Salinity Tolerance in Nile Tilapia (*Oreochromis niloticus*)

Biboon Withyachumnarnkul

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Aquaculture (International Program) Prince of Songkla University 2017

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การทนความเค็มของปลานิล *Oreochromis niloticus*
ผู้เขียน นายใบบุญ วิทยชำนาญกุล
สาขาวิชา การเพาะเลี้ยงสัตว์น้ำ (นานาชาติ)
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บทคัดย่อ

การเพาะเลี้ยงร่วมกัน (co-culture) ระหว่างปลานิล (*Oreochromis niloticus*) และกุ้งทะเลได้เป็นแนวทางปฏิบัติที่เกิดมากขึ้นเนื่องจากมีหลักฐานว่าการเพาะเลี้ยงร่วมกันแบบนี้เป็นหนึ่งในวิธีการไม่กี่วิธีที่จะป้องกันการเกิดโรคของกุ้งได้ โดยเฉพาะอย่างยิ่ง โรค acute hepatopancreatic necrosis disease ที่ได้สร้างปัญหาให้แก่การเพาะเลี้ยงกุ้งทะเลทั่วโลก เนื่องจากโดยพื้นฐานปลานิลเป็นปลาน้ำจืด ดังนั้นในการเพาะเลี้ยงร่วมกับกุ้งทะเลนั้นปลานิลจึงต้องมีการปรับตัวให้เข้ากับความเค็ม ซึ่งปลานิลมีความสามารถในการปรับตัวเช่นนั้นอยู่แล้ว การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาการเปลี่ยนแปลงทางด้านเลือดและภูมิคุ้มกันในปลานิลที่เพาะเลี้ยงภายใต้ความเค็มที่สูงขึ้นพร้อมกับการศึกษาความยากง่ายของการติดเชื้อโรค โดยใช้ปลานิล น้ำหนักตัวเริ่มต้น 180 ± 30.20 กรัม มาเพาะเลี้ยงภายใต้ความเค็มที่ค่อยๆ สูงขึ้น จาก 0 ถึง 30 ส่วนในพัน ภายในระยะเวลาหนึ่งเดือน โดยการเพิ่มความเค็มขึ้น 3 ส่วนในพันในทุกๆ 3 วัน และหลังจากที่ได้อยู่ภายใต้ความเค็ม 30 ส่วนในพัน ซึ่งเป็นความเค็มเท่าน้ำทะเล แล้ว ปลานิลเหล่านั้นยังคงถูกเพาะเลี้ยงต่อภายใต้ความเค็ม 30 ส่วนในพันไปอีกหนึ่งเดือน เมื่อครบ 60 วันของการทดลอง พบว่าจำนวน 51.3 ± 15.5 เปอร์เซ็นต์ ของปลานิลยังรอดชีวิตอยู่ และการตายของปลานิลส่วนใหญ่เกิดขึ้นในช่วงเดือนที่สอง ในขณะที่ปลานิลเหล่านั้นอยู่ภายใต้ความเค็ม 30 ส่วนในพัน อัตราการเจริญเติบโตช้าลงเหลือ 60 เปอร์เซ็นต์ เมื่อเทียบกับปลานิลกลุ่มควบคุม ที่เพาะเลี้ยงในน้ำจืด ปลานิลที่เลี้ยงในน้ำความเค็มสูงนี้มีสัดส่วนของเม็ดเลือดแดงอัดแน่นลดลง (24.1 ± 1.2 เปอร์เซ็นต์) ปริมาณเม็ดเลือดขาวรวมมากขึ้น (35,000 ± 5,000 เซลล์ต่อไมโครลิตร) โดยเม็ดเลือดชนิดลิมโฟซัยต์และทรอมโบซัยต์มากขึ้น (13,500 ± 1,700 เซลล์ต่อไมโครลิตร และ 17,700 ± 2,300 เซลล์ต่อไมโครลิตร ตามลำดับ) และปฏิกิริยาของไลโซไซม์ในซีรัมมากขึ้น (49.3 ± 19.3 ยูนิตต่อไมโครลิตร) อย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับปลานิลที่เลี้ยงในน้ำจืด ได้ตรวจการติดเชื้อที่เป็นตัวแทนเชื้อโรคสามชนิด ได้แก่ เชื้อไวรัส infectious spleen and kidney necrosis virus (ISKNV) เชื้อแบคทีเรีย *Francisella noatunensis* และ *Streptococcus agalactiae* ในปลานิลที่อยู่ในน้ำจืดและที่อยู่ในน้ำเค็ม โดยใช้วิธี polymerase chain reaction (PCR) ผลการตรวจพบปลานิลมีการติดเฉพาะเชื้อ ISKNV เท่านั้น โดยปลานิลที่อยู่ในน้ำจืดมีการติดเชื้อ 1 ตัว ใน 10 ตัว ในขณะที่ปลานิลที่อยู่ในน้ำเค็มมีการติดเชื้อ 6 ตัว

ใน 10 ตัว การติดเชื้อนั้นพบได้ในอวัยวะต่างๆ ของปลา ได้แก่เหงือก สมอง ตับ อวัยวะสืบพันธุ์ ผิวหนัง ไต และม้าม โดยมีปริมาณเชื้อโรคในระดับปานกลางในไตและมากในม้าม ทำการยืนยันการติดเชื้อ ISKNV ด้วยวิธี *in situ* hybridization ในอวัยวะสืบพันธุ์และไต โดยใช้การเพิ่มปริมาณดีเอ็นเอด้วยวิธี loop-mediated DNA amplification และแสดงออกด้วยสาร digoxigenin ที่ก่อปฏิกิริยากับสารสีที่ทำให้เกิดสีน้ำตาลเข้มอันแสดงออกถึงการติดเชื้อ ISKNV ในเนื้อเยื่อที่ให้ผล PCR เป็นบวก ผลของการวิจัยเหล่านี้บ่งบอกว่ามีปลานิลจำนวนมากที่สามารถมีชีวิตอยู่ได้ในน้ำที่มีความเค็มระดับน้ำทะเล โดยที่มีการเพิ่มความเค็มอย่างช้าๆ และภายใต้สภาวะเช่นนั้น ปลานิลเกิดการเปลี่ยนแปลงบางประการในเลือดและในระบบภูมิคุ้มกัน ซึ่งอาจจะเป็นสาเหตุของการเกิดการเพิ่มจำนวนของเชื้อ ISKNV ที่มีอยู่แล้วในปลานิล โดยที่ปลานิลที่ติดเชื้อนั้นและอยู่ในน้ำจืดไม่มีอาการแต่อย่างใด แต่การเพิ่มปริมาณของเชื้อโรคนี้ในปลานิลที่อยู่ในน้ำเค็มอาจเป็นสาเหตุให้ปลานิลเสียชีวิตได้

คำสำคัญ ปลานิล *Oreochromis niloticus* การทนความเค็ม ค่าแสดงถึงเลือดและภูมิคุ้มกัน เชื้อไวรัส ISKNV การเพิ่มปริมาณ DNA ด้วยวิธี loop-mediated DNA amplification

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ABSTRACT

Co-culture of Nile tilapia, *Oreochromis niloticus*, with marine shrimp has been an increasing farm practice as evidence has suggested that the co-culture is one of a few ways to mitigate shrimp diseases, especially the acute hepatopancreatic necrosis disease that has affected shrimp culture industry worldwide. Since Nile tilapia are basically freshwater species, having them in co-culture with marine shrimp therefore requires a certain degree of salinity adaptation, in which the fish are readily capable to. The present study was aimed at studying certain hemato-immunological changes of the fish reared under elevated salinity, as well as their susceptibility to pathogens. Nile tilapia (180 ± 30.20 g initial body weight) were reared under gradually increasing salinity within one month from 0 to 30 ppt at 3 ppt/3d and maintained for another month in 30-ppt salinity. At the end of the 60-day experimental period, 51.3 ± 15.5% of the fish survived with mortality occurred mainly during the second month when the salinity was at 30 ppt. Their growth rate was retarded to 60% of that of the control fish maintained under freshwater (0 ppt). The fish reared under elevated salinity also had significantly lower hematocrit (24.1 ± 1.2%), higher total white blood cell counts (35,000 ± 5,000 cells/µL), higher absolute numbers of lymphocytes and thrombocytes (13,500 ± 1,700 cells/µL and 17,700 ± 2,300 cells/µL, respectively) and higher serum lysozyme activity (49.3 ± 19.3 unit/mL), compared to those reared under freshwater. Presence of three representative pathogens: infectious spleen and kidney necrosis virus (ISKNV), *Francisella noatunensis* and *Streptococcus agalactiae*; were determined in the fish reared under freshwater and in those reared under elevate salinity, using polymerase chain reaction (PCR) method. Of the three pathogens, only ISKNV was detected in 1/10 of the freshwater and 6/10 of the elevated-salinity fish. In both groups, the virus was present in the gills, brain, liver, gonads, skin, kidney and spleen, with moderately and severely positive reaction observed in the kidney and

spleen. The detection of ISKNV was confirmed by *in situ* hybridization of the gonads and kidney, using loop-mediated DNA amplification with digoxigenin, which clearly showed dark-brown stain of the positive signals in the PCR-positive samples. These results suggest that a substantial number of *O. niltoicus* could survive elevated salinity up to full-strength seawater provided that the salinity had been gradually elevated. And under that situation, changes in hemato-immunological functions of the fish did occur, which probably caused lethal proliferation of pre-existing ISKNV that otherwise would remain dormant.

Keywords: *Oreochromis niloticus*; salinity tolerance; hemato-immunological parameters; ISKNV; loop-mediated DNA amplification

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LIST OF ABBREVIATIONS AND SYMBOLS

ADG	average daily growth
AHPND	acute hepatopancreatic necrosis disease
BW	body weight
cm	centimeter
d	day
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DO	dissolved oxygen
dUTP	deoxyuridine triphosphate
EMS	early mortality syndrome
FAO	Food and Agricultural Organization
FCR	feed conversion ratio
Fig.	figure
fL	femtoliter
g	gram
GH	growth hormone
GIFT	genetically improved farmed tilapia
GMO	genetically modified organism
h	hour
H&E	hematoxylin and eosin
Hct	hematocrit
HSP	heat shock protein
Ig	immunoglobulin
ISDL	*In situ* hybridization using loop-mediated DNA amplification with digoxigenin labelling
ISKNV	infectious spleen and kidney necrosis virus

kg	kilogram
L	liter
LAMP	loop-mediated DNA amplification
m	meter
MCV	mean corpuscular volume
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MCP	major capsid protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	nitro-blue tetrazolium
ng	nanogram
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
nm	nanometer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear
ppm	part per million
ppt	part per thousand
PRL	prolactin
RBC	red blood cell
rpm	rounds per minute
SEAFDEC	Southeast Asian Fisheries Development Center
sec	second
SGR	specific growth rate
SOD	superoxide dismutase
sq.m.	square meter
TAN	total ammonia nitrogen

U	unit
WBC	white blood cell
μL	microliter
μM	micromolar

CHAPTER 1
INTRODUCTION

1.1 Background and Rational

In recent decades, tilapia has become one of the major aquaculture species around the world, at production of 4.0 million tons yearly and worldwide production of tilapia is only second to that of carp production (FAO, 2012). Nile tilapia, *Oreochromis niloticus* (Fig. 1), which has their origin in the Nile and its tributaries of Africa, was introduced into Thailand in 1965. Fifty individuals of the fish were given by Prince Akihito of Japan to King Bhumibhol (https://th.wikipedia.org/wiki/ปลานิล); the fish were first reared in a pond within the King's palace (Jitrladda Palace). As the king foresaw that the fish were relatively easy to grow and tolerated well in wide range of water conditions with low cost of production, and thus could be a cheap source of protein for Thai people, they were therefore distributed in all parts of Thailand. Since then, the Department of Fisheries, Ministry of Agriculture, Thailand, has taken full activities in developing programs leading to fast growth and large marketable size [≥0.8 kg body weight (BW)], such as Jitrladda-strained tilapia, for commercial distribution. Many large corporations and fishery centers in the country and worldwide have been engaging in tilapia selective breeding program and produced wide varieties of tilapia for commercial production. For instance, one of the popular strain of Nile tilapia known for their fast growth and large size is genetically improved farmed tilapia, or GIFT, being produced by the Southeast Asian Fisheries Development Center (SEAFDEC) in the Philippines (WorldFish Center, 2004).

