A peer-reviewed version of this preprint was published in PeerJ on 18 December 2014.

Avarre J, Guinand B, Dugué R, Cosson J, Legendre M, Panfili J, Durand J. 2014. Plasticity of gene expression according to salinity in the testis of broodstock and F1 black-chinned tilapia, *Sarotherodon melanotheron heudelotii*. PeerJ 2:e702 https://doi.org/10.7717/peerj.702

Plasticity of gene expression according to salinity in the testis of broodstock and F1 black-chinned tilapia, *Sarotherodon melanotheron heudelotii*

Jean-Christophe Avarre, Bruno Guinand, Rémi Dugué, Jacky Cosson, Marc Legendre, Jacques Panfili, Jean-Dominique Durand

The black-chinned tilapia *Sarotherodon melanotheron heudelotii* Rüppell 1852 (Teleostei, Cichlidae) displays remarkable acclimation capacities. When exposed to drastic changes of salinity, which can be the case in its natural habitat, it develops quick physiological responses and keeps reproducing. The present study focused on the physiological impact of salinity on male reproductive capacities, using gene expression as a proxy of acclimation process. Two series of experimental fish were investigated: the first one was composed of fish maintained in freshwater for several generations and newly acclimated to salinities of 35 and 70, whereas the second one consisted of the descendants of the latter born and raised under their native salinity. Expression patterns of 43 candidate genes previously identified from the testes of wild males was investigated in the three salinities and two generations. Twenty of them showed significant expression differences between salinities, and their predicted function revealed that most of them are involved in the osmotic tolerance of sperm cells and/or in the maintenance of sperm motility. A high level of expression variation was evidenced, especially for fish maintained in freshwater. In spite of this, gene expression patterns allowed the differentiation between fish raised in freshwater and those maintained in hypersaline water, in both generations. Altogether, the results presented here suggest that this high variability of expression is likely to ensure the reproductive success of this species under varying salinities.

Plasticity of gene expression according to salinity in the testis of broodstock and F1 black-chinned tilapia, *Sarotherodon melanotheron heudelotii*

Jean-Christophe Avarre[1*], Bruno Guinand[1], Rémi Dugué[1], Jacky Cosson[2], Marc Legendre[1], Jacques Panfili[3], Jean-Dominique Durand[3]

[1] Institut des Sciences de l'Evolution de Montpellier, UMR 226 IRD-CNRS-UM2, Montpellier, France

[2] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic

[3] Ecologie des Systèmes Marins Côtiers, UMR 5119 IRD-UM2-CNRS-IFREMER, Montpellier, France

***Corresponding author**:

Jean-Christophe Avarre

Institut des Sciences de l'Evolution de Montpellier, IRD, UMR 226

Université Montpellier II, Bat 22, CC 065

Place Eugène Bataillon

34095 Montpellier cedex 05, France

Tel: +33467143480, fax: +33467143622

jean-christophe.avarre@ird.fr

Short title: salinity and gene expression in tilapia testis

Introduction

The black-chinned tilapia, *Sarotherodon melanotheron heudelotii* Rüppell 1852 (Teleostei, Cichlidae), is a mouth-brooding fish that mainly occurs in estuarine and lagoon ecosystems of West Africa, but also sometimes in isolated, natural or artificial ponds. This species is an important local fish resource, accounting for a large part of catches in this area. Because of reduced freshwater input and intense evaporation that has occurred over the last years (Pagès & Citeau 1990; Savenije & Pages 1992), it is regularly exposed to changes of salinity in its natural habitats. This led to physiological modifications of osmoregulation (Lorin-Nebel et al. 2012; Tine et al. 2011) and reproductive strategies (Gueye et al. 2012; Legendre et al. 2008; Panfili et al. 2004; Panfili et al. 2006).

Analysis of the gene expression patterns in the gills of this species evidenced a clear differentiation of sub-populations along the Sine Saloum estuary (Senegal) linked to the ambient salinity (Tine et al. 2012). It was also demonstrated that salinity induced phenotypic modifications of the mechanisms involved in the activation of sperm cell motility, one of the major indicators of male fitness (Fauvel et al. 2010; Lahnsteiner et al. 1998). Indeed, the osmolality that enabled sperm activation in the black-chinned tilapia increased significantly with the salinity at which broodfish were maintained (Legendre et al. 2008). This finding was also recently reported for another estuarine species, *Fundulus grandis* (Tiersch & Yang 2012), indicating that increased knowledge in *S. m. heudelotii* could also benefit other euryhaline species.

Most of the studies focusing on the response of aquatic animals to alternative osmotic (Evans & Somero 2008; Larsen et al. 2007; Whitehead & Crawford 2006; Whitehead et al. 2011) or hypoxic (Gracey 2007; Rathburn et al. 2013; Tiedke et al. 2014) environments addressed this question through functional genomics of the gills and/or liver. Nevertheless, the fitness of individuals does not only rely on short-term, direct physiological responses to environmental

challenges, but also on their capacity to produce viable gametes and offspring under a wide range of environmental conditions (Breckels & Neff 2013; Dorts et al. 2012). Salinity has been shown to significantly modify some reproductive traits such as length at first sexual maturity, fecundity and oocyte size in the wild (Diouf et al. 2009; Panfili et al. 2004; Panfili et al. 2006; Whiterod & Walker 2006). However, very few studies attempted to investigate how gene expression in gonads responded to salinity challenges, even though gametogenesis and gamete quality may be highly influenced by salinity (Alavi & Cosson 2006; Bobe & Labbé 2010; Cosson 2004).

Using a high-throughput transcriptomic approach, Avarre et al. (Avarre et al. 2014) validated a *de novo* qPCR assay complying with the MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines (Bustin et al. 2009) for 43 candidate and 11 reference gene transcripts in the testes of mature males sampled in Senegal at locations displaying salinities of 40 and 95. The aim of the present study was to examine the putative involvement of the expression pattern of these 43 candidate genes in the acclimation of male reproductive capacities to salinity changes, over two generations. More specifically, it investigated (*i*) how transfer from low (0) to high salinities (35 and 70) induced specific changes of gene expression in testes within a single parental generation, and (*ii*) if these variations persisted in the next generation (F_1). Two series of experimental fish were analyzed: the first one was composed of fish maintained in freshwater and newly acclimated to salinities of 35 and 70 ("transferred fish", T), whereas the second one consisted of the F_1 descendants of T fish. These F_1 individuals were born and raised in each salinity condition ("born fish", B). Levels of gene expression were compared between the two generations in order to bring insights into the mechanisms that allow *S. melanotheron* males to respond to salinity changes without compromising the success of reproduction.

Material and methods

Fish samples

The fish used in this study came from a single freshwater strain of *Sarotherodon melanotheron heudelotii*. They originated from a population of ~50 juveniles sampled in the Niayes (natural freshwater ponds) of Dakar (Senegal) and transferred to our facilities (Montpellier) nearly 15 years ago. Since then, fish have been reared in freshwater recirculation systems (*i.e.* in the same salinity as that of their natural environment). In order to minimize the loss of genetic diversity, 3-10 mate pairs were used to obtain a new generation, and one generation corresponded to approximately 18 months. Twelve mature males and twelve mature females (approximately 18 month old) from this pool (hereafter referred to as T) were transferred to 3 independent water recirculation systems dedicated to a specific salinity (*i.e.* 0, 35 and 70). These salinities mimic fresh, saline and hypersaline waters in which *S. melanotheron heudelotii* may be frequently encountered in the wild. Yet, because of technical limitations, experiments with higher water salinities were not implemented here. Each system comprised two breeding tanks (polyester tanks of 2.5 m length x 0.53 m width x 0.30 m depth). Water salinity was gradually increased at a rate of about 1 day^{-1} by the addition of synthetic sea salt ("Instant Ocean", Aquarium system, Sarrebourg, France) until the target salinities of 35 and 70 were reached. Following a 5-week period of acclimation at the final salinities, fish were monitored for their reproductive behaviour over 18 weeks. Specifically, male reproductive success was assessed through the number of incubating males per tank (related to the number of available couples), the effective fertilization of incubated eggs and the viability of progenies. Finally, they were anaesthetized (Eugenol, 0,1 mL/L) and then killed by an overdose of anaesthetic (Eugenol, 0,5 mL/L) in ice (in accordance with the EU Directive 2010/63/EU) for dissection and testis collection. The body weight (Wb), fork length (FL) and gonad weight (Wg) were recorded for each fish, and the gonadosomatic index (GSI) was calculated as follow: Wg / Wb x 100. Condition factor K was also calculated,

according to the standard formula 100 x Wb / FL^3. During this period, about 50 hatchlings from 6-8 spawning events per salinity were transferred to another series of tanks. They were grown at salinities of 0, 35 and 70 until they were 9-11 months old, *i.e.* about 3 months after they became sexually mature. From these large pools of animals (referred to as B), 12 males and 12 females per salinity condition were randomly selected and transferred to the same breeding tanks used for T animals. Again, their reproductive activity was monitored for 18 consecutive weeks before they were processed for testis collection as indicated above. Testes were placed in RNA later (Ambion) overnight at 4°C and then stored at -20°C until use. In total, testes from 33 T and 35 B fish were collected (Figure 1). All the experimental procedures took place in our facilities in Montpellier, under the laboratory agreement for animal experimentation number A-34-172-24 and the author's personal authorization for animal experimentation number 34-188, both delivered by the French government.

