Myo-inositol supplement helps the performance of seawater Nile tilapia, *Oreochromis niloticus*

Behnam Foroutan

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy
(International Program)
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Author	Behnam Foroutan
Major Program	Aquaculture

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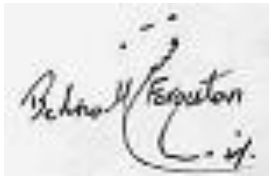
Major Advisor



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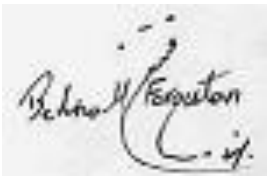

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ABSTRACT

A population of seawater (SW)-acclimated Nile tilapia, *Oreochromis niloticus*, have been generated for six generations since 2017 and maintained under 30-ppt salinity. This fish population, however, has lower growth and survival than *O. niloticus* reared under freshwater (FW); this phenomenon was likely due to the osmotic stress of the animals. Two attempts were tried to improve the growth and survival of SW-acclimated *O. niloticus* were providing feed supplemented with lipid or *myo*-inositol (MI), a natural compatible osmolyte. The first attempt was based on a hypothesis that increasing lipid intake would provide more energy to deal with the osmotic stress, while the second hypothesis was that MI might support the cellular metabolism of the fish and allow the fish cells to create appropriate mechanisms against the osmotic stress. In the first trial, salmon, soybean, and palm oil were tired for one month and all failed to improve the growth and survival of the SW-acclimated fish. In the second trial, MI supplements at 250, 500, and 750 mg/kg pellets were provided to SW-acclimated *O. niloticus* for one month. At the end of the experiment, the fish supplemented with 500 mg MI showed significantly higher survival and biomass increase than those of the SW-acclimated fish, with significantly less FCR. At 500-mg MI supplement, the rise in plasma osmolality and Na^+ observed in the SW-acclimated fish were significantly attenuated. Plasma Cl^- value was decreased in all the SW-acclimated fish and further suppressed by 500-mg MI supplement. Plasma K^+ value was decreased in the SW-acclimated fish but restored to normal values by MI supplement. The transcript *MIPS250* of the FW- and SW-acclimated fish was comparable, but that of the SW-acclimated fish receiving MI supplement was 1.7x to 4.1x fold up-regulated, compared with the FW-acclimated fish. In contrast, the transcript *MIPA1* of the SW-acclimated fish was 323x fold higher than that of the

FW-acclimated one. The up-regulation was maintained by MI supplement at 250 and 750 mg; however, at 500-mg supplement, this up-regulation was attenuated significantly. The study suggests that exogenous MI at optimum dose helped to maintain cellular metabolism of the SW-acclimated *O. niloticus* and allow the fish cells to respond more efficiently to the osmotic stress.

Keywords: osmotic stress; SW-acclimated *O. niloticus*; lipid supplement; *myo*-inositol; *MIPS*; *MIPA*

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AAF	AquaAcademy Farm
ACC	acetyl-CoA carboxylase
AHPND	acute hepatopancreatic necrosis disease
AOAC	Association of Official Agricultural Chemists
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BW	body weight
cDNA	complementary deoxyribonucleic acid
°C	degree celsius
Cl^-	chloride
cm	centimeter
CPF	Charon Pokphand Foods
CPT I	carnitine palmitoyltransferase I
d	day
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DO	dissolved oxygen
ECF	extracellular fluid
e.g.	for example
et al.	and others
EMS	early mortality syndrome
f	final
FAO	Food and Agricultural Organization
FCR	feed conversion ratio
Fig.	figure
FW	freshwater
g	gram
GH	growth hormone
GI	gastrointestinal

GIFT	genetically improved farmed tilapia
Gr.	group
Grs.	groups
h	hour
H^+	hydrogen ion
HCO_3^-	bicarbonate
H&E	hematoxylin and eosin
HSI	hepatosomatic index
i	initial
ICF	intracellular fluid
i.e.	that is
ISKNV	infectious spleen and kidney necrosis virus
K^+	potassium ion
kg	kilogram
L	liter
m	meter
m^2	square meter
m^3	cubic meter
mg	milligram
Mg^{++}	magnesium ion
MI	*myo*-inositol
MIB	*myo*-inositol biosynthesis
min	minute
MIPA	gene encoding *myo*-inositol phosphatase
MIPS	gene encoding *myo*-inositol phosphate synthase
mL	milliliter
mOsm	milliosmole
Na^+	sodium ion
Na^+/K^+-ATPase	sodium-potassium adenosine triphosphatase
ng	nanogram
NKCC	$Na^+/K^+/2Cl^-$ co-transporter

N	number
nm	nanometer
PCR	polymerase chain reaction
pH	potential of hydrogen
ppm	part per million
ppt	part per thousand
PRL	prolactin
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
R^2	R-squared
RNA	ribonucleic acid
ROMK	renal outer medullary potassium
rpm	rounds per minute
RT-PCR	reverse transcription polymerase chain reaction
s	second
SW	seawater
SGR	specific growth rate
TAN	total ammonia nitrogen
TSH	thyroid-stimulating hormone
U	unit
USA	United State of America
USB	United State Biologicals
US$	United State dollar
vs.	versus
v/v	volume by volume
µL	microliter
µm	micron
x g	unit of relative centrifugal force, stands for times gravit

CHAPTER 1
INTRODUCTION

1. Background and significance of this research

Nile tilapia (*Oreochromis niloticus*) is commercially cultured worldwide and its production, ~3.5 million tons yearly, is second only to Chinese carp (FAO, 2020). The fish grows to marketable size (0.8-1.0 kg) within 6-8 months. World production of tilapia has reached around 4 million tons in 2013 and is increasing at 3-5% per year, with China as the leading production and exporting country. While being a freshwater species by nature, the fish could tolerate salinity up to 15 ppt without morbidity or significant mortality (Suresh and Lin, 1992; Kamal and Mair, 2005; Luan *et al.*, 2008; Schofield *et al.*, 2011). If salinity of the rearing freshwater was gradually increased for 1 ppt/d, the fish might be able to tolerate salinity up to 30 ppt (full-strength seawater), or even higher (Schofield *et al*. 2011; Withyachumnarnkul *et al*., 2017). It was found that the salinity-tolerated fish survived at about 50% and their growth rate about 60% of that of the freshwater fish (Withyachumnarnkul *et al*., 2017).

The ability to survive in full-strength seawater may be attributed to the expression of certain specific genes that enable the “seawater” Nile tilapia to modify their osmoregulation mechanisms (Hwang *et al*., 1989; Breves *et al*., 2010). Based on that, it is therefore possible to selectively breed Nile tilapia that tolerates high salinity from the freshwater stocks (Suresh and Lin, 1992). In fact, the idea of selective breeding program for Nile tilapia was suggested a decade ago by Luan *et al*. (2008). The reason why this idea has not been pursued is probably because there is no good reason to create seawater Nile tilapia. Doing so requires investment and the results may not be rewarding enough, considering that the fish grow slower than the freshwater ones. Besides, if one prefers seawater tilapia then they could rear red tilapia, which tolerate high salinity and is already commercially available. Red tilapia is a hybrid strain obtained from the crossing of *O. niloticus* x *O. mossambicus* or *O. aureus* x *O. mossambicus* (Watanabe *et al*., 1990; Hashim *et al*., 2002). The tilapia *O. mossambicus* also tolerate high salinity and are found in abundance in brackish water in many tropical countries.

However, at present, there are a few reasons that make the breeding program of seawater-acclimated Nile tilapia become appealed to tilapia farming industry. In Thailand, and probably in many other countries, tilapias are raised in net cages placed in canals, rivers and lakes, which are natural waterways. While the practice ensures low investment and thus high profit for farmers, it also pollutes environment and obstructs water traffics. Because of these problems, Thai government has prohibited cage culture of tilapia and other aquatic species in public waterways. Although this regulation has not been fully reinforced, farmers are looking for private facilities to place net cages. The problem is an investment on inland freshwater pond facilities and the sale price of tilapia could hardly covers the investment and operational expenses. The available shrimp farms widely distributed in Thailand, with extensive areas of reservoir, therefore, may serve this purpose. It is possible to set up net cages for tilapia culture in reservoirs of shrimp farms, along with shrimp farming activity. In fact, the culture of fish, especially tilapia, in shrimp farms has long been practiced, and there are scientific supports that the co-culture makes the shrimp healthier than being mono-cultured (Yi and Fitzsimmons, 2004).

Outbreaks of acute hepatopancreatic necrosis disease (AHPND), commonly known as Early Mortality Syndrome (EMS), in marine farmed shrimp (Tran *et al*., 2013) and the findings that tilapia-shrimp co-culture could reduce the problem of AHPND, both in its prevalence and severity (Withyachumnarnkul, 2013), have attracted some farmers to divert the monoculture to the co-culture. While scientific explanation for the protection is still unclear, the co-culture practice has been widely spread in Thailand.

In marine shrimp-fish culture, farmers have already employed red tilapia and *O. mossambicus* in farms, both intentionally and non-intentionally. The downside of having red tilapia in shrimp farms is its bright red or pink color that attracts birds. Fish-eating birds, like egrets, in shrimp farms could bring diseases to shrimp by incidentally dropping diseased shrimp they pick up from diseased ponds into the healthy shrimp ones. The most dangerous pathogen that birds possibly bring to the farm is white-spot disease. The tilapia *O. mossambicus*, on the other hand, has small size and thus do not have much commercial value. In addition, the fish breed rapidly

in brackish and seawater, the uncontrollable increase in *O. mossambicus* offspring consumes feed pellets resulting in high feed-conversion ratio (FCR) of the fish. Therefore, both red tilapia and *O. mossambicus* are not considered best choice to raise in the shrimp farm.

Taking all together, seawater-acclimated Nile tilapia, *O. niloticus*, may be a better choice than red tilapia and *O. mossambicus* as a commercial commodity in shrimp farms, or in any brackish/seawater ponds.

As mentioned, the problem of raising seawater-acclimated Nile tilapia, among others, is their slow growth and low survival (Withyachumnarnkul *et al*., 2017). It is possible that energy from nutrient that the fish receive from the feed is diverted to activities for osmoregulation, and thus may not be adequate to support the fish survival. In this thesis research, the seawater-acclimated Nile tilapia was orally supplemented with lipid, to provide extra energy, and *myo*-inositol to protect the fish cells from osmolality stress.

1.2 Literature review

1.2.1 Tilapia

The word, tilapia, is used as a common name for over 70 species of fish, subdivided into as many as 10 genera. Three Genera: *Tilapia*, *Sarotherodon* and *Oreochromis*; are important tilapia for aquaculture production. By taxonomy, these three genera belong to the Kingdom Animalia, Phylum Chordata, Class Actinopterygii, Order Perciformes, and Family Cichlidae, which makes all the fishes in this Family being called cichlids. Tilapias in the Genus *Tilapia* are characterized by being substrate spawning and nest-guarding, while those belonged to *Sarotherodon* and *Oreochromis* are mouthbrooding, i.e., taking eggs in their mouth chamber during hatching and nursing processes (McAndrew *et al*., 2016). The *Sarotherodon* species are characterized as either paternal or biparental mouthbrooders and were originally restricted to West Africa. The *Oreochromis* species are all characterized as maternal mouthbrooders and were originally found in central and east African rivers and lakes. Four *Oreochromis* species, *O. niloticus* or Nile tilapia, *O. aureus* or blue tilapia, *O. mossambicus* or Mozambique tilapia, and *O. hornorum* (Fig. 1), are the most

significant species in aquaculture production. Another type of commercial tilapias are red tilapias, *Oreochromis* spp., which are hybrid fish of *O. niloticus* vs. *O. aureus* cross or *O. niloticus* vs. *O. mossambicus* cross (Watanabe *et al*., 1990; Hashim *et al*., 2002). Both Nile and red tilapias are cultured worldwide and constitute the two most important farmed tilapias.

