

# Distinct hormonal regulation of $\text{Na}^+, \text{K}^+$ -atpase genes in the gill of Atlantic salmon (*Salmo salar* L.)

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## Abstract

It has recently become evident that maintenance of ionic homeostasis in euryhaline salmonids involves a reciprocal shift in expression of two isoforms of the gill  $\text{Na}^+, \text{K}^+$ -atpase  $\alpha$ -subunit when the surrounding salinity changes. The present study investigated the regulation of this shift between the  $\alpha 1a$  (freshwater (FW) isoform) and the  $\alpha 1b$  (seawater (SW) isoform) by cortisol, Gh, prolactin (Prl) and Igf1. Injection with cortisol into FW salmon increased  $\alpha 1a$  expression, while Gh had no effect. Conversely, both cortisol and Gh stimulated  $\alpha 1b$  expression, and a significant synergy was observed. *igf1* expression was increased by Gh in both gill and liver, and inhibited by cortisol in the liver. Gill *igf1* and *gh* receptor expression increased in response to cortisol. Injection with Prl into SW salmon compromised their

hypo-osmoregulatory performance, selectively reduced the expression of the  $\alpha 1b$  isoform and decreased enzymatic  $\text{Na}^+, \text{K}^+$ -atpase activity in the gill. Cortisol and Prl reduced gill and liver *igf1* expression, and both hormones stimulated gill *igf1* receptor expression. In a short-term experiment with incubation of FW gill cell suspensions, cortisol stimulated  $\alpha 1a$  and  $\alpha 1b$  expression, while Igf1 stimulated only  $\alpha 1b$ . The data elaborate our understanding of Prl and Gh as being antagonists in the control of gill ion regulation, and support a dual role for Gh involving endocrine and paracrine Igf1 action. Gh and Prl may be the decisive stimuli that direct cortisol-aided mitochondrion-rich cell development into either secretory or absorptive types.

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## Introduction

The gill epithelium of euryhaline teleosts has an extraordinary functional plasticity, as it is able to interchange between modes of ion uptake in freshwater (FW) and ion excretion in seawater (SW). In both environments, mitochondrion-rich cells (MRCs) and their adjoining accessory and pavement cells are fundamental to compensatory ion transport for which the driving force is delivered by the  $\text{Na}^+, \text{K}^+$ -atpase and in the FW mode possibly complemented by an apical V-type  $\text{H}^+$ -ATPase (see Hirose *et al.* 2003). The recent discovery of different  $\text{Na}^+, \text{K}^+$ -atpase  $\alpha$ -1 isoforms in the salmonid teleost gill ( $\alpha 1a$ ,  $\alpha 1b$  and  $\alpha 1c$ ; Richards *et al.* 2003) has offered new insight into transepithelial ion transport in the gill. The mRNA expression levels of these isoforms have been investigated during salinity acclimation in several salmonids, and it is remarkable how salinity induces a reciprocal switch between  $\alpha 1a$  and  $\alpha 1b$  isoforms,  $\alpha 1a$  being augmented in FW and  $\alpha 1b$  in SW (Richards *et al.* 2003, Mackie *et al.* 2005, Bystrianski *et al.* 2006). This has led to the interesting suggestion that  $\alpha 1a$  is a FW isoform driving ion uptake, whereas  $\alpha 1b$  is a SW isoform driving ion excretion. Recently, this hypothesis has gained support based on structural and thermodynamical considerations (Jorgensen 2008). We demonstrated the specific localization of the  $\alpha$  isoform

transcripts in the gill (Madsen *et al.* 2009) and recognized  $\alpha 1b$  as a good marker for SW type MRC and their putative precursor cells deeper in the filament, while the possibility that  $\alpha 1a$  plays a dual role in both FW and SW could not be excluded.

An array of signalling mechanisms mediates the physiological changes necessary to counter changes in environmental salinity. Characteristically for salmonid teleosts, transfer to SW induces increased plasma Gh and insulin-like growth factor-I (Igf1) along with reduced prolactin (Prl) levels (Avella *et al.* 1990, Young *et al.* 1995, Poppinga *et al.* 2007). There are reports that cortisol levels increase temporarily after both hyper- and hypo-osmotic challenges (see McCormick 2001), reflecting its role in the general stress-response. More specific roles associated with activation of ion-uptake and ion-secretory mechanisms respectively have also been assigned to cortisol (see Evans *et al.* 2005). An important role of the Gh-Igf1 axis and cortisol in promoting SW tolerance and increasing overall gill  $\text{Na}^+, \text{K}^+$ -atpase activity has been clearly documented in salmonids (Björnsson *et al.* 1987, Madsen 1990, McCormick 1996, Seidelin *et al.* 1999). It was early on established that Prl has an ion-retaining effect in teleosts (Pickford & Phillips 1959). Several years later, it was demonstrated that Prl opposes SW acclimation and the effect of Gh when injected into trout (Madsen & Bern 1992,

Seidelin & Madsen 1997). Thus, Prl and Gh appear as antagonists with respect to control of osmoregulatory status. In FW, cortisol may stimulate gill ion uptake (Laurent & Perry 1990), possibly in combined action with Prl (Zhou *et al.* 2004), but the molecular details of the interaction of these osmoregulatory hormones are not yet clear.

The main aim of the present study was to clarify the effects of Gh, Igf1, Prl and cortisol on the expression level of the assumed FW- and SW-type Na<sup>+</sup>,K<sup>+</sup>-atpase isoforms  $\alpha 1a$  and  $\alpha 1b$  respectively. In addition to analysing the effects of these hormones separately, especially the interaction with cortisol of the three peptide hormones was intriguing. The effect of cortisol was tested in two-factorial injection experiments with Gh in FW salmon, with Prl in SW salmon and with Igf1 directly in isolated gill cells from FW salmon *in vitro*. The Na<sup>+</sup>,K<sup>+</sup>-atpase  $\beta_1$ -subunit that is needed for membrane targeting and functional maturation of the Na<sup>+</sup>,K<sup>+</sup>-atpase in general and the Na<sup>+</sup>,K<sup>+</sup>,2Cl<sup>-</sup> cotransporter (Nkcc)-1a, a marker of secretory MRCs in salmon gill (Mackie *et al.* 2007), were analyzed in parallel with  $\alpha 1a$  and  $\alpha 1b$ . In order to approach the mechanism of hormonal actions and interactions, we examined the expression levels of *gh* receptor type I (*ghr1*) and *igf* receptor type I (*igfr1*) in the gill together with expression of *igf1* in both liver and gill.