RNA extraction and cDNA synthesis

RNA was extracted with the Nucleospin-8 total RNA isolation kit (Macherey-Nagel). Fifteen to twenty mg of testis preserved in RNA later were weighed and transferred into 2ml tubes containing a 5mm steel bead (Qiagen) as well as 360 µl lysis buffer supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). Tissues were homogenized with a tissue lyzer (Qiagen) for 2 min at 50 Hz. Tubes were then centrifuged for 5 min at full speed and the supernatants were transferred to new tubes and kept at -20°C overnight. RNA was extracted the following day according to the manufacturer's instructions, using a Janus automated workstation (Perkin Elmer), and eluted in 70 µl RNase-free H_2O. In order to remove any trace of contaminating genomic DNA, RNA eluates were subjected to a second DNase treatment: a mix of 0.2 µl of RNase-free DNase and 2 µl of reaction buffer (Macherey-Nagel) was added to 20 µl of each

RNA eluate, and digestion was carried out for 15 min at 37°C. RNA quantity was measured by UV spectrophotometry (Nanodrop 1000, Thermoscientific), and its integrity was verified by capillary electrophoresis (Agilent Bioanalyzer 2100). Each RNA sample was diluted to a concentration of 50 ng.µl^{-1} in H_2O and stored at -80°C.

Reverse transcription was performed with oligodT primers on 250 µg RNA, using the transcriptor first strand cDNA synthesis kit (Roche). A template-primer mixture consisting of 250 µg RNA and 2.5 µM oligodT was denatured at 65°C for 10-min and immediately cooled on ice. The reaction (in 20 µl final) was supplemented with reaction buffer (1X), dNTPs (1 mM each), RNase inhibitor (20 U) and reverse transcriptase (10 U), incubated for 1 hr at 50°C, then heated for 5 min at 85°C and immediately cooled on ice. The resulting cDNAs were diluted 10 times with 180 µl H_2O and stored at -20°C until use.

Gene sequence annotation

The candidate and reference genes investigated here were identified using a high-throughput digital gene expression approach (Avarre et al. 2014). Their raw sequences can be found under the SRA study accession number SRP022935, whereas the assembled sequences can be accessed through a freely accessible interactive database (http://asahttp.drim.com/tilapia/tilapia_menu.php). Annotation of the corresponding sequences was therefore needed to infer their putative functions. This was realized with Blast2GO v2.6.6 (Conesa et al. 2005). Sequences were used as a query to search the non-redundant protein database available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) using the BlastX algorithm with an E-value cutoff set at 10^{-6}. Sequences were then functionally annotated by mapping against gene ontology (GO) resources. Sequences that were not assigned any GO term were checked for conserved domains using the CD-search tool (Marchler-Bauer &

Bryant 2004). Likewise, sequences for which the number of BlastX hits was < 5 were re-aligned using the BlastN algorithm, and their description was corrected when necessary.

Gene expression analysis

The expression of 43 candidate genes previously validated for their potential as being involved in testis response to salinity was analyzed by qPCR at the 3 salinities and for the 2 fish generations. PCR amplifications were carried out in 384-well plates with a LightCycler 480 (Roche) in a final volume of 6 µl containing 3µl of SYBR Green I Master mix (Roche), 2 µl of cDNA and 0.5 µM of each primer (Avarre et al. 2014). Amplifications were performed in duplicate with an initial denaturation step of 10 min at 95 °C followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s and elongation at 72°C for 10 s. Amplifications were followed by a melting procedure, consisting of a brief denaturation at 95°C for 5 sec, a cooling step at 65°C for 1 min and a slow denaturation to 97°C. Amplification products were validated by analyzing the shape of their corresponding melting curve and by measuring their size on agarose gel electrophoresis. For each given sample, all the genes were amplified simultaneously in the same 384-well plate, and each plate contained a no-template control for every primer pair. Cycle of quantification (Cq) values were calculated with the LightCycler software, using the second derivative method. Results were expressed as changes in relative expression according to the $2^{-\Delta\Delta Cq}$ method (Pfaffl 2001). Cq values were first corrected with the amplification efficiency of each primer pair according to the following equation: $Cq_{E=100\%} = Cq_E (\log(1+ E) / \log(2))$, where E is the efficiency and Cq_E the uncorrected Cq values. Then the corrected Cqs of each gene of interest were normalized (ΔCq) with the mean Cq of 4 validated reference genes (Avarre et al. 2014) , and ΔCq values were related to the average ΔCq value of all samples.

Statistical analyses

T and B animals maintained in freshwater were initially analyzed as 2 different groups; however, because they belonged to the same salinity treatment and were kept in the same conditions, they were also considered as one single group for statistical purposes. Comparison of the two situations showed that variations in gene expression followed the same trend, indicating that pooling did not mislead interpretation (not shown). All statistical analyses were performed with the GenEx Pro package (MultiD analyses, Sweden). The normality of data distribution was first verified for each series of samples using the Kolmogorov-Smirnov test. Since more than 90% of series turned out to be normally distributed, a one-way ANOVA test with a Tukey–Kramer's post-test was applied to infer significant differences between salinities, using a confidence level of 0.95 ($p < 0.05$). *P*-values were corrected for multiple testing using the false discovery rate (Benjamini & Hochberg 1995). Concurrently, expression levels were also compared by salinity pairs with a *t*-test, using the same confidence level. Finally, a principal component analysis (PCA) was also carried out on the two series of fish, according to the expression pattern of the investigated genes.

Results

In spite of their age difference, average GSI for the two series of fish (T and B) were comparable, with mean (±SD) values of 0.27 ± 0.13 and 0.26 ± 0.12 for T and B males, respectively. Condition factor calculated for each salinity group showed similar values, ranging between 1.99 ± 0.11 and 2.04 ± 0.14. Moreover, spermatozoa produced by the fish investigated in this study led to successful fertilization with viable offspring in all experimental conditions, indicating that salinity did not impair the ability of male to successfully reproduce.

Differences in gene expression between salinities and generations

A collective analysis of raw Cq values for the 43 candidate and 4 reference genes in the 33 T and 35 B fish with geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) software indicated that Contig_Tilapia_90_13722 (R1), Contig_Tilapia_90_7452 (R2), Contig_Tilapia_90_3058 (R3) and Transcript_AVA3_453 (R4) were the most stably expressed genes. This confirmed that these four genes were appropriate to use as reference in the present conditions, as was already demonstrated on wild fish (Avarre et al. 2014).

Among the 43 tested candidate genes, 20 showed significant variations between fish kept at different salinities in at least one of the 2 generations investigated in this study (Table 1). The number of genes that showed significant variations in their expression levels between salinities was higher in T (18) than in B (10) animals. The relative expression levels of these genes are displayed in Figures 2 and 3. Generally, fold-changes in relative expression between the different salinity conditions were quite low, as the highest ratio was 3.86. Conversely, inter-individual variations among salinity groups were rather high. Interestingly, these variations were uppermost in fish kept in fresh water. Within each generation, the largest differences were observed between the most extreme salinities, *i.e.* between 0 and 70, and to a lesser extent between 0 and 35, as indicated by Tukey–Kramer pairwise comparisons. In T fish, the number of genes showing significant differences was 11 between fresh and seawater, 15 between fresh and hypersaline water and 2 between saline and hypersaline water. In B fish, these numbers amounted 5, 11 and 2, respectively. Likewise, the largest fold-change differences were also observed between salinities 0 and 70, for the two series of animals. Overall, directions of expression differences between salinities were comparable in both generations.

