UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF DIFFERENCE TEMPERATURE ON ACUTE AMMONIA TOXICITY IN JUVENILE HYBRID GROUPER

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THE EFFECTS OF DIFFERENCE TEMPERATURE ON ACUTE AMMONIA TOXICITY IN JUVENILE HYBRID GROUPER

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It is hereby certified that we have read this project paper entitled "The effects of difference temperature on acute ammonia toxicity in juvenile hybrid grouper", by Abdul Karim Zaidan bin Ab Aziz and in our opinion it is satisfactory in term of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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DEDICATION

I dedicate this thesis to:

My dearest parent:

Ab Aziz bin Ab Rahman

Noor Hafizah Tan binti Abdullah

My supervisor:

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My co-supervisor:

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ABSTRAK

Abstrak daripada kertas kerja yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada kursus VPD 4999 –Projek.

Kesan perbezaan suhu kepada ketoksikan ammonia secara akut terhadap anak ikan kerapu hibrid.

Oleh

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2018

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Ammonia adalah toksik kepada semua haiwan vertebra termasuklah ikan dan ini telah mendapat perhatian yang besar di dalam industri perikanan aquakultur. Ammonia apabila di dalam larutan cecair terbahagi kepada ammonia yang tidak diionkan dan ammonia yang telah diionkan. Perkadaran ammonia yang tidak diionkan dan yang telah diionkan di dalam air bergantung kepada suhu dan pH air itu tersendiri. Di Malaysia, suhu naik dan turun sepanjang tahun disebabkan keadaan cuaca yang panas dan lembab. Oleh itu, objektif kajian ini adalah untuk mengetahui kesan perbezaan suhu kepada ketoksikan ammonia secara akut terhadap anak ikan kerapu hibrid. Lapan puluh ekor anak ikan kerapu hibrid (TGGG) bersaiz lingkungan 28.3 g dengan jumlah kepanjangan 11.9 cm

telah digunakan untuk menilai kemampuan mereka terhadap pelbagai tahap ammonia dan suhu yang berbeza. Kesemua ikan dibahagikan kepada 8 kumpulan; Kumpulan A1 (1.5mg/L NH3-N at 29°C), Kumpulan A2 (2.5mg/L NH3-N at 29°C), Kumpulan A3 (3.5mg/L NH3-N at 29°C), Kumpulan B1 (1.5mg/L NH3-N at 25°C), Kumpulan B2 (2.5mg/L NH3-N at 25°C), Kumpulan B3 (3.5mg/L NH3-N at 25°C). Kumpulan kawalan negatif dibahagikan pada suhu 25°C dan 29°C dengan tiada penambahan ammonia. Hasil keputusan menunjukkan jumlah ammonia di dalam air bertambah mengikut dengan masa, tetapi perkadaran ammonia yang tidak diionkan menurun mengikut dengan masa di kesemua kumpulan. Tambahan pula, tiada kematian didapati di kesemua kumpulan pada suhu 25°C dan 29°C dengan tahap ammonia yang berbeza. Hal ini disebabkan keupayaan ikan mengatasi dan bertahan dengan peningkatan tahap ammonia di dalam air. Di samping itu, fisiologi ikan itu sendiri dipercayai mampu menukarkan ketinggian tahap ammonia di dalam air kepada bahan yang kurang tosik, seperti glutamin dan urea. Kesimpulannya, metabolisma anak ikan kerapu hibrid mempunyai keupayaan untuk bertahan dengan peningkatan tahap ammonia di dalam air pada suhu yang berbeza.

Kata kunci: Perbezaan suhu, Ammonia, Kerapu hibrid

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 –Project.

The effects of difference temperature on acute ammonia toxicity in juvenile hybrid grouper.