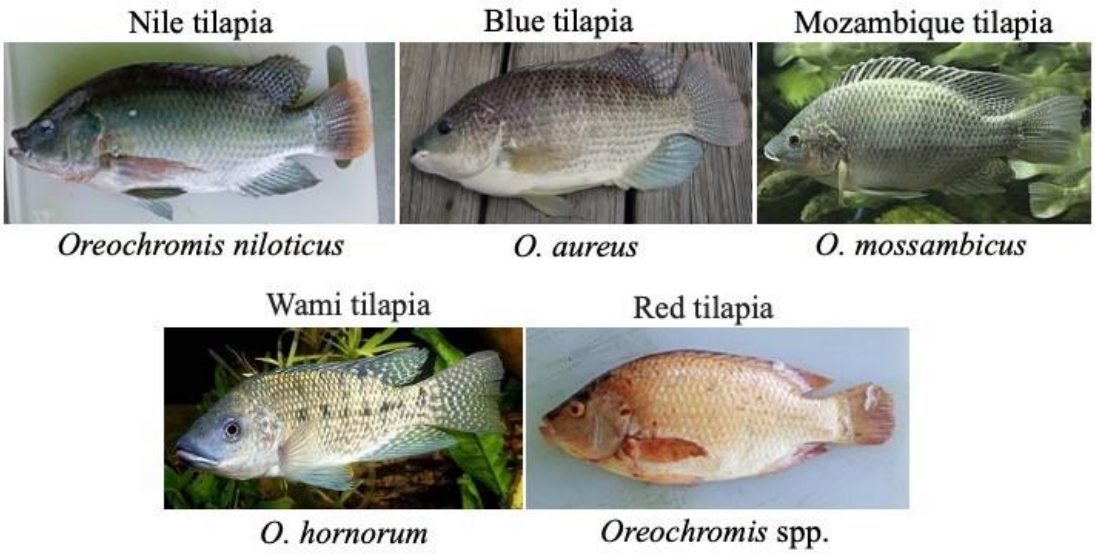

Figure 1. The four *Oreochromis* species and red tilapia (The pictures of *O. aureus*, *O. mossambicus* and *O. hornorum* are from https://en.wikipedia.org/wiki/Nile_tilapia)

The two most common types of farmed tilapia, Nile and red tilapia are the products of the breeding program for rapid growth, cold tolerance, and salinity tolerance, which have been continued for generations. The breeding program for growth performance of Nile tilapia has been successfully carried out especially the program setup in the Philippines called the genetically improved farmed tilapia (GIFT) program. The GIFT strain of Nile tilapia has been distributed commercially in several tropical countries, including Thailand. In low-temperature water, like in Israel and China, blue tilapia, *O. aureus*, is preferable to *O. niloticus* since they could tolerate low temperature better than *O. niloticus*. For brackish water or seawater, *O. mossambicus* is the species of choice since they could tolerate a wide range of salinity, or being euryhaline, while *O. niloticus* thrive well in freshwater, and in

salinity less than 10 ppt; they are thus stenohaline. The disadvantage of *O. mossambicus* is that they grow much slower than *O. niloticus*, and cannot reach the size comparable to *O. niloticus*. To achieve both growth rate and salinity tolerance, a hybrid between *O. niloticus* and *O. mossambicus* has been generated, resulting in red tilapia, which could grow at the rate comparable to *O. niloticus* and tolerate elevated salinity, even in seawater (Watanabe *et al*., 1990; Hashim *et al*., 2002; Pongthana *et al*., 2010).

Another problem of tilapia farming is due to their high fecundity, which results in tremendous number of offspring in the pond at the uncontrollable level. This large number of fish consumes more feed and grows slower than expected because of increasing population density. This undesirable effect of tilapia culture is overcome by stocking all-male tilapia, which could limit or completely avoid the fish production of offspring. Moreover, male tilapias grow faster than the females. Either one of the two methods are employed to achieve this goal: one is by feeding testosterone to the fish at the early days of their lives, the first 3 weeks after hatching, the other is through the breeding program. Testosterone induces the primordial germ cells of the fish to differentiate to the male type, resulting in the formation of testis and spermatogenesis. All-male production of tilapia could be achieved by cross breeding between female *O. niloticus* and male *O. aureus* or between female *O. mossambicus* and male *O. hornorum* (FAO, 2014).

The development of tilapia farming in Thailand started in 1965 when Prince Akihito from Japan gave 50 pairs of Nile tilapia to His Majesty King Rama IX. The King then raised the fish in a small pond in Jitraladda palace, and later the Department of Fisheries further improved the growth performance of the fish through breeding program and finally achieves the fast growing Nile tilapia strain, which they named Jitrladda-strain to honor the fish original stocking place in the country.

1.2.2 Tilapia culture

Since Nile and red tilapia are the two most-cultured types of tilapia, the culture techniques of these two types of fish are now addressed. Commercial production of these fish requires hatchery/nursery production of fry/fingerlings and grow-out phase.

The former aims at producing genetically improved all-male fry/fingerlings, and the latter aims at the production of marketable size fish, varying from a few hundred-gram to ~one-kg size.

Fry/fingerling production

Mating of male and female broodstock is accomplished by stocking the fish at a ratio preferably more females than male, e.g. female to male of 3:1 in the earthen pond, or hapa, or in the concrete/canvas tank, at the density preferably not more than 6 individuals / m^3 (Tahoun *et al.*, 2008). Every 7-15 days, the females are checked for the presence of fertilized eggs in their mouth (Fig. 2). If present, the eggs are gently taken out by shaking the fish head under water, and the eggs are collected and processed in the hatchery, which takes a few days before hatching. The offspring, or swim-ups, are sex-reverted to all-male population by testosterone treatment (Megbowon and Mojekwu, 2014), if not being reversed to all-male from the genetic program. They are then further nursed in in-door tanks to reach 30-50g body weight (BW), before being stocked in grow-out ponds.

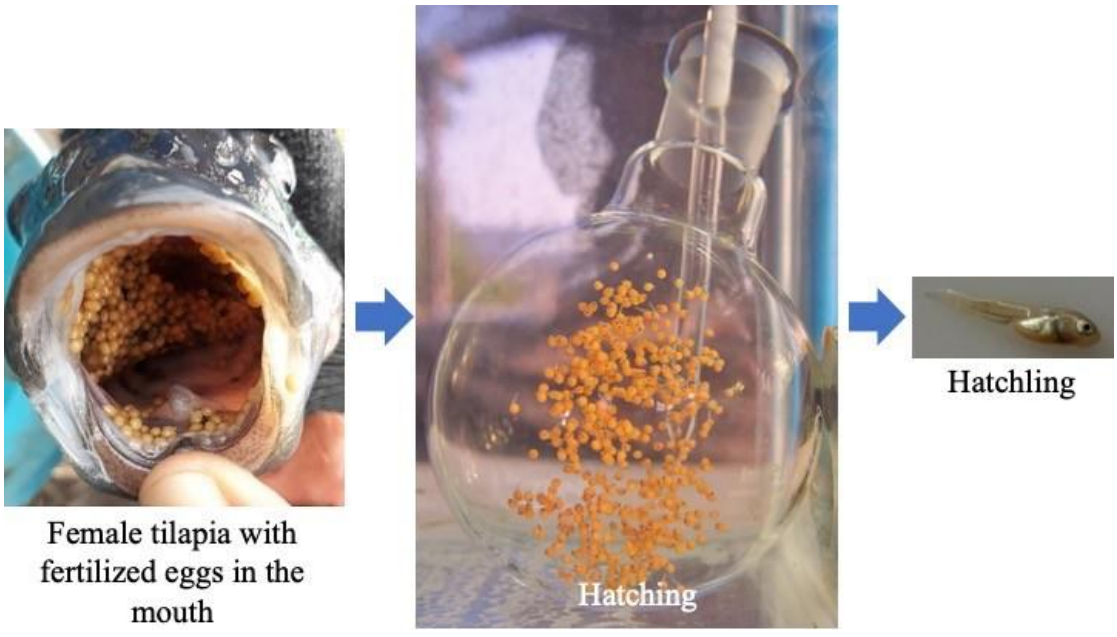

Figure 2. Fertilized eggs in the mouth of the female tilapia, "mouth brooder", which are artificially hatched

Grow-out phase production

Juveniles of *O. niloticus* are usually reared in earthen ponds, with either by stocking directly into the pond or stocking in floated net cages in public waterways, such as in rivers, canals, and lakes. From 30-50g BW, the fish can grow up to ~1.0-kg BW within 6 months, depending on the stocking density. With good water quality, i.e., high dissolved oxygen (DO), low nitrogen waste, optimum pH and temperature, the stocking density could be as high as 30-50 kg/m^3. For general farmers who employ river cage culture, the production usually reaches 10-20 kg/m^3.

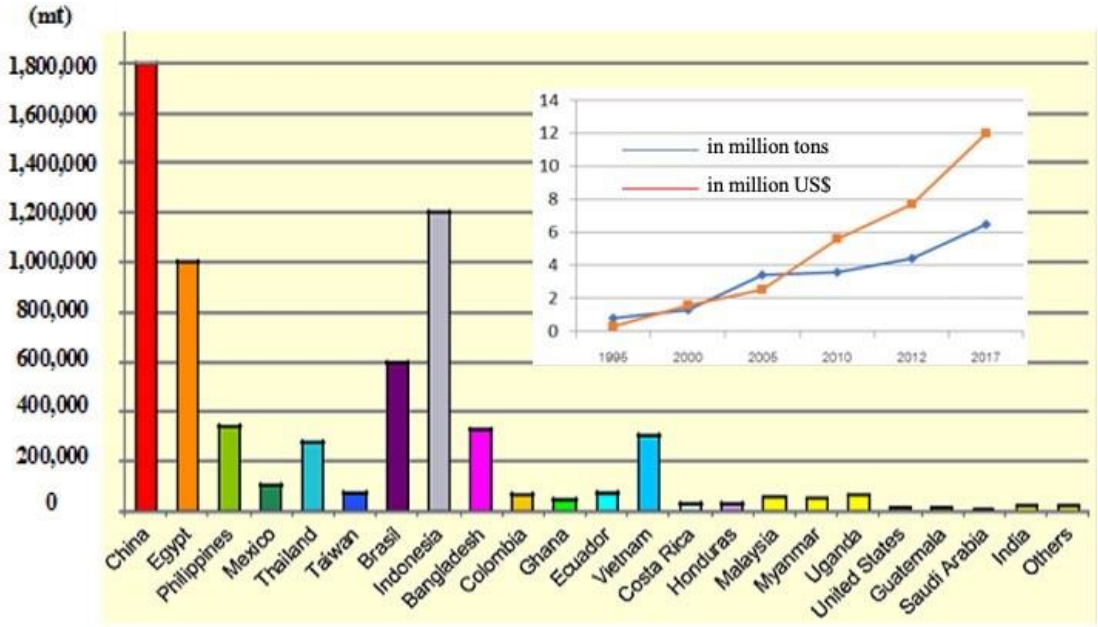

Figure 3. World tilapia production

World production of farmed tilapia, which is mostly Nile tilapia, is more than 6 million tons per year in 2017, and ~90% of the production is by Asian Countries (Little, 2000), with China is at the top (Fig. 3).

One of the problems stemmed around tilapia culture is “off-flavor” taste of the fish meat. The fish has a muddy smell and that makes tilapia less appealing to customers. This “off-flavor” smell comes from the presence of one of the two substances (Fig. 4): geosmin and 2-methyl isoborneol, in the fish meat and fat. The two substances are synthesized naturally in certain micro-organisms present in the water, especially in freshwater; these micro-organisms include proteobacteria,

cyanobacteria, actinomycetes, and fungi (Gerber 1968; Auffret *et al*., 2011). Tilapia grown under seawater is unlikely to have off-flavor since in seawater the micro-organisms that synthesize geosmin and 2-methyl isoborneol are not present in significant amount.

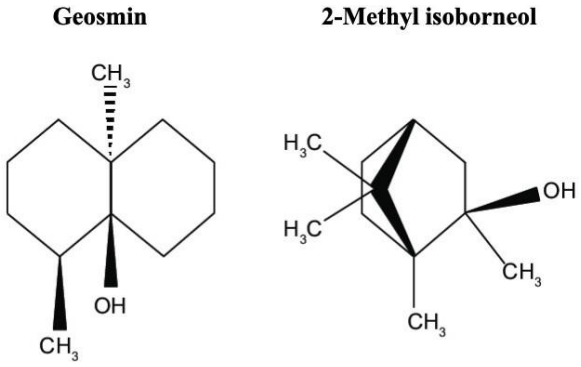

Figure 4. Chemical structure of Geosmin and 2-Methyl isoborneol

As mentioned in the Introduction, it is possible to culture Nile tilapia, *O. niloticus*, under elevate salinity. And culture of seawater (SW)-acclimated Nile tilapia should be prepared, particularly in Thailand because: 1. New government regulations are curving on cage culture of tilapia in public waterways; 2. Several deserted shrimp farms in Thailand should be revitalized by making the otherwise useless areas to culture SW-acclimated Nile tilapia; 3. The culture of SW-acclimated Nile tilapia may revitalize marine shrimp culture. Also, the advantage of SW-acclimated Nile tilapia over freshwater (FW)-acclimated ones is its absence of off-flavor.

Since most fish species, including tilapia, use fat in their body as energy reserve, it is possible that under elevated salinity, SW-acclimated Nile tilapia may increase the use of their storage fat to produce energy in order to overcome osmotic stress. Therefore, lipid metabolism in tilapia and other fish species is now addressed.

1.2.3 Lipid metabolism in Nile tilapia

In mammals, excess energy is reserved as triglyceride, which is deposited principally in fat cells or adipocytes, as well as in the liver (Du *et al.* 2013). In case of deficiency, de novo lipid biosynthesis takes place, but the sites of lipogenesis are species-specific; in pigs, it is the adipose tissue, while in avian species, it is the liver (Gonzalez *et al.*, 2014). Lipogenesis and lipolysis play key roles in maintaining lipid homeostasis. Metabolic imbalance between the two processes leads to obesity, diabetes, and other

pathological conditions (Unger *et al.*, 2010). Many key enzymes and transcriptional factors are involved in these metabolic processes (Chen *et al*., 2015).

