Pituitary hormone mRNA expression in European sea bass *Dicentrarchus labrax* in seawater and following acclimation to fresh water

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Abstract

The mRNA expression of pituitary prolactin (prl), growth hormone (gh), somatolactin (sl), proopiomelanocortin (pomc), and gonadotropins (gthI and gthII) was quantified by real-time PCR, in sea bass, *Dicentrarchus labrax*, adapted for 1 month to seawater (SW) or freshwater (FW). In addition, IGF-I (igfl) mRNA expression in liver and branchial Na⁺/K⁺-ATPase activity were determined. L17 ribosomal protein (rpL17) and elongation factor 1 α (efl1 α) were validated as reference genes in real-time PCR in the experimental context. The real-time PCR assays were validated for the different hormone genes considered. Expression of pituitary pomc, gthI, gthII, gh, and

liver igfl was not significantly different between FW and SW fish. Pituitary prl was 4.5-fold higher in FW than in SW, whereas pituitary sl was 1.8-fold higher in SW- compared with FW-adapted fish. Gill Na⁺/K⁺-ATPase specific activity was 2.3-fold higher in FW sea bass compared with SW fish. Plasma cortisol levels were 6.5-fold lower in SW- than in FW-adapted specimens. The results are discussed in relation to the osmoregulatory strategy of this euryhaline SW species, which displays features that do not fit present models based on salmonids and FW euryhaline teleosts.

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Introduction

Endocrine control of osmoregulation has been studied only in a limited number of teleostean species, with an emphasis on salmonids. These studies underline the importance of pituitary hormones, such as prolactin (Hirano *et al.* 1987, Manzon 2002) or growth hormone (i.e. the gh/igfl axis; Sakamoto *et al.* 1993, McCormick 2001) in the adaptation to gradual or rapid salinity changes; also in the ontogenetic acquisition of salinity tolerance, these hormones play a pivotal role (Bœuf 1993, Varsamos *et al.* 2005). The hormones target ionocytes (Pisam & Rambourg 1991, Sakamoto *et al.* 2001) and the ion transporters therein (mainly Na⁺/K⁺-ATPase), in osmoregulatory tissues (Marshall 1995, McCormick 1995, Manzon 2002). They also affect mechanisms involved in the control of water balance (Fuentes & Eddy 1997).

The European sea bass (*Dicentrarchus labrax* Linnaeus 1758) is a marine teleost fish which has aroused significant socioeconomic and scientific interest, especially around the Mediterranean Sea (Pickett & Pawson 1994). Its remarkably strong euryhalinity – it thrives in fresh water (FW), seawater (SW) and even concentrated SW – makes this species an excellent model to study adaptive ecophysiology. In previous works, we have determined the tolerance of sea bass to hyper-saline SW (Varsamos 2002), explored the fundamental

ultrastructural and physiological bases for its euryhalinity (Varsamos *et al.* 2002b) and described the development of its osmoregulatory capacity throughout post-embryonic development (Varsamos *et al.* 2001, 2002a, 2004). Interestingly, the osmoregulatory strategy of sea bass during adaptation to FW differs from that of other euryhaline teleosts. In fact, when sea bass moves from SW to FW, its branchial ionocytes undergo morphological changes (Varsamos *et al.* 2002b) that remind of phenomena normally seen when euryhaline fish move oppositely, i.e. from FW to SW. Indeed, these changes are thus opposite to those seen in salmonids that return to FW for spawning (Pisam & Rambourg 1991). Hence, an investigation into the endocrine control of the osmoregulatory processes could further our understanding of the adaptive strategy employed by this marine species to enter FW habitats.