In general, Nile tilapia tolerates wide range of water qualities. The fish survive in a wide range of temperature from 12 - 42 $^{\circ}$C and thus can be raised in both tropical and sub-tropical countries, can tolerate pH ranging from 3.8 to 10.2 and under dissolved oxygen (DO) as low as 3-ppm (Popma and Lovshin, 1996; Sifa *et al*., 2002; Luan *et al*., 2008; Rebouças *et al*., 2015). Due to its capacity to tolerate wide ranges and varieties of environmental conditions, Nile tilapias are therefore raised in many parts of the world, including in Asia, Africa, North and South America, Australia and in Europe (Watanabe *et al*., 2002; Dalsgaard *et al*., 2013).

Nile tilapia, *O. niloticus*, is considered freshwater species; however, under certain conditions, the fish can survive and grow well in brackish and seawater. When the fish were suddenly shifted from freshwater to higher-salinity water, they did not survive beyond 20 ppt; however, with gradual salinity increase (chronic condition), at 5 ppt/week, the fish grew and survived up to 40 ppt (Schofield *et al*., 2011). The adaptation to salinities of *O. niloticus* was found to be temperature-dependent; in summer (30 °C), the increasing salinities did not affect growth, survival and reproduction, but in winter (14 °C), all the parameters were affected. This surprising tolerance to salinities of *O. niloticus* was reported by other researchers as well (Kamal and Mair, 2005; Luan *et al*., 2008).

Figure 1. Nile (*O. niloticus*), red (*Oreochromis* sp.) and Mossambique (*O. mossambicus*) tilapia

For many years, Nile tilapia, *O. niloticus*, and hybrid red tilapia, *Oreochromis* sp., have been co-cultured with marine shrimp (Yi and Fitzsimmons, 2004; Kamal and Mair, 2005). The purpose of the co-culture practice is for the fish to become by-products in shrimp culture or, by unknown reasons, to increase growth and survival of the shrimp. After the incidence of a devastating disease called acute hepatopancreatic necrosis disease (AHPND), commonly known as early mortality syndrome (EMS), in cultured marine shrimp, especially in *Litopenaeus vannamei* and *Penaeus monodon* culture (Figs. 2, 5), it was found that co-culturing the shrimp with tilapia could somehow lessen severity of the disease (Tran *et al*., 2013; Withyachumnarnkul, 2013). Therefore, co-culturing marine shrimp with tilapia seems to have more meaningful purpose.

Figure 2. Shrimp pond and the two most cultured species, the black tiger shrimp, *P. monodon*, and the Pacific whiteleg shrimp *L. vannamei*

Since farmed marine shrimp are normally reared under brackish or seawater, the fish being co-cultured need to be able to grow and survive under the same salinities. Although it is possible for *O. niloticus* to grow and survive in brackish and even in seawater, no commercial production of salinity-tolerant strain of the fish is available at present. It is therefore desirable to have mass production of this special strain of *O. niloticus* for shrimp farming, and maybe for other purpose as well. In order to achieve that goal, selective breeding program of high-salinity tolerance should be set up for the species. But before reaching this goal, basic knowledge on how the fish adapt to salinity changes and any pathologies afflicted on the fish should be known; and, for these reasons, the thirst of this knowledge forms the basis of this study.

1.2 Objectives

The general objective of this study is to produce salinity-tolerant Nile tilapia, *O. niloticus*, for co-culturing with marine shrimp. The production will be incorporated into the selective breeding program for fast growing and disease-resistant traits of seawater Nile tilapia in the future. The scope of this thesis research, however, is confined to finding out a possibility of selecting high-salinity survivors from existing freshwater Nile tilapia.

From previous studies, it is expected that a certain proportion of freshwater Nile tilapia could survive seawater salinity. Further assumption is that the fish that tolerate elevated salinity would need to have some kind of physiological adjustment and therefore may have hemato-immunological parameters different from those of Nile tilapia reared under freshwater. The specific objectives of this study were therefore to find out first the percentage of Nile tilapia that could survive elevated salinity up to full-strength seawater (30 ppt), and then to compare hemato-immunological parameters between the seawater tilapia and freshwater ones. Since it has recently shown that Nile tilapia, as well as red tilapia, could harbor multiple pathogens without clinical manifestations, it was therefore interesting to find out if the fish, under salinity stress and probably with hemato-immunological dysfunctions, could become more susceptible to pathogens already residing in the fish bodies. The presence of representative pathogens was determined by polymerase chain reaction methods in

various organs of both types of fish and was confirmed by *in situ* hybridization method.

1.3 Review of Literature

1.3.1 Tilapia Industry

Nile tilapia require 6-10 months to grow from hatching to marketable size and the price varies from 45 to 85 baht (1.5-2.5 US$/kg) in Thailand during the past decade. The fish reproduce freely in the pond and take 2.5-3.0 months to reach sexual maturity, with BW around 70 g. Naturally the female brooders take fertilized eggs in their mouth cavity and hatching takes place inside the mouth, after which the newly hatched fish, or swim-ups, stay in and out of the mother's mouth cavity for about 1-2 weeks before swimming out from their safe place (WorldFish Center, 2004). In commercial hatcheries, eggs are taken out from the brooders' mouth and hatching is accomplished through external incubation by clean, circulating water for a few days (Fig. 3). After hatching, swim-ups are fed testosterone-containing feed for 3 weeks to make them all-male (Jo *et al*., 1995). However, some (2-5%) fish may still be females, which is not 100% sex-conversion. This process is carried out because the male grows faster and has larger size than the female.

The use of testosterone for sex conversion has raised a caution on its adverse effects on human by consumption of the testosterone-treated fish, as well as on the environment. However, it was found that the hormone was undetectable in the fish at 5 days after cessation of the treatment. Therefore, human consumption of the fish that usually occurs a few months after the cessation of the hormone treatment should not affect the consumer (Megbowon and Mojekwu, 2014). The release of testosterone from hatchery drainage into natural waterways may affect other aquatic lives but, again, it was also shown that the hormone is mostly degraded by soil and sediment via mineralization, or being reacted with minerals such as calcium and becomes inactive compound (Shore and Shemesh, 2003).

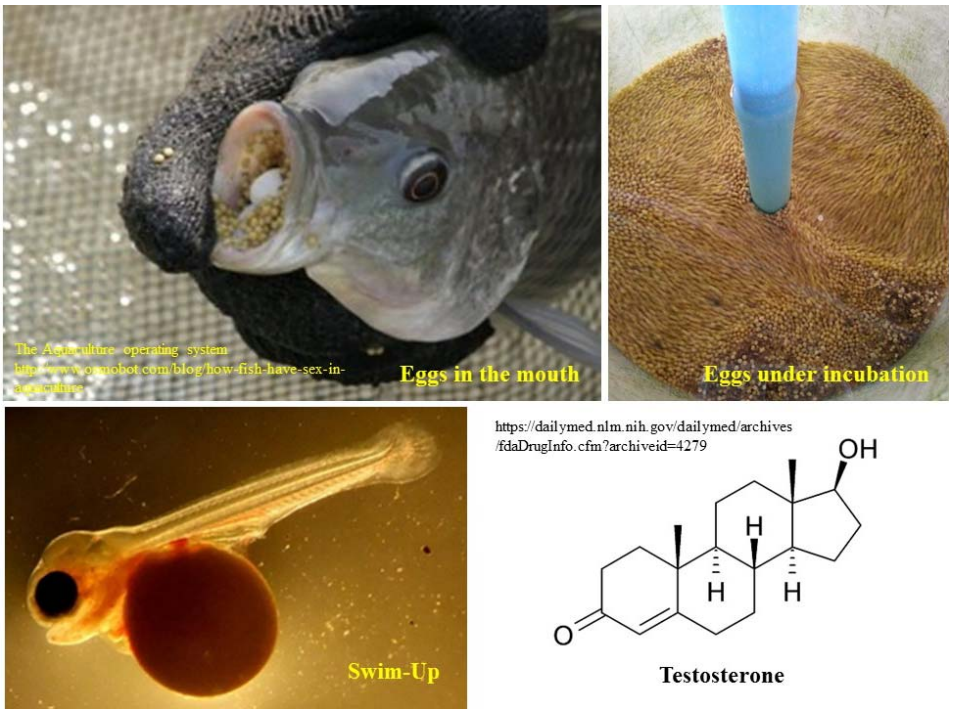

Figure 3. Female Nile tilapia with eggs in the mouth, eggs under incubation, swim-up and testosterone structure. The eggs are pooled and incubated with freshwater for 4-5 days and hatch to become swim-up, which are sex-conversed to all males by testosterone ingestion.

1.3.2 Tilapia-Marine Shrimp Co-culture

Shrimp farming in Thailand began in 1920s as an extensive-typed culture (cited by Hossain and Lin, 2001). The extensive type of shrimp rearing was followed by intensive culture of the black tiger shrimp *P. monodon* in 1980s and then by the Pacific white leg shrimp *L. vannamei* in 2010s. At present, both shrimp species from farming industry have become an important export commodities for Thailand, with more production of *L. vannamei*.

The production, despite a steady increase over the years, was periodically interrupted by disease outbreaks, starting in 1990s, with yellow-head disease (YHD), followed by white spot disease (WSD) (Wongteerasupaya *et al.*, 1995) (Fig. 4). Several diseases caused by infections from many types of viruses, bacteria, fungi and other pathogens resulting in mortality or morbidity of farmed shrimp have been reported (Flegel, 2006). In 2011, AHPND occurred, starting with the outbreaks in

China, then in Vietnam, Malaysia, Thailand and Mexico (Fig. 5). This disease is caused by the destruction of hepatopancreas of the shrimp by plasmid-induced toxins released from the bacteria *Vibrio parahaemolyticus*, and possibly by other types of bacteria as well (Tran *et al*., 2013; Joshi *et al*., 2014). In 2014, shrimp production in Thailand dropped to 40% of its maximum production and could no longer maintain its leadership status as shrimp exporter in the world.

Figure 4. Yellow-head disease (YHD) and white spot disease (WSD) in cultured shrimp

In response to AHPND, researchers have been trying to solve the problem by various means, such as by stocking healthy seed being checked to be free of *V. parahaemolyticus* toxins, using probiotics or biofloc in shrimp ponds to compete against the causative bacteria and using tilapia in co-culture with the shrimp. For the last method, the rationale has come from the findings that rearing water of tilapia could prevent experimental AHPND infection and low incidence of AHPND have been reported in tilapia-shrimp co-culture ponds (Tran *et al*., 2013; Withyachumnarnkul, 2013). As tilapia-shrimp co-culture is a convenient method to

prevent AHPND, farmers in Thailand and nearby countries have employed the method with relatively successful results.

