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A transcriptomic approach of salinity response in the euryhaline teleost, Dicentrarchus labrax

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Abstract:

Euryhaline teleosts possess the capacity to osmoregulate under various environmental conditions (freshwater to hypersaline water). This physiological capacity is generally monitored using enzyme activity assays (Na+/K+-ATPase…), hormones quantification (prolactine, growth hormone) or their mRNAs expression. To date, few studies addressed the genetic correlates of adaptation to varying salinity at a molecular level in such fish. In the sea bass Dicentrarchus labrax, genetic differentiation was observed at specific allozyme loci between lagoon- and open-sea populations. In the present study, we investigated transcriptomic response of D. labrax to salt- and freshwater acclimation in two organs involved in osmoregulation, gill and intestine. By using suppression subtractive hybridisation, we characterised 586 partial cDNA sequences encoding proteins potentially involved in the metabolism of sea bass acclimated to salt- or freshwater under experimental conditions. Using these results, we first characterised complete genomic sequence of a carbonic anhydrase and then analysed mRNA expression of genes potentially involved in osmoregulation mechanisms (Na+/K+-ATPase, carbonic anhydrase, angiotensin-converting enzyme and claudin-3), cell-cycle regulation (secretagogin) and immune system (nephrosin) in gill and intestine of wild fish from open sea and lagoons. Our analyses indicate a strong tissue- and environmental-dependant expression pattern for all the genes studied. A transcriptomic approach such as described in the present paper provides thus a first description of genes involved in metabolic or structural functions important for coping with environmental salinity variations in a euryhaline fish like the common sea bass D. labrax. It should be supplemented by proteomics to check the direct involvement of the gene products at the protein level, and by polymorphism analyses if one is to understand population or individual fluctuations in acclimation to salinity variation.

Keywords: Sea bass; mRNA expression; Osmoregulation; Carbonic anhydrase; Suppression subtractive hybridisation

1. Introduction

Terrestrial and aquatic organisms have to control and maintain the osmotic presure of their cells by regulating fluxes of ions and water through the cell membrane, often with some metabolic cost. The ability of an aquatic organism to tolerate wide variation of salinity without compromising life processes is called euryhalinity (in opposition to stenohalinity). Marine teleost fishes tend to lose water through osmosis and to gain ions (essentially Na^+ and Cl^-) through diffusion (ingestion of seawater, excretion of small volume of urine and active excretion of salt through the gill), whereas the reverse mechanism occurs in freshwater fishes (excretion of relatively dilute urine, active uptake of salt across the gill and possibly some ingestion of salt in the food) (Alderdice, 1988).

Numerous studies have investigated the effects of salinity changes in teleosts at a physiological level (osmoregulation enzyme activity and hormones quantification, urine volume measurement...) and in term of specific mRNA expression variations (Na^+/H^+ (NH_4^+) exchanger, $Na^+/K^+/2Cl^-$ cotransporter) (Hirose et al., 2003). The activity of ion-transporters is particularly well studied in seawater challenged fishes. A seawater transfer of salmons, *Onchorhynchus kisutch* or *Salmo salar*, and brown trout, *Salmo trutta*, resulted in a gradual adjustment of Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter expression in gill epithelial cells (Tipsmark et al., 2002). It was observed that some Na^+/K^+-ATPase subunits mRNA levels increased with environmental salinity, while some others decreased, in tilapia *Oreochromis mossambicus* and rainbow trout *Onchorhynchus mykiss* tissues (Richards et al., 2003). Besides, the responses of fishes to freshwater challenges are particularly studied through the quantification and the expression of the hormone prolactin that is involved in adaptation to fresh water. Prolactin plays a major role in osmoregulation in freshwater fishes by regulating water and salt fluxes through the gills and kidney (change in membrane permeability and development of chloride cells) (Forsyth and Wallis, 2002). As was observed for several hormones, prolactin becomes efficient after its binding to the prolactin receptor that activates intra-cellular signalling pathways such as tyrosine kinase, MAPK (mitogen-activated protein kinase), Src kinase and the activation of ion channels (Freeman et al, 2000). In laboratory conditions, tilapia showed a strong increase of prolactin concentration in response to a salinity decrease (Weber et al., 2004). In the tilapia *O. mossambicus* exposed to various salinities (from fresh water to seawater), Streelman and Kocher (2002) demonstrated a relationship between (1) the length of different microsatellite alleles in the promoter region of the prolactin gene, (2) the gene expression, and (3) the ability of fishes to survive under different salinity conditions. This work provided one of the first *in vivo* evidence that differences in microsatellite length among individuals may indeed affect gene expression and that variation in expression has concomitant physiological consequences.

The common (or European) sea bass *Dicentrarchus labrax* L. (Teleost, Perciformes, Moronidea) is a marine teleost of great economical importance in the Mediterranean area able to survive under various salinity conditions (Jensen et al., 1998). The sea bass acquires tolerance to fluctuating salinity very early during development, already present at hatching, and increasing throughout development (Varsamos et al., 2001, Hirose et al., 2003). Physiologically, this capacity is characterised by the presence of mitochondria-rich cells in the gill epithelium, named chloride cells, and capable of regulating ion content during salinity changes (Varsamos et al., 2002). *D. labrax* is able to colonise coastal lagoons and estuaries as well as the open sea, but mating and spawning occur only at sea (Barnabé, 1980). Previous works demonstrated a significant genetic differenciation between lagoon and marine sea bass (Lemaire et al., 2000) and before and after freshwater acclimation under experimental conditions (Allegrucci et al., 1995). To date, the response to salinity changes in teleosts has primarily been examined at a physiological level. Recently, Nebel et al. (2005) have dissected some of the physiological correlates of individual variation in the capacity of seabass to adapt to freshwater.

In the present study, we investigated the response of the sea bass *D. labrax* to freshwater and seawater acclimation under experimental conditions by a transcriptomic approach. We first determined the differentially regulated genes after a six-month period in saltwater and in fresh water using a suppression subtractive hybridisation (SSH) method. We then investigated the mRNA expression of Na^+/K^+-ATPase, angiotensin-converting enzyme (ACE), secretagogin, nephrosin and claudin-3, according to their role in various cellular metabolism, by semi-quantitative RT-PCR in sea bass from open sea (near Sète, French Mediterranean coast) and a nearby coastal lagoon (Ingril, Mediterranean). We also characterised gene sequence and mRNA expression of carbonic anhydrase in experimentally

acclimatised to fresh- and saltwater sea bass and in sea- and lagoon-caught individuals. We discuss the validity of these different genes as markers of adaptation of sea bass to various salinity conditions.