Fish accumulate lipid in several storage sites, including liver, muscle, mesenteric fat, and subcutaneous fat. Different marine fish species accumulate fat in different sites, with markedly different hepatosomatic index (HSI), suggesting that fishes have their own rate of fat metabolism (Ando *et al*., 1993). In marine fish, demersal fish, which stay most of the time on the seabed without active movements, have more fat content in the liver (Takama *et al*., 1994). In *O. niloticus*, total fat content in the liver was reported to be approximately 12% (Jia *et al*., 2020), and those that were fed with low protein feed (<30%) had a tendency to have more fat in the liver and muscle (Chen *et al*., 2010).

It is possible that SW-acclimated Nile tilapia may use more energy from their fat, and therefore, the fat content in the muscle and liver may be decreased.

Under elevated salinity, all marine fish species face osmotic stress. The osmolality of their habitat, if in 30 ppt seawater, is about 3 times higher than the osmolality of their plasma and intracellular fluid (~300 mOsm or 10 ppt); i.e. they are under hypertonic environment. On the opposite, freshwater fish species are in the habitat where the surrounding osmolality is less than that of their plasma and intracellular fluid; i.e., they are under hypotonic environment. How the fish under these two different situations manage to withstand the positive or negative osmotic stress imposed on them is an interesting subject. Besides, certain fish species, like salmon, sea bass, Mozambique tilapia (*O. mossambicus*), etc., could, or have to, spend their life in both hypertonic and hypotonic environments, i.e., being euryhaline. At this point, we should address some physiological features of osmoregulation of the fish in general.

1.2.4 Fish osmoregulation

Freshwater fish have the osmolarity of their plasma and intracellular fluid at higher concentrations than that of the environment. Therefore, ions (Na^+, K^+, Mg^{++}, Cl^-, and other) in their plasma and intracellular fluid tend to out flux into the water, and at the same time, water tend to influx into the fish body. If this happens, the fish would suffer from their metabolic imbalance and cannot survive. To prevent this to

happen, the fish have to do two things: one is to limit the out flux of ions and the other is to excrete more water out from their bodies.

Osmoregulation through the gills

Although the whole picture of fish osmoregulation is not clear at present, research has revealed some light on the mechanisms. In the gills and opercular membrane, a number of specialized cells called ionocytes (previously called chloride cells) are located. These cells contain a rich population of mitochondria therefore, they are sometimes called mitochondrion-rich cells (Jumah *et al*, 2016; Uchida *et al*., 2000). The feature also indicates that the cells are highly energetic and produce tremendous amount of ATP, the molecules that provide energy for the cell. These cells also contain high level of the enzyme Na^+/K^+-ATPase (Hwang *et al*., 1989), the enzyme that helps pushing Na^+ out from the cell cytoplasm to extracellular space, in exchange for K^+ being transferred into the cell; 3 Na^+ out and 2 K^+ in. Not only Na^+, but Cl^-, is secreted out of the cell as well. In addition, ionocytes in several fish species also contain Na^+, K^+, $2Cl^-$ co-transporter (NKCC) that helps secreting Na^+, K^+ and Cl^- out of the cell; a good example is the ionocytes of the killifish that also show a complex control of NKCC through other integral proteins of its cell membrane acting as the osmosensing complex (Marshall *et al*., 2008). The ionocytes could increase their size when the fish is under hypertonic condition, and decrease their size when the fish is in the hypotonic condition. Not only the size, the number of the ionocytes in both *O. niloticus* and *O. mossambicus* are also increased under hypertonic, and decreased under hypotonic condition (Jumah *et al*, 2016; Uchida *et al*., 2000). These changes in the size and number of ionocytes according to environmental salinities suggest that the fish excrete less amount of Na^+ out when they are in FW and more when they are in SW.

In addition, ionocytes in salmon are composed of two types with two opposite functions. One is for secreting Na^+ out (when the fish is in SW) and the other was for shipping Na^+ into (when the fish is in FW) the cell (Uchida *et al*., 1996; 1997).

Osmoregulation through the kidney

Fish kidney also plays a very important role in osmoregulation. It creates urine, which is composed of water and ions. Both water and ions, while passing through the

long tubule inside the kidney, are reabsorbed into the plasma, and the rest is excreted as urine. In the rainbow trout and killifish, NKCC is also localized in the kidney tubules (Katoh *et al*., 2008), suggesting that Na^+, K^+, and Cl^- are also excreted from the kidney of these two anadromous fish. The kidney of FW fish would reabsorb more ions than water, while the SW fish would reabsorb more water than ions. The result is that the urine of FW fish is more diluted than that of the SW fish (Gonzalez, 2011). While in the kidney tubule, water are reabsorbed into the blood, and K^+ is excreted out from the blood into the tubular lumen and comes out with urine.

Osmoregulation through the intestine

As mentioned, marine fish drink lot of seawater and retained the water in, while secreting ions out, the body. The seawater that has high levels of Na^+, K^+, and Cl^- passes through the gastrointestinal (GI) tract of the fish. The first part of the GI tract would absorb these ions into the fish blood, and allow the diluted seawater that has low osmolality to pass into the middle and last parts of the GI tract, where the diluted seawater is absorbed into the fish blood. The absorbed ions are secreted out from the gills into the environment by the aforementioned mechanisms (Smith, 1930).

Since seawater has the pH of 8.0 or higher, the absorbed seawater may increase the pH of the fish body fluid, which is normally at 7.0. This problem seems to be solved by the finding that HCO_3^- is excreted from the middle and posterior parts of the GI tract, in exchange for the absorption of Cl^- (Grosell *et al*., 2001; Yan *et al*., 2013).

In summary, marine fish drinks lot of seawater, Na^+ and K^+ of the seawater is absorbed from the anterior part of the intestine into the blood, which is then excreted from the blood through the gills. The diluted seawater (after ions are taken out) is absorbed into the fish body in the middle and posterior parts of the intestine, while HCO_3^- is excreted from the blood to the intestinal lumen, in exchange for Cl^-. In the kidney, all ions are excreted from the blood into the tubular lumen and passed out as urine (Fig. 5).

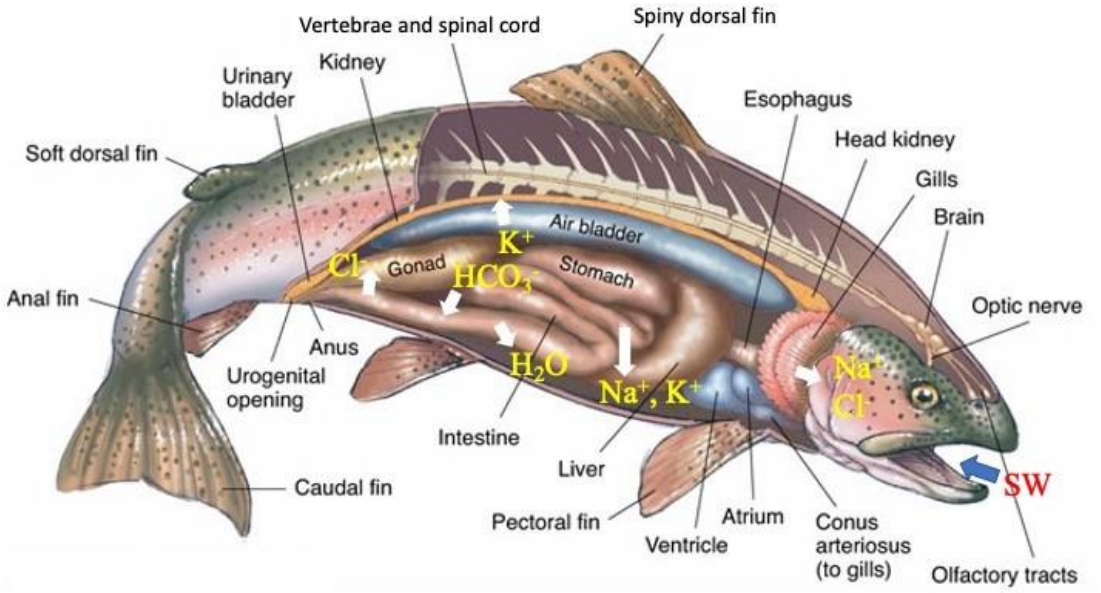

Figure 5. Summary of ions and water movements between the extracellular space of the fish body and the lumen of the intestine, kidney tubule, and from the gills to the external environment (the diagram is drawn according to the findings by Grosell *et al*., 2001and Yan *et al*., 2013)

Hormones involved in the osmoregulation

Besides cortisol, other hormones involved in the osmoregulation of the fish include growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin (PRL) (McCormick, 2001; Sakamoto and McCormick, 2006), and insulin-like growth factor 1 (IGF-1). GH, a hormone produced by the pituitary gland, induces the synthesis of IGF-I in the fish liver. Both hormones help the fish to survive in SW (Mancera and McCormick, 1999). They also help stimulate activities of Na^+/K^+-ATPase and NKCC in the gills (Pelis *et al*., 2001). PRL, the hormone produced int eh pituitary gland, helps the fish to cope with low salinity or freshwater (Pisam *et al*., 1993). It reduces the size and number of SW-typed ionocytes and increase the those of the FW-typed.

Compatible osmolytes

While SW-acclimated fish has mechanisms to adjust the levels of ions influx from seawater into its body, another defense mechanism comes into play. This is by

the *de novo* production, or accumulation, of compatible osmolytes, which increase cytoplasmic osmolality without disturbing the essential functions of the cell (Burtle and Lovell, 1988; Deng *et al*., 2002; Wang and Kültz, 2017). Several osmolytes have been reported thus far, including taurine, *myo*-inositol (MI), N-acetyl-histidine, N-acetyl-aspartate, betaine, threonine-phosphoethanolamine, and serine-phosphoethanolamine; however, the most studied osmolyte in the fish has been MI.

Myo-inositol biosynthesis (MIB) pathway is through the conversion of glucose-6-phosphate to *myo*-inositol 3-phosphate, which is dephosphorylated to MI (Geiger and Jin, 2006; Parthasarathy *et al*., 2006). The first step employs myo-inositol-3-phosphate synthase and the second step is catalyzed by myo-inositol mono-phosphatase, both of which are encoded by *MIPS* and *MIPA*, genes, respectively (Fig. 6). The two transcripts are present in the gills, brain, kidney, and intestine of several teleosts, including tilapia (Sacchi *et al*., 2013; Kalujnaia *et al*., 2016; Wang and Kültz, 2017).

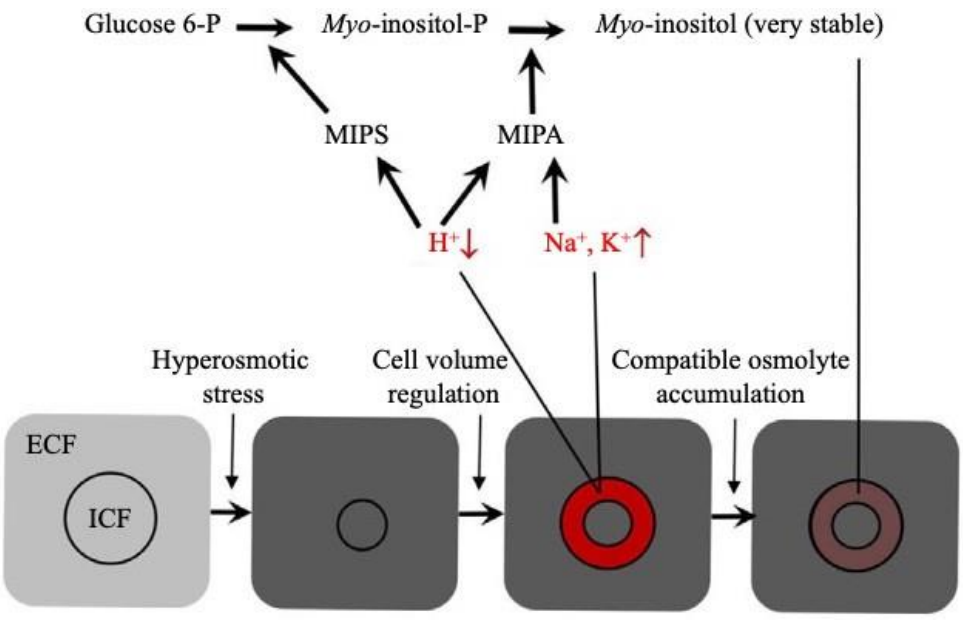

Figure 6. Direct ionic regulation of the *myo*-inositol biosynthesis (MIB) pathway in tilapia: ECF, extracellular fluid; ICF, intracellular fluid

When the Mozambique tilapia, *O mossambicus*, was transferred from freshwater to seawater, its plasma osmolality increases and causes hyperosmotic stress, which leads to cellular dehydration and shrinkage (Kültz, 2015). Cell shrinkage is compensated by regulatory volume increase, a process that increases intracellular Na^+ and K^+, but decreases H^+ (increases pH). Cells do not tolerate the change in the concentration of these cations for long and, thus, the excess ions are replaced by MI, which is an organic osmolyte that is compatible with cell function. Elevated intracellular Na^+, K^+, and pH directly stimulate the enzymatic activity of *myo*-inositol phosphate synthase (MIPS) and inositol monophosphatase (IMPA), which constitute the MIB pathway.

