypoxia. As the salinity of the acclimation medium had an impact on both the control levels and the response to hypoxia, salinity groups were analyzed individually (see *Statistical analysis* for more details): 28 ‰, ■; 21 ‰, ▲; 14 ‰, ◆; 7 ‰, ▼. * significantly different from the normoxic level ($P \leq 0.05$).

of the acclimation medium ($P>0.05$). The response over time was influenced by the intensity of hypoxia ($P \leq 0.01$), and this difference between the two treatments was significant at $t=360$ min.

### *Plasma metabolites*

During normoxia, plasma lactate and glucose levels were not significantly influenced by salinity ($P>0.05$), but plasma pyruvate concentration was ($P \leq 0.01$; Fig. 6).

During the hypoxic trial, the level of hypoxia ($P \leq 0.01$) but not the salinity ($P>0.05$) influenced plasma lactate concentration. Statistical analysis revealed a clear effect of time ($P \leq 0.01$) as well as a significant TIME×HL interaction ($P \leq 0.01$). The difference in the time course between the two hypoxic treatments was significant after 1 h of hypoxia ($P \leq 0.01$).

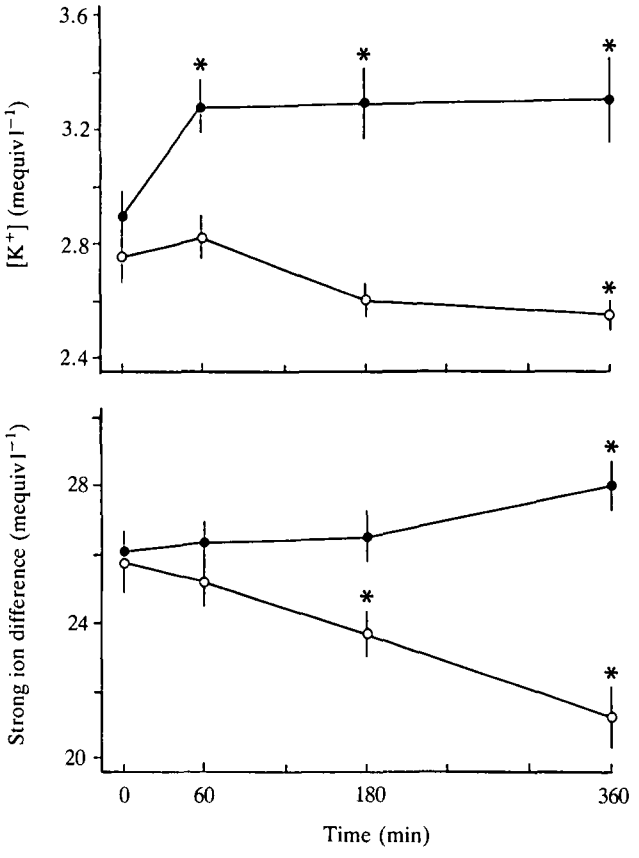


Fig. 5. Plasma potassium concentration and strong ion differences in cod acclimated to various water salinities and exposed to 8.0 kPa (open symbols) and 4.0 kPa (filled symbols) hypoxia. As no differences between salinity treatments were observed in hypoxia, salinity treatments were pooled (see *Statistical analysis* for more details). * significantly different from the normoxic level ($P \leq 0.05$).

Owing to the initial differences, plasma pyruvate during hypoxia was analyzed as the difference from the normoxic level. Both the intensity of hypoxia and salinity had a significant impact on pyruvate levels ($P \leq 0.01$). Pyruvate concentration also changed with time ($P \leq 0.01$), but no TIME×SAL interaction was found ($P > 0.05$), showing that there was no further effect of salinity in addition to that reported during normoxia. There was a positive TIME×HL interaction ($P \leq 0.01$). The time courses between hypoxic treatments became significantly different at $t = 60$ min ($P \leq 0.01$).