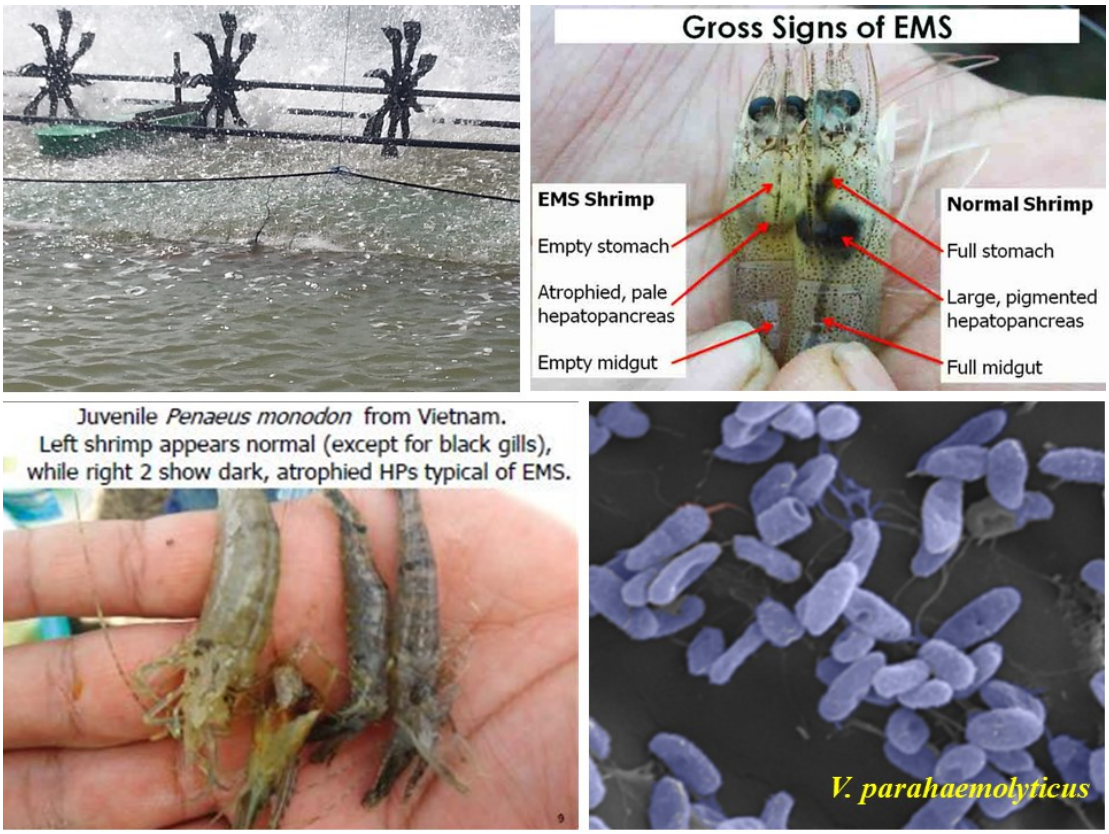

Figure 5. Acute hepatopancreatic necrosis disease (AHPND) or Early Mortality Syndrome (EMS) caused by toxins released from bacteria, especially *Vibrio parahaemolyticus* (http://www.seafood.vasep.com.vn/Daily-News/53_8788/EMS The-Perfect-KillerA-Webinar)

In the past, before the occurrence of AHPND, tilapia-shrimp co-culture has been performed and farmers found the method being beneficial in term of having both shrimp and fish at the same time. In addition, the fish also reduce waste in the pond and probably prevents certain shrimp diseases (Yi and Fitzsimmons, 2004; Kamal, 2005; Luan, 2008). After the AHPND incidence, the co-culture has been employed even more routinely. Most farmers stock a few hundred of tilapia in one-rai (1,600 sq.m.) area of pond, while the shrimp, either *P. monodon* or *L. vannamei* are stocked at their usual, or slightly lower than, normal stocking density. For low salinity pond,

<15 ppt seawater, Nile, Mossambique (*O. mossambicus*) or red tilapia can be stocked with the shrimp without any problem. Red tilapia are hybrid *O. niloticus* male x *O. mossambicus* female (mutant reddish-orange), or produced by crossing between red-gold *O. mossambicus* male with normal colored *O. hornorum* female (Behrends *et al*., 1982; Galman and Avtalion, 1983); thus, they are scientifically named *Oreochromis* sp. (Fig. 1). Both Mossambique and red tilapia tolerate high salinities better than Nile tilapia. Therefore, for shrimp farms that have water salinity >15 ppt, Mossambique and red tilapia are preferred to Nile tilapia.

A problem of red tilapia is on their color, which is brightly red or orange or yellow, and they become very attractive to birds. Nets need to be installed in shrimp ponds being co-cultured with red tilapia (Fig. 6); this installation increases the investments. Although in many farms, bird nets have been installed to prevent disease outbreaks but to make the installation an obligatory measure are not very desirable by farmers. Another problem of using red tilapia in shrimp ponds is that the fish are generally weaker than Nile tilapia. They tend to catch diseases more easily than Nile tilapia, or die easily after sudden weather changes.

The problem of stocking *O. mossambicus* is that the fish has not been sterilized and thus reproduce freely in the pond. The over-populated fish could consume shrimp feed and thus cause an increase in feed conversion ratio (FCR), which is not desirable. And *O. mossambicus*, not having been genetically improved, cannot economically grow up to a large size generally acceptable by consumers, i.e., 0.8-1.2 kg size.

Therefore, Nile tilapia with high salinity tolerance, up to full-strength seawater (30 ppt), is probably the best choice of tilapia species to co-culture with marine shrimp. And it is preferable to have sex-reversed all-male tilapia, 1-5 g BW, being stocked directly in the shrimp pond at the beginning of the culture. The use of all-male tilapia would prevent further breeding in the shrimp pond, which would subsequently increase the fish number that consume shrimp feed.

The idea of producing high salinity-tolerated Nile tilapia is not new, it has been reported previously (Watanabe *et al*., 1984; Suresh and Lin, 1992; Popma and Lovshin, 1996; Schofield *et al*., 2011). The fish can survive full-strength seawater provided the salinity was gradually increased. In general, growth rate and reproductive function were lowered by increasing salinity, especially when being reared above 15-

ppt salinity. However, growth rate under high salinities seems to be highly variable and some researchers reported no significant reduction in growth rate of *O. niloticus* reared under full-strength seawater (Schofield *et al*., 2011). It is agreed upon that the reproductive function is decreased as salinity increases, and probably more on the females rather on the males (Schofield *et al*., 2011).

In our pilot study, it revealed that tilapia reared under high salinity can acclimatize well in low-salinity water, e.g., the fish in 30ppt seawater can directly be released into 0-ppt water without mortality. On the contrary, tilapia reared under low salinity required gradual adjustment in order to be placed in higher-salinity water, e.g., in order to acclimatize tilapia reared under 0-ppt water to 15-ppt seawater, the salinity needs to be increased by 3-5 ppt per day. Therefore, it takes a few days to accomplish without any mortality. However, we occasionally found that under 15-20 ppt salinity, some *O. niloticus* developed skin lesions and eventually died (Fig. 7). And one way to cure fish with skin lesions is to place the fish back into freshwater for a week or so. The reason that returns the fish back to freshwater is the freshwater fish those are acclimated in seawater may have skin lesion caused by the diversion of energy for ion osmoregulation rather than for growth. (Watanabe *et al*., 1985; Suresh and Lin, 1992; Ridha, 2008). Returning them back into freshwater may re-adjust their energy for growth, rather than ion osmoregulation, for cure the lesions.

Figure 6. Bird nets constructed over shrimp ponds

In co-culture with marine shrimp, since the fish are intended only to protect the shrimp against AHPND, therefore farmers usually do not feed the fish. Some of the fish, however, could grow to marketable size at the end of shrimp culture and become by-product. Profit from the fish sold commercially, although low compared to that of shrimp, can still compensate for some parts of the investment, e.g. for electricity bill. Additionally, tilapia raised in shrimp ponds with brackish or seawater salinity usually have no off-flavor, and thus are more preferable than tilapia raised in freshwater.

Figure 7. Moribund Nile tilapia being reared under high salinities

Off-flavor in fish comes from the water that the fish are reared in; it is caused by blue-green algae or bacteria that release chemicals called geosmin and/or 2-methylisoborneol (Avault Jr., 1994; Schrader *et al*., 2005, Guttman and van Rijn, 2008) (Fig. 8). Most off-flavored fish originate from stagnant freshwater. For instance, fish from dams that are omnivores or herbivores usually have high degree of off-favor. Waters with movement like rivers have less or no off-flavor being due to the fact that

the blue-green algae and other geosmin/2-methylisoborneol-producing microorganisms do not grow well.

The slow growth of Nile tilapia being reared under high salinities may be attributed to an increase in energy for osmoregulation. However, there are Nile tilapia that survive and grow relatively well under high salinities. The question is what makes this small number of *O. niloticus* different from the majority. Recent research data has raised some interesting explanation on that.

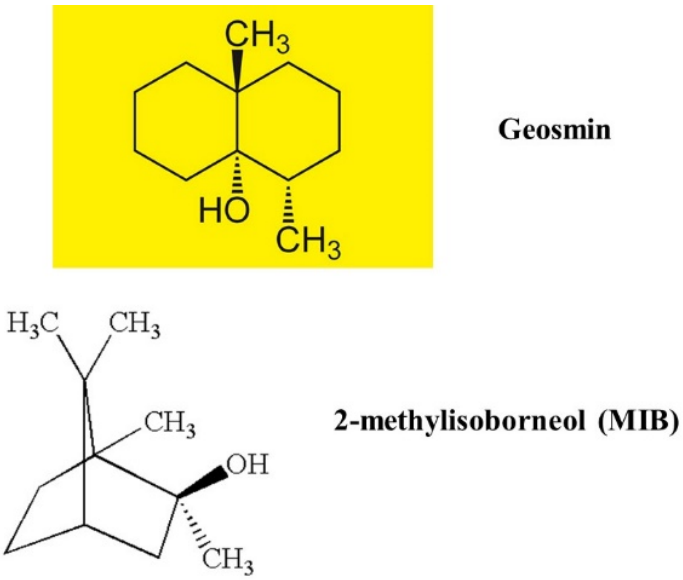

Figure 8. Geosmin and 2-methylisoborneol, the two chemicals that cause off-flavor in fish (from Schrader *et al*., 2005)

1.3.3 Mechanism of Salinity Tolerance in Tilapia

As mentioned, *O. mossambicus* tolerate salinity very well, while *O. niloticus* do not. When both species are living in freshwater, they survive well. As the salinity increases, *O. mossambicus* seems to be able to maintain their blood osmolality, or concentration of their ionic levels in the blood, to their physiological optimum. As salinity increases, salt, especially sodium, will enter the fish blood and extracellular fluid. The fish that are salinity-adaptable could effectively eliminate the extra salt in their blood and extracellular fluid by excreting ions (e.g., Na^+, K^+) out from their bodies, mostly through the gills, which serve as the osmo-regulatory organ of the fish. The sodium pump-out mechanism may require a set of yet unknown

complicated control system. One key enzyme on the cell membrane functioning to pump excessive sodium out of the cell is $Na^+/K^+/2Cl^-$ cotransporter (NKCC), which pumps sodium, potassium and chloride out from the body fluid (Breves *et al*., 2010). The expression of this enzyme is from a translational process from genes that control the production of this enzyme. When *O. mossambicus* is placed in high salinity water, the genes are up-regulated and thus the fish can maintain its osmolality levels in the blood. This mechanism may be absent or the expression of the genes may be too slow or too sluggish in *O. niloticus*. However, some *O. niloticus* individuals may be able to up-regulate their genes, thus making them similar to *O. mossambicus* in their ability to survive in high-salinity water.

When tilapia are transferred from freshwater to brackish or seawater, other biochemical and physiological changes occur in the fish. One of the most mentioned change is a decrease in prolactin (PRL) (Breves *et al*., 2010), a hormone that, in mammals, helps the mother to produce milk for the baby and makes the mother to change her behavior to take care of her offspring; the hormone is thus called nursing hormone. The function of this hormone, PRL, in fish is, of course, not related to milk production (as the fish do not produce milk), but is related to the change in osmolality of the body fluid. The finding of PRL receptors in the gills suggests that PRL may have some function in the gills, and possibly concern the osmo-regulation. The finding that PRL in tilapia is decreased as the fish are placed in brackish and seawater suggests that PRL exerts its function when the fish are in freshwater environment.

Another hormone mentioned in the fish being adapted to high salinities and is structurally related to PRL is growth hormone (GH). Contrary to PRL, tilapia that are transferred from freshwater to brackish water has an increase in GH, as well as an increase in GH receptors in the gills (Breves *et al*., 2010). Therefore, the two hormones seem to be active under two opposite environments; PRL is active when the fish is in freshwater and GH is active when the fish is placed in high salinities. However, the changes in PRL and GH levels in response to changes in salinities do not help *O. niloticus* survive better in high salinities; therefore, the two hormones are not the factors that help the fish to tolerate salinities. It is likely that the tolerance is based on the ability to express certain genes, like NKCC.

Another gene related to increasing osmolarity of tilapia is that regulating the enzyme Na^+-K^+-ATPase. The gene is expressed in a particular cell type called chloride cells, which are salt-secretory cells (Foskett and Scheffey, 1982). Increasing number of chloride cells in the gills and increasing activity of Na^+-K^+-ATPase was shown in *O. massambicus*, following adaptation in high salinities, as well as following cortisol treatment (Dang *et al*., 2000). Another gene, aquaporin-3, was found being related to changes in water salinities in *O. mossambicus* and the gene was localized in the gill chloride cells as well (Watanabe *et al*., 2005).

