Global Veterinaria 3 (1): 15-21, 2009
ISSN 1992-6197

Expression Analysis on Some Genes in Hybrid Tilapia Following Transfer to Salt Water

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Abstract: The growth enhancement and osmoregulatory roles of growth hormone (GH), prolactin (PRL) and Insulin –like growth factor-1 (IGF-1) were examined in selected and non-selected tilapia in relation to water salinity with special emphasis on its impact on plasma IgM level.Three groups of fish (*Oreochromis niloticus, O. aureus* and their hybrid) were raised in 50% salinity treatment for one week, then transferred to full-strength saltwater (35 ppt) for 30 days. Growth rates were examined, plasma GH, PRLs, IGF-1 and IgM levels were assayed by ELISA. RT-PCR was performed on liver and pituitary tissues to examine the role of GH, PRL and IGF-1 in salinity. Results showed that growth was significantly increased in sea water (SW) than fresh water (FW) in all groups with higher value in F2 compared to non-selected fish. Plasma and mRNA GH and IGF-1 exhibited increased values in SW with higher values in the F2 individuals, where Plasma levels of PRL_{177} decreased in SW as compared to FW, in contrast to PRL mRMA levels which elevated in SW. It's suggested that GH and PRL_{177} have the same role in growth promoting but the priority for GH in SW. as well as in osmoregulation, but the priority in SW for PRL_{177}. Plasma IgM was significantly higher in non-selected tilapia compared to F2. It can be concluded that F2 individuals were of higher growth rates and salinity tolerants, but their immune response indicated by IgM value was lower than the F1 fish

Key words: Selective breeding · Growth hormones · Saltwater · RT-PCR

INTRODUCTION

Tilapia species of fish have positive aquacultural characteristics due to they are among the most important world's aquacultural finfish [1], their tolerance to poor water quality and the fact the they eat a wide rang of natural food organisms, in addition to their acceptable taste for the consumer. A multitude of studies have addressed means to grow bigger tilapia faster, through experimentation with water salinity, temperature, hybridization, administration of growth-promoting hormones and transgenesity [2-4].

Selective breeding is a strategy which would improve the quality and performance of fish in commercial aquaculture; also genetic markers are important tools which allow the tracking of percentage and performance lines in breeding programs.

Growth hormone (GH) and prolactin (PRL) belong to a family of hormones that share similarities in structure and function [5]. Based on the similarity in structure, function and consequently gene sequences; it has been proposed that these hormones evolved from a common ancestral gene through duplication and subsequent divergence [6]. GH regulates growth in all vertebrates, including fish [7]. Recently, GH has been implicated in seawater osmoregulation of salmonid and cichlid teleosts [8]. The action of the GH is mediated to a high degree through insulin-like growth factor (IGF-1) and its primary source is the liver [9].

Insulin-like growth factor (IGF_s) constitutes a family of polypeptides that interacts with GH and PRL to regulate the cell proliferation and other physiological functions [10]. PRL is the most versatile of the pituitary hormones. It shows lactogenic, luteotropic, mitogenic, somatotropic, metamorphic, antimetamorphic and osmoregulatory activities [11,12]. Among teleosts, The most prominent action of PRL is its osmoregulation in fresh-water adaptation [12].

Two forms of prolactin have been identified in the tilapia, PRL_{177} and PRL_{188}. Studies demonstrated that both hormones facilitate osmoregulatory adaptation to fresh water [13]. Moreover, PRL and GH are known to enhance the immune functions in fish [14], as well as mitotic activity of leucocytes of the chum salmon (*Oncorhynclius*

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keta) and are necessary to maintain circulating levels of immunoglobulin M (1gM) in the rainbow trout (*O. mykiss*) [15, 16]. In many teleost species, PRL maintain hydro-mineral balances in freshwater, whereas GH has been found to facilitate acclimation to seawater in several euryhaline species including salmonides and mosambique tilapia (*O. mossambicus)* [17]. In salmonides the immuonomodulatory effects of the GH seem to be related to its role in osmoregulation [18]. In this concept, the role of prolactin (PRLs), growth hormone(GH) and Insulin-like growth factor (IGF-1) genes family in growth promoting, salinity resistance and administration of some immune functions will be studied in the F_2 hybrid of females of *Oreochromis niloticus* and males of *O. aureus* compared to non selected individuals to evaluate the process of selective breeding.

