



## **Vacuolar-Type H<sup>+</sup>-ATPase and Na<sup>+</sup>, K<sup>+</sup>-ATPase Expression in Gills of Atlantic Salmon (*Salmo salar*) during Isolated and Combined Exposure to Hyperoxia and Hypercapnia in Fresh Water**

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# Vacuolar-Type H<sup>+</sup>-ATPase and Na<sup>+</sup>, K<sup>+</sup>-ATPase Expression in Gills of Atlantic Salmon (*Salmo salar*) during Isolated and Combined Exposure to Hyperoxia and Hypercapnia in Fresh Water

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**ABSTRACT**—Changes in branchial vacuolar-type H<sup>+</sup>-ATPase B-subunit mRNA and Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit mRNA and ATP hydrolytic activity were examined in smolting Atlantic salmon exposed to hyperoxic and/or hypercapnic fresh water. Pre-smolts, smolts, and post-smolts were exposed for 1 to 4 days to hyperoxia (100% O<sub>2</sub>) and/or hypercapnia (2% CO<sub>2</sub>). Exposure to hypercapnic water for 4 days consistently decreased gill vacuolar-type H<sup>+</sup>-ATPase B-subunit mRNA levels. Salmon exposed to hyperoxia had either decreased or unchanged levels of gill B-subunit mRNA. Combined hyperoxia+hypercapnia decreased B-subunit mRNA levels, although not to the same degree as hypercapnic treatment alone. Hyperoxia generally increased Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit mRNA levels, whereas hypercapnia reduced mRNA levels in presmolts ( $\beta$ ) and smolts ( $\alpha$  and  $\beta$ ). Despite these changes in mRNA levels, whole tissue Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was generally unaffected by the experimental treatments. We suggest that the reduced expression of branchial vacuolar-type H<sup>+</sup>-ATPase B-subunit mRNA observed during internal hypercapnic acidosis may lead to reduction of functional V-type H<sup>+</sup>-ATPase abundance as a compensatory response in order to minimise intracellular HCO<sub>3</sub><sup>-</sup> formation in epithelial cells.

**Key words:** salmon, acidosis, gill, H<sup>+</sup>-ATPase, Na<sup>+</sup>,K<sup>+</sup>-ATPase

## INTRODUCTION

The gill is the major iono-regulatory organ in teleosts. In the current model for active Na<sup>+</sup> uptake across tight epithelia, such as frog skin and the gills of freshwater (FW) teleosts, the driving force is thought to be set up by two membrane ion-pumps acting in series: an apical H<sup>+</sup>-ATPase and a basolateral Na<sup>+</sup>,K<sup>+</sup>-ATPase (Ehrenfeld and Garcia-Romeu, 1977; Avella and Bornancin, 1989). The H<sup>+</sup>-ATPase extrudes protons to the surrounding dilute medium thereby creating the electrochemical gradient that enables Na<sup>+</sup> to diffuse in through apical Na<sup>+</sup>-channels. Baso-laterally, Na<sup>+</sup> is pumped into the blood by the action of the Na<sup>+</sup>,K<sup>+</sup>-ATPase. Furthermore, the activity of the H<sup>+</sup>-ATPase may energise the uptake of Cl<sup>-</sup>, as proton extrusion leads to a build up of HCO<sub>3</sub><sup>-</sup>, which may then be

exchanged for Cl<sup>-</sup> via the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> antiporter in the apical membrane of gill epithelial cells (Krogh, 1938; Fenwick *et al.*, 1999). In FW fish, this model is supported by the presence of immunoreactivity for vacuolar(V)-type H<sup>+</sup>-ATPase (Lin *et al.*, 1994; Sullivan *et al.*, 1995), Na<sup>+</sup>,K<sup>+</sup>-ATPase (Ura *et al.*, 1996; Witters *et al.*, 1996), and carbonic anhydrase (Rahim *et al.*, 1988), as well as Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> antiporter mRNA expression (Sullivan *et al.*, 1996) in both primary filament and secondary lamellae gill cells.

In FW teleosts, the gill also constitutes the major site for acid-base regulation through the differential regulation of Na<sup>+</sup> and Cl<sup>-</sup> transport mechanisms. During a respiratory acidosis induced by environmental hypercapnia or hyperoxia, plasma HCO<sub>3</sub><sup>-</sup> levels are increased as a means of regulating plasma pH back towards its original value (review by Heisler, 1993). The compensation has in some cases been shown to involve modulation of Na<sup>+</sup> fluxes, but in most cases the compensation is mainly Cl<sup>-</sup> mediated (Wood *et al.*, 1984; Jensen and Weber, 1985; Larsen and Jensen, 1997; review by Heisler, 1993). Recently, regulation of the teleost gill V-type H<sup>+</sup>-ATPase has been studied during acid-base disturbances. Despite some disagreement on the cellular location of this protein (Lin *et al.*, 1994; Sullivan *et al.*, 1995), gill V-type H<sup>+</sup>-ATPase