## Materials and Methods

### Fish and sampling procedure

One-year-old Atlantic salmon (mixed sex *Salmo salar*, Danish Centre for Wild Salmon, Randers, Denmark) were kept in outdoor tanks with flowing tap water at environmental temperature and exposed to natural daylight through smoltification. In July, they were transferred to indoor conditions in 500 l glass fibre tanks at 14 °C at 12 h light:12 h darkness cycle. During standard sampling, the fish were anaesthetized in buffered MS222 (3-aminobenzoic acid ethyl ester) and blood was collected with a heparinized syringe from the caudal vessels, after which the fish was killed and further tissues were excised. In injection experiments, the fish were anaesthetized in buffered MS222 before injection. Upon sampling, one second gill arch was dry frozen immediately in liquid nitrogen for RNA extraction, and the other second arch was frozen in sucrose-EDTA-imidazole buffer (300 mM sucrose, 5 mM Na<sub>2</sub>EDTA, 50 mM imidazole, pH 7.3) for analysis of Na<sup>+</sup>,K<sup>+</sup>-atpase enzymatic activity. One first arch was dissected into 3 mm blocks and frozen on dry ice in optimal cutting temperature compound (Miles Inc., Eikhardt, IN, USA) for further analysis by *in situ* hybridisation (ISH). The liver was excised and frozen in liquid nitrogen for RNA extraction. The experimental protocols were approved by the Danish Animal Experiments Inspectorate and in

accordance with the European convention for the protection of vertebrate animals used for experiments and other scientific purposes (#86/609/EØF).

### Experimental protocols

Three two-factorial experiments were set up to investigate the effects of and interaction between 1) cortisol and Gh, 2) cortisol and Prl and 3) cortisol and Igf1 on osmoregulatory and endocrine targets in gill and liver. All hormone doses were chosen to be within the range commonly used in studies of osmoregulatory endocrinology in fish. Based on studies of hormone dynamics and clearance values in a variety of fish species (Hirano & Utida 1971, Weisbart *et al.* 1987, Sakamoto *et al.* 1990, Duan & Hirano 1991, Mancera *et al.* 1994, Shrimpton & Randall 1994), the chosen doses were expected to give temporarily supra-physiological serum levels and metabolic half-lives in the range of 3–12 h.

**Effect of cortisol and Gh** FW-acclimated fish were used in order to analyse the hormonal induction of hypo-osmoregulatory mechanisms in fish being in a hyper-osmoregulatory condition. In September, four groups of fish (15–25 g) were injected intraperitoneally with either saline (0.9% NaCl, 0.4% BSA), 4 µg cortisol/g (F; Na-hydrocortisone hemisuccinate; Sigma), 0.2 µg recombinant trout Gh/g (Novozymes Biopharma AU Ltd, Adelaide, Australia) or a combination of 4 µg cortisol and 0.2 µg Gh/g. Injections were repeated 2 days later, and the experiment terminated by sampling on the following day.

**Effect of cortisol and Prl** In this experiment, fish (30–40 g) acclimated to SW (2 weeks in October) were used in order to analyse the hormonal induction of hyper-osmoregulatory mechanisms in fish being in a hypo-osmoregulatory condition. Four groups were injected intraperitoneally with either saline, 4 µg F/g, 0.2 µg salmon Prl/g or a combination of 4 µg cortisol and 0.2 µg salmon Prl/g. The Prl was purified from chum salmon pituitaries and a gift from Dr S Moriyama (Kitasato Univ., Japan). Injections were repeated after 2 days, and the experiment terminated by sampling 1 day later.

**Direct effect of cortisol and Igf1** In this experiment, we used gill cell suspensions from FW-acclimated fish (60–80 g) in order to analyze the direct effect of expected hypo-osmoregulatory hormones in gills from fish being in a hyper-osmoregulatory condition. In December, gill cell suspensions were prepared as described by Tipsmark & Madsen (2001). The cells were incubated 12 h in salmon Ringer's solution equilibrated with 99% O<sub>2</sub>/1% CO<sub>2</sub> (in mM: 140 NaCl, 15 NaHCO<sub>3</sub>, 3.5 KCl, 1.5 CaCl<sub>2</sub>, 1.0 NaH<sub>2</sub>PO<sub>4</sub>, 0.8 MgSO<sub>4</sub>, 10.0 D-glucose and 5.0 N-2-hydroxyethyl-piperazine propanesulphonic acid; 500 IU

penicillin/ml; 250 µg/ml streptomycin; 4 mg/ml BSA; osmolality: 305 mOSM; pH 7·8) with either vehicle, 10 µg F/ml, 0·1 µg Igf1/ml (recombinant human Igf1 was supplied by Chiron Corporation, Emeryville, CA, USA) or a combination of 10 µg cortisol and 0·1 µg Igf1/ml. After incubation, cells were isolated by centrifugation (5000 g, 1 min) and sonicated in TRIzol solution (Invitrogen) for extraction of mRNA.

#### Analytical techniques

##### Plasma osmolality and Na<sup>+</sup>,K<sup>+</sup>-atpase activity

Plasma osmolality was measured by freezing point depression osmometry (Osmomat 030; Gonotec, Berlin, Germany). Gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity was measured according to McCormick (1993) using a microplate reader (SPECTRAMax PLUS, Molecular Devices, Sunnyvale, CA, USA) and normalized to protein content of the homogenate (Lowry *et al.* 1951).