1.3.4 Gene Insertion into Nile Tilapia Makes the Fish Tolerate High Salinities

Another way to make *O. niloticus* tolerate high salinity is to insert genes of seawater fish into *O. niloticus* genome and allow *O. niloticus* to produce proteins that help their osmo-regulation when being placed in high salinity. Of course, this is to make the tilapia being genetically modified organism (GMO), and may not be suitable commercially. El-Zaeem *et al*. (2011) inserted a part of DNA extracted (salted tolerated gene) from sea beam and *Artemia* into the gonads of *O. niloticus* and found that the tilapia offspring could tolerate salinities up to 32 ppt, with normal growth and survival rate. An interesting finding in their study was that the GMO tilapia had silver skin color without black stripes. In our experience, we also found that *O. niloticus* that survived high salinities also had silver skin color and without black stripes. This is an interesting co-incidence. It is possible that some *O. niloticus* may possess genes similar to those of sea beam or *Artemia* that make them survive high salinities, and therefore the production of salinity-tolerated *O. niloticus* may be by simply select this special strain out of what already exist in nature, it is not necessary to produce GMO tilapia.

1.3.5 Tilapia Hematology and Defensive Mechanisms

Blood components of tilapia include red (RBC) and white (WBC or leucocytes) blood cells. Blood parameters that suggest health of the fish are the proportion of red blood cells in the whole blood volume (or hematocrit, Hct), the size and shape of the red blood cells, the amount of leukocytes and the percentage of each type of leucocytes, or differential WBC counts (Chen *et al*., 2004) (Fig. 9). Decrease in Hct

has been suggested as a sign of bacterial infections of Nile tilapia as well (McNulty *et al*., 2003; Benli and Yildiz, 2004; Shoemaker *et al*., 2006).

The leucocytes can be differentiated into several types: polymorphonuclear (PMN) leucocytes, basophils, eosinophils, monocytes, lymphocytes and thrombocytes. Among these, PMNs and monocytes are considered most important since they function as first-line defense against foreign bodies (or pathogens). Foreign bodies are engulfed (or being phagocytized) by these cell types (Adeyemo *et al*., 2002), destroyed in the cytoplasm by reactive oxygen intermediates (called respiratory burst), like superoxide, hydrogen peroxide and hydroxyl radicals, produced by the cells (Secombes and Fletcher, 1992). The leucocytes also release lysozyme and myeloperoxidase into the fish serum that could kill pathogens.

These properties are shared by all normal fish, thus considered innate defense. The innate defense is sometimes called non-specific defense, to separate it from specific defense that aim at attacking certain particular pathogen. Environmental factors affect non-specific defense of tilapia. At high temperature (33 $^{\circ}$C), *O. niloticus* lysozyme activity in the plasma was decreased. However, at high salinities (12 and 24 ppt) or under low pH (pH 4) condition, the plasma lysozyme activity was increased (Dominguez *et al*., 2005). In *O. mossambicus*, the total leucocyte count, the respiratory burst and phagocytic activity were decreased at low (19-23 $^{\circ}$C) and high (35 $^{\circ}$C), while the lysozyme activity was increased significantly at 31 and 35 $^{\circ}$C (Ndong *et al*., 2007).

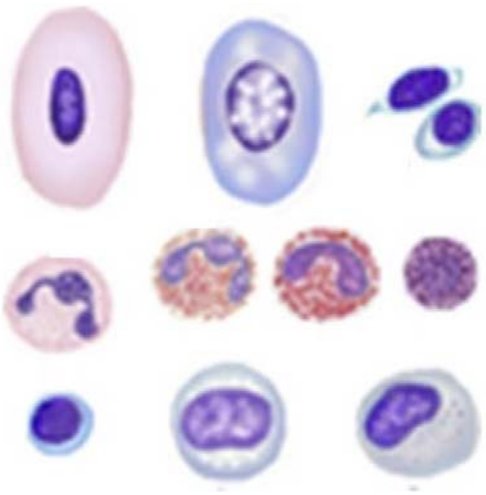

Figure 9. Various cell types in the peripheral blood of fish (from Shoemaker *et al*., 2006)

Among several ways to test the non-specific defense of the fish is to find out if the fish serum could inhibit growth of bacteria placed into the serum. The test is directly called the bacteria inhibition test. This simple method is performed by incubating the fish serum with some types of bacteria, such as *Streptococcus agalactiae*, and, after a certain period, examine the number of bacteria left in the serum. If the number of bacteria left in the serum is decreased, then it indicates that certain components of the serum have an ability to inhibit the growth of the bacteria. The percentage of that inhibition is calculated, by comparing to the growth of the bacteria if no fish serum is added.

Specific defense is usually produced by the production of antibodies against specific foreign bodies or pathogens. In general, the antibodies are composed of immunoglobulin (Ig) M, IgG, IgA and IgE; but in fish, only IgM is produced. Nile tilapia IgM was increased with increasing water temperature from 18 to 28 $^{\circ}$C, and decreased as the temperature reached 33 $^{\circ}$C (Dominguez *et al*., 2004). This suggests that when the water temperature is too warm, as in summer, the immunological defense of tilapia may be lower than normal. This phenomenon agrees well with the observation that the fish has a tendency to catch diseases in summer, like having streptococcal infections.

Salinities, but not pH or suspended solid, also affects IgM level. It was found that Nile tilapia raised in salinity at 24 ppt had significantly higher IgM level than those raised at 12-ppt salinity (Dominguez *et al*., 2004). If this increase was due to the infection of the fish, then the increase level of IgM might be simply the response of the fish to the infection. But if the increase occurred without the infection, the fish might actually have better defense as the salinity increased. This interesting point requires more research.

1.3.6 Serious Pathogens in Tilapia

Several fish diseases have caused significant fish mortalities and economic losses in fish farming. Fish pathogens are variety of micro-organisms from different taxonomic groups, including viruses, bacteria, fungi and protozoa. The most common pathogens associated with mortality in tilapia are *Streptococcus agalactiae* (more in Eastern countries), *S. iniae* (more in Western countries), *Aeromonas hydrophila*, *A. sobria*, *Flavobacterium columnare* and *Francisella* spp. Most of these pathogens are

extracellular bacteria, except *Francisella* spp., which is intracellular bacteria. Few viruses have been associated with tilapia, although viral infection in tilapia has become an important issue recently, such as the infectious spleen and kidney necrosis virus (ISKNV) (Suebsing *et al*., 2013) and tilapia lake virus (TiLV) (Eyngor *et al*., 2014).

Francisella noatunensis belongs to Francisellaceae family. These facultative intracellular bacteria are aerobic, non-motile, coccobacilli gram-negative. The disease caused by infection with *F. noatunensis* and other *Francisella* spp. has been termed francisellosis. The infected fish develop granulomas formation in liver, spleen and kidney. Some infected fish may have clinical manifestations, including swimming error, skin hemorrhage and death, while some may be healthy carriers.

Streptococcosis caused by an infection with *Streptococcus* spp. has been reported as the most prevalent disease in tilapia farming in Thailand (Suanyuk *et al*., 2008, 2010). Clinical signs and symptoms of the infected fish include loss of orientation and erratic swimming, unilateral or bilateral exophthalmia, anorexia, eye opacity, abdominal distention, darkening of skin and hemorrhaging skin around the anus or at the base of the fins (Amal and Zamri-Saad, 2011; Amal *et al*., 2013; Karsidani *et al*., 2010).

Infectious spleen and kidney necrosis virus belongs to the Genus Megalocytivirus in the family Iridoviridae (Wang *et al*., 2007; Whittington *et al*., 2010; Kurita and Nakajima, 2012). The virus has been reported to cause serious mortality in tilapia during its early stages of development (McGrogan *et al*., 1998; Gua *et al*., 2012; Shin *et al*., 2014). One characteristics of ISKNV infection is the presence of hypertrophic cells in the infected organs, such as in the spleen, kidney and connective tissue. Obviously, the disease and this pathogen have been largely unknown, especially in tilapia.

CHAPTER 2
RESEARCH METHODOLOGY

2.1 Experimental Animals

Nile tilapia, *O. niloticus*, were obtained from a commercial source and reared in canvas tanks under freshwater condition at the land belonged to the Prince of Songkla University, Chaiya, Surat Thani, from newly-hatched stage. The rearing tank was round, 6 m in diameter and 1 m high, and stocked with freshwater up to 0.8 m deep. Right after yolk sac resorption, a few days after hatching, they were sex-reversed by feeding on testosterone-mixed feed for three weeks, and grown up to 150-200g size with commercial pellets. Then, 120 fish were randomly sampled and stocked in six 2-ton canvas round tanks, with 2-m diameter and 1-m high (Fig. 10). The tanks are filled up with freshwater to 60-cm depth. Each tank contained 20 fish. Three tanks were designated control group, in which the fish (0-ppt fish) were reared under freshwater throughout the experiment; and 3 tanks were experimental groups, in which the fish (0-ppt fish) were reared under stepwise increase in salinities from 0 to 30 ppt.

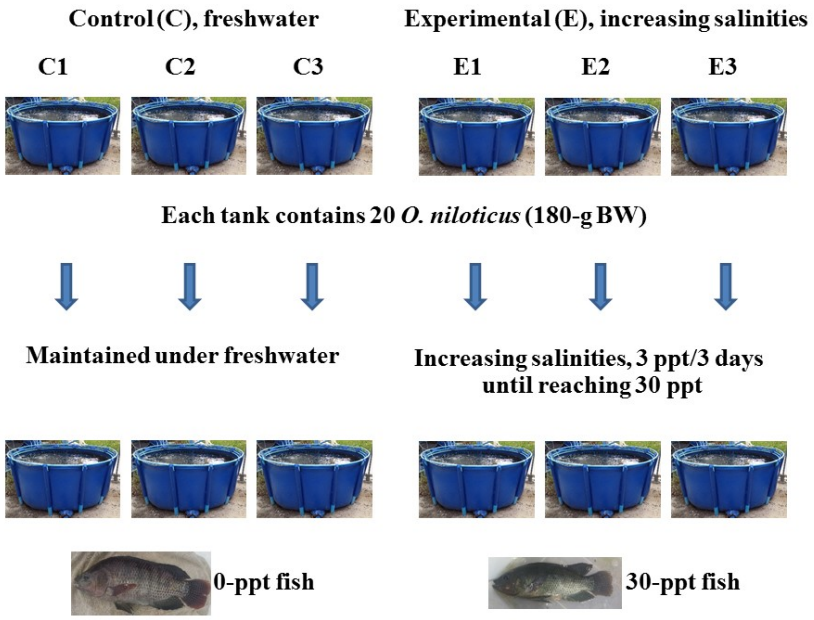

Figure 10. Grouping and rearing conditions in the salinity tolerance study of Nile tilapia, *O. niloticus*

At the beginning of the experiment, the fish were acclimatized in freshwater for 3 days, with adequate aeration. Water qualities; DO, total ammonia nitrogen (TAN), total nitrite, alkalinity, pH and temperature were monitored daily at the first 3 days and then at every 4 days afterward. Water exchange, 70-90%, was carried out whenever TAN and/or total nitrite reach 1 ppm. The DO was maintained at 4 ppm or higher. Temperature, alkalinity and pH were determined. The tanks were covered by shading that shield 70% of sunlight to prevent extreme temperature changes.

The freshwater was from underground water, which was 0 ppt, with pH at 8.0-8.5, with alkalinity at 180-250 ppm and TAN and total nitrite <0.5 ppm. Seawater was from the reservoir that had seawater pumped from the Gulf of Thailand. The alkalinity was at 150-200 ppm, pH at 8.0-8.5, and TAN and total nitrite <0.5 ppm.

The fish were given commercial pellets (containing 25% crude protein) *ad libitum* (approximate 3.5% biomass/d), three times a day (08.00 h, 13.00 h and 18.00 h) and were individually weighed at every 5 days. At the end of the two-month experiment, average daily growth (ADG) was calculated as weight gain (g)/rearing period (60 d), and specific growth rate (SGR) was calculated as: [ln (W2) – ln (W1)] x 100/t; where W2 = final BW, W1 = initial BW, and t = experimental period (60 d).

2.2 Salinity Adjustment

After the acclimatization period, salinity in the experimental tanks was increased successively by 3 ppt, from 0 to 30 ppt. Therefore, 10 steps of salinity adjustment: 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 ppt, all together took 30 days to reach the final salinity of 30 ppt. The fish were thus maintained under each level of salinity for 3 days. The salinity adjustment was carried out by taking away certain amount of the existing rearing water and filling up with new 30-ppt seawater.