MATERIALS AND METHODS

Selective Breeding: Individuals of F1 reared in earthen bonds of Abbassa Fish Farm were selected for breeding in condition that females' *Oreochromis niloticus* and males *O. aureus* were allowed to hybridize.

Experimental Design: A total number of 300 fish of three groups were used; group one (100 fish) *O. niloticus*, group two (100 fish) *O. aureus* of 15-60 g body weight and group three (100 fish) F2 progeny (produced from hybridization between *O.s niloticus* and *O.s aureus*) with an average weight of 30 g. These fish were collected from El-Abbassa fish farms in February 2007, transmitted to the NRC Laboratory in glass tanks. They were acclimated for two weeks prior to the experiments, supplied with a continuous flow of fresh water (FW) at 20 ± 2°C under natural photoperiod and fed daily with commercial dry diet approximately 3% of body weight per day. Fish were examined for growth rates and plasma hormones (GH, PRLs and IGF-1 factor) and IgM levels were assayed.

The Effect of Transfer from Fresh Water (Fw) to Salt Water (SW): Comparative studies between selected (F2 progeny) and non selected (*O. niloticus* and *O. aureus*) were carried out to examine different gene expression of GH, PRLs,, IGF-1 factor and IgM in relation to water salinity, 30 days after transfer from fresh water. Fish (No = 243) from the three mentioned groups with average weight of 15-60 g were raised in 50% salinity treatment in separate 100-liter tanks for one week, then transferred to full-strength saltwater (35 ppt) for 30 days. Temperature was maintained at 22±2°C.

Growth Rates Analysis: Growth rates were determined by measuring the mass of the fish (F2 progeny,*O. niloticus* and *O. aureus*) at 10 days intervals as described by [19].

Plasma hormones, plasma IgM and pituitary expression of PRLs GH and IGF-1: Afrer 30 days from the transfer from fresh to seawater, blood was collected by syringe treated with heparin, plasma was separated by centrifugation at 4.000 rpm for 10 min and stored at -20°C until analyzed. Plasma IGF-1 levels were assayed by a specific enzyme-linked immunosorbent assay enzymeconjugated to horseradish peroxidase using kits from DRG Diagnostics, Frauenbergstrasse, Germany as described by [20] with analytical sensitivity was calculated from the mean minus two standard deviations of ten replicate analyses of standard 0 and was found to be 1.292 ng/mL, CVs were 6.62 and 7.7% for intera and inter-assay-variation, respectively. Plasma levels of GH, PRLs and IgM were detected using kits from BioVendor GmbH, Germany. For GH, the analytical sensitivity was 1.6 pg/ml, Cvs were 3.65 and 3.93% for intera and inter-assay-variation, respectively. For PRL, measurement was within the range of 0.78–50 ng/ml, with respective Cvs of 2.3 and 4.6%. The range of sensitivity for IgM was 3.9 -250 ng/ml, results were read using a DigiScan ELISA Reader.

RNA Extraction and cDNA Synthesis for Expression Analysis: Total RNA from pituitaries and liver tissues from selected and non-selected individual formally frozen at -80°C were extracted using TRIzol reagent Promega as described by the manufacturer, quantified by spectrophotometery and reverse transcribed with power-script TM reverse transcriptase Clontech, PCR Primers were designed using the software primer 5 to amplifythe primers (Table 1).