Neither salinity nor hypoxia intensity had a significant effect on the overall plasma glucose level. However, changes in glucose concentration were time-dependent ($P = 0.02$) and this response to time was modified by the level of hypoxia (TIME×HL; $P = 0.02$). In this case, salinity did not play any significant

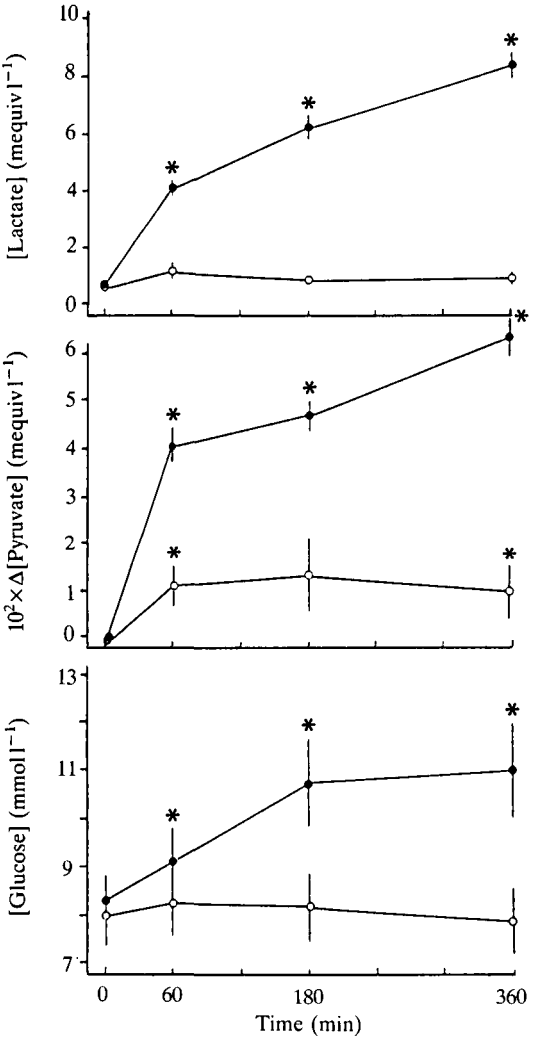


Fig. 6. Plasma metabolite concentrations of cod acclimated to various environmental salinities and exposed to 8.0 kPa (open symbols) and 4.0 kPa (filled symbols) hypoxia. As there was no effect of salinity during hypoxia, salinity treatments were pooled (see *Statistical analysis* for more details). * significantly different from the normoxic level ($P \leq 0.05$).

role. The difference in the time course of plasma glucose level between hypoxic groups was significant after 1 h ($P \leq 0.01$).

### *Tissues metabolites*

Owing to the relatively small number of fish involved and to the high variability in the results, no clear general trends emerged from the tissue data, even when they were analyzed as differences between normoxic and hypoxic levels (Table 2).

Table 2. *Heart, liver and white muscle metabolite concentrations in cod acclimated to various salinities, under control conditions and after 6 h of mild (8.0 kPa) or deep (4.0 kPa) hypoxia*