To date, mainly semi-quantitative approaches have been used to monitor the gene expression in fish and only limited data have been generated by real-time quantitative PCR, a powerful technique for profiling gene expression (Bustin *et al.* 2005). Although semi-quantitative methods are reliable, they are generally complex and their results cannot be easily compared with those obtained in other species or with those of independent experiments. Implementation of standard real-time PCR protocols should enhance developments in

comparative physiology. To study the involvement of pituitary hormones known to be involved in the adaptation to different salinities, we made a quantitative profile of a series of pituitary mRNAs by real-time quantitative PCR of sea bass adapted for 1 month to SW or FW. We considered hormones with established effects on osmoregulatory processes in teleosts, viz. prolactin (prl), growth hormone (gh), somatolactin (sl), proopiomelanocortin (pomc); the gonadotropins (gthI and II) were considered as well, as reproduction is known to compromise osmoregulation and vice versa. Liver insulin-like growth factor-I (igfl) mRNA was also assessed as a second parameter of the gh/igfl axis; the liver is generally considered as the main source of circulating igfl (Plisetskaya 1998), although other peripheral sites of production are known (Sakamoto & Hirano 1993). Plasma cortisol (widely considered the SW-adapting hormone) levels were also determined. Branchial Na^+/K^+ -ATPase activity was assayed to determine potential correlations between pituitary hormone expression and activity of this key enzyme in ion transport and osmoregulation.

Materials and Methods

Fish and experimental design

Fish were kept in the aquaculture facilities of the Station Biologique de Sète (Hérault, France) in early 2004. Thirty fish (weight: 250 ± 50 g) of identical genetic origin and reared in SW from hatching were randomly split into two batches of 15 fish transferred to two 2 m^3 tanks containing SW (salinity: 35‰). In one tank, salinity was reduced by the addition of dechlorinated FW to reach 0.3‰ after 1 week. Temperature in both tanks ranged between 11 and 14 °C. The fish were fed commercial pellets, at a ration of 2% of the estimated body weight per day. They were starved 24 h before sampling (routine precaution before fish handling).

Sampling, RNA isolation, and cDNA synthesis

After 1 month of adaptation, ten fish from each salinity were randomly netted and rapidly sacrificed by decapitation. Pituitary glands were dissected on ice, placed in individual vials containing RNAlater (Ambion, Cambridgeshire, UK) to preserve RNA for molecular analyses and frozen at -20°C until further processing; the liver was rapidly taken out and small portions ($1\text{--}2 \text{ mm}^3$) treated similarly.

A commercial kit (SV Total RNA Isolation System; Promega), which combines the protective properties of guanidine thiocyanate and β -mercaptoethanol to inactivate RNases and includes a DNase treatment to remove contaminating DNA, was used to extract total RNA from individual pituitary and liver samples. RNA was finally eluted with 15 μl nuclease-free H_2O and stored at -80°C .

RNA concentration, integrity, and purity of each sample were determined with an RNA Bioanalyzer 2100 (Agilent

Technologies, Palo Alto, CA, USA). The method employs electrophoretic analysis with microfluidics RNA Nano-chips (Agilent, USA) and fluorescence monitoring. For each total RNA sample, the concentration was measured in duplicate on 1 μl aliquots. Electrophoresis was conducted inside the Agilent Bioanalyzer and results were analyzed with Agilent 2100 Bio Sizing software.

For synthesis of first strand cDNA, an 'alien' gene (mRNA of chlorophyll A/B-binding protein (CAB) from *Arabidopsis thaliana*) was used to calibrate reverse transcription (Varlet-Marie *et al.* 2004): 0.6 μg total RNA and 1 μl CAB ($4 \cdot 10^8$ copies; Stratagene) were then reverse transcribed with oligo-dT as primer and SuperScript II reverse transcriptase (SuperScript II first-strand synthesis kit, Invitrogen). The resultant cDNAs were checked by conventional PCR and stored at -20°C .

Primer design for target and reference genes

Sequences available on the GenBank database allowed primer design for most of the genes used in the present work (Table 1). Two to four primer sets (purchased from Sigma Genosys, UK) were designed for each gene with Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi>) and different PCR conditions were tested for each couple of primers to determine the most efficient set for PCR and real-time quantitative PCR. Control or invariant internal 'house-keeping' genes were necessary for the global normalization of the quantification by real-time PCR. The candidate control genes were the L17 ribosomal protein (rpL17), a sea bass house-keeping gene validated previously (Varsamos *et al.* 2003) and elongation factor 1 α (efl α). To determine sea bass efl α -specific primers, PCR was performed on cDNA obtained from sea bass pituitary RNA with degenerate primers designed on the basis of consensus efl α sequences (GenBank; <http://www.ncbi.nlm.nih.gov/Genbank/index.html>).

