Original Research Article

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# Stress Dampening Effect of Common Salt on *Oreochromis Niloticus* During Transport

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## Abstract

The present investigation was carried out to study the prolactin and growth hormones genes expression, interleukin (IL-1β) and Transforming growth factors (TGFβ-1a) genes expression of Nile tilapia (*Oreochromis niloticus*). Three groups; control (not transported), post transport with no salt and post transport with addition of the salt in an extremely 5-hour transport model were used. Inclusively, the studied parameters were intensely varied in the post transport with no salt fish group than post transport with salt fish group. PRL188 gene was significantly down regulated in the post-transport with no salt (0.67) and post-transport with the salt (0.88) fish groups**.** GH gene expression was significantly down regulated in the post-transport with no salt (0.37) and post-transport with salt (0.79) groups. IL-1β was up-regulated to 2.27 folds in the post-transport with no salt group and to 1.15 folds in the post-transport with the salt group and TGFβ-1a was also up-regulated to 9.65 folds in the post-transport with no salt and to 4.32 folds in the post-transport with the salt group. All results revealed that the transport has bad effects on the skin and the gene expression of growth and prolactin hormones, while the addition of 5g NaCl/L transport water mitigated the bad effect of transport moderately and preserved the surface skin features of transported *O. niloticus*.

**Keywords:** *O. niloticus*, Gene expression, Growth hormone, Prolactin hormones, Interleukin (IL-1β), Transforming growth factors (TGFβ-1a).

## Introduction

*Oreochromis niloticus* (Nile tilapia) is economic freshwater fish with annual global production exceeding 2.6 million metric tons in 2014 (FAO, 2016). Many fish aquaculture operations involve transportation of fish from one facility to another or during restocking practices, from a hatchery to rivers, lakes, or ponds. Transportation is thought to cause stress to fish and ends up in variety of physiological responses regard to endocrine, osmoregulatory, respiratory and immune systems, in addition to the fish behavior (Barton, 2011). Fish transport leads to many physiological responses including corticosteroids and glucose release that commonly were used as a marker for the stress in fish (Pankhurst, 2011 and Pottinger, 2008). A number of authors stated that gene expression of stress proteins is moderated by reaction to stress and the transcriptional reactions of these genes can be used as thoughtful biomarkers in bio monitoring of aquatic environs (Zhou *et al*., 2010; Sinha *et al*., 2012),

Transport procedures should be minimizing the stress (Dobšikova *et al.*, 2009). Adding of NaCl to transport water is a common practice in freshwater fish farms to mitigate the bad effect of transport (Takata & Luz, 2015), salt is cheap and simply used in fish farms and alleviates osmoregulation troubles throughout

transport (Crosby *et al.*, 2006 and Oyoo-Okoth *et al.*, 2011).

The purpose of this work is to examine the response of *O. nloticus* due to stress and the mitigation effects of 5g/L NaCl in transporting water through studying the cortisol level and the prolactin hormone, growth hormone genes expression, the pro-inflammatory interleukin 1β (IL-1β) and the anti-inflammatory transforming growth factors (TGFβ-1a).

## Materials and Methods

Experiments fish transportation:

The fish were gained from the tilapia breeding farm in the province of Asyut (Egypt). Fish were 100 grams, fish were sampled before (control) and after five hours transport incident (The transported groups were fish after transport in water without sodium chloride and the group after transport in water with 5 g/liter sodium chloride). The transport water was taken directly from the raft on which the fish were kept. The Fish were not anesthetized through transport. Twenty fish were sampled from each test group. Fish were sedated by MS-222 (Tirawat *et al.*, 2021) prior to blood and skin collection; Blood samples were collected from the caudal blood vessels using a non-heparinized sterile 3 mL needle and 1.5 mL Eppendorf tubes, the blood samples were allowed to coagulate at room temperature, then centrifuged at 1200xg for ten minutes, the serum was gently collected and kept at -80ºC until analysis. The fish is euthanized by spinal cord transection and the skin dissected, skin samples were conserved in RNA later and kept at -80 °C until analysis(Hoseini *et al*., 2019).