##### Extraction of RNA and cDNA synthesis

Total RNA was extracted by the TRIzol procedure (Invitrogen) according to the manufacturer's recommendation and using maximally 100 mg of tissue or cells per ml TRIzol. Total RNA concentrations were determined by measuring A<sub>260</sub> in duplicate with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A<sub>260</sub>/A<sub>280</sub> varied from 1·8 to 2·0. One microgram RNA was treated with 0·7 unit RQ1 DNase (Promega) for 30 min at 37 °C followed by addition of RQ DNase stop solution and 10 min at 65 °C to inactivate the DNase. cDNA was synthesized by reverse transcription carried out on 1 µg DNase-treated RNA with oligo(dT)<sub>15</sub> primers using DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland), according to the manufacturer's recommendations. At the end, the cDNA was diluted to a total volume of 50 µl with milli-Q H<sub>2</sub>O.

**Primers** Primer3 software was used to design primers detecting the deduced sequences and checked using NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences were tested for non-specific product amplification and primer-dimer formation by analysis of melting curve analysis, and amplicon size was verified by agarose gel electrophoresis. Na<sup>+</sup>,K<sup>+</sup>-atpase and elongation factor 1a (*ef1a*) primers have been reported previously (Madsen *et al.* 2009). Additional primers used were (5'-3'): *igf1* (forward, ACTGTGCCCTGT-CAAGTCT; reverse, CTGTGCTGTCTACGCTCTG; based on acc. no. EF432852), *igf1* (forward, AGCCA-CCTGAGTCACTACG; reverse, CTCCTCAGCCATC-TGAATAA; based on acc. no. AY049954), *ghr1* (forward, TCCCAACATGCAGCTGTAGA; reverse, TGTGG-CACCTTGAAGAACAG; based on acc. no. AY462105) and *nkcc1a* (forward, TCCATCGACATGAAGGAC; reverse, CGTTCATCATCGTCACCT; based on acc. no.

DQ864492; Mackie *et al.* 2007). Several primer pairs (based on acc. no. DQ163908) failed to detect *ghr2* in any other tissues than liver and, therefore, only *ghr1* was examined.

##### Real-time QPCR

Quantitative PCR analysis was carried out on a Mx3000p instrument (Stratagene, La Jolla, CA, USA) with SYBR Green detection using standard software settings with adaptive baseline for background detection, moving average and amplification-based threshold settings with built-in FAM/SYBR filter (excitation wavelength: 492 nm; emission wavelength: 516 nm). Reactions were carried out with a cDNA amount equivalent of 20 ng total RNA, 150 nM forward and reverse primer, 1× SYBR Green JumpStart (Sigma) in a total volume of 25 µl. Cycling conditions were 95 °C for 30 s and 60 °C for 60 s in 50 cycles. Melting curve analysis was carried out routinely with 30 s for each 1 °C interval from 55 to 95 °C. For all primer sets, a pooled cDNA sample was diluted and analyzed by QPCR to establish amplification efficiency (*E<sub>a</sub>*). This was in the range 1·85–2. For normalization of gene expression, *ef1a* was used in accordance with Olsvik *et al.* (2005). Relative copy numbers of the target genes were calculated as  $E_a^{-C_t}$ , where *C<sub>t</sub>* is the threshold cycle number and *E<sub>a</sub>* is the amplification efficiency. Normalized units were obtained by dividing relative copy number of target genes with relative copy number of *ef1a*. Negative control reactions using DNase-treated total RNA from representative samples were used to analyze carry-over of genomic DNA. In all samples and with all primers, there was no genomic contamination.

##### In situ hybridisation

The dissected gill specimens were stored and desiccated at –80 °C until further processing. Frozen sections (8–10 µm) were cut on a cryostat at –14 °C, transferred to Superfrost Plus glass slides (Erie Scientific Company, Portsmouth, NH, USA) and dried for 1 h at 37 °C. The sections were then transferred to –80 °C until further processing. ISH was performed using the protocol described by Madsen *et al.* (2009), which is based on the method by Lambertsen *et al.* (2001). In short, gill sections were incubated with 3 pmol/ml alkaline phosphatase (AP)-labelled synthetic 28-mer cDNA probes recognising the *α1a* and *α1b* isoforms of the Na<sup>+</sup>,K<sup>+</sup>-atpase *α*-subunit (Madsen *et al.* 2009). Hybridisation conditions were: 35% formamide, 4× saline sodium citrate buffer (4× SSC: 30 mM NaCl, 3 mM sodium citrate), 1× Denhardt's solution (0·2 mg/ml Ficoll, 0·2 mg/ml polyvinyl pyrrolidone, 0·2 mg/ml BSA), 10% dextran sulphate, 10 µg/ml single-stranded salmon sperm DNA overnight at 37 °C. Post-hybridisation washes were: 3×30 min changes of 3× SSC (22·5 mM NaCl, 2·3 mM sodium citrate, pH 8·0) at 37 °C followed by two 10 min rinses in post-hybridisation buffer (0·01 M Tris-HCl, 0·15 M NaCl, pH 9·5) at room temperature. Finally, the specimens were incubated in freshly made AP-developer buffer (0·32 mg/ml nitro blue tetrazolium (Sigma), 0·17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 0·1 M Tris-HCl, 0·1 M NaCl and 0·05 M MgCl<sub>2</sub>·6H<sub>2</sub>O, pH 9·5) for

**Table 1** Comparison of plasma osmolality, gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity and relative mRNA expression levels in freshwater- (FW) and seawater- (SW)-acclimated Atlantic salmon. All mRNA expression levels are calculated relative to expression of elongation factor-1a (*ef1a*). Ratio is calculated as the ratio between SW and FW value

Parameter		FW	SW	Ratio	P value*
Osmolality		313.8 ± 6.7	299.2 ± 6.7	0.95	0.14
Gill Na <sup>+</sup> ,K <sup>+</sup> -atpase		2.10 ± 0.12	4.24 ± 0.45	2.0	0.0003
Gill mRNA	<i>α1a</i>	9.31 ± 1.78	0.40 ± 0.03	0.04	0.0002
	<i>α1b</i>	0.57 ± 0.11	3.72 ± 0.45	6.5	<0.0001
	<i>β1</i>	0.45 ± 0.03	0.80 ± 0.09	1.8	0.001
	<i>nkcc1a</i>	0.065 ± 0.006	1.12 ± 0.12	17	<0.0001
	<i>igf1</i>	0.0023 ± 0.0004	0.022 ± 0.009	9.6	<0.03
	<i>igfr1</i>	0.0004 ± 0.0001	0.0108 ± 0.0017	27	<0.0001
	<i>ghr</i>	0.051 ± 0.007	0.10 ± 0.02	2.0	<0.03
Liver mRNA	<i>igf1</i>	12.3 ± 1.7	1.02 ± 0.22	0.08	<0.0001
	<i>ghr</i>	2.47 ± 0.40	8.16 ± 1.03	3.3	0.0001

\*P value is obtained from a two-tailed Student's *t*-test. FW, *n* = 8; SW, *n* = 7.