After an initial denaturing step at 95°C for 2 min, PCR was performed on 1 μl template cDNA during 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. A final extension step at 72°C for 2 min was carried out. PCR products were analyzed by electrophoresis on a 1.5% agarose/ethidium bromide gel, cloned and sequenced.

Quantification of target and reference gene expression

Quantification of *prl*, *gh*, *sl*, *pomc*, *gthI*, and *gthII* gene expression on sea bass cDNAs in pituitary gland, of igfl expression in liver and of rpL17 and efl α in both pituitary gland and liver, was carried out by means of real-time quantitative PCR using a Light Cycler (Roche) according to Varsamos *et al.* (2003).

Dilutions of a reference sample were used to obtain the calibration curve, demonstrating a linear relationship between threshold cycle (C_t) and $\log_{10}$ of template availability. Ten microliter reactions were run containing 2 μl PCR Mix

Table 1 Primers sequences for quantification of sea bass (*Dicentrarchus labrax*) hormone expression by real-time quantitative PCR (W=A/T)

	Primers	Primers sequences (5'-3')	Expected size (bp)	Gene sequence references/ accession no
Genes				
Prolactin (<i>prl</i>)	PRL _{DL2-F}	GCTCTGACACACTGCACTCC	219	Doliana <i>et al.</i> (1994)
	PRL _{DL2-R}	AGGATTACAAGGGGGTCTCG		X78723
Growth hormone (<i>gh</i>)	GH _{DL1-F}	GACAAGCACGAGACACAACG	195	Doliana <i>et al.</i> (1992)
	GH _{DL1-R}	CTGTGAGGGAACATCTCTGC		
Proopiomelanocortin (<i>pomc</i>)	Dlp-F	AAGCCTGTTGGAGCAAAGC	191	Varsamos <i>et al.</i> (2003)
	Dlp-R	GAGCCATCCTTCTTCTCGTG		AY691808
Somatolactin (<i>sl</i>)	SL _{DL1-F}	CATACCAAGCCCTTACCC	300	Company <i>et al.</i> (2000)
	SL _{DL1-R}	GGCACATCATACTGGAATAGGC		AJ277390
Gonadotropin I (<i>gthI</i>)	GTHI _{DL2-F}	GTGCTACCACGAGGATCTGG	208	Mateos <i>et al.</i> (2003)
	GTHI _{DL2-R}	GGGTATGTCTCCAGGAAAGC		AF543314
Gonadotropin II (<i>gthII</i>)	GTHII _{DL1-F}	CAGATGATGTTCCCTTGG	203	Mateos <i>et al.</i> (2003)
	GTHII _{DL1-R}	TGACAGGGTCCTTAGTGATGC		AF543315
Insulin-like growth factor I (<i>igfI</i>)	IGFI _{DL1-F}	CGCTGCAGTTTGTGTGTGG	207	AY800248
	IGFI _{DL1-R}	CTCTTGGCATGTCTGTGTGG		
Elongation factor 1 α (<i>ef1α</i>)	EF1 _{DL1-F}	GGCTGGTATCTCCAAGAACG	239	Present study
	EF1 _{DL1-R}	GTCTCCAGCATGTTGTCWCC		
Ribosomal protein L17 (<i>rpL17</i>)	DlrpL17-F	CTGGCTTGCCTTCTTGACT	201	AF139590
	DlrpL17-R	GAGGACGTGGTGGTTCATCT		
Chlorophyll A/B-binding protein (CAB)	CAB-F	GCATTTGTTGAGCACCAGAG	259	Varlet-Marie <i>et al.</i> (2004)
	CAB-R	TATCGCCAATGTTGTTGTGC		

All except the last (i.e. CAB) gene sequence references are from sea bass. Primers codes are arbitrary.

