

Influence of hyperosmotic culture conditions on osmoregulatory ions, gill chloride cells and Na^+/K^+ -ATPase activity of Nile tilapia, *Oreochromis niloticus*

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Abstract. Aquaculture of Nile tilapia, *Oreochromis niloticus* L. in saline environment is a new development in the industry intending to expand the production of this fish species in countries with less freshwater resources but with large areas of productive saltwater coastlines and estuarine environments. Tilapia species are typical freshwater fishes but are known to tolerate a wide range of salinities making them ideal species for culture in brackish and seawater environments. Information on the physiological response of *O. niloticus* upon exposure to higher salinity conditions is essential in optimization of culture conditions of this fish in sea water-based culture system. The present study investigates the osmoregulatory capacity and the changes of the gill chloride cells as a physiological response of juvenile Nile tilapia exposed to brackish (15‰) and seawater (30‰) conditions. Juvenile tilapia was exposed to two levels of salinities the brackish (15‰) and the seawater (30‰) conditions for 30 days. Each treatment groups were done in triplicates following a completely randomized design. The physiological responses of the fish were measured at the middle of the culture and at the last day of the exposure period. Results suggest a significantly higher chloride cell count in fish reared in seawater than in brackish water both in the middle and at the end of the experiment. Significant enlargement of these chloride cells were also observed in seawater treatment at 15 days until the end of the experiment. This was accompanied with the significantly increased of Na^+/K^+ -ATPase (NKA) activity on the gills and the continual rise of plasma sodium (Na^+) and chloride (Cl^-) until the end of the experiment in treatment groups reared in seawater. No mortalities were observed in the treatment groups. Collectively, the present findings suggest that Philippine strain of Nile tilapia could tolerate salinity conditions up to 30 ppt and this adaptation is associated with the increase in gill chloride cell (CC) density, size, enhanced activity Na^+/K^+ -ATPase, and balanced level of blood Na^+ and Cl^- ions. Although these results are promising, the influence of seawater salinity on energetics and growth of this fish are aspects that need further investigations.

Key Words: ionic regulation, saline tilapia, osmoregulatory enzyme, brackishwater.

Introduction. Nile tilapia, *Oreochromis niloticus* L. culture in less saline environment is a new development that intends to expand the production of this commercially important fish species, especially in a country that has long seawater coastlines and productive estuarine environments like the Philippines. Tilapia culture, just like in other several countries, is one of the top priority of the Philippine National Government as an effort that is necessary to supply cheap food protein source for the fast growing Filipino population. Tilapia has been coined as the Fish of the Millenium since it is highly favored for aquaculture. Could survive in almost all types of environment, tolerant to a wide range of environmental conditions, highly efficient in feed conversion, and exhibits fast growth rates are the traits that marked this species as ideal for aquaculture production (El-Sayed 2006). However, the current culture technology for Tilapia is based on freshwater culture systems that is not applicable to archipelagic countries like the Philippines where fresh water resource is basically limited. Brackish water aquaculture

and open sea cage aquaculture of tilapia is seen as a potential system in the expansion of tilapia aquaculture. Unlike most cultured carnivorous fish that requires high protein diets to attain maximum growth and requires complicated hatchery system to produce fry for culture, tilapia has low technology for hatchery, exhibits high feed efficiency and requires a lower protein in feed. Currently, it is suggested that salinity is the major limiting factor in the expansion of tilapia culture towards the open sea. Exposure to salinities beyond the optimum levels to survive has been documented which result in a significant mobilization of protein reserves and catabolism for energy production to compensate the needs for osmoregulation and ionic balance (Jumah & Traifalgar 2015; Wedemeyer 1996).

Understanding the physiological responses of tilapia to salinity exposure and establishing the limit for survival and growth is essential in the development of culture techniques of this species in brackish and seawater conditions (Ashley 2007). Work on the effects of salinity on the osmoregulatory functions of the gills in Nile tilapia has been initially investigated by Guner et al (2005) but only on a short term exposure period and the fish were acclimatized to the test salinities. Tilapia has been known to adapt and tolerate a wide range of salinities (Mjoun et al 2010) but the physiological responses relating to increase environmental salinities on the long term effect are not fully evaluated to date. Thus, the aim of this study is to determine the effect of hypersaline exposure on the plasma Na^+ , Cl^- , Na^+/K^+ -ATPase (NKA), gill chloride cell (CC) density and gill chloride cell size of Nile tilapia as an adaptive strategy of this economically important cultured species to survive in a long term culture period.