2. Materials and methods

2.1. Sea bass conditioning and collection

Two groups of *D. labrax* juveniles (both male and female seabass weighing 20 ± 1.5g), progeny originated from a complete factorial sheme 3 x 3 obtained by artificial breeding at IFREMER (Palavas-les-Flots, Hérault, France) were reared in recirculating 20°C salt water, under natural photoperiod. Transfer to fresh water were carried out according to the protocol described by Nebel et al. (2005). After a six-month period (i.e. complete acclimation), surviving fishes in fresh water (n=10) and seawater (n=10) were collected, dissected, and intestine and gill tissues were sampled and stored at -80°C until used. During the experiment, the fish were fed daily on adapted commercial pellets (Le Gouesnant, Lamballe, France). For all experiments, the principles of laboratory animal care were followed. The fish were anesthetised in a solution of phenoxy-2-ethanol (150µg/l) prior to any manipulation.

Adult wild sea bass were collected in December 2004 from the Mediterranean Sea (35‰; ≈ 11ºC; collected about 3km offshore; n=5; Hérault, France) and from the Ingril lagoon (28.5‰ at collection time; ≈ 5ºC; n=5; Hérault, France). If salinity is roughly constant in the sea across the year, data have shown that salinity may vary from 3.5‰ up to 41‰ in the Ingril lagoon which is very shallow (2004 survey; Syndicat Intercommunal des Etangs Littoraux, *pers. comm.*). All individuals were dissected and intestine and gill tissues were collected in tubes and stored at -80°C until RNA extraction.

2.2. Suppression subtractive hybridisation

Total RNA was extracted from the intestine and gills tissues of saltwater and freshwater acclimated fishes (n=10) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Poly(A+) mRNA was isolated from total RNA using the Nucleotrap® mRNA midi purification kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. Two subtracted libraries per salinity condition were generated. Two libraries contained partial transcripts of genes up-regulated in intestine and gills of saltwater-acclimated fishes, while the two other libraries contained those that were up-regulated in intestine and gills of freshwater-acclimated fishes. The libraries were obtained by subtracting out the cDNA common to both the saltwater acclimated fishes and freshwater acclimated fishes, leaving the differentially expressed partial transcripts from the saltwater and freshwater fishes to be amplified. First and second strand cDNA synthesis, *Rsa*I endonuclease enzyme digestion, adapter ligation, hybridisation, and PCR amplification were performed as described in the PCR-select cDNA subtraction manual (BD Biosciences). The differentially expressed PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). Ligation mixtures were transformed into DH5α *Escherichia coli* competent cells. Three hundred white colonies per library were isolated, grown in Luria-Bertani medium (containing 100µg/l ampicilline) and sequenced (MWG-Biotech AG, Ebersberg, Germany). All sequences were subjected to a homology search through the BLASTX program (www.ncbi.nlm.nih.gov/BLAST).

2.3. Cloning and sequencing of carbonic anhydrase cDNA, gene and proximal promoter region

Total RNA was extracted from the gill tissue of one experimentally saltwater acclimated sea bass using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The procedures for the generation of carbonic anhydrase cDNA 5' and 3' untranslated regions (UTR) were carried out according to the commercial 5'/3' rapid amplification of cDNA ends protocol (5'/3' RACE Kit, Roche, Mannheim, Germany) using specific antisense (5'-TGT AAC TCC ATG GGA TAG CGC TTT CCA TC-3') and sense (5'-AGC TGG AAC ACT TTG ACC TCC TAC CTG GC-3') primers for the 5' and the 3' UTR, respectively. The 3' UTR was amplified as follows: 200 ng of reverse transcription product was submitted to amplification using one cycle at 94°C for 2 min, 58°C for 2 min, 72°C for 2 min 30, then 35 cycles at 94°C for 15 s, 58°C for 30 s, 72°C for 2 min and a final step at 72°C for 10 min with

2 mM $MgCl_2$ and 10 pmol each of PCR anchor primer (5'-GAC CAC GCG TAT CGA TGT CGA C-3') and specific primer. Amplification of the 5' UTR was carried out according to the following procedure: denaturation at 94°C for 2 min, then 10 cycles at 94°C for 15 s, 58°C for 30 s, 72°C for 1 min, then 30 cycles at 94°C for 15 s, 58°C for 30 s, 72°C for 1 min with an increase of 20 s per cycle for the elongation time, and a final step at 72°C for 15 min with 2 mM $MgCl_2$ and 10 pmol each of oligo dT anchor primer (5'-CGC TCT AGA ACT AGT GGA $TCT_{(16)}$-3') and specific primer. The resulting cDNA fragments corresponding to the 5' and 3' UTRs were cloned into pGEM-T vector (Promega), transformed in DH5α *E. coli* competent cells and sequenced (MWG-Biotech AG).

Total genomic DNA was isolated from sea bass muscle using phenol/chloroform/isoamyl alcohol (25:24:1). The resulting preparation was amplified with primers (5'-GGA GGT ACA GGT GCG TCA AAG ATG AAC TGG-3' and 5'-CCT GTA AAC TTA GTG CAA GTG CAT TCC TGC C-3') using *UptiTherm* DNA polymerase (Interchim). Two hundred ng of DNA were submitted to amplification using denaturation at 94°C for 5 min, then 15 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 3 min, then 15 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 3 min with an increase of 10 s per cycle for the elongation time, and a final step at 72°C for 30 min with 2 mM $MgCl_2$, and 10 pmol of each primer. The resulting product was cloned into pGEM-T vector (Promega), transformed in *E. coli* DH5α competent cells and finally sequenced (MWG-Biotech AG).

The characterisation of the proximal promoter region was conducted by using a modified GenomeWalker protocol as follows. First, 10µg of genomic DNA was partially digested by the *Pvu*II restriction enzyme in a 100µl-final reaction volume for 2h at 37°C. A 20µl volume was removed every 30 min, stopped with 1µl of EDTA/glycogen mix and stored on ice until use. The digested DNA was then purified with 150µl of phenol-chloroform-isoamylic alcohol (25:24:1), precipitated with 4M ammonium acetate and ethanol 100, and dried. The DNA pellet was suspended in 10µl of water. Digested DNA was then ligated overnight at 16°C with two different adapters (5'-CGA GCG GCC GCC CGG GCA GCA G-3' and 5'-CTG CTG CCC GGG CGG CCG CTC G-3'). The reaction was stopped by incubating the mix 5 min at 72°C, and diluted in 100µl of dilution buffer (20mM HEPES-HCl pH 8.3, 50mM NaCl, 0.2mM EDTA pH 8). PCR amplification was performed by using Advantage® cDNA PCR kit (BD Biosciences) as follow: one cycle at 75°C for 5 min, one cycle at 94°C for 1 min, 30 cycles at 94°C for 30 s, 57°C for 40 s and 72°C for 4 min with PCR primer (5'-CGA GCG GCC GCC CGG GCA GCA G-3') and a reverse carbonic anhydrase primer (5'-TGT AAC TCC ATG GGA TAG CGC TTT CCA TC-3'). A nested PCR was performed on the first PCR product (1:10 diluted) as follow: 30 cycles at 94°C for 30 s, 59°C for 40 s and 72°C for 3 min and a final extension of 15 min at 72°C with PCR primer (10pmol) and a reverse carbonic anhydrase primer (10pmol) (5'-CCA GTT CAT CTT TGA CGC ACC TGT ACC TCC-3'). PCR product was separated on 1.5% agarose gel using 0.5X TBE-buffer and visualised on UV after Ethidium Bromide staining. A 1000 bp fragment was obtained and the band was excised from gel and extracted using the QIAEX II purification kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purified product was then ligated into pGEM-T vector (Promega), transformed in *E. coli* DH5α competent cells and the alkaline lysis minipreparation was finally sequenced (MWG-Biotech AG).