| | Salinity 28‰ | | | Salinity 21‰ | | | Salinity 14‰ | | | Salinity 7‰ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normoxia | Mild hypoxia | Deep hypoxia | Normoxia | Mild hypoxia | Deep hypoxia | Normoxia | Mild hypoxia | Deep hypoxia | Normoxia | Mild hypoxia | Deep hypoxia |
| *N* | 5 | 3 | 7 | 4 | 3 | 7 | 4 | 3 | 8 | 5 | 3 | 7 |
| Heart | | | | | | | | | | | | |
| Lactate | 1.84±0.36 | 2.09±0.79 | 17.55±1.19 | 1.63±0.15 | 1.76±0.38 | 15.20±2.00 | 1.87±0.20 | 2.44±0.76 | 15.37±2.50 | 1.35±0.19 | 1.64±0.18 | 12.65±1.69 |
| Pyruvate | 0.71±0.26 | 0.20±0.05 | 0.19±0.02 | 0.34±0.08 | 0.29±0.10 | 0.23±0.05 | 0.18±0.01 | 0.26±0.13 | 0.34±0.07 | 0.28±0.04 | 0.25±0.01 | 0.29±0.03 |
| Glucose | 4.53±0.37 | 6.26±1.54 | 3.91±1.22 | 4.28±1.37 | 5.08±0.74 | 4.99±1.60 | 7.40±0.97 | 5.46±1.74 | 5.15±1.01 | 2.78±0.16 | 6.17±1.13 | 4.54±1.02 |
| Glycogen | 13.82±4.73 | 9.45±6.73 | 8.55±2.00 | 12.91±6.18 | 28.55±10.73 | 12.91±1.09 | 3.82±0.36 | 12.73±4.55 | 10.73±1.09 | 1.27±0.73 | 1.09±1.09 | ND |
| Liver | | | | | | | | | | | | |
| Lactate | 1.20±0.33 | 0.81±0.13 | 3.16±0.23 | 1.50±0.31 | 0.93±0.14 | 3.09±0.49 | 1.24±0.11 | 1.06±0.23 | 3.62±0.47 | 0.78±0.21 | 1.84±0.07 | 3.58±0.46 |
| Pyruvate | 0.52±0.23 | 0.23±0.04 | 0.39±0.08 | 0.65±0.47 | 0.14±0.02 | 0.15±0.02 | 0.23±0.03 | 0.17±0.05 | 0.26±0.05 | 0.19±0.05 | 0.26±0.06 | 0.32±0.07 |
| Glucose | 2.44±0.37 | 3.32±0.91 | 3.77±0.98 | 3.40±0.89 | 2.98±1.40 | 4.60±1.64 | 4.90±0.44 | 2.91±0.99 | 6.72±1.53 | 2.16±0.56 | 4.13±0.27 | 8.09±0.94 |
| Glycogen | 30.55±4.91 | 35.64±7.82 | 68.55±21.27 | 53.09±20.00 | 97.27±31.27 | 49.82±17.45 | 36.55±4.01 | 106.72±38.00 | 70.00±20.91 | 23.64±8.18 | 41.27±10.55 | 36.91±11.64 |
| White muscle | | | | | | | | | | | | |
| Lactate | 4.98±0.99 | 7.24±0.38 | 12.50±1.43 | 5.77±0.89 | 7.53±2.89 | 13.58±1.89 | 4.65±1.04 | 7.08±1.11 | 11.74±1.57 | 4.68±1.20 | 3.95±0.64 | 9.91±1.23 |
| Pyruvate | 0.15±0.02 | 0.25±0.05 | 0.23±0.03 | 0.19±0.02 | 0.22±0.03 | 0.18±0.03 | 0.11±0.01 | 0.20±0.07 | 0.13±0.02 | 0.10±0.02 | 0.09±0.01 | 0.12±0.01 |
| Glucose | 0.48±0.26 | 0.80±0.29 | 2.20±0.42 | 0.82±0.48 | 0.63±0.13 | 2.48±0.68 | 1.21±0.26 | 1.02±0.41 | 2.15±0.72 | 0.58±0.19 | 1.58±0.05 | 1.44±0.39 |
| Glycogen | 12.55±2.18 | 11.82±2.73 | 16.36±4.36 | 15.45±3.45 | 26.54±10.73 | 10.73±1.82 | 4.55±0.73 | 10.18±0.91 | 9.45±2.36 | 1.27±0.55 | 0.54±0.36 | 1.27±0.73 |

ND, non detectable.

Values are mean±S.E.M. and are expressed as $\mu$mol $g^{-1}$ wet mass.

Under normoxic conditions, heart ($P \leq 0.01$) and liver glucose ($P \leq 0.05$) and white muscle glycogen concentration ($P \leq 0.001$) were the only variables to be significantly affected by salinity. Statistical analysis revealed that cod acclimated to 7 ‰ had significantly lower heart and liver glucose concentrations and lower white muscle glycogen levels than animals in full-strength sea water. The highest glucose values were observed in the heart and liver at 14 ‰, while the highest glycogen concentrations were in the white muscle of the fish acclimated to 21 ‰ salinity. During hypoxia, none of the metabolic variables exhibited a significant salinity effect that could not be attributed to the differences during normoxia (TIME× SAL; $P>0.05$). When testing the metabolic responses during hypoxia, all salinity treatments were therefore pooled.