1–2 days at room temperature until appropriate colour deposition was observed. After a rinse in distilled water (30 °C, 30 min) to stop the colorimetric reaction, coverslips were mounted using Aquatex (Merck). Images were taken at 250 and 400× magnification using a Leica microscope (DM R, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) mounted with a Leica digital camera (DC200).

#### Statistical analysis

FW and SW control values were compared using a Student's *t*-test. In the injection experiments, a two-factorial ANOVA was used to analyze for overall effects of hormones and their interaction. When interaction between hormones was significant, multiple comparisons were performed. Differences between means were assessed by Tukey's hydroxysteroid dehydrogenase multiple comparison test or Dunn's post test, as appropriate. When required, transformation of data was done to meet the ANOVA assumption of homogeneity of variances as tested by Bartlett's test, and if this was unattainable, Kruskal–Wallis nonparametric one-way ANOVA was employed. All statistical analyses were performed using GraphPad Prism 4.0 software (San Diego, CA, USA), and significant differences were accepted when *P* < 0.05. Asterisks indicate significance level (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001).

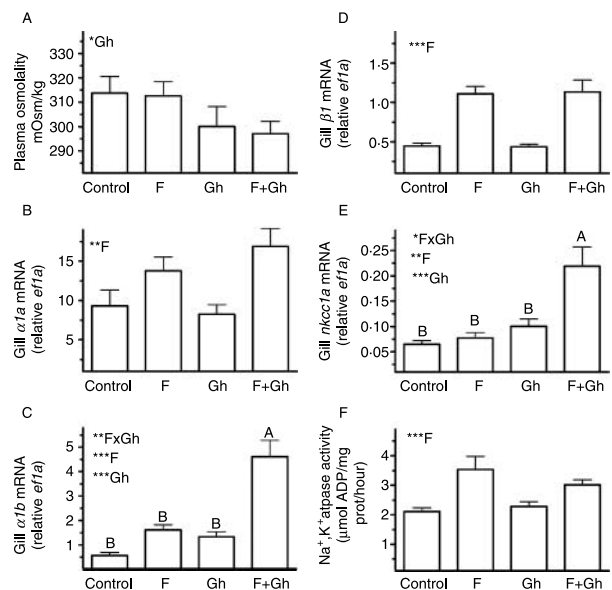
## Results

### Effect of salinity

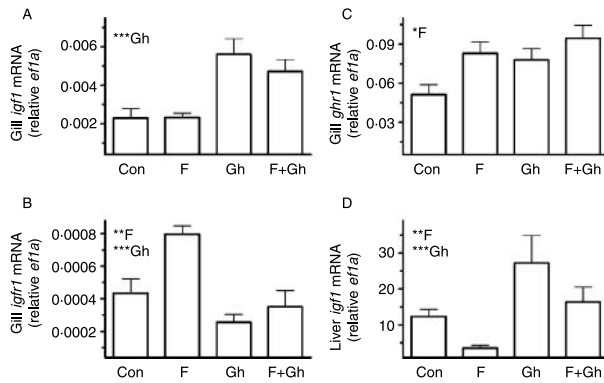
FW and SW control values from the two injection experiments are compared in Table 1. Plasma osmolality was not significantly different, whereas all other parameters showed major differences with respect to salinity. Gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity, *α1b*, *β1*, *nkcc1a*, *igf1*, *igfr1*, *ghr* and liver *ghr* mRNA levels were all higher in SW than in FW. Gill *α1a* and liver *igf1* mRNA were lower in SW than in FW.

### Effect of cortisol and Gh in FW fish

Gh induced a significant decline in plasma osmolality of ~10 mOsm/kg (Fig. 1A). Overall, cortisol stimulated *α1a*, *β1* mRNA and gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity, but no significant effect of Gh was observed on these parameters (Fig. 1B, D and F). Both cortisol and Gh stimulated gill *α1b* and *nkcc1a* expression (Fig. 1C and E) with a significant synergistic interaction. Gill *igf1* expression was stimulated by Gh (*P* < 0.001), while cortisol had no effect (Fig. 2A). Both gill



**Figure 1** Effects of cortisol (F) and Gh on plasma osmolality (A) and expression of *α1a* (C), *α1b* (D), *β1* (D), *nkcc1a* (E) and Na<sup>+</sup>,K<sup>+</sup>-atpase activity (F) in the gill of FW salmon. Asterisks next to F or Gh indicate significant overall effect. Asterisks next to F × Gh indicate significant interaction, in which case different letters above bars indicate significant difference between groups. Values are means ± S.E.M. (*n* = 8–10).

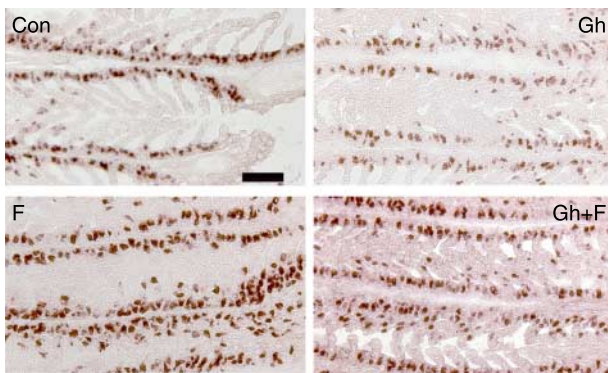


**Figure 2** Effects of cortisol (F) and Gh on expression of gill *igf1* (A), gill *igfr1* (B) and gill *ghr1* (C) and liver *igf1* (D) in FW salmon. Asterisks next to F or Gh indicate significant overall effect. Values are means  $\pm$  S.E.M. ( $n=8-10$ ).