By

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2018

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Ammonia is a toxic to all vertebrae including fish and can caused a major concerned in aquaculture industries. Ammonia expressed as unionized ammonia and ionized ammonia when in aqueous form. The proportion of unionized and ionized ammonia level in the water are depends on the temperature and pH of the water. In Malaysia, the temperature was rise and fall through all the year due to the hot and cold climate. Therefore, the objective of this study is to determine the different temperature on acute ammonia level in the juvenile hybrid grouper. Eighty juvenile hybrid grouper (TGGG) size around 28.3g with total length of 11.9cm were used to assess their capabilities to respond towards ammonia level and different temperature. All fish were

divided into 8 groups; Group A1 (1.5mg/L NH_3-N at 29°C), Group A2 (2.5mg/L NH_3-N at 29°C), Group A3 (3.5mg/L NH_3-N at 29°C), Group B1 (1.5mg/L NH_3-N at 25°C), Group B2 (2.5mg/L NH_3-N at 25°C), Group B3 (3.5mg/L NH_3-N at 25°C). The negative control group was assigned at temperature 25°C and 29°C with no ammonia added. The result showed that the amount of total ammonia increasing by times, but the proportion of the unionized ammonia reducing following times in all treatment groups. In addition, the result also showed that no mortality were observed in temperature group 25°C and 29°C with different level of ammonia. This was due to the ability of the fish in respond and tolerant to the elevated ammonia level in the water. Moreover, the physiological of the fish is believed in converting the higher level of ammonia to less toxic substances, such as glutamine and urea. In conclusion, the metabolism of juvenile hybrid grouper had the ability to tolerate with elevated ammonia level in the water at different temperature.

Keyword: different temperature, ammonia, hybrid grouper

1.0 Introduction

According to FAO, aquaculture in Malaysia had developed since 1920's and become an important activity currently. Fishery industries had moving from capture fished practice to aquaculture practice. According to Shariff, (2009), aquaculture industries in Malaysia up grow from 6% of total fish production in 1991 to 74.7%. Moreover, grouper species covered 1% of total Malaysia aquaculture product in 2010. The number will continuing growing by times. In 2006, The Borneo Marine Institute (IPMB) of Universiti Malaysia Sabah reared a new group of hybrid grouper called as TGGG. The TGGG is cross bred between the eggs of Tiger Grouper (*Epinephelus fuscoguttatus*) with the sperm of Giant Grouper (*Epinephelus lanceolatus*) via external fertilization. It is widely raised in aquaculture industry of Peninsular Malaysia and covered 70% of Malaysia grouper production.

In aquaculture industry, ammonia is one of the major threats to the survival of the fish. Ammonia is one of the nitrogenous wastes from the fish and was excrete from the gill. Another fish nitrogenous waste was urea. Ammonia is toxic to all vertebrate. According to United State Environmental Protection Agency report in 1984 and 1989, the average mean acute ammonia toxicity for 17 seawater species was ay 1.86 mg NH3-N/L. Ammonia in aqueous solution express in two form either unionized ammonia (NH_3) or ionized ammonia (NH_4^+). Unionized ammonia is the more toxic of the two forms, as it diffuses through the epithelial membranes of aquatic animals more readily than the ionized ammonia (United State Environmental Protection Agency (USEPA), 1999)

The proportion of unionized and ionized ammonia was determined by the water temperature and pH. As the water increase is the pH or temperature, the unionized ammonia increases in the percentage. According to Økelsrud & Pearson (2007), the ratio of unionized to ionized ammonia increased 10 fold per pH unit rise and approximately 2 fold for each 10 rise in the 0-30°C range. The research establish that unionized ammonia species of total aqueous ammonia is relatively more toxic than the ionized ammonia (Thurston & Emerson, 1979). Thus, the total ammonia toxicity upon fish will be increased with the pH and temperature.

The water temperature in Malaysia was rise and fall throughout the year due to the monsoon and hot humid climate. The water temperature influences the fish body temperature as the fish body temperature was according to the surrounding water temperature. From Morvan *et al.,* (1997), fish have a body temperature that is essentially the temperature of the surrounding water (Fry, 1967) so that their entire physiology, including immune functions, is influenced by environmental temperature. From the unpublished data by Fish Research Institute Tanjung Demong, the range of water temperature in the fish farms from East Peninsular of Malaysia was at 22°C to 30°C in year 2017. For TGGG hybrid grouper, it was exhibited relatively better growth form and condition at 26°C and 30°C (Moumita *et al*, 2016).