(containing SYBR Green and Taq DNA polymerase, purchased from Roche), 0.5 μ l of each primer (20 μ M), 6 μ l nuclease-free H₂O and 1 μ l template DNA (measured in duplicate). The thermal profile used for real-time PCR consisted of a step at 95 °C for 10 min and 40 cycles of denaturing at 95 °C for 15 s, annealing at 62 °C for 4 s and elongation at 72 °C for 8 s. After the last cycle, temperature in the Light Cycler chamber increased to 95 °C and then decreased to 62 °C for 30 s. Then it was increased gradually to 95 °C to obtain the melting curves of the amplified fragments. Absence of non-specific PCR products and primer dimers was checked by the melting curve analysis and electrophoresis on 8% acrylamide/SYBR Green gel.

Quantification and analysis of the results were performed using Light Cycler Relative Quantification Software 1.0 (Roche) and calculations were done according to Rasmussen (2001). Both 'second derivative' and 'fit point' methods were applied to the data set. C_t values from target genes were normalized to CAB C_t values for each individual sample. The quantification of the gene expression is presented both in terms of absolute number of copies of mRNA per microgram total RNA and relative to the expression of house-keeping genes.

Plasma cortisol levels

Plasma concentrations of cortisol were determined in triplicate by RIA according to Arends *et al.* (1998) using a commercial antiserum (Bioclinical Services Ltd, Cardiff, UK). The cortisol antibody cross-reactivity with 11-deoxycortisol, cortisone acetate, cortisone, and 17 α -OH-progesterone was 5.9, 0.16, 2.6, and 0.4% respectively. Standards and samples (10 μ l) in RIA

buffer (phosphate-EDTA buffer containing 0.1% 8-anilino-1-naphthalenesulfonic acid and 0.1% (w/v) bovine γ -globulin) were incubated with 100 μ l antiserum (in RIA buffer containing 0.2% normal rabbit serum) for 4 h. Samples were incubated overnight with 100 μ l iodinated cortisol (2000 disintegration per minute; Amersham Nederland BV, 's Hertogenbosch, The Netherlands). Bound and free hormone were separated by adding 1 ml ice-cold precipitation buffer (phosphate-EDTA buffer containing 2% (w/v) BSA and 5% (w/v) polyethylene glycol). The tubes were centrifuged at 4 °C (2000 g, 20 min), the supernatants removed and counted in a gamma counter (LKB Wallac, Finland).

Gill Na⁺/K⁺-ATPase activity

The first right side gill arch was removed and rinsed in a solution (pH 7.4) containing 300 mM sucrose, 20 mM Na₂EDTA and 100 mM imidazol (Zaugg 1982), placed in tubes containing the same solution and stored at -20 °C until use. During the whole procedure, samples were kept on ice.

Stored samples were thawed at room temperature and briefly centrifuged. The preservation medium was then removed, branchial arch cartilage discarded and 2 ml isotonic isolation medium (IIM: 250 mM sucrose, 5 mM MgCl₂ and 5 mM Hepes; pH 7.4) were added in each tube. Samples were then homogenized in a glass potter homogenization device and the homogenate obtained was subsequently centrifuged at 3000 g for 5 min at 4 °C to eliminate debris. The supernatant containing the plasma membrane fragments was transferred to new tubes. During the extraction procedure, samples were maintained at 4 °C on ice.

Enzyme specific activity was expressed per milligram protein. To this end, protein was determined by a colorimetric method (Bio-Rad) using BSA as reference. Na⁺/K⁺-ATPase specific activity was assessed as the difference of ATP hydrolysis in the presence of Na⁺, K⁺, Mg²⁺, and ATP, and that in the same medium without K⁺, but with an optimal concentration of ouabain (1 mg/ml; Flik *et al.* 1983). The amount of phosphate released was assessed colorimetrically against a certified standard (Sigma). The enzyme specific activity was expressed in μmol P_i/h per mg protein.

Statistical analysis

From each of the FW and SW tanks, ten fish were sampled and analyzed, as described above. In two of the FW-adapted fish, pituitary total RNA was too low following RNA extraction, reducing *n* to 8. Data are expressed as mean ± s.d. and were checked for normal distribution. Comparisons in gene expression and gill Na⁺/K⁺-ATPase activity between SW and FW fish were statistically analyzed by Student's *t*-test; statistical significance was accepted when *P* < 0.05.

