**Improving salinity tolerance in tilapias: A REVIEW**

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**Abstract**

With increasing scarcity of fresh water available for aquaculture, especially in arid regions, development of tilapias that tolerate high salinity would increase fish (and hence, animal protein) production. We review culture practices, nutrition, physiology and genetics, and propose approaches to improving salinity tolerance in tilapias. Dietary supplementation with NaCl and optimized acclimation protocols are immediate and practical ways to improve salt tolerance. Inter-specific variation in salinity tolerance may be used to select salt-tolerant species and develop salt-tolerant hybrids. Physiological studies of biochemical pathways underlying phenotypic differences in salt tolerance can lead to genetic studies of intra- and inter-specific variation. Molecular technology can lead to studies on osmoregulation-related biochemical pathways, for which the euryhaline tilapia is an attractive model. Functional genomics and proteomics are powerful tools for studying the molecular bases of environmental adaptation and metabolic connections to osmoregulatory physiology. Both provide avenues for discovering novel pathways related to osmoregulation with relevance to aquaculture. In the long term, quantitative trait loci associated with, or genes involved in saltwater tolerance may facilitate marker-assisted or gene-assisted selection for this trait in tilapia.

We recently examined the possible interrelationships between the allelic polymorphism of the tilapia Prolactin gene and growth performance in brackish water*.* Comparative sequencing revealed one amino-acid substitution at a highly conserved site adjacent to the receptor binding site, where the conserved leucine in *O. mossambicus* is replaced by phenylalanine in *O. niloticus*. We also studied allelic variation in a microsatellite marker at the promoter region of the Prolactin 1 gene. Two distinct alleles in *O. niloticus* as well as two additional alleles in *O. mossambicus* were detected. Parental

crossing was performed between *O. mossambicus* and *O. niloticus* in search for correlations between the allelic composition and growth performance of F2 families in brackish water. Correlation between genotypes (allelic composition) and growth performance was found in some but not all families. One allele appears to be associated with good growth in brackish water.

**INTRODUCTION**

With the increasing scarcity of freshwater available for aquaculture in general, and for tilapia culture in arid regions (like Israel) in particular, tilapias tolerating high salinity would increase global tilapia production (and hence, animal protein production) by expanding the range of production in many regions of the world. Cnaani and Hulata (2011) have recently reviewed the subject, aiming to show what can be learned from the past experience in areas of culture management practices and nutrition as well as physiology and genetics, and to propose the best approaches for improvement of salinity tolerance in tilapias. The present paper brings the highlights from that review, focusing on the more recent findings, and some results of additional work carried out recently in our laboratory.

**Physiological studies**

Control of salt and water balance within a narrow limit is critical to life in all multicellular organisms, including teleost fishes. Salt tolerance is a term describing the overall fitness, or productivity, of the fish in a saline environment. It is a combination of different quantitative traits, such as metabolism, growth, osmoregulation, immunocompetence and fecundity.

Inter-specific variation in salinity tolerance may be used to select salt-tolerant species and develop salt-tolerant hybrids. Growth of *O.* *niloticus* at high salinity is significantly lower than that in freshwater (Fineman Kalio, 1988), whereas survival is not affected by salinity. High salinity does seem to suppress, or at least delay, onset of reproduction in *O.* *niloticus,* thus presenting a practical method of population control.

Chloride cells (known also as Mitochondrion Rich Cells - MRC) in the gill epithelium are important osmoregulatory sites in all fish species. Their large surface area at both sides, the apical and basolateral, is a placement of ion transporting proteins such as sodium-potassium ATPase (Na+/K+-ATPase) and Na+/K+/2Cl− co-transporter (NKCC). Studies on changes in chloride cells characteristics and function in response to salinity challenges, mostly in *O. mossambicus*, revealed a significant increase in the abundance of chloride cells and in ion transporters activity in the gills (Fiess et al., 2007). Differences in ion transporters type and membrane location on the chloride cells were also found between fresh- and salt-water challenged fish.