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B-subunit mRNA level showed a transient rise after 2 hr exposure of rainbow trout to hypercapnic water but otherwise stayed at pre-hypercapnia levels until the end of a 12 hr-experiment (Perry *et al.*, 2000). In contrast to the current debate concerning the specific cells involved in ion uptake across the FW-teleost gill (e.g. Goss *et al.*, 1992), the excretion of ions across the seawater (SW)-teleost gill is generally agreed to be associated with chloride cells (CCs) in the primary filament, where the Na<sup>+</sup>,K<sup>+</sup>-ATPase located in the baso-lateral membrane provides the driving force for the trans- and para-cellular excretion of ions (Silva *et al.*, 1977).

The juvenile anadromous salmon develops gill ion excretory mechanisms during the parr-smolt transformation. This metamorphosis-like transformation takes place in FW and changes the stenohaline parr-fish into the euryhaline smolt (for review see Hoar, 1988). During the parr-smolt transformation, gill CCs mature and their numbers increase (Pisam *et al.*, 1988; Ura *et al.*, 1997) elevating the total amount of Na<sup>+</sup>,K<sup>+</sup>-ATPases and specific Na<sup>+</sup>,K<sup>+</sup>-ATPase activity (e.g. Ura *et al.*, 1997). In a recent study, cDNA fragments of the Atlantic salmon gill V-type H<sup>+</sup>-ATPase B-subunit, Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit, and Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\beta$ -subunit were cloned, and their regulation in smolting Atlantic salmon was reported (Seidelin *et al.*, 2001). In salmon restocking programs, bulk transportation of juveniles to the release site often results in conditions of combinations of hyperoxia and hypercapnia. Little is known about the physiological consequences of this treatment to the salmon in relation to the stage of the parr-smolt cycle. Recently we reported on the organismal responses of exposing Atlantic salmon pre-smolts, smolts and post-smolts to hyperoxia and hypercapnia and its influence on subsequent SW-tolerance (Brauner *et al.*, 2000). The aim of the present study was to investigate at the mRNA level, how vacuolar-type H<sup>+</sup>-ATPase B-subunit and  $\alpha$ - and  $\beta$ -subunits of the Na<sup>+</sup>,K<sup>+</sup>-ATPase were affected in the gills of just the same fish as used in the study of Brauner *et al.* (2000).

## MATERIALS AND METHODS

### Fish Maintenance

Four hundred immature upper-mode (>13 cm length, mixed sex) pre-smolts of Atlantic salmon, *Salmo salar* (1 year old, first generation hatchery fish of the Irish Burrishoole River stock), were obtained in late February 1997 from the Salmon Rearing and Research Station (Randers, Denmark). The fish had been hatched and reared in in-door tanks under simulated natural photoperiod and water temperature (minimum temperatures during winter 4°C). The fish were brought to the Odense University Campus and held in outdoor 500-L flow-through freshwater (FW) tanks supplied with Odense tap water (in mM: [Cl<sup>-</sup>]=1.39, [Na<sup>+</sup>]=1.46, [K<sup>+</sup>]=0.16, [Mg<sup>2+</sup>]=0.64, [Ca<sup>2+</sup>]=3.03, [HCO<sub>3</sub><sup>-</sup>]=5.4, pH 8.3). They were fed a 2% (body weight)<sup>-1</sup> diet of commercial trout pellets three times a week.

### Experimental protocol

Feeding was withheld 4 days prior to experimentation. Upon start of an experiment, 24 fish were placed in each experimental tank containing 30 L of fresh water at 10°C with a 12-hr light:12-hr dark-rhythm. The water surface was covered with a piece of polystyrene and the water bubbled with either air (control), 100% O<sub>2</sub> (hyperoxia), 2% CO<sub>2</sub> and 98% air (hypercapnia), or 2% CO<sub>2</sub> and 98% O<sub>2</sub> (hyperoxia +hy-