Results of the PCA, based on the expression pattern of the 20 investigated genes, are displayed in figure 4. The two first PC axes accounted for 87.20% and 5.40% of variation. Because the variation explained by the second axis accounted for approximately 17 times less than the first one, an ANOVA with a Tukey-Kramer's pairwise comparison was performed on the PC scores of

the first axis only. It revealed that freshwater males could be significantly differentiated from T35 ($P = 0.024$), T70 ($P = 0.0015$) and B70 ($P = 0.0004$) animals, but not from B35 ones ($P > 0.05$). Moreover, there were no significant differences between T35 and B35 animals or between T70 and B70 animals ($P > 0.05$).

Predicted function of the differentially expressed and reference genes

Except for Contig_Tilapia_90_2321 which returned no significant blastX hit and Transcript_AVA3_33497 which matched a hypothetical protein, all 24 genes could be attributed either a known or a predicted function, with rather low E-values. Among these, 21 could be assigned at least one GO description (Table 2). The list contains proteins for which a putative role in spermatogenesis has already been proven in other organisms (MORC family CW-type zinc finger 2 protein, 28 kDa heat- and acid-stable phosphoprotein, seminal plasma glycoprotein), as well as proteins involved in energy metabolism (NADH dehydrogenases, phosphatase), in stress response and osmoregulation (heat shock proteins, sodium potassium ATPase), or in axonemal activity (calcium-binding protein, beta-tubulin). The predicted function of the 23 remaining genes, which did not show any significant variations according to salinity, is displayed in Supplementary Table 1.

Discussion

This study aimed to analyze how mature males originating from a single freshwater population responded to transfer in saline (salinity 35) or hypersaline (salinity 70) water, and how F_1 individuals born in these new environments expressed the same genes. Salinity changes were shown to noticeably modify the life-histories and reproductive strategies of populations (Gueye et al. 2012; Legendre et al. 2008; Panfili et al. 2004; Panfili et al. 2006), and to impact their osmoregulatory capacities (Lorin-Nebel et al. 2012; Tine et al. 2012) and their stress response (Tine et al. 2010). Nevertheless, gene expression variation in reproductive organs like testes was

never investigated, although they are necessary to the preservation of male fitness and contribute to the demographic features of populations. Previous results have shown that salinity of the water under which fish were raised had a major effect on sperm characteristics and on the conditions for activation of spermatozoa motility. Particularly, higher osmolality and higher concentrations of extracellular calcium were required for activation of spermatozoa in fish maintained in saline/hypersaline water (Legendre et al. 2008 and unpublished results). The precise mechanisms, and especially the molecular basis behind these physiological adaptations, still need further investigations. However, as intra-testicular spermatozoa and other testicular cell types are particularly difficult to separate, and the quantification of gene expression for each single cell type difficult to reach, studying gene expression variation at the testis level is a necessary step for deciphering the genes involved in salinity acclimation. Physiological changes related to environmental salinity were observed not only at the spermatozoa level (motility activation), but also at the gonad level (*e.g.* ionic content and osmolarity of the seminal fluid involved in the protection of spermatozoa during storage in the reproductive system) (Legendre et al., unpublished results), indicating that a tissue level approach is necessary.

Global expression patterns of the 20 genes distinguished fish that only experienced freshwater from their counterparts acclimated to saline and hypersaline conditions (T35 and T70). This reflected a shift - *i.e.* a plastic response - in mean levels of gene expression from a standard freshwater environment to a new environment, with no significant differences in global gene expression patterns between T35 and T70 males. Among B animals (*i.e.* born in a specific salinity), gene expression patterns were found significantly different only between freshwater and B70 males. Inter-individual variation in mRNA levels was found highest for T and B fish maintained in freshwater, *i.e.* the only animals that did not undergo any environmental change for many generations. Concurrently, inter-individual variation in gene expression was around twice lower in T35, B35 and T70 males, and 3 times lower in B70 fish. Elevated inter-individual

variations in mRNA levels have been proposed as a possible source of variation to enable future evolution in reaction to rapid environmental changes (Aubin-Horth et al. 2005; Oleksiak et al. 2002; Whitehead & Crawford 2005; Whitehead & Crawford 2006), since production of better adapted protein orthologs does not fit such short time-scales (Hofmann & Todgham 2010). By contrast with gene expression levels, no differences in fertilization capabilities were observed between all investigated fish. If salinity increase induced a shift in the osmolarity at which sperm cells were activated (not shown), it yet did not affect sperm motility itself, and spermatozoa produced by all the fish led to successful fertilization with viable offspring in all experimental situations. This indicates that salinity changes and salinity itself did not impair the ability of males to reproduce successfully. Combined together, these findings suggest that the genes investigated here are involved in the mechanisms of acclimation to salinity. They also support the hypothesis that naturally-occurring expression variation contributes to the phenotypic plasticity of male black-chinned tilapia, which ensures its reproductive success under varying salinities. Nevertheless, this plasticity may differ between T and B fish. Indeed, T fish demonstrated an ability to respond to a punctual, context-dependent change in environmental conditions after being raised in a common environment, a process known as phenotypic flexibility (Piersma & Drent 2003). In contrast, B fish 'accomodated' their respective saline environment since hatching, and differences in gene expression across treatments might partly originate from the developmental component of gene expression plasticity (West-Eberhard 2003). Yet, the present experimental design does not permit to conclude whether the differences found between T and B animals (in terms of gene expression) are due to phenotypic flexibility itself or to the co-occurrence of both types of plasticity. Detailed studies of these two components of plasticity warrant further investigations in the black-chinned tilapia.

Among the 20 genes showing differential expression in the testes, many encode proteins that have a link with the general oxidation-reduction level of sperm cells, and/or participate to plasma

membrane channel activity through differential regulation of ion content. Both activities play an important role in the osmotic tolerance of sperm cells (Morita et al. 2011) and in the maintenance of sperm motility in fish (Alavi & Cosson 2006). The potential involvement of some of these genes is discussed below.

Among the largest variations in gene expression that were observed, gene 1 encodes a protein homologous to type-IV ice-structuring protein, also known as antifreeze protein type-IV (AFPIV). The AFPIV has already been reported in many fishes from cold, temperate and warm waters (Lee et al. 2011), and its role is still subject to question. It was indeed shown to have actual antifreeze ability. However, its low plasma level measured in the longhorn sculpin (*Myoxocephalus octodecimspinosis*) suggests another function for this protein, such as a role in lipid transport due to its close structure relatedness with that of fish apolipoproteins (Gauthier et al. 2008). Recently, AFPIV was shown to be abundantly synthesized in ovaries of the Atlantic cod (*Gadus morhua*), especially during late stages of vitellogenesis, and was proposed to be involved in lipid transport and/or metabolism (Breton et al. 2012), in spite of a proven low concentration in the blood of adults (Gauthier et al. 2008). Finally, expression of AFPIV was also demonstrated in embryos of the gibel carp (*Carassius auratus gibelio*), and the authors proposed a potential role in the embryonic patterning (Liu et al. 2009). Beyond fish, it is known that antifreeze proteins participate to the osmotic resistance of spermatozoa by reducing mechanical stress to the cell membrane. They are hence often used in sperm cryopreservation (Prathalingam et al. 2006). Genes or more generally factors regulating osmolality and ion content are central to sperm motility (Cosson et al. 2008). In both T and B fish, expression of gene 1 significantly decreased with the salinity to which tilapia fish specimens were exposed. Experimental evidence is now required to elucidate the potential role of an antifreeze type IV-related protein in the adaptation to salinity, especially with regard to male gonadic activity and fertility. This is the first time that expression of an AFPIV is reported to occur in testes.