Material and Method. The experiment was conducted on September 12 until October 12, 2014 at the Institute of Aquaculture Hatchery, College of Fisheries and Ocean Sciences, University of the Philippines Visayas (UPV), Miagao, Iloilo, Philippines. One hundred forty four Nile tilapia with a total length of 14.67 ± 0.12 cm and weighing 44.57 ± 0.48 g were used in the study. These were taken from the fishpond of Freshwater Aquaculture Station, UPV. Fish were conditioned in 200 L fiberglass tank at a density of 1 fish 10 L^{-1} and fed twice daily with Tateh® commercial floating feed for 30 days prior to experiment proper. The desired salinity level was obtained by mixing fresh water and seawater (30 ppt) stored from a reservoir tank. Two experimental treatments in triplicates following a completely randomized design were carried out for 30 days. The first treatment group was exposed to 15 ppt, while the second fish group was exposed to 30 ppt. The experimental fish were maintained in a 60 L rectangular plastic tank with 12 fish per tank. Experimental tanks received continuous aeration, 70% of water was changed twice daily and uneaten feeds and wastes were removed daily to maintain good water quality. The fish were fed twice daily at 3% ABW with Tateh® commercial feeds. Blood sampling was done twice in the study. Water parameters including dissolved oxygen, pH and ammonia were maintained to optimum levels throughout the experimental duration.

Plasma sodium (Na^+) and chloride (Cl^-). Feeding was stopped 24 hours before sampling. Prior to blood extraction, organisms were anesthetized using 2-phenoxyethanol (1 mL L^{-1}) as described by Morgan et al (1997). One to 1.5 mL blood was taken from the caudal vessel using heparinized (26 gauge) 1 mL syringe. Collected blood was centrifuged at 3000 rpm for 10 minutes to collect blood plasma using refrigerated centrifuge (HETTICH 4903-02-0). Plasma samples were stored at -80°C in ultra-low freezer (ILSHIN NKH10579) until analyzed. The plasma Na^+ and Cl^- determination was done using the Volhard method.

Na^+/K^+ -ATPase (NKA) activity. Gill tissues were homogenized in phosphate buffer saline (1XPBS) at pH 7.4. The gill membranes were separated from the underlying cartilage by homogenizing and the homogenate was centrifuged at 3000 rpm for 20 minutes at 4°C using refrigerated centrifuge (HETTICH 4903-02-0). Gill NKA activity was measured using an enzyme-link immunosorbent assay (ELISA) kit following the manufacturer's protocols (SunLong Biotech Co., LTD).

Gills histological examination. Three fish specimens were sacrificed from each tank after 15 and 30 days of experiment in order to examine the natural structural components of the gill tissues in response to the treatment. The first and second gill arches from the left or right side of the fish were excised immediately after the caudal puncture. Gills were immediately immersed in container containing 5 mL Bouin's solution for fixation. Histology was performed to the gill tissues and were stained with normal haematoxylin and eosin staining method. The gill tissues were examined under the compound microscope (Motic model) for gill chloride cell density and size at 100x magnification under oil immersion. Digital photos were taken using Moticam® installed in the microscope and the digital images were analyzed using ImageJ program in the laptop.

Statistical analysis. Analysis of data was carried out using *t*-test in SPSS 20. Data on plasma Na⁺, Cl⁻, gill NKA, CC density and CC size were presented as means±SE, of triplicate groups. Level of significance was set at 5% and homogeneity test was determined using Levene's test.

Results and Discussion. Plasma sodium (Na⁺) and chloride (Cl⁻) were found significantly higher at 30 ppt salinity level than in the brackish salinity during the first sampling at 15 days. During the 30th day, plasma sodium levels in the 15 ppt has increased to a level that is similar to that observed during the 15th day of sampling while the 30 ppt group has plasma sodium level that is lower than that of the group exposed at 15 ppt. In contrast, the 30th day of salinity exposure group at 30 ppt still exhibited a significantly higher plasma chloride than that of the group exposed at 15 ppt. However, the level of plasma chloride in the 30th day at 15 ppt was 3-fold higher than that found on the 15 days of sampling but the levels on the group exposed to 30 ppt at 15 days was found similar to that of the group after 30 days of salinity exposure (Table 1).