2.3 Determinations of Water Qualities

The above water qualities were determined every 4 days periodically using test kits and spectrophotometric methods. As mentioned, water exchange was carried out when any of the parameters was not in optimum range. After the water exchange, salinities were carefully restored to the assigned levels.

2.4 Procedure during Fish Rearing

With increasing salinity, a number of the 30-ppt fish had become weaken, moribund and dead. All the tanks were observed twice daily at 09.00 and 17.00 h and dead and moribund fish were taken out from the tank. The number of dead fish observed daily in the experimental tanks was more than those of the control, and, if without any intervention, the stocking density of the experimental tanks would be less than that of the control tanks. With lower density, the fish would have grown faster, and therefore the data on growth rate might not be relevant as stocking density influences growth rate. To avoid that problem, normal fish in the control tanks were removed out to make the number equal to that in their experimental counterpart tanks. The fish that removed from the 3 control tanks were placed in separated 500-L tanks with proper aeration and care.

At the end of the experiment, 10 each of the apparently healthy 0-ppt and 30-ppt fish were randomly sampled and tested for the presence of pathogens: infectious spleen and kidney necrosis virus (ISKNV), *Francisella noatunensis* and *Streptococcus agalactiae*, using polymerase chain reaction (PCR) method, positive results was confirmed by *in situ* hybridization method. The presence of pathogens was further confirmed by localization, using *in situ* hybridization methods.

At the end of the 60-day experiment, the 0-ppt and 30-ppt fish that survived and apparently healthy were randomly sampled for determinations of hematological parameters and serum enzyme levels. Blood, 100 μL, was withdrawn from tail veins of individual fish and immediately mixed with heparin (200 unit/mL) for the determinations of hematological parameters. For the determinations of serum enzyme levels, 1-mL of blood was withdrawn from the tail vein without anti-coagulant and transferred to 1-mL Eppendorf tube. Serum was isolated by centrifugation at 4,000x g for 10 min and stored at -20 $^{\circ}$C until analysis.

2.5 Determinations of Hematological Parameters

The withdrawn blood was immediately determined for Hct, total RBC counts, total and differential WBC counts and mean corpuscular volume (MCV). Hematocrit was determined by placing well-mixed blood into capillary tubes and centrifuged at 12,000 rpm for 5 min, and calculated as percentage of packed cells volume against

whole blood volume (Goldenfarb *et al*., 1971). The RBC and WBC counts were determined by adding Yokoyama staining solution (Yokoyama, 1947) into the whole blood and placing well-mixed stained blood under Neubauer chamber and counted (Silva *et al*., 2009). Mean corpuscular volume (MCV) was determined by using the formula: MCV (fL) = (Hct x 10)/total RBC (x10^6 cells/μL). WBC differential counts were done by smearing whole blood onto microscopic slides, stained by Wright–Giemsa and examined under light microscopy for neutrophil, lymphocyte, monocyte and thrombocyte. At least 200 cells were counted for differential WBC determinations (Stoskoph, 1993). From the total WBC count and percentage of each cell types, absolute counts of neutrophil, lymphocyte, monocyte and thrombocyte were calculated.

2.6 Determinations of Serum Enzymes

2.6.1 Lysozyme Activity

Lysozyme activity of serum was determined following the procedures described by Chang *et al*. (2012) with some modifications. Briefly, 100 μL serum was mixed with an equal volume of 0.3 mg/mL suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in sodium phosphate buffer. Optical density was measured at 540 nm using a microplate reader (iMark Microplate Reader, Bio-Rad, USA) at 0 min and 10 min after incubation at 25 °C using sodium phosphate buffer as blank. For negative control, sodium phosphate buffer was used instead of serum. A unit of lysozyme activity was expressed as the amount of serum causing a decrease in absorbance of 0.001 unit/min

2.6.2 Alkaline Phosphatase Activity

Alkaline phosphatase activity was determined following the procedures described in Caipang *et al*. (2014) with some modifications. Briefly, 20 μL of 0.5 mg/mL *p*-nitrophenyl phosphate (Merck Millipore, USA) was added to an equal volume of serum in a microtube. It was then incubated at 25 °C for 30 min, followed by an addition of 3 N NaOH (100 μL) to terminate the reaction. Optical density was read at 405 nm using a spectrophotometer (Biochrome Libra S22, UK).

2.6.3 Myeloperoxidase Activity

Myeloperoxidase was assayed following the procedures described (Caipang *et al*., 2014) with some modifications. Briefly, 35 µL of plasma was added in 96-well plates. The serum sample in each well was added with 35 µL of 20 mM 3,3′,5,5′-tetramethyl benzidine hydrochloride (Amresco, USA) and 5 mM H_2O_2. After 2-min incubation period, 35 µL of 4 M sulfuric acid was added to stop the reaction, and the absorbance was read using a spectrophotometer (iMark Microplate Reader, Biorad, USA) at 450 nm. Deionized water, instead of serum, was identically treated and used as control. Stimulation index was calculated by dividing each sample value by its means control value (Diaz-Rosales *et al*., 2006)**.**

2.6.4 Bactericidal Activity

The capability of serum in inhibiting proliferation of *Vibrio parahemolyticus* was determined using a MTT assay using the procedure modified from Budiño *et al*. (2006). Briefly, 100 µL of serum was mixed with 10 µL of bacteria suspension to make final concentration of 10^6 CFU/mL and incubated at 35 °C for 24 h. After incubation, 25 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in PBS (MTT; 2 mg/mL) was added to each well and mixed. The MTT reduction was read at 630 nm after incubated for 15 min at 25 °C with a spectrophotometer (Biochrome Libra S22, UK). Bactericidal index was expressed as absorbance of each sample divided by the absorbance of the control, which was the bacterium incubated in the culture medium without the fish serum.

2.7 PCR Procedure

Three types of representative pathogens: ISKNV (virus), *F. noatunensis* (intracellular bacteria) and *S. agalactiae* (extracellular bacteria), were determined in normal 0-ppt and 30-ppt *O. niloticus*, 10 from each group, using PCR method. The three pathogens were found in apparently healthy Nile and red tilapia in commercial culture (Suebsing *et al*., 2013, 2015, 2016; Pradeep *et al*., 2016).

2.7.1 DNA Extraction

Total DNA was extracted from brain, gills, kidney, liver, spleen, skin, testis, and ovary by using 200 µL of 0**.**005 N NaOH**.** All tissue samples were homogenized and incubated at 95 °C for 5 min, then placed on ice for 5 min and centrifuged at 12,000

rpm for 15 min, the clear solution was collected in a new tube**.** The DNA was precipitated with an equal volume of cold isopropanol. The mixture was incubated at –20 °C for 1 h. The mixture was then spun down at 12,000 rpm for 5 min and the supernatant discarded. The DNA pellet was then washed with 75% ethanol and dried. The dried DNA was dissolved in 30-100 µL of H_2O, kept at 4 °C until use. The volume of H_2O added was depended on the amount of DNA obtained.

The DNA concentrations were measured by using NanoDrop™ to prepare the final concentration of 100 ng/µL DNA and stored at -20 °C until use**.** The PCR procedure was accomplished by using specific primers for each pathogen (Table 1).

2.7.2 PCR Procedure for ISKNV and *F. noatunensis* Detection

The PCR reactions were performed in a 25 µL of total reaction mixture containing 1x PCR buffer, 10 mM dNTP mixture (Promega, USA), 50 mM $MgCl_2$, 10 µM each primer (Table 1), template DNA, and 1 unit of *Taq* DNA polymerase (Thermo Scientific, USA). The PCR program consisted of initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing of primers at 60 °C for 20 sec and primer extension for 72 °C for 30 sec. To complete the chain extension, the reaction mixture was incubated for 3 min at 72 °C after the last cycle. The PCR products were determined by 1.5% agarose gel electrophoresis and ethidium bromide staining.

2.7.3 PCR Procedure for *S. agalactiae* Detection

The oligonucleotide primers were selected from the DNA coding for the superoxide dismutase gene for *S. agalactiae* (GenBank: HM004089 – HM004094) based on previously published paper (Suebsing *et al*., 2013). Primers used were forward primer, and reverse primer. The PCR reactions were performed in a 25 µL of total reaction mixture containing 1x PCR buffer, 10 mM dNTP mixture (Promega, USA), 50 mM $MgCl_2$, 10 µM each primer (Table 1), 100 ng template DNA, and 1 unit of *Taq* DNA polymerase (Thermo Scientific, USA). The PCR program consisted of initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing of primers at 63 °C for 20 sec and primer extension for 72 °C for 30 sec. The PCR products were visualized by the same procedure used in section 2.7.2.

Table 1. Specific primers used for PCR for the detections of three representative pathogens. IS, ISKNV; FN, *F. noatunensis*; SA, *S. agalactiae*; HSP, heat shock protein; MSP, major capsid protein; SOD, superoxide dismutase

Primer Name	Sequence (5' to 3')	Target Gene	Amplified Product (bp)	Reference
IS F3	TGTAGCCAGACTGTTTGCTG	MSP	400	Suebsing *et al*., 2016
IS B3	AGATCCCCTCCATCACATCC			
IS FIP	ACCGACACCTCCTCCACTAGAT/ TTTT/GGAGAACAGCTACATCCGC		300	Suebsing *et al*., 2016
IS BIP	TTAACGACCTGGTGGCACAGAC/ TTTT/CATGCAGGCGTTCCAGAAG			Suebsing *et al*., 2016
IS LF	GCATCAGATTGTCGCACCA		200	Suebsing *et al*., 2016
IS LB	CCTCACCAGCGAGTTCCT			
FN F	TGTTTCTAAGTCAGGTAAAGC	HSP	200	Pradeep *et al*., 2016
FN R	CCTAGATCTTCAGAGATAACTGT			
SA F3	ATATGATGCGCTTGAGCC	SOD	200	Suebsing *et al*., 2013
SA B3	ACCACCGTTATTGATGACTG			

2.8 *In situ* Hybridization using Loop-mediated DNA Amplification with Digoxigenin Labelling (ISDL)

As the results of pathogen detections by PCR revealed that ISKNV was the only pathogen detected, further investigation to confirm the presence of ISKNV in the fish tissue was performed. Representative samples from ovary, testis, and kidney that were found positive for the presence of ISKNV by PCR methods were randomly selected for paraffin sectioning with hematoxylin and eosin (H&E) stain and by *in situ* hybridization of the amplifying product from loop-mediated DNA amplification (LAMP) with digoxigenin (DIG) or ISDL (Jitrakorn *et al*., 2016). ISKNV specific primers for LAMP method were designed according to the published sequences of the major capsid protein (MCP) gene (GenBank accession no. AB669096). A set of six primers composed of outer (ISKNV-F3 and B3), inner (ISKNV-FIP and BIP) and loop (ISKNV-LF and LB) primers was designed as previously reported in Suebsing *et al*.

(2016) (Table 1). Briefly, a routine digestion step was replaced by heat denaturation (100 °C, 2 min). A total of 150 µL of LAMP-DIG labeling mixture was placed onto each histological section and then covered with a Frame–Seal™ Incubation Chamber (Bio-Rad Laboratories, Philadelphia, PA, USA). The slides were set on a heat block warmed at 65 °C and *in situ* LAMP reaction was conducted for 45 min. The slides were subsequently performed according to the traditional *in situ* hybridization using anti-DIG labelled with alkaline phosphatase (Roche, Germany) and color was developed with a nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (Roche, Germany). The slides were then stained with 0.5% Bismarck brown Y. The resulting slides were examined under light microscopy for the presence of dark purple to black staining against the brown counterstain, which indicated hybridization with target DNA of MCP. Negative control was prepared under identical process without adding DIG-labelled dUTP in the LAMP-DIG labeling mixture. The entire process of ISDL was depicted in figure 11.