2.4. Gene expression analysis by using semi-quantitative RT-PCR in wild sea- and lagoon-caught bass

Total RNA was extracted using Trizol from experimental individuals (acclimated to saltwater and fresh water, n=5 each, carbonic anhydrase expression analysis only), open sea and Ingril lagoon individuals (n=5 each, environmental characteristics in section 2.1). Ten µg of RNA were submitted to reverse transcription using an oligo dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). To perform semi-quantitative RT-PCR, the total amount of isolated total RNA was quantified by UV-spectroscopy at 260 nm. Amplification of Na^+/K^+-ATPase, claudin-3, ACE, nephrosin and secretagogin were performed in a 25µl-final volume containing 2mM $MgCl_2$ and 10 pmol of each primers (Table 1) as follows: one cycle at 94°C for 2 min, 55°C for 2 min and 72°C for 1 min 30s; between 25 and 40 amplification cycles (according to the gene, see table 1) at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min 20 followed by a final 7 min extension at 72°C. The optimal number of PCR cycle was determined for each gene studied as the number of PCR cycle necessary to obtain a PCR product in the exponential phase. A PCR amplification control (28S ribosomal DNA) was used for all experiments and was amplified as described above with primers described in table 1. PCR product was separated on 1.5%

agarose gel using 0.5X TBE-buffer and photographed after ethidium bromide staining. Quantification of band intensity was made using Gene Profiler Software v. 4.03 (Scanalytics, Inc.). Statistical analysis (non-parametric test Kruskall-Wallis) was performed with Statistica software v. 6.0.

3. Results

3.1. Identification of salinity-regulated genes

SSH libraries were generated from intestine and gills of freshwater- or seawater-acclimated *D. labrax*. The search for homology using the BLASTX program revealed 586 different sequences and amongst them 450 (about 77%) corresponded to unidentified genes (unknown function or no hits). Four tables list the sequences obtained from the SSH libraries: freshwater up-regulated genes in intestine (Table 2), seawater up-regulated genes in intestine (Table 3), freshwater up-regulated genes in gills (Table 4) and seawater up-regulated genes in gills (Table 5). These results indicate that saltwater and freshwater acclimation involved at least eight major cellular physiological functions during the experiment: osmoregulation, protein regulation (including protein synthesis, transport and degradation), nucleic acid regulation (including transcription and metabolism of nucleic acid components), cell cycle regulation, respiratory chain and energetic metabolism, lipid metabolism, cell communication (including immune system, cell protection and membrane receptors), and cytoskeleton.

3.2. Carbonic anhydrase gene characterisation

The sequence of the carbonic anhydrase (*CA)* gene is 2947 bp in length and contains 5 coding exons of 408, 96, 79, 164 and 183 bp in length (Fig. 1). All the intron borders of *CA* start and end with the consensus GT and AG splicing signals, respectively. The corresponding amino acid sequence encodes a protein with a molecular weight of 32.97 kDa and an isoelectric point of 6.65. The proximal promoter region (1034 bp) contained an identified TATA box (TATAAA), and several ATTTA(G) motifs (involved in mRNA stability) are present in the UTRs; there are three ATTTG and three ATTTA motifs in the 5'UTR and four ATTTA and three ATTTG motifs in the 3'UTR. We also found seven putative N-glycosylation motifs translating as Asn-Xaa-Thr/Ser in the coding region of *D. labrax CA* gene.

3.3. Gene expression analysis

Carbonic anhydrase. Carbonic anhydrase expression analysis in sea bass showed a tissue- and condition expression pattern. CA has a significantly higher expression level in gills than in intestine in saltwater acclimated fish and individuals from open sea (Fig. 2 and Table 6). In freshwater individuals (experimental and lagoon), CA expression is not significantly different between tissues (Fig. 2 and Table 6).

Claudin-3. Expression of claudin-3 (involved in cell-cell adhesion and regulation of solute transport) is not significantly different between the two organs studied (Fig. 3A and B). The claudin-3 mRNA expression exhibits a strong environmental effect in gill tissues (Fig. 3A). The expression is higher in open sea individuals than in those from lagoon.

Na^+/K^+-ATPase. This enzyme shows a strong tissue- and environmental-dependant expression. The expression is lower in open sea- than in lagoon-caught *D. labrax* in the two tissues but Na^+/K^+-ATPase is more expressed in gills than in intestine (Fig. 3A and B).

Angiotensin converting enzyme. The angiotensin converting enzyme is known to be involved in the activation of angiotensin I to active angiotensin II, a strong regulator of blood presure. The expression of this enzyme is not significantly different between tissues and fish origin (Fig. 3A and B).

Secretagogin. The secretagogin expression pattern (a protein involved in cell-cycle regulation through Ca-dependant processes) is strongly environment- and tissue-dependent. In the gills, expression is lower in open sea than in lagoon-originating fishes (Fig. 3A), while it shows an opposite trend in intestine (Fig.3B). We also observed that secretagogin expression is higher in intestine than in gills in individuals from the sea, and strongly higher in gills than in intestine in lagoonal individuals. We also identify two

Ca^{2+}-binding sites in the 388bp secretagogin partial cDNA sequence obtained in our SSH (GenBank accession no CX660758).
Nephrosin. Nephrosin is a secreted protease involved in immune function in fish. No significant nephrosin expression differences were observed between tissues. Open sea individuals showed a higher expression pattern than lagoonal individuals in intestine (Fig. 3B) but not in gill tissues (Fig. 3A). The nephrosin partial cDNA sequence obtained in the SSH (GenBank accession no CX660754) contains the specific zinc-binding motif generally observed in the astacin familly members HE*XX*H*XX*GF*X*HE*XX*R*X*DR.

4. Discussion

4.1. Identification of salinity-regulated genes

The SSH method has previously been used to identify up-regulated genes in teleost and mollusc species (Straub et al., 2004; Boutet et al., 2004) submitted to various environmental challenges, mostly pollutants. In the present report, we use the same method to characterise the transcriptomic response of a marine euryhaline teleost, the common (or European) sea bass *D. labrax* to salinity acclimation under experimental conditions. We obtained 586 different partial sequences of cDNA, encoding proteins involved in the metabolism of sea bass acclimated to saltwater and fresh water in two organs known to be involved in osmoregulation (intestine and gills).

