Molecular characterization and transcriptional regulation of the Na⁺/K⁺ ATPase α subunit isoforms during development and salinity challenge in a teleost fish, the Senegalese sole (*Solea senegalensis*)

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Abstract:

In the present work, five genes encoding different Na⁺/K⁺ ATPase (NKA) α -isoforms in the teleost *Solea senegalensis* are described for the first time. Sequence analysis of predicted polypeptides revealed a high degree of conservation across teleosts and mammals. Phylogenetic analysis clustered the five genes into three main clades: α 1 (designated *atp1a1a* and *atp1a1b*), α 2 (designated *atp1a2*) and α 3 (designated *atp1a3a* and *atp1a3b*) isoforms. Transcriptional analysis in larvae showed distinct expression profiles during development. In juvenile tissues, the *atp1a1a* gene was highly expressed in osmoregulatory organs, *atp1a2* in skeletal muscle, *atp1a1b* in brain and heart and *atp1a3a* and *atp1a3b* mainly in brain. Quantification of mRNA abundance after a salinity challenge showed that *atp1a1a* transcript levels increased significantly in the gill of soles transferred to high salinity water (60 ppt). In contrast, *atp1a3a* transcripts increased at low salinity (5 ppt). In situ hybridization (ISH) analysis revealed that the number of ionocytes expressing *atp1a1a* transcripts in the primary gill filaments was higher at 35 and 60 ppt than at 5 ppt and remained undetectable or at very low levels in the lamellae at 5 and 35 ppt but increased at 60 ppt. Immunohistochemistry showed a higher number of positive cells in the lamellae. Whole-mount analysis of *atp1a1a* mRNA in young sole larvae revealed that it was localized in gut, pronephric tubule, gill, otic vesicle, yolk sac ionocytes and chordacentrum. Moreover, *atp1a1a* mRNAs increased at mouth opening (3 DPH) in larvae incubated at 36 ppt with a greater signal in gills.

Abbreviations

- aa, amino acid;
- cDNA, CDS, coding sequence, DNA complementary to RNA;
- DPH, days post hatch;
- DEPC, diethyl pyrocarbonate;
- ECD, extracellular domain;
- IC, intracellular domain;
- ISH, *in situ* hybridization;
- IHC, immunohistochemistry;
- kb, kilobase(s);
- kDa, kiloDaltons;
- ML, maximum likelihood;
- NKA, Na⁺,K⁺ ATPase;
- nt, nucleotide(s);
- PBS, phosphate buffered saline;
- PK, protein kinase;
- ppt, parts per thousand;
- SEM, standard error of the mean, SSC, saline-sodium citrate;
- TMD, transmembrane domain, WISH, Whole mount ISH

Keywords : Senegalese sole ; Na⁺,K⁺ ATPase ; *atp1a1a* ; *atp1a1b* ; *atp1a2* ; *atp1a3a* ; *atp1a3b* ; osmoregulation

1. Introduction

Sodium-Potassium ATPase (NKA) is an ubiquitously expressed integral membrane protein that couples the exchange of two extracellular K⁺ ions for three intracellular Na⁺ ions, a process that is linked to the hydrolysis of one molecule of ATP (Mobasher et al., 2000). The Na⁺,K⁺ pump contains equimolar amounts of α and β subunits and belongs to the P-type class of ATPases (Lingrel and Kuntzweiler, 1994 and Blanco and Mercer, 1998). The α subunit contains the catalytic site, which is phosphorylated in each pump cycle and its structure is stabilized by the β subunit, which also routes the $\alpha\beta$ complex to the plasma membrane (Schoner and Scheiner-Bobis, 2007). A γ subunit has also been identified as a third putative subunit that in mammals is predominantly present in the kidney and is essential for blastocyst development (Jones et al., 1997). In eukaryote cells, Na⁺/K⁺ transport plays a key role in the maintenance of ionic homeostasis and it also provides a transmembrane Na⁺ gradient for other cellular activities such as Na⁺-dependent nutrients transport or signal transduction (Lai et al., 2013). Moreover, NKA can also display other non-ion transport functions (Krupinski and Beitel, 2009 and Chen et al., 2011).

Four α isoforms ($\alpha 1$ to $\alpha 4$) have been identified in mammals that are encoded by a multigene family and despite sharing 90% sequence similarity they are under different transcriptional regulation and exhibit different tissue expression patterns (Lingrel and Kuntzweiler, 1994).

Blanco and Mercer, 1998). The $\alpha 1$ isoform is ubiquitous and occurs in most cell types; $\alpha 2$ is the principal isoform of the skeletal muscle, but is also detected in brain and heart; the $\alpha 3$ isoform is mainly expressed in neural tissue; and the expression of $\alpha 4$ is restricted to the testes germ cells (Lingrel and Kuntzweiler, 1994; Blanco and Mercer, 1998). The functional divergence of the different NKA α isoforms still remains to be fully characterized. In fish, only $\alpha 1$ to $\alpha 3$ subunit-encoding genes have been identified although according to species and subunit type there may be several paralogous genes. In zebrafish, nine distinct NKA α -subunit genes (six $\alpha 1$, one $\alpha 2$, and two $\alpha 3$) have been reported (Rajarao et al., 2001; Serluca et al., 2001; Blasiolo et al., 2002; Canfield et al., 2002; Liao et al., 2009). In Atlantic salmon and rainbow trout, seven NKA α -subunit genes were identified (four $\alpha 1$, one $\alpha 2$, and two $\alpha 3$) (Richards et al., 2003; Gharbi et al., 2005). These NKA α subunits showed specific expression patterns in adult tissues (Rajarao et al., 2001; Richards et al., 2003), during embryogenesis (Canfield et al., 2002) and in response to salinity (Richards et al., 2003; McCormick et al., 2009) suggesting that a subfunctionalization process occurred during their evolution. The identification of the complete set of NKA α subunits and the evaluation of their expression patterns in embryos and juveniles is essential to understand the evolution of this complex gene family and their functional specialization.

Teleosts are exposed to a constant osmotic challenge from the surrounding water and osmoregulation maintains internal ion homeostasis when teleosts are in freshwater or seawater (Sakamoto et al., 2001; Marshall, 2002). Osmoregulatory plasticity varies between species with euryhaline teleosts being able to maintain almost constant blood osmolality even in the face of wide variations in external salinity. Most of these euryhaline teleosts exhibit higher NKA activity when they reside in seawater and are able to modulate NKA abundance when challenged by a salinity shift (Jensen et al., 1998; Marshall and Bryson, 1998; Lin et al., 2004; Laiz-Carrión et al., 2005). In teleost gills, NKA is located mainly in a specialized cell type known as mitochondrion-rich cells or ionocytes, the site of active ion transport. In the ionocytes, α subunits are differentially expressed and respond differently to osmotic challenges and acclimation to seawater and freshwater (Richards et al., 2003; Liao et al., 2009; Madsen et al., 2009). The Senegalese sole (*Solea senegalensis* Kaup 1858) is an euryhaline species and inhabits environments with wide seasonal fluctuations in salinity. In coastal marshes of the southern Iberian Peninsula salinity varies from 12.5 to 61 ppt in January and August respectively with significant salinity fluctuations throughout the year (Imsland et al., 2003; Arias and Drake, 2005; Yufera and Arias, 2010). Previous studies carried out in sole juveniles confirmed a high tolerance to a wide range of salinities (5-55 ppt). Soles are able to acclimate very quickly (~3 h) or over longer periods (17 days) by adjusting cortisol levels and energy allocation, with a linear relationship existing between environmental salinity and gill NKA activity (Arjona et al., 2007;

Herrera et al., 2012). Curiously, Senegalese sole larvae incubated at low salinities (10 ppt) develop severe malformations including undeveloped jaws, abnormal lower jaw development, and absence of an upper jaw or gaping jaws (Salas-Leiton et al., 2012) suggesting their aetiology may somehow be linked to the larvae's osmoregulatory capacity. To evaluate the role of NKA α subunits on acclimation of sole to salinity, the aims of this work were: 1) identification and characterization of NKA α subunits in sole, 2) establishment of the relative abundance of NKA α subunits in juvenile tissues and during larval development, 3) transcriptional analysis of NKA α subunits responsiveness to salinity challenge in juveniles, and 4) mapping the change in the *atpla1a* transcript amounts in developing larvae and in the gills of juveniles under a salinity challenge. The knowledge generated is the starting point to understand how this multigene family contributes to the variable osmoregulatory capacity of teleost fish. Moreover, since Senegalese sole is an economically important species both in fisheries and aquaculture, understanding the molecular mechanisms governing salinity adaptation will contribute to improvement of hatchery management techniques and growing procedures.

2. Materials and methods

2.1 Source of fish and experimental rearing conditions

Juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) samples to study tissue distribution of NKA α subunits were those reported previously (Manchado et al., 2008b; Manchado et al., 2009). Briefly, individuals (average mass = 23.2 ± 3.6 g; $n=3$) were obtained from the facilities of IFAPA Centro *El Toruño* (El Puerto Santa María, Cádiz, Spain). They were sacrificed by immersion in 2-phenoxyetanol (300 ppm for 10 min). Liver, spleen, brain, gills, intestine, head kidney, heart, skeletal muscle, and skin were rapidly dissected out, frozen in liquid nitrogen, and stored at -80°C .