1.1 Justifications

According to United State Environmental Protection Agency (USEPA) report in 1984 and 1989, the average mean acute ammonia toxicity for 17 seawater species was at

1.86 mg NH3-N/L, but Grouper (*Epinephelus sp*.) was not included in this report. Furthermore, juvenile hybrid grouper TGGG is a new type of grouper that being introduced to the industry. In addition, Malaysia is tropical country which the temperature was rise and fall throughout along the year due to the changed seasons from hot climate to cold monsoon weather. Thus, we would like to examine the acute lethal effects of different temperature on ammonia toxicity in juvenile hybrid grouper.

1.2 Objective

We would like to examine the acute lethal effect of different temperature on ammonia toxicity in juvenile hybrid grouper.

1.3 Hypotheses

1. H_0: There is no significant different on the acute ammonia toxicity in juvenile hybrid grouper at different temperature.

2. H_A: There is significant different on the acute ammonia toxicity in juvenile hybrid grouper at different temperature.

2.0 Literature review

2.1 Ammonia

Ammonia is a compound of nitrogen and hydrogen with the formula of NH_3. Ammonia is toxic to all vertebras. In fish and aquatic organism, ammonia is an important toxicant. Ammonia when dissolved in water, it exists as unionized and ionized form (Thurston, 1979). The aqueous ammonia equilibrium can be written as;

$NH_3 + H_20 = NH_3\text{-}H_20 = NH_4^+ + OH^- + H_20$

The equilibrium is determined by the water pH, temperature and ionic strength of the aqueous solution (Emerson *et al*, 1979). From Erickson (1985), the unionized ammonia increased 10 fold with the increased of one unit of pH and 2 fold if the temperature increased by 10°C. According to Chipman (1934), supported by Wuhrmann *et al.,* (1947) and Waker (1948) said that unionized ammonia (NH_3) is more toxic than NH_4^+.Based on this finding, a water quality criterion of 0.02 mg/liter NH3 has been established by the U.S. Environmental Protection Agency (USEPA) to protect freshwater aquatic life in (1977).

In addition, Tabata (1962) has attributed toxicity to fishes and aquatic invertebrates to the NH_4^+ fraction as well as to the NH_3 fraction, and has estimated NH4+ to be perhaps 1/50th as toxic as NH_3. However, Armstrong *et al*., (1978) reported that the toxicity of NH_4^+ to be greater than that reported by Tabata. Even so, there is no doubt that unionized ammonia is much more toxic to the fish than ionized ammonia.

2.1.1 Ammonia productions

In fishes, nitrogenous waste was ammonia and urea, with ammonia compromising about 80% of it in teleost (Smith, 1929; Wood, 1958). Ammonia is a byproduct of protein metabolism and excretes directly from the fish gill into water (William, n.d.). This kind of fish were also classified as ammoniotelism which able to excrete their nitrogenous waste primarily as ammonia.

Dietary protein is a major source of amino acids in animals. The intestines of carnivorous fishes are adapted to process diets that are high in protein and low in carbohydrate (Buddington *et al*., 1997). The major pathway for the production of ammonia is through the transamination of various amino acids (Forster & Goldstein, 1969; Watts and Watts, 1974). The primary site for ammonia production is probably the liver (Pequin & Serfaty, 1963). Amino acids in excess of those required for protein synthesis are converted to ammonia in the liver.

Ammonia in fish is produced by the catabolism of amino acid with require little of energy. The amino group of any amino acid is transferred to alpha-ketoglutarate to form glutamate and alpha-keto acid. The glutamate then released the amino group as ammonia by the enzyme glutamate dehydrogenase (Helen, n.d).

The ammonia that being produced will be excrete out from the body. They were excreted by gill primarily and also by kidney. The mechanism is the ammonia is diffuse passively into the water by transcellularly over the leaky junction in gill and actively transport by replace K^+ in Na^+-K^+-ATPase and H^+ in H^+-Na^+-Exchanger.