4.2. Molecular characterisation and expression of carbonic anhydrase.

CA is an ubiquitous enzyme, catalysing the reversible hydration of CO_2, and constitutes a family of zinc metalloenzymes found in nearly all organisms (Sly and Hu, 1995). These isozymes are found in many different tissues and are involved in a number of homeostatic processes, including carbon dioxide transport, ion exchange and acid-base balance (Geers and Gros, 2000). Because of both its general function and its universal presence in gill tissue of every species examined, CA is believed to be critical to the osmoregulatory function of the gills of all aquatic invertebrates (Henry, 1988). In this study, we characterised the complete sequence of the gene encoding *CA* in *D. labrax*. The coding region of the *CA* gene is interrupted by four introns. The *CA* sequence exhibits multiple ATTTA(G) motifs in its untranslated regions. These motifs are known to be correlated with transcript stability (Shaw and Kamen, 1986), and have been found in other cDNAs (Fucci et al., 1995; Tanguy et al., 2005). We also observed seven putative N-glycosylation motifs Asn-Xaa-Thr/Ser (Marshall, 1974) in the coding region of *D. labrax CA* gene, as described in the mouse *CA* gene (Tamai et al., 1996).
We also investigated the *CA* mRNA expression in gills and intestine of sea bass acclimated to salt- or fresh water under experimental conditions. We observed that mRNA level is higher in gill of saltwater- than freshwater-acclimated sea bass. Earlier studies conducted on the rainbow trout showed a higher *CA* mRNA expression rate as well as higher enzyme activity in red blood cells than in all other tissues studied, such as kidney, gill or muscles (Esbaugh et al., 2004). Our study shows a higher expression of *CA* in the gills than in the intestine. It is however noteworthy that the gill tissue contained numerous red blood cells which were not removed before RNA extraction. Our results also indicated that *CA* expression in gill (or gill and associated red blood cells) is higher in wild animals from higher salt concentrations. These results are in agreement with those reported for the in tilapia. In this species, Kültz et al. (1992) showed that CA and Na^+/K^+-ATPase activities are greatly increased under high salinity conditions. Flouders, however, did not show this regulation (Sender et al., 1999). These two studies measured CA activity levels. Our results suggest that the regulation of CA expression could be at the level of transcription, and possibly at the level of translation. It has been demonstrated that the large increase in CA activity in response to low-salinity exposure could be a result of regulatory processes operating at either the transcriptional or translational level in the green crab *Carcinus maenas* (Henry et al., 2003). Few other studies on *CA* mRNA level in response to salinity variations are published to date. Hirata et al. (2003) studied *CA* mRNA in Osorezan dace (*Tribolodon hakonensis*) in response to acidic water conditions and showed that *CA* mRNA level is strongly increased in the gill and intestine, suggesting an important role of CA in acid adaptation. Further investigations are necessary to elucidate how *CA* mRNA

expression intervenes in protein activity regulation (total translation into protein?) in *D. labrax* gills, but expression analysis of *CA* in this organ seems to be a good indicator of fish from the sea.

4.3. Osmoregulation genes expression in wild sea bass from the sea and the lagoon.

Amongst the different genes encoding proteins involved in osmoregulation, we particularly focused on the Na^+/K^+-ATPase. This enzyme, present in branchial, renal and intestinal epithelia, plays an important role in maintaining osmotic homeostasis in freshwater- as well as saltwater-acclimated fish. Our study indicates a higher mRNA expression in lagoon-caught sea bass both in the gill and the intestine. Scott et al. (2004) explored the expression patterns of several genes encoding ion transport proteins in euryhaline killifish *Fundulus heteroclitus* after transfer from near-isosmotic brackish water [10 parts/thousand (ppt)] to either fresh water or saltwater. They demonstrated that many changes in response to saltwater transfer were transient. Increased mRNA expression occurred 1 day after transfer for the Na^+/K^+-ATPase-α1a gene (3-fold). The responses to freshwater transfer were quite different from those to saltwater transfer. In particular, freshwater transfer increased Na^+/K^+-ATPase-α1a mRNA expression and Na^+/K^+-ATPase activity to a greater extent than did transfer to saltwater (Scott et al., 2004). However few studies have attempted to determine wheter Na^+/K^+-ATPase α-isoforms play a role in facilitating freshwater or seawater acclimation (Richards et al., 2003) and, in addition, there is discordance among studies in the degree and timing of changes in Na^+/K^+-ATPase activity, α-subunit mRNA expression and protein abundance.

We also analysed expression pattern of claudin-3, a protein family involved in cell-cell adhesion through tight junctions (TJ) (Tsukita et al., 2001). TJ form a continuous intercellular seal that restricts and regulates the paracellular transport of water, small solutes, and immune cells (Heiskala et al., 2001). We observed a higher level of mRNA encoding this protein in individuals maintained in seawater in the laboratory (SSH, compared to freshwater acclimated fish) as well as in open sea-caught individuals. It has been suggested that tissue- and cell-type-specific expression of claudins determine the composition of the resulting intercellular complex and, in turn, the characteristic permeability properties observed for different epithelia and endothelia (Heiskala et al., 2001). While the intercellular seal provided by TJ acts as a barrier for the diffusion of large molecules, it allows the selective transport of water and, depending on the specific claudins, small solutes. Claudins 2 and 4, for example, have been linked to intercellular Na^+ and K^+ permeability (Amasheh et al., 2002), whereas claudin 16 is critical for renal re-absorption of Ca^{2+} and Mg^{2+} (Muller et al., 2003), indicating that claudins can function as highly selective paracellular ion channels.

Another enzyme studied in the present work is the angiotensin-converting enzyme (ACE). This enzyme, generally present in endothelia, plays a major role in the renin-angiotensin system (RAS) by converting angiotensin I (Ang I) to vasoactive angiotensin II (Ang II) (Olson, 1992). This system is involved in blood presure regulation in vertebrates. In euryhaline teleosts, circulating Ang II levels are higher in saltwater- than in freshwater-acclimated fish (Rankin et al, 2001; and references therein), indicating a possible role of RAS (and consequently of ACE) in osmoregulation in teleosts. Surprisingly, our analysis in wild sea bass indicated that there is no significant difference of levels of ACE mRNA expression between open sea and lagoon individuals in the gills.

Differential expression is not enough to conclude on the actual role of these proteins in the acclimation of *D. labrax* to various environmental conditions. However, our analysis provides a first basis for the development of osmoregulation markers amenable to quantitation in wild individuals.