During moderate hypoxia, lactate concentration did not change significantly ($P>0.05$) in the three tissues, whereas during deep hypoxia, lactate levels were significantly elevated above control normoxic levels in every case ($P<0.001$).

As a salinity effect was observed during normoxia, glucose data were transformed, to give differences between hypoxia and normoxia, and pooled. Both liver and white muscle glucose levels increased during severe hypoxia ($P<0.01$), whereas no significant changes were observed during moderate hypoxia for any of the three tissues analyzed ($P>0.05$). Pyruvate and glycogen concentrations were not affected by hypoxia ($P>0.05$).

### *Mortality*

While all the fish facing mild hypoxic conditions survived ($N=12$), six out the 29 fish exposed to severe hypoxia died between the third and fourth hour of the experiment (i.e. two fish from each of the groups acclimated to 28, 21 and 14 ‰). Though differences were observed in some variables between dead and surviving fish at $t=180$ min (i.e. lower ventilation frequency, higher SID and plasma lactate concentration), they were not significant ($P>0.05$), probably partly because of the relatively small number of fish. For the remainder of the statistical analysis, deep hypoxia data were pooled according to the salinity of the acclimation medium.

## Discussion

This study describes the physiological changes occurring in cod during a 6-h hypoxic period, and investigates whether acclimation to low water salinities influences the efficiency of the various regulatory mechanisms. A high degree of euryhalinity has been reported in cod by Odense *et al.* (1966) and J.-D. Dutil, J. Munro, C. Audet and M. Besner (in preparation).

Moderate hypoxia ($Pw_{O_2}=8.0$ kPa) induced only ventilatory (Table 1) and possibly circulatory rearrangements, which were presumably efficient enough to avoid further physiological perturbations. Deep hypoxia ($Pw_{O_2}=4.0$ kPa), in contrast, resulted in pronounced disturbances at various physiological levels. Sundnes (1957) and Saunders (1963) reported the critical $O_2$ level (below which $O_2$ consumption becomes dependent upon water $O_2$ concentration) as being approxi-

mately 8.0 kPa in cod, while Jobling (1988) mentioned 2.5 kPa as being the limit below which cod asphyxiate. In our experimental conditions, no antagonism was noticed between the osmoregulation processes on the one hand and the oxygen transport or acid–base regulation mechanisms on the other. Indeed, acclimation of fish to low water salinity was never accompanied by dramatic alterations in the time-dependent changes in the physiological variables during hypoxia.

### *Ventilation and blood gases*

During normoxia, the observed changes in blood $Pa_{O_2}$ in relation to water salinity may, at least partly, be associated with the energetic cost of osmoregulation. Indeed, low $Pa_{O_2}$ values were observed in fish acclimated to middle-range salinities (i.e. 21 and 14‰), whereas extreme water salinities (i.e. 28 and 7‰) resulted in high blood oxygen tensions. Low blood $P_{O_2}$ may thus indicate a reduced oxygen requirement as water salinities decrease towards the isosmotic point (Rao, 1968; Farmer and Beamish, 1969; Maceina *et al.* 1980; Maxime *et al.* 1990). The increased blood $P_{CO_2}$ with decreasing salinity can be attributed to higher $CO_2$ production rates in conjunction with osmoregulatory processes and/or to reduced excretion rates. However, no change in red blood cell carbonic anhydrase activity, blood $P_{CO_2}$, $[HCO_3^-]$ or gill carbonic anhydrase activity were observed in flounder (*Platichthys flesus*) transferred from sea water (SW) to fresh water (FW) (Carter *et al.* 1976) or in rainbow trout (*Oncorhynchus mykiss*) transferred from FW to SW (Milne and Randall, 1976).