If the expression of the Na+/K+ ATPase (NKA) gene has been extensively studied in the gills of fish exposed to different salinities (Havird et al. 2013), including *S. melanotheron* (Lorin-Nebel et al. 2012; Tine et al. 2008; Tine et al. 2012), this is only the first report of its expression in the testes (gene 3). In gills, NKA plays an essential role in osmoregulation through branchial ionocytes to actively uptake/excrete ions from/to environmental water, respectively. Since testes have no ionocytes, it is likely that NKA plays other roles in the male gonads, and this requires further investigations. Sequence 4 matches a portion of the catalytic subunit of a serine-threonine protein phosphatase. A modulatory role of serine-threonine protein phosphatase in osmoregulation has been demonstrated in fish (Marshall et al. 2005), but details are scarce and the mechanisms still poorly explained, probably because of the myriad of reactions controlled by serine-threonine protein phosphatases (Shi 2009). The involvement of several serine-threonine protein phosphatases in the regulation of sperm motility was recently demonstrated in humans (Fardilha et al. 2013); this research has to be extended to fish. Sequence 5 is similar to that of a seminal plasma glycoprotein that contains both a partial von Willebrand factor type D domain and a zona pellucida (ZP) domain. This high molecular weight glyco-protein was shown to have a sperm-binding activity and a sperm-immobilizing activity (Mochida et al. 2002).

The predicted function of gene 15 points to a possible calcium-binding activity. A significant raise of its abundance was observed with salinity in T animals, suggesting an increase of Ca^{2+} metabolism in the testes of fish exposed to higher salinities. Earlier studies on another euryhaline tilapia, *Oreochromis mossambicus*, suggested that acclimation of sperm motility to salinity was associated with a modulation of the Ca^{2+} flow in order to increase its intracellular concentration (Morita et al. 2004). More recent studies on the black-chinned tilapia showed that the osmolality that enabled sperm activation increased significantly with the salinity at which broodfish were maintained. It was also found that increasing amounts of calcium in the sperm activation medium were needed to initiate sperm motility as a function of fish rearing salinity (Legendre et al. 2008).

Sequence 16 matches a beta-tubulin, which involvement in the flagellar motility, especially through post-translational modifications, has been shown for a wide range of organisms (Huitorel et al. 1999). The description of gene 17 matches a MORC family CW-type zinc (Zn) finger protein, which absence was first shown to trigger the stop of spermatogenesis in mice (Watson et al. 1998). Zinc is a trace element essential to reproduction in both sexes of numerous mammalian species including humans (Bedwal & Bahuguna 1994). Its involvement in spermatogenesis was recently shown in the Japanese eel (*Anguilla japonica*), by activating Zn-finger proteins and modulating transcription factor genes containing Zn-finger motifs (Yamaguchi et al. 2009). It allows for the regulation of mitotic cell proliferation and meiosis, the activation/inactivation of sperm motility, and may also affect the regulation of steroid hormone receptors including androgens (Yamaguchi et al. 2009). .

Among the 24 (reference and differentially expressed) genes analyzed in this study, four encode predicted NADH dehydrogenase subunits: two of them showed differential expression between salinities (genes 9 and 10), whereas the other two were used as reference (R1 and R2). Expression of NADH dehydrogenase was already demonstrated to significantly vary with salinity in the gills of the black-chinned tilapia (Tine et al. 2010; Tine et al. 2008; Tine et al. 2012)), but transcripts were not annotated precisely at that time. The two differentially regulated transcripts found in this study correspond to subunits ND1 and NDUFB3/B12, whereas the two sequences used as reference correspond to subunits NDUFV1 and NDUFA10. All of these NADH dehydrogenase subunits are part of a complicated multiprotein complex located in the inner mitochondrial membrane, the NADH:ubiquinone oxidoreductase (complex I). It plays a central role in oxidative phosphorylation and its main function is the transport of electrons by oxidation of NADH followed by reduction of ubiquinone, which is accompanied by the translocation of protons from the mitochondrial matrix to the inter-membrane space (Loeffen et al. 1998). In humans, correlations between sperm quality or sperm motility and mitochondrial activities

including oxidative phosphorylation have been known for a while (Piomboni et al. 2012; Ruiz-Pesini et al. 1998). Complex I is composed of 45 different subunits, regulated by both nuclear and mitochondrial genomes (Lazarou et al. 2009). It is therefore not surprising that different subunits may be subjected to differing regulation pathways, depending on available substrates and on the physico-chemical conditions in which they operate, as reported in humans (Piomboni et al. 2012). This was recently shown in fish by a study analyzing the transcriptional regulation during the ovarian development of the Senegalese sole (*Solea senegalensis*) (Tingaud-Sequeira et al. 2009), but data on male-specific tissues such as testes are lacking. Variations in complex I activity have been reported in many species, especially in the case of altered environments, and a reduction of its activity with water temperature was recently shown in *Fundulus heteroclitus* (Loftus & Crawford 2013). In the present study, expression of the two above-mentioned subunits significantly decreased with salinity. This differential expression could be related to the link of complex I with reactive oxygen species (ROS) (Maranzana et al. 2013), which are known to be involved in the control of sperm motility, both in mammals (de Lamirande et al. 1997) and fish (Shaliutina et al. 2014). Indeed, a recent study pointed out a relationship between the oxidation-reduction level and the phosphorylation status of an 18-kDa superoxide anion scavenger protein in the sperm cells of *Oreochromis mossambicus*, and showed that ROS-dependent mechanisms contributed to the osmotic tolerance of this other euryhaline tilapia (Morita et al. 2011).

Conclusion

The present study enabled the identification of 20 candidate genes likely involved in the acclimation to salinity changes of the reproductive physiology of *Sarotherodon melanotheron heudelotii*. It evidenced the potential role of unexpected transcripts (such as that encoding an antifreeze protein type-IV), and supported the hypothesis that elevated variations in gene expression may contribute to the remarkable plasticity of this species. Now, the relative

383 contribution of phenotypic flexibility and developmental plasticity has to be investigated in more
384 details, in order to gain further understanding on the fitness consequences of such changes in
385 testis gene expression.

386 **Acknowledgements**

387 We are very grateful to Mr. Christophe Cochet for his strong involvement in the maintenance of
388 fish welfare. This is publication IRD-DIVA-ISEM-2014-190.

389 **References**

390 Alavi SMH, and Cosson J. 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: A
391 review. *Cell Biology International* 30:1-14.

392 Andersen CL, Jensen JL, and Ørntoft TF. 2004. Normalization of Real-Time Quantitative
393 Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to
394 Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets.
395 *Cancer Research* 64:5245-5250.

396 Aubin-Horth N, Landry CR, Letcher BH, and Hofmann HA. 2005. Alternative life histories shape
397 brain gene expression profiles in males of the same population. *Proceedings of the Royal*
398 *Society B: Biological Sciences* 272:1655-1662.

399 Avarre JC, Dugue R, Alonso P, Diombokho A, Joffrois C, Faivre N, Cochet C, and Durand JD.
400 2014. Analysis of the black-chinned tilapia *Sarotherodon melanotheron heudelotii*
401 reproducing under a wide range of salinities: from RNA-seq to candidate genes.
402 *Molecular Ecology Resources* 14:139-149.

403 Bedwal RS, and Bahuguna A. 1994. Zinc, Copper and Selenium in Reproduction. *Experientia*
404 50:626-640.

405 Benjamini Y, and Hochberg Y. 1995. Controlling the false discovery rate - a practical and
406 powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B-*
407 *Methodological* 57:289-300.
408 Bobe J, and Labbé C. 2010. Egg and sperm quality in fish. *General and Comparative*
409 *Endocrinology* 165:535-548.
410 Breckels RD, and Neff BD. 2013. The effects of elevated temperature on the sexual traits,
411 immunology and survivorship of a tropical ectotherm. *Journal of Experimental Biology*
412 216:2658-2664.
413 Breton TS, Anderson JL, Goetz FW, and Berlinsky DL. 2012. Identification of ovarian gene
414 expression patterns during vitellogenesis in Atlantic cod (*Gadus morhua*). *General and*
415 *Comparative Endocrinology* 179:296-304.
416 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl
417 MW, Shipley GL, Vandesompele J, and Wittwer CT. 2009. The MIQE Guidelines:
418 Minimum Information for Publication of Quantitative Real-Time PCR Experiments.
419 *Clinical Chemistry* 55:611-622.
420 Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, and Robles M. 2005. Blast2GO: a
421 universal tool for annotation, visualization and analysis in functional genomics research.
422 *Bioinformatics* 21:3674-3676.
423 Cosson J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa.
424 *Aquaculture International* 12:69-85.
425 Cosson J, Groison AL, Suquet M, Fauvel C, Dreanno C, and Billard R. 2008. Studying sperm
426 motility in marine fish: an overview on the state of the art. *Journal of Applied Ichthyology*
427 24:460-486.
428 de Lamirande E, Jiang H, Zini A, Kodama H, and Gagnon C. 1997. Reactive oxygen species and
429 sperm physiology. *Rev Reprod* 2:48-54.