Gene expression studies using RT-qPCR

The RNA kit (Qiagen) is used to extract total RNA from the tissue sections (both challenged and well fish) according to the producer's protocol. RNA purity and concentration are determined at 260/280 nm in nanodrops (Thermo Scientific). cDNA synthesis is performed and assayed by 1.5% agarose gel electrophoresis by PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) according to manufacturer's protocol using 2.5 mg RNA as templates. The synthesized cDNA is then diluted 40-fold in nuclease-free water and stored at -80°C for further use. The expression of the Cytokines (IL-1β, Transforming growth factors (TGFβ-1a)) Prolactin and Growth hormone was studied in the Control, PT-S and PT+S groups by RT-qPCR using specific primers (Table 1). The comparative expression level of the genes will be determined agreeing to Pfaffl method (Pfaffl, 2001) as previously described (Tacchi *et al.*, 2013).

Oligonucleotide primers used in this study for qPCR

**Table 1:** Oligonucleotide primers used in SYBR Green real time PCR.

| Gene | Primer sequence (5'-3') | Reference |
|---|---|---|
| *EF-1α* | CCTTCAACGCTCAGGTCATC | Gröner *et al.*, 2015 |
| | TGTGGGCAGTGTGGCAATC | |
| *PRL-188* | AATGTGCCACACCTCCTCTC | Almanza 2014 |
| | CGGTAGGGCAGTGAAGTGAT | |
| *GH* | GAACTGATGCCAGCCATGA | Ber and Daniel, 1992 |
| *IL-1ß* | GCTGGAGAGTGCTGTGGAAGAACATATAG | Castro *et al.*, 2011 |
| | CCTGGAGCATCATGGCGTG | |

Statistical Analysis

Data are expressed as mean ± standard error. Data were submitted to one-way ANOVA test to identify statistically significant differences between groups. Statistically significant differences were considered if $p<0.05$.

## Results

Prolactin hormone gene expression (PRL188)

Prolactin hormone (PRL188) was significantly down regulated in the post-transport with no salt and post-transport with addition of the salt groups and recorded 0.67 and 0.88. The greater down-regulation was recorded in the post-transport with no salt group (Table 2 - Figures 1 & 2).

**Table 2:** Prolactin hormone gene expression in different fish groups after one way ANOVA ($p<0.0001$).

| Fish groups | Sample | Elongation factor 1 alpha (EF1α) | Prolactin (PRL 188) | |
|---|---|---|---|---|
| | | Cycle threshold (CT) | CT | Fold change |
| Control group | A1 | 19.23 | 20.09 | - |
| | A2 | 19.28 | 20.12 | |
| | A3 | 19.38 | 20.18 | |
| | Mean | 19.30 | 20.13 | |
| Post transport in water without salt fish group (PT-S) | B1 | 18.65 | 19.89 | 0.75 |
| | B2 | 19.14 | 20.58 | 0.66 |
| | B3 | 19.88 | 21.30 | 0.66 |
| | B4 | 19.27 | 20.77 | 0.63 |
| | Mean | 19.24 | 20.64 | 0.67** |
| Post transport in water with 5g/L salt fish group (PT+S) | C1 | 19.15 | 20.09 | 0.93 |
| | C2 | 20.47 | 21.53 | 0.85 |
| | C3 | 18.29 | 19.42 | 0.81 |
| | C4 | 21.11 | 22.03 | 0.94 |
| | Mean | 19.76 | 20.77 | 0.88** |

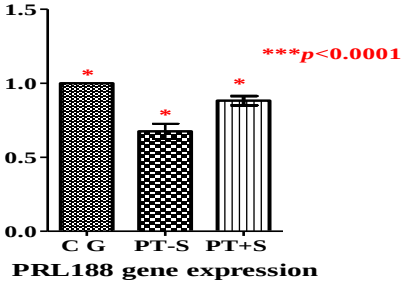


**Figure 1:** Changes in prolactin hormone (PRL188) gene expression in PT-S and PT+S skin measured by RT-qPCR. Data are expressed as the mean fold-change compared to the control skin group. Bars represent means ± standard error. There were highly significant differences between groups after One way ANOVA ($p$<0.0001).