Results

Normalization and high quality RNA are crucially important to produce reliable quantification by real-time PCR. Measurements of RNA concentrations using the Agilent 2100 Bioanalyzer confirmed the quality of RNA isolation procedure and allowed the same amount of RNA to be used for reverse transcription (RT) of the individual samples. The 'alien' gene used in this study (CAB) allowed normalization of the results of the RT by correction for target gene *C_t* values with the *C_t* values of the CAB for each individual sample. Quantification of expression data obtained by either the 'second derivative' or the 'fit point' methods resulted in a similar outcome; data obtained by the 'second derivative' method are presented here. Abundance of mRNA is expressed in terms of number of copies per microgram RNA as well as in values relative to the house-keeping genes.

The efficiencies of the real-time PCR for all the genes examined in this study ranged between 1.804 and 1.996 (Table 2).

The house-keeping genes tested in the present work were validated as controls for experiments involving sea bass adapted to SW and FW, since no significant variation in their expression was found, either in pituitary glands or in liver, independent of ambient salinity. In terms of number of copies per microgram RNA (Fig. 1), expression levels of *rpL17* in the pituitary of SW and FW fish were 962 000 ± 40 485 and 1 192 671 ± 136 999 respectively (*P* = 0.14). No differences were found in *rpL17* expression levels in liver (approximately 600 000 copies per microgram RNA; *P* = 0.8). Expression levels of *ef1α*, in terms of the number of copies per microgram RNA, in the pituitary of SW and FW fish were 1 094 934 ± 82 131 and 1 488 963 ± 239 786 respectively (*P* = 0.15; Fig. 1).

Table 2 Efficiencies of real-time quantitative PCR applied for quantification of sea bass (*Dicentrarchus labrax*) hormone expression in fish maintained in SW and FW

Genes	Efficiencies
<i>ef1α</i>	1.953
<i>rpL17</i> (pituitary)	1.883
<i>prl</i>	1.884
<i>gh</i>	1.961
<i>sl</i>	1.907
<i>pomc</i>	1.804
<i>gthI</i>	1.958
<i>gthII</i>	1.843
<i>igfI</i> (liver)	1.973
<i>rpL17</i> (liver)	1.996

Efficiency = 10e(− 1/*a*); *a*, slope of the standard curve.

Pituitary *prl* mRNA expression dramatically increased after acclimation of sea bass to FW. In terms of number of copies per microgram RNA (Fig. 1), the expression level of *prl* in FW fish was 4.5-fold higher (*P* < 0.0001) compared with SW fish (about 30 millions copies in SW and 136 millions copies in FW). Relative to *rpL17* or *ef1α*, *prl* expression was significantly lower (*P* < 0.0001) in SW fish than in FW fish (Table 3). The *prl* gene had the second highest transcriptional level in FW fish, after POMC (in FW and SW fish, see below) compared with the other target genes (Fig. 1).

The number of copies of *gh* per microgram RNA also tended to be increased in pituitary gland of FW fish (1.9-fold higher than in SW fish; Fig. 1), but this difference was just not statistically significant, neither for the number of copies, nor for the expression relative to *rpL17* or *ef1α* (Table 3). Expression of *igfI* mRNA in liver did not significantly differ between FW and SW sea bass (Table 3). The number of copies per microgram RNA of this hormone mRNA in the liver was very low in both SW and FW conditions (325 ± 57 and 408 ± 47 respectively).

Pituitary *sl* mRNA expression significantly decreased after acclimation of sea bass to FW. The number of copies per microgram RNA in FW fish (10 326 914 ± 920 540) was 1.8-fold lower (*P* < 0.05) compared with SW fish (18 709 872 ± 3 499 884) (Fig. 1). The mRNA expression relative to *rpL17* or *ef1α* of SW and FW fish was also significantly different (*P* < 0.05; Table 3).

Expression levels of *pomc* mRNA were similar in FW and SW sea bass (Table 3). The number of copies per microgram RNA of this prohormone in the pituitary of SW and FW fish was very high (about 250 millions copies); the abundance of this prohormone was much higher than any of the other target genes tested (Fig. 1). Expression of *gthI* and *gthII* mRNA in the pituitary gland also did not significantly differ between sea bass in FW or SW (Table 3). In terms of number of copies per microgram RNA, mean mRNA expression level of *gthI* in SW fish was 2.1-fold lower compared with FW, but there was no statistically significant difference (Fig. 1).