RT-PCR: Single-chain cDNA was used for semi-quantitative reverse transcription-PCR (RT-PCR)

Table 1: Designed primers to amplify PRL177, PRL188, GH, IGF-1 and Beta-actin

1.PRL177	(5′ -CTATAGA GGG TT C GCG -3′, forward) (5′-GCA GGACAGCAGTTT GGTAA-3′, reverse).
PRL_{188}	(5′-GTCAGATTTGATGTCCCTGG-3′, forward) (5′-GCAGGACAGCAGAAAGTTGA-3′,reverse).
GH	(5′-CAGCTGTCGGTTGTGTGTTT-3′-forward) (5′-CAGCAGCAA GATTCCCGTTT-3′-reverse).
IGF-1	: (5′-ATAAACCAACAGGCTATGGC-3′ forward) (5′-TTCTGGT GGACTTCCTTGA-3′-reverse).
β-actin	(5'-GGTGGGTATGGGTCAGAAAGA-3′ forward) (5'-GCTGTCGTGAAGGAGTAG-3′-reverse).

reactions.The RT-PCR reaction was conducted using the sets of the forward and reverse primers designed, the PCR cycling parameters for PRL177, GH and β-actin were, 94°C for 5 min., 35 cycles of 94°C for 30 seconds, 56°C-59°C for 30 seconds, 72°C for 1 min and a final 72°C for 5 min. PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide, β-actin (a housekeeping gene) a commonly used internal standard product was used as control to ensure approximate quantities of templates in the reaction.

The gel images were digitally captured with a digital camera and analyzed with the NIH Imager beta version 2 program. The quantity and base pair size of the PCR generated DNA fragments were estimated relative to DNA ladder standards. The optical density (OD) was quantified using a scanning densitometer. RT-PCR values are presented as a ratio of the specified gene's signal in the selected linear amplification cycle divided by the β-actin signal (housekeeping gene) which was performed by measuring the photo stimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Japan).

Statistical Analysis: Statistical analysis were performed using Microsoft® Excell 2003

RESULTS

After the transfer from fresh water to saltwater, a significant(P= 0.05) increase in the mean body mass was detected in the species under investigation (Fig. 1) as compared to the control (Fish remained in the fresh water). These rates either in FW or in SW were higher in the F2 followed by O. *niloticus* then the O. *aureus*.

To investigate the mechanisms that might regulate the observed increase in the body mass after the transfer to the salt-water, plasma levels of GH, PRLs and IGF-1 factor were measured using ELIZA. Plasma levels of GH and IGF-1 were elevated (P > 0.05) in SW transferred fish as compared with the control or the non-transferred fish (Fig. 2 (1, 4)). Plasma levels of PRL_{177}were lower (P=0.05 in the SW transferred fish compared to the control (Fig. 2(2)), in contrast to the plasma values of PRL_{188} which showed no significant difference in the groups under investigation (Fig. 2(3)). Slight increase in the plasma level of IgM was noticed in SW transferred fish if compared to the control fish in the three groups under investigation. There was a higher immune response in *O.s niloticus* expressed by the plasma level of IgM followed by F2 individuals and finally the *O. saureus* (Fig. 2(5)).

RT-PCR showed that GH and liver IGF-1 mRNA levels using exhibited the same picture of the elevated values which was observed in the plasma after 30 days of FW transfer, where as higher (P<0.05) GH and liver IGF-1 mRNA levels were detected compared to the control fish (Fig. 3 (A and D)). Pituitary PRL_{177} and PRL_{188} mRNA levels exhibited an elevated values (P<0.05) in fish transferred to SW compared to that remained in FW (Fig. 3 (B and C)).

The picture of elevated plasma levels of GH and IGF-1 and the mRNA of these hormones were the same in the three groups of fish under investigation (*O. niloticu*; O. *aureus* and F_2 progeny), with same variations in the level of hormones and mRNA levels of expression, where after the 30 day transfer to the saltwater, the values of plasma GH, IGF-1 and PRLs increased in the three species but the most increased value was in *O. nitoticus* followed by F_2 progeny and finally the *O.s aureus*.

The results of RT-PCR showed an increase (P=0.05) in the mRNA values of GH, PRL177 and IGF-1 in SW transferred fish compared to the FW fish in the F_2 individuals followed by *O. niloticus* and finally the *O. aureus*.