4.4. Genes involved in cell-cycle regulation

We characterised several cDNA encoding proteins involved in cell-cycle regulation in sea bass as a response to salinity condition acclimatisation. We first examined the sequence encoding secretagogin and its expression in wild individuals. Secretagogin is a recently described member of the EF-hand Ca^{2+}-binding protein involved in several Ca-dependant processes, cell-cycle regulation, development and apoptosis (Gartner et al., 2001 and references therein). The partial 388bp-secretagogin cDNA sequence obtained in our SSH library (up-regulated gene in gills of saltwater acclimated fish) contained two of the six characteristic Ca^{2+}-binding sites described in human secretagogin gene (Wagner et al., 2000). Its

mRNA expression is largely regulated by environmental factors in wild sea bass. In *D. labrax* from the sea, secretagogin mRNA is more abundant in the intestine than in the gills, while an opposite pattern is observed in individuals from Ingril lagoon. Recent studies on human indicated that secretagogin is abundantly expressed (mRNA) and secreted in central nervous system and neuroendocrine cells and to a minor extent in intestine, colon and stomach (Zhan et al., 2003). Its expression in pancreas is specific to the islets, and it is thought to be involved in KCl-stimulated calcium flux and the regulation of cell proliferation. The current study highlights the potential importance of this newly described protein, whose function has been little studied (Cras-Meuneur et al., 2004). Our results indicate that cell-cycle regulation seems to be potentially dependant on salinity conditions in sea bass acclimated to salt- and fresh water. Interestingly, it is the first report a regulation of secretagogin at a transcriptomic level in fish on one hand and in another organ than pancreas, the gills, on the other hand.

4.5. The case of nephrosin, a gene potentially involved in regulation of immune system

Nephrosin is a newly discovered member of the astacin family. It is a secreted proteinase and is present in carp head kidney, posterior kidney, and spleen, all of which are responsible for immune and haematopoietic functions in fish (Tsai et al., 2004). We characterised nephrosin as an upregulated gene in gill of saltwater acclimated sea bass. The partial *D. labrax* nephrosin sequence obtained in our SSH (GenBank accession N° CX660754) contains the specific zinc binding motif generally observed in the astacin familly members HE*XX*H*XX*GF*X*HE*XX*R*X*DR (Hung et al., 1997), indicating that this partial cDNA encodes nephrosin in sea bass. Our semi-quantitative RT-PCR expression analysis indicated a similar level of nephrosin mRNA in gill and intestine and a higher intestinal expression rate in individuals from the sea than those from Ingril lagoon. In zebrafish, it has been demonstrated that nephrosin is specifically expressed in granulocytes (Song et al., 2004). Our SSH results support the activation of the immune system by salinity variations in sea bass. Some hormones involved in osmoregulation, such as prolactin (PRL) and growth hormone (GH) are known to enhance immune functions in fish as in mammals (Harris and Bird 2000). The phagocytic activity of fish leucocytes is stimulated by administration of PRL or GH (Harris and Bird 2000). In salmonids, the immunomodulatory effect of GH seems to be related to its role in osmoregulation. We have observed an increase of PRL mRNA in gill of sea bass from Ingril lagoon (Boutet et al., unpublished data). The indirect effect of environmental salinity and osmoregulatory hormones on the immune systems could be used as a potential marker of acclimatisation or migration of sea bass to new environmental conditions generally observed in lagoon and during the fish life-cycle.

Conclusion

Osmoregulatory mechanisms and acclimation to salinity environmental conditions in euryhaline fishes are known to be under control of hormonal factors such as prolactin and cortisol. In euryhaline teleosts, these hormones increase in plasma levels usually within a few days after freshwater or seawater entry and seem to drive the osmoregulatory system into the appropriate direction. Previously described differentially expressed proteins (Sakamoto et al., 2001; and references therein) may play a role for the subsequent survival of the fish, although changes in mRNA levels do not necessarily reflect different amounts of the encoded proteins. The salinity-regulated genes characterised in the present study, which mRNA levels in gill and intestine change according to environmental conditions, may serve as convenient entry points to study the molecular basis of environmental adaptation. The physiological role of many of theses genes in environmental adaptation remains to be explored. A further step would be to address the question of how the environmental factors trigger regulation of expression of these genes. Identification of regulation polymorphisms should be performed as a next step to understand the basis of inter-individual variation to salinity response observed in this species.

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Table 1. Combinations of primers used in semi-quantitative RT-PCR expression analysis and optimal number of PCR cycles (analysis in exponential phase).

Genes	Optimal number of PCR cycles	Primer sequences
Carbonic anhydrase	40	sense GATGGAAAGCGCTATCCCATGGAGTTACA antisense CCTGTAAACTTAGTGCAAGTGCATTCCTGCC
Angiotensin-converting enzyme	30	sense GGGCCTCTACATACCTGCGACATCTA antisense CCAAGGCTTACTGAAGCCCAGCTTCAT
Na^+/K^+-ATPase	35	sense GACATCATGAAGAGACAGCCCAGAAA antisense GCCAGAGCTGTCTCCTCAAACAGTCC
Claudin-3	25	sense GTGGTGATCGATGCCCAGCGCAGGGA antisense TGGGTTCGGGGAGCATGGCGGCACAG
Secretagogin	30	sense GACTTGGCCAGGATCTTGGCTTTAGA antisense GGTTTAGGTTTGACTCCAAGACACAG
Nephrosin	40	sense CCCTGCACTGCCAGAGGATGCAAGTGG antisense ACACGAGCAATGTCATTGACACTCAT
28S ribosomal DNA	25	sense CTCAGTAGCGGCGAGCGAAGAGGGAAG antisense AGGTACTTGTCGACTATCGGTCTCGTG

Table 2. Identified SSH up-regulated clones in intestine of freshwater-acclimated fishes.