Hormones of the neuroendocrine system are essential players in the control of osmoregulatory mechanisms, and extensive studies on endocrine pathways involved in osmoregulation clarified the role of prolactin (PRL) and growth hormone (GH) in osmoregulation. PRL and GH are closely related and thought to be derived from the same ancestral gene. They exhibit a variety of functions in growth, development, osmoregulation and reproduction that are variously distinct, overlapping or opposing (Sakamoto and McCormick, 2006; Mancera and McCormick, 2007). Tilapias possess two PRLs that are encoded by separate genes (Specker et al., 1985). One form of PRL, PRL177, binds to the GH receptor and has somatotropic actions of stimulating growth and cell proliferation (Shepherd et al., 1997). GH is produced and secreted from the anterior pituitary gland and has been shown to have an osmoregulatory role in seawater, where it promotes ion regulation by stimulating chloride cell proliferation and up-regulating ion transporters tied to extrusion pathways (Sakamoto and McCormick, 2006; Mancera and McCormick, 2007). Several isoforms of the PRL receptor exist in tilapia, with unique intracellular signaling pathways (Fiol et al., 2009). It is becoming increasingly apparent that the existence of an array of receptor subtypes has a major role in the pleiotropic nature of GH and PRL.

Osmoregulation, somatic growth, and reproduction are among the most energetically costly metabolic activities engaged by teleost fishes. Boeuf and Payan (2001) discussed four possible pathways of interaction between osmoregulation and growth: (1) difference in standard metabolic rate, (2) increase in food intake, (3) increase in digestibility, and (4) hormonal stimulation. These four pathways can interact, and none can be considered as a unique route connecting osmoregulation and growth.

Growth and development are directed by an integration of environmental, physiological and genetic factors. The high energetic cost of osmoregulation, usually estimated as 25-50% of metabolic output, means that there is a link between osmoregulatory and growth capacities. This might explain the observation that growth and osmoregulation are governed by many of the same hormones, notably PRL and GH. It has been demonstrated that genetic variation in the tilapia PRL gene is associated with differential gene expression and growth rate in saline water (Streelman and Kocher, 2002).

Functional genomics (i.e., the field of molecular biology that attempts to answer questions about the function of DNA at the levels of genes, RNA transcripts, and protein products) and proteomic (i.e. the study of the entire complement of proteins, particularly their structures and functions) approaches represent powerful tools for gaining insight into the molecular bases of environmental adaptation. Gene transcripts for ion transporters, enzymes, hormones and components of cellular stress signaling were characterized in the brain, gill, gut and kidney of Mozambique tilapia (Fiol et al., 2006) and black-chinned tilapia (D’Cotta et al., 2006; Tine et al., 2008). The transcriptional response to tilapias salinity challenge was studied for the immediate and long term response in two highly salinity tolerant species. In the Mozambique tilapia genes involved in the immediate hyperosmotic stress response were analyzed in gill epithelial cells. Most genes show an immediate response with peak levels observed between 2 and 8 h after seawater transfer. Pathway analysis of the newly identified genes revealed that more than half of the identified immediate hyperosmotic stress genes interact closely within a cellular stress response signaling network. The genes cluster together in six molecular processes that are rapidly activated in tilapia gills upon salinity transfer: (1) stress response signal transduction, (2) compatible organic osmolyte accumulation, (3) energy metabolism, (4) lipid transport and cell membrane protection, (5) actin-based cytoskeleton dynamics, and (6) protein and mRNA stability (Fiol et al., 2006). In the black-chinned tilapia genes whose transcription is induced by 45 days acclimation to either hyper-saline waters or to fresh water were analyzed in the gills. The suppression subtractive hybridization (SSH) resulted in the isolation of a wide spectrum of differentially expressed genes, classified according to functional annotations. These genes were clustered into 14 functional categories of biological processes. Cellular processes, metabolic processes, and localization were the most abundant categories in the high salinity library (D’Cotta et al., 2006; Tine et al., 2008).

**Approaches to improving salt tolerance in tilapias**

***Adding salt to feed*:** Saltwater survival of *O. mossambicus* improved by 84%after two weeks of feeding the salt diet, and that of the *O. aureus* x *O. niloticus* hybrids by 62%. Three weeks of feeding the salt diet were required to improve survival of *O. spilurus* by 50%. Contrary to the sudden increase in plasma osmotic concentration recorded in the fish transferred directly from freshwater to 60% sea water, feeding the high-salt diet prior to the transfer resulted in only a slight increase in the plasma osmotic concentration in sea water (Al-Amoudi, 1987).In all-male tilapia hybrids (*O. aureus* X *O. niloticus*) fed with feed that did not contain fish meal, 3% dietary salt supplementation resulted in about 20% improvement in specific growth rate and feed conversion ratio while cultured in freshwater for two months (Cnaani et al., 2010).