2.1.2 Ammonia toxicity

Acute ammonia toxicity mainly effects the central nervous system of vertebrae. This is by causing convulsion following to death. High level of ammonia in brain lead to increase extracellular glutamate by increasing glutamate release and decreasing glutamate synaptic reuptake (Rao *et al.*, 1992; Bosman *et al.,* 1992*;* Schitmdt *et al.,* 1993). From Marcaida *et al.,* (1992), excessive activation of NMDA glutamate receptor that mediated the ammonia toxicity. This will lead to influx of calcium and sodium ions that eventually lead to cell death. Previously, ammonia toxicity was claimed to be caused by increased in brain extracellular which lead to excessive activation of NMDA receptors and subsequently neuronal cell death (Randall & Tsui, 2002). However, recently Hermengildo *et al.,* (2000) showed that activation of NMDA receptors preceded the increase in extracellular glutamate levels. One of the consequences of excessive NMDA receptor activation is ATP depletion, which reverses the sodium-dependent glutamate uptake mechanism. From Binstock & Lecar (1969), NH_4^+ can substitute K^+. Therefore, the primary cause of ammonia toxicity lead to depolarization effect of NH_4^+ on neurons leading ton excessive activation of NMDA receptors and death of the cell.

2.2 Fish defense mechanism against ammonia in water

Elevated ammonia level will cause increase in the body ammonia level. Most fish cannot tolerate high environmental ammonia level but there are some species of fish able to tolerate with it and avoid ammonia toxicity (Tsui & Randall, 2002) by converting the ammonia into less toxic substance.

Many fish detoxify ammonia into glutamine when being exposed to elevated ammonia level in the environment. This was proved by Levi *et al.,* (1974) that goldfish brain glutamine level had liner correlation with the ambient ammonia level when being exposed to ammonia chloride. The mechanism is starting from the alpha-ketoglurate detoxify ammonia ions to form glutamate. Glutamate than detoxify another ammonia ion by enzyme glutamine synthesize to form glutamine. Glutamine can be stored in the tissue. The disadvantage of this mechanism is it required 2 mole of ATP to be hydrolyzed.

Another mechanism is the fish able to convert the ammonia into urea via ornithine urea cycle (Wood *et al.*, 1995). The ammonia will bind with the bicarbonate to form carbomyl-phosphate. The carbomyl-phosphate will become the enzyme to convert the ornithine into citruline. The cycle will be end until urea was being produce and was excrete by the gill or kidney.

2.3 Hybrid Grouper TGGG

Hybrid grouper TGGG was a cross bred between tiger grouper (*Epinephelus fuscoguttatus)* and giant grouper (*Epinephelus lanceolatus)* by external fertilization. It was first produce by Universiti Malaysia Sabah Borneo Marine Research Institute on 2006. Both of the species is widely cultured in Southeast Asia but there were constrain on culture tiger grouper due to difficulties to obtain high quality sperm while difficulties in obtain high quality egg in giant grouper. Thus, after few researches being done, a hybrid grouper TGGG being produced.

The hybrid grouper has many attributes including fast growth, higher resilience to environmental variation, better disease resistant and excellent organoleptic quality with survival rate was as high up till 40% (Rossita, 2016). Study by Amni *et al.,* (2015) show that hybrid grouper TGGG has a wide range of salinity tolerance. Liang *et al.,* (2013) stated that this grouper could with stand low water pH and salinity. However, still a trace study has been done on this new hybrid grouper species may due to fish was just newly introduced to the aquaculture industries despite their higher demand in the market.

2.4 Guideline of toxicity test

The acute toxicity studies were follow the standard guideline by the USEPA was namely the exposure of the organism to the toxicant with static condition using starved, resting and unstressed animals. This standardize can help to compare between the studies that being done. This is due to the internal ammonia level in fish is increase when the fish was fed, swim and under stress which need to be avoided during acute toxicity test.