Homolog (protein); blastx value	**GenBank accession no**
Osmoregulation (2.6%)	
Aquaporin; 3e-20	CX660607
Thiazide-sensitive Na-Cl cotransporter; 3e-30	CX660616
Angiotensin-converting enzyme; 2e-45	CX660620
Amiloride binding protein 1; 4e-46	CX660623
Cell cycle regulation (1.9%)	
Tpt-1 protein; 2e-16	CX660591
Protein tyrosine phosphatase; 3e-05	CX660603
gastrula specific embryonic protein 1; 6e-11	CX660609
Cytoskeleton (0.6%)	
Myosin light chain alkali ; 8e-74	CX660592
Energetic metabolism and respiratory chain (4.5%)	
Cytochrome c oxidase subunit Va; 2e-29	CX660594
Cytochrome c oxidase subunit III; 0.001	CX660445
Cytochrome c oxidase subunit I; 5e-24	CX660601
Cytochrome b; 6e-78	CAA57262
Fructose-biphosphate aldolase B; 2e-67	CX660600
Succinate dehydrogenase C; 7e-26	CX660602
Glyceraldehyde-3-phosphate dehydrogenase; 2e-05	CX660611
Protein regulation (1.3%)	
Spermidine/spermine N1-acetyltransferase ; 7e-16	CX660595
Alpha-aspartyl dipeptidase ; 3e-27	CX660615
Lipid metabolism (3.2%)	
Very-long-chain acyl-coA synthetase; 3e-70	CX660598
Intestinal fatty acid binding protein; 6e-18	CX660610
Soluble epoxide hydrolase; 1e-08	CX660617
C1q-like adipose specific protein; 7e-11	CX660618
Cellular retinol-binding protein 1; 6e-22	CX660621
Cell communication, immune system, cell protection (3.2%)	
Antifreeze protein LS-12; 3e-26	CX660592
X-transporter protein 2; 1e-66	CX660597
Activated protein kinase C; 2e-60	CX660599
Hexosaminidase B; 2e-37	CX660606
Elastase 1; 9e-67	CX660608
Detoxification (2.6%)	
Glutathione S-transferase theta 3; 8e-46	CX660604
Peroxiredoxin 5; 3e-11	CX660613
Aflatoxin B1 aldehyde reductase; 8e-17	CX660614
Ferritin H; 5e-72	CX660619
Nucleic acid regulation (0.6%)	
Elongation factor-2; 1e-35	CX660612
Ribosomal proteins (5.8%)	CX660734-CX660742
Unidentified sequences[a] (73.4%)	CX660624-CX660733

[a] Sequences presented no significant alignment (<0.001) or significant alignment with an unknown protein.

Table 3. Identified SSH up-regulated clones in intestine of seawater-acclimated fishes.

Homolog (protein); blastx value	**GenBank accession no**
Cytoskeleton (0.7%)	
Myosin light chain 4; 2e-51	CX660886
Energetic metabolism and respiratory chain (2.2%)	
Cytochrome c oxidase subunit Vb; 8e-49	CX660885
Glucosamine-6-phosphate deaminase; e-106	CX660894
ATP synthase FO subunit 6; 4e-31	CX660904
Protein regulation (3.6%)	
Solute carrier family 15; 4e-13	CX660890
Proteasome subunit beta 3; 2e-62	CX660895
Dipeptidylpeptidase 4b; 2e-85	CX660897
Aminopeptidase N; 1^{e}-37	CX660898
Cystatin; 3e-19	CX660899
Lipid metabolism (0.7%)	
Apolipoprotein B; 5e-07	CX660896
Cell communication, immune system, cell protection (5.1%)	
Notch 2; 5e-08	CX660886
Immune-associated nucleotide-binding protein 9; 1e-05	CX660888
Uromodulin; 5e-08	CX660891
Major histocompatibility complex class I; 2e-13	CX660892
Antifreeze polypeptide; 3e-09	CX660900
serum lectin isoform 2; 1e-08	CX660902
Small inducible cytokine; 2e-23	CX660904
Detoxification (0.7%)	
Cu/Zn superoxide dismutase; 2e-78	CX660893
Unidentified sequences[a] (86.9%)	CX660905-CX661022

[a] Sequences presented no significant alignment (<0.01) or significant alignment with an unknown protein.

Table 4: Identified SSH up-regulated clones in gills of freshwater-acclimated fishes.

Homolog (protein); blastx value	**GenBank accession no**
Osmoregulation (0.7%)	
Na+K+ ATPase alpha 4; 7e-81	CX660460
Cell cycle regulation (0.7%)	
Neural proliferation differenciation and control protein 1; 5e-11	CX660470
Cytoskeleton (4.6%)	
Cytokeratin; 2e-70	CX660438
Keratin 18; 5e-17	CX660441
Gelsolin; 6e-28	CX660443
Collagen type I alpha 2; 7e-13	CX660451
Type I keratin S8; 5e-09	CX660454
Type I keratin E7; 6e-04	CX660461
Type II basic cytokeratin; 3e-73	CX660466
Energetic metabolism and respiratory chain (2%)	
Isocitrate dehydrogenase; 6e-42	CX660439
ATP synthase c-subunit; 4e-42	CX660449
Ecto-ATPase; 4e-12	CX660457
Protein regulation (1.3%)	
Arginase; 5e-19	CX660463
Ornithine decarboxylase antizyme small isoform; 7e-10	CX660465
Cell communication, immune system, cell protection (7.9%)	
MHC class II alpha; 2e-62	CX660440
class I helical cytokine receptor number 26; 3e-07	CX660444
tetraspan 3; 1e-33	CX660447
Alpha 3-fucosyltransferase; 2e-51	CX660452
Receptor for activited kinase C; 2e-75	CX660453
Cathepsin; 9e-53	CX660458
JAK1 tyrosin kinase; 6e-05	CX660459
Ficolin; 3e-10	CX660462
Growth factor independence-1B; 3e-09	CX660464
Integrin beta 4; 1e-62	CX660467
Regulator of G-protein signalling 4; 2e-14	CX660468
Lymphocyt protein tyrosin kinase; 1e-19	CX660471
Detoxification (0.7%)	
Glutathione peroxidase; 1e-70	CX660442
Nucleic acid regulation (3.3%)	
Splicing factor 3B; 1e-37	CX660437
eukaryotic translation initiation factor 3; 1e-72	CX660446
Int-6 protein; 1e-32	CX660450
Nuclease diphosphate kinase B; 5e-69	CX660455
Signalosome component COPS2; 3e-38	CX660469
Ribosomal proteins (3.3%)	CX660586-CX660590
Unidentified sequences[a] (75.5%)	CX660472-CX660585

[a] Sequences presented no significant alignment (<0.01) or significant alignment with an unknown protein.

Table 5: Identified SSH up-regulated clones in gills of seawater-acclimated fishes.