***Acclimation***: *O. mossambicus* can be acclimated in a single step (intermediate salinity directly to final full sea water salinity) and requires only one day at the intermediate salinity for sea water acclimation with no mortality, *O. aureus* requires four days and *O. niloticus* eight days for acclimation (Perschbacher, 1992). Yao et al. (2008) investigated the best conditions for transfer of Nile tilapia (*O. niloticus*) from freshwater to salt water. Fingerlings (8 to 12 g) were transferred, either directly or gradually, from freshwater to water of variable salinities, and survival was monitored after 3 weeks. Survival of fish transferred directly to saline water was high (84.3% to 96.8%) until 17 ppt, but mortalities were significant (60-70%) above that salinity. High rate of survival (78 to 81%) was, however, achieved by gradual acclimation to salinity of 30 ppt over two days.

These management practices are useful, yet genetic approaches may be more sustainable.

***Variation among species and hybrids:*** Villegas (1990) found that *O. niloticus* was significantly less saline-tolerant than *O. mossambicus* and their reciprocal F1 hybrids. Similarly, Kamal and Mair (2005) evaluated *O. niloticus, O. mossambicus* and *O. mossambicus*x*O. niloticus*hybrids over a series of salinities. *O. niloticus* exhibited faster growth at low salinity and *O. mossambicus* at the higher salinities; the hybrid was superior to *O. mossambicus* at all salinities and to *O. niloticus* at salinities above 10 ppt.

Several red tilapias, such as the Taiwanese (Cheong et al., 1987), Florida (e.g., Thourad et al., 1990; Watanabe et al., 1990; Ernst et al., 1991; Head et al., 1996), Philippine (Romana-Eguia and Eguia, 1999) and Thai (Yi et al., 2002) strains, originating by hybridization of either *O. niloticus* or *O. mossambicus*, are also considered saline-tolerant. Significant differences in the growth among five strains of Asian red tilapia (*O. mossambicus* or *O. mossambicus*-*hornorum* hybrid crossed with *O. niloticus*) were found when grown in fresh, brackish and salt water (Romana-Eguia and Eguia, 1999) using *O. mossambicus* as a reference strain. However, they also observed a significant interaction between strain and rearing condition. Ignoring the interaction effects, their results suggested that overall growth in length was more rapid in brackish water (17 ppt) than in either freshwater or salt water (34 ppt).

***Variation within species*:** To the best of our knowledge, there is not much divergence for salt tolerance within Nile tilapia (*O. niloticus*). Selection of more salt-tolerant strain(s)/population(s) of *O. niloticus* would be based on documenting the salinity tolerance of various wild stocks in their native waters, and comparing them under standard conditions. Basiao et al. (2005) evaluated three commercial strains of *O. niloticus* for growth rate in freshwater and saline water (32 ppt) relative to an 'internal reference' population and found significant strain effects on specific growth in standard length in both saline and freshwater environments.

***Hybridization*:** A saline-tolerant hybrid, produced by crossing the salt-tolerant *O. mossambicus* with a commercial ND9 line is characterized by good growth rate and high salinity tolerance. Males of this F3 hybrid were crossed with orange-colored females of the ND5 commercial line, resulting in a mostly homogenous red tilapia with good growth rate and salinity tolerance, termed ND60. The good performance qualities were confirmed in growth trials in tanks (compared to a commercially-cultured hybrid of *O. niloticus* x *O. aureus*) and in commercial sea-cages. Consequently, this hybrid was introduced into a brackish water farm in Surinam and a marine farm in Guatemala (Lahav and Ra'anan, 1997).A synthetic strain of fast-growing tilapia with high salinity tolerance that breeds naturally in brackish water was developed in the Philippines starting in 1999 through a series of repeated backcrosses of the saline-tolerant *O. mossambicus* to the hybrids, coupled with selection for growth rate, and was named "molobicus". The first stage of the project produced a hybrid population that was 1/3 *O. niloticus*, 2/3 *O. mossambicus* with a good salinity tolerance (Mateo et al., 2004; Rosario et al., 2004).Another inter-generic hybrid of interest regarding salinity tolerance was produced by artificial propagation - the two reciprocal hybrids between the fast-growing *O. niloticus* and the highly euryhaline *S. melanotheron* (Toguyeni et al., 1997; Baroiller et al., 2000). Both hybrids were viable and fertile, and their growth rate was intermediate to that of the two parental species, but their relative salinity tolerance was not reported.