Diouf K, Guilhaumon F, Aliaume C, Ndiaye P, Do Chi T, and Panfili J. 2009. Effects of the environment on fish juvenile growth in West African stressful estuaries. *Estuarine Coastal and Shelf Science* 83:115-125.

Dorts J, Grenouillet G, Douxfils J, Mandiki SNM, Milla S, Silvestre F, and Kestemont P. 2012. Evidence that elevated water temperature affects the reproductive physiology of the European bullhead Cottus gobio. *Fish Physiology and Biochemistry* 38:389-399.

Evans TG, and Somero GN. 2008. A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish Gillichthys mirabilis: osmosensors to effectors. *Journal of Experimental Biology* 211:3636-3649.

Fardilha M, Ferreira M, Pelech S, Vieira S, Rebelo S, Korrodi-Gregorio L, Sousa M, Barros A, Silva V, da Cruz e Silva OA, and da Cruz e Silva EF. 2013. "Omics" of human sperm: profiling protein phosphatases. *Omics* 17:460-472.

Fauvel C, Suquet M, and Cosson J. 2010. Evaluation of fish sperm quality. *Journal of Applied Ichthyology* 26:636-643.

Gauthier SY, Scotter AJ, Lin F-H, Baardsnes J, Fletcher GL, and Davies PL. 2008. A re-evaluation of the role of type IV antifreeze protein. *Cryobiology* 57:292-296.

Gracey AY. 2007. Interpreting physiological responses to environmental change through gene expression profiling. *The Journal of Experimental Biology* 210:1584-1592.

Gueye M, Tine M, Kantoussan J, Ndiaye P, Thiaw OT, and Albaret J-J. 2012. Comparative analysis of reproductive traits in black-chinned tilapia females from various coastal, marine, estuarine and freshwater ecosystems. *Plos One* 7:e29464.

Havird JC, Henry RP, and Wilson AE. 2013. Altered expression of Na(+)/K(+)-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: a meta-analysis of 59 quantitative PCR studies over 10 years. *Comp Biochem Physiol Part D Genomics Proteomics* 8:131-140.

455 Hofmann GE, and Todgham AE. 2010. Living in the Now: Physiological Mechanisms to Tolerate
456 a Rapidly Changing Environment. *Annual Review of Physiology* 72:127-145.
457 Huitorel P, Audebert S, White D, Cosson J, and Gagnon C. 1999. Role of tubulin epitopes in the
458 regulation of flagellar motility. In: Gagnon C, ed. *The male gamete: from basic science to*
459 *clinical applications*. Vienna, IL: Cache River Press, 475-491.
460 Lahnsteiner F, Berger B, Weismann T, and Patzner RA. 1998. Determination of semen quality of
461 the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters,
462 and spermatozoal metabolism. *Aquaculture* 163:163-181.
463 Larsen PF, Nielsen EE, Williams TD, Hemmer-Hansen J, Chipman JK, Kruhoffer M, Gronkjaer
464 P, George SG, Dyrskjot L, and Loeschcke V. 2007. Adaptive differences in gene
465 expression in European flounder (Platichthys flesus). *Molecular Ecology* 16:4674-4683.
466 Lazarou M, Thorburn DR, Ryan MT, and McKenzie M. 2009. Assembly of mitochondrial
467 complex I and defects in disease. *Biochimica Et Biophysica Acta-Molecular Cell*
468 *Research* 1793:78-88.
469 Lee JK, Kim YJ, Park KS, Shin SC, Kim HJ, Song YH, and Park H. 2011. Molecular and
470 comparative analyses of type IV antifreeze proteins (AFPIVs) from two Antarctic fishes,
471 *Pleuragramma antarcticum* and *Notothenia coriiceps*. *Comparative Biochemistry and*
472 *Physiology B-Biochemistry & Molecular Biology* 159:197-205.
473 Legendre M, Cosson J, Hadi Alavi SM, and Linhart O. 2008. Activation of sperm motility in the
474 euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859) acclimatized to
475 fresh, sea and hypersaline waters. *Cybium*:181-182.
476 Liu JX, Zhai YH, and Gui JF. 2009. Molecular characterization and expression pattern of AFPIV
477 during embryogenesis in gibel carp (*Carassius auratus gibelio*). *Molecular Biology*
478 *Reports* 36:2011-2018.

Loeffen JLCM, Triepels RH, van den Heuvel LP, Schuelke M, Buskens CAF, Smeets RJP, Trijbels JMF, and Smeitink JAM. 1998. cDNA of Eight Nuclear Encoded Subunits of NADH:Ubiquinone Oxidoreductase: Human Complex I cDNA Characterization Completed. *Biochemical and Biophysical Research Communications* 253:415-422.

Loftus SJ, and Crawford DL. 2013. Interindividual Variation in Complex I Activity in *Fundulus heteroclitus* along a Steep Thermocline. *Physiological and Biochemical Zoology* 86:82-91.

Lorin-Nebel C, Avarre JC, Faivre N, Wallon S, Charmantier G, and Durand JD. 2012. Osmoregulatory strategies in natural populations of the black-chinned tilapia *Sarotherodon melanotheron* exposed to extreme salinities in West African estuaries. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* 182:771-780.

Maranzana E, Barbero G, Falasca AI, Lenaz G, and Genova ML. 2013. Mitochondrial Respiratory Supercomplex Association Limits Production of Reactive Oxygen Species from Complex I. *Antioxidants & Redox Signaling* 19:1469-1480.

Marchler-Bauer A, and Bryant SH. 2004. CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* 32:W327-331.

Marshall WS, Ossum CG, and Hoffmann EK. 2005. Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. *Journal of Experimental Biology* 208:1063-1077.

Mochida K, Matsubara T, Andoh T, Ura K, Adachi S, and Yamauchi K. 2002. A novel seminal plasma glycoprotein of a teleost, the Nile tilapia (*Oreochromis niloticus*), contains a partial von Willebrand factor type D domain and a zona pellucida-like domain. *Molecular Reproduction and Development* 62:57-68.

503 Morita M, Nakajima A, Takemura A, and Okuno M. 2011. Involvement of redox- and
504 phosphorylation-dependent pathways in osmotic adaptation in sperm cells of euryhaline
505 tilapia. *Journal of Experimental Biology* 214:2096-2104.
506 Morita M, Takemura A, and Okuno M. 2004. Acclimation of sperm motility apparatus in
507 seawater-acclimated euryhaline tilapia *Oreochromis mossambicus*. *Journal of*
508 *Experimental Biology* 207:337-345.
509 Oleksiak MF, Churchill GA, and Crawford DL. 2002. Variation in gene expression within and
510 among natural populations. *Nature Genetics* 32:261-266.
511 Pagès J, and Citeau J. 1990. Rainfall and Salinity of a Sahelian Estuary between 1927 and 1987.
512 *Journal of Hydrology* 113:325-341.
513 Panfili J, Mbow A, Durand JD, Diop K, Diouf K, Thior D, Ndiaye P, and Lae R. 2004. Influence
514 of salinity on the life-history traits of the West African black-chinned tilapia
515 (*Sarotherodon melanotheron*): Comparison between the Gambia and Saloum estuaries.
516 *Aquatic Living Resources* 17:65-74.
517 Panfili J, Thior D, Ecoutin JM, Ndiaye P, and Albaret JJ. 2006. Influence of salinity on the size at
518 maturity for fish species reproducing in contrasting West African estuaries. *Journal of*
519 *Fish Biology* 69:95-113.
520 Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR.
521 *Nucleic Acids Research* 29:6.
522 Piersma T, and Drent J. 2003. Phenotypic flexibility and the evolution of organismal design.
523 *Trends in Ecology & Evolution* 18:228-233.
524 Piomboni P, Focarelli R, Stendardi A, Ferramosca A, and Zara V. 2012. The role of mitochondria
525 in energy production for human sperm motility. *Int J Androl* 35:109-124.