To study the effects of osmotic conditions on mRNA abundance of the NKA α isoforms and their localization in the gill, we selected 150 juveniles (mean mass = 29.9 ± 0.8 g) that were acclimated in a tank at a temperature ranging from 17.6 - 18.3°C and a salinity that oscillated between 35.0 - 35.4 ppt. Ten days before experiment started, the temperature was reduced to 16.5°C (ranging 16 - 17°C) to fit better the environmental temperature conditions (January-February 2008) and to ensure the water temperature was maintained at 16 - 17°C during the experiment. During this acclimation period, animals were fed dry pellets (Skretting LE-2 Elite) provided by automatic feeders (approx. 1% biomass daily). Soles were maintained in a flow-through circuit under automatic control of temperature with 300% water renovation daily. Water supply was from the so-called "Rio San Pedro". Before starting experiment, animals were distributed in six

100 L tanks (25 sole per tank) at a salinity of 35 ppt (35.0-35.3 ppt) under a natural photoperiod. After an acclimation period (24 h), water was completely replaced in less than half an hour establishing three different salinities in duplicate tanks: low-salinity water (5 ppt), control (35 ppt) and high-salinity water (60 ppt). Expected experimental salinities (low-salinity, control and high-salinity) were achieved by mixing seawater with dechlorinated tap water or marine salt depending on the condition. No food was provided during the experiment. Two individuals from each tank were initially sampled before salinity change (0 h); then three individuals per tank were sampled at 24, 48, 72, 96 and 168 h after salinity change. Animals were euthanized by phenoxyethanol overdose (300 ppm for 10 min). Gills, kidney and intestine were rapidly dissected out, frozen in liquid nitrogen and stored at -80 °C. Moreover, gill tissue from the 96 h sampling was fixed overnight in 4% paraformaldehyde at 4 °C for ISH.

For larval studies, fertilized eggs from naturally spawning Senegalese sole broodstock (IFAPA Centro *El Toruño*) were collected in spring (April 2006) and separated by buoyancy. Water temperature for the broodstock tank (25 animals ratio 2 M:1F) during spawning ranged between 17.5-20.2 °C and salinity between 33.7-36.9 ppt. Breeders were fed polychaeta, mussels and squid. Eggs were incubated at a density of 2,000 eggs L⁻¹ in 300 L cylindro-conical tanks in an open circuit containing gently aerated water at 20 °C and a salinity of 36 ppt until 3 days post hatching (DPH). Larvae were then transferred to two 300 L tanks at an initial density of 45 to 50 larvae L⁻¹ with a 16L:8D photoperiod and light intensity of 300 lux. Larvae were fed rotifers (*Brachionus plicatilis*), previously enriched with *Isochrysis galbana* (T-ISO strain) cultivated at exponential phase, from 3 DPH until 9 DPH. T-ISO cells were also added (2 mg dry mass L⁻¹ d⁻¹) directly to the larval culture tanks during the rotifer feeding stage. From 7 DPH up until the end of the experiment sole larvae were fed artemia metanauplii enriched with T-ISO. Larvae were sampled at 2, 3, 6, 7, 8, 9, 10, 11, 13, 15, 16, 19 and 22 DPH. In each sampling, three independent pools per tank were randomly collected. Each pool (from 10 to 100 larvae depending on the age) was collected using a 350-µm-mesh net, washed with DEPC water, placed in separate eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C. To evaluate effects of salinity in early stages, we collected eggs from a natural spawn and separated fertilized eggs by buoyancy. After that, we incubated embryos (in gastrula stage) at three salinities (10, 20 and 36 ppt) in 15 L cylinder conical tubes coupled to a recirculation system to keep constant temperature (20°C) and target salinities. Initial density was 1,500-2,000 embryos L⁻¹ and experiment was done in duplicate tanks. At 1 and 3 DPH, three pools of larvae were collected from each tank as described above, washed with DEPC water, frozen directly in liquid nitrogen and stored separately at -80 °C until analysis. In addition, some larvae were fixed overnight in 4% paraformaldehyde at 4 °C for whole mount analysis.

2.2 Cloning and sequencing of Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* genes.

Partial coding sequences of Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a*, and *atp1a3b* were obtained from the EST libraries built in the Pleurogene project (Cerdeira et al., 2008) and SoleaDB (www.aquagenet.eu; Benzekri et al., in preparation). To obtain full-length transcripts isoform-specific primers were designed using Oligo v6.89 software (Medprobe; Table 1) and PCR reactions were performed using 1 µl of cDNA prepared from an extract of gill total RNA as previously described (Manchado et al., 2007; Manchado et al., 2008a). PCR amplification conditions were: 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 68 °C and 90 s at 72 °C, and a final elongation step at 72 °C for 7 min.

For Senegalese sole *atp1a1a*, four primer pairs (*SseNKAa1a•1/SseNKAa1a•2*, *SseNKAa1a•3/SseNKAa1a•4*, and *SseNKAa1a•5/SseNKAa1a•6*, *SseNKAa1a•7*, *SseNKAa1a•9*) were used to amplify the full-length cDNA (Table 1). Specific primers were also used to amplify Senegalese sole *atp1a1b* (*CL436•1*, *CL436•2*, *SseNKA436•1*, *SseNKA436•2*, *SseNKA436•3*, *SseNKA436•5* and *SseNKA436•6*), *atp1a2* (*SseNKAa2•1*, *SseNKAa2•2*, *SseNKAa2•3* and *SseNKAa2•4*), *atp1a3a* (*SseNKAa3a•1/SseNKAa3a•2*, *SseNKAa3a•3/SseNKAa3a•2*, *SseNKAa3a•5/SseNKAa3a•6*, *SseNKAa3a•5/SseNKAa3a•8*) and *atp1a3b* (*SseNKAa3b•1/SseNKAa3b•2*, *SseNKAa3b•3/SseNKAa3b•4*, *SseNKAa3b•5/SseNKAa3b•6*) using the conditions indicated above. The full-length *atp1a1b*, *atp1a2*, and *atp1a3b* transcripts were obtained using a SMARTerTM RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. For *atp1a1b*, the specific primer *SseNKA436GSP1* was employed in RACE-PCR using the Advantage 2[®] polymerase mix (Clontech) for the first amplification and the primer *SseNKA436GSP2* for the nested PCR. For *atp1a2*, RACE-PCR the specific primer *NKAa2GSP1* was employed. For *atp1a3b*, the primer *SseNKAa3b•GSP1* was used to obtain the 3'UTR and the primers *SseNKAa3b•7*, *SseNKAa3b•8* and *SseNKAa3b•9* were used to complete and validate the target template. Full-length Senegalese sole *atp1a3a* was amplified using specific primers (*SseNKA3a3R1* and *SseNKA3a3R2*) and a 3' RACE kit (Invitrogen) and the primer *SseNKA3a3R3* was used for consecutive nested PCR. The SMARTerTM 5' RACE kit (Clontech) was used with a specific primer *NKAa3bGSP1* to obtain the 5'-UTR sequence.

Additional primers were used for sequencing of *atp1a3a* (Table 1)

Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a*, and *atp1a3b* double-stranded DNA products were purified using NucleoFast96 PCR plates (Macherey-Nagel) and cloned using the TOPO TA Cloning[®] kit for sequencing (Invitrogen). Recombinant colonies were screened by PCR with universal oligonucleotides primers M13 forward and M13 reverse in a final volume of 25 µL.

PCR amplification conditions were: 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 15 s at 52 °C and 1 min at 72 °C. Double-stranded DNA products were purified as previously described, and used for direct cycle sequencing using the BigDye[®] Terminator v3.1 kit (Applied Biosystems). All sequencing reactions were performed according to the manufacturer's instructions on a 3130 Genetic Analyzer (Applied Biosystems) using the universal primers T3 and T7 as well as the specific primers employed for PCR. In the case of *atp1a2* (*SseNKAa2•5* and *SseNKAa2•7*) and *atp1a3a* (*SseNKAa3aseq1*, *SseNKAa3aseq2*, *SseNKAa3aseq3* and *SseNKAa3aseq4*) primer walking was used to ensure full sequence coverage. As some sequences were from SoleaDB and generated and assembled from Illumina short reads, some further primers were used to validate *atp1a2* sequences including, *SseNKAa2•F1*, *SseNKAa2•F2*, *SseNKAa2•R1* and *SseNKAa2•R2*. Nucleotide sequences were analyzed using Seqman v5.53 (DNASTAR) and the identity of the sequenced cDNA confirmed by Blasting against the NCBI database.

The predicted NKA protein sequence was determined with EditSeq v8.1.3 software from Lasergene (DNASTAR). The Motif Scan tool at the Pfam site (<http://pfam.jouy.inra.fr/>) was used to identify domains in the predicted NKA protein sequences. Multiple sequence alignment of the predicted amino acid sequence of Senegalese sole NKAs with sequences from other chordates was performed in MegAlign (DNASTAR) and sequence identities were determined. NKA cDNA of diverse chordates were retrieved from GenBank/EMBL/DDBJ and Ensembl (<http://www.ensembl.org/index.html>) and used for multiple sequence alignments and phylogenetic analyses. Maximum likelihood (ML) phylogenetic analysis was performed and the best-fit model of sequence evolution was determined to be LG+I+G (-lnL = -27256.69) using the ProtTest v1.4 with a gamma distribution shape parameter (four rate categories) of 0.746. The PHYLIP package was then employed to estimate the bootstrap values using SEQBOOT (1000 replicates) and the data were analyzed using the software PHYML. The consensus phylogenetic tree was then obtained (CONSENSE). Trees were drawn using the TreeViewX program v0.5.0. The *Ciona intestinalis* atpase sequence (Ensembl accession no. **ENSCING00000023652**) was used as an outgroup to root the tree.