DISCUSSION

Although it is well established that the tilapia raised in SW, grow faster than those in FW [16] one of our goals in this investigation was to study the effect of transfer from freshwater to saltwater on the selected fish (F_2 individuals) compared to non-selected fish in relation to the growth and osmoregulation processes. The present work showed increased growth rates of F2 fish as compared to F1 after SW transfer which indicates that the hybridization process product of fish was of higher growth rates than the non-selected individuals. These findings were consistent with the previous findings which showed that FW-reared tilapia grow more slowly than those reared in SW [19, 21].

Salinity was found to increase the food intake in tilapia especially, when fed on a limited ration, tilapia raised in SW grew to twice the size as those raised in FW, Furthermore, doubling the feeding ration significantly increased the growth of tilapia in SW, but not in FW [22]. In the present study, the tilapia of same body mass showed an increased growth rates after SW transfer, this suggests that it may be a consequence of an increase in food intake, or / and the availability of the energy needed for growth as the metabolic rate was lower in SW compared to FW [21,22]. This study is consistent also with the study of [23] who found that the transfer of

tilapia mossambicus from SW to FW for 40 days decreased the growth rates.

The analysis of the effect of SW transfer on growth rates and GH/ IGF-1 and PRLs in tilapia is difficult because these hormones are also involved in SW adaptation [8]. Moreover, [24] found that, in addition to its action on growth, GH also regulates energy metabolism. In fish, osmoregulation has been estimated to consume a high portion, 25 to 50% of the available basal metabolic energy in SW [25]. In the present study, fish transferred to SW exhibited an increased plasma and mRNA GH and IGF-1. So there is a positive correlation between the growth rates and the plasma and mRNA of GH and IGF-1. This condition may be explained in light of the low metabolic cost of osmoregualation in the F_2 fish than that of *O. niloticus* followed by *O.s aurens.*

It was found that when rainbow trout (*onceorhynchus mykiss*) was injected by bovine GH, it showed an increased oxygen consumption in FW [26]. Also, [21, 22] found that osmoregulation in FW is more metabolically costive than in SW, this may be in consistent with our findings, where the metabolic cost for osmoregulation in SW might be lower than in FW and indicates the availability of the metabolic cost of growth energy.

Insulin-like growth factor (IGF-1) is known to mediate several of the growth promoting actions of GH in teleosts [27-29]. Recently, IGF-1 has been suggested to play a role also in SW osmoregulation [30]. In this study, IGF-1 levels were significantly higher in SW transferred fish than those remained in FW after 30 days of the transfer, which seems to be in conflict with it's role in the stimulation of growth [19]. Plasma IGF-1 was significantly correlated with somatic growth during salmon smoltification, a time when salmon grow rapidly [31], plasma GH and IGF-1 levels are significantly elevated during puberty, a time of rapid growth and then return to pre puberty levels there after [32], these results are in consistent with our findings, the highest values of plasma GH and IGF were found in F_2 individuals followed by *O. niloticus* and finally the *O. aureus*, which might indicate the higher growth promoting action of GH in F2 individuals compared to the non-selected ones.

PRL is a member of the same hormone family as GH and have osmoregulatory actions in tilapia and other teleosts, treatment with PRL was found to reduce gill Na^+, K^+, ATPase levels that are consistent with its role as the freshwater osmoregualtory hormone [33].

Radioreceptor studies have undertaken by [5]to understand how GH and PRL exert their biological activity in tilapia and identified high-affinity, low-capacity binding sites for GH in tilapia liver t PRL_{177} binds to these sites with lower affinity than tGH. $tPRL_{188}$ doesn't compete for t GH binding sites, this study indicated also that $tPRL_{177}$ but not $tPRL_{188}$ can displace labeled tGH from it's receptor given the structural similarity of these hormones and their receptors.

In the present study plasma levels of PRL_{177} decreased in SW compared to FW remained fish, which might increase the level of the gill Na^+, K^+ and ATP ase that are in consistent with seawater adapting role of this hormone, this result is in consistent with studies of [5,33] on *O. niloticus*, they suggested that $tPRL_{177}$ in addition to its freshwater osmoregulatory role, may also be somatotropic in fresh water tilapia, where circulating levels were high. Collectively, these findings point to the existence of a GH/ PRL-IGF-1 axis in the tilapia. In contrast to plasma levels of PRL, GH and IGF-1, pituitary mRMA levels of GH, PRLs and IGF-1 elevated in SW transferred fish, which may be explained on the bases of the competition between GH and PRL_{177} on the binding sites.