percapnia). Gas mixtures were supplied from Wösthoff (Bochum, Germany) gas mixing pumps. The actually measured partial pressures (in mmHg) for the different treatment groups were (mean±SEM): control (PO<sub>2</sub>: 152±2.2; PCO<sub>2</sub>: 2.2±0.35), hyperoxia (PO<sub>2</sub>: 618±18.1; PCO<sub>2</sub>: 2.2±0.43), hypercapnia (PO<sub>2</sub>: 143±3.7; PCO<sub>2</sub>: 13.7±1.20), hyperoxia+hypercapnia (PO<sub>2</sub>: 597±14.6; PCO<sub>2</sub>: 14.6±1.29). Half the tank volume of water was changed twice daily during the exposure through a slow inflow of fresh water. The partial pressure levels of both O<sub>2</sub> and CO<sub>2</sub> stayed constant during this water exchange in all treatment groups. The exposure time was 4 days in pre-smolts (Series 1; starting March 21), 4 and 1 day in smolts (Series 2, starting April 27 and Series 3, starting May 5, respectively) and 4 days in post-smolts (Series 4, starting September 12). At the end of the exposure, 8–12 fish from each group were sampled. The initiation of each experimental series was decided by monitoring the progress of smoltification in the same stock of Atlantic salmon (held under similar conditions) by classical 24-hr SW-challenge tests and subsequent determination of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activities and deflections in muscle water content (Seidelin *et al.*, 2001).

### Sampling

The fish were stunned by a blow to the head, whereupon blood was drawn from the caudal vessels into heparinised syringes. The fish were then decapitated, and samples of the gills were taken. From 4–6 fish in each group, 1 first gill arch, 2 third and 2 fourth gill arches were immediately homogenised in 2.5 mL of ice-cold denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M  $\beta$ -mercaptoethanol, and 0.3% Antifoam (Sigma), pH 7.0). In addition, the second gill arches were dissected out from all fish and placed in buffer (300 mM sucrose, 20 mM EDTA, 50 mM imidazole, pH 7.3). All samples were immediately frozen in liquid N<sub>2</sub> and stored at -80°C until analysis.

### Analysis

Gill V-type H<sup>+</sup>-ATPase B-subunit, Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit mRNA levels were analysed by northern blotting. Gill total RNA was isolated as described by Madsen *et al.* (1995). Samples of 20  $\mu$ g total gill RNA from each fish were loaded on 1% agarose / 6.5% formaldehyde-gels and electrophoresed at 5 V/cm in 1×MOPS buffer (20 mM 3-(N-morpholino) propanesulfonic acid, 5 mM NaAc, 0.5 mM EDTA, pH 7.0). Total RNA from pre-smolts (n=5–6) and post-smolts (n=4) were run on the first gel, total RNA from smolts (n=6) were run on the second gel, and total RNA from fish of different developmental stages were run on the third gel to allow for comparison of relative gill mRNA expression between all fish. Ribonucleic acids were transferred by capillary blotting onto nylon membranes (Zeta probe, Bio-Rad, Hercules, CA, USA). To assess RNA integrity, RNA was stained by soaking the membranes in 0.04% methylene blue / 0.5 M NaAc (pH 5.2) for a few minutes and then destained in milliQ H<sub>2</sub>O. Membranes were subsequently hybridized, stripped and probed with radiolabelled specific Atlantic salmon gill cDNA-probes of the V-type H<sup>+</sup>-ATPase B-subunit, the Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit (Genbank accession numbers AJ250811, AJ250809, AJ250810, respectively: Seidelin *et al.* 2001) and rainbow trout  $\beta$ -actin (Madsen *et al.*, 1997). Each hybridization was performed as follows. The Northern blot membrane was pre-hybridized for 4 hr at 47°C in 10 ml of pre-hybridization buffer containing 50% deionised formamide, 5×SSC (0.75 M NaCl, 75 mM Na<sub>2</sub>citrate, pH 7.0), 1% SDS, 5×Denhardt's (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% BSA), 0.1% Na<sub>3</sub>P<sub>3</sub>i, 1 mM EDTA, 5 mg denatured calf thymus DNA, and 2 mg denatured yeast transfer RNA. Radioactive cDNA probes were made by random primer extension ( $\alpha$ <sup>32</sup>P-dCTP; Oligolabelling kit, Pharmacia, Uppsala, Sweden), separated from the unincorporated nucleotides on a G-50 micro column (ProbeQuant™, Pharmacia), denatured, 1.25×10<sup>6</sup> cpm of specific probe activity added per mL of prehybridization-buffer, and hybridized for 16 hr. Radioactivity was detected by phosphor imaging exposing screens after 1–4 days (Storm, Molecular Dynamics, Sunny-

vale, CA, USA). All probes used hybridize with transcripts of only one size in gills of Atlantic salmon (see Seidelin *et al.*, 2001). Relative band intensities were analysed by the ImageQuaNT 4.1 software (Molecular Dynamics).

Gill  $\text{Na}^+, \text{K}^+$ -ATPase activities were analysed at 25°C in crude homogenates by the method of McCormick (1993) using a plate reader (Spectramax, Molecular Devices, Sunnyvale, CA, USA). Protein content was measured by the method of Lowry *et al.* (1951) also using plate reader.