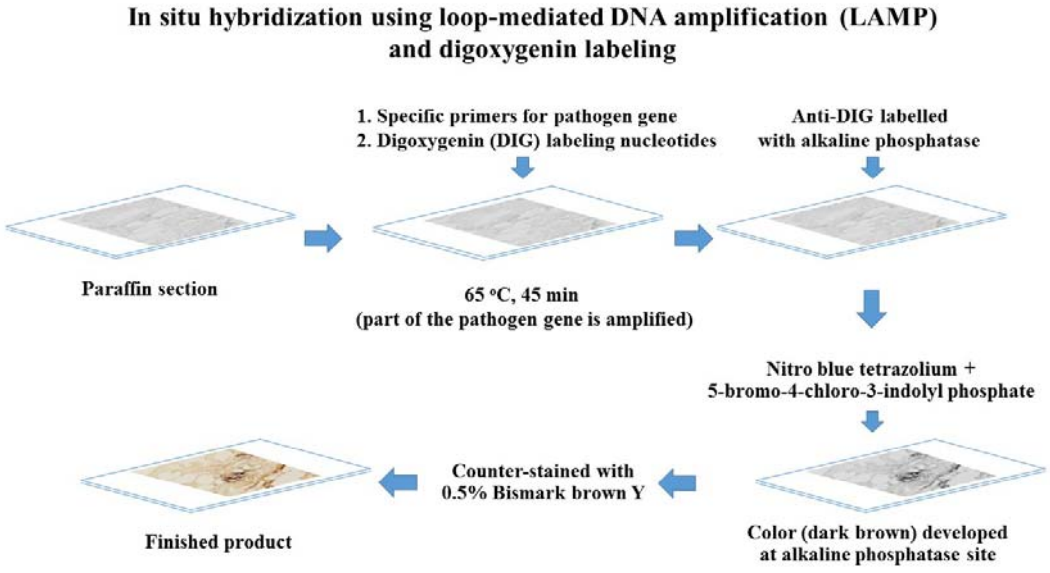

Figure 11. Procedure of *in situ* hybridization using loop-mediated DNA amplification with digoxigenin labeling (ISDL)

2.9 Data Analysis

The data are analyzed by Student t-test and significant levels of difference is indicated at 95% (significant) and 99% (highly significant) levels.

CHAPTER 3
RESULTS

3.1 Water Qualities

During the experiment, water qualities, except salinity, were similar in all tanks, either in freshwater or in elevated salinity groups, and no statistically significant difference was detected between the two groups (Fig. 12). Water temperature was around 28 °C in the morning (08.30 h) and slightly increase in the afternoon (16.00 h) to 31 °C. This level of temperature is considered optimum for tilapia and for most types of tropical fish. Dissolved oxygen level was above 6 ppm in all tanks, which is considered adequate for tilapia since the fish are normally healthy at DO around 3-4 ppm (Popma and Lovshin, 1996). Water pH was around 8 in both freshwater and elevated salinity groups. For freshwater, this level of pH is considered high, though not unusual. It is most likely due to the use of underground water with high alkalinity level, around 240 ppm. However, these levels of pH and alkalinity do not normally pose any problem to the fish (Sifa *et al*., 2002; Luan *et al*., 2008; Rebouças *et al*., 2015).

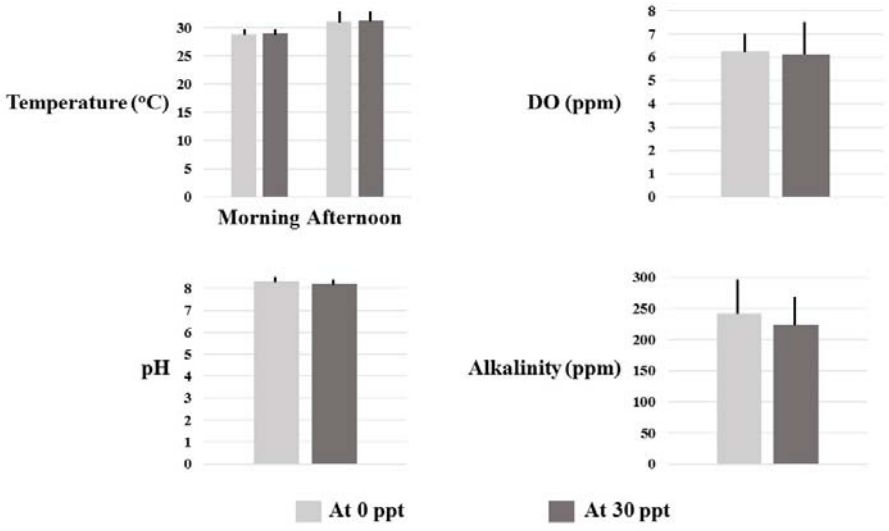

Figure 12. Water temperature, dissolved oxygen (DO), pH and alkalinity in rearing tanks of 0-ppt and 30-ppt *O. niloticus*

Since water exchanges were performed regularly to ensure that TAN and total nitrite were at safety levels for the fish, both levels were therefore always below 0.5 ppm. In addition, the stocking density, at less than 20 individuals per tank, or around 2.5-kg biomass per ton of water, is considered low and did not pose any risk of nitrogen waste accumulation.

3.2 Survival Rate of the Fish

Under elevated salinity, the 30-ppt fish became moribund and dead when the salinity was increased to 18-ppt and the mortality was increase when salinity reached 27 ppt (Fig. 13). The 0-ppt fish survived well throughout the 60-day experiment. Statistically significant difference ($p<0.05$) was detected from day 40 onward when salinity was at 30 ppt. At day 60, survival rate of the 0-ppt fish, was 94.3 ± 3.1 (n, 3) %, whereas that of the 30-ppt fish was at 51.3 ± 15.5 (n, 3) %. Some of the 30-ppt fish that were found weak and moribund suffered skin lesion, with descaling, with or without subcutaneously hemorrhage.

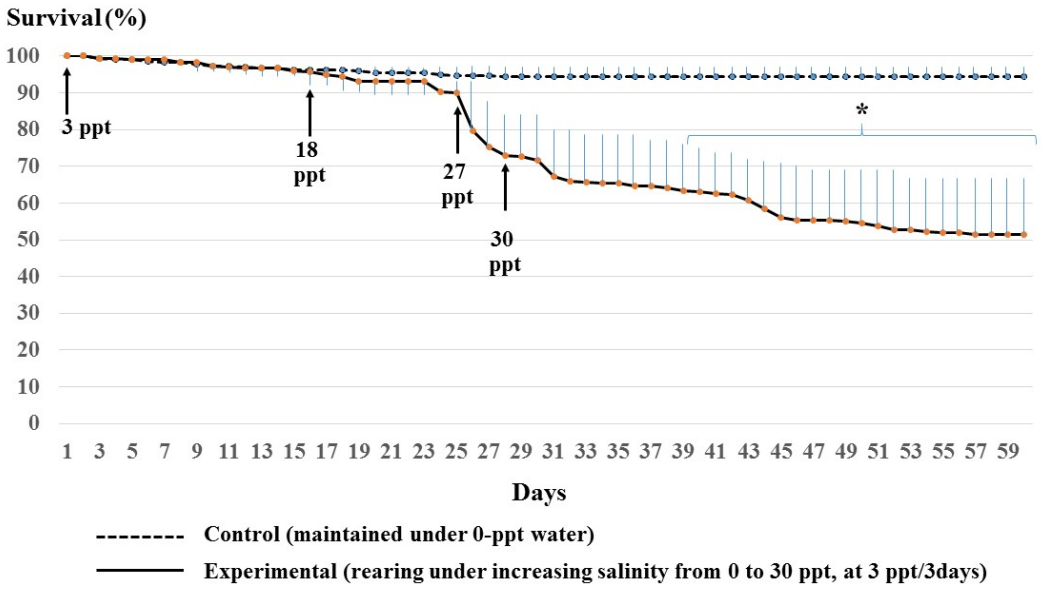

Figure 13. Survival rate of 0-ppt and 30-ppt *O. niloticus* over 60-day course. Each point is mean and standard deviation from 3 replicates. *$p<0.05$, compared to the 0-ppt fish.

3.3 Growth Rate of the Fish

The initial BWs of the fish in all tanks, both the 0-ppt and 30-ppt fish, were 180 ± 30.20 g, and not significantly different among the two groups (Fig. 14). As mentioned in Chapter 2, comparison of the fish BWs was made between experimental group (combined E1, E2 and E3) and control group (combined C1, C2 and C3), the fish BW of the experimental group was significantly ($p<0.01$) lower than that of the control group, beginning at day 16 when the salinity was at 12 ppt.

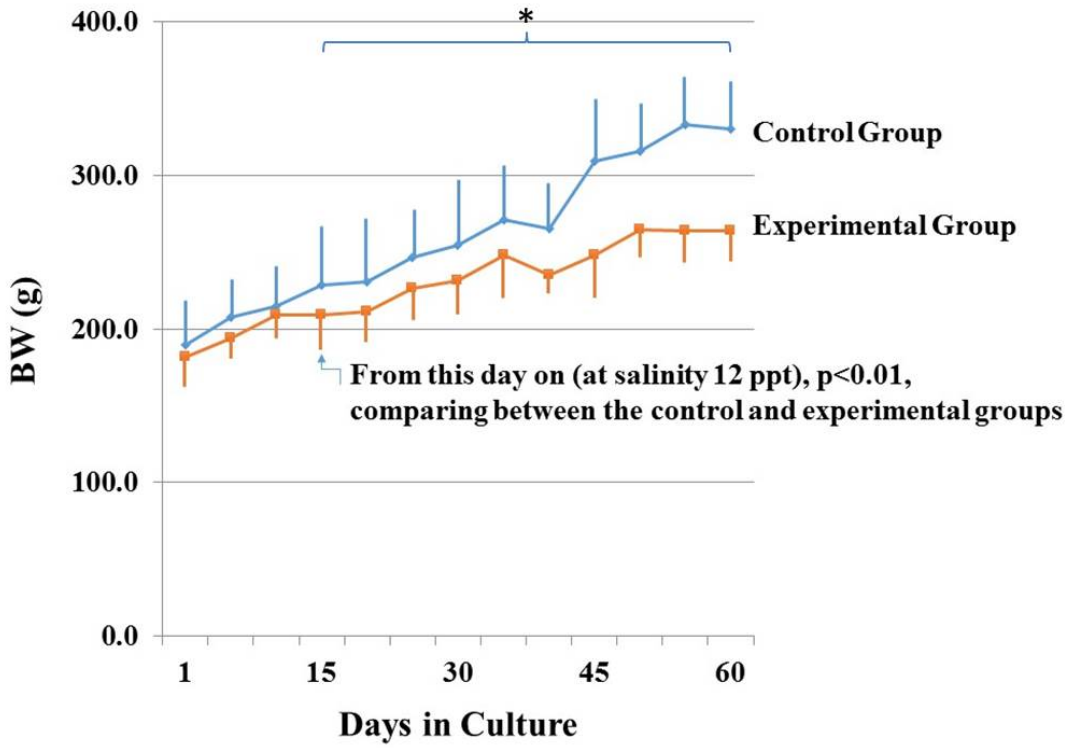

Figure 14. Body weights of the 0-ppt (combined C1, C2 and C3) and 30-ppt *O. niloticus* (combined E1, E2 and E3) over the 60-day course. Each point represents mean and standard deviation of 20 fish.

At day 60, average daily growth of the 0-ppt and 30-ppt fish was 2.34 ± 0.20 (n, 3) and 1.37 ± 0.43 (n, 3) g/d, respectively; the difference was statistically significant ($p<0.05$). The specific growth rate was 0.93 ± 0.07 % (n, 3) and 0.62 ± 0.16 % (n, 3) %/d, respectively. Therefore, the growth rate of the 30-ppt fish was about 60% of that of the 0-ppt fish at the end of the experiment. The standard deviations of the ADG and SGR of the 30-ppt fish were higher than those of the 0-ppt fish, with coefficient of

variation of ADG at 8.6% and 31.4% for the 0-ppt and 30-ppt fish, respectively. This indicates marked size difference among the 30-ppt fish. And by observation, the 30-ppt fish had more variation in size than the 0-ppt fish.

Gross morphology of the 30-ppt fish, besides being smaller, was different from that of the 0-ppt fish. The 0-ppt fish normally had pinkish color at the periphery of the caudal and pectoral fins while the 30-ppt fish did not (Fig. 15). The skin color at the operculum and belly of the 30-ppt fish also had less pink color, compared to the 0-ppt fish. Another feature found frequently in the 30-ppt fish was that the periphery of the caudal fin was partly torn and had irregular border.