2.3 RNA isolation and gene expression analysis

Homogenization of juvenile tissues and larvae was carried out with the Lysing Matrix D (Q-BioGene) for 40 s at speed setting 6 in the Fastprep FG120 instrument (Bio101). Total RNA was isolated from 50 mg of tissues or pooled larvae using the RNeasy Mini Kit (Qiagen). For skeletal muscle, heart and skin the RNeasy Fibrous Tissue Mini Kit (Qiagen) was employed. All RNA isolation procedures were performed in accordance with the manufacturer's protocols. In all

cases, total RNA was treated twice with DNase I using the RNase-Free DNase kit (Qiagen) for 30 min to avoid amplification of genomic DNA. RNA sample quality was checked in agarose gels and quantification was performed with a NanoDrop 8000 spectrophotometer (Thermo Scientific). Total RNA (1 µg) from each sample was reverse-transcribed with the iScript™ cDNA Synthesis kit (Bio-Rad) following the manufacturer's protocol. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

Real-time analysis was carried out on a CFX96™ Real-Time System (Bio-Rad) using Senegalese sole specific primers for each NKA transcript (Table 1). Real-time reactions were accomplished in a 10-µl volume containing cDNA generated from 10 ng of original RNA template, 300 nM each of specific forward and reverse primers, and 5 µl of iQ™ SYBR Green Supermix (Bio-Rad). The amplification protocol used was as follows: initial 7 min denaturation and enzyme activation at 95°C, 40 cycles of 95°C for 15 s and 70°C for 30 s. Each PCR assay was performed in duplicate. For normalization of cDNA loading, all samples were run in parallel with the reference genes glyceraldehyde-3-phosphate dehydrogenase (*gapdh2*; larvae), 18S rRNA (juvenile tissues) or ubiquitin (*ub52*; salinity challenge in juveniles), which have been previously demonstrate to be suitable as reference genes (Manchado et al., 2007; Infante et al., 2008b). Reference genes for normalization were selected according to their expression stability within each experiment. To estimate efficiency, a standard curve was generated for each primer pair based on 10-fold serial dilutions corresponding to cDNA transcribed from 100 to 0.01 ng of total RNA. Calibration curves exhibited a correlation coefficient higher than 0.99, and the corresponding real-time PCR efficiencies (E) were 1.89, 2.04, 1.90, 2.0 and 2.0 for *CL196-1/CL196-2*, *CL436-1/CL436-2*, *SseNKAa2•1/SseNKAa2•2*, *SseNKAa3a•1/SseNKAa3a•2*, and *SseNKAa3b•1/SseNKAa3b•2*, respectively. Relative mRNA expression was determined using the $(1+E)^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001; Manchado et al., 2007). Samples corresponding to larvae at 2 DPH and liver were used as a calibrator in larval development and tissues analyses, respectively. In the salinity challenge experiment in juveniles, the calibrator was the 35 ppt group at 24 h after salinity change. In the embryos experiment, the calibrator was 1 DPH larva incubated at 10 ppt.

Results were expressed as mean $\pm$ SEM. Significant differences during larval development, in juvenile tissues and in larvae incubated at different salinities were determined with a Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Fisher's Least Significant Difference (LSD) test. Statistical analyses were performed with the Statgraphics Plus v5.1 software. For the salinity challenge in juveniles, changes in mRNA abundance over time and salinity were analyzed by two-way ANOVA followed by Tukey's post hoc analysis for pair-wise comparisons when applicable. A Kolmogorov-Smirnov normality test was performed to evaluate normality

distribution. Statistical analyses were performed with the SPSS v21 software. Significance was accepted at $p < 0.05$.

2.4 Whole mount (WISH) and section in situ hybridization (ISH)

To synthesize the ISH probe for *atp1a1a*, a fragment of 1102 bp was PCR amplified with specific primers (5'-GACCCCGGAGAACCTGGACGATT-3' (F) and 5'-CAACGATGAAAGACCGGGCTGCT-3' (R)). The PCR product was cloned in TOPO-TA vector and a sense and anti-sense probe were prepared using 20 U T3 and T7 polymerase in transcription buffer (Promega) with 1 μ l digoxigenin-RNA labeling mix (Roche Diagnostics, Mannheim, Germany) as described in Campinho et al. (2007).

For ISH experiments, gills of four individuals at 96 h for each salinity were fixed in 4% PFA overnight at 4 °C washed several times in 1XPBT (1XPBS+0.1% Tween20) and stored in 100% methanol at -20 °C. Tissues were embedded in paraffin as described previously (Campinho et al., 2007) and serial 5- μ m-thick transverse section were cut and mounted on slides coated with 3-aminopropyltriethoxysilane. For ISH sections were dewaxed in xylene and rehydrated through an ethanol series (100% to 0%). Slides were washed several times in 1XPBT and pre-hybridized 2 h at 56 °C in hybridization mix without the riboprobe (Campinho et al., 2007) and then hybridized overnight at 56 °C in hybridization mix containing 0.25ng/mL of antisense or sense DIG-labelled probe for Senegalese sole *atp1a1a*. Slides were washed using high stringency conditions, 56 °C for 30 min in 0.2XSSCT. Incubated with anti-DIG serum (1/5000; Roche) overnight at 4 °C and then washed 6x15 min at 22 °C in malic acid buffer/triton (0.1%), before incubation in the developing buffer for 15 min. Development was performed at room temperature in developing buffer containing NBT/BCIP (Roche) accordingly to the manufacturer's instructions. Slides were washed several times in 1xPBT and mounted in glycerol/gelatin (Sigma). Images were taken in a Leica DM2000 microscope fitted with a DFC480 camera.

For WISH analyses of embryos, samples were fixed in 4% (wt/vol) paraformaldehyde in 1X PBS overnight at 4 °C, dehydrated in methanol 100% and conserved at -20 °C until processing. Fixed embryos were depigmented and permeabilized (10 μ g/ml proteinase K for 16 min at 37 °C) before being soaked in the digoxigenin-labeled *atp1a1a* probe as previously described (Thisse and Thisse, 2008). Excess probe was then removed according to the same protocol through successive washes in 2XSSC and 0.2XSSC at 70 °C and a last wash in 1XPBT. Hybrids were detected using anti-DIG serum (1/5000; Roche) and developed using the chromogens NBT/BCIP (Roche). Embryos were photographed in 80% glycerol using a Olympus SZX9 microscope and DFK31AU03 camera (The Imaging Source).

2.5 Immunohistochemistry

Ionocytes were detected in gills by immunohistochemistry (IHC) in serial paraffin wax sections adjacent to those used for in situ hybridization. IHC was performed as previously described (Einarsdóttir et al., 2011). In brief, sections were dewaxed in xylene and rehydrated in an ethanol series (100% ethanol to water). Sections were blocked at room temperature for 2 h in phosphate buffered saline (PBS buffer pH 7.4) containing k-carrageenan and 0.5% Triton X-100 and then in blocking solution containing anti- Na^+/K^+ atpase α (1/1000, NK α 5, Developmental Studies Hybridoma Bank, University of Iowa, USA) for 3 h at room temperature. The secondary antisera was goat anti-mouse IgG (H+L) conjugated with HyLite-596 (1/400, AnaSpec) and nuclei were counterstained by immersion for 5 min in 4', 6-diamidino-2-phenylindole (DAPI 0.7 nmol/ml, Roche, Germany) in 1X PBS. Slides were washed in several changes of 1X PBS, and mounted in glycerol (Sigma) and sealed. Samples were observed with a Leica DM2000 epifluorescent microscope and photographed with a Leica DFC480 digital camera. Images were adjusted for brightness and contrast in Image J 1.48d (National Institutes of Health, USA).

To count the number of ionocytes in the primary filaments and secondary lamellae in juveniles exposed to three salinities, we examined a gill section of 4 specimens after performing ISH and 3 individuals for IHC. Photos (four per fish) were taken randomly in the mid region of the gill filament at 40 $\times$ magnification. In general, photos spanned 0.18-0.28 mm of filament length over which there would be 7–12 lamellae; a single filament and associated lamellae were analyzed per photo. Digital images were managed using Image J software. Numbers of ionocytes were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis for pair-wise comparisons. Analyses were performed using SPSS software.

3. Results

3.1 Molecular characterization and phylogeny of Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b*

Five complete sequences encoding NKA α subunits, referred to as *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b*, were cloned and sequenced in the Senegalese sole. A partial *atp1a1a* sequence deposited in GenBank/EMBL/DDBJ database (Acc n° **JX508624**) with a 99% identity with the clone isolated in the present study was identified that differed at only 3 nt positions without any change in amino acid composition. No sequences corresponding to Senegalese sole *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* were identified in public databases. The full-length cDNA sequences have been deposited in GenBank/EMBL/DDBJ under accession Nos. **AB759891**,

AB759892, **AB862096**, **AB759894**, and **AB759893** for Senegalese sole *atpl1a*, *atpl1b*, *atpl1a2*, *atpl1a3a* and *atpl1a3b*, respectively.

The main features of the cDNAs encoding NKA α subunits are summarized in Table 2. cDNA sizes oscillated between 3,281-3,697 for *atpl1a* and *atpl1b*, respectively. The initiator ATG was identified in all the NKA α subunits and was in an optimal context for initiating translation with a purine at position -3. The translated amino acid (aa) sequences were between 1,010 and 1,025 aa for *atpl1a3b* and *atpl1a1b*, respectively.

Transmembrane domains (TMD) were identified by sequence comparison with NKA α subunit sequences from mammals (Blanco and Mercer, 1998). All subunits contained 10 hydrophobic segments that corresponded to membrane spanning domains TMD1 to TMD10 with both N- and C-termini located on the cytoplasmic side of the membrane (Fig. 1). They were linked by alternating hydrophilic extracellular and intracellular domains (ECD1 to ECD5 and ICD1 to ICD4, respectively) that were 4 (ECD 5) to 431 (ICD 2) aa in length.