526 Prathalingam NS, Holt WV, Revell SG, Mirczuk S, Fleck RA, and Watson PF. 2006. Impact of
527 antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw.
528 *Theriogenology* 66:1894-1900.
529 Rathburn CK, Sharp NJ, Ryan JC, Neely MG, Cook M, Chapman RW, Burnett LE, and Burnett
530 KG. 2013. Transcriptomic responses of juvenile Pacific whiteleg shrimp, *Litopenaeus*
531 *vannamei*, to hypoxia and hypercapnic hypoxia. *Physiological Genomics* 45:794-807.
532 Ruiz-Pesini E, Diez C, Lapena AC, Perez-Martos A, Montoya J, Alvarez E, Arenas J, and Lopez-
533 Perez MJ. 1998. Correlation of sperm motility with mitochondrial enzymatic activities.
534 *Clinical Chemistry* 44:1616-1620.
535 Savenije HHG, and Pages J. 1992. Hypersalinity - a Dramatic Change in the Hydrology of
536 Sahelian Estuaries. *Journal of Hydrology* 135:157-174.
537 Shaliutina A, Gazo I, Cosson J, and Linhart O. 2014. Comparison of oxidant and antioxidant
538 status of seminal plasma and spermatozoa of several fish species. *Czech Journal of*
539 *Animal Science* In press.
540 Shi YG. 2009. Serine/Threonine Phosphatases: Mechanism through Structure. *Cell* 139:468-484.
541 Tiedke J, Thiel R, and Burmester T. 2014. Molecular Response of Estuarine Fish to Hypoxia: A
542 Comparative Study with Ruffe and Flounder from Field and Laboratory. *Plos One*
543 9:e90778.
544 Tiersch TR, and Yang HP. 2012. Environmental salinity-induced shifts in sperm motility
545 activation in Fundulus grandis. *Aquaculture* 324:145-150.
546 Tine M, Bonhomme F, McKenzie DJ, and Durand J-D. 2010. Differential expression of the heat
547 shock protein Hsp70 in natural populations of the tilapia, *Sarotherodon melanotheron*,
548 acclimatised to a range of environmental salinities. *BMC ecology* 10:11-11.

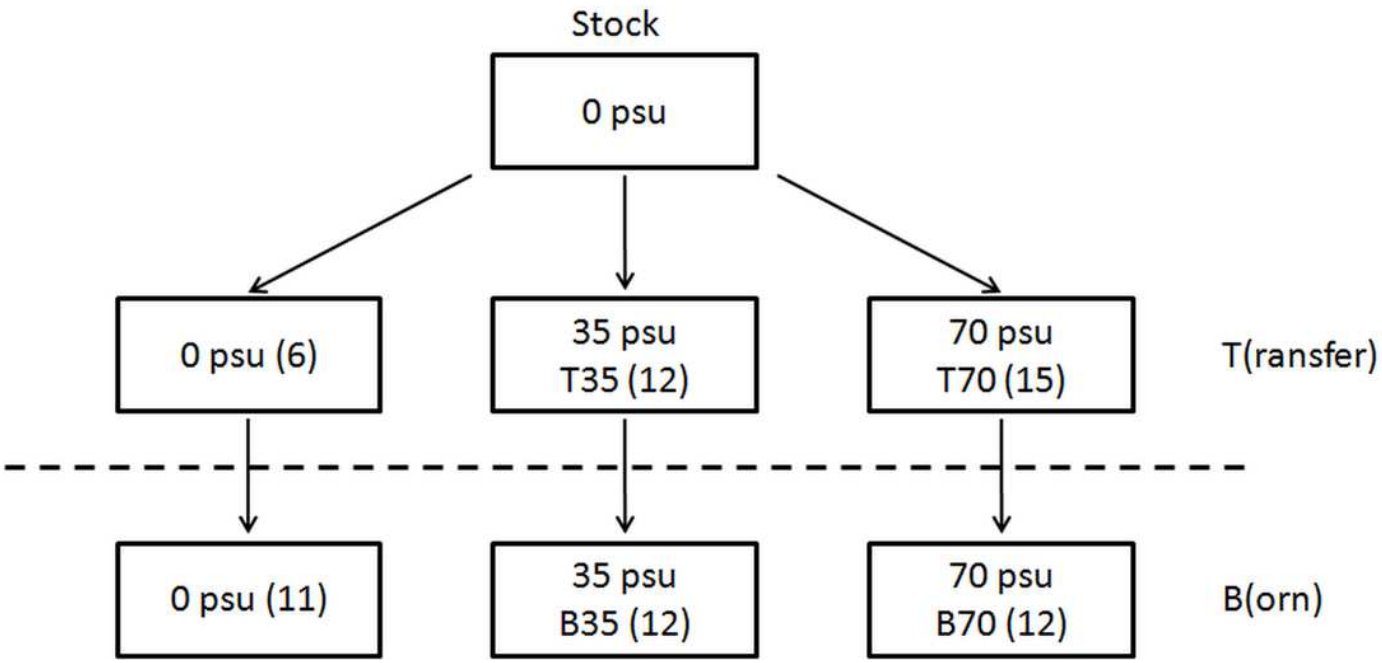
549 Tine M, de Lorgeril J, D'Cotta H, Pepey E, Bonhomme F, Baroiller JF, and Durand JD. 2008.
550 Transcriptional responses of the black-chinned tilapia *Sarotherodon melanotheron* to
551 salinity extremes. *Marine Genomics* 1:37-46.
552 Tine M, Guinand B, and Durand JD. 2012. Variation in gene expression along a salinity gradient
553 in wild populations of the euryhaline black-chinned tilapia *Sarotherodon melanotheron*.
554 *Journal of Fish Biology* 80:785-801.
555 Tine M, McKenzie DJ, Bonhomme F, and Durand J-D. 2011. Salinity-related variation in gene
556 expression in wild populations of the black-chinned tilapia from various West African
557 coastal marine, estuarine and freshwater habitats. *Estuarine Coastal and Shelf Science*
558 91:102-109.
559 Tingaud-Sequeira A, Chauvigne F, Lozano J, Agulleiro M, Asensio E, and Cerda J. 2009. New
560 insights into molecular pathways associated with flatfish ovarian development and atresia
561 revealed by transcriptional analysis. *BMC Genomics* 10:434.
562 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F.
563 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric
564 averaging of multiple internal control genes. *Genome Biology* 3:research0034.0031 -
565 research0034.0011.
566 Watson ML, Zinn AR, Inoue N, Hess KD, Cobb J, Handel MA, Halaban R, Duchene CC,
567 Albright GM, and Moreadith RW. 1998. Identification of morc (microrchidia), a mutation
568 that results in arrest of spermatogenesis at an early meiotic stage in the mouse.
569 *Proceedings of the National Academy of Sciences* 95:14361-14366.
570 West-Eberhard MJ. 2003. *Developmental Plasticity and Evolution*. Oxford, UK: Oxford
571 University Press.
572 Whitehead A, and Crawford DL. 2005. Variation in tissue-specific gene expression among natural
573 populations. *Genome Biology* 6:14.