3.0 Materials and Methods

3.1 Animal screening

Hundred juvenile hybrid groupers were transported from the nearby farm. Four fishes were chosen randomly for screening purpose. All the fishes being anesthetized by using Tricaine Methanesulfonate (MS222) at 50ppm. The fishes were remove from water when achieve stage II anesthesia.

The weight of the fished was weighing using electronic table top weight machine. By using string and ruler, the total length of the fish was calculated. Total length is defined as the measurement taken from the anterior-most part of the fish to the end of the caudal fin rays when compressed dorsa-ventrally (Anderson and Gutreuter 1983).

All the four fishes then being euthanasia by using decapitation (bleeding) technique. The method is by sever the fish's head at the junction of the skull and first vertebra with a scalpel blade explain by Queen's University Animal Care Committee in 2014. Then, by using the cover slip, scrape through the mucous of the fish from the lateral part of the body up till the tail end. Scrape the skin in the direction of the scales. Put a drop of saline of the glass slide before placing the cover slip on top of the drop of the water. The drop of the water came from the water of the fish tank. This is do prevent the parasite from being crushed. Next, open the operculum to reveal the opercular cavity. Prepare another glass slide with a drop of water. By using scissor, cut the gill filament and place on the glass slide and cover up with cover slip. Examine both glass slide under the compound microscope under 4x, 10x and 40x. Both techniques were used to examine the present of ectoparasite on the fish.

The fishes undergo post mortem check. The fishes were incised along the ventral surface immediately anterior to the anus. Insert scissors and cut forward to the base of the pectoral fins. Cut through the pectoral girdle and then cut up the edge of the operculum to the top of the abdominal cavity and then back towards the vent so that the flap of the body can be removed. Make sure to ensure that the internal organs are not ruptured. The fish is check for ascites. The liver, spleen, heart, stomach, intestines, abdominal fat, swim

bladder, peritoneum, muscles and kidney were observed grossly. Abdominal organs are check for colour, friability, adhesions, hemorrhages, necrosis, nodules, cysts and parasites.

Sample was collected from the kidney and liver for bacteria culture. An incision was made through the liver using a sterile scalpel blade. A sterile cotton swab was used to swab inside the liver. Rub and roll the swab firmly inside the liver. The cotton swab then swabs on the Trypticase Soy Agar (TSA) and Thiosulfate Citrate Bile Salts (TCBS) agar. TSA is a solid medium recommended for use in isolation and cultivation of wide variety of organisms. It contains casein and soy peptone that provide nitrogen, amino acid and peptides necessary for the growth of wide variety of organisms. The source of energy comes from dextrose whereas sodium chloride supplies essential electrolytes and maintains osmotic equilibrium. The TCBS then highly selective for the isolation of *Vibrio spp* either *V. cholerae* or *V. parahaemolyticus.* It has very high pH at 8.5-9.0 which suppress growth of intestinal flora other than *Vibrio spp.* The bile salts in the agar inhibit growth of gram-positive microorganisms. The agar is left in room temperature for 24 hours to see the growth of the bacteria.

After the screening was done, the remaining fish was acclimatized in 2 water tanks, each was place 48 fishes. The water tank was clean prior acclimatized 2 days earlier with fresh water and Dettol. The water tank is aerated with oxygen and water flowing. The fish was fed with protein rich pellet twice a day, morning and evening.

3.2 Acute toxicity test

Eight glass aquariums were prepared for this experiment. Prior experiment, all the glass aquarium was clean with fresh water and Dettol. This is to kill all the parasite and bacterial present in the aquarium that may affect the fishes during the experiment. 50 liter of sea water was filled in all the aquarium. The sea water comes from the Pantai Tanjung Demong, which was pumped and filtered using sand filter. The amount of water was calculated by multiply the width, height and length of the aquarium.

The day before the experiment, the fish was fasting just to prevent the buildup of the ammonia inside the water from the fish. This may affect the result of the experiment.