A search for consensus sequences identified two potential N-glycosylation sites located in the ICD1. One of them was common to all NKA α isoforms (N²¹⁴ in NKA α 1a, N²¹⁶ in NKA α □b, N²⁰³ in NKA α □, N²¹⁵ in NKA α □□ and N²⁰² in NKA α □b), whereas the second one was only detected in NKA α □a and NKA α □ (N²⁴⁰ and N²²⁹ respectively). In ICD2, several potential sites for N-glycosylation were also identified: N⁴⁰⁴, N⁵⁵⁸, N⁶³⁶ in NKA α □□; N⁴⁰⁶, N⁴⁹⁵, N⁶⁴⁰ in NKA α □□; N⁶²⁵ and N⁶⁷¹ in NKA α □; N⁶³⁸ and N⁶⁸⁴ in NKA α □□, and N⁶²⁵ and N⁶⁸⁴ in NKA α □b. Moreover, a total of 21 protein kinase (PK) phosphorylation sites were found in NKA α 1a, NKA α 1b and NKA α 2, 23 in NKA α □□, and 25 in NKA α □b; such sites are targets for casein kinase II, protein kinase C, tyrosine kinase and cAMP-dependent protein kinase (Fig. 1). Most of the structural differences in the amino acid sequence across the NKA α subunits were located in the N-terminal region, ICD2 and ECD4. A lysine-rich sequence that acts as an ion-selective gate during cation binding and occlusion (Shull et al., 1986) was detected (Fig. 1) with NKA α 1a and NKA α 1b containing the greatest number of lysine residues. Moreover, the putative three Na⁺ and two K⁺ binding sites (referred to as Na⁺I,II,III and K⁺I,II in TMD4, TMD5 and TMD6; Fig.1) were fully conserved across the five NKA α subunits (Ogawa and Toyoshima, 2002; Chen et al., 2013). Other conserved domains were: a) a highly hydrophobic residue (L¹⁰² in NKA α 1a, L¹⁰⁴ in NKA α 1b, L⁹¹ in NKA α 2, L¹⁰³ in NKA α 3a and L⁹⁰ in NKA α 3b) with an important function during ion occlusion (Bublitz et al., 2010) in TMD1 (Fig1 number 1); b) a putative binding site to ankyrin repeats (Zhang et al., 1998) in ICD1 (box number 2); c) the cytoplasmic TGES/A motif in ICD1 (box number 3), critical for the catalytic cycle of all P-type ATPases and for stabilization of the E2P form (Horisberger et al., 1991; Bublitz et al., 2010); d) the DKTGT signatory region in ICD2 important for phosphorylation of the α subunit with an

aspartic acid residue involved in the conformation during potassium binding (box number 4) (Horisberger et al., 1991); e) the GDGVNDSP motif associated with the phosphorylation site and stabilization of phosphoenzyme intermediates (Horisberger et al., 1991) (box number 5); f) an arginine residue important for Na^+ binding and stabilizing one of the Na^+ sites (Henriksen et al., 2013) (number 6); g) the motif SYGQ, crucial to $\alpha\beta$ subunit interaction (Horisberger et al., 1991; Colonna et al., 1997) in ECD4 (box number 7); h) the conserved serine and arginine residues (box number 8) within the consensus XRRXSX for PKA phosphorylation, which is thought to modulate pumping activity by changing accessibility to the ion-binding site (Serluca et al., 2001; Toustrup-Jensen et al., 2009; Poulsen et al., 2012); i) a PGG motif (box number 9) preceded by two arginine residues to sense the membrane potential that are followed by an extension of eight residues contain the KETYY motif as a modulator of Na^+ affinity (Morth et al., 2007; Toustrup-Jensen et al., 2009).

Sequence identities between Senegalese sole NKA α subunits and those identified in *Danio rerio* and *Oryzias latipes* were calculated (Table 3). Overall, sequence identities were lower for DNA than for the predicted protein and the highest sequence variation was located at the third codon position. Identities between *S. senegalensis atp1a* paralogous genes ranged between 72.3 and 84.4% for *atp1a1a/atp1a2* and *atp1a1a/atp1a1b* respectively for DNA sequences and between 81.3 and 92.6% for *atp1a2/atp1a3a* and *atp1a3a/atp1a3b* respectively for aa sequences. The overall aa sequence identity of NKA α subunits of Senegalese sole relative to other fish ranged from 74.2% (between Senegalese sole NKA α 2 and *D. rerio* NKA α 1.5) to 96.2% (between Senegalese sole NKA α 3a and *O. latipes* NKA α 3a). In relation to human isoforms, the lowest aa sequence identity was found in all comparisons with human NKA α 4 (data not shown). The highest aa sequence identity of Senegalese sole NKA α 1b, NKA α 2, NKA α 3a and NKA α 3b were with their orthologous genes in *O. latipes* (95%, 89.3%, 93.8% and 96.2%, respectively) and in the case of NKA α 1a with the *D. rerio* NKA α 1.4 (Table 3). Comparison of the aa sequence identity of N-, C- and TM- domains between species, indicated the lowest sequence identity were located in the N-terminal domain (average 49.3% Senegalese sole NKA α isoforms and other species) and the highest aa sequence identity in the TM-domain (average 90.7%). The Maximum Likelihood phylogenetic tree (Fig. 2) showed that *S. senegalensis atp1a* paralogous genes clustered in three major and consistent clades: α 1 clade that grouped vertebrate *atp1a1* genes, α 2 clade that grouped vertebrate *atp1a2* genes and α 3 clade that grouped vertebrate *atp1a3* genes and were supported by 85%, 82% and 93% bootstrap values, respectively. Moreover, in α 1 and α 3 clades, the Senegalese sole and other teleost sequences clustered into two separate groups (referred to as type-a/type-b). The α 4 clade was only identified in tetrapod sequences.

3.2 Transcript levels of Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* during larval development

Transcript abundance of the Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* paralogous genes were detected very early during development (at 2 DPH) and had divergent expression profiles during development (up to 22 DPH; Fig. 3). *atp1a1a* mRNA levels peaked at 3 DPH (2.62-fold, Fisher's LSD $p < 0.05$) and decreased until 10 DPH remaining constant thereafter. In contrast, *atp1a1b* increased slightly until 9 DPH and dropped progressively until metamorphosis. *atp1a3a* and *atp1a3b* shared similar expression patterns with a progressive decrease of their mRNA abundance during development. The transcript levels at 22 DPH were 0.62-, 0.60- and 0.65-fold lower than at 2 DPH for *atp1a1b*, *atp1a3a* and *atp1a3b*, respectively. Finally, *atp1a2* mRNA abundance increased progressively from 2 DPH to 22 DPH, reaching the highest transcript levels at the end of metamorphosis (11.7-fold, Fisher's LSD $p < 0.05$).

3.3 Transcript levels of Senegalese sole *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* in juvenile tissues

Steady-state levels of *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* transcripts were quantified in nine different tissues from juvenile soles (Fig. 4). The *atp1a1a* gene was highly expressed in osmoregulatory organs: gills (~17-fold higher than in liver; Fisher's LSD $p < 0.05$), intestine (~23-fold higher than in liver; Fisher's LSD $p < 0.05$), and head kidney (~18-fold higher than in liver; Fisher's LSD $p < 0.05$). The *atp1a1b* was most abundant in brain (~555-fold higher than in liver; Fisher's LSD $p < 0.05$) and heart (186-fold higher than in liver; Fisher's LSD $p < 0.05$). The *atp1a2* was detected mainly in skeletal muscle (~5,000-fold higher than in liver; Fisher's LSD $p < 0.05$) and *atp1a3b* and *atp1a3a* were more abundant in brain (~2,600- and 158,000-fold higher than in liver, respectively; Fisher's LSD $p < 0.05$). *atp1a3b* mRNA levels were also abundant in heart (~400-fold higher than in liver), gills (~95 fold higher than in liver) and kidney (41-fold higher than in liver), although not significant with respect to other tissues.

3.4 Effect of a salinity shift on transcript levels of *atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b* and physiological localization in gills

As the five NKA α -paralogous genes had detectable mRNA levels in the gill, we evaluated their transcriptional response after transferring soles to 5, 35 and 60 ppt from seawater. Although quantification and comparison of mRNA levels between paralogues could be problematic under our experimental approach (small differences in amplification efficiency and relative sensitivity factor K_{RS} (Stahlberg et al., 2003)), Ct values were used as an approximate estimator of their

relative abundance. The lowest Ct values (the most abundant isoforms) in the gills from the control group (35 ppt 24-72h) were found for *atpl1a1a* and *atpl1a3b* (22.6 ± 0.3 and 21.5 ± 0.3 , respectively) whereas the highest Ct values (the lowest abundant isoform) for *atpl1a3a* (33.2 ± 0.4).

No significant differences in transcript abundance between salinities or time were found for *atpl1a1b* and *atpl1a2* (Fig. 5). For *atpl1a3b*, small but significant differences in mRNA levels across time were detected but not between salinities (Two-way ANOVA; Salinity: $F_{2,30}=1.96$, $p=0.16$; Timepoint: $F_{4,30}=15.54$, $p<0.001$; salinity*timepoint interaction: $F_{8,30}=3.40$, $p<0.01$). In contrast, *atpl1a1a* mRNA levels increased significantly in gills with salinity (Two-way ANOVA; Salinity: $F_{2,57}=55.14$, $p<0.001$; timepoint: $F_{4,57}=0.83$, $p=0.51$; salinity*timepoint interaction: $F_{8,57}=3.67$, $p<0.01$), with a significantly increased of transcript levels in soles exposed to 60 ppt at 72 h (8.8-fold higher than 35 ppt), 96 h (11.7-fold) and 7 days (9.2-fold) (ANOVA 72 h: $F_{2,15}=16.04$, $p<0.001$; 96 h: $F_{2,15}=40.94$, $p<0.001$; 7 d: $F_{2,15}=53.49$, $p<0.001$). No significant differences in transcript abundance in the gill of soles exposed to 5 and 35 ppt were found during the experiment. Moreover, *atpl1a3a* mRNA levels also modified with salinity and time (Two-way ANOVA; Salinity: $F_{2,30}=7.14$, $p<0.01$; Timepoint: $F_{4,30}=5.35$, $p<0.01$; salinity*timepoint interaction: $F_{8,30}=1.40$, $p=0.24$), with significantly higher transcript levels at 5 ppt than 35 and 60 ppt (ANOVA; 24 h: $F_{2,6}=5.39$, $p<0.05$; 48 h: $F_{2,6}=3.53$, $p=0.10$). Finally, *atpl1a1a* mRNA abundance was also evaluated in intestine and kidney but no significant changes were detected at any time point (data not shown).