***Selective breeding*:** Four *Oreochromis* species were used in an evaluation of salinity tolerance conducted by Tayamen et al. (2002) in the Philippines. A diallel cross of the different species/strains was carried out involving *O. spilurus*, *O. aureus*, *O. mossambicus* and three genetically improved strains of *O. niloticus*,namely: sixth-generation improved GIFT strain, FAC selected line (FaST), and all-male YY tilapia. Progenies from the 27 cross combinations (5 purebreds and 22 crossbreds) were evaluated in 10 environments with different salinity levels and agro-climatic conditions using a communal rearing design. Among the different cross combinations reared across environments, *O. aureus x O. spilurus gave the highest body weight and O. mossambicus x O. spilurus, the highest survival rate.* Tayamen et al. (2004) continued the selection program by breeding the selected fish and testing the progeny in different culture systems. Salinity tolerance in terms of growth and survival was positively influenced by having *O. spilurus* as sires, while *O. niloticus* FaST dams contributed most to increased growth rate. Different rankings in terms of growth and survival were obtained across environments. The second stage of the "molobicus" project was initiated in 2003 with selection process based on a simple within-family selective breeding scheme in a saline environment (Rosario et al., 2004). Fish of the first selected generation of "molobicus" currently are used on a small scale in the Philippines, while the selection process is going on (P. Morissens, CIRAD, France, and W. Rosario, BFAR-NIFTDC, Philippines, pers. comm.).

Armas-Rosales (2006) took a quantitative genetics approach to evaluate genetic effects influencing tilapia salinity tolerance using a diallel mating design. Six parental strains were used [*O. aureus*, *O. mossambicus*, *O. niloticus*, Stirling red *O. niloticus*, Florida red tilapia (originated from an *O. urolepis hornorum* x O. *mossambicus* male hybrid) and a commercial hybrid (originated from the Rocky Mountain White® tilapia)], resulting in 36 genetic groups. Twenty-four salinity levels were used in the growth trial. Salinity tolerance was determined for all strains and crosses, and genetic effects influencing salinity tolerance were estimated. Several lines exhibited highly significant line and maternal effects. Several crosses exhibited highly significant heterosis effects. The results suggest that improvement in salinity tolerance could be accomplished by developing a breeding program combining selection, hybridization and backcrossing among *O. aureus, O. mossambicus* and Florida red tilapia. Experiments were conducted at the Research Institute for Aquaculture No.1 (RIA1), northern Vietnam, to evaluate the growth and survival of the GIFT and Vietnamese strains of Nile tilapia in fresh and brackish water earthen ponds. The heritability estimates for harvest weight in both test environments were moderate (~0.2) for both brackish and fresh water. The genetic correlations of harvest body weight and survival were relatively low (>0.4) between the two test environments. The results suggest a substantial additive genetics variance for the traits that can be further exploited through a selective breeding program. However, in view of the strong genotype by environment interaction for harvest weight and survival traits observed, separate breeding programs should be considered for Nile tilapia in fresh and brackish water farming (Luan et al., 2008).

***Genomic approaches***: Genomic approaches may offer contribution to aquaculture over a longer term. With the application of modern molecular biology techniques, it may be possible to identify genes encoding specific proteins active in salt-tolerant species that are lacking or are less active in less-tolerant species, or specific proteins that are induced under salt stress. One such gene is prolactin1 (*prl1*); this gene has a central role in adaptation of marine species to freshwater by reducing Na+/K+-ATPase activity and consequently increasing the osmotic level of the plasma (e.g., Sakamoto et al., 1997). Streelman and Kocher (2002) reported that microsatellite polymorphism in the tilapia *prl1* promoter is associated with differences in *prl1* gene expression and growth response of salt-challenged fishes. They crossed females of the salt-tolerant *O. mossambicus* (homozygous for long alleles) with a freshwater-adapted *O. niloticus* male heterozygous for microsatellite alleles that differed by 17 repeat units (CA31 vs. CA14). Fish homozygous for the long allele grew more slowly at 16 ppt and their weight was only half those of the other two genotypes, while in freshwater growth rate did not differ significantly among the three genotypes.