Figure 1 Number of copies per microgram total RNA of pituitary prl, gh, sl, pomc, gthI, gthII, rpL17, ef1 α , and of liver igfI, rpL17 (mean $\pm$ s.d.; $n=8-10$), in sea bass (*Dicentrarchus labrax*) maintained in SW (35‰) or adapted for 1 month in FW (0.3‰). Symbols indicate significant differences between the two salinity conditions, * $P<0.05$, † $P<0.0001$.

Plasma cortisol levels were 6.5-fold lower ($P<0.001$) in SW- than in FW-adapted specimens (4.0 ± 1.9 and 26.0 ± 8.5 nM respectively).

Gill Na^+/K^+ -ATPase-specific activity was 2.3-fold higher in FW than in SW: it was 7.00 ± 0.71 and 3.03 ± 0.56 $\mu\text{mol P}_i/\text{h}$ per mg protein respectively.

Discussion

The aim of this study was to quantitatively compare mRNA levels of pituitary hormones in sea bass kept in FW or SW, by measuring mRNA levels by real-time PCR. Although this

technique has become one of the most appropriate ways to detect and quantify mRNA expression, normalization is necessary at different steps of its implementation to ensure data reliability and consistency (Bustin *et al.* 2005). We first normalized the rate of the RT by introducing an 'alien' gene, i.e. CAB (see Materials and Methods section). To quantify the level of mRNA expression of a target gene, it is also necessary to measure the expression level of a constitutively transcribed house-keeping gene treated identically as the target gene prior to measurements. However, significant differences may exist (up to tenfold) in the expression levels of endogenous reference genes that have commonly been used to normalize mRNA expression

Table 3 Hormone mRNA expression (mean $\pm$ s.d.; $n=8-10$) in sea bass (*Dicentrarchus labrax*) maintained in SW (35 ‰) or adapted for one month to FW (0.3 ‰)

	Salinity	Relative to L17 mRNA expression	P	Relative to ef1 α mRNA expression	P
Hormone <i>prl</i>	SW	1.30 ± 0.03	<0.0001	1.22 ± 0.02	<0.0001
	FW	1.50 ± 0.02		1.40 ± 0.01	
<i>gh</i>	SW	1.16 ± 0.02	0.37	1.09 ± 0.02	0.42
	FW	1.20 ± 0.02		1.11 ± 0.02	
<i>sl</i>	SW	1.27 ± 0.02	<0.05	1.20 ± 0.02	<0.05
	FW	1.20 ± 0.01		1.12 ± 0.02	
<i>pomc</i>	SW	1.51 ± 0.03	0.81	1.42 ± 0.02	0.64
	FW	1.50 ± 0.03		1.40 ± 0.03	
<i>gthI</i>	SW	1.11 ± 0.03	0.20	1.04 ± 0.03	0.24
	FW	1.17 ± 0.03		1.09 ± 0.03	
<i>gthII</i>	SW	1.12 ± 0.05	0.50	1.05 ± 0.05	0.57
	FW	1.17 ± 0.03		1.09 ± 0.04	
<i>igfI</i> (liver)	SW	0.61 ± 0.003	0.24	—	—
	FW	0.62 ± 0.004		—	

For each hormone, mRNA expression has been normalized on the expression of the alien gene and is expressed in values relative to the expression of the house keeping genes.

of target genes (Bustin 2000). There is increasing evidence that genes encoding structural ribosomal proteins or translation factors are among the genes with the lowest transcriptional regulation (Gray & Wickens 1998, Frost & Nilsen 2003). In the present study, we demonstrate that expression levels of rpl17 and e1f1 α did not vary between SW and FW sea bass (*D. labrax*); these genes were consequently considered suitable for internal calibration of real-time PCR data in this species. Since co-regulation of rpl17 (structural component of the ribosome) and of e1f1 α (involved in translation) seems unlikely, the use of both as reference genes should make quantification completely reliable.