ISH identified *atpl1a1a* transcripts in relatively large cells mainly localized at the base of the primary gill filaments and in the interlamellar region of fish acclimated to 5 and 35 ppt (Fig. 6). Moreover, the number of positive cells was higher at 35 ppt than at 5 ppt ($F_{2,9}=18.98$, $p=0.001$) and they were absent in the secondary lamellae at 5 ppt but were present in low abundance at 35 ppt (4.2 ± 2.8 ionocytes/mm filament; Fig. 7). Nevertheless, under hyperosmotic conditions, a significant increase of the number of cells expressing *atpl1a1a* was observed in the interlamellar basal region and throughout the length of the lamellae (110.22 ± 39.78 ionocytes per mm filament at 60 ppt; Fig. 6 and 7). Localization of NKA immunoreactive cells matched the number of ionocytes found for ISH in the primary filament. Nevertheless, unlike ISH, we detected ionocytes in the secondary lamellae at 5 ppt (25.9 ± 7.0) that also increased significantly with salinity (ANOVA $F_{2,6}=103.18$, $p<0.001$) reaching 88.6 ± 9.1 at 35 ppt and 223.9 ± 27.6 at 60 ppt. These results together with qPCR results (section 3.3 and 3.4) suggest that *atpl1a1a* is an important isoform expressed in gill ionocytes and regulated by salinity.

3.5 Effect of salinity on *atp1a1a* transcript levels in newly hatched larvae and localization by whole-mount ISH (WISH).

As *atp1a1a* transcripts were mainly detected in osmoregulatory organs, we selected this paralogous gene for further characterization. To know if *atp1a1a* transcript levels were modulated by salinity in early developmental stages, mRNA was quantified in 1 to 3 DPH larvae after incubation of embryos at 10, 20 and 36 ppt. The expression profile at 36 ppt was similar to that observed in the first experiment (Fig. 3) with a peak at 3 DPH at mouth opening (Fig. 8). Furthermore, at 3 DPH embryos at 36 ppt had significantly higher (Fisher's LSD $p < 0.05$) *atp1a1a* transcript levels compared to embryos at 10 and 20 ppt. In 1 DPH embryos maintained at 20 ppt, *atp1a1a* transcripts were significantly (Fisher's LSD $p < 0.05$) more abundant than in embryos at 36 ppt.

WISH analysis at 1 DPH showed that *atp1a1a* mRNAs were mainly detected in gut and pronephric tubule although hybridization positive signals were also observed in gills, otic vesicle, and developing chordacentrum of the notochordal sheath (supplementary file 2). At 3 DPH, the expression profile was similar but with intense staining of the pronephric tubule, chordacentrum, yolk sac ionocytes (ysi) and gills (Fig. 9). WISH signal in gut tended to decrease between 1 DPH and 3 DPH. Among salinities, differences were not very pronounced and we only detected a stronger signal in gills at 36 ppt compared to 10 ppt in 3 DPH larvae. It appeared that expression in gut was reduced between 1 and 3 DPH in larvae incubated at 36 ppt but not in larvae incubated at 10 or 20 ppt.

4. Discussion

The complete cDNA sequences of the five genes encoding the NKA α subunits (*atp1a1a*, *atp1a1b*, *atp1a2*, *atp1a3a* and *atp1a3b*) are characterized for the first time in the euryhaline teleost fish *S. senegalensis*. Previous studies have revealed that the membrane-spanning NKA α -subunit is the main catalytic unit and contains critical binding sites for ATP and Na^+ and K^+ and promotes active ion translocation against an electrochemical gradient and in the "alternating-access" model is suggested to involve extensive conformational changes tightly regulated by kinases (Palmgren and Nissen, 2011; Poulsen et al., 2012). The five NKA α -subunits have fully conserved the Na^+ or K^+ binding sites. Moreover, a typical domain organization for P-type NKA α -subunits was also identified: ten membrane-spanning α -helices (TMD1-TMD10) comprising the ion transport domain, five extracellular domains (ECD1-ECD5) with the β subunit binding domain, and four intracellular domains (ICD1-ICD4) containing the ATP binding and phosphorylation sites. Moreover, the highly conserved TGES (A-domain), DKTGT and

GDGVNDSP (P-domain) motifs associated with the phosphorylation and stabilization of phosphoenzyme were identified (Horisberger et al., 1991; Bublitz et al., 2010). Overall, the structural analysis of the five Senegalese sole NKA α predicted polypeptides suggests they are functional as they contain all the necessary elements for proper integration in the membrane and function.

ATPase α subunits are highly conserved throughout the animal kingdom (Martin, 2005). Identity between Senegalese sole isoforms ranged between 81.3 and 92.6% and compared to other teleosts between 74.2-96.2%. Identities with respect to humans oscillated between 77.1 and 89.6%. Most of the sequence divergence observed between Senegalese sole α subunits and that of other teleosts and humans was localized in the N-terminal region. In the N-termini, several protein kinase putative target sites are concentrated suggesting that this region plays a regulatory role in all isoforms (Feschenko and Sweadner, 1994; Blanco et al., 1995). In addition, the central part of ICD2 near the potassium-binding site was highly variable and this may be associated with different ion affinity and binding of each type of α subunit (Brodsky and Guidotti, 1990; Munzer et al., 1994). Interestingly, the region proximal to the SYGQ motif in ECD4 is highly variable between sole isoforms suggesting that they may have differing affinities for cations. Such differences in cation affinity between NKA isoforms have been associated with adaptation to physiological requirements and tissue or cell-type specific functions (Blanco et al., 1995). The highest identity occurred between the TMDs of the Senegalese sole subunits. Structural P-type ATPase studies identified the highly conserved transmembrane α -helices as key structures for reorganization within the plane of the membrane during ion-translocation (Bublitz et al., 2010). Phylogeny depicted a tree topology in which NKA α sequences were arranged in four major clades, referred to as $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$. Senegalese sole NKA α subunits clustered in the $\alpha 1$, $\alpha 2$, and $\alpha 3$ clades and the fourth clade was tetrapod-specific. The set of NKA α isoforms in sole, including the duplicate isoforms of $\alpha 1$ (*atpla1a* and *atpla1b*) and $\alpha 3$ (*atpla3a* and *atpla3b*) are presumably a result of the three rounds of large-scale gene duplications (referred to as 1R, 2R, and 3R) that occurred during the fish evolutionary lineage (Voff, 2005). The 3R or teleost specific whole-genome duplication, which occurred after splitting from the tetrapod lineage about 350 million years ago, presumably originated the paralogous genes identified in the $\alpha 1$ and $\alpha 3$ clades (Taylor et al., 2003; Meyer and Van de Peer, 2005). These three whole genome duplications have shaped fish genomes and the NKA paralogues join a range of other genes including glycolytic enzymes, hormones, their receptors and binding proteins, as well as structural and immune-related genes for which teleost specific paralogues exist (Steinke et al., 2006; Manchado et al., 2007; Infante et al., 2008a; Infante et al., 2008b; Zhou et al., 2008; Manchado et al., 2009; Infante et al., 2011). Moreover, some species such as zebrafish, Atlantic

salmon or rainbow trout, have an even higher number of NKA α subunits than Senegalese sole (Serluca et al., 2001; Richards et al., 2003; Gharbi et al., 2005; Liao et al., 2009). Most of these duplicated NKA α genes mainly belong to the highly diversified clade $\alpha 1$ (6 copies in zebrafish, 5 in salmon, 5 in tilapia (Ensembl genome browser), 3 in climbing perch) (Gharbi et al., 2005; Liao et al., 2009; Ip et al., 2012). Genome comparison across fish species identified that most of these NKA $\alpha 1$ copies were co-localized in tandem arrays. Moreover, tandem organization of Dre1a.1 (b-type) and Dre1a.4 (a-type) orthologues was well conserved across species (Liao et al., 2009). Our sequences for NKA $\alpha 1$ in *S. senegalensis* clustered together with these conserved gene copies although further genomic analysis would be necessary to confirm if they group together in tandem in the genome and if additional uncharacterized copies exist as reported in zebrafish (Serluca et al., 2001).