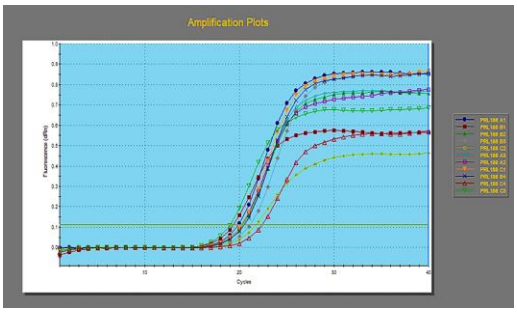


**Figure 2:** A diagram shows amplification plots of prolactin hormone (PRL188) gene.

## Growth hormone gene expression (GH)

Growth hormone expression was significantly downregulated in the post-transport with no salt and post-transport with salt groups. Greater down-regulation (0.37) was recorded in the post-transport with no salt group compared to post-transport with salt group (0.79) (Table 3 - Figures 3& 4).

**Table 3:** Growth hormone gene expression in different fish groups after one way ANOVA (p<0.0001).

| Fish groups | Sample | Elongation factor 1 alpha (EF1α) | Growth hormone (GH) | |
|---|---|---|---|---|
| | | Cycle threshold (CT) | CT | Fold change |
| Control group | A1 | 19.23 | 19.62 | - |
| | A2 | 19.28 | 19.70 | |
| | A3 | 19.38 | 20.00 | |
| | Mean | 19.30 | 19.77 | |
| Post transport in water without salt fish group (PT-S) | B1 | 18.65 | 20.62 | 0.35 |
| | B2 | 19.14 | 20.83 | 0.43 |
| | B3 | 19.88 | 21.70 | 0.39 |
| | B4 | 19.27 | 21.46 | 0.30 |
| | Mean | 19.24 | 21.15 | 0.37*** |
| Post transport in water with 5g/L salt fish group (PT+S) | C1 | 19.15 | 19.93 | 0.81 |
| | C2 | 20.47 | 21.36 | 0.75 |
| | C3 | 18.29 | 19.20 | 0.74 |
| | C4 | 21.11 | 21.80 | 0.86 |
| | Mean | 19.76 | 20.57 | 0.79*** |

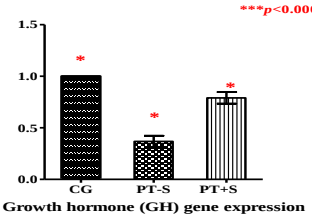


**Figure 3:** Showed the growth hormone (GH) gene expression in PT-S and PT+S skin measured by RT-qPCR. Data are expressed as the mean fold-change compared to the control skin group. There were highly significant differences between groups after One way ANOVA(p<0.0001).

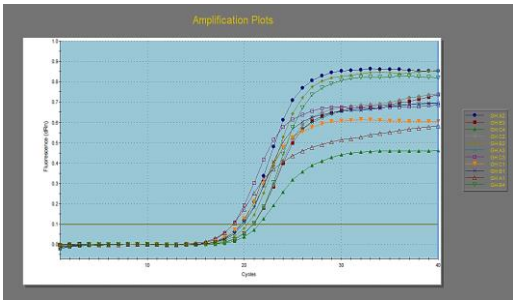


**Figure 4:** Amplification of growth hormone gene (GH) plots.

## Interleukin-1β and Transforming growth factors -β-1a genes expression.

The expression of pro-inflammatory cytokines such as interleukin (IL-1β), was up-regulated to 2.27 folds in the post-transport with no salt group and to 1.15 folds in post-transport with salt group. However, the expression of the anti-inflammatory cytokine Transforming growth factors (TGFβ-1a) was also up-regulated to 9.65 folds in the post-transport without salt group PT-S and to 4.32 folds in the post-transport with salt group (Table 4 - Figures 5 & 6).

**Table 4:** Skin cytokines genes expression in different fish groups according to Two-way ANOVA (p<0.0001).

| Fish groups | Sample | *Elongation factor 1 alpha (EF1α)* | Interleukin-1β *(IL-1β)* | | Transforming growth factor *(TGFβ-1a)* | |
|---|---|---|---|---|---|---|
| | | Cycle threshold (CT) | CT | Fold change | CT | Fold change |
| Control group | A1 | 19.23 | 21.39 | - | 22.28 | - |
| | A2 | 19.28 | 21.41 | | 22.32 | |
| | A3 | 19.38 | 21.55 | | 22.41 | |
| | Mean | 19.30 | 21.47 | | 22.35 | |
| Post transport in water without salt fish group (PT-S) | B1 | 18.65 | 19.85 | 1.96 | 18.48 | 9.32 |
| | B2 | 19.14 | 20.22 | 2.13 | 18.91 | 9.71 |
| | B3 | 19.88 | 20.61 | 2.71 | 19.57 | 10.27 |
| | B4 | 19.27 | 20.20 | 2.36 | 19.10 | 9.32 |
| | Mean | 19.24 | 20.22 | 2.27*** | 19.02 | 9.65*** |
| Post transport in | C1 | 19.15 | 21.19 | 1.09 | 20.15 | 4.14 |
| | C2 | 20.47 | 22.30 | 1.27 | 21.33 | 4.56 |