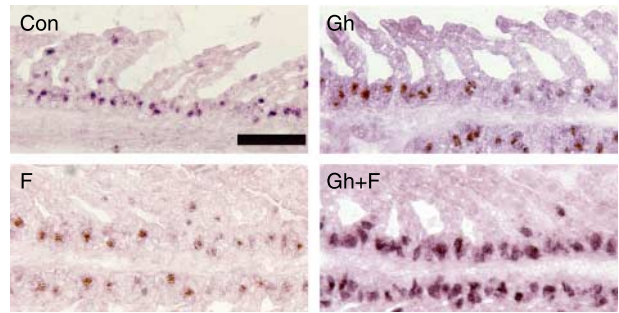
*igf1* and *ghr1* mRNA levels were stimulated by cortisol, while Gh depressed the *igf1* level (Fig. 2B and C). Hepatic *igf1* was inhibited by cortisol and stimulated by Gh, and the effects were additive (interaction:  $P>0.05$ ; Fig. 2D). The  $\alpha 1a$  transcript was most abundant in large cells in the interlamellar (IL) filament region and occasionally on the lamellae in gills of FW control fish (Fig. 3). No major effect on this localization pattern was observed after treatment with F and/or Gh. The  $\alpha 1b$  transcript was mostly present in small cells below the surface of the IL filament region in FW control fish (Fig. 4). The cellular staining intensity, size and abundance increased markedly after F+Gh treatment.

#### Effect of cortisol and Prl in SW fish

Injection with Prl induced a very significant increase in plasma osmolality of  $\sim 50$  mOsm/kg (Fig. 5A). Prl induced a decrease in gill  $\alpha 1b$  and *nka1a* expression, while no effect of cortisol on these transcripts was observed (Fig. 5C and E).



**Figure 3** *In situ* hybridisation of gill cryosections using an AP-conjugated 28-mer antisense probe detecting the  $\alpha 1a$  isoform of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$ -subunit. Con, control; F, cortisol; Gh+F, Gh+cortisol. Bar represents 100  $\mu$ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0281>.



**Figure 4** *In situ* hybridisation of gill cryosections using an AP-conjugated 28-mer antisense probe detecting the  $\alpha 1b$  isoform of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$ -subunit. Con, control; F, cortisol; Gh+F, Gh+cortisol. Bar represents 100  $\mu$ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0281>.

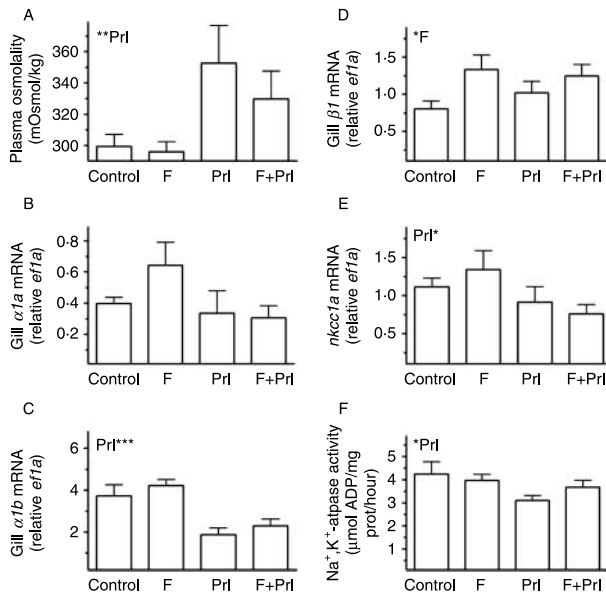
Overall, cortisol stimulated gill  $\beta 1$  mRNA levels, while no effect on  $\alpha 1a$  was observed (Fig. 5B and D). An overall decrease in gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity was observed in response to Prl (Fig. 5F). Gill *igf1* expression was decreased by cortisol, while Prl had no effect (Fig. 6A). Gill *igf1* was stimulated by both Prl and cortisol, while *ghr1* was inhibited by cortisol (Fig. 6B and C). A nonparametric ANOVA showed that hepatic *igf1* was inhibited by Prl, while cortisol had no effect (Fig. 6D). The  $\alpha 1a$  transcript was localised in small cells below the surface of the IL filament and occasionally in the lamellar epithelium of SW control fish (Fig. 7). F treatment stimulated the number of positive cells, whereas there was no obvious effect of Prl or F+Prl treatment. The  $\alpha 1b$  transcript was abundant in cells in the IL filament and occasionally on the lamellae (Fig. 8). Prl treatment induced weaker staining and smaller cells.

#### Effect of cortisol and Igf1

Incubation of FW gill cells with cortisol for 12 h stimulated  $\alpha 1a$ ,  $\alpha 1b$  and  $\beta 1$  mRNA (Fig. 9A–C). In contrast, *igf1* only stimulated  $\alpha 1b$  of the three Na<sup>+</sup>,K<sup>+</sup>-atpase genes examined. Both cortisol and Igf1 stimulated gill cell *igf1* expression (Fig. 9D). *igf1* was stimulated by cortisol, an effect that was blocked by *igf1* (Fig. 9E; antagonistic effect:  $P<0.05$ ). *ghr1* mRNA levels were also stimulated by cortisol, an effect that was significantly antagonized, albeit not blocked by Igf1 (Fig. 9F;  $P<0.01$ ).

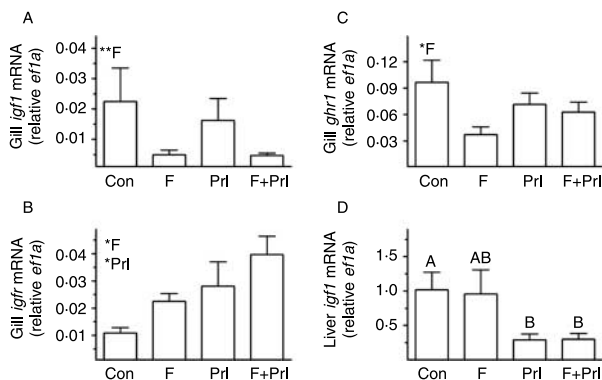
## Discussion

Changes in environmental salinity induce intricate molecular and cellular changes in the gill of euryhaline teleosts. As a fundamental part of the response in salmonids, a distinct switching occurs between Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$  isoforms *1a* and *1b* (Richards *et al.* 2003), which may change the functional properties of the enzyme (Jorgensen 2008) in order to maintain the appropriate ionic gradients across the gill.