### Statistics

For each series, we used a 2×2-factorial statistical design where factor 1 was either normoxia or hyperoxia, and factor 2 was either normocapnia or hypercapnia. Statistical differences were analysed using SYSTAT 5.03 (Systat, 1991, Evanston, IL, USA). When necessary, transformations of data were performed to meet the parametric ANOVA assumption of homogeneity of variances (evaluated by residual-plots). In each series, data for individual parameters were analysed by two-way ANOVA. If the interaction among the two factors was significant, differences among individual groups were analysed by Tukey's Honestly Significant Difference (HSD) Test. A Tukey-Kramer adjustment of the Tukey's HSD Test was performed

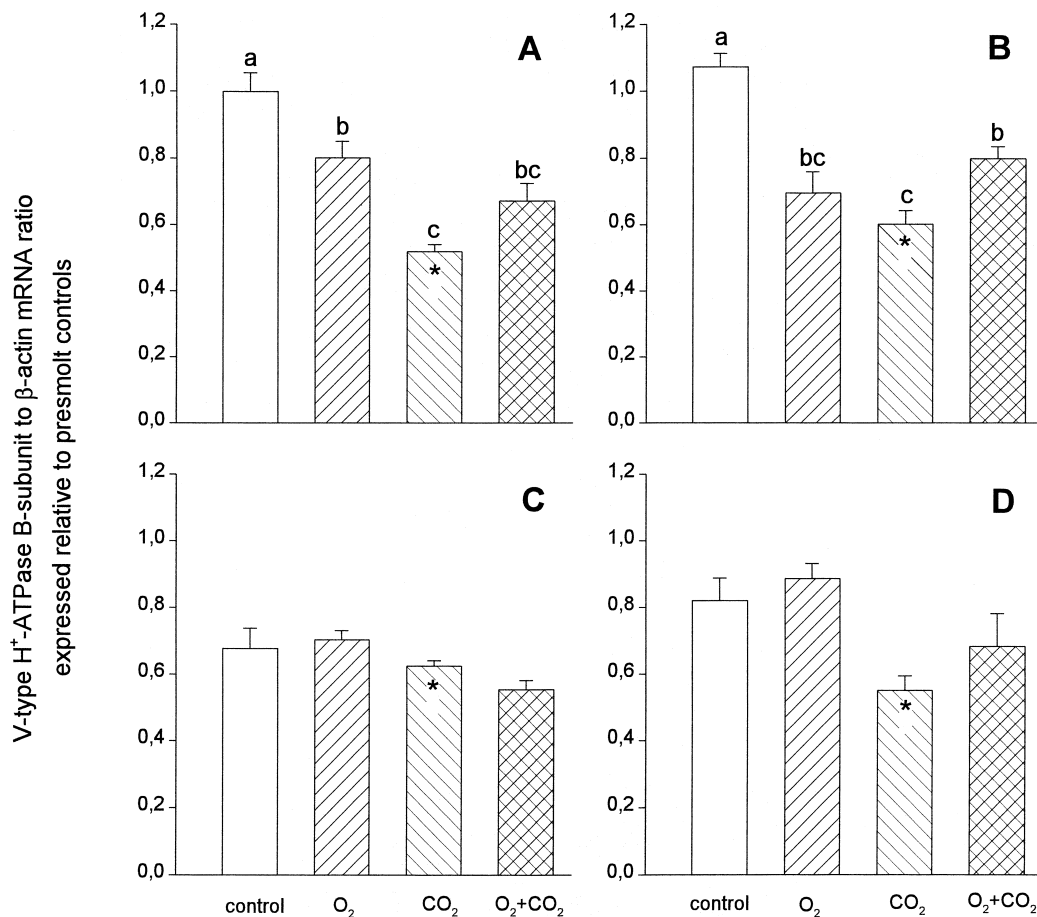
when the dataset contained unequal numbers in treatment groups. In all cases, a significance level of  $P \leq 0.05$  was used.

## RESULTS

Smolting is a gradual process and salmon used on May 5 (1-d exposure) were more fully developed smolts than those on April 27 (4-d exposure). Therefore, the differences in the response to experimental treatments between these two groups can not unequivocally be ascribed to duration of the experimental treatment.