1.3 Objectives

The general objectives of this research were to improve growth and survival of SW-acclimated *O. niloticus*, through feed supplement. Specifically, the research plan was as follow:

1. Comparison of baseline data between the FW- and SW-acclimated fish regarding lipid contents in the fish liver, muscle, and general histology of the liver
2. To determine basic osmoregulation of the SW-acclimated fish, plasma osmolality and ions between the two groups of fish were compared.
3. Testing any benefit of lipid supplement in the feed on growth and survival of the SW-acclimated fish; three types of lipids were tried; salmon oil, soybean oil, and palm oil.
4. To find out if MI supplement would improve the growth and survival of the SW-acclimated fish
5. To find out how MI supplement modifies the expressions of transcripts regulating MI

CHAPTER 2
RESEARCH METHODOLOGY

2.1 Experimental animals

All experimental animals in this study were SW-acclimated Nile tilapia, *O. niloticus*, raised in AquaAcademy Farm (AAF), a private aquatic farm in Tha Chana District, Surat Thani. The fish were at their 5^{th}-6^{th} generations originally selected from a mixed population of commercial sources intended for FW rearing. They had been continuously selected for growth and survival under SW (30 ppt in average) in the farm. In the breeding program, the SW-acclimated fish chosen to become the brood-stock were acclimated to 0ppt and underwent the breeding program described earlier (Withyachumnarnkul, *et al*., 2017). The reason was that proper embryogenesis of *O. niloticus* could not occur under salinity >15 ppt (Withyachumnarnkul, *et al*., 2022).

At its 5^{th}-6^{th} generation, the survival rates of FW- and SW-acclimated *O. niloticus* were determined by stocking 14 fish (3-5g BW) in 40L plastic tanks; 6 tanks contained FW (0 ppt) and 6 tanks contained SW (27-30 ppt). They were reared for 30 days and provided with floating commercial pellets (35% protein; CPF, Thailand) at 4% biomass daily. The water qualities in all tanks [total ammonia nitrogen (TAN), nitrite, dissolved oxygen (DO), pH, alkalinity, salinity, Ca^{++}, and Mg^{++}] were monitored daily to ensure optimum conditions for the fish. Water exchange at 70% was carried out daily, using either FW or SW in respect to the fish groups.

2.2 Proximate analysis of the fish muscle, the determination of the hepatosomatic index, and the histology of the fish liver

To find the difference in the composition of the fish muscle between the FW- and SW-acclimated *O. niloticus*, proximate analysis was performed. In this study, another set of fish that were reared from 3-5g BW under FW or SW to approximately 300g BW in approximately 2 months was employed. The fish were stocked in 5m x 5m x 1.5m floating net cages at the density of 10 individuals/m^3. The cage stocking the FW-acclimated fish was in the freshwater reservoir while the cage stocking the

SW-acclimated fish was in the seawater reservoir. They were reared under the same conditions as mentioned in 2.1.

As the main purpose of this study was to find out how to increase the growth and survival rates of the SW-acclimated fish through diet supplements, and, among others, an organ that is involved in the growth/survival of the fish is the liver, as metabolism of most nutrients is processed in this organ. Therefore, the relative size of the liver, or hepatosomatic index (HSI), and histology of the liver of the FW- and SW-acclimated *O. niloticus* were compared.

Both FW- and SW-acclimated *O. niloticus* sub-adults (200-400g BW), 30 individuals of each, were anesthetized with 2-phenoxyethanol (200 mg/L), weighed and sacrificed. The muscle was isolated, as fillet, and kept frozen at -20 ^{o}C for subsequent proximate analysis. The liver was isolated, individually weighed, and its relative wet weight, HSI, was determined as the percentage of the BW. The small pieces (10-20 mg) of individual livers were fixed in 10% neutral formalin for histological examination.

For proximate analysis, muscles (fillets) of three fish were combined for one determination and stored at -20 °C for proximate composition analyses (Fig. 7) conducted by the standard method of AOAC (2005). Moisture content was determined by oven drying at 105 °C until constant weight and expressed as a percentage. Crude protein (nitrogen x 6.25) was determined by the Kjeldahl method using the Auto Kjeldahl system (FOSS KT260, Switzerland), which includes three steps: digestion, distillation and titration. Crude lipid was determined by an ether extraction system (Ankom XT15, USA). The ash content of the samples was determined by burning 1g samples in clean, weighed silica crucibles overnight in a muffle furnace (GALLEN KAMP, size 2, UK) at 600 °C. The crude fiber content is estimated as the difference between the pre-ash weight and the post-ash weight.[1] Carbohydrate content was determined by calculating the difference, i.e., the sum of protein, lipid, fiber, and ash content were subtracted from 100 %.

[1] The crude fiber procedure has many sources of error and, therefore, is the most unsatisfactory principle of proximate analysis.

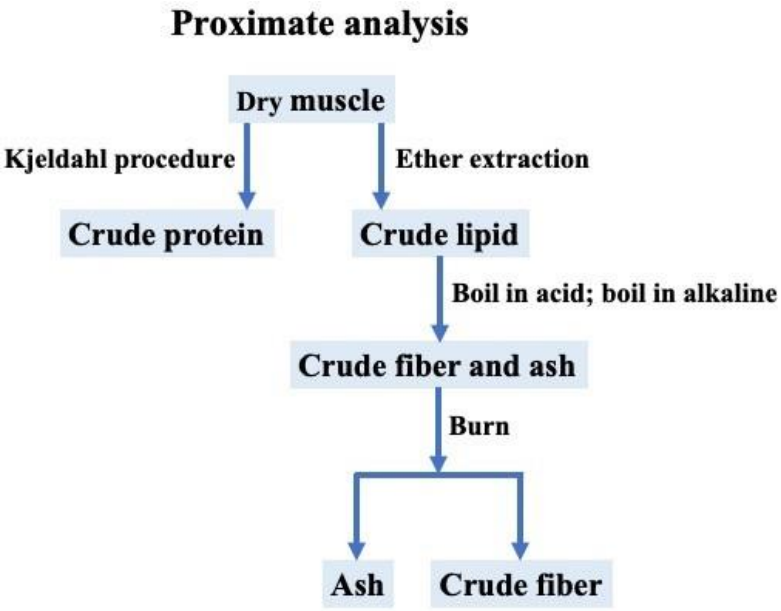

Figure 7. The process of proximate analysis

For histological study of the liver, the piece of liver was washed and placed in 70% ethanol after 12 h of fixation, and kept under 4 °C until being processed through standard paraffin sectioning. The procedure includes the dehydration steps in series of ethanol, followed by the embedding step in paraffin blocks. The blocks were sectioned at 5 µm-thickness stained with hematoxylin and eosin (H&E), and examined under light microscopy.

2.3 Lipid content in the liver

During the study, it was found that the liver of SW-acclimated *O. niloticus* was significantly smaller than that of the FW-acclimated fish, therefore, a study was carried out to determine the level of total lipid in the liver.

Liver were isolated from the same set of fish used in section 2.2, and were determined for total lipid content according to the method described by Bligh and Dyer and modified by Breil *et al*. (2017). The tissue was frozen immediately by dried ice and kept at -80 ^{o}C until use. At time of total lipid determination, frozen liver tissues (~ 0.6 g) were homogenized in 1 mL distilled water and transferred to glass test tubes. Mixture of 1.25 mL chloroform and 2.5 mL methanol was added into the

glass tube to make the ratio of chloroform: methanol: water at 1:2:0.8 (v/v/v). The mixture was incubated at room temperature for 1 h. Then, 1.25 mL of chloroform and 1.25 mL of water were added into the mixture to make a new ratio of chloroform: methanol: water at 1:1:0.9 (v/v/v). The solution was mixed well using vortex and incubated until settling at 4 °C overnight. The settling solution was centrifuged at 1500 x g for 15 min at 4 °C and the three phases of solution was appeared. The bottom phase containing lipids and chloroform was carefully collected and transferred to a new glass tube using a glass Pasteur pipette. The glass tube containing the sample was heated at 40 °C and the lipids were dried under nitrogen stream in a fume hood and stored in -20 °C freezer until use. The weight of dried lipid was calculated from the subtraction of weight of the glass test tube. The total lipid of sample was calculated as percentage of the lipid dry weight in the frozen tissue.

2.4 The lipid supplement tests

The fish was stocked in four net cages, 0.6m x 0.6m x 0.8m, in a SW reservoir (27-33ppt) of AAF (Fig. 8). They were divided into four groups, each consisting of 3 replicates, according to the different types of lipid supplement: Control (normal feed) and 3 types of oil; salmon, soybean, and palm. Each cage was stocked with 20 SW-acclimated *O. niloticus* (3-5g BW). The lipid was supplemented at the rate of 6 mL/kg feed by thoroughly mixing 1 kg pellets with 6 mL of of lipid.

The fish were fed three times a day (at 08.00, 13.00, and 17.00 h), initially at 4% biomass, and was increased according to the fish's demand. Each experiment lasted for one month. Initial and final body weights of individual fish were determined, and mortality was recorded daily. Parameters of water quality (TAN, total nitrite, alkalinity, pH and salinity) were monitored daily throughout the whole experiments.

It was later found out that palm oil supplement was probably the best among the four groups, further test was then performed to find out the optimum amount of palm oil supplement. The new sets of fish (3-5g BW) were divided into five groups, receiving 0, 3, 6, 9, and 12 mL of palm oil supplement/kg feed, and each group was composed of 3 replicates. Again, the initial and final body weights of individual fish were determined, and mortality was recorded daily.

Figure 8. Floating net cages in the reservoir of AquaAcademic Farm (AAF)

2.5 Supplementation with *myo*-inositol (MI)

As it revealed later that lipid supplementation did not improve the performance of SW-acclimated *O. niloticus*, another strategy was to use MI as a supplemented material. The problem of SW-acclimated fish is probably not the inadequate intake of calories, but rather the osmotic stress. As mentioned, MI is a compatible osmolyte and might help the fish to survive and grow better under high salinity environment. In this study, FW-acclimated *O niloticus* was added as another group of control, so that the performance of MI-supplements of SW-acclimated fish could be compared to that of the fish reared under FW.

At the time of breeding, SW-acclimated *O. niloticus*, reared under 30ppt, were acclimated to FW (0ppt) since the embryos could not survive at high salinity (Withyachumnarnkul *et al*., 2022). After hatching, the fish were reared under FW until reaching 0.3-0.5g BW. They were then gradually acclimatized to 30-ppt for 30 days and reared further until reaching sexually mature adults.

In this experiment, the fish (2-4g BW) were divided into 5 groups of 6 replicates each (Fig. 9). The experiment began after the fish of all groups, except Gr. 1, had gone through a complete SW-acclimation according to the method described, which lasted for one month (Withyachumnarnkul *et al*., 2017). Gr. 1 (FW-control) remained under FW from hatching and received normal pellets before and during the experiment. Gr. 2 (SW-control) also received normal pellets before and during the experiment. Grs. 3, (MI-250), 4 (MI-500), and 5 (MI-750) received MI (USB, Cleveland, OH) supplement at 250, 500, and 750 mg/kg pellets, respectively. The supplement was prepared by top-dressing the pellets with MI (USB, Cleveland, OH) dissolved in distilled water at three different concentrations (5, 10, and 15 mg/mL). Fifty mL of the solution was thoroughly mixed with 1-kg pellets to make the assigned supplementation of 250, 500, and 750 mg MI/kg pellets, respectively. The pellets were further coated with 6-mL squid oil/kg to prevent leaching.

Each replicate consisted of 13-14 fish stocked in 60L polyvinyl circular tanks. The rearing water was aerated to maintain DO at 6 ppm. The water quality; TAN, total nitrite, pH, and alkalinity, were monitored daily to ensure optimum conditions for the fish. Water exchange was performed daily at a 70% rate during 07.00-08.00 h.

Floating commercial pellets (CPF, Thailand), with 35% protein, was provided to apparent satiation twice daily at 09:00 and 17:00 h. The amounts of pellets consumed by fish in individual tanks were recorded daily to calculate the feed conversion ratio (FCR).

The feeding trial was carried out for 4 weeks. Dead fish were recorded and discarded. At the end of the experiment, the fish was anesthetized with 2-phenoxyethanol (200 mg/L), individually weighed, and blood withdrawn from the caudal vein was mixed with 100-U heparin and placed in the 1.5mL plastic tube. The tubes were centrifuged at 4 ºC, 1,500x g, for 10 min; the plasma was separated into a new tube and stored at −70 °C for the determination of osmolality and concentrations of Na^+, K^+, and Cl^-. To assure enough volume (1 mL) of plasma for each determination, plasma from 2 fish of the same replicate was combined for one determination. Each replication, 10-14 fish, therefore comprised 5-7 determinations, the average of which represented the value of that particular replicate. The gills of individual fish, approximately 30 mg, were isolated and fixed in 1mL TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) for determination of *MIPS250* and *MIPA1* transcripts by quantitative polymerase chain reaction (qPCR) method. The number of determinations per replicate was thus equal to the number of fish that survived; all the values obtained were averaged to represent the relative expression of the replicate.