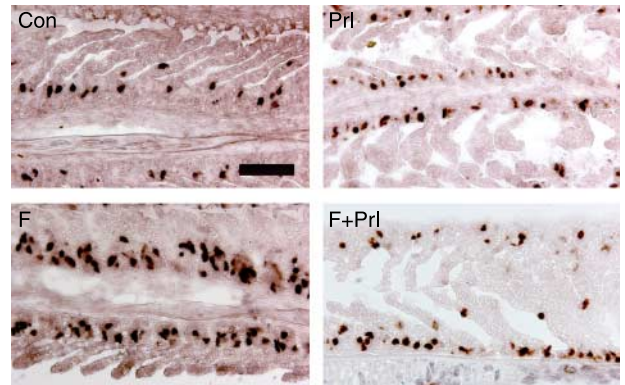


**Figure 5** Effects of cortisol (F) and Prl on plasma osmolality (A) and expression of  $\alpha 1a$  (B),  $\alpha 1b$  (C),  $\beta 1$  (D), *nkcc1a* (E) and Na<sup>+</sup>,K<sup>+</sup>-atpase activity (F) in the gill of SW salmon. Asterisks next to F or Prl indicate significant overall effect. Values are means  $\pm$  S.E.M. ( $n=8-10$ ).

The primary focus of our study was the endocrine and paracrine regulation of this particular isoform switch, and the experiments were performed under both hypo- and hyper-osmotic conditions in anticipation of maximal effects of the applied hormonal treatments. Accordingly, the initial expression level of  $\alpha 1a$  was higher in FW than in SW, and the level of  $\alpha 1b$  and *nkcc1a* was higher in SW than in FW, reflecting the characteristic ion-regulatory modus of the gill epithelium in the two situations.



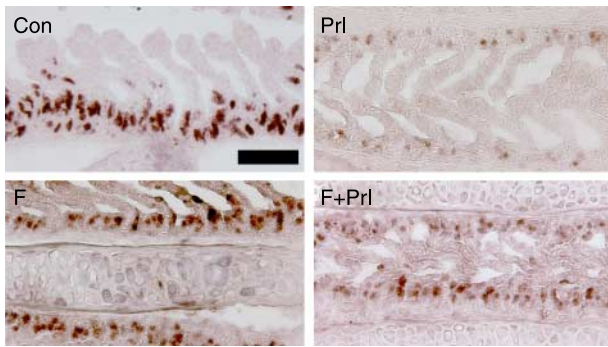
**Figure 6** Effects of cortisol (F) and Prl on expression of gill *igf1* (A), gill *igf1* (B), gill *ghr1* (C) and liver *igf1* (D) in SW salmon. Asterisks next to F or Prl indicate significant overall effect. The data in panel D were subject to nonparametric analysis, and groups marked with different letters are significantly different. Values are means  $\pm$  S.E.M. ( $n=8-10$ ).



**Figure 7** *In situ* hybridisation of gill cryosections using an AP-conjugated 28-mer antisense probe detecting the  $\alpha 1a$  isoform of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$ -subunit. Con, control; F, cortisol; Prl, prolactin; F+Prl, cortisol+prolactin. Bar represents 100  $\mu$ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0281>.

#### Hormonal effect on $\alpha 1a$ and $\alpha 1b$ isoforms in the gill

Several studies have shown mineralocorticoid actions of cortisol in teleosts (see Mommsen *et al.* 1999). Cortisol is known to stimulate gill MRC proliferation and overall gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity, and it has been puzzling for many years how cortisol in doing so may both facilitate acclimation to hyper- and hypo-osmotic conditions. The discovery by Richards *et al.* (2003) that two isoforms of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$ -subunit contribute to overall enzymatic activity in the gill has led to the hypothesis, that  $\alpha 1a$  is primarily expressed in MRCs responsible for absorption of monovalent ions (and possibly Ca<sup>2+</sup>), while  $\alpha 1b$  is the dominant isoform in secretory-type MRCs. This has gained support from ISH analyses of transcript localisation in the salmon gill (Madsen *et al.* 2009). In the present study, both isoforms were stimulated by cortisol *in vivo* and *in vitro*. This explains how cortisol may simultaneously facilitate both FW and SW acclimation by increasing the general ion transport capacity of the gill. A salinity-specific effect of cortisol is, however, only attained by interaction with additional hormones: Gh or Prl. The present data show a very significant synergy with Gh specifically on the  $\alpha 1b$  isoform, which was confirmed by a strong increase in cellular ISH staining intensity and abundance of large  $\alpha 1b$ -positive cells in the FW salmon gill. A similar synergism is seen with respect to the *nkcc1a* transcript level, which is specifically involved in ion secretion of SW MRCs (Wu *et al.* 2003), thus underlining the specific importance of  $\alpha 1b$  in this cell-type. The data nicely explain in molecular terms how cortisol and Gh in salmonids exert a strong synergistic effect on the ability to regain osmotic balance after FW to SW transfer (Madsen 1990, McCormick 1996). In earlier studies, the functional effect of cortisol and Gh was associated with stimulatory effects of the two hormones on overall gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity, which was either synergistic (Madsen 1990) or additive



**Figure 8** *In situ* hybridisation of gill cryosections using an AP-conjugated 28-mer antisense probe detecting the  $\alpha 1b$  isoform of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$ -subunit. Con, control; F, cortisol; Prl, prolactin; F+Prl, cortisol+prolactin. Bar represents 100  $\mu$ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0281>.

(McCormick 1996). The lack of interaction on overall Na<sup>+</sup>,K<sup>+</sup>-atpase activity in the present experiment and in a previous study by McCormick (1996) is likely to adhere to masking by the dominant gill isoform in FW  $\alpha 1a$ , which was insensitive to Gh treatment but stimulated by cortisol. In addition, the present lack of interaction on enzyme activity may be due to the short duration of the experiment (3 days).