Table 1
Influence of hyperosmotic culture conditions on plasma Na⁺, Cl⁻, CC density and CC size of Nile tilapia at 15 and 30 days of culture

	Na ⁺ (mmol L ⁻¹)	Cl ⁻ (mmol L ⁻¹)	CC density	CC size
Day 15				
15 (‰)	52.17±17 ^b	53.52±16 ^b	4.00±1.00 ^b	0.0009±0.001 ^b
30 (‰)	369.56±178 ^a	374.65±180 ^a	7.33±0.58 ^a	0.0017±0.001 ^a
p < 0.05	0.037	0.037	0.007	0.006
Day 30				
15 (‰)	369.57±178 ^b	247.88±25 ^b	1.00±0.00 ^b	0.0009±0.00 ^b
30 (‰)	243.47±26 ^a	402.81±23 ^a	4.67±0.58 ^a	0.0011±0.00 ^a
p < 0.05	0.001	0.001	0.008	0.000

Data are presented as means±SE, n = 3. Means having the same superscript letter on the same column are not significant (p > 0.05).

The present data suggest the adaptation of this animal to salinity exposure. The rise in the Na⁺ and Cl⁻ in 15 days has almost similar levels of these ions in 30 days without significant increase as the salinity is increased (15-30). This indicates that ionic stabilization in the animal has already fully compensated its physiological processes to balance the removal due to the influx of these ions from the environment (Felder et al 2007). Significant increase of plasma Na⁺ and Cl⁻ values at higher salinity after 24 hours exposure to higher salinity was also reported in Mozambique tilapia, *Oreochromis mossambicus* indicating a state of osmotic stress at the beginning of this saline exposure. Also, Vonck et al (1998) suggested that increasing salinity increased drinking rate of tilapia which further contributes to Na⁺ and Cl⁻ influx. Average decreased percentage composition of Na⁺ and Cl⁻ in the blood was observed in the succeeding sampling periods as compared with initial sampling day, suggesting that the organisms tried to reduce excess ions influx. The gradual rise of plasma Na⁺ and Cl⁻ in the treatment groups

sampled at 15 and 30 days indicates osmoregulatory imbalance that gradually normalize with time (Kammerer et al 2010). The present data imply that net influx of these ions from the water at the early period of exposure was enhanced indicating that seawater imposes a Na^+ and Cl^- burden (Vonck et al 1998) but at the 30th day it appears that the levels tend to stabilize indicating osmoregulatory homeostasis. This result contradicts with other studies on euryhaline teleost, wherein salinity adaptation and ionic balance is achieved and the animal recovers to the original condition at a very short time (Mancera et al 1993). Our data suggest that Nile tilapia – although hardy and able to tolerate this salinity exposure, takes about 30 days to fully attain physiological and ionic balance. Also the present results can be attributed to the limited ability of the Nile tilapia gill to excrete excess ions in a hyperosmotic environment. This might be due to a limited capacity to up-regulate the expression of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) towards the recruitment of seawater type chloride cells (Breves et al 2010). In agreement with the present findings, similar response was also observed when euryhaline fish species were exposed to extreme salinities for long periods of time. Sampaio & Bianchini (2002) considered this response as an osmotic strategy to decrease the negative impacts of ionic imbalance brought about by the changing environmental salinities. It was observed in flounder's *Paralichthys orbignyanus* that plasma Cl^- concentration reared in freshwater for 90 days were significantly lower than in those maintained in seawater. Transfer of this fish in sea water condition also manifested a lower plasma Cl^- ions. This earlier report indicates the rapid physiological response of the euryhaline fish to changes in environmental salinities. This species is noted to be a euryhaline teleost and its adaptation to salinity to reach cellular homeostasis is extremely rapid (Imsland et al 2008; Morgan et al 1997).