The high diversity and complexity of the gene family encoding the NKA α subunits suggests that during evolution they may have acquired new functions (neofunctionalization), or divided their ancestral function between paralogues (subfunctionalization) contributing to a variable number of genes in each species. Previous studies have shown that NKA α subunits can display different kinetic properties, ion affinities and expression patterns depending on the isoform and species (Canfield et al., 2002; Blanco, 2005). Moreover, NKA $\alpha 1$ paralogues have a differential distribution in gill ionocytes responding specifically to environmental ion levels (Liao et al., 2009). These data suggest that the NKA α subunits are able to “fine-tune” regulation to adapt to fluctuating conditions of environmental salinity. As the number of paralogues is species-specific, it will be important to evaluate their role and function in each species. In Senegalese sole juveniles, NKA α subunits had a differential tissue distribution. Thus, *atp1a1a* was mainly expressed in osmoregulatory organs (gill, intestine and kidney), *atp1a1b* in brain and heart, *atp1a2* in skeletal muscle and *atp1a3a* and *atp1a3a* in brain. Previous studies in zebrafish and trout also demonstrated that each isoform had a tissue-specific distribution suggesting differences in their functional properties within specific cell and tissue types (Rajarao et al., 2001; Richards et al., 2003). Nevertheless, tissue expression profiles of *atp1a1b*, *atp1a2* and *atp1a3* appear to have been conserved during evolution and for example zebrafish *atp1a1b* is also mainly located in brain (Rajarao et al., 2001; Serluca et al., 2001; Canfield et al., 2002). In mammals, *atp1a2* is predominantly expressed in skeletal muscle, but also in the heart and in astrocytes of the central nervous system (Lingrel and Kuntzweiler, 1994; Blanco and Mercer, 1998). In zebrafish, the *atp1a2* was also the most prominent isoform in skeletal muscle and the second most abundant form in brain and heart and has an essential role in skeletal and heart muscle function (Canfield et al., 2002; Doganli et al., 2012). Similarly in trout, the *atp1a2* also was expressed mainly in white muscle, red muscle, brain and eye (Richards et al., 2003). Concerning to *atp1a3*, this isoform has

been defined as a specific subunit of neural tissues in mammals (Blanco and Mercer, 1998). Most neurons express the ubiquitous *atp1a1* as well as the more selectively expressed *atp1a3* although they have revealed specific distributions with a mutually exclusive distribution in motoneurons (Edwards et al., 2013). *atp1a3* also participates in the potential firing of the neurons at a faster rate than *atp1a1* and this difference is thought to be determinant for a selective expression of each isoform within the neuron types (Azarias et al., 2013; Edwards et al., 2013). In teleosts, both *atp1a3a* and *atp1a3b* were mainly detected in brain (Rajaroo et al., 2001; Canfield et al., 2002; Feng et al., 2002) as in the Senegalese sole.

WISH detected *atp1a1a* transcripts in osmoregulatory organs including yolk sac ionocytes, pronephros tubule, gut and gills as well as in the otic vesicle and in presumptive chordacentrum. In zebrafish, the *atp1a1a.2*, *atp1a1a.4* and *atp1a1a.5* paralogues were the predominant α subunits expressed in pronephros, mucous cells and otic vesicle (Blasiole et al., 2002; Canfield et al., 2002). Moreover, *atp1a1a.2*, *atp1a1a.5* and *atp1a1a.1* paralogues were also detected in gill ionocytes (Liao et al., 2009). The most striking is the localization of *atp1a1a* signals in the presumptive chordacentrum, not previously described. In teleosts, this structure is the precursor of vertebral bodies and forms through mineralization of the notochordal sheath (Grotmol et al., 2005; Bensimon-Brito et al., 2012). Previous studies on skeletal development in sole have described an unsegmented and uniform sheath notochord as the main axial suspension structure at early developmental stages (till start of metamorphosis) (Gavaia et al., 2002; Padrós et al., 2011). Although no mineralization have been reported at such early stages, the expression pattern of *atp1a1a* indicates that notochord sheath segmentation at the molecular level appears very early. A similar segmental expression of alkaline phosphatase activity in the chordacentrum was also observed in Atlantic salmon and was related to the advance in mineralization within the notochordal sheath (Grotmol et al., 2005). This is the first time that NKA has been associated with chordacentrum formation and reveals that it is a very precocious process in sole. Further research will be necessary to clarify the role of NKA in early formation of vertebrae, its regulation by environmental conditions and its relevance for the production of high-quality larvae with a lower impact of malformations.

NKA activity can change in the teleost gill in response to the environmental salinity and provides the driving force to adapt to low (ion uptake) and high (salt excretion) salinity. These changes in activity have been associated to a differential regulation of NKA isoforms in the gill. In salmonids, NKA α 1a is predominantly expressed in freshwater and the NKA α 1b in seawater (Richards et al., 2003; McCormick et al., 2009). In other species such as milkfish and tilapia, NKA isoforms (NKA α 1 and α 3) modified their relative abundance in gills after a salinity shift (Feng et al., 2002; Tang et al., 2009). A previous study in sole juveniles demonstrated that gill

NKA activity has a linear correlation with environmental salinity and that significant changes in NKA activity were detected 3 days after salinity transfer both at low and high salinity. In contrast, kidney NKA activities were largely unaffected (Arjona et al., 2007). Our data indicate that *atp1a1a* mRNA levels increased significantly in the gills of juvenile soles 3 days after transfer to high salinity water (60 ppt), although no change was detected in the intestine or kidney. The pre-existence of a bulk of cytoplasmic and membrane NKA pumps would facilitate initially the fast response for hydromineral balance. However, a later induction of transcriptional responses at 72 h after salinity shift would facilitate an active secretion of Na^+ and Cl^- in the gill maintaining plasma homeostasis and allowing for an adaptation to environmental salinity for a long-term response (Evans et al., 2005). Moreover, the high correspondence between qPCR data and the number of positive staining signals using both ISH and IHC confirmed that *atp1a1a* transcription and translation are coupled at least in the filament, and that this isoform should be considered as the major gill isoform for ion secretion in sole. Also, a small but significant increased of *atp1a3a* mRNA levels at 5 ppt in gills was observed. However, the low mRNA abundance of *atp1a3a* in gill (highly expressed in brain) suggests a secondary role in low salinity adaptation. In fact, sole has been considered to be a partially euryhaline due to the inability of juveniles to survive more than 24 h in freshwater and 5 ppt is the tolerance limit in this species (Arjona et al., 2007). Further research will be necessary to elucidate the role of this isoform in the adaptation to low salinity

Sole larvae fully develop their osmoregulatory capacity after mouth opening. Larvae incubated at 33 ppt until mouth opening and then transferred to low salinity (10 ppt) are able to grow, develop and complete metamorphosis. In contrast larvae incubated at low salinity (5 ppt) die just after hatching and those maintained at 10 ppt develop severe mouth deformities, which reduce their viability (Salas-Leiton et al., 2012). In embryonic stages of teleosts, ionocytes can be found in several organs including epithelia covering the yolk and body, kidney, gut and precursor of gills (Canfield et al., 2002; Varsamos et al., 2002; Sucre et al., 2011). Nevertheless, yolk sac membrane and enteric ionocytes seem to play a major role in embryonic osmoregulation until other organs such as the gill are formed and become functional later after hatching. In sole, gills are underdeveloped and only second and third gill arches can be distinguished at hatching. The first and fourth gill arches develop later after mouth opening (Padrós et al., 2011). In this study, WISH results demonstrated that *atp1a1a* mRNAs were located in the main osmoregulatory organs of young larvae including gut, yolk sac, pronephros and gills. Before mouth opening, the gut seems to play a major role in early osmoregulatory processes. *atp1a1a* WISH signal decreased between 1 and 3 DPH in the gut of larvae incubated at 36 ppt and increased in the gill and skin; in larvae maintained at lower salinity (10 and 20 ppt) the shift from a predominantly

gut to gill and skin expression pattern was delayed. At mouth opening (3 DPH), *atplala* transcription increased and was associated to a higher number of cells expressing this paralogue in the gill suggesting that this first contact with seawater is essential for ionocyte maturation and adaptation to salinity as reported by Salas-Leiton et al (2012). Moreover, the skin ionocytes have been suggested to play a role in the provision of Ca^{2+} to the bones and formation of fin rays and scales (Varsamos et al., 2002). The expression of *atplala* in the chordacentrum suggests that this gene could also play a role in calcium uptake and regulation at this site and potentially other sites, such as the jaw that has an increased incidence of malformations in high and low salinity (Salas-Leiton et al., 2012).

Three main distribution patterns of gill ionocytes have been reported according to their immunolocalization and response to environmental salinity: group A includes those species (i.e. eels) possessing lamellar ionocytes in freshwater but not in seawater; group B includes species (i.e. salmonids) with ionocytes in both filaments and lamellae at any salinity (in the case of Atlantic salmon, it is classified into both groups according to age: smolts, group A and parr, group B); and group C include only euryhaline fish belonging to Acanthopterygii in which ionocytes are found only in the filaments and rarely in the lamellae both in freshwater and seawater (Hiroi and McCormick, 2012). ISH localization of *atplala* in the gill of sole showed that this gene was mainly localized in ionocytes distributed in primary gill filaments and in the interlamellar region of fish exposed to brackish and seawater. Immunolocalization confirmed a similar pattern in the number of positive cells in the filament although the number of ionocytes detected in the lamellae at low and seawater salinity was much higher than in ISH (not detectable at 5 ppt). The possible cross-reactivity of the pan- $\alpha 5$ antibody to NKA α -subunit isoforms in fish (McCormick et al., 2009) could explain these differences between ISH and IHC suggesting that other isoforms could express in the lamellae at these salinities. The number of ionocytes in the lamellae increased significantly with salinity from 25.9 at 5 ppt to 223.9 cells/mm of filament at 60 ppt. In the Hawaiian goby (group B), ionocytes were detected both in the filament (~90-130 cells/mm filament) and the lamellae (~40-50 cells/mm filament) and although the number of ionocytes increased in the filament at higher salinity as occurred in sole, they did not vary in the lamellae (McCormick et al., 2003). The observation of ionocytes by IHC in both filaments and lamellae suggests that the sole may fit better in the B group along with the Hawaiian goby (McCormick et al., 2003; Hiroi and McCormick, 2012).