Homolog (protein); blastx value	**GenBank accession no**
Osmoregulation (2.1 %)	
Claudin 3; 3e-05	CX660743
Carbonic anhydrase 4; 8e-17	CX660749
Endothelin receptor A; 2e-25	CX660777
Cell cycle regulation (4.2%)	
Pescadillo; 1e-53	CX660747
PTTG-binding factor; 2e-14	CX660750
Chromosome segregation protein SMC1; 2e-40	CX660756
Vesicle-associated membrane protein; 5e-41	CX660764
Caspase 3; 1e-59	CX660770
Secretagogin; 1e-43	CX660758
Cytoskeleton (2.1%)	
Beta actin; 5e-28	CX660456
Alpha-tubulin; 1e-34	CX660448
Type II keratin E3; 3e-93	CX660773
Energetic metabolism and respiratory chain (1.4%)	
Mitochondrial ATP synthase gamma subunit; 8e-63	CX660761
Cytochrome c oxidase subunit III; 1e-42	CX660445
Protein regulation (3.5%)	
Ubiquitin like protein; 8e-35	CX660748
Epidermis specific serine protease; 8e-31	CX660751
Protein disulfide isomerase related protein; 1e-29	CX660755
Rhomboid, veinlet-like 2; 4e-61	CX660757
Importin 7; 3e-90	CX660769
Cell communication, immune system, cell protection (10.5%)	
Chemokine ligand 4; 6e-09	CX660744
Mpx protein; 2e-51	CX660745
RW1 protein; 3e-18	CX660746
Interferon regulatory factor; 1e-60	CX660752
GDP dissociation inhibitor 2; 8e-31	CX660759
RHCG-2; 3e-74	CX660762
Glutamic acid-rich protein; 3e-07	CX660763
IFN-inducible and antiviral protein; 1e-55	CX660765
ISG12 protein; 7e-05	CX660766
Ovary-specific C1q-like factor; 6e-15	CX660767
Cadherin 1; 9e-29	CX660768
Protective protein for beta-galactosidase; 8e-38	CX660771
VHSV-induced protein-3; 3e-05	CX660775
Rab-7; 2e-24	CX660776
Nephrosin; 3e-31	CX660754
Detoxification (0.7%)	
Glutathione S-transferase; 2e-33	CX660774
Nucleic acid regulation (1.4%)	
RNA helicase-DEAD box protein; 9e-22	CX660753
Nuclear matrix protein NXP2; 9e-51	CX660772
Ribosomal proteins (2.1%)	CX660882-CX660884
Unidentified sequences[a] (72.2%)	CX660778-CX660881

[a] Sequences presented no significant alignment (<0.01) or significant alignment with an unknown protein.

Table 6. Summary of the results obtained in expression analysis of carbonic anhydrase in wild and experimental individuals.

	Gill	Intestine
SW / FW	*	*
SW / Sea	NS	*
SW / Lag	*	*
FW / Sea	*	NS
FW / Lag	*	NS
Sea / Lag	*	*

(NS) non significant. (*) significant ($P<0.05$). (SW) saltwater acclimated fish. (FW) freshwater acclimated fish. (Sea) fish from open Mediterranean sea. (Lag) fish from Ingril Lagoon.

Fig. 1. Nucleotide sequence of carbonic anhydrase gene of *D. labrax*. The coding region is in upper case and the non-coding regions are in lower case. The corresponding amino acid sequence is bold characters. Identified TATA box is boxed, ATTTA(G) motifs (involved in mRNA stability) are underlined and putative N-glycosylation motifs Asn-Xaa-Thr/Ser are double-underlined.

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actactagcagcaactccagtaaaatattatagtttctggaaattaagctgataacacctactacaactacc    72
atactactgtgtaaattgtatttcattcagtcatgtttgtggttctagactcagaacctgttaacatgtttt   144
ttgctactcaaagaagaacagtgaagtgggacccttgattgatccctgaatgttgtaaatagaaataatact   216
tcctggaactatacacccacatgaatagtcaggaacgcaaccaggaattaaagttctactttaatactaacc   288
tcaaaaacccaagttctgttgcttcctgcagctcgacggagctttatagtgaacttcacctcattgtttagc   360
tgttagaccacaactttaatgttttggtccaggctcacaaccctcttagtgtcatttatggctgttttcagt   432
gcaaacgctatacaagccgaacgttcagctcagcaccaaattgctgacagacacagttaaccactagagaga   504
gagatttgaggatatgaactagtttctgggttcctggaaaaatccttgtggatgaaacaaagatccaaacat   576
cacctgggtttggaaatgccatttactcctgtttTATAAAcagtagcctacgttgttttgatgcttaataaa   648
taaatacgtgtgtttttaacattttaaagatttaaaattcatacaaaatttgagtttatcttatttttaa    720
actggctgcaatttaattttactgtgtaatttatttgcataaacctacacttgcaatactgctgtcagattt   792
atgcttcctgttattactcattcatccatacctatgcatgtttaaacaaataaataaacaaaaaaacaaata   864
cctcctctgcttattagccccagaacatttttcaggaagctaatggccaagtcagatgtcacatagaggcag   936
tgttagacagctgggggttgcctgagcgaataataaggggaggaggatgaggccctttagccattagcggac  1008
                              M   N   W   L   V   A   A   L   A   V   C      11
actgaggaggtacaggtgcgtcaaag ATG AAC TGG CTT GTA GCT GCG CTT GCT GTG TGC    1067
 V   L   V   P   S   A   N   C   A   S   D   S   V   A   W   C   Y   H      29
GTC CTA GTG CCC AGC GCA AAC TGT GCT TCA GAC TCA GTC GCC TGG TGT TAT CAT   1121
 Q   P   S   C   N   D   T   T   W   P   T   I   A   A   K   Y   C   N      47
CAG CCA AGC TGC AAT GAC ACT ACC TGG CCA ACC ATC GCT GCT AAG TAT TGC AAT   1175
 G   T   R   Q   S   P   I   N   I   V   S   A   S   A   E   P   N   A      65
GGC ACC CGA CAG TCT CCC ATT AAC ATC GTC TCA GCA TCT GCG GAA CCT AAC GCC   1229
 N   L   T   E   F   T   F   Q   N   Y   G   D   T   S   I   L   K   K      83
AAC CTG ACT GAA TTC ACC TTT CAG AAC TAC GGC GAC ACC TCC ATC TTG AAA AAG   1283
 I   L   N   T   G   K   T   V   Q   V   S   L   G   S   G   V   S   I     101
ATC CTA AAC ACT GGC AAG ACA GTN CAA GTC AGC TTG GGC AGT GGC GTT AGC ATT   1337
 S   G   G   D   L   S   E   A   Y   D   S   L   Q   F   H   L   H   W     119
TCA GGG GGA GAT CTG TCT GAG GCA TAT GAC AGC CTG CAG TTC CAC TTG CAC TGG   1391
 G   K   G   S   S   I   P   G   S   D   G   K   R   Y   P   M   E         136
GGT AAA GGC TCC TCC ATC CCC GGC TCC GAT GGA AAG CGC TAT CCC ATG GAG  gta   1445
cagtctttgttttcatgttgagctatatgttagagatttgttaacacggattatattatatacaagtattta  1517
                          L   H   I   V   N   S   K   S   T   F   N   G     148
attagttctcttttcttattcccagTTA CAC ATT GTA AAC AGC AAG TCA ACC TTT AAT GGG  1578
 N   T   T   L   A   V   K   D   S   T   G   L   A   A   L   G   F   F     166
AAC ACA ACT CTA GCT GTT AAA GAC TCT ACA GGA CTT GCT GCT CTT GGT TTC TTT   1632
 I   E                                                                     168
ATT GAG  gtgagaggggtttttgtaatgccaatatactgattgaagttgctctaagtatcagacttattt  1701
caaagtattcttcaaaagtcaaatgctacttttttttcaacactgcattatttatgatctctttaagttggt  1773
gctccactctttatttattcatgccaaagccccatgaagtatttttccccctccactgatacggaaacaaac  1845
                              E   T   S   G   N   E   T   Q   Q   P   A   S     180
actgaagtgtgtgcacatttcagGAA ACG TCA GGC AAT GAA ACT CAG CAA CCT GCA AGC   1904
 W   N   T   L   T   S   Y   L   A   N   I   T   N   S                     194
TGG AAC ACT TTG ACC TCC TAC CTG GCC AAC ATC ACA AAC AGT G gtaaggagatgctt  1961
ttgttttttttgttgaaaccgtgatctaatgttgcgaaaggaagtgccctcacagttcacatttctacccctt  2033
  G   D   S   V   S   I   A   P   G   I   S   L   D   D   L   L   V   G     212
agGT GAC TCT GTT TCA ATT GCA CCT GGA ATT TCA TTG GAT GAC CTC CTG GTC GGG  2088
 V   D   R   T   K   Y   Y   R   Y   L   G   S   L   T   T   P   Q   L     230
GTG GAT CGT ACC AAA TAT TAC CGC TAT CTT GGT TCC TTG ACC ACC CCC CAA TTG   2142
 Q   E   A   V   V   W   T   V   F   K   D   S   I   K   V   S   K   D     248
CAA GAG GCT GTG GTT TGG ACT GTG TTC AAG GAT TCG ATC AAA GTC AGC AAA GAT   2196
 L                                                                         249
TTG  gtgagatttaatttttcctactgtgtaataagtaaaatgctctcaacagtgcttcaccaaactaact  2266
                          I   D   L   F   S   T   T   V   H   V   S   N   T     262
ggctcaatatcctgcctccagATT GAC CTC TTC AGC ACA ACA GTA CAC GTC TCC AAC ACC  2326
 S   S   P   L   M   T   N   V   F   R   N   V   Q   P   A   Q   P   V     280
TCG TCA CCT TTG ATG ACC AAT GTC TTC AGA AAC GTC CAG CCA GCA CAA CCA GTC   2380
 T   T   Q   A   A   S   S   S   A   T   S   K   T   C   Y   S   L   G     298
ACA ACA CAG GCT GCC AGC AGT AGC GCT ACC TCC AAA ACC TGT TAC TCT CTG GGG   2434
 L   M   A   L   S   L   A   L   G   R   S   *                           310
TTG ATG GCA CTG AGC CTG GCT CTG GGG AGG AGT TAG  ggccaatgaagaatgggggggga  2493
gaattattgtggtggtttggcaacttatggtgctgctggtaaacctgttagtatacacctatctcacttatt  2565
atcctctttcaatgtgatttgtctgacttaaacctccaaattgttagggcgcagttttgcttggatttgact  2637
aacgccgcaagaacttttgttgtgttccagtctagctctctatatatttgtcataatgaattgttcacaaac  2709
attaaatgtgataatggctatagttgtccatattgcttccaggagtatgaaaagctataaagaatttatcaa  2781
tctgattttaatctgttgctcactatatttatgttaaacattgtgcacaagaatgaacatgttttgcagaat  2853
gactggttcaggcacagctaatgactgcgcttgagctgtcaaagctacatttaggagcctgaggcaggaatg  2925
cacttgcactaagtttacagga                                                    2947
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Fig. 2. Semi-quantitative RT-PCR determination of expression levels of carbonic anhydrase in gills (black) and intestine (white) of sea bass (n=5 for each condition). Results are presented as the ratio OD CA / OD 28S. The four conditions tested are sea bass experimentaly acclimated to seawater (SW) or fresh water (FW) and wild sea bass (Mediterranean Sea [Sea] and Ingril lagoon [Lag]).