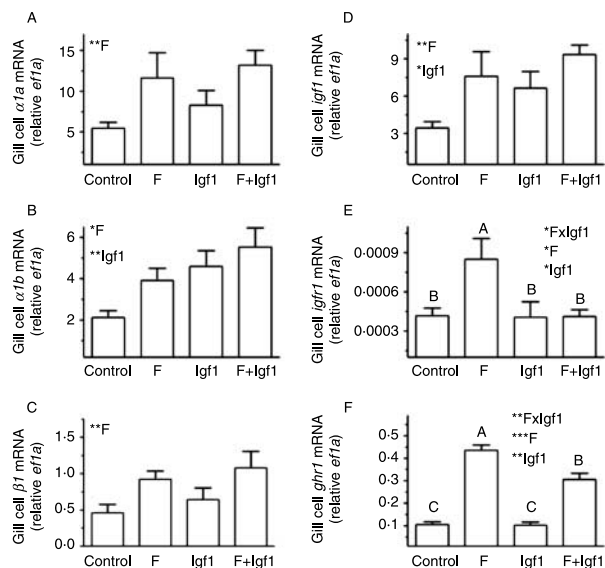
In the SW experiment, Prl induced a substantial osmotic imbalance underlining its hyper-osmoregulatory action. Our data suggest that this is causally related to a selective reduction of  $\alpha 1b$  isoform expression in the Na<sup>+</sup>,K<sup>+</sup>-atpase, while the  $\alpha 1a$  isoform is unaffected. Consequently, gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity and the secretory capacity of the gill decreased. The anti-SW effect is further substantiated by the parallel downregulation of the secretory *nkc1a* and the much weaker ISH-staining intensity in gills from Prl-treated fish. IL filament cells staining positive for  $\alpha 1b$  expression were much reduced in size and retrieved from the mucosal surface of the epithelium. Taken together, the data suggest that Prl impairs the functional status of secretory MRCs, thereby supporting histological evidence in SW tilapia gill and opercular membrane, where Prl induced a changed morphology of the putative ion-secretory  $\alpha$ -type MRC into the typical FW MRC morphology (Herndon *et al.* 1991, Pisam *et al.* 1993). The observed effect of Prl may either be due to anaplasia of existing SW-type MRCs, transformation of SW-type MRCs into FW-type MRCs or inhibition of recruitment of SW-type MRCs (Fig. 10). Transformation into FW-type MRCs does not seem likely from the present data, as there was no evidence that the  $\alpha 1a$  isoform was stimulated by Prl.

#### Endocrine and paracrine mechanisms of action

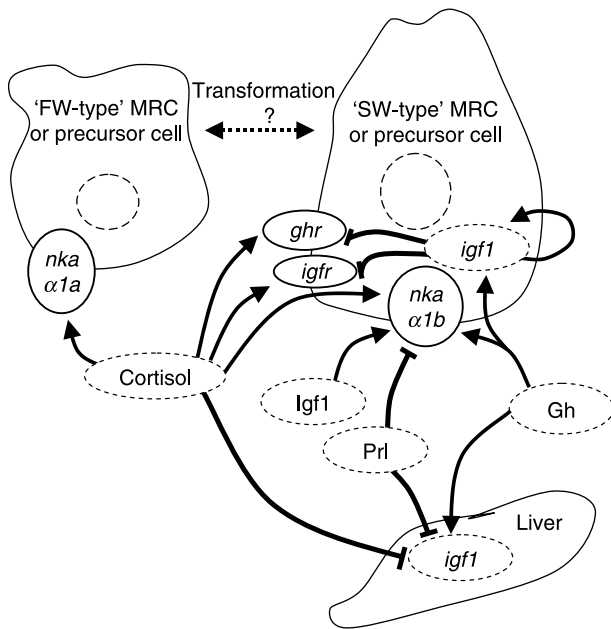
All the prerequisites for a dual-effector mode-of-action of Gh in regulating gill MRC development are present in salmonids. MRCs are responsive to Gh, they express the *igfr*, they express *igf1* in response to Gh, and Igf1 and its receptor colocalise

with the Na<sup>+</sup>,K<sup>+</sup>-atpase enzyme in MRCs (Reinecke *et al.* 1997, Tipsmark *et al.* 2007). Radioreceptor assays have shown specific Gh binding to gill membranes (Gray *et al.* 1990), but the precise cellular localisation of the Ghr in gills still awaits examination. Gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity is stimulated by both Gh and Igf1 (Madsen & Bern 1992, McCormick 1996), and here we show explicitly that this is due to specific stimulation of the  $\alpha 1b$  isoform of the pump. In the isolated-cell experiment, the  $\alpha 1a$  isoform expression was solely stimulated by cortisol, while the SW-type,  $\alpha 1b$ , was sensitive to both cortisol and Igf1. This partially explains the selective effect of Gh on  $\alpha 1b$  found in the injection experiment, where endocrine and paracrine *igf1* expression was elevated. However, the strong synergistic effect of Gh and cortisol cannot be explained by these findings, but is likely to result from Gh-enhanced sensitivity to Igf1 (Madsen & Bern 1992) and cortisol (Shrimpton & McCormick 1998), or cortisol enhanced sensitivity to Igf1 and Gh as suggested by the current data.

The effect of environmental salinity on additional elements of the Gh–Igf1 axis of the salmon was further investigated. *igf1* expression, which, as expected, was much higher in the liver than the gill, responded to SW with a tenfold increase in the gill and a tenfold decrease in the liver. The increase in the gill confirmed the reports in rainbow trout by Sakamoto & Hirano (1993) and Poppinga *et al.* (2007), and suggests paracrine actions of Igf1 to be involved in modification of ion transport in the gill. The decrease in the liver was surprising and is at variance with Poppinga *et al.* (2007), but



**Figure 9** Effects of cortisol (F) and Igf1 on expression of  $\alpha 1a$  (A),  $\alpha 1b$  (B),  $\beta 1$  (C), *igf1* (E) and *ghr1* (F) in isolated gill cells from FW salmon. Asterisks next to F or Igf1 indicate significant overall effect. Asterisks next to FxIgf1 indicate significant interaction, in which case different letters above bars indicate significant difference between groups. Data are pooled from two separate experiments, and values are means  $\pm$  S.E.M. ( $n=8$ ).