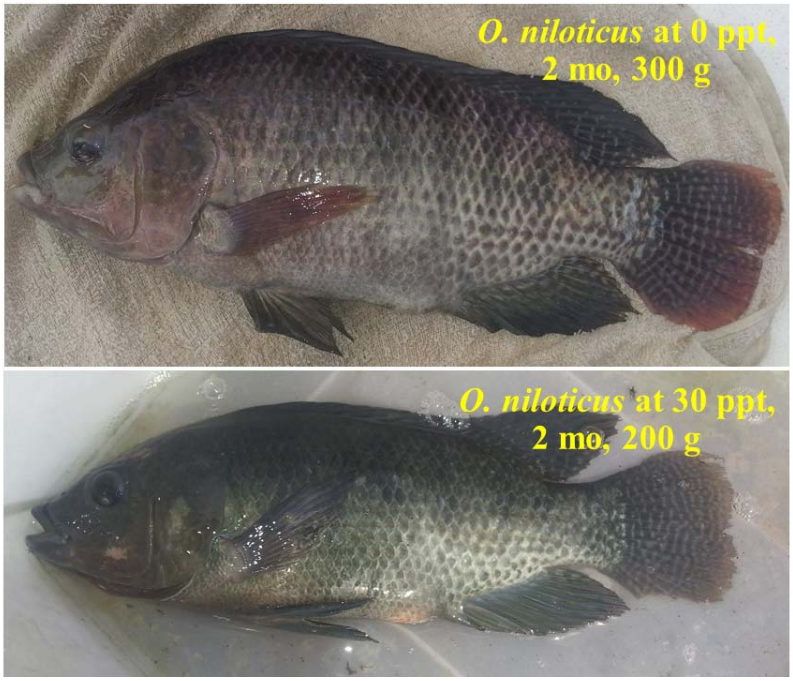

Figure 15. Gross morphology of the 0-ppt and 30-ppt *O. niloticus*. The 30-ppt fish showed difference in size, color of fins and skin, as well as tearing of caudal fin.

3.4 Hematological Parameters

Hematological parameters were compared between the apparently healthy 0-ppt and healthy 30-ppt *O. niloticus*. The parameters determined included Hct, total RBC count, MCV, WBC count, percentage and absolute amounts of lymphocytes, monocytes, neutrophils and thrombocytes.

The mean Hct of the 0-ppt fish was 28.8 ± 3.2% and that of 30-ppt fish was 24.1 ± 1.2% (Fig. 16); although it seems that the difference was not much but, statistically, the two values significantly differ ($p<0.01$).

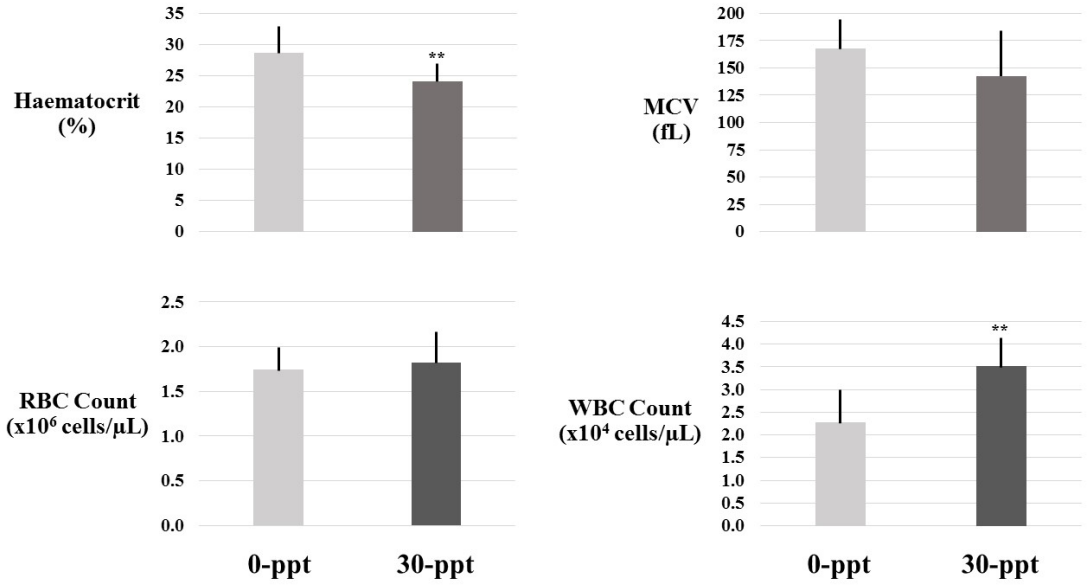

Figure 16. Hematocrit, total red blood cell (RBC) count, mean corpuscular volume (MCV) and total white blood cell (WBC) count of the 0-ppt and 30-ppt *O. niloticus*. **$p<0.01$, compared to the 0-ppt value.

As the total WBC was determined in both groups of fish, it revealed that the 0-ppt fish had an average of 23,000 ± 6,000 cells/µL of blood, while that of the 30-ppt fish was at 35,000 ± 5,000 cells/µL of blood (Fig. 16). The difference of about 1.5 times higher in total WBC count in the 30-ppt than in the 0-ppt fish was highly significant ($p<0.01$). Since the WBC determined in this study were blood cells that were not RBC, and the count included thrombocyte; it might be stated that the count was actually total WBC plus thrombocytes.

The differential counts revealed that the highest percentage was in the lymphocytes and thrombocytes, followed by neutrophils and monocytes (Table 2). In the 0-ppt fish, the percentage of both lymphocytes and thrombocytes was similar, and together accounted for about 80% of the total cell count. In the 30-ppt fish, almost

90% of the cell counts was the lymphocytes and thrombocytes, and more thrombocytes (>10% higher) were observed than lymphocytes. The percentage of thrombocytes in the 30-ppt fish was also >10% higher than that of the 0-ppt fish; and the difference was statistically significant. The percentage of monocytes, the least WBC population, in the 30-ppt fish, however, was significantly lower than that in the 0-ppt fish ($p<0.05$).

Table 2. Percentage of cell types (besides red blood cells) in the peripheral blood of 0-ppt and 30-ppt *O. niloticus*. Numbers in the parentheses are numbers of determination.

Cell type	Different WBC (%)	
	0 ppt	30 ppt
Neutrophil	9.55 ± 7.03 (10)	7.35 ± 3.93 (10)
Monocyte	7.20 ± 2.46 (9)	4.15 ± 1.44 (10)*
Lymphocyte	42.00 ± 14.18 (10)	37.95 ± 7.38 (10)
Thrombocyte	37.39 ± 13.63 (9)	50.55 ± 7.78 (10)*

*$p<0.05$, compared to 0 ppt

With the total WBC count and differential count, absolute numbers of each cell types were calculated, which represented the number of cells/μL in the peripheral blood. By doing that, it revealed that the absolute number of neutrophils/μL of the blood in the 0-ppt and 30-ppt fish was comparable, with a slightly, but non-significantly, higher level in the 30-ppt fish (Fig. 17). High variation among individual values was also observed. The absolute number of lymphocytes of the 30-ppt fish was averaged at 13,500 ± 1,700 cells/μL, comparing to 9,400 ± 1,600 cells/μL in the 0-ppt fish. Statistically, the difference, which was 1.4x time higher in the 30-ppt fish, was highly significant ($p<0.01$). For monocytes, although the percentage of cells in the 30-ppt fish was significantly lower than that of the 0-ppt fish (Table 2), but since the total WBC in the 30-ppt fish was significantly lower than that of the 0-ppt fish, when calculated into absolute number, the absolute values of monocytes in the 30-ppt and 0-ppt fish has become comparable and no statistically significant difference was observed. The percentage of thrombocytes was already significantly higher ($p<0.05$) in

the 30-ppt fish, compared to that of the 0-ppt fish (Table 2), and when being converted to the absolute number, the difference had become highly significant ($p<0.01$). The levels of thrombocytes in the 30-ppt fish was 17,700 ± 2,300 cells/μL, while that of the 0-ppt fish was 9,400 ± 2,600 cells/μL; the value of the 30-ppt fish was almost twice that of the 0-ppt fish.

In conclusion, the absolute numbers of both lymphocytes and thrombocytes in the peripheral blood were significantly increased in the 30-ppt fish, compared to those of the 0-ppt fish.

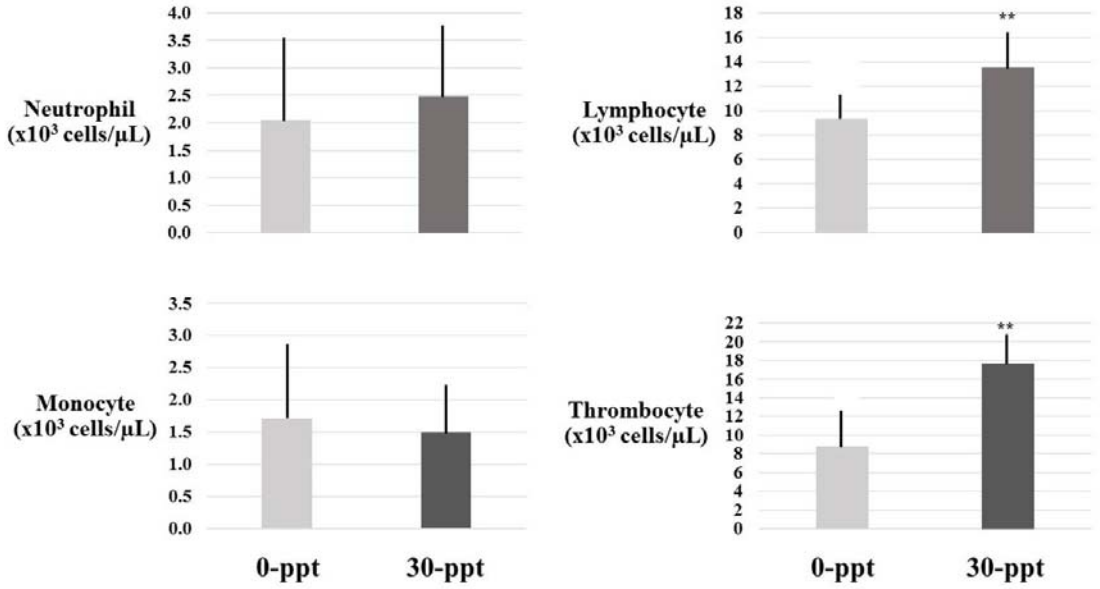

Figure 17. Absolute number of peripheral neutrophils, monocytes, lymphocytes and thrombocytes of the 0-ppt and 30-ppt *O. niloticus*. **$p<0.01$, compared to the 0-ppt value.

3.5 Serum Enzyme Levels

The following serum enzyme activities were determined in apparently healthy 0-ppt and 30-ppt *O. niloticus*: lysozyme, myeloperoxidase and alkaline phosphatase. In addition, the bactericidal index was tested in both groups of fish to find out any difference in the fish ability to eliminate bacteria from their blood.

The test revealed that, among the three serum enzymes studied, only lysozyme activity was found significantly different between the two groups, and that of the 30-ppt fish was about 1.7x times higher than that of the 0-ppt fish (Table 3). The bactericidal index of both groups was also similar, and without significant difference.

A large variation in all the serum parameters tested was observed among individual fish, with the coefficient of variations were between 12 to 46% and no difference of these values between the 0-ppt and 30-ppt fish. This probably reflects genetic variations among individual fish, which do not depend on water salinity.

Table 3. Lysozyme, myeloperoxidase, alkaline phosphatase and bactericidal index of 0-ppt and 30 ppt *O. niloticus*. Numbers in the parentheses are numbers of determination.

Determined parameters	**Enzyme level**	
	0 ppt	**30 ppt**
Lysozyme (unit/mL)	28.8 ± 13.1 (8)	49.3 ± 19.3 (9)*
Myeloperoxidase (stimulation index)	1.25 ± 0.15 (10)	1.46 ± 0.30 (10)
Alkaline phosphatase (405 nm absorbance)	0.19 ± 0.05 (10)	0.16 ± 0.04 (7)
Bactericidal index	0.89 ± 0.19 (10)	0.93 ± 0.29 (8)

*$p<0.05$, compared to 0 ppt

3.6 PCR

The presence of ISKNV, *F. noatunensis* and *S. agalactiae* was determined in apparently normal 0-ppt and 30-ppt fish by PCR. The PCR was performed in the gills, brain, liver, testis (gonad), kidney and spleen, but not in the skin of the fish. The results revealed that, among the three pathogens checked, only ISKNV was detected. Six out of 10 of the 30-ppt fish tested were positive, while only one out of 10 of the 0-ppt fish was positive (Table 4). The positive signals as revealed by the width and density of the amplification product bands at 200 bp were arbitrarily judged as negative (-), lightly (+), moderately (++) and severely (+++) positive. The single 0-ppt fish that was positive for ISKNV showed the presence of ISKNV amplification

product in the gills, brain, liver, testis, kidney and spleen, but not in the skin. Severely positive signal was found in the kidney and spleen, while moderately positive signal was found in the liver and testis. All the six 30-ppt fish that were ISKNV-positive had positive signals in most tissues studied, with variable degree of band density; however, no tissue was considered having severely positive signals. Moderate signal was detected mostly in the ovary and kidney, whereas the skin was the least frequently detected.