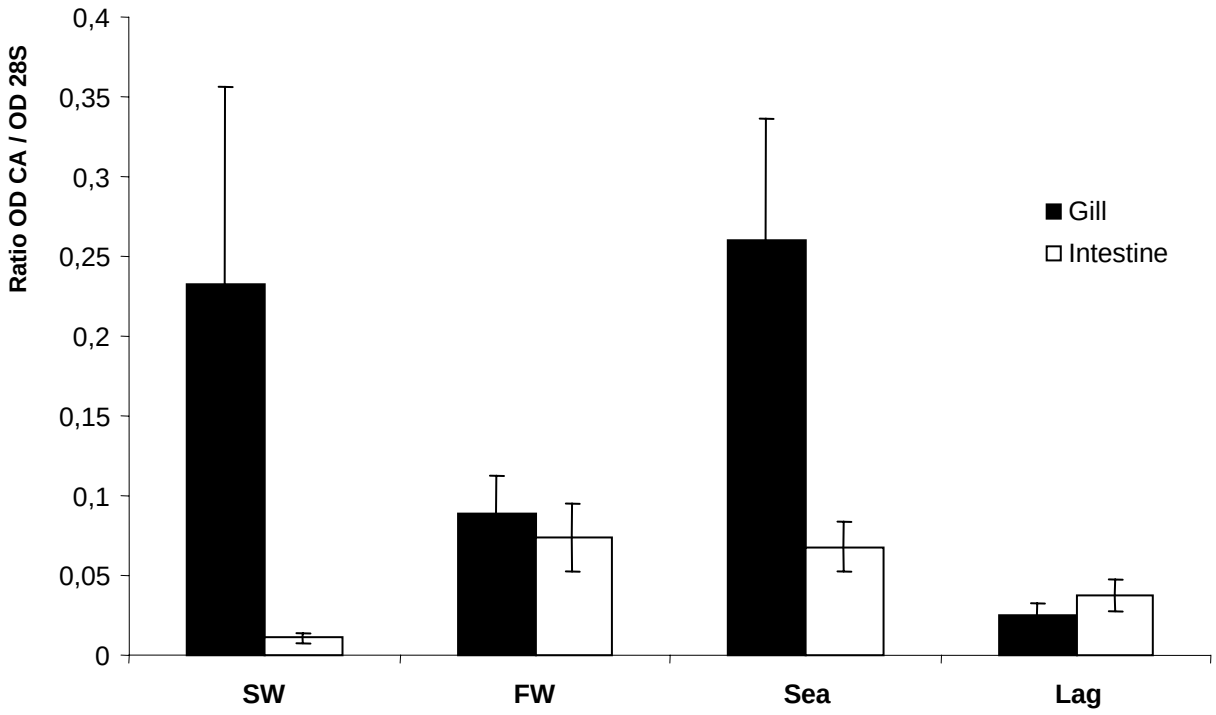

Fig. 3. Semi-quantitative RT-PCR determination of expression levels of Na^+/K^+-ATPase, angiotensin-converting enzyme, claudin-3, nephrosin and secretagogin in gill (A) and intestine (B) of sea bass (n=5 for each condition). Results are presented as the ratio OD gene / OD 28S. The animals were collected in Mediterranean Sea [Sea] (grey) and Ingril Lagoon [Lag] (white). Significant values at 5% are shown by an asterisk (*).