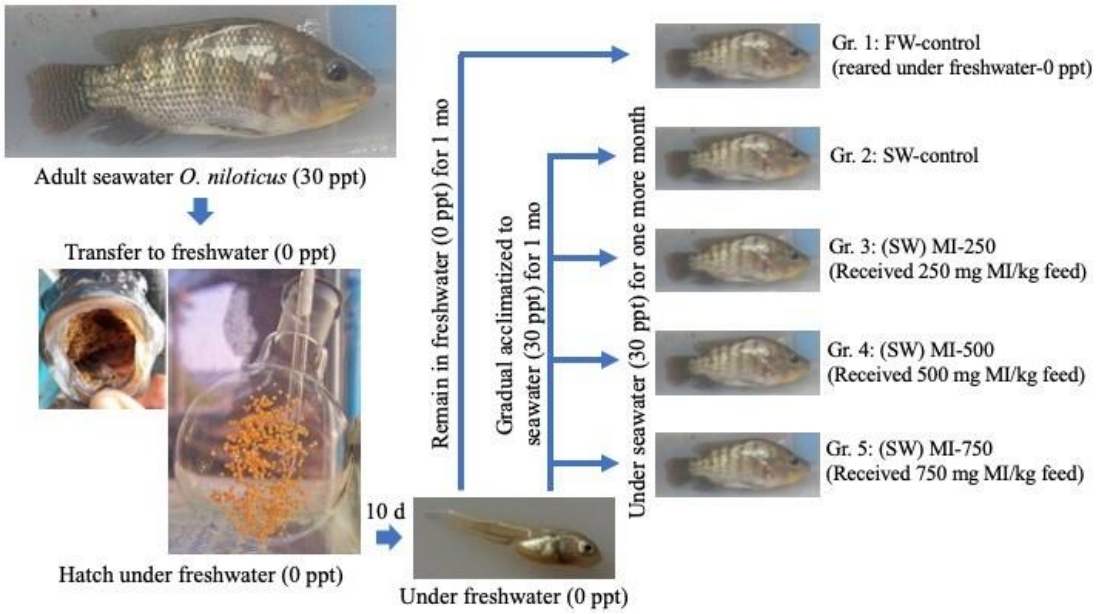

Figure 9. The production of seawater-acclimated *O. niloticus* and grouping of the animals, with 6 replicates each. BW, body weight; FW, freshwater; SW, seawater; MI, *myo*-inositol

2.5.1 Plasma osmolality and ions

Plasma osmolality and concentrations of Na^+, K^+, and Cl^- were determined by a micro-osmometer (Fiske® Micro-Osmometer 210, Thermo Fisher Scientific, Waltham, MA) and an electrolyte analyzer (Caretium® XI-9318, USA), respectively.

2.5.2 The transcripts *MIPS250* and *MIPA1*

Since the transcripts *MIPS* and *MIPA* are directly involved in MI biosynthesis, it is interesting to find out how these two transcripts were modified in *O. niloticus* that were reared under SW. The transcript *MIPS* has two variants, *MIPS160* and *MIPS250*, with *MIPS250* being the major one, at least in tilapia (Sacchi *et al.*, 2013). The transcript *MIPA* also has two variants, *MIPA1* and *MIPA2*, and in *O. mossambicus*, *MIPA1* plays a more important role than *MIPA2* (Zhu *et al.*, 2021). Therefore, *MIPS250* and *MIPA1* were determined in the gills of all the fish groups in this study.

To determine the relative expressions of *MIPS250* and *MIPA1* transcripts, total RNA was extracted from gill tissues using TRI reagent. The amount of RNA was measured using absorbance at 260 nm. One microgram of RNA was treated with 1 U of DNase I to eliminate the genomic DNA and subjected to cDNA synthesis with random hexamers (Invitrogen, USA) and SuperScript® III Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. The cDNA was then used as the template for qPCR reaction. Relative expressions of *MIPS250* and *MIPA1* were measured in the samples by qPCR using a 7500 RT-PCR system (Applied Biosystems, CA, USA). The transcript β-*actin* was used as an internal control gene. The reaction was performed in a 96-well plate with a 20 µL reaction volume of 1 µL of cDNA from each sample, 10 µL of KAPA SYBR® FAST qPCR Master Mix (2x) Kit (KAPA Biosystems, Cape Town, South Africa), 0.4 µL of specific primers (10 µM), and 8.2 µL sterile distilled water. The thermal cycle profile for qPCR was performed with one cycle at 95 °C for 3 min, 40 cycles at 95 °C for 3 sec for denaturation, 60 °C for 30 sec for annealing/extension, and extended for one cycle at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 sec, and 60 °C for 15 sec, respectively. All reactions were analyzed in triplicates. Details of the primers used in this experiment are presented in Table 1. The comparative CT method ($2^{-\Delta\Delta CT}$ method)

was performed to analyze the relative expression levels of the *MIPS250* and *MIPA1* transcripts (Livak and Schmittgen, 2001). The production of specific products was confirmed by performing a melting curve analysis of the samples.

Table 1. Sequences of primers used in qPCR analysis

Name of Primer	**Sequence 5' to 3'**
IMPA1 F	CGAAACTCTCCTAAGCAAGCCCCC
IMPA1 R	CCAGCTTTCCTAATTTCCGCGCCA
MIPS-250 F	GTGCATGATCTTCCAGATGGAGCG
MIPS-250 R	AGAAGCGCTCGGTGTTGGCG
β-actin F	CCACAGCCGAGAGGGAAAT
β-actin R	CCCATCTCCTGCTCGAAGT

CHAPTER 3
RESULTS

3.1 Survival of the fish

During 30 days of rearing in the 40L plastic tanks, the water quality was optimum for the fish, i.e., TAN and total nitrite were <0.5 ppm, DO was >5 ppm, alkalinity of the FW was 50-60 ppm and that of the SW was 130-150 ppm, pH of the FW was 7.0-7.5 and that of the SW was 8.0-8.5. At day 30, the difference on the survival rate of FW- and SW-acclimated *O. niloticus* was not statistically significant (Fig. 10). However, approximately 10-20% of the SW-acclimated fish suffered from skin hemorrhage, ulcerative lesion of the skin, and exophthalmos (Fig. 11).

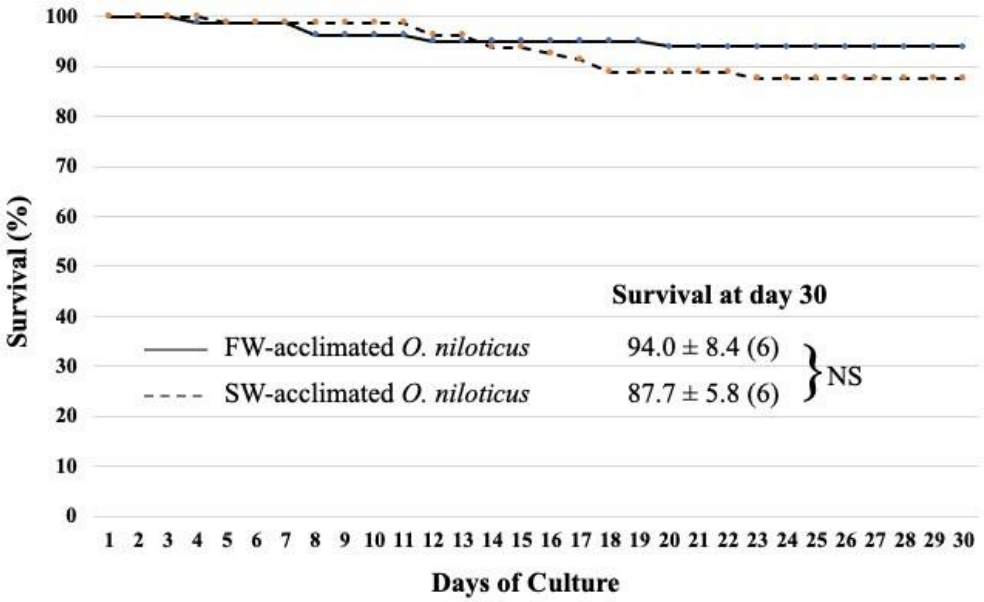

Figure 10. Survival of the 5th generation of FW- and SW-acclimated *O. niloticus* during 30-day rearing in 40L plastic tanks, NS, non significant

3.2 Proximate analysis of the muscle

At the comparable BW of the FW- and SW-acclimated *O. niloticus*, the muscle of both groups showed comparable levels of moisture and ash (Table 2), while the fiber content of the SW-acclimated fish was significantly higher ($p<0.01$) than that of the FW-acclimated ones. The protein content of the SW-acclimated fish (79.5%) was

slightly but significantly less ($p<0.05$) than that of the FW-acclimated ones (81.1%). The lipid content of the SW-acclimated fish (5.98%), however, was significantly higher ($p<0.05$) than that of the FW-acclimated ones (4.87%), which was about 20% higher. The carbohydrate content of the SW-acclimated fish (8.98%) was 12% higher and significantly different ($p<0.01$) from that of the FW-acclimated ones (8.00%).

Figure 11. Lesions on the surface of SW-acclimated *O. niloticus*, including skin hemorrhage (a), skin ulcers (b, c), and exophthalmos (d)

Table 2. Proximate analysis of the muscle of FW- and SW-acclimated *O. niloticus*. N = 10. NS, non-significant difference

	FW-acclimated	**SW-acclimated**	**Statistical analysis**
Body weight	272.5 ± 86.7	300.2 ± 91.1	NS
Moisture (%)	77.8 ± 0.9	78.3 ± 1.2	NS
Ash (% dry weight)	5.75 ± 0.34	5.85 ± 0.23	NS
Fiber (% dry weight)	0.09 ± 0.03	0.19 ± 0.09	$p<0.01$
Protein (% dry weight)	81.1 ± 1.4	79.5 ± 1.3	$p<0.05$
Lipid (% dry weight)	4.87 ± 0.41	5.98 ± 1.28	$p<0.05$
Carbohydrate (% dry weight)	8.00 ± 1.53	8.98 ± 1.84	$p<0.01$

3.3 Hepatosomatic index (HSI)

The BWs of the FW- and SW-acclimated *O. niloticus* samples were comparable between the same sex, while HSI of both sexes was significantly lower in the SW-acclimated fish (Fig. 12). The difference is more significant in the male ($p<0.01$) than that in the female ($p<0.05$). In the female, the HSI of the SW-acclimated fish was about 70%, while in the male it was about 56% of that of the FW-acclimated fish.

3.4 Lipid content of the liver

Comparing between the FW- and SW-acclimated *O. niloticus* of the same BW, the percentage of total lipid in the liver of the SW-acclimated fish was approximately 1.5x fold of, and significantly higher ($p<0.01$) than, that of the FW-acclimated fish (Fig. 13). Together with the results in figure 12, it can be stated that although the liver of SW-acclimated *O. niloticus* was smaller than that of the FW-acclimated fish, it contained more lipid.

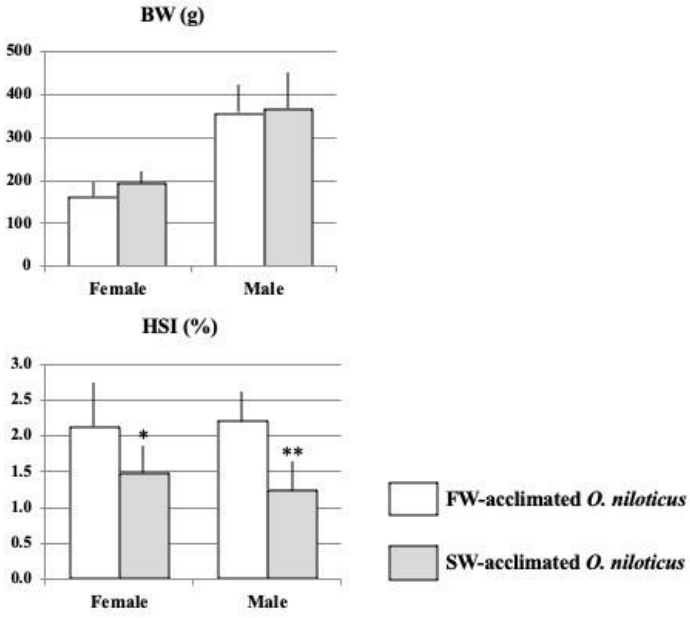

Figure 12. The BW and HSI of FW- and SW-acclimated *O. niloticus*

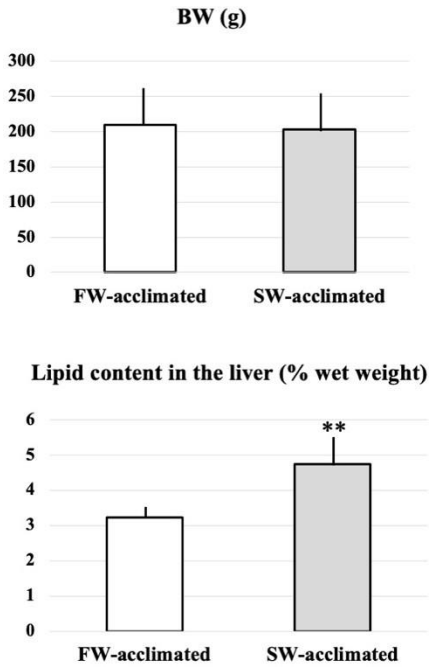

Figure 13. The percentage of total lipid in the liver of FW- and SW-acclimated *O. niloticus*

3.5 Histology of the liver

The fish liver normally consists of liver parenchyma, or hepatocytes, which lie as cords of double cell layers, covered by thin endothelial cells that lie the space called sinusoids. Compared to the mammalian liver, the hepatic cords of the fish do not have a clear cord arrangement but rather look like a homogenous distribution of the liver cell parenchyma or hepatocytes. The sinusoids are branches of the hepatic portal vein and thus contain plasma and sometimes red blood cells. They join to become a common larger vessel, or hepatic venule, many of which are joined to become hepatic vein. Several glandular structures are scattered within the hepatic parenchyma, these are intra-hepatic pancreas.