In the present data, the pattern of gill NKA activity was similar to that observed for the blood osmoregulatory ions. Group exposed to sea water salinities has significantly higher levels of NKA activity than those exposed at 15 ppt (Figure 1). Both treatment groups exhibited an increased level of NKA activity from the 15th day and to the 30th day of sampling. The present data suggest that as salinity increased, osmoregulatory capacity and greater ion transport capacity for water absorption is required as observed by Sardella & Brauner (2008) in 'California' Mozambique tilapia *Oreochromis mossambicus* x *O. urolepis hornorum*. In relevance, the increased NKA activity agrees with Fiess et al (2007) who reported on Mozambique tilapia that there is a need for fish in higher salinity to extrude excess ions on the gills. Gill NKA activity plays an important role in ion transportation in juvenile GIFT tilapia *Oreochromis niloticus* (Qiang et al 2013). NKA or Na^+ pump energizes salt transport across both absorptive and secretory epithelia by pumping out, for every molecule of ATP hydrolyzed, three Na^+ ions in exchange for two K^+ ions against their electrochemical gradients (see review in Hirose et al 2003). Higher NKA activity in salinity above their natural medium was considered common physiological response even in euryhaline fishes such as rabbitfish, *Siganus rivulatus* (Saoud et al 2007), Senegalese sole, *Solea senegalensis* (Arjona et al 2007) and obscure puffer *Takifugu obscurus* (Li et al 2014) but only in the adjustment period. This suggests that fish spends more amount of osmoregulatory energy in higher environmental salinities where they evolved to live in (Saoud et al 2007). Consistent result was also reported in euryhaline species (Arjona et al 2007). This result was also similar to preceding findings in embryonic and larval tilapia *O. mossambicus* during the course of early development to maintain osmoregulatory homeostasis (Kosztowny et al 2008). In contrary, juvenile milkfish, *Chanos chanos* exhibited highest NKA activity in freshwater than seawater which is thought to improve the osmoregulatory capacity of the milkfish in hyposaline environments (Lin et al 2003). This suggests that NKA activity rises whenever the fish is exposed to changes in environmental salinities whether it is above or lower than the salinity that the fish is currently exposed.

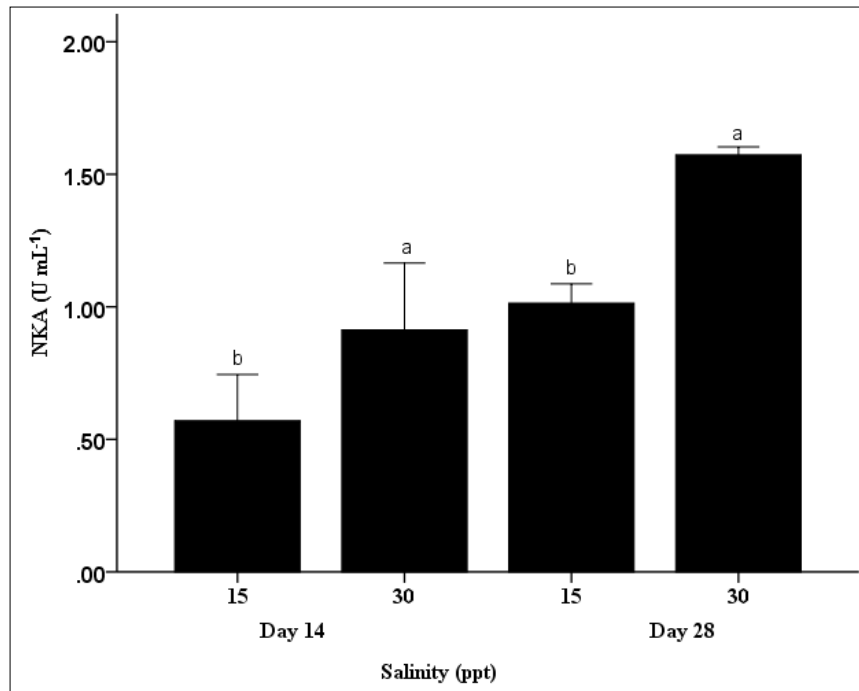


Figure 1. Influence of hyperosmotic culture conditions on gill NKA of Nile tilapia at 15 and 30 days of culture.