Hyperosmotic conditions provoked changes in the number of cells expressing *atplala* similarly to NKA immunoreactive cells with a similar distribution pattern (interlamellar region, lamellae basal region and in the whole lamellae itself indicating that *atplala* was the main isoform modulated at high salinity for ion excretion. The pattern of immunoreactive cells under

hypersaline conditions has not been explored extensively in other species. In seabream (group C), animals transferred to 60 ppt have ionocytes always restricted to the primary filament in gills in spite of changes in their number, size and shape (Laiz-Carrión et al., 2005). In contrast, in seabass (group A), a salinity of 70 ppt increased the ionocytes number and they invaded both lamellae and filaments. This high increase in the number of ionocytes in the lamellae at 60 ppt should be considered as a major adaptive mechanism to hypersaline conditions in the gills to compensate the excess of salt ingested and to increase ion extrusion capacity. Moreover, these adjustments of ion transport capacity are usually linked to altered permeability properties to reduce the water loss and diffusive gain of salts (Lavery and Skadhauge, 2012).

In summary, in this study the sequence and main features of the five cDNAs encoding NKA α isoforms in the Senegalese sole are reported. A high level of structural conservation occurred with functional mammalian isoforms suggesting that the sole NKA α genes encode functional proteins. Phylogeny clustered the five paralogous sole genes into three main clades ($\alpha 1$, $\alpha 2$ and $\alpha 3$) with the orthologues from other fish. The five genes exhibited different expression patterns during larval development, tissue specific expression in juveniles and after a salinity challenge. qPCR, ISH and IHC confirmed *atpla1a* as a major NKA α isoform in gills during salinity adaptation in this euryhaline species. In larvae, WISH identified *atpla1a* mainly localized in osmoregulatory organs and strikingly in the chordacentrum of developing sole (3 DPH) indicating it may have a role in skeletal formation.

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Figure captions

Figure 1. Comparison of Senegalese sole NKA α proteins. Dots represent identity, and dashes represent gaps with respect to *atplala*. The ten predicted transmembrane regions (TMD1–TMD10), the five extracellular (ECD1-ECD5) and four intracellular domains are shaded in shaded in blue, purple and yellow, respectively. Triangles indicate the lysine- rich region. Potential N-glycosylation sites are highlighted in pink. Potential phosphorylation sites and motifs for different kinases are highlighted in green (casein kinase II), brown (protein kinase C), red (tyrosine kinase) and orange (cAMP-dependent protein kinase). Conserved domains (boxed) and single conserved residues (highlighted) are numbered from 1 to 10. Na⁺ I,II,III and K⁺ I,II in TMD4, TMD5 and TMD6 refer to Na⁺ and K⁺ binding regions. The putative catalytically phosphorylated aspartyl residue is marked with an asterisk.

Figure 2. Phylogenetic relationships among the predicted sequence of Senegalese sole NKA α subunit isoforms and the corresponding deduced amino acid sequences from other vertebrates (see supplementary file 1) using the Maximum Likelihood method. *Ciona intestinalis* NKA α subunit was used as outgroup to root tree. Only bootstrap values higher than 50% are indicated on each branch. The scale for branch length (0.2 substitutions/site) is shown below the tree.

Figure 3. Transcript abundance of NKA α genes during larval development (from 2 to 22 DPH) in Senegalese sole. Expression values were normalized to those of *gapdh2*. Data are expressed as the mean fold change (mean $\pm$ SEM, n = 3) from the calibrator group (2 DPH). Different letters denote days that are significantly different ($P < 0.05$) analyzed using a Kruskal-Wallis test. Double and triple letters indicate intermediate states of statistical significance between the corresponding single and double letters, respectively. The interval for the metamorphic process is shaded.

Figure 4. Transcript abundance of NKA α genes in different tissues of Senegalese sole juveniles. Expression values were normalized to those of 18S rRNA. Data were expressed as the mean fold change (mean $\pm$ SEM, n = 3) from the calibrator group (liver). Different letters denote tissues that are significantly different from liver ($P < 0.05$) groups were determined with the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Fisher's Least Significant Difference (LSD) test L: liver; Sp: spleen; B: brain; G: gills; I: intestine; St: stomach; HK: head kidney; H: heart; M: muscle; Sk: skin.

Figure 5. Transcript abundance of NKA α genes after salinity transfer (5, 35 and 60 ppt) in the gill of Senegalese sole juveniles. Expression values were normalized to those of *ub52*. Data were expressed as the mean fold change (mean $\pm$ SEM, n = 3 except for *atplala* at 72 h, 96 h and 7 d n=6) from the calibrator group (24 h-35ppt). Significant differences in the relative expression intensity by time or salinity were tested using a two-way ANOVA ($p < 0.05$). Lowercase letters denote significant differences between sampling times and uppercase letter between salinities at a specific time.

Figure 6. Localization of *atplala* expression by ISH (a-f) and IHC (g-i) in longitudinal sections of gills from *S. senegalensis* juveniles adapted to 5 (a, d, g), 35 (b, e, h) and 60 (c, f, i) ppt environmental salinities. Arrows denote positive signals for ISH. NKA-positive cells are depicted in green and DAPI nuclear staining is shown in magenta. F, filament; L, lamellae.

Figure 7. Quantification of the number of ISH *atplala* positive cells (A) and NKA-immunoreactive ionocytes (B). Positive signals on the primary filament and secondary lamellae were counted and analyzed separately. Uppercase letters denote significant differences in the putative ionocyte number in the filament and lowercase letters in the lamellae. Groups with different letters were significantly different from one another ($p < 0.05$, Tukey test). Values are

expressed as means $\pm$ STD. Four and three individuals in each treatment group for ISH and IHC were measured, respectively.

Figure 8. Relative expression levels of *atp1a1a* in young larvae incubated at 10, 20 and 36 ppt. Expression values were normalized to those of *gapdh2*. Data were expressed as the mean fold change (mean $\pm$ SEM, n = 3) from the calibrator group (10 ppt 1 DPH). Different letters denote significant differences among salinities for each day ($P < 0.05$) using a Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Fisher's Least Significant Difference (LSD) test.

Figure 9. WISH of *atp1a1a* in sole larvae at 3 DPH incubated at 10, 20 and 36 ppt. Whole larvae and lateral and ventral views are shown. A riboprobe from the sense strand was used as a control in 3 DPH larvae incubated at 36 ppt. (pt) pronephric tubule, (ch) chordacentrum, (gi) gills, (gu) gut, (ysi) yolk sac ionocytes, (ov) otic vesicle

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Table 1. Primers used for qPCR, cloning and sequencing.