Acute effects of ammonia on juvenile hybrid grouper were investigated in a static nonrenewal 96-h experiment that involved a strict pH and temperature regime. Water temperature was assigned at 25°C and 29°C. Group A0, A1, A2 and A3 was assigned their temperature was at around 29°C±1.0 along the experiment. Group B0, B1, B2 and B3 was assigned their temperature was at around 25°C±1.0 along the experiment. To maintain the water temperature at 25°C, ice pack and ice cube was used to lower the temperature. On other hand, heater probe was used to maintain the water temperature at 29°C. Mercury thermometer was used along the experiment to monitor the water temperature. It was checked every 8 hours. Water pH was monitor by using the pH meter to maintain the pH at around 8.0 for all the aquarium. Sodium bicarbonate was prepared for this occurrence. If water pH is reducing, an amount of sodium bicarbonate will be adding into to water to raise the pH back to be at around 8.0.

Dissolved oxygen and salinity also was strictly monitoring to prevent the death of fish due to both of this factor. Two oxygen line each was set up at every aquarium and was monitored by using the YSI ProDSS. The dissolved oxygen must be maintained at above 5.00 ppm. The salinity of the waste was also monitor by the YSI ProDSS to maintain at range 29-32 ppm.

Before the experiment started, the water pH and temperature were recorded to make sure at the desire value. Then, amount to ammonia chloride was added into the water according to the treatment group requirement. Group A1 and B1 was assigned for 1.5mg/L of ammonia, group A2 and B2 was assigned for 2.5mg/L of ammonia, group A3 and B3 was assigned for 3.5mg/L of ammonia in the water. Group A0 and B0 was assigned as negative control thus no added ammonia in the water. The amount of the ammonia chloride needed to achieve the desire ammonia level in the water was calculated using the formula by Thurston *et al.,* (1979).

After the ammonia chloride was added into the water, the fishes were randomly picked and assigned into the treatment group A0, B0, A1, A2, A3, B1, B2, B3. Then by using YSI ProDSS, all the water parameter like temperature, pH, dissolved oxygen and salinity was recorded in the monitor form. The result was recorded every 12 hours interval along the experiment.

Then, a small beaker of 60ml was used to collect water from each aquarium. The water will then be used to determine the total ammonia nitrogen level in the water by using the salicylate method. This process was done every 24 hours interval for 96 hours.

3.2.1 YSI ProDSS

YSI ProDSS (Digital Samping System) is a portable water quality multi-parameter instrument for the measurement of several critical parameters such as dissolved oxygen (optical), turbidity, pH, ORP, conductivity, specific conductance and salinity. It is designed for use in applications such as surface water, groundwater, coastal/estuarine, aquaculture, and wastewater. YSI ProDSS cable assemblies with 4 sensors. All sensors were used to read the water parameters. The reading will show up in the main display (Run screen) when the cable was introduced into the water.

3.2.2 Salicylate method

The salicylate method is a variation of the Berthelot-Phenate method but does not required the use and disposal of toxic phenol. The salicylate method involves a three-step reaction sequence. The first reaction step involves the conversion of ammonia to monochloroamine by the addition of chlorine. The monochloroamine then reacts with salicylate to form 5-aminosalicylate. Finally, the 5-aminosalicylate is oxidized in the presence of sodium nitroferricyanide (a catalyst) to form a blue-green colored dye that absorbs light at 650nm.

Firstly, one sample cell is prepared with 10ml distilled water as the blank. Next, all the water sample that was collected from the aquarium was filled into the sample cell at 10ml volume. Pipette was used to make sure correct amount of volume was taken. Then add in one sachet of ammonia salicylate reagent powder to each sample cell. The cell was capped and shake for the reagent to dissolve. Leave to sample cell for three minutes for

the reaction occur. After three minutes, add another one sachet of ammonia cyanurate reagent powder to each sample cell. The cell was capped again and shake to dissolve the reagent. At 15 minutes required for the reaction to occur. After 15 minutes, if there is present ammonia nitrogen, a green colour will be present. Then, place the blank sample cell into the cell holder of laboratory turbidimeter for zeroing. After zeroing, all other sample cell was place into the cell holder to read the total ammonia nitrogen that will show in mg/L NH_3-N. This turbidimeter has only able to read the result that less than 3.5 mg/L NH_3-N. Thus, if the result is more than 3.5mg/L NH_3-N, dilution method will be used to dilute the sample to x10 dilution.