574 Whitehead A, and Crawford DL. 2006. Neutral and adaptive variation in gene expression.
575 *Proceedings of the National Academy of Sciences of the United States of America*
576 103:5425-5430.
577 Whitehead A, Roach JL, Zhang S, and Galvez F. 2011. Genomic mechanisms of evolved
578 physiological plasticity in killifish distributed along an environmental salinity gradient.
579 *Proceedings of the National Academy of Sciences of the United States of America*
580 108:6193-6198.
581 Whiterod NR, and Walker KF. 2006. Will rising salinity in the Murray-Darling Basin affect
582 common carp (Cyprinus carpio L.)? *Marine and Freshwater Research* 57:817-823.
583 Yamaguchi S, Miura C, Kikuchi K, Celino FT, Agusa T, Tanabe S, and Miura T. 2009. Zinc is an
584 essential trace element for spermatogenesis. *Proceedings of the National Academy of*
585 *Sciences of the United States of America* 106:10859-10864.
586

1

Figure 1

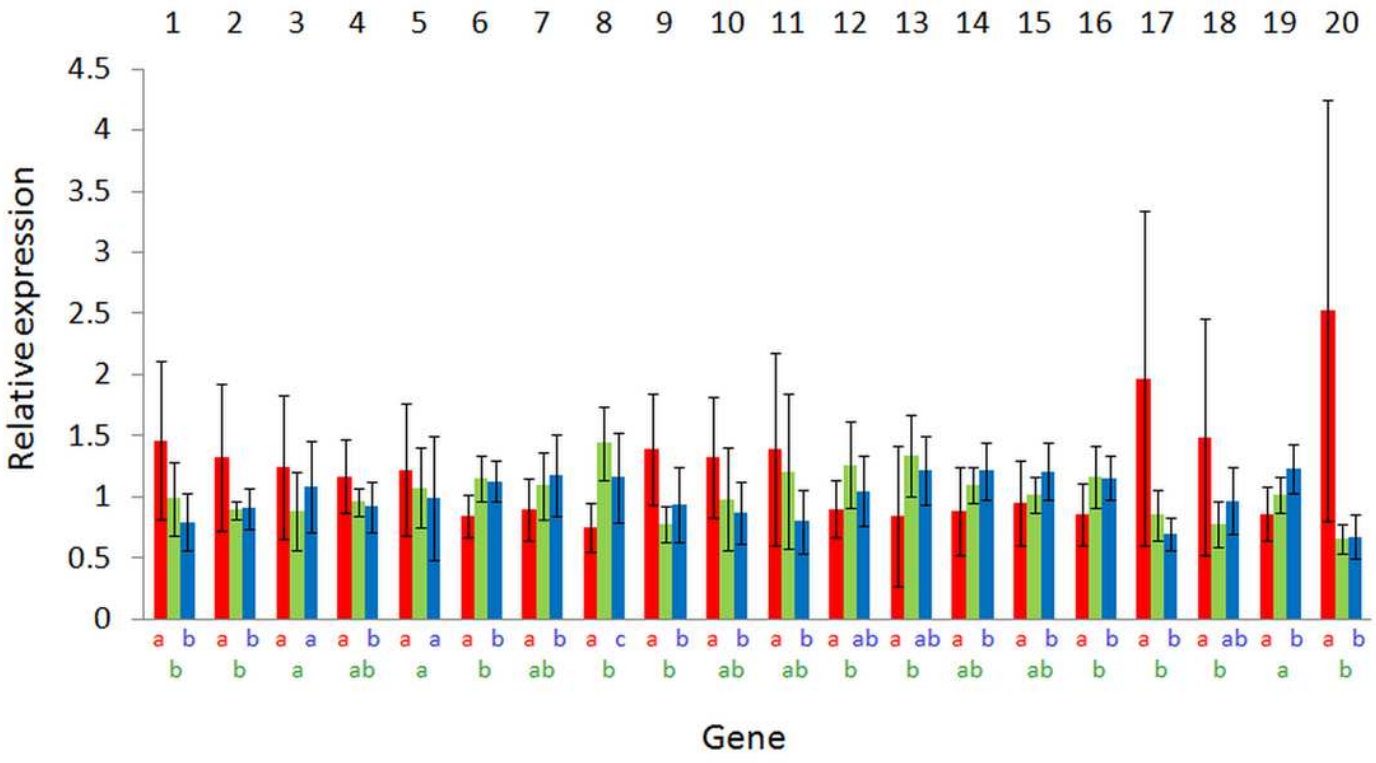
Origin of the 2 series of experimental animals considered in this study, which both originated from a single stock maintained in captivity in freshwater since ~15 years (see text for details). T animals result from a transfer from fish of this stock to the same (0) or to different salinity conditions (35 and 70). B animals represent first-generation male offspring from the reproduction of each category of T fish under their respective salinity conditions (0, 35 or 70). Number of T and B males collected from each salinity condition is reported in brackets.



2

Figure 2

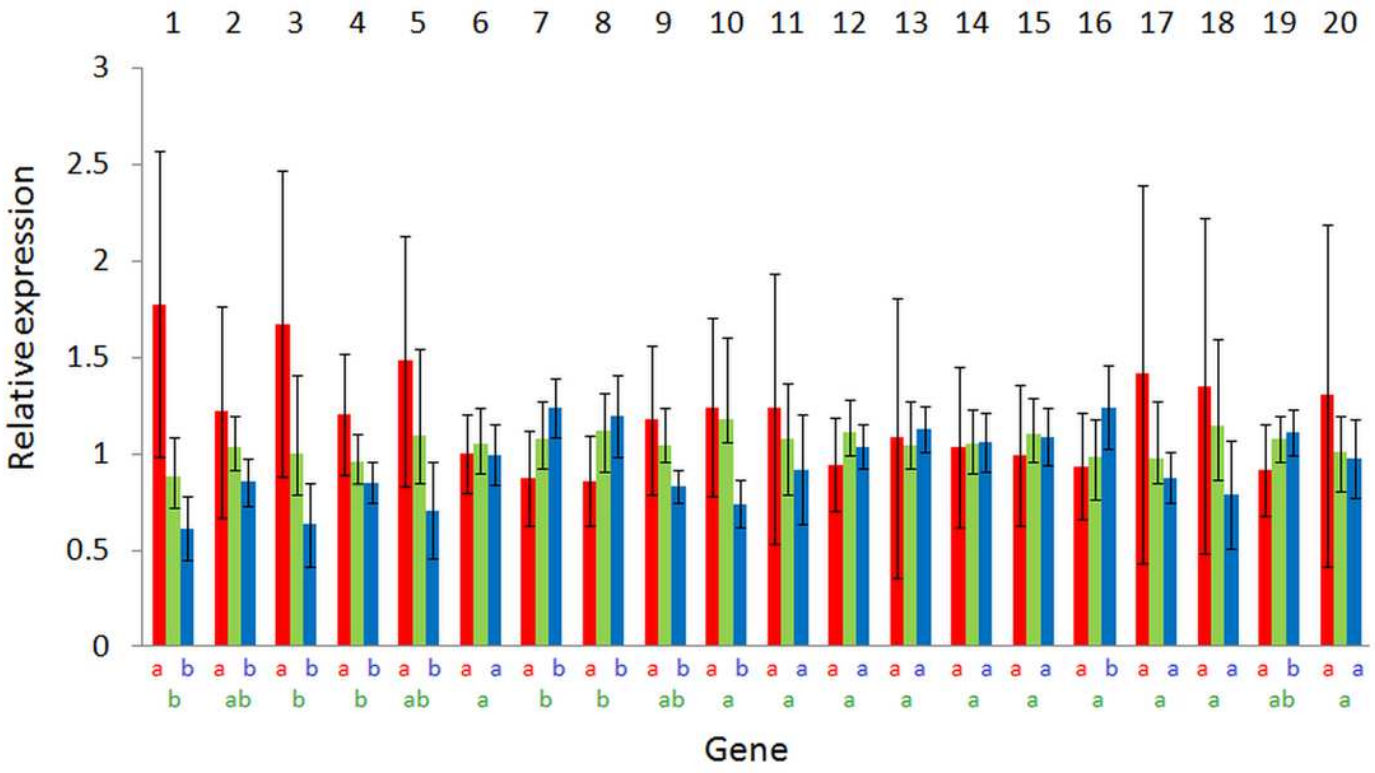
Expression variations according to salinity of the 20 significant genes for T animals. Values are expressed as relative expression ± SD. Original sequence names may be found in table 1. Bars are colored according to the salinity condition (red: freshwater; green: seawater; blue: hypersaline water). Identical letters indicate no significant differences (according to a Tukey–Kramer's post-ANOVA test) between salinities.



3

Figure 3

Expression variations according to salinity of the 20 significant genes for B animals. Values are expressed as relative expression ± SD. Original sequence names may be found in table 1. Bars are colored according to the salinity condition (red: freshwater; green: seawater; blue: hypersaline water). Identical letters indicate no significant differences (according to a Tukey–Kramer's post-ANOVA test) between salinities.



4

Figure 4

Principal component analysis of the 6 fish groups according to their gene expression pattern. The first axis accounted for 87.20% of the total variance and the second axis for 5.40%. The ellipses include 95% of the variance within each group, and the stars represent the gravity center of each ellipse. According to a Tukey-Kramer's pairwise comparison test performed on the principal component scores of the first axis, freshwater animals (group 0) are significantly differentiated from those of groups T35 (P = 0.024), T70 (P = 0.0015) and B70 (P = 0.0004).