Cod responded to hypoxia with a rapid increase in ventilation frequency (Table 1) and in ventilatory stroke volume, though this was not directly measured. Nevertheless, the breathing movements observed during hypoxia always appeared to be more exaggerated than during normoxia. This hyperventilatory response led to greater $CO_2$ excretion and resulted in a respiratory alkalosis (Fig. 1). Despite the increased ventilation rate, $Pa_{O_2}$ decreased significantly and, together with $Pa_{CO_2}$, stabilized at a level that was linked to the intensity of hypoxia (Table 1). This difference in $Pa_{CO_2}$ in the two hypoxia groups further suggests that the ventilation/perfusion ratio of the gills may have been different. The differences in arterial blood $P_{O_2}$ and $P_{CO_2}$ observed between salinity treatments in normoxia persisted throughout hypoxia. Ventilation frequency, in contrast, was unaffected by environmental salinity. Breathing frequency, however, can be misleading as it has been observed on many occasions that fish can respond to hypoxia by changing their tidal volume with essentially no modifications in breathing frequency (Burggren and Randall, 1978; Kerstens *et al.* 1979; Smith and Jones, 1982).

### *Acid–base and erythrocytic responses*

The hyperventilatory response observed during mild hypoxia resulted in an extracellular respiratory alkalosis that persisted throughout the 6-h trial; in more severe conditions, a weak metabolic acidosis was also observed. There were significant changes in plasma $[HCO_3^-]$ during the first hour of hypoxia. Though

plasma bicarbonate level only decreased slightly during this period in moderate hypoxic conditions, a twofold decrease was observed during deep hypoxia, suggesting that a marked release of protons into the blood circulation had occurred. Thereafter, the decreases in plasma bicarbonate concentration were similar in both treatments (Fig. 1). Two main sources of metabolic protons may have been involved: (i) red blood cell (RBC) $Na^+/H^+$ ionic exchangers and (ii) white muscle anaerobic metabolism.

The plasma acidosis that occurred during the first 10–15 min of deep hypoxia may have been related to the activation of RBC $\beta$-adrenergic $Na^+/H^+$ exchange. Such an exchange process has been observed in many species of fish under stress conditions and is associated with the preservation of the haemoglobin oxygen-transport properties (Nikinmaa, 1983, 1986; Boutilier *et al.* 1986, 1988; Claireaux *et al.* 1988). Cod exposed to similar hypoxic conditions (4.0 kPa) have also been shown to exhibit increased plasma catecholamine concentrations (Fritsche and Nilsson, 1990). The increased Hct, together with the decreased MCHC, recorded in our experiments after the first hour of deep hypoxia (Fig. 2), is indicative of such stimulation of RBC ionic exchangers (Baroin *et al.* 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986). Moreover, when $Pw_{O_2}$ reached 5.5 kPa (i.e. $Pa_{O_2}$=3.5–4.0 kPa) the fish always went through a short period of violent struggling. This phenomenon has also been reported in rainbow trout when arterial blood oxygen saturation reached 50 % and was thought to be an emergency signal for the setting of the catecholamine-mediated regulation mechanisms (Thomas *et al.* 1988).

After 1 h of deep hypoxia, although tissue lactate concentrations had increased sharply (Table 2), cod displayed a good capacity to reduce the extent of the potential extracellular metabolic acidosis. Indeed, even though a decrease in the whole-blood bicarbonate concentration was observed, the level was still fairly high after 6 h of severe hypoxic conditions. This suggests either that the protons produced by anaerobic metabolism were not transferred as such into the extracellular space or that, if they were, they were subsequently disposed of at the gill. For example, lactate may diffuse into the blood without parallel movements of $H^+$ (Heisler, 1986), in which case protons would be buffered within the white muscle cells and later removed *via* metabolic pathways. Protons are also likely to be removed into the external medium *via* gill $Na^+/H^+$ exchange (see below).

In our experiments, repetitive blood sampling is likely to have had an effect, as indicated by the decreased blood Hct and [Hb], together with an unchanged MCHC, observed during mild hypoxia. During deep hypoxia, this effect was offset by the adrenergic regulation mechanisms described above.