| | | | | | | |
|---|---|---|---|---|---|---|
| water with 5g/ salt fish group (PT+S) | C3 | 18.29 | 20.55 | 0.94 | 19.09 | 4.76 |
| | C4 | 21.11 | 22.84 | 1.36 | 22.20 | 3.89 |
| | Mean | 19.76 | 21.72 | 1.15*** | 20.69 | 4.32*** |

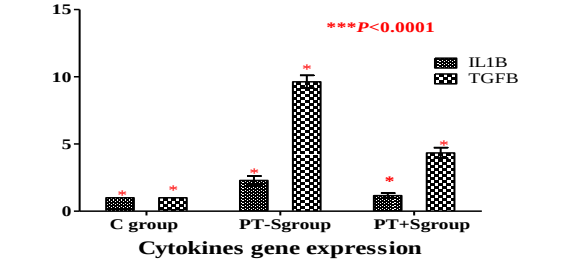


Figure 5: Changes in cytokines gene expression of IL-1β and TGFβ-1a in PT-S and PT+S skin measured by RT-qPCR. Data are expressed as the mean fold-change compared to the control skin group. Bars represent means ± standard error. There were highly significant differences between the groups.

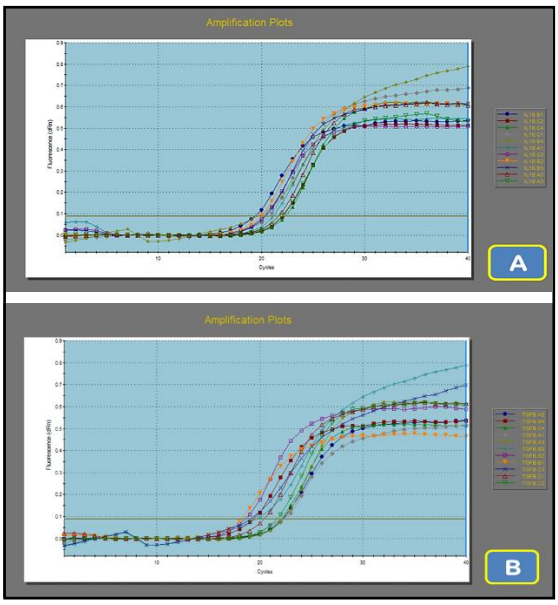


**Figure 6:** A & B diagrams show amplification plots of IL-1β and TGFβ-1a genes respectively.

## Discussion

Transportation of live fish is an unavoidable preparation in fish aquaculture (Harmon, 2009 and Vanderzwalmen *et al.*, 2019). Transportation processes include several pre-transport procedures and during transport procedures that represent stressful conditions to fish (Pakhira *et al.*, 2015). In the current study, Prolactin hormone (PRL) gene expression was significantly down regulated in the in the post-transport with no salt group and in the post-transport with salt groups and the greater down - regulation was recorded in the in the post-transport with no salt group (0.67 fold) compared to the in the post-transport with salt group (0.88 fold), these results may be due to the elevated level of plasma cortisol inhibited the PRL release from the pituitary gland (Uchida *et al.*, 2004). PRL helps in osmoregulation by controlling the activities of gill and the decrease in prolactin reduces the osmotic permeability of gills as well as increased mucus secretion (Saha, *et al.*, 2021).