Considering the precautions mentioned above, our data more accurately compare mRNA expression of genes between experimental conditions (the main thrust of this work) and to a lesser degree estimate differences in mRNA expression between genes within individuals. We focused on the comparison of pituitary hormone mRNA synthesis under two steady-state conditions, proceeding from the notion that significant differences in the gene expression must correspond to anticipated and well-known differences in protein productions and secretion.

The eventual physiological interpretation of the protein output of cells requires aspects such as differential storage and post-translational processing, phenomena we did not address here. However, this study is unique as it is the first to address absolute mRNA quantification of European sea bass hormone genes by real-time quantitative PCR. Most studies so far and mentioned below concern data on hormone mRNA expression in fishes obtained by means of semi-quantitative methods (blotting, RNase protection assays) that allow only qualitative comparisons.

An interesting finding of the present study is the differential expression of sl mRNA in sea bass in SW and FW. To the best of our knowledge, this is the first report on a putative role of pituitary sl in teleost osmoregulation. Sl is a recently (early 1990s of the former century) discovered member of the gh/prl family, produced in the pituitary pars intermedia (Ono *et al.* 1990). Most of the studies published on sl concern only a single group of fish, viz. salmonids, and although these studies highlight the pleiotropic character of the hormone (Ono & Kawachi 1994, Kakizawa *et al.* 1997, Pérez-Sánchez *et al.* 2002), still little is known on sl physiology. Although a second sl gene has been recently found in zebrafish (Zhu *et al.* 2004), in sea bass as well as in most of the studied species, only one sl gene has been reported to date (Company *et al.* 2000). We report here that pituitary mRNA level of this hormone was significantly higher (1.8-fold) in SW than FW sea bass. Down-regulation of sl mRNA in FW suggests an involvement in hyposmotic regulation in this species.

Prl and gh, the two other members of the pleiotropic sl/prl/gh gene family, appear to antagonize each other in salinity adaptation (reviews in McCormick 2001, Manzon 2002). Indeed, in sea bass too, pituitary prl mRNA increased 4.5-fold in FW sea bass compared with SW, congruent with the established key role of this hormone in hyperosmotic regulation.

Remarkably, in both FW and SW, prl mRNA levels were relatively higher than the mRNA expression of most other genes quantified in this work, which probably underlines the wide range of biological activities of this hormone (Björnsson *et al.* 2002, Pérez-Sánchez *et al.* 2002) and a particular role for prl in SW sea bass. Although the role of prl in FW adaptation varies among species, it is well established as the hormone preventing loss of ions (particularly Na⁺ and Cl⁻) and decreasing integumental permeability to water of osmoregulatory organs in euryhaline teleosts (reviews in Hirano *et al.* 1987, Manzon 2002). A good correlation between prl mRNA expression in the pituitary and prl plasma concentration has been shown in Atlantic salmon *Salmo salar*, since both are elevated during smoltification and decrease after entry of completely smoltified fish in SW (Bœuf 1993, Agústsson *et al.* 2003). Moreover, both mRNA levels and plasma protein decrease after transfer from FW to isosmotic (brackish) water in channel catfish *Ictalurus punctatus* (Tang *et al.* 2001) as well as in the tilapia *Oreochromis niloticus* (Auperin *et al.* 1994). Our results show that increased pituitary prl levels are part of the osmoregulatory strategy in FW adaptation of sea bass.

In salmonid species, prl may antagonize the SW-adaptive actions of gh (Sakamoto *et al.* 1993, McCormick 1995, Seidelin & Madsen 1997). Some (if not all) of the osmoregulatory effects of gh are mediated by igfl which is known, indeed, to interact with both prl and gh (Mancera & McCormick 1998, Fruchtmann *et al.* 2001, Kajimura *et al.* 2002, Pérez-Sánchez *et al.* 2002). We found no significant difference either in sea bass pituitary gh mRNA level, or in liver igfl mRNA expression, whether the fish were in FW or SW. Yet, unaffected pituitary gh or liver igfl mRNA levels in SW and FW were also reported for the euryhaline Mozambique tilapia *Oreochromis mossambicus* (Ayson *et al.* 1994) and the rainbow trout *Oncorhynchus mykiss* (Sakamoto & Hirano 1993), which could indicate that the SW-adaptive actions of gh/igfl are particular to parr-smolt transformation of salmonids. Interestingly, the pituitary gh mRNA level (mean value) was about twofold higher in FW sea bass, and although the difference was not statistically significant because of the high variability between samples, it is a response to FW adaptation different to what one would predict from salmonid responses. In Mozambique tilapia, Riley *et al.* (2003) postulated that transfer from SW to FW could activate the gh/igfl axis. Absence of significant differences in gh and igfl mRNA expression does not allow to conclude that these hormones are not involved in sea bass osmoregulation. It is very likely that in this species when in FW or SW, metabolic clearance and distribution space for gh and igfl differ, but we do not know how these processes and properties relate to mRNA expression, nor how the kinetics of the activation/deactivation of the gh/igfl axis are during salinity challenges.