### Gill vacuolar-type $\text{H}^+$ -ATPase B-subunit mRNA levels

Control groups of pre-smolt and smolt fish (exposed on April 27) had higher gill V-type  $\text{H}^+$ -ATPase B-subunit mRNA levels than smolts exposed on May 5 and post-smolt (Fig. 1). Hypercapnia exposure (hypercapnia and hypercapnia + hyperoxia groups) consistently induced a significant reduction in  $\text{H}^+$ -ATPase B-subunit mRNA levels compared to the



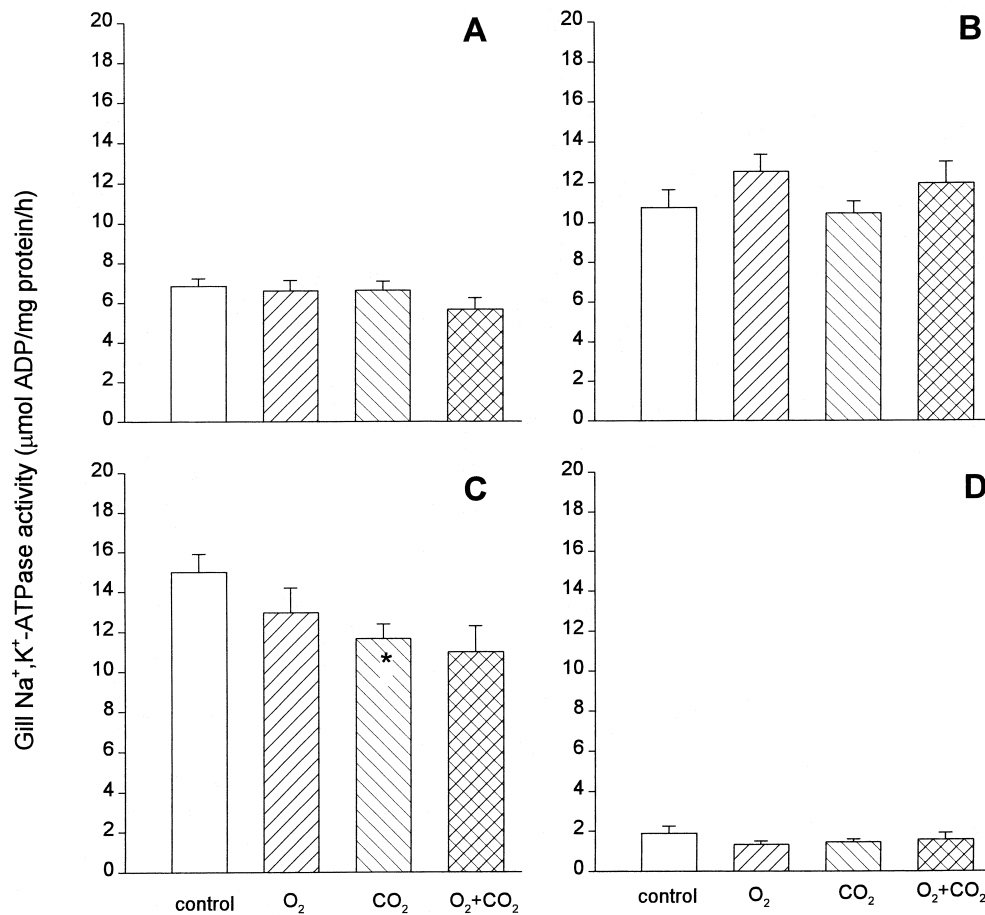
**Fig. 1.** Beta-actin normalised levels of gill vacuolar-type  $\text{H}^+$ -ATPase B-subunit mRNA in Atlantic salmon exposed to either control (normoxic+normocapnic), hyperoxic (100%  $\text{O}_2$ ), hypercapnic (2%  $\text{CO}_2$ ), or hyperoxic+hypercapnic fresh water for 4 days (A: pre-smolts,  $N=5-6$ ; B: smolts,  $N=6$ ; D: post-smolts,  $N=4$ ) or 1 day (C: smolts,  $N=6$ ). Data are shown as means + SEM. All values were normalised to the control pre-smolt values. An asterisk indicate an overall effect of the hypercapnic water exposure within that series (two-way ANOVA,  $P \leq 0.05$ ). There was a significant interaction among the two factors, only for pre-smolts and smolts exposed for 4 days; values with shared letters are not significantly different from each other ( $P > 0.05$ ).

normocapnic treatments (control and hyperoxia groups). Exposure of pre-smolts and smolts to hyperoxic water for 4 days induced lower levels of B-subunit mRNA, whereas mRNA abundance was unchanged relative to controls in smolts exposed for 1 day and in post-smolts exposed for 4 days. Non-additive effects of hyperoxia and hypercapnia on B-subunit mRNA levels were observed only in pre-smolts and smolts exposed for 4 days. However, in pre-smolts, post-smolts and smolts exposed for 1 day, the combined exposure resulted in similar levels of gill V-type H<sup>+</sup>-ATPase B-subunit mRNA as in the hypercapnic condition.

### Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase expression

Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was elevated in smolts (Fig. 2B, C) compared to pre-smolts (Fig. 2A). The activity of the smolt series 3 started at May 5 (Fig. 2C) showed even higher levels than the smolt series 2 started at April 27 (Fig. 2B). In post-smolts sampled in September, enzyme activity (Fig. 2D) was decreased to the same level as observed in parr-fish at

the onset of the parr-smolt transformation (Seidelin *et al.*, 2001). In fish exposed to hyperoxic and/or hypercapnic water for 4 days, gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was the same as in control fish. However, in smolts exposed for 1 day to hypercapnic and hyperoxic+hypercapnic water, enzyme activity was reduced compared to normoxic and hyperoxic fish. The ratios of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit mRNA levels were near constant in all groups of fish (Table 1). Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit mRNA levels in smolt control fish were slightly higher than in pre-smolts and more than twice the values in post-smolts. Only in series 3 smolts, started on May 5 (smolt-1d), were gill Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit mRNA levels clearly increased above levels observed in series 2 smolts, started on April 27 (smolt-4d). Smolts exposed to hypercapnic water for 1 and 4 days had lower levels of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit than controls. Hypercapnic pre-smolts also had lower expression of the  $\beta$ -subunit mRNA. Hyperoxia for 4 days caused an overall increase in Na<sup>+</sup>,K<sup>+</sup>-ATPase subunit mRNA levels in both pre-smolts and smolts.



**Fig. 2.** Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity levels in Atlantic salmon exposed to either control (normoxic+normocapnic), hyperoxic (100% O<sub>2</sub>), hypercapnic (2% CO<sub>2</sub>), or hyperoxic+ hypercapnic fresh water for 4 days (A: pre-smolts, N=10–12; B: smolts, N=9–12; D: post-smolts, N=8–11) or 1 day (C: smolts, N=12). Data are shown as means + SEM. An asterisk indicate an overall effect of 1 day hypercapnic water exposure in smolts (two-way ANOVA, P≤0.05). There were no exposure effects for pre-smolts, smolts and post-smolts exposed for 4 days (P>0.05).

**Table 1.** Beta-actin normalised levels of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -subunit mRNA in Atlantic salmon exposed to either control (normoxic+normocapnic), hyperoxic (100 % O<sub>2</sub>), hypercapnic (2 % CO<sub>2</sub>), or hyperoxic+hypercapnic fresh water for 4 days or 1 day. All values were normalised to control pre-smolt values. Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit mRNA levels were not affected by hyperoxic and/or hypercapnic water exposure in post-smolts.

		Pre-smolts		Smolts-4d		Smolts-1d		Post-smolts	
Na <sup>+</sup> ,K <sup>+</sup> -ATPase $\alpha$ -subunit mRNA	Control	1.00±0.059		1.17±0.026	<b>a</b>	1.59±0.136	<b>a</b>	0.61±0.047	
	O <sub>2</sub>	1.19±0.059	*	1.21±0.051	<b>*a</b>	1.41±0.059	<b>ab</b>	0.89±0.129	
	CO <sub>2</sub>	0.99±0.061		0.94±0.077	<b>b</b>	1.01±0.046	<b>*c</b>	0.77±0.052	
	O <sub>2</sub> +CO <sub>2</sub>	1.09±0.079		1.36±0.038	<b>a</b>	1.22±0.030	<b>bc</b>	0.75±0.047	
Na <sup>+</sup> ,K <sup>+</sup> -ATPase $\beta$ -subunit mRNA	Control	1.00±0.042		1.21±0.073		1.19±0.058	<b>a</b>	0.48±0.042	<b>b</b>
	O <sub>2</sub>	1.24±0.069	*	1.26±0.123	*	1.01±0.075	<b>ab</b>	0.88±0.057	<b>*a</b>
	CO <sub>2</sub>	0.84±0.027	*	0.65±0.048	*	0.76±0.033	<b>*c</b>	0.55±0.041	<b>b</b>
	O <sub>2</sub> +CO <sub>2</sub>	0.98±0.066		0.99±0.061		0.90±0.060	<b>bc</b>	0.64±0.066	<b>b</b>

Data are shown as means  $\pm$  SEM; pre-smolts: N=5–6; smolts-1d: N=6; smolts-4d: N=6; post-smolts: N=4.

\* indicates an overall effect of the hyperoxic or hypercapnic water exposure within that series (two-way ANOVA,  $P \leq 0.05$ ). For each series where a significant interaction among the two factors was found, values with shared letters are not significantly different from each other (Tukey's HSD Test,  $P > 0.05$ ).