Increasing CCs density with increasing salinity was prominently observed in the higher salinity and at the 30th day, CCs density tends to revert back similar to that observed in the 15th day of sampling indicating reversion to normal condition. This physiological pattern correlates well with the levels of osmoregulatory ions (chloride and sodium). In terms of CCs size the group exposed to 30 ppt has a significant increase in size as compared to that exposed at 15 ppt observed at all sampling periods. The current data suggests that once ionic regulation homeostasis is achieved the size of CCs stabilizes and as a response to salinity changes both CCs density and size are influenced (Table 1, Figures 2 and 3).

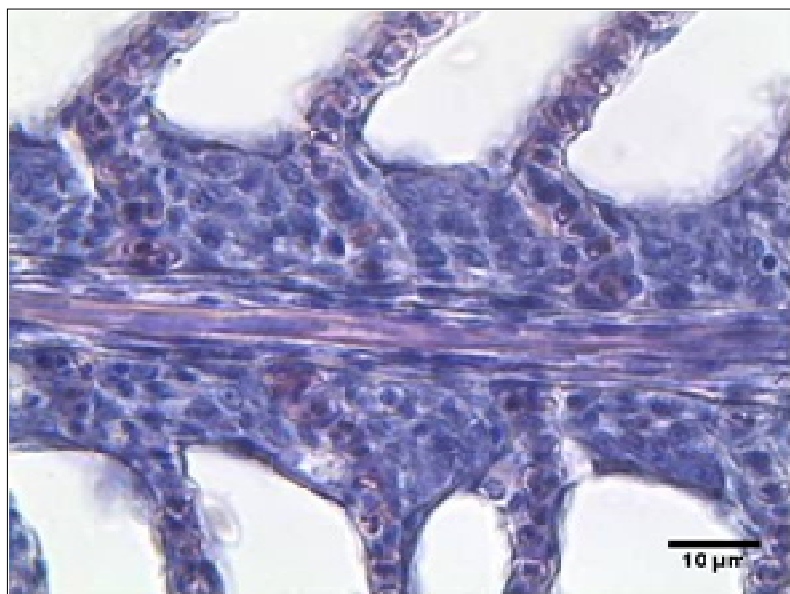


Figure 2. Influence of saline condition (15‰) on gill histology of Nile tilapia at 30 days of culture.

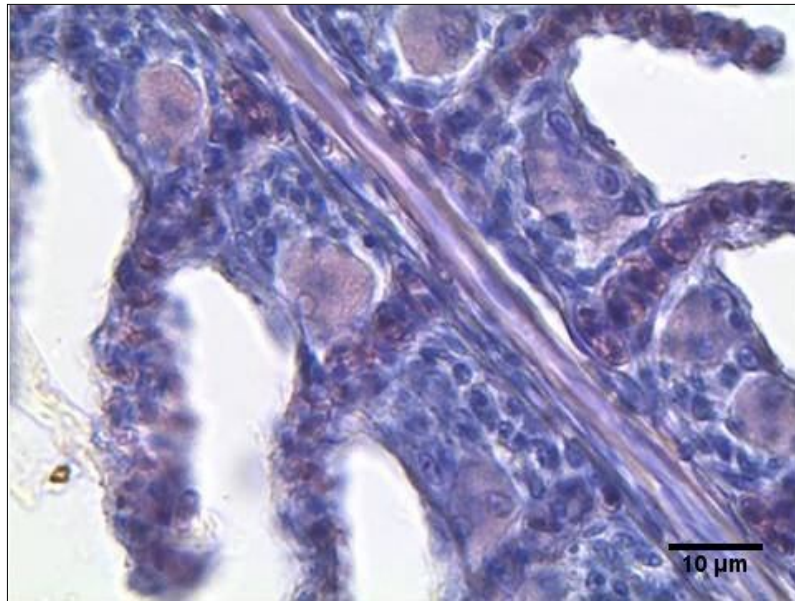


Figure 3. Influence of hypersaline condition (30‰) on gill histology of Nile tilapia at 30 days of culture.