The histology of the FW- and SW-acclimated *O. niloticus* did not show any remarkable difference (Fig. 14). Probably, more pyknotic nuclei of hepatocytes were observed in SW-acclimated fish than in the FW-acclimated ones (Fig. 15a). Other features, bile ducts, hepatic arteries, and hepatic veins were normally present in both groups of fish.

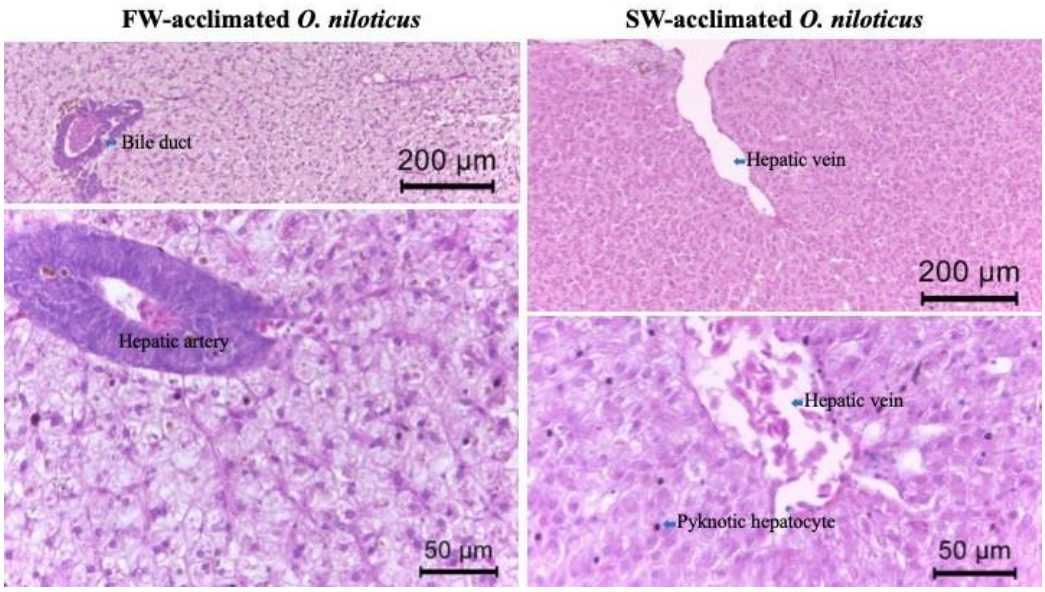

Figure 14. Histology of the liver of the FW- and SW-acclimated *O. niloticus*, with more cytoplasmic vacuolation in the FW-acclimated fish. H&E stain.

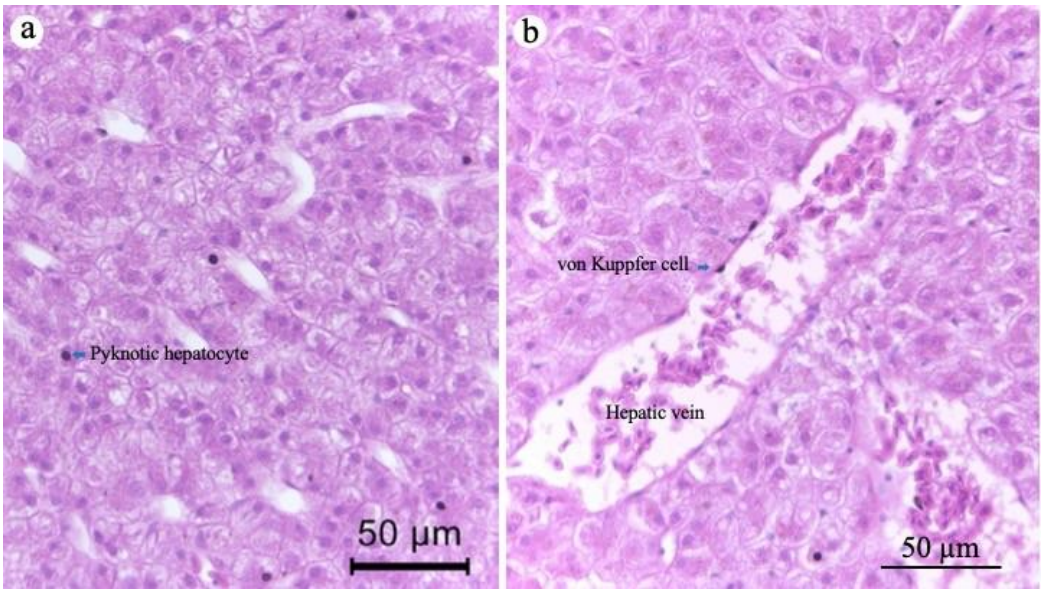

Figure 15. Histology of the liver of SW-acclimated *O. niloticus* showing pyknotic nuclei of hepatocytes and von Kuppfer cells. H&E stain

The cytoplasm of the hepatocytes of the SW-acclimated fish with densely eosinophilic stain was observed clearly in figure 15. Triangular-shaped nuclei lining

the liver sinusoid were frequently observed; these cells were most likely the phagocytic von Kuppfer cells (Fig. 15b).

In the liver parenchyma of the SW-acclimated fish, more red blood cells were found scattering inside the sinusoids, suggesting vascular congestion (Fig. 16a & b). In some areas, groups of red blood cells were observed, suggesting focal hemorrhage (Fig. 16c). Occasionally, groups of basophilic rods appeared among the parenchyma, suggesting an invasion by certain type of bacteria (Fig. 16d).

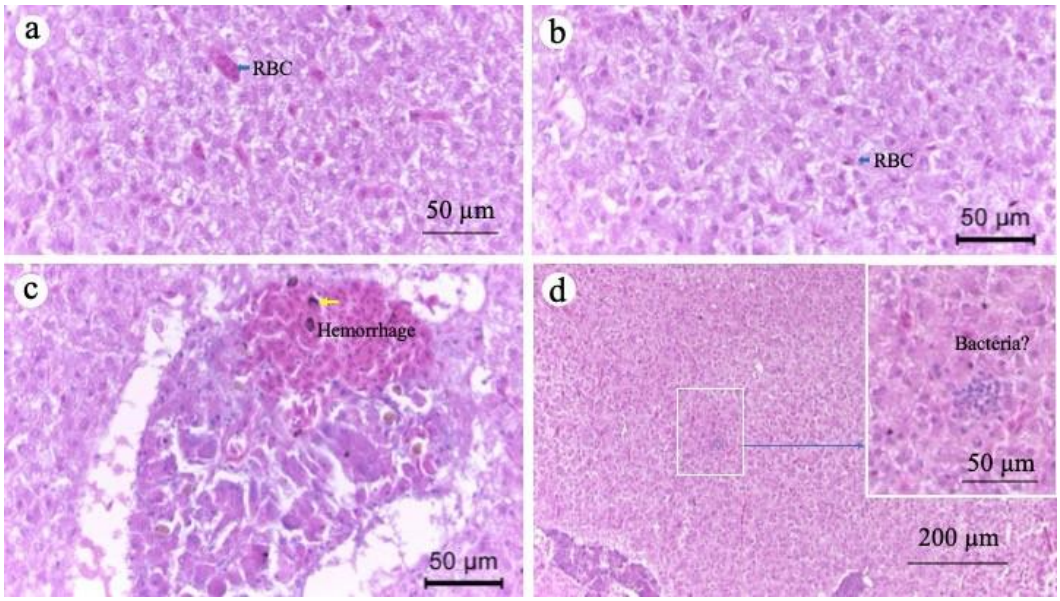

Figure 16. Histology of the liver of SW-acclimated *O. niloticus* showing red blood cells in the liver parenchyma and sinusoids (a & b), and a cluster of red blood cells, suggesting hemorrhage, as well as brownish stained materials (c, arrow), suggesting hemosiderin engulfed by macrophages. Groups of basophilic rods appeared among the parenchyma, suggesting an invasion by certain type of bacteria (d, inset). H&E stain.

3.6 Selection of the lipid type

In this net cage experiment, three types of lipids; salmon, soybean, and palm oil, supplemented in feed at 6 mL/kg feed were provided to the SW-acclimated *O.*

niloticus. It revealed that only the group receiving palm oil supplement survived at the rate, 70%, significantly higher ($p<0.05$) than that of the control group, 57% (Fig. 17).

However, the final BWs of all the lipid supplemented groups were significantly lower ($p<0.01$) than that of the group receiving no supplement. The percentage of biomass increase, which was calculated from the difference between the final and the initial biomass were not significantly different in all groups. Judging from the results, palm oil was selected as the best among the three types of lipid supplement.

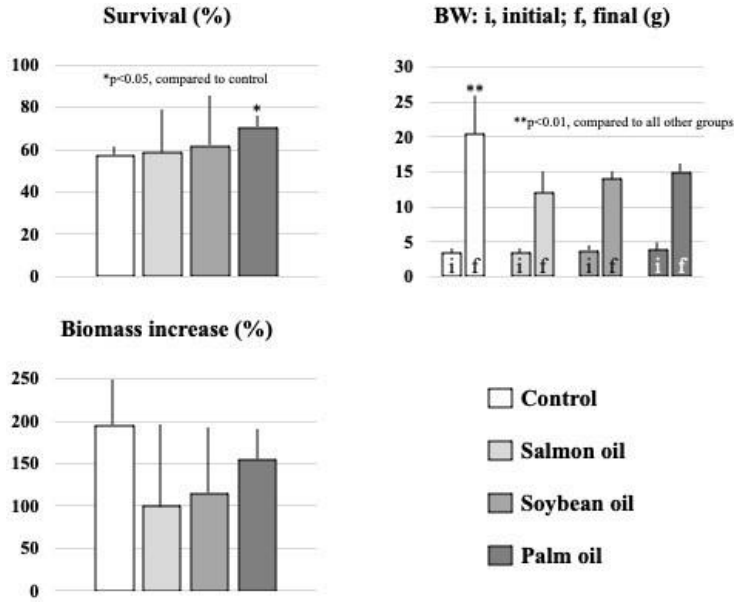

Figure 17. Survival, BW, and biomass increase of SW-acclimated *O. niloticus* receiving feed supplemented with salmon, soybean, and palm oil, at 6 mL/kg feed, compared to the control that received no supplement.

3.7 The optimum level of palm oil supplement

Under the same net-cage arrangement, the test for the optimum level of palm oil supplements (0, 3, 6, 9, and 12mL/kg feed) was performed. The experiment revealed that the palm oil supplement at any levels did not increase the survival of fish (Fig. 18). The final BW of the 6mL supplement group did not differ from that of the control; and both the control and the 6mL supplement groups had the final BW significantly higher ($p<0.05$) than that of the other supplement groups. The biomass increase in all the groups did not differ statistically.

The results of 3.6 and 3.7 suggest that palm oil supplement had very little, if any, to improve growth and survival of SW-acclimated *O. niloticus*.

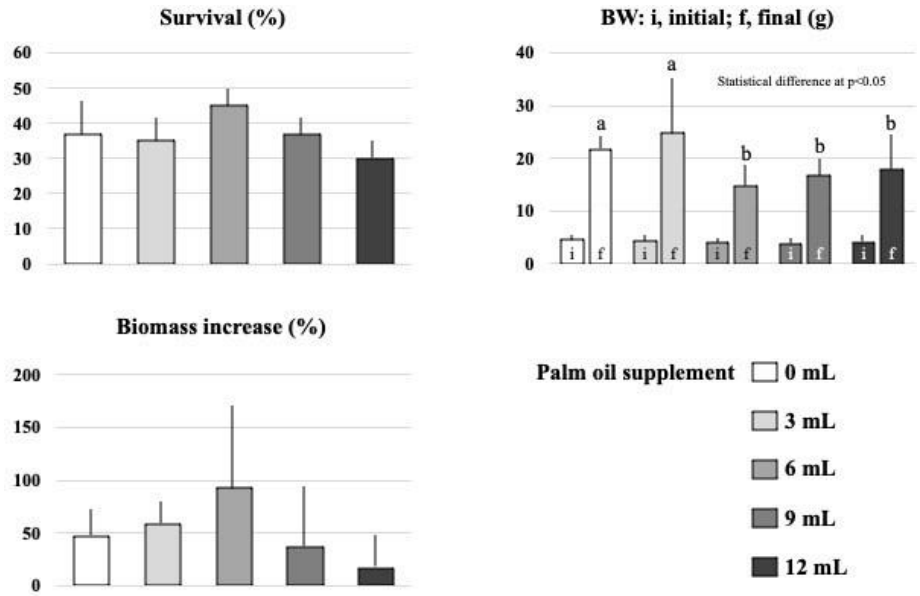

Figure 18. Survival, BW, and biomass increase of SW-acclimated *O. niloticus* receiving feed supplemented with palm oil, at 3, 6, 9, and 12 mL/kg feed, compared to the control that received no supplement.