## DISCUSSION

### Parr-smolt transformation

The upper-mode Atlantic salmon used in the present study clearly underwent the parr-smolt transformation during the course of this study as judged from changes in gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and mRNA levels in FW (this study), and from plasma osmolality and ion deflections in fish transferred to SW for 24 hr (reported in Brauner *et al.*, 2000). The basic level of gill V-type H<sup>+</sup>-ATPase B-subunit mRNA (i.e. in normoxic + normocapnic water) appears high in pre-smolts and early smolts (April 27), and was lower in smolts (May 5) and post-smolts (Fig. 1). These smoltification-related changes are very similar to our previous report (Seidelin *et al.*, 2001) using the same stock of fish. We suggested that V-type H<sup>+</sup>-ATPase expression is high during the development of gill ion-excretory mechanisms in order to counter an increased ion-loss in FW. This idea is supported by the observation that trans-epithelial Na<sup>+</sup> fluxes increase during the parr-smolt transformation (Primmitt *et al.*, 1988), and gill V-type H<sup>+</sup>-ATPase immunoreactivity of SW-acclimated rainbow trout is lower than that of FW-acclimated trout (Lin *et al.*, 1994). Thus the lower levels of V-type H<sup>+</sup>-ATPase B-subunit mRNA in smolts exposed on May 5 (Fig. 1C) compared to those exposed on April 27 (Fig. 1B) may indicate that the former fish were more fully developed smolts. This is also supported by the more elevated levels of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in these fish (Fig. 2C).

### Gill vacuolar-type H<sup>+</sup>-ATPase expression

Exposure to hyperoxia and/or hypercapnia leads to an internal respiratory acidosis that is compensated by modulation of the transfer of acid-base relevant ions across the gill epithelium (review by Heisler, 1993). The compensatory accumulation of HCO<sub>3</sub><sup>-</sup> in body fluids is primarily Cl<sup>-</sup> mediated (cf. Introduction). This was also the case in the present exposures (reported in Brauner *et al.*, 2000), as large decreases were seen in plasma [Cl<sup>-</sup>] during hypercapnia and/or hyperoxia. The present data using a homologous V-type H<sup>+</sup>-ATPase B-

subunit probe demonstrate that compensation of an internal respiratory acidosis induced by exposure to hypercapnic water is associated with a consistent decrease in V-type H<sup>+</sup>-ATPase B-subunit expression in gills of Atlantic salmon after 1–4 days (Fig. 1A, B, D). Even though we do not know how this change affects the protein level within the present time frame, the observation is in accordance with Lin *et al.* (1994). They showed that exposure of rainbow trout to hypercapnic fresh water for 40 hr decreased gill V-type H<sup>+</sup>-ATPase 70 kDa A-subunit protein levels. Their estimate of the relative abundance of the 70 kDa A-subunit was, however, complicated by the presence of additional high molecular weight positive staining material in the gel. In contrast, the V-type H<sup>+</sup>-ATPase 31 kDa E-subunit protein level was increased during maximum net acid-secretion in rainbow trout exposed to hypercapnia for 18 hr (Sullivan *et al.*, 1995). Sullivan *et al.* (1996), using a bovine 28-mer oligonucleotide V-type H<sup>+</sup>-ATPase E-subunit probe, suggested that the increased protein level was associated with an increased mRNA level in pavement cells. Perry *et al.* (2000) recently showed that in rainbow trout (N=2) exposed to hypercapnic water, gill V-type H<sup>+</sup>-ATPase B-subunit mRNA levels increased transiently at 2 hr, whereas values were back to control levels at 6 hr and 12 hr. Although we cannot exclude the possibility that H<sup>+</sup>-ATPase mRNA expression is increased in certain gill epithelial cells at the sampling times used in the present study, we consider this unlikely during long-term compensation of a respiratory acidosis, on the basis of the consistent and marked decrease in B-subunit mRNA expression in fish exposed to hypercapnia for 4 days (Fig. 1A, B, D). The less pronounced decrease in gill V-type H<sup>+</sup>-ATPase B-subunit mRNA levels in the fish exposed to hyperoxic water as compared to hypercapnic exposure correlates with lower HCO<sub>3</sub><sup>-</sup> accumulation in this situation (see Brauner *et al.*, 2000). Atlantic salmon exposed to combined hyperoxia and hypercapnia showed depressed V-type H<sup>+</sup>-ATPase B-subunit mRNA levels but generally not to the same degree as fish exposed to hypercapnia (Fig. 1). This result is difficult to interpret, as it is not known how well compensated the respiratory acidosis

was in this study.