There is a physiological link between stress and growth-related genes in the different fish species (Reinecke *et al.*, 2005). The present study clearly showed that the growth hormone gene expression was significantly downregulated in the in the post-transport with no salt group and in the post-transport with salt groups. Greater down-regulation was recorded in the fish of the in the post-transport with no salt group. Auperin *et al.* (1997) clarified that the confinement stress decreased the plasma GH in Nile tilapia. Nakano *et al*., (2013), Zahedi *et al.*, 2019 and Aksakal and Ekinci, (2021) stated that the acute physiological stress can down-regulate the expressions of growth-related genes. The GH gene expression down regulation of the *Nile tilapia* in in the post-transport with salt group was lower than that of in the post-transport with no salt group and was closer to the control group, this may be due to the adding of sodium chloride to the transport water of in the post-transport with salt group acted as a critical contributor to the stress and alleviates the negative effects of stress on the fish and may be involved in reallocation of metabolic energy from supporting growth toward the maintenance of homeostasis (Fox *et al*., 2006). The down regulation of GH was always companied with elevated levels of cortisol in the examined fish, this findings come in contact with Wehrenberg, *et al*., (1992).

The interleukin-1β (IL-1β) plays a central role in the initiation and regulation of immune and inflammatory responses (Hong *et al*., 2003); it is produced predominantly by lymphocytes, macrophages, and monocytes in response to microbial infections and acts as pro-inflammatory cytokine that promotes the inflammatory reactions. Our results showed that the IL-1β gene expression was significantly up-regulated to 2.27 folds in the post-transport with no salt group compared to the control group and revealed that the transported *O. niloticus* were exposed to stress and/or infection during transport. The greater and significant

up regulation of IL 1β in the post-transport with no salt group than that of the post-transport with salt group indicated that the transported *O. niloticus* in water without salt may be exposed to stress and bacterial invasion that stimulated the IL 1β production that acts as immune and inflammatory responses mediator and plays an important role in bactericidal activity in fish (Ma *et al.,* 2016; Hong *et al*., 2003 and Ren *et al*., 2020). The IL-1β was slightly up regulated to 1.15-fold in the post-transport with salt group in relation to the control group, this slight up regulation may be attributed to the stress mitigation effect of salt that alleviates the skin inflammatory reactions and decrease the fish pathogens attack.

Transforming growth factor-β (TGF-β 1a) constitutes of dimeric proteins that regulate the growth, differentiation and metabolism of many fish body cell types and plays a role in the inflammation (Funkenstein *et al.,* 2010). TGFβ-1a gene expression was greatly and significantly up-regulated in the post-transport with no salt group reporting 9.65 folds compared to the control group and significantly up regulated in the post-transport with salt group groups recording 4.32 folds comparing to the control group. The avoidance of a local inflammatory response in the skin of the post-transport with salt group fish group may be partially attributed to the induction of anti-inflammatory cytokine TGFβ-1a. Our result was confirmed by Tacchi *et al.,* (2015) who reported that anti-inflammatory effect in the skin of trout after transport in the water containing salt coupled with the up regulation of TGFβ-1a, it is possible that penetration of skin bacteria into the epithelium led to the induction of TGFβ-1a expression.

Generally, the results of this study reported a prominent anti-inflammatory effect in the skin of *Nile tilapia* transported for five hours in non-salt water (post-transport with no salt group) combined with greater up regulation of IL-1 and TGF-1a gene expression and greater downregulation of PRL and GH gene expression in addition to a significant increase in cortisol levels compared to both *O. niloticus* control and waterborne Fish group with 5gram NaCl/L transport water. A slight anti-inflammatory effect was observed in the post-transport with salt fish group compared to the *O. niloticus* control group and the post-transport with no salt fish group. These results showed that the post-transport with no salt *O. niloticus* group was subject to higher transport stress effect and microbial intrusion than the other two groups, the adding of salt to the transport water diminished the transport stress and reduced the likelihood of bacterial intrusion by restoring mineral equilibrium between fish and water and preserve the skin's superficial surface.

## Conclusion

The study results are of great importance for fish farming sector and underline the importance of skin health through transport, so we recommend the use of Sodium chloride (5gram/l transport water) through the transport of *O. niloticus*, especially when the fish are transported for long distances, as the benefits of salt during transport appear to reduce the stress effects of transport on *Oreochromis niloticus.*.

## Conflict of interest

The authors haven't conflict of interest to declare.

## Ethical approval

The study protocol is ethically approved by the Veterinary Medical Research Ethics Committee, Faculty of Veterinary Medicine, Sohag University, Approval number: Soh.un.vet/00011R.

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