### *Ionic variables*

During normoxia, concentrations of plasma $[Na^+]$ and $[Cl^-]$ were maintained within a narrow range in the fish acclimated to 28, 21 and 14 ‰ but decreased in the 7 ‰ salinity group. If low environmental salinity were involved, these changes

would also be within the range of the seasonal variation reported for this species (C. Audet, J.-D. Dutil, J. Munro and M. Besner, in preparation).

During deep hypoxia, haemoconcentration probably occurred, as water is likely to have shifted into the white muscle cells in response to the increased concentration of the osmotically active lactate anion. However, the potent effect of salinity on plasma ion concentrations suggests that the redistribution of water within the fish is not the only mechanism involved. The main site for ionic and acid–base regulation in both seawater and freshwater fish is the gill (McDonald *et al.* 1982; Heisler, 1986) and this regulation is thought to be achieved primarily *via* $HCO_3^-/Cl^-$ and/or $H^+(NH_4^+)/Na^+$ ionic exchangers (Heisler, 1986). Although in freshwater animals, transepithelial proton extrusion mechanisms are dependent on, if not limited by, the availability of corresponding counter ions in the medium, marine fishes are not faced with such limitations. However, the possibility of a reduction in the activity of the $HCO_3^-/Cl^-$ and/or $H^+(NH_4^+)/Na^+$ exchangers arising from the decreasing external salinity cannot be ruled out. Indeed, decreasing water salinity to 7‰ resulted in a reduction in the plasma $Na^+$ and $Cl^-$ concentrations during normoxia, as well as in the amplitude of the ionic response to hypoxia. This decreased availability of external chloride may also participate in the apparent decrease in $CO_2$ excretion rate observed in the fish acclimated to 7‰ (McDonald and Prior, 1988). In our experimental conditions, ionic movements were mainly dedicated to plasma pH regulation as SID changes were found to be salinity-independent but linked to the level of hypoxia (Fig. 6). Differential permeability of the gill to $Na^+$ and $Cl^-$ was also involved, as illustrated by the observation that only plasma $[Na^+]$ changes were found to be linked to the level of hypoxia (Isaia *et al.* 1978; McDonald *et al.* 1989). Similarly, exhaustion of the flathead sole (*Hippoglosoides elassodon*) results in a greater increase in plasma $[Cl^-]$ than in plasma $[Na^+]$ (Turner *et al.* 1983). McDonald and Rogano (1986) also showed that infusing freshwater rainbow trout with $10^{-4}$ mol $l^{-1}$ adrenaline caused a large increase in branchial electrolyte permeability and stimulated $Na^+$ efflux about threefold more than $Cl^-$ efflux. In a hyperosmotic medium, increased electrolyte permeability of the gills of cod would result in increased $Na^+$ and $Cl^-$ influxes, as well as in increased water efflux, which would explain the observed changes in ion concentrations (Figs 3 and 4). Reducing water salinity towards the isosmotic point would, accordingly, reduce the ionic diffusion and osmotic gradients and eventually result in net $Na^+$ and $Cl^-$ losses at salinities below 12‰. By determining the degree of swelling of the gill epithelial cells, salinity can also determine the size of the paracellular diffusing channels (McDonald and Prior, 1988).

Unlike plasma $[Na^+]$ and $[Cl^-]$, plasma potassium concentration during normoxia was not affected by water salinity. The time course and amplitude of the $K^+$ changes during hypoxia were found to be independent of the salinity. During deep hypoxia, plasma $[K^+]$ increased sharply during the first hour and then stabilized (Fig. 5). If outward shifts of water partially determine the plasma $K^+$ concentration, this accumulation certainly also reflects an extrusion of $K^+$ from

the muscle cells to the extracellular space in response to an intracellular acidosis. In moderate hypoxia, in contrast, slightly decreased plasma [$K^+$] suggests that intramuscular anaerobic metabolism was not stimulated.

### *Metabolic responses*

The salinity of the acclimation medium had no effect on the level of plasma metabolites (Fig. 6) and hardly influenced the tissue metabolite levels (Table 2) under normoxic condition. Moreover, where variations were observed (plasma pyruvate, heart and liver glucose and white muscle glycogen), the possibility of a seasonal, salinity-independent effect could not be discounted. Indeed, Eliassen and Vahl (1982) have demonstrated marked seasonal changes in the biochemical composition and energy content of the liver, gonad and white muscle in cod.