Kidney and gonads seemed to the two organs heavily infected by ISKNV. In the kidney, 4 out of 7 fish showed moderate PCR signal, while in the gonads, 3 out of 7 fish had moderate PCR signal, while one was negative.

Table 4. Pathogens detections by PCR of 0-ppt and 30-ppt *O. niloticus*, from 10 randomly sampled fish from each group.

A. Number of positive fish/total number of fish examined

Pathogen	**0 ppt**	**30 ppt**
Infectious spleen and kidney necrosis virus (ISKNV)	1/10	6/10
Francisella noatunensis	0/10	0/10
Streptococcus agalactiae	0/10	0/10

B. Tissue distribution and intensity of ISKNV-positive *O. niloticus*. -, negative; +, lightly positive; ++, moderately positive; +++, severely positive

Salinity	**Fish**	**Tissue**						
	No.	**Gills**	**Brain**	**Liver**	**Gonads**	**Skin**	**Kidney**	**Spleen**
0 ppt	1	+	+	++	++	-	+++	+++
30 ppt	1	+	+	+	++	-	+	-
	2	+	-	+	++	-	++	+
	3	+	++	-	+	-	++	+
	4	+	-	+	+	-	+	+
	5	+	+	-	+	-	++	+
	6	+	+	+	-	+	+	+

3.7 ISDL

Tissue samples of the ovary, testis and kidney that were found moderately positive for the presence of ISKNV by PCR methods were further processed for *in situ* hybridization, using ISDL technique to amplify the specific product in the tissue and visualized by peroxidase reaction (Fig. 18). In the ovary, positive signals were detected at both the periphery and within the nuclei of mature oocytes. In the testis, positive signals were observed in the lumen of the seminiferous tubules. It seemed that positive cells were large-sized, unidentified cells localized inside the testis. In the kidney, it was observed that the signals were localized in some nuclei of the epithelial cells, while some were in the cytoplasm and intercellular space, and in other non-epithelial cells.

The enlarged positive cells were similar to the infected cells infected by ISKNV, which is a type of megalocytic virus reported previously (Dong *et al*., 2008).

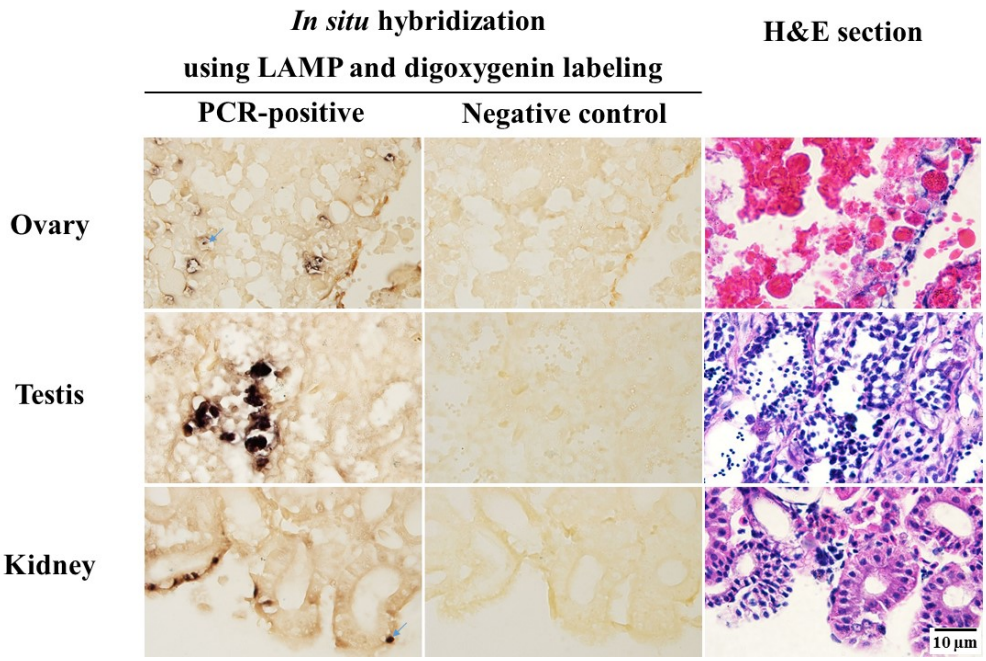

Figure 18. Photomicrograph of tissue sections processed through ISDL showing the presence of ISKNV as dark brown precipitates in sections of ovary, testis and kidney of the 30-ppt *O. niloticus*. Arrows point to ISKNV-positive signal in nuclei of oocyte and kidney epithelium. All figures have the same magnification.

CHAPTER 4
DISCUSSION

The survival and growth rates of *O. niloticus* reared under elevated salinity reported herein were similar to those reported by Kamal and Mair (2005) and Luan *et al*. (2008). Kamal and Mair (2005) reported that *O. niloticus* reared under 20-ppt water and higher had low survival, probably being due to osmotic stress and diseases. In the study of genotype x environment interaction of *O. niloticus* in fresh and brackish water, Luan *et al*. (2008) reported a slightly decreased in survival (<5%) and growth (<10%) rates of the brackish tilapia reared under 20 ppt, compared to those under 0 ppt. More importantly, they showed that the genetic correlations of harvest body weight and survival were relatively low between the two tested environments. In other words, the fish that grew or survived well under freshwater may not do so under brackish water. Therefore, it is likely that two separated sets of genes are responsible for these two traits and, to select brackish *O. niloticus* for growth and survival, a separate selective breeding program is recommended.

In this study, it is clearly shown that salinity affected survival and growth of *O. niloticus*, with retarded growth rate starting as early as 6 ppt, and both survival and growth were significantly affected when salinity reached 30 ppt. It also showed that around 50% of the fish could survive full-strength seawater. Together with the separate breeding program suggested by Luan *et al*. (2008), it is possible to improve growth and survival of the brackish (or seawater) *O. niloticus*, which would make them more suitable to co-culture with marine shrimp.

In this study, the Hct of the 30-ppt *O. niloticus* was significantly lower than that of the 0-ppt fish. To our knowledge, decreased Hct level has not been reported in *O. niloticus* reared under elevated salinity. It was shown, however, that the fish became anemic under certain bacterial infection, e.g., by *Mycobacterium marinum* (Ranzani-Paiva *et al*., 2004). The anemic condition in this case was apparently due to the smaller size of RBC, not the reduced number of total RBC. It suggests that RBC production in the 30-ppt fish is not affected by the salinity. The reduced size of RBC could be due to reduced amount of hemoglobin or reduced water content of the cells. It

is likely that the plasma of the 30-ppt fish might have increased osmolality, compared to the 0-ppt fish, water efflux from RBC might occur through osmotic pressure.

In this study, increased level of peripheral WBC, with significant increase in lymphocyte population was observed in the 30-ppt fish. The finding, again, has not been reported in *O. niloticus* reared under elevated salinity, but rather in bacterial infectious conditions, like being infected by *M. marinum*, *Enterococcus* sp. and by *Flavobacterium columnare* (Ranzani-Paiva *et al*., 2004; Martins *et al*., 2009; Sebastião *et al*., 2011).

Among all cell types of WBC of tilapia, lymphocyte population was the highest (Hrubec *et al*., 2000; Gültepe *et al*., 2014). In fish, as well as in mammals, lymphocytes are cells that could be transformed to mediate both cellular and humeral immunity (Moss *et al*., 2009). In this study, it is possible that, with elevating salinity, pre-existing pathogen (in this case, ISKNV) may flare up and activate lymphocyte proliferation. This reaction may be one of the immunological responses of the 30-ppt fish to the viral infection.

In this study, thrombocytes were also significantly increased in the 30-ppt fish, compared to that of the 0-ppt fish. In mammals, it is well known that thrombocytes are responsible for clotting mechanism. Doggett and Harris (1989) reported that thrombocytes of *O. mossambicus* were also responsible for clotting mechanism. In addition, in fish, thrombocyte may be involved in defense mechanisms against pathogens and phagocytic activity (Bozzo *et al*., 2007; Jatoba´ *et al*., 2011). Nile tilapia consuming probiotics was reported to have a significant increase in both lymphocytes and thrombocytes, suggesting a possible involvement of both cell types in the immunological function (Jatoba´ *et al*., 2011).

Regarding the fish serum enzymes in this study, lysozyme, but neither myeloperoxidase nor alkaline phosphatase, was increased in the 30-ppt fish. In fish, lysozyme functions as an opsonin and activates the complement system and phagocytes and is produced in phagocytic cells such as monocytes (or tissue macrophages) and neutrophils (Scapigliati *et al*., 2002). Myeloperoxidase plays a role in killing microorganisms (Johnston, 1978) and is produced by neutrophils (Ueda *et al*., 2001). Alkaline phosphatase is localized in the liver cells and its increasing level in fish serum suggests liver damage (Firat *et al*., 2011). It is not known what caused

the increased level of lysozyme in the 30-ppt fish in this study. Since the enzyme is produced by monocytes and neutrophils and with normal level of peripheral monocytes in this study, it is possible that the tissue monocytes, or macrophages, might be increased being due to ISKNV invasion. The tissue monocytes could, as well, produce and release lysozyme into the blood circulation. The normal level of myeloperoxidase was correlated well with the normal level of neutrophils. Likewise, the comparable level of serum alkaline phosphatase between the 0-ppt and 30-ppt fish suggests that the liver of the 30-ppt fish were relatively normal. And this was supported by normal histology of the fish liver in both groups (data not shown).

Serum bactericidal activity is a mechanism noted for the killing and clearing of pathogenic organisms in fish, especially bacteria (Ellis, 2001). In this study, this activity in the 0-ppt and 30-ppt fish did not differ. It is possible that both groups of fish had not encountered any bacterial invasion, either endogenously or exogenously, that would make them respond differently in term of the bactericidal index.

Both results from PCR and ISDL revealed ISKNV presence in various tissues of *O. niloticus* in this study, with more prevalence in the 30-ppt fish than in the 0-ppt fish. Since the virus infect spleen and kidney in particular (Suebsing *et al*., 2016), severely infected condition was therefore observed in the 0-ppt fish. The low prevalence in the 0-ppt fish suggests a possibility that many of the sub-clinically infected fish contained low number of copies of ISKNV and thus escaped the detection limit of the PCR methods employed. And under elevated salinity the virus replicated increasingly, resulting in more positive cases being detected. Alternately, it is also possible that the 30-ppt fish had become more susceptible to the virus being transmitted horizontally from the ISKNV-infected fish. The elevated salinity might lower immunological potency of the fish and that allowed the pre-existing ISKNV to replicate out of the host ability to control.

In this study, kidney is also another target tissue of ISKNV. As head kidney is well known to be an important tissue of the fish to generate immuno-potency cells, its damage would make the fish more susceptible to pathogens, either those already inside the fish body or in the environment. Interestingly, the virus was also present in the gonads of both the 0-ppt and 30-ppt fish suggesting vertical transmission of the virus, a possibility being reported earlier (Suebsing *et al*., 2016).

CHAPTER 5
CONCLUSIONS AND SUGGESTION

5.1 Percentage of 51.3 ± 15.5 of Nile tilapia *O. niloticus* could survive a gradual increase in salinity from 0 to 30 ppt in one month and further for another month under full-strength seawater. Their growth rate was about 60% of that of the fish in 0-ppt environment.

5.2 Decreasing hematocrit, increasing total white blood cells, increasing peripheral lymphocytes and thrombocytes, and increasing serum lysozyme activity were observed in *O. niloticus* reared under elevated salinity.

5.3 Rate of PCR detection for the infectious spleen and kidney necrosis virus was increased in *O. niloticus* reared under elevated salinity, and the infection was confirmed morphologically by *in situ* hybridization method.

5.4 Further research to improve growth rate of seawater Nile tilapia, probably through appropriate nutrition, should be carried out. Selective breeding program aiming for fast growth trait for the fish should be carried out in parallel to the nutrition study.

CHAPTER 6
REFERENCES

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