Dilution method to x10 dilution was done by adding 1ml of water sample and mix with 9ml of distilled water. The result that shown then will be multiply by 10.

3.2.3 Monitor sheet

The results of the water temperature, pH, dissolved oxygen and salinity were recorded every 12 hours for 96 hours along the experiment using YSI ProDSS. The results of total ammonia nitrogen were recorded every 24 hours for 96 hours along the experiment using salicylate method and turbidimeter. The fishes in the aquarium was monitored every 8 hours to record their number of mortality. The fish remaining motionless when removed confirmed mortality. The proportion of the unionized ammonia and ionized ammonia was calculated using the table by Emerson *et al.,* (1979) according to the current water temperature and pH. All results were recorded in a monitor sheet.

3.3 Post experiment

All fishes were then returned back to Fish Research Institute (FRI) Tanjung Demong. The carcass was disposed following FRI procedure.

4.0 Results

During earlier screening on the juvenile hybrid grouper, the mean size of the fish was 28.34g with range of 24.55g to 33.23g. The total length of the fish was 11.87 cm with range between 11.6cm to 12.3cm. All four fishes were free from ectoparasite on the gill and body mucus when examine under the microscope. There was no growth on the TSA and TCBS agar for the kidney and liver swab confirming the fish is free from bacteria.