Target	Primer name	Sequence	Amplicon size
<i>atp1a1a</i>	<i>CL196.1</i>	5'-CATCATCCAGCAAGGAATGAAGAACCGT-3' (F)	157
	<i>CL196.2</i>	5'-GGAGTAAGGGAAGGCACAGAACCACCAAC-3' (R)	
	<i>SseNKAa1a•1</i>	5'-GAGAGAGGCGGAGTAGGAAAGTTGC-3' (F)	
	<i>SseNKAa1a•2</i>	5'-GTGGGCCACGGTCATCCTGTTCTG-3' (R)	
	<i>SseNKAa1a•3</i>	5'-GTCGCCAACGTGCCAGAAGGTCTCC-3' (F)	
	<i>SseNKAa1a•4</i>	5'-GAGTCGTTGACGCCGTACCTGT-3' (F)	
	<i>SseNKAa1a•5</i>	5'-ATGACCCCGGAGAACCTGGACGA-3' (F)	
	<i>SseNKAa1a•6</i>	5'-GCCAGAGCCGTCTCCTCAAACAGC-3' (R)	
	<i>SseNKAa1a•7</i>	5'-GTGCTGTCTGCCGTCTCATCATC-3' (F)	
<i>atp1a1b</i>	<i>SseNKAa1a•9</i>	5'-CGCCTCCTTCGACAGAAGCTC-3' (R)	115
	<i>CL436.1</i>	5'-GCCTTCCCTTACTCCCTGCTCATCTTCATC-3' (F)	
	<i>CL436.2</i>	5'-GCTGACGGGGTCTTTAATAGTAGGTCTCGC-3' (R)	
	<i>SseNKA436•1</i>	5'-AGAGCAGCCCTGGATGACGAGATGA-3' (F)	
	<i>SseNKA436•3</i>	5'-GAGAACCCTGTGCTTCATTGGCCTCATGTCC-3' (F)	
	<i>SseNKA436•2</i>	5'-GGAGATGGCGGGAACCATGT-3' (R)	
	<i>SseNKA436•5</i>	5'-CACCGGCGTCGAGGAAGGTCGTC-3' (F)	
	<i>SseNKA436•6</i>	5'-ATGATCAGATCGGCCACTGCAC-3' (R)	
	<i>SseNKA436GSP1</i>	5'-GGGATGTTTACAGACGAGCGGCGATGT-3' (R)	
<i>atp1a2</i>	<i>SseNKA436GSP2</i>	5'-CACTTCCCGACAGCGTCAGGCACAG-3' (R)	93
	<i>SseNKAa2•1</i>	5'-TCCTCTCCTACTGCCCTGGAATGGACA-3' (F)	
	<i>SseNKAa2•2</i>	5'-TGAGAAGACTGTACGGGAAGGCACAGAA-3' (R)	
	<i>SseNKAa2•3</i>	5'-CATGAAACGCCAGCCGAGGA-3' (F)	
	<i>SseNKAa2•4</i>	5'-CTATAACAGGCTAAACAGGCCACAA-3' (R)	
	<i>SseNKAa2•5</i>	5'-CTCGGTGTGGTTTCTCAGCAGGTCGT-3' (R)	
	<i>SseNKAa2•7</i>	5'-TGGATTGAGAGCTGGTACTTGTGTGGTGA-3' (R)	
	<i>NKAa2GSP1</i>	5'-GAGCTTGGATCATACCTATTGTCCGTAG-3' (R)	
	<i>SseNKAa2•F1</i>	5'-CCCCAGAGCACCGTCCCTCA-3' (F)	
<i>atp1a3a</i>	<i>SseNKAa2•F2</i>	5'-CGGCACACGGCATCGTGATAGC-3' (F)	159
	<i>SseNKAa2•R1</i>	5'-ATGTGGATGAAGTGTTCAATCTCGATGTT-3' (R)	
	<i>SseNKAa2•R2</i>	5'-GAATGTGACCAGAGGGATTGTCTCCAC-3' (R)	
	<i>SseNKAa3a•1</i>	5'-TCTGTGCGTTCCCGTACAGTTTCTCTCA-3' (F)	
	<i>SseNKAa3a•2</i>	5'-GAGTTCAGGGGACAGGGATGACGGAGT-3' (R)	
	<i>SseNKAa3a•3</i>	5'-AACGCCTATTTGGAAGTGGGAGGACT-3' (F)	
	<i>SseNKAa3a•5</i>	5'-AAGGCTTGTGTGATCCACGGCACAGA-3' (F)	
	<i>SseNKAa3a•6</i>	5'-GAGAGATAGGGGTTAGAGAAGGAGGGATG-3' (R)	
	<i>SseNKAa3a•8</i>	5'-CCCGTAACTGTCTCCAGGTCGTTGT-3' (R)	
<i>atp1a3b</i>	<i>SseNKA3a3R1</i>	5'-GGCATCCGGCTCAACTG-3' (F)	97
	<i>SseNKA3a3R2</i>	5'-GACAGTTACGGGCAGCAGTG-3' (F)	
	<i>SseNKA3a3R3</i>	5'-TGTTCCAGCAGGGCATGAAG-3' (F)	
	<i>SseNKAa3a•GSP1</i>	5'-GGGATGTTGAGGCGTGCAGCAATGT-3' (R)	
	<i>SseNKAa3a•GSP2</i>	5'-GATGATGCCACACCCCTAGCAATG-3' (R)	
	<i>SseNKAa3aseq1</i>	5'-GAAGGAAGTGCCATAATGGAACAC-3' (F)	
	<i>SseNKAa3aseq2</i>	5'-GCAGGTAAGGGCTTGTGTTTGTGTC-3' (R)	
	<i>SseNKAa3aseq3</i>	5'-ACGGCTGCAAGGTGGACAACATC-3' (F)	
	<i>SseNKAa3aseq4</i>	5'-GCTGCTCCTTGCCCTGCATCAT-3' (R)	
<i>atp1a3b</i>	<i>SseNKAa3b•1</i>	5'-TCCGAAAACCTGCTCATCCGTCGAAACC-3' (F)	97
	<i>SseNKAa3b•2</i>	5'-AGATGAGGGGAGTTTGAGCCAAGTTGC-3' (R)	
	<i>SseNKAa3b•3</i>	5'-GGTAGGGAGTGACGCGGCAGTAGG-3' (F)	
	<i>SseNKAa3b•4</i>	5'-ATGAGGGCCAGGACGAAAAAGGTGA-3' (R)	
	<i>SseNKAa3b•5</i>	5'-CGCTACGCTGACCTCTGGCCTGGA-3' (F)	
	<i>SseNKAa3b•6</i>	5'-TCTGAGATGATGCCACGCCCTTA-3' (R)	
	<i>SseNKAa3b•7</i>	5'-GACCTTCCCGTGTGCTGTG-3' (F)	
	<i>SseNKAa3b•8</i>	5'-GTCTTTAAGGGGCGAGACATCC-3' (R)	
	<i>SseNKAa3b•9</i>	5'-TCTGGCTCCGACGTGTCCAAACA-3' (F)	
TOPO® TA	<i>SseNKAa3b•GSP1</i>	5'-ATAGGCATTTCGGCTAAATTGGGACGAC-3' (F)	
	T7	5'-TGTAATACGACTCACTATAGGGCGAATTGG-3'	
	T3	5'-TTGATTACGCCAAGCTCGAAATTAACCCTCA-3'	
	M13fw	5'-GTAACACGACGCGCCAG-3'	
	M13rv	5'-CAGGAAACAGCTATGAC-3'	

Table 2. Main features of the cDNAs encoding Senegalese sole Na⁺,K⁺ATPase alpha subunit isoforms

Locus	Accession no.	cDNA length (nt)	5'-UTR length (nt)	Coding sequence	3'-UTR length (nt)	3' poly-A signal
<i>atp1a1a</i>	AB759891	3,281	51	52-3,120	161	3,258-3,263
<i>atp1a1b</i>	AB759892	3,697	219	234-3,311	386	3,655-3,660
<i>atp1a2</i>	AB862096	3,443	66	67-3,099	344	3,421-3,426
<i>atp1a3a</i>	AB759894	3,637	368	369-3,440	197	3,436-3,441
<i>atp1a3b</i>	AB759893	3,543	273	274-3,306	237	3,457-3,462

Table 3

Percentage of amino acid (above diagonal) and DNA (below diagonal) sequence identity among Senegalese sole (Sse), *Danio rerio* (Dre), and *Oryzias latipes* (Ola) NKA subunits. The nomenclature 1a, 1b, 2, 3a and 3b refers to the NKA isoform. Comparisons between NKA isoforms in *S. senegalensis* are shaded. *D. rerio* and *O. latipes* NKA isoforms are separated by a line.

	Sse1a	Sse1b	Sse2	Sse3a	Sse3b	Dre1a1	Dre1a2	Dre1a3	Dre1a4	Dre1a5	Dre1b	Dre2	Dre3a	Dre3b	Ola1a	Ola1b	Ola13	Ola2	Ola3a	Ola3b
Sse1a		89.8	82.9	82.8	83.3	89.9	80.7	84.1	91.4	79.4	89.2	81.8	83.7	82.7	88.9	88.8	90.4	81.3	82.3	82.8
Sse1b	84.4		83.1	84.2	84.2	93.1	79.8	82.8	89.4	78.2	92.8	82.7	84.3	83.3	86.0	95.0	88.1	81.3	83.5	83.4
Sse2	72.3	74.7		82.4	81.3	82.9	75.4	78.1	82.3	74.2	83.1	87.1	82.2	81.7	80.4	83.1	81.9	89.3	81.9	81.7
Sse3a	74.3	76.9	74.8		82.6	83.8	76.3	78.7	82.7	74.3	84.5	82.1	95.9	93.7	80.6	83.4	82.4	80.5	96.2	91.4
Sse3b	74.1	75.6	72.3	92.6		84.0	76.4	78.5	83.5	74.4	84.3	80.3	92.4	93.0	81.1	83.1	82.7	79.8	91.3	93.8
Dre1a1	77.1	80.2	71	73.3	73.6		79.8	84.1	90.1	79.1	91.9	82.4	84.6	83.5	86.1	92	87.8	81.7	83.1	83.3
Dre1a2	72	71.3	65	67.3	68.2	76.3		82.4	83.1	77.1	79.7	75.8	76.6	75.3	79.7	79.3	80.6	73.7	75	75
Dre1a3	74.3	72.8	71.3	68.2	65	67.3	76.3		86.1	87.8	83.6	76.8	79.2	78.3	81.6	83.2	83.1	77.1	78	78.4
Dre1a4	77.3	76.6	70	70.6	71.5	81.7	82.1	84.7		81.2	89.2	81.7	83.6	83.2	88	89.2	89.5	81.5	82.1	82.5
Dre1a5	70.8	69.9	65.7	65.8	67.2	74.8	78.8	89.4	82.1		78	73	74.9	74.1	77.1	78.2	78.6	73.6	73.8	74.8
Dre1b	75.4	77.6	70.1	72.5	73.2	77.4	70.6	73	75.6	70.4		82.3	84.6	83.4	85.5	91.8	87	81.4	83.5	83.4
Dre2	70.5	73	76.7	72.7	70.3	70.7	65.4	67.2	69.2	65.1	70.2		81.8	81.6	80	82.2	81.4	85.1	81.8	80.7
Dre3a	74	76.3	73	83.7	81.2	72.9	68.8	69.3	71.5	67.0	72.5	73.3		93.9	81.3	83.4	82.6	81.0	94	91.7
Dre3b	70.9	72.3	71.3	81.3	79.5	71.7	66.7	68.4	70.7	65.8	71.8	70.8	82.1		80.7	82.4	82.3	80.5	93.3	92.5
Ola1a	81.7	78.2	71.1	73.2	73.5	76	72.2	73.5	77	70.5	74	69.2	72.2	70.2		85.0	86.5	81.2	82.6	83.1
Ola1b	80.3	87.1	74.6	75.7	74	79.3	70.5	72.8	75.7	70	77.3	72.6	74.2	71.8	79		94.5	79.3	80.6	80.8
Ola13	82.6	79.4	71.3	73.4	74.2	76.8	73.0	73.9	77.8	71	75.3	69.5	72.8	70.8	79.8	95.5		80.6	81.9	82.3
Ola2	71.9	72.6	83.3	73.1	71.9	70.4	65.2	67.2	69.5	65.3	70.3	75.3	72.8	69.3	73.4	70.7	71		81.4	80.3
Ola3.a	73	74.4	73.4	87.2	80.6	72.3	67.2	68.6	71.3	66.4	71.4	70.4	81.4	79.5	73.8	71.5	72.4	72.4		90.6
Ola3.b	71.7	72.9	71.3	80.3	85.2	71.3	66.3	68.8	69.8	66.7	71.5	68.3	78.3	78.5	72.7	71.5	72.5	70.4	78.5	