3.8 *Myo*-inositol supplement

The fish were divided into 5 groups, 6 replicates of each. Gr. 1 was the FW control and received no supplement. Gr. 2 was the SW control and received no supplement. All other groups were SW-acclimated fish that received 250-, 500-, and 750-mg MI/kg feed, and were assigned as MI-250, MI-500, and MI-750, respectively.

3.8.1 Rearing performance

After rearing under specified water salinity and with or without MI supplements for 30 days, all groups of Nile tilapia grew at a comparable rate, with comparable initial and final BWs (Fig. 19). Most groups survived at comparable rates, but that of MI-500 was 12.2% higher than, and significantly different from, that of SW-control. The biomass increase, which is the difference between the initial and final biomass, was highest in FW-control. All the SW-acclimated fish receiving MI had comparable biomass increase, except that MI-500 had the value at 23% more, and significantly

different from, that of SW-control. The biomass increase in MI-500 was comparable to that of FW-control.

The FCR of MI-500 was the lowest; the value was 11.2% lower and significantly different from, that of SW-control.

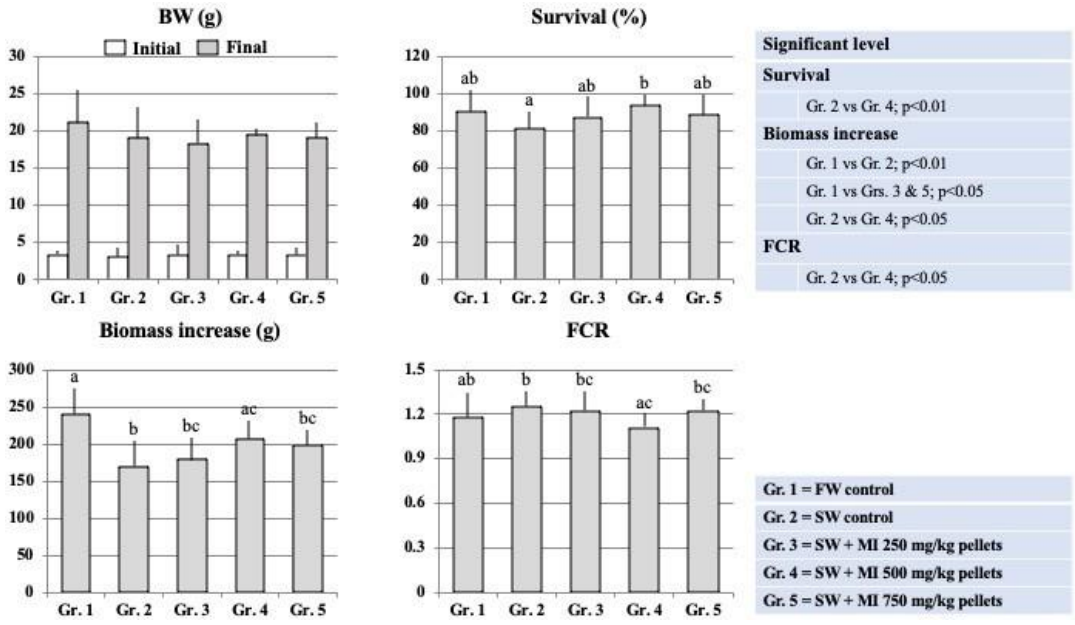

Figure 19. Body weights (initial and final, N = 10-14), survival (N = 6), biomass increase (N = 6), and feed conversion ratio (N = 6) of FW- and SW-acclimated *O. niloticus*, and the SW-acclimated fish receiving myo-inositol supplement. Different superscripts indicate a statistically significant difference. FCR, feed conversion ratio; FW, freshwater; SW, seawater; MI, *myo*-inositol

3.8.2 Plasma osmolality and electrolytes

Plasma osmolality of *O. niloticus* reared in SW (SW-control) was significantly higher ($p<0.01$) than that of the fish reared in FW (FW-control) (Table 3). The increasing value of plasma osmolality was maintained in SW-acclimated fish supplemented with 250 mg MI (MI-250), and 750 mg MI (MI-750), with the values of both groups significantly higher ($p<0.01$) than that of FW-control. However, at MI supplement of 500 mg (MI-500), the value was lowered to the level comparable to

that of FW-control but significantly lower ($p<0.01$) that that of SW-control, MI-250, and MI-750.

Plasma Na^+ values of FW-control and SW-control did not differ. With MI supplement at 250 and 750 mg, the values were slightly, but significantly, higher ($p<0.05$) than that of the FW-control and SW-control. Similar to the osmolality value, MI supplement at 500 mg also made plasma Na^+ value significantly lower ($p<0.01$) than the other two groups receiving MI supplement. And the value was even significantly lower ($p<0.01$) than that of the FW-control and SW-control.

The plasma K^+ value of SW-control was significantly lower ($p<0.01$) that that of FW-control. With MI supplement, either at 250, or 500, or 750 mg, plasma K^+ values rose to level comparable to that of FW-control.

The plasma Cl^- value of SW-control was significantly lower ($p<0.01$) than that of FW-control. And the values remained significantly lower ($p<0.01$) in all groups supplemented with MI. Further suppression of plasma Cl^- value was observed, again, in MI-500, which had the value significantly lower ($p<0.01$) than that of M-250 and M750.

Table 3. Plasma osmolality and ions of *O. niloticus*, without and with MI supplement. Different superscripts of the values in the same column indicate statistical significance. See Figure 9 for the different treatments of each group.

Group	Osmolality (mOsm/kg)	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)
FW-control	343 ± 5 (6)^a	168.8 ± 0.8 (8)^a	2.87 ± 0.57 (8)^a	140.1 ± 1.4 (8)^a
SW-control	358 ± 4 (7)^b	169.6 ± 1.7 (5)^a	1.78 ± 0.67 (5)^b	136.7 ± 1.2 (5)^b
MI-250	360 ± 8 (5)^b	171.2 ± 2.0 (5)^b	3.17 ± 0.40 (5)^a	134.9 ± 1.7 (5)^b
MI-500	347 ± 1 (5)^a	163.4 ± 1.5 (6)^c	3.09 ± 0.76 (6)^a	124.7 ± 1.1 (6)^c
MI-750	359 ± 4 (5)^b	172.4 ± 2.1 (7)^b	3.12 ± 0.73 (7)^a	132.4 ± 1.5 (7)^b

3.8.3 Relative expressions of *MIPS250* and *MIPA1*

After optimization of qPCR conditions for *MIPS250*, *MIPA1*, and β-*actin*, the corresponding Ct values had a linear relationship with sample concentration (R^2>0.996), amplification efficiency was high (85.2–98.8%), and melt curve analysis confirmed the presence of a single qPCR product in each case.

No significant difference was observed in the relative expression of *MIPS250* between FW- and SW-control (Fig. 20). The expressions of all MI-supplement groups were significantly higher than that of SW-control, which were 1.9x, 1.7x, and 4.1x fold of that of the SW-control, in MI-250, MI-500, and MI-750, respectively. The relative expression of MI-750 was also significantly higher than that of MI-250 and MI-500. Contrary to *MIPS250*, the relative expression of *MIPA1* in SW-control was 323x fold of that of the FW-control, a markedly significant up-regulation of the transcript. No significant difference of *MIPA1* relative expression among those of SW-control, MI-250, and MI-750; but the level in MI-500 was significantly attenuated, to 0.36x fold of that of SW-control.

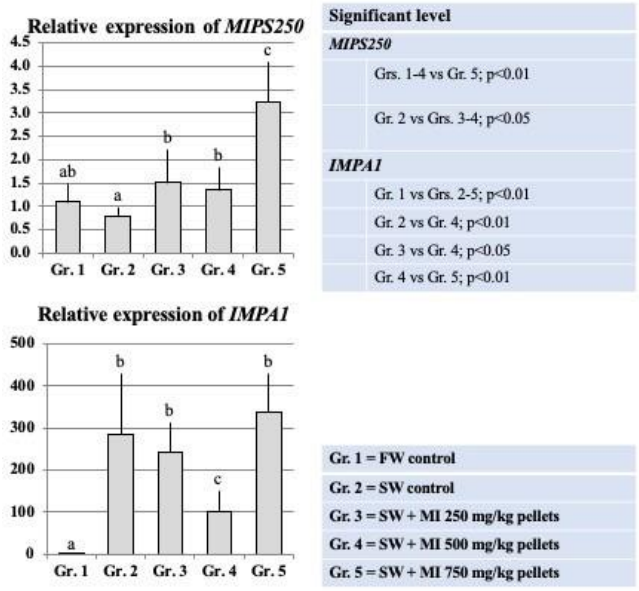

Figure 20. Relative expression of *MIPS250* and *MIPA1* from the gills of FW- and SW-acclimated *O. niloticus*, and the SW-acclimated fish receiving *myo*-inositol supplement. Different superscripts indicate a statistically significant difference. N = 6. FW, freshwater; SW, seawater; MI, *myo*-inositol

CHAPTER 4
DISSCUSION

4.1 Survival of the fish

The survival of the SW-acclimated *O. niloticus* in the 40L plastic tanks (Fig. 10) was not different from that of the FW-acclimated fish, which was quite an improvement from what previous described in the 1st generation of the SW-acclimated fish (Withyachumnarnkul *et al*., 2017). However, this level of survival was not observed when the fish were reared in the net cages (Fig. 17). The fish of the two experiments were similar in size, 3-5 g at the beginning, and the period of rearing was the same, one month. The stocking density of the tank rearing (0.35 individuals/L) was much higher than that of the cage rearing (0.06 individuals/L), therefore, the better survival in the tank could not be explained by stocking density. At the moment, it may not be possible to answer what is the cause of the difference. But it should be clarified that the cages were placed in the shrimp farm reservoir, and exposed to natural conditions, including fluctuation in temperature, salinity, and waste released from other aquatic animals in the reservoir, while conditions in the tanks were less fluctuating and without interference from other aquatic species. From this difference in the survival, it is possible that, under control conditions, the SW-acclimated *O. niloticus* could survive at the rate comparable to that of the FW-acclimated fish.

Despite a good survival achieved in the 5th-6th generation of SW-acclimated *O. niloticus*, a fraction of the fish (<20%) still suffered from skin lesions and exophthalmos. It was shown that most skin lesions, but not exophthalmos, were recovered when the fish were brought back to FW (Withyachumnarnkul *et al*., 2017). Exophthalmos was due to the infection in the brain and the ophthalmic veins that prevent venous blood return from the ocular bulb, and was a permanent damage (Palang *et al*., 2020).

4.2 Proximate analysis of the muscle

The proximate analysis revealed that the muscle of the SW-acclimated *O. niloticus* contained more fibers, lipid, and carbohydrate, but less protein content than those of the FW-acclimated fish. The reasons for this difference cannot be explained at present unless detailed studies on the metabolism of these three nutritional compounds in this type of fish are pursued in the future. However, it is worthwhile to explore the difference between the contents of protein, lipid, and carbohydrate in the muscle of the freshwater and marine fish.

As revealed in literature, levels of crude protein, lipid, and carbohydrate in tilapia muscle were variable in different reports. Crude protein level varied from 50-70%, lipid from 10-15%, and carbohydrate from 1-10% of dry weight (Al-Ghanim *et al*, 2017; Moses *et al*., 2018). Many reports provided units of proximate analysis as percentage of wet weight, which makes it difficult to compare. The results in this study revealed higher level of protein (approximately 80%) and lower level of lipid (approximately 5-6%) in the muscle. This difference could be due to the difference in technical analysis, or in sample preparation, or in different strain of tilapia, or even in the feed intake. It is not possible to discern this discrepancy. Rather, it may be more important to discuss that this study suggests that *O. niloticus* reared under SW may have less protein content and more lipid in the muscle, compared to those rear under FW.

From the results of nutrient analysis in the fish muscle of approximately 10 species, Li *et al*. (2011) suggested that that marine fish have more lipid content in the muscle than the freshwater fish. However, Lukasik *et al*. (2020) had reviewed several studies and concluded that nutritional contents in the muscle, especially protein and lipid, in fish were quite variable, even within the same species. The contents depend on feed intake, fish behavior, temperature of water, etc., which makes it difficult to generalize.

To our knowledge, there has been no report on the nutritional values of muscle in any particular species of tilapia reared under different salinity, therefore, this report is the first one showing that *O. niloticus* reared under SW had less protein but more lipid content in their muscle, compared to their FW counterparts. This difference,

despite being low, may be due to a possibility that more energy expenditure is needed for the SW-acclimated fish to adapt to the osmotic stress. Since the energy the fish use is mainly through lipid, it is possible that the conversion of muscle protein to lipid is a part of their adaptation. Further study on lipid metabolism of SW-acclimated *O. niloticus* should be able to reveal this interesting notion.