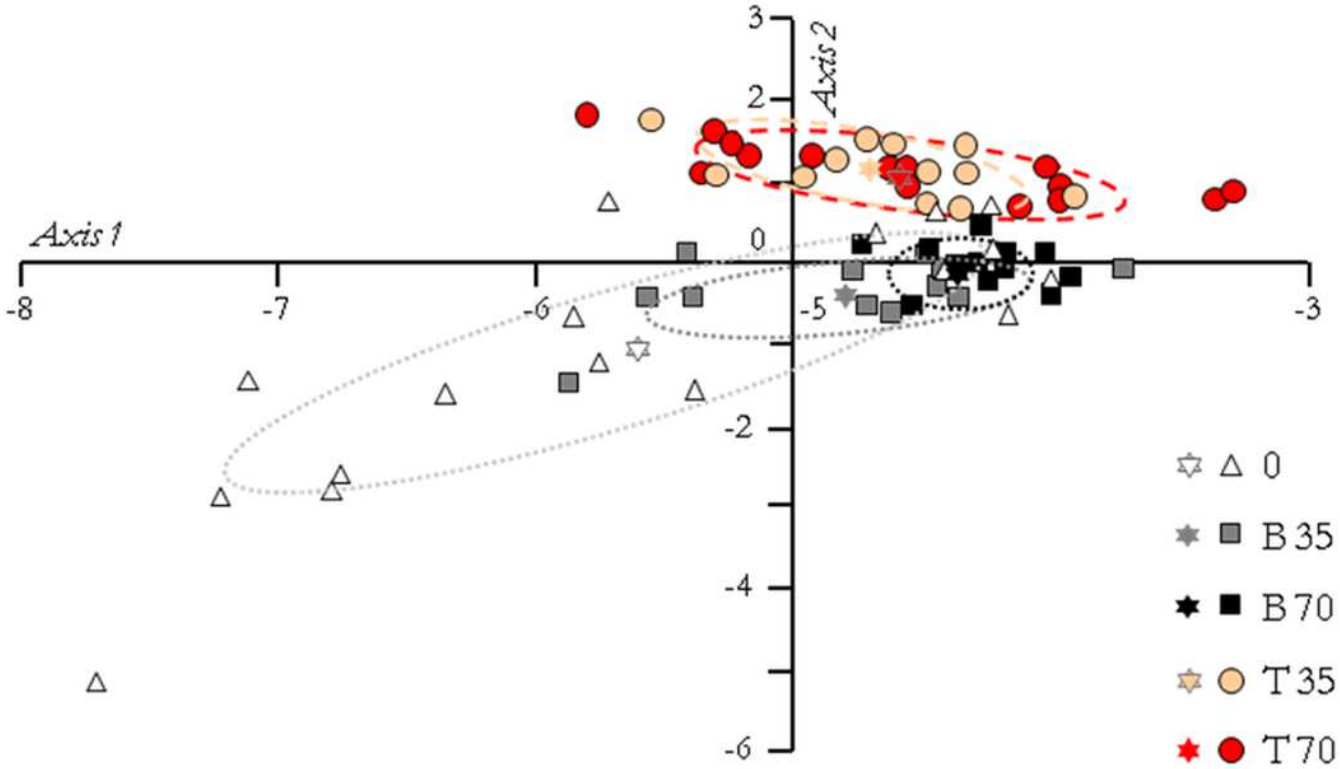


Table 1(on next page)

Table 1

List of genes showing significant differential expression between salinities in the two fish generations (T and B) and corresponding statistical values

Table 1. List of genes showing significant differential expression between salinities in the two fish generations (T and B) and corresponding statistical values

Original sequence name[a]	Gene #[b]	Corrected ANOVA *p*-values	
		T animals	B animals
Contig_Tilapia_90_6346	1	1.34E-03	3.12E-05
Contig_Tilapia_90_8891	2	8.76E-03	7.60E-02
Contig_Tilapia_90_947	3	1.46E-01	6.70E-04
Contig_Tilapia_90_6938	4	1.29E-02	1.97E-03
Contig_Tilapia_90_21432	5	3.84E-01	3.17E-03
Contig_Tilapia_90_1393	6	7.53E-05	7.67E-01
Contig_Tilapia_90_10643	7	3.13E-02	1.06E-03
Transcript_AVA3_33497	8	1.28E-05	1.60E-03
Transcript_AVA1_24409	9	2.02E-04	1.75E-02
Contig_Tilapia_90_26617	10	1.29E-02	1.03E-02
Contig_Tilapia_90_2414	11	3.38E-02	3.35E-01
Contig_Tilapia_90_2253	12	1.29E-02	1.08E-01
Contig_Tilapia_90_2777	13	1.29E-02	9.62E-01
Contig_Tilapia_90_8343	14	1.05E-02	9.73E-01
Transcript_AVA1_58357	15	3.38E-02	5.67E-01
Contig_Tilapia_90_26561	16	1.34E-03	1.07E-02
Contig_Tilapia_90_27008	17	7.76E-04	1.08E-01
Contig_Tilapia_90_1736	18	1.32E-02	1.08E-01
Contig_Tilapia_90_7359	19	1.54E-04	2.65E-02
Contig_Tilapia_90_2321	20	7.53E-05	3.35E-01

[a] Names of the sequences as they appear at http://asahttp.drim.com/tilapia/tilapia_menu.php.

[b] This gene numbering is used in Table 2, figures 2 and 3 and all along the manuscript in order to facilitate reading.

Table 2(on next page)

Table 2

Annotation features of the 20 responsive and 4 reference genes

Table 2. Annotation features of the 20 responsive and 4 reference genes

Gene #	Sequence length (nt)	#Blast X hits[a]	Protein description[b]	Best hit accession	E-Value	#GO terms
Differentially expressed genes						
1	353	16	PREDICTED: type-4 ice-structuring protein-like	XP_004549213	1.06533E-61	2
2	584	20	PREDICTED: tubulin-specific chaperone A-like	XP_003455155	4.68328E-50	5
3	524	20	sodium/potassium-transporting ATPase alpha-1 subunit	AGO02179	4.12826E-87	20
4	289	20	Serine/threonine-protein phosphatase 6 catalytic subunit	ELW48549	1.44788E-46	6
5	164	20	PREDICTED: sperm plasma glycoprotein 120	XP_004574936	4.12231E-23	0
6	849	20	PREDICTED: proteasome subunit alpha type-5-like	XP_003441568	8.42686E-175	19
7	459	20	PREDICTED: neuroserpin-like	XP_004561427	1.13288E-76	4
8	177	1	Hypothetical protein	XP_004563130	5.19086E-18	0
9	260	20	PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3-like	XP_003457472	8.68852E-26	2
10	179	20	NADH dehydrogenase subunit 1	ADR10264	3.32857E-21	4
11	604	20	PREDICTED: hypoxia-induced gene domain family member 1A-like	XP_003438136	4.44514E-47	1
12	545	20	heat-shock protein 90	CAX33858	8.51839E-92	34
13	1010	20	heat shock protein 70	ACI42865	0.0	3
14	506	20	PREDICTED: glutathione S-transferase theta-1-like	XP_004572434	1.57519E-28	1
15	321	20	PREDICTED: calcium-binding protein 39-like isoform X1	XP_004573664	6.54477E-61	8
16	230	20	beta tubulin	BAD11697	7.71535E-48	7
17	313	20	PREDICTED: MORC family CW-type zinc finger protein 2A-like	XP_004544577	5.08988E-31	2
18	1105	20	PREDICTED: nucleolar protein 56-like	XP_004545283	3.59979E-151	8
19	465	20	PREDICTED: 28 kDa heat- and acid-stable phosphoprotein-like	XP_003443172	4.92124E-54	4
20	512	0	-	-	-	0
Reference genes						
R1	291	20	PREDICTED: NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial-like	XP_003452502	5.14723E-58	8
R2	299	20	PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial-like	XP_004571622	7.27748E-52	6
R3	265	20	PREDICTED: tubulin beta-4B chain	XP_004005609	1.57111E-59	21
R4	323	20	PREDICTED: cytochrome c oxidase subunit 6C-1-like	XP_003451899	3.40807E-36	6

[a] The number of BlastX hits was limited to 20.

[b] According to the best blast hit.