The present study revealed a negative correlation between the body mass and the plasma levels of PRL_{177} but there was a positive correlation between the body mass and the pituitary mRNA of PRL_{177}, these results suggests that transferring from FW to SW, activates certain aspects of the PRL_{177} but it's role in growth is still depending on the competition results with the GH on the biding receptors sites, at the mean time the role of PRL177 in osmoregulation may need further studies on K^+, Na^+ and ATPase activities. It seems also that GH and PRL_{177} have different roles in osmoregulation in FW and SW, the two hormones have the same role in growth promoting but the priority will be for GH in case of SW transferred fish. GH and PRL_{177} may have the same role also in osmoregulation, but the priority here in SW transferred fish will be for PRL_{177}.

It is not clear why we failed to observe an elevated values for the PRL_{188}, where there was no detectable difference in the plasma PRL188 in SW compared to FW inter the species, the three species under investigation, but the highest value of plasma PRL188 was detected in *Oreochromis niloticus* followed by F2 individuals and finally the *Oreochromis aureus*, this might need also further studies.

This study also clarified the impact of the SW transfer on the immune system of the selected and non selected individuals exemplified by the plasma IgM to evaluate the process of the selective breeding regarding the immune function, in this study, it was clear that the immune response of the *O. niloticus* was higher than the F2 individuals followed by the *O. aureus* in SW. This

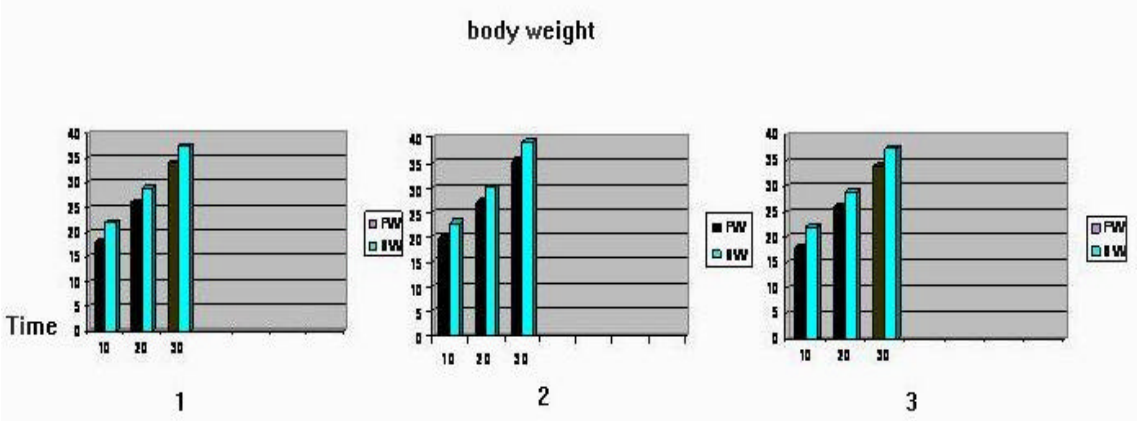

Fig 1: Changes in body weight of F2 progeny(1), *O. niloticus* (2)and *O. aureus*(3) following 30 days transfer from freshwater to salt- water; Dark column: freshwater; light column: salt-water