4.3 The liver of SW-acclimated *O. niloticus*

The results revealed that HSI of the SW-acclimated *O. niloticus* of both sexes was significantly lower than that of the FW-acclimated fish, however, the total lipid content in the liver was significantly higher in the SW-acclimated fish than in the FW-acclimated ones. Physiologically, the livers of fish are responsible for the same basic metabolic functions as in mammals, including processing, and storage of nutrients, the synthesis of enzymes and other cofactors, bile formation and excretion, production of vitellogenin, and the metabolism of xenobiotic compounds (Bruslé and Anadon, 1996). The reason for the decrease in size of the liver in the SW-acclimated fish cannot be explained from the present data. The histology of the liver of both groups did not differ, but probably a subjective view of increasing pyknotic nuclei of the hepatocytes in the SW-acclimated fish may suggest that the hepatocytes in that group were decreasing in number.

Liver histology of different fish species were found to be variable in pattern of hepatocytic arrangement (Sales *et al*., 2017) and hepatocytes of the fish could be either glycogen-rich or lipid-rich (Akiyoshi *et al*., 2001). In this regard, further study by special staining to identify glycogen or lipid should be done in the future. From this study, the SW-acclimated *O. niloticus* had significantly higher level of total lipid more than that of the FW-acclimated fish. It is therefore interesting to find out what types of hepatocytes dominate in the two groups of fish.

Hemosiderin is an iron pigment in the ferric form (Fe^{+++}) and results from the breakdown of red blood cells, which is often found in fish liver (Wolke, 1992; Leknes, 2015). Increased hemosiderin in hepatocytes (hemosiderosis) was found in teleosts under stress (Pronina *et al*., 2014). Since the SW-acclimated *O. niloticus* had been reared under disturbed environment, i.e., osmotic stress, it is possible that

hemosiderosis may be higher in the SW-acclimated fish. Here, it was already shown that pyknotic nuclei of this group of fish were higher than that of the FW-acclimated one. Vascular congestion, hemorrhage, and a possible invasion by bacteria shown in the histology of SW-acclimated fish suggested that the fish may have low defense mechanisms from being under osmotic stress, a condition similar to an infection by the virus infectious spleen and kidney necrotic virus described earlier (Withyachumnarnkul, 2017).

Why the total lipid content was increase in the SW-acclimated fish was interesting. Since lipid is a more important source of energy for the fish, compared to carbohydrate (Tocher, 2003), the increase in fat content in SW-acclimated fish suggests that the fish need more energy to survive. The liver accumulates lipid through either increasing lipogenesis or decreasing lipolysis (Fig. 21). The important enzymes involved in lipogenesis are acetyl-CoA carboxylase and fatty acid synthase, and those involved in lipolysis are carnitine palmitoyltransferase 1 and fatty acid dehydrogenase (Kerner and Hoppel, 2000; Cheng *et al.*, 2011; Chen *et al.*, 2015). It is, therefore, interesting to explore lipid metabolism, as well as lipid components, of the FW- and SW-acclimated *O. niloticus* in the future.

4.4 Lipid supplement

In the trial of three types of lipids; salmon, soybean, and palm oil, only palm oil supplement increased the survival of the fish, but the final BW was decreased by the supplement, resulting in a non-significant difference of the biomass increase among all groups. Further trial on the increasing dose of palm oil also resulted in decreasing final BW of the fish, without any significant change in the survival and biomass increase. These results suggest that lipid supplements in the SW-acclimated fish as a way to increase energy source for the defense against osmotic stress is unlikely to work. The fish may not be able to use exogenous lipid while being under osmotic stress, unless the cellular function was put back into normal function.

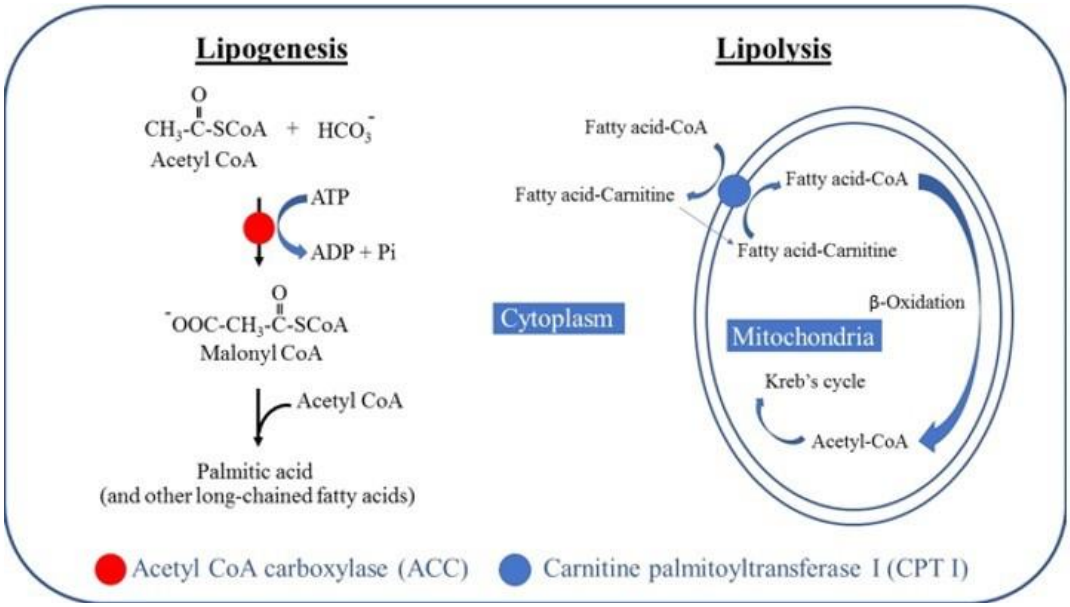

Figure 21. Schematic pathways of lipogenesis and lipolysis (β-oxidation) in the cell

4.4 *Myo*-inositol supplement

This study revealed no significant difference in growth and survival rates of FW- and SW-control. The findings were different from what previously reported when the fish were in their first generation of SW acclimation (Withyachumnarnkul *et al*., 2017), in which SW-acclimated *O. niloticus* grew at the rate of 60% and with a survival rate of 50% of the FW-acclimated fish. This is probably because all the fish in this study belonged to the 6th generation in the breeding program aiming at growth and survival improvement; thus, the process might have narrowed the difference in the performance between the FW- and SW-acclimated fish. However, the slight, but non-significant, decrease in growth and survival of the SW-control that still existed had resulted in a significant decrease in its biomass production, compared to that of FW-control.

The decrease in biomass production was partially reversed by MI supplement, especially at 500 mg/kg pellets. It revealed that, with that level of MI supplement, the SW-acclimated *O. niloticus* did not differ from that of FW-acclimated fish regarding their biomass increase. The beneficial effect of MI supplement has not been reported in *O. niloticus* reared in SW. Peres *et al.* (2004) reported that MI supplement did not affect growth, survival, and FCR of *O. niloticus* reared under FW. It is possible *O. niloticus* reared in FW may not face osmotic stress to the level as the SW-acclimated fish do, therefore, they may not require the supplement. In hybrid red tilapia rearing under elevated-salinity environment, it was reported that MI supplemented at 400 mg/kg diet improved its growth rate (Shiau and Su, 2005; Zaki *et al*., 2010), but also caused abnormality in the fish ovary and liver (Zaki *et al*., 2010). These findings support our current report, and also suggest that the level of MI supplement need to be optimum. In other fish species, either in FW or SW, the beneficial effects of MI supplement have been reported to increase growth, reduce susceptibility to pathogens, and prevent physical abnormalities (Khosravi *et al*., 2015; Shirmohammad *et al*., 2016; Yang *et al*., 2021).

The 23% increase in biomass production by 500 mg MI/kg pellets supplement in this report is considered a commercial significance. In addition, the significant reduction in FCR suggests that this increment was due to the better utilization of nutrients, not the increasing feed intake.

In this study, the increased osmolality was maintained in MI-250 and MI 750, but attenuated in MI-500. The values of plasma Na^+ also follow the similar pattern; i.e., being increase in the SW-acclimated group but were attenuated in MI-500.

However, plasma Cl- values were all decreased in the SW-acclimated fish, but again, was further lowered in MI-500.

The levels of plasma osmolality and electrolytes in this study were in the range reported in *O. niloticus* by other investigators (Chen *et al*., 2003; Lim *et al*., 2006). The increasing plasma osmolality in SW-control, compared to that of FW-control, in this study was corresponded to that report in *O. mossmabicus*, when the fish were transferred from FW to SW (Uchida *et al*., 2000).

Excretion of ions is accomplished by various membranous transport proteins in the gills, kidney, and intestine, including Na^+/K^+-ATPase, renal outer medullary K^+ channel (ROMK), Na^+-K^+-2Cl co-transporter (NKCC) (Haas and Forbush, 2000; Uchida *et al*., 2000; Furukawa *et al*., 2012; Ghahremanzadeh *et al*., 2014; Jumah *et al*., 2016). In principle, excess ions are transported from blood into excretory cells at their basolateral membrane and then from the cells into excretory space (as in kidney tubule and intestinal lumen) or into the external environment (as in the gills) through their apical membrane. In the gills, specialized cells that perform this excretory function are ionocytes, previously termed chloride cells or mitochondrion-rich cells, which could modify their morphology and number according to the salinity of the environment (Uchida *et al*., 2000).

Hwang *et al*., (1989) reported that the increase in plasma osmolality and ions in *O. mossambicus* when being transported from FW to SW were observed only within the first 24 h, thereafter the levels returned to those of FW levels. This phenomenon was different from that of *O. niloticus* in this study, in which the increasing levels were observed at least for 30 days. As *O. mossambicus* is a euryhaline species, its

physiological mechanisms in dealing with salinity changes were likely more advanced than those of *O. niloticus*, regarding as a stenohaline species. The elevated salinity might have up-regulated mechanisms, e.g., Na^+/K^+-ATPase and other transport proteins, to the levels that the influx/efflux of ions were completely balanced, while in *O. niloticus*, the mechanisms might be partially induced. MI supplement, as a compatible metabolite, might have helped stabilize the cell functions and allow the cells to up-regulate mechanisms on the ion excretion.

In this study, plasma K^+ was significantly decreased in SW-control, compared to that of FW-control, and its levels were restored by MI supplement. The control of plasma K^+ in fish has not been well documented. Very low level of K^+ was observed in the urine of marine fish (Smith, 1930; Hickman, 1968), therefore, it is unlikely that the kidney functions as key organ in K^+ excretion in fish. Furukawa *et al*. (2012) reported that the important route of K^+ excretion in *O. mossambicus* was through ionocytes in the gills, employing ROMK as the main transport protein. As in this study, Furukawa *et al*. (2012) also found that SW-acclimated *O. mossambicus* had significantly lower plasma K^+ than that of the FW-acclimated fish. In sea bass, *Lates calcarifer*, providing exogenous K^+ through diet helped increase the survival of the fish being transferred from FW to SW (Partridge and Lymbery, 2008). It is, therefore, possible that hypokalemia could be a factor leading to low survival of the SW-acclimated *O. niloticus*.

This study revealed that the relative expression of the transcript *MIPS250* in FW- and SW-control did not differ, while that of *MIPA1* was markedly increased in SW-control. The result was different from that of Kalujnaia *et al*. (2016), which

showed a 6- to 32- fold increase of *MIPS* splice variants in SW-acclimated *O. niloticus*. However, this study was similar to that reported by Sacchi *et al*. (2013) that showed a modest increase in *MIPS* but markedly increase in *MIPA1* in the gills of *O. mossambicus* when the fish were transferred from FW to SW.

Following MI supplement, up-regulation of *MIPS250* relative expression was observed in this study; this phenomenon has not been reported in tilapia. In the marine turbot *Scophthalmus maximus*, exogenous MI did not alter the expression of *MIPS* and *MIPA1* in the gills and kidneys of the fish (Ma *et al*., 2019). The up-regulation of *MIPS250* in our study suggests that exogenous MI might have induced the conversion of glucose-6-phosphate to *myo*-inositol-1-phosphate. The transcript *MIPA1*, which was markedly up-regulated in SW-control, was attenuated by exogenous MI, and again, in MI-500. This attenuation could be the negative feedback of the intracellular MI on the transcriptional level of MI biosynthesis, as suggested by Wang and Kültz (2017). This intracellular MI could be the combination of the *de novo* synthesis of MI and exogenous MI.

CHAPTER 5
CONCLUSIONS AND SUGGESTIONS

Feed supplement with salmon, soybean, and palm oil failed to improve growth and survival of SW-acclimated *O. niloticus*. However, *myo*-inositol (MI) supplemented through diet at 500 mg/kg pellets increased the survival and biomass of juvenile seawater (SW)-acclimated Nile tilapia *O. niloticus* to the levels comparable to the fish reared under freshwater. The supplement also attenuated the rise in plasma osmolality and sodium normally observed in the SW-acclimated fish. In the gills, both *MIPS250* and *MIPA1*, two important genes encoding for the enzymes responsible for MI biosynthesis, were up-regulated, with markedly more on *MIPA1*. Exogenous MI further up regulated the transcript *MIPS250*, while attenuating the up-regulation of *MIPA1*. Supplement of MI at the optimum dose is, therefore, one way to increase commercial production of *O. niloticus* reared under brackish/seawater. Based on this study, it is therefore recommended that MI supplement should be provided to SW-acclimated *O. niloticus* for commercial production, with probably adjustment of the dose of MI in the feed for the marketable-sized fish. More study on the MI *de novo* synthesis at transcription level and lipid metabolism of the fish should be performed in the future to improve the culture performance of this important commercial fish.

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