These CCs were localized in the filament specifically in interlamellar regions. The same observation was reported by Pelis & McCormick (2001) suggesting that chloride cells on the primary filament are involved in ion secretion, whereas chloride cells on the secondary lamellae take up ions. This signifies the osmoregulatory function of chloride cells at higher salinities (Estudillo et al 2000). The present result corroborates well with these earlier findings. Significant enlargement of the CC in the basal filament is observable in the seawater exposed group, suggesting active excretion of chloride and sodium ions in response to a higher salinity condition at 30 ppt. Increasing occurrence of filaments CCs with increasing salinity was reported in previous studies (Sterzelecki et al 2013; Ouattara et al 2009) which are believed to be involved in ion transport. The present results are also in agreement with results obtained in the amphidromous Hawaiian goby, *Stenogobius hawaiiensis* (McCormick et al 2003) as well as in anadromous Atlantic salmon, *Salmo salar* (Pelis & McCormick 2001). Similar result was also observed in other teleost species such as; grey mullet *Liza aurata* (Khodabandeh et al 2009), and cichlid *Etroplus maculatus* (Virabhadrachari 1961) during adaptation to different salinities. This suggests that Nile tilapia could adapt to the salinity changes of the environment through changing the quantity of branchial chloride cells which play a key role for the osmoregulation in the waters with high salinity.

Increasing CCs size was highly evident at higher salinity during the last sampling at 30th day in the present study. Hirose et al (2003) reviewed that enhanced expression of NKA is consistent with the increased size of CCs in seawater, which is accompanied by expansion of the tubular network where NKA is incorporated. This reflects the generation of shallow leaky junctions and chloride cell complexes with subsequent increased ion permeability in response to the need for increased turnover rates of Na^+ and Cl^- (Fielder et al 2007). Enlargement of CCs size suggests cell complexes formed by ionocytes and accessory cells indicating osmoregulation activities at higher salinities (Ouattara et al 2009).

Similar findings were observed in other teleost species such as rainbow trout, *Oncorhynchus mykiss* and brown trout, *Salmo trutta* forma *fario* (Kizak et al 2013), *Pseudosciaena crocea* (Ruanchengxu et al 2014), Persian sturgeon, *Acipenser persicus* (Kazemi et al 2003). This attributes to the considerable enlargement of the basolateral tubular system and the presence of a distinct apical crypt and has been speculated, that this could be due to a higher need for ion pumps required by fish reared at hypertonic media (Mylonas et al 2009). This indicates adaptation for osmoregulation and it appears to be the gill's enhanced capability to excrete excess Na^+ and Cl^- (Erkmen & Kolankaya

2009). Lastly, this physiological adaptation could be attributed to the high survival rate of the experimental fish > 90% at the end of the experimental period suggesting the important roles of gill chloride cells and gill NKA pump in maintaining the osmotic homeostasis in Nile tilapia when exposed to salinity changes.

Conclusions. Collectively, the present findings suggest that Nile tilapia could withstand and tolerate seawater conditions (30 ppt) with physiological and ionic homeostasis attained at 30 days period. Enhancement of gill NKA pump coupled with the recruitment of gill filament chloride cells and enlargement of basal gill chloride cells are physiological adaptive strategy employed by Nile tilapia to attain ionic balance in response to exposure to higher salinity conditions.

Acknowledgements. The main author would like to mention the heart felt gratitude to the following institutions such as the Office of the Vice Chancellor for Research and Extension of the University of the Philippines Visayas (OVCRE-UPV), Mindanao State University-Tawi-Tawi College of Technology and Oceanography (MSU-TCTO) for funding the experiment. This research paper would not become a reality without also the financial aid from the Philippine government, the Department of Science and Technology-Accelerated Science and Technology Human Resource Development Program (DOST-ASTHRDP).

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Received: 20 March 2016. Accepted: 08 May 2016. Published online: 23 May 2016.

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How to cite this article:

Jumah Y. U., Traifalgar R. F. M., Monteclaro H. M., Sanares R. C., Jumah D. S. U., Mero F. F. C., 2016 Influence of hyperosmotic culture conditions on osmoregulatory ions, gill chloride cells and Na⁺/K⁺-ATPase activity of Nile tilapia, *Oreochromis niloticus*. *AAFL Bioflux* 9(3):498-506.