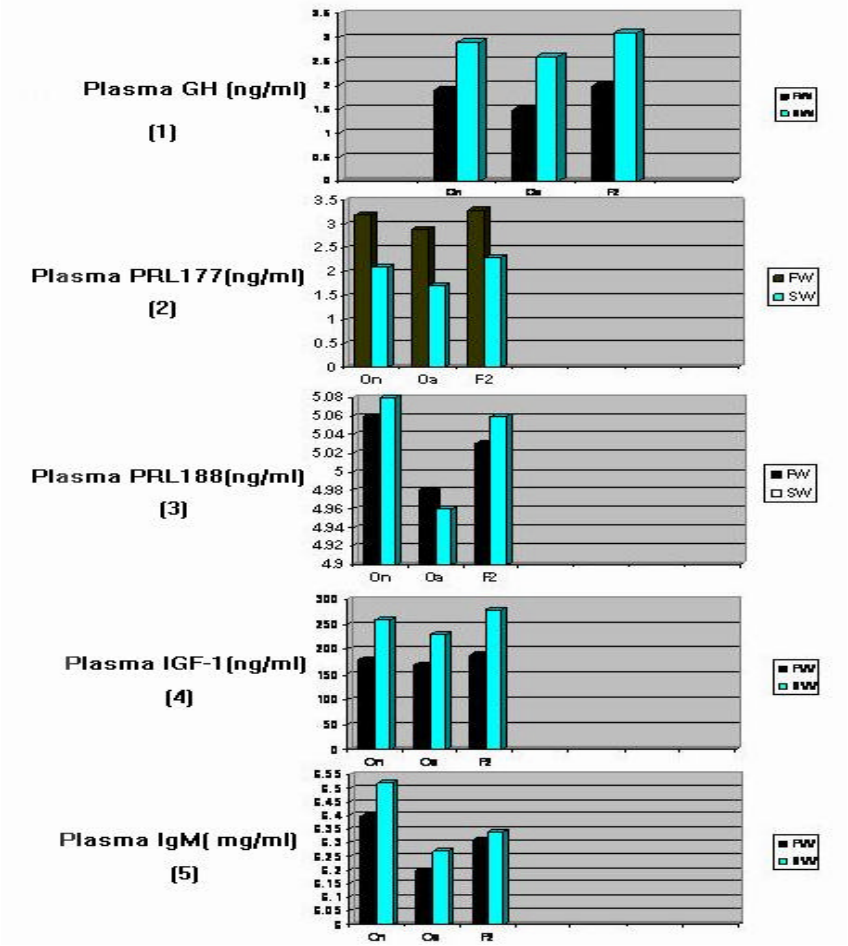

Fig. 2: Effect of SW transfer on plasma level of GH (1), PRL177(2), PRL188(3), IGF-1(4), and IgM (5) in O.n: *O.s niloticus*; *O. aureus* (O.a) and F2 progeny . Dark column freshwater; light column: saltwater. Significantly different from control fish in FW at $P<0.05$

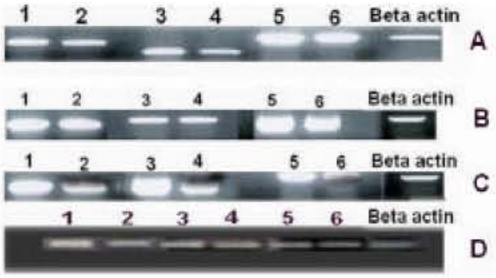

Fig. 3: Expression of pituitaries GH mRNA(A); PRL177(B); PRL188(C);and IGF-1(D) of F2 {SW:1, FW:2}; *O. niloticu* {SW:3, FW:4} and *O. aureus* {SW:5, FW:6}; β actin

result need further studies on the immune response against SW transfer. However, enhancement of the immune functions in high environmental salinity has been observed in salmonides [16]. An increase in the secretion rate of GH estimated from the clearance of administrated GH was observed during the course of SW acclimation of rainbow trout [8], this increased secretion in SW seems to facilitate not only osmoregulation but also immune function [18]. Stimulation by exogenous GH in parallel with the enhancement of hypo-osmoregulation in SW [16] tilapia is another euryhaline species in which environmental salinity stimulates GH secretion with GH having a hypo-osmoregulatory or SW-adapting effect [17].

it could be concluded that selective breeding between males of *O.s niloticus* and females of *O. aureus* as a genetic strategy to improve tilapia aquaculture, could result in high growth rates of F2 individuals, salinity tolerants, but the immune response of these individuals exemplified by the IgM level was lower than the F1 fish individuals.

ACKNOWLEDGMENT

This research was supported by a grant from NRC project No. 7120301

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(Received: 08/09/2008; Accepted: 25/10/2008)