In the tight epithelium of the renal collecting duct, Bastani *et al.* (1991) reported that total V-type 31kDa H<sup>+</sup>-ATPase E-subunit mRNA and protein levels were unchanged in acidotic rats. Instead, there was a marked increase in collecting duct intercalated cells showing apical H<sup>+</sup>-ATPase immunoreactivity over cells with diffuse or basal staining. Evidently, intercalated cells may rapidly change their acid-secretory potential without changing the expression of V-type H<sup>+</sup>-ATPase subunits. Other mechanisms may involve recruitment by assembly and disassembly of the more than 10 different V-type H<sup>+</sup>-ATPase subunits and their isoforms, as well as kinetic changes of the enzyme by regulatory proteins (review by Gluck *et al.*, 1996). Similar non-expressional regulatory mechanisms may exist in the teleost gill as indicated above, but this and earlier studies (Lin *et al.*, 1994; Sullivan *et al.*, 1995, 1996; Perry *et al.*, 2000) clearly indicate that expression levels are altered during compensation of a respiratory acidosis in fish. During the initial phase of a respiratory acidosis (0–24 hr), V-type H<sup>+</sup>-ATPase vesicles may be inserted into the apical membrane (Laurent *et al.*, 1994; Sullivan *et al.*, 1995), and new protein may be synthesised in parallel with increasing mRNA levels (Sullivan *et al.*, 1995, 1996; Perry *et al.*, 2000). In contrast, long-term compensatory mechanisms (1–4 days) seem to involve a decreased level of the gill V-type H<sup>+</sup>-ATPase protein (Lin *et al.*, 1994), a decrease or return of the messenger level (this study, Perry *et al.*, 2000) and possibly endocytosis of plasma membrane V-type H<sup>+</sup>-ATPases as suggested by Lin *et al.* (1994). A reduced H<sup>+</sup>-ATPase activity may minimise the formation of HCO<sub>3</sub><sup>-</sup> in the epithelial cells and decrease cytosolic [HCO<sub>3</sub><sup>-</sup>], thereby limiting the loss of HCO<sub>3</sub><sup>-</sup> to the water through the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger, or even allowing a shift to a HCO<sub>3</sub><sup>-</sup> (influx)/Cl<sup>-</sup> (efflux) mode in water with high [HCO<sub>3</sub><sup>-</sup>] as used in this study (cf. Larsen and Jensen, 1997). In accordance with this hypothesis, plasma [Cl<sup>-</sup>] is strongly reduced, whereas plasma [Na<sup>+</sup>] stays at the same or an only slightly elevated level during compensation of a respiratory acidosis in salmonids.

### Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase expression

Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity is generally positively correlated with hypo-osmoregulatory ability in developing salmon smolts (review by McCormick, 1995). This trend was also obvious in the present study (Fig. 2 and Brauner *et al.*, 2000). Additionally, smolts exposed on May 5 were more fully developed than smolts on April 27, as reflected by increased gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity levels (Fig. 2), and a slightly better SW-tolerance (Brauner *et al.*, 2000). As reported by Brauner *et al.* (2000), exposure to hyperoxia generally decreased SW-adaptability whereas hypercapnia improved it. These differences were largely accompanied by unchanged gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity (Fig. 2A, B, D). Only smolts exposed to hypercapnia for 1 day showed a decrease in enzyme activity (Fig. 2C). These data suggest that exposure of fish to hyperoxic and/or hypercapnic water may rather affect branchial ion-permeability or the activation of ion-excretory mecha-

nisms when subsequently exposed to SW. Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase α- and β-subunit mRNA levels (Table 1) in control-fish were comparable to levels in the same stock of salmon sampled throughout the spring (Seidelin *et al.*, 2001), with slightly elevated levels in smolts and much lower levels in post-smolts compared with pre-smolts. Hyperoxia increased, whereas hypercapnia decreased Na<sup>+</sup>,K<sup>+</sup>-ATPase subunit mRNA levels - changes that were not transmitted to the level of enzymatic activity within the time course of the study. The reason for these effects on gill Na<sup>+</sup>,K<sup>+</sup>-ATPase expression is unclear at present but when studying the response in the whole gill there is always a chance that the overall expression of Na<sup>+</sup>,K<sup>+</sup>-ATPase subunits may mask changes in expression within individual cell types with putative differential osmoregulatory functions (Pisam *et al.*, 1988; Ura *et al.*, 1997; Seidelin *et al.*, 2000).

### Conclusion

Hyperoxic and/or hypercapnic water induced a respiratory acidosis in the young Atlantic salmon which was at least partly compensated (Brauner *et al.*, 2000). This was accompanied by a paradoxical but consistent reduction in the level of gill V-type H<sup>+</sup>-ATPase B-subunit mRNA in groups exposed to hypercapnia (all groups 1–4 days) and hyperoxia (pre-smolts and smolts exposed for 4 d). From the present data set and recent publications in the field, there is an emerging picture of a biphasic response during acclimation to hypercapnia. An early (<24hr) increase in the expression seems to be followed by a reduced expression during long-term compensation of the internal hypercapnic acidosis. Further studies are clearly needed to clarify the dynamics of expressional and non-expressional regulation of the gill V-type H<sup>+</sup>-ATPase and other ion-transporters involved in branchial acid/base-regulation.

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