**Figure 10** Tentative model for hormonal regulation of Na<sup>+</sup>,K<sup>+</sup>-atpase isoform expression in mitochondrion-rich cells (MRC) in salmon gill epithelia. Stimulation is indicated by arrows and inhibition by block-ended lines. Two types of differentiated MRCs (or their associated precursor cells) are suggested. One difference between these is the abundance of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha 1a$  isoform in the 'FW-type' versus the dominance of the  $\alpha 1b$  isoform in the 'SW-type' MRC. Upon the appropriate endocrine/paracrine stimulation, these may develop into fully functional ionocytes ('FW-type' or 'SW-type'). The possibility of transformation between the functionally differentiated MRCs is currently speculative. Cortisol is a stimulator of both isoforms of the Na<sup>+</sup>,K<sup>+</sup>-atpase  $\alpha$ -subunit; however, only in the SW-type cell does cortisol interact in synergy with Gh and Igf1 (local and systemic). In addition, cortisol sensitizes this cell type by increasing Gh and Igf1 receptor levels. Prl interferes with this development by blocking the expression of  $\alpha 1b$  and also by suppressing hepatic *igf1* production.

may be explained by our additional observation that cortisol has a strong inhibitory effect on hepatic *igf1* expression. Cortisol was not measured in the present experiment but is known to increase when FW salmonids are transferred to SW (see McCormick 2001). Additional factors such as the time course of sampling and nutritional status may also add variance when endocrine responses are compared between experiments. The *ghr1* was also expressed at higher levels in the liver than in the gill and showed an increase in the liver in response to SW. This is in accordance with Poppinga *et al.* (2007) and with increased total *ghr* number after SW transfer in rainbow trout (Sakamoto & Hirano 1991). On the contrary, *igf1* receptor type I mRNA was found in the gill but not detected in liver. The absence in liver agrees with Greene & Chen's (1997) observation in juvenile and adult trout, but is at variance with early stages of shi drum (Radaelli *et al.* 2003) and barramundi (Drakenberg *et al.* 1997), where hepatic expression was reported. Gill *igf1* expression increased very significantly in response to SW,

suggesting elevated sensitivity to *igf1*. In trout and striped bass, *igf1* also increased after SW transfer although lasting only for 1–2 days (Poppinga *et al.* 2007, Tipsmark *et al.* 2007). Altogether, these data support our conception of involvement of the Gh–Igf1 axis in SW acclimation of the salmon.

Gh injection elevated both liver and gill *igf1* mRNA, and thus parallels findings in injection experiments in rainbow trout (Sakamoto & Hirano 1993) and *in vitro* data from coho salmon hepatocytes (Duan *et al.* 1993). Meanwhile, cortisol inhibited *igf1* expression in the liver both with and without exogenous Gh induction. This effect is in full agreement with *in vitro* observations on primary culture of salmon hepatocytes, where dexamethasone blocks Gh-induced *igf1* expression possibly due to decreased *ghr* expression (Pierce *et al.* 2005). In the gill, cortisol had either no effect (*in vivo*, FW) or a stimulatory effect (*in vitro*, FW) on *igf1* expression, which agreed well with the elevated *ghr1* levels induced by the steroid. The sensitivity of the gill to Gh and its secondary effector, Igf1, are thus likely to be elevated by cortisol in FW since both *ghr1* and also the *igf1* were stimulated by the steroid. Therefore, the strong synergistic effect of cortisol and Gh on  $\alpha 1b$  expression may relate to elevated sensitivity to Gh and Igf1 in putative SW MRCs or precursor cells (Fig. 10). The synergy may be further reinforced by enhanced cortisol sensitivity found in Atlantic salmon gill upon Gh treatment (Shrimpton & McCormick 1998). Curiously, in the SW gill, the effect of cortisol is totally different: *igf1* and *ghr1* mRNA levels decrease, and  $\alpha 1b$  and *nkcc* are not affected.

One possible mode of action of Prl may be via reduced *igf1* expression in the liver assuming systemic Igf1 is a requirement for sustaining secretory mode of the gill. However, Prl receptors are expressed in salmon gill (Nilsen *et al.* 2008), and a direct effect of Prl could also be involved. In previous injection studies performed in hyper-osmoregulating trout in FW, Prl antagonized the hypo-osmoregulatory effect induced by cortisol and Gh (Madsen *et al.* 1995, Seidelin & Madsen 1997). In these experiments, Prl had no isolated effect on gill Na<sup>+</sup>,K<sup>+</sup>-atpase activity but solely opposed the stimulatory action of Gh and F. The Prl receptor has been localised in FW MRCs (tilapia: Sandra *et al.* 2000), and it has been hypothesized that Prl works together with cortisol to promote development/maintenance of FW MRC (McCormick 2001). The present data gave no such indication, as neither Prl alone nor in interaction with cortisol stimulated the alleged FW  $\alpha 1a$  isoform of the Na<sup>+</sup>,K<sup>+</sup>-atpase. Instead, Prl had a negative effect on elements of SW-type MRCs. Taken together, Gh and Prl may be the decisive stimuli that direct cortisol-aided MRC development into either secretory or absorptive types. The effects of Gh/Pril may occur at the level of stem cell differentiation or proliferation of smaller differentiated progenitor cells (deeper in the epithelium) into large functional MRCs in contact with the apical surface.

As summarized in Fig. 10, the present data give functional support to a dual-effector mode of action of Gh on establishment of a hypo-osmoregulatory modus of the salmon



gill. Cortisol stimulates the general ion transport capacity of the gill, reflected by its stimulation of both  $\alpha 1a$  and  $\alpha 1b$  isoforms of the Na<sup>+</sup>,K<sup>+</sup>-atpase. Through a synergistic interaction with cortisol, Gh specifically potentiates equipment of the gill with secretory transport proteins. This action is in part mediated by paracrine/endocrine *igf1* and abolished by Prl. Even though the present data do not preclude that FW and SW MRCs may transformed back and forth upon the appropriate stimuli, future studies should focus on clarifying the mechanisms responsible for early stem cell differentiation into MRCs in the gill.

### Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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