During hypoxia, the lactate build-up in the plasma is undoubtedly related to the stimulation of anaerobic metabolism (Dunn and Hochachka, 1987). Though white muscle is known to be a probable source of lactate, increased lactate concentrations were also observed in the heart and the liver. Studies have suggested that 'aerobic tissues', such as heart and liver, can take up lactate from the blood and oxidize it (Bilinski and Jonas, 1972; Milligan and Wood, 1986). Similarly, Lanctin *et al.* (1980) and Farrell *et al.* (1988) have shown that exogenous glucose and lactate are effective substrates for rainbow trout heart. Driedzic (1978), however, has shown that high levels of lactate are detrimental to cardiac function in a hypoxic heart preparation of the dogfish (*Mustellus canis*). Similarly, Dunn and Hochachka (1986) suggested that rainbow trout heart is a lactate exporter and does not survive well under hypoxic stress.

As in rainbow trout (Dunn and Hochachka, 1986) or in flounder (Jorgensen and Mustafa, 1980), the increases in lactate and glucose concentration were not accompanied by parallel decreases in liver glycogen content. What is the metabolic origin of these metabolites? Dunn and Hochachka (1987) reported that glucose turnover was not increased during hypoxia in trout and they suggested that liver glycogen was not mobilized into glucose (to be later distributed *via* the blood circulation), but rather that endogenous sources had to be present in each organ to fuel the glycolysis. In our case, the absence of any depletion of liver glycogen together with the relatively moderate increase in plasma glucose concentration and the marked rise in liver and white muscle glucose content favour this hypothesis. However, the possibility of a slowing down of anaerobic metabolism, to reduce the metabolic acidosis, must not be discounted (Van Den Thillart, 1982; Van Den Thillart and Van Waarde, 1985). Indeed, experiments on isolated liver of both anoxia-tolerant bullhead catfish (*Ictalurus nebulosus*) and anoxia-sensitive rainbow trout showed that glycolysis was not enhanced to compensate for the decline in aerobic ATP production (i.e. the absence of a Pasteur effect), implying a reduction in the metabolic demand (Heath, 1988).

### *Mortality*

In severe hypoxic conditions, six out of 29 fish died between the fourth and the

fifth hour of the experiment. The cause of death was difficult to establish because of the small number of fish. Nevertheless, no differences were observed at $t$=180 min in any of the variables measured ($P$>0.05) when fish that died before the end of the trial ($t$=360 min) were compared to those that survived. As reported in severely exercised rainbow trout by Wood *et al.* (1983), none of the physiological variables studied here could clearly be related to the low tolerance to hypoxia.

In conclusion, acid–base and metabolic perturbations are only seen during severe hypoxia. Water salinity has little influence on the tolerance of cod to low levels of oxygen as it only determines the ion and water movements. Although six of the 29 fish died during the deep hypoxic trial (two for each of the groups acclimated to 28, 21 and 14 ‰), all of the fish acclimated to 7 ‰ ($N$=7) survived the exposure to severe conditions, suggesting no direct antagonism between low-salinity acclimation and hypoxia tolerance. Atlantic cod can tolerate very low oxygen concentrations for up to 6 h. This gives them the ability to cope temporarily with poorly oxygenated waters such as may occur in their habitat. Dunbar *et al.* (1980) and Sameoto and Herman (1990) have shown that low oxygen concentrations are to be found in deeper water layers in areas where cod are present. Though oxygen levels reported in these studies are not lethal, they may become physiologically relevant when considering fish in their environment. Cod searching for food near the sea floor or swimming away from predators may encounter oxygen-poor waters. Increased oxygen demand during digestion may also require that they avoid such poorly oxygenated waters. Furthermore, dissolved oxygen concentration, together with salinity and temperature, may be a major factor to be taken into account when considering distribution and small-scale migration of cod. These aspects are currently being investigated in our laboratory.

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