Prl and gh plus igfl, interact with cortisol, widely considered the SW-adapting hormone (reviews in Sakamoto *et al.* 1993, McCormick 1995). Interestingly, plasma cortisol levels were higher in FW-adapted than in SW maintained sea bass specimens. The endocrine control of cortisol secretion in

teleosts is dominated by the pituitary gland, in particular by acth and α -msh plus β -endorphin (reviews in Mayer-Gostan *et al.* 1987, Wendelaar Bonga 1997). These peptides are derived from the same precursor, pomc. In sea bass, no difference was found in pituitary pomc mRNA level in SW or FW fish. In accordance with a previous study (Varsamos *et al.* 2003), pituitary pomc mRNA levels are very high ($2\text{--}3 \times 10^8$ copies/ μg RNA) under both water salinity conditions. Unfortunately, we did not discriminate between pituitary pars distalis (acth cells) and pars intermedia (msh cells), as the small acth cell volume did not allow the anticipated analyses. Obviously, differences in acth-cell pomc mRNA expression in FW and SW fish may have gone undetected in our set up, and this aspect requires further experimentation.

There is some evidence for a complex interaction between the gnrh-gth sex-steroid axis and the gh/igfl axis, given a demonstrated role of gh in salmonid reproduction (Björnsson *et al.* 2002). Moreover, sex maturation and treatment with sex steroids is known to affect SW adaptation of some species (McCormick 1995, Riley *et al.* 2002). Our findings concerning pituitary gth mRNA expression do not substantiate an involvement of these hormones in salinity adaptation. Although sea bass can live and grow in FW, it neither does nor can reproduce in FW (Pickett & Pawson 1994). Moreover, our fish may be considered sexually immature, which further precludes differences in expression of these genes. More investigations on salinity effect on gonad maturation and/or on a putative role of reproductive hormones in osmoregulation will be necessary.

Branchial Na^+/K^+ -ATPase activity was 2.3-fold higher in FW sea bass compared with SW fish. We (Varsamos *et al.* 2002a,b) have demonstrated that the euryhalinity of this species relies in part on the phenotypic plasticity of branchial ionocytes, also called mitochondria rich cells (MRC) and their Na^+/K^+ -ATPase content that differs drastically between FW and SW. Prl, gh, igfl, and cortisol are known to affect MRC number and size as well as Na^+/K^+ -ATPase activity throughout the post-embryonic development of teleosts (reviews in McCormick 2001, Sakamoto *et al.* 2001, Varsamos *et al.* 2005). Clearly, the sea bass endocrine osmoregulatory repertoire differs from that of most other teleosts studied so far. Following transfer from SW to FW, numbers and size of MRCs increase, as does Na^+/K^+ -ATPase activity, concomitantly with elevated prl expression and plasma cortisol levels. This is counterintuitive to the well-documented inhibitory actions of prl on these cells and this enzyme in other euryhaline teleosts (Flik *et al.* 1994, Manzon 2002). Moreover, sl appears to be implicated in sea bass osmoregulation, prompting further investigations on this hormone in teleost salinity adaptation. At last, our results do not allow the exclusion of a putative involvement of gh in FW adaptation of this species. The present study thus constitutes a first step towards the assessment of pituitary hormonal messengers involved in control of osmoregulation in the European sea bass *D. labrax*. More work is needed on hormone expression and/or concentrations of the proteins of these hormones in plasma and tissues, especially for prl, gh, and sl. For the mRNAs involved we now have a starting point of view.

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