We have recently re-examined this association in nine F2 families of *O. mossambicus* X *O. niloticus* hybrids (Velan et al., 2011). Both parental fish were heterozygous for different alleles (CA33 and CA38 in *O. mossambicus*, CA30 and CA35 in *O. niloticus*, resulting with PCR products of 253, 263, 247 and 257 bp, respectively). The association reported earlier by Streelman and Kocher (2002) was observed in only three of the nine families. In two of those three families, full-sibs were also grown in freshwater where no correlation between the genetic polymorphism and growth was found. In these two families, fish carrying the allelic combination 247/253 grew better in saline water and worst in fresh water (Figure 1).



**Figure 1.** Weight of the four genotypes of offspring in one family, grown in salt water (dark bars) and fresh water (light bars). Groups sharing the same letter are not significantly different (α = 0.05).

We concluded that this variation is probably not a major contributor to the total genetic variation in salinity tolerance, and that there may be a large environmental influence underlying the differential growth in saline water. We have sequenced the *prl1* gene in the parental species and discovered one

point mutation, within a conserved motif, that cause substitution of phenylalanine with leucine and forming two isoforms of the tilapia *prl1* (Figure 2).



**Figure 2.** Comparison of *prl1* sequences between *O. niloticus* (top) and *O. mossambicus* (bottom) alleles.

The F31L mutation within a conserved motif is marked.

Rengmark et al. (2007) identified several candidate genes associated with salt tolerance in tilapia through a study that identified a number of genes differentially expressed in saltwater and freshwater – *beta haemoglobin, Ca2+‏ transporting plasma membrane ATPase, pro-opiomelanocortin* (all up-regulated in saltwater) and *beta-actin* (down-regulated). Rengmark and Lingaas (2007) investigated the role of transferrin, an iron-binding glycoprotein known to have an important role in the immune system, on salinity tolerance. They cloned and sequenced the entire transferrin gene of tilapia, and identified two microsatellites closely linked to the gene as well as many single nucleotide polymorphisms (SNPs) within it. Studies of the segregation of alleles in these two closely-linked microsatellite loci showed that they defined two haplotypes (combinations of alleles); salt-tolerant individuals showed a strong tendency to possess haplotype 2, whereas the less salt-tolerant ones tended to possess haplotype 1. Expression levels of transferrin were compared in saltwater- and freshwater-reared tilapia using real-time PCR. Transferrin showed an 85% up-regulation in tilapia kept in saltwater compared to freshwater, suggesting that transferrin may be involved in saltwater tolerance or that closely-linked genes may be directly involved in saltwater tolerance. This gene was partially cloned and mapped to linkage group (LG) 21 of the tilapia linkage map by Cnaani et al. (2002), LG18 in the more recent Lee et al. (2005) map.

**ConclusionS**

Studies on the molecular basis of osmoregulatory properties of the gills, kidney, gut and brain have revealed a wealth of genomic knowledge that can lead to genetic studies of intra- and inter-specific variation for salinity tolerance. Once relevant genes are identified, genetic polymorphisms can be searched for in cultured and natural populations. The emerging knowledge of quantitative trait loci (QTL) associated with, or genes directly involved in saltwater tolerance may facilitate marker-assisted or gene-assisted selection for this trait in tilapia in the future. Hence, the two routes that hold the keys for improving salinity tolerance are: (1) exploring and revealing biochemical pathways and gene networks involved in osmoregulation, thereby realizing a better understanding of both the salt tolerance phenotype and the genotypic background; and (2) screening domesticated and natural populations, searching for genetic variation in the biochemical pathways that underlie the observed phenotypic differences. Knowledge so gained can be exploited in selective breeding of tilapia stocks performing well in saline waters.

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