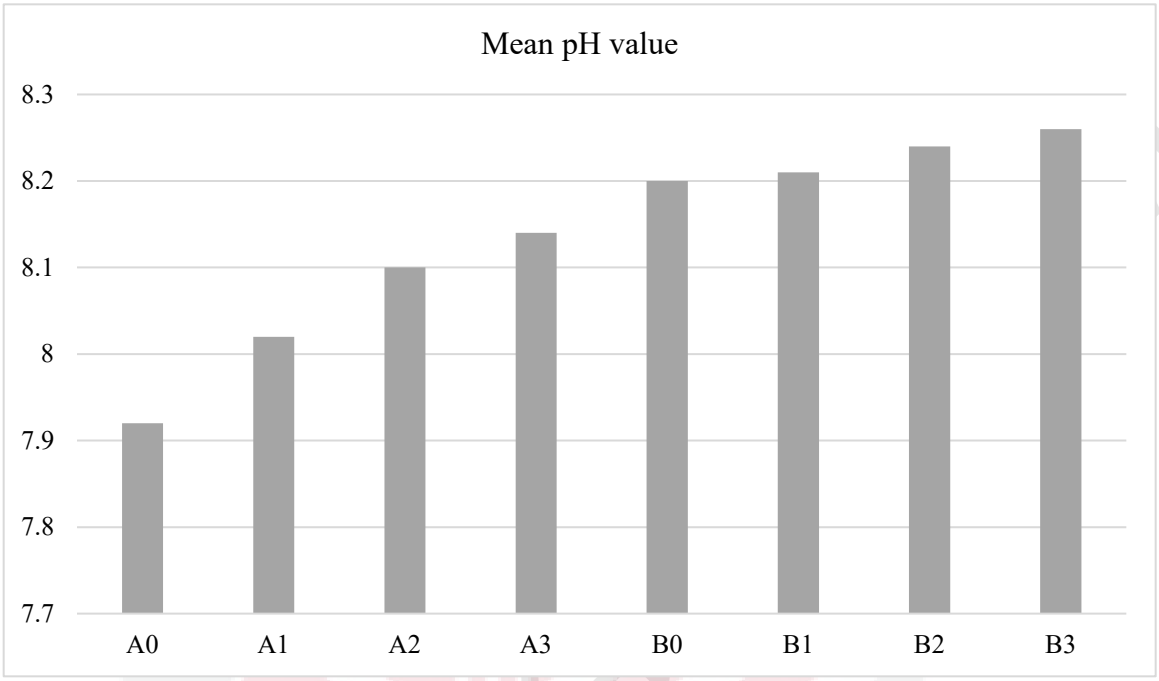

Figure 1: Mean value of water pH

Upon along the acute ammonia toxicity testing, the mean pH for all the treatment groups was at 8.13 with the range for the mean of each group was at 7.92 to 8.26. The average mean for group A0, A1, A2, and A3 that was assigned to maintain temperature at 29°C were slightly lower than the group B0, B1, B2, and B3 that were assigned to maintain the temperature at 25°C. The range of mean pH of group that was assigned temperature at around 29°C±1.0 were at 7.92 to 8.14. The range of mean of pH of group that assigned was assigned temperature at around 25°C±1.0 were at 8.20 to 8.26.

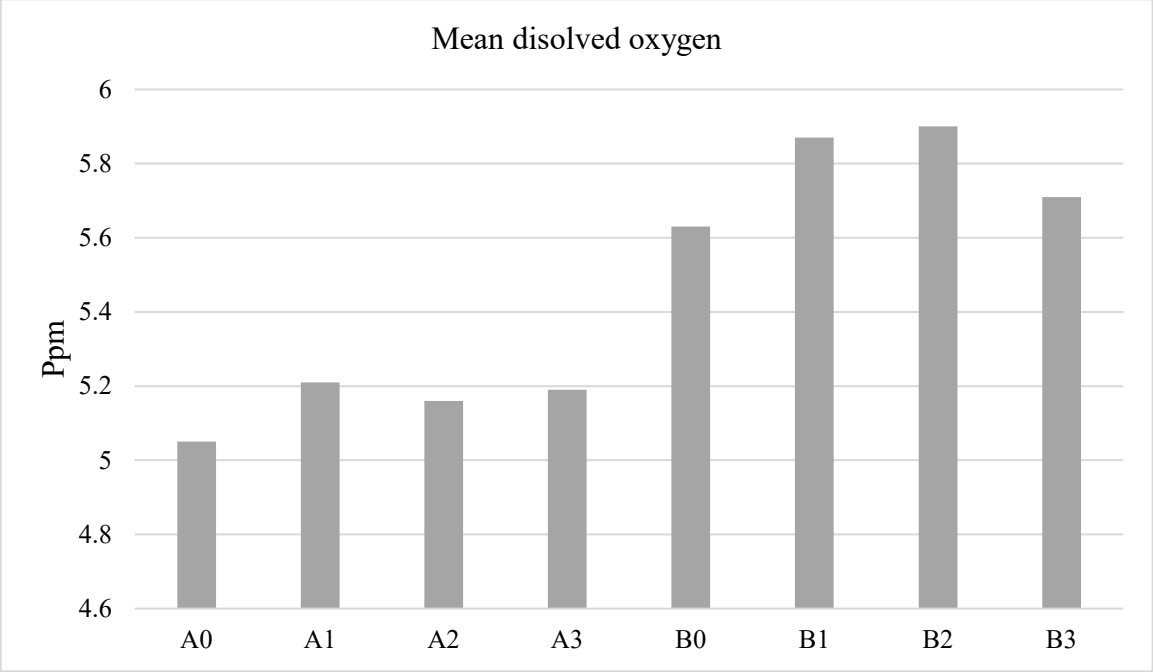

Figure 2: Mean value of water dissolved oxygen

The mean dissolve oxygen for all treatment groups was at 5.47ppm with the range of 5.09ppm to 5.90ppm. Once again, the average mean of dissolved oxygen for group A0, A1, A2, and A3 that was assigned to maintain temperature at 29°C were slightly lower than the group B0, B1, B2, and B3 that were assigned to maintain the temperature at 25°C. The range of mean dissolved oxygen for group A0, A1, A2, and A3 was 5.09ppm to 5.19ppm. The range of mean dissolved oxygen for group B0, B1, B2, and B3 was 5.63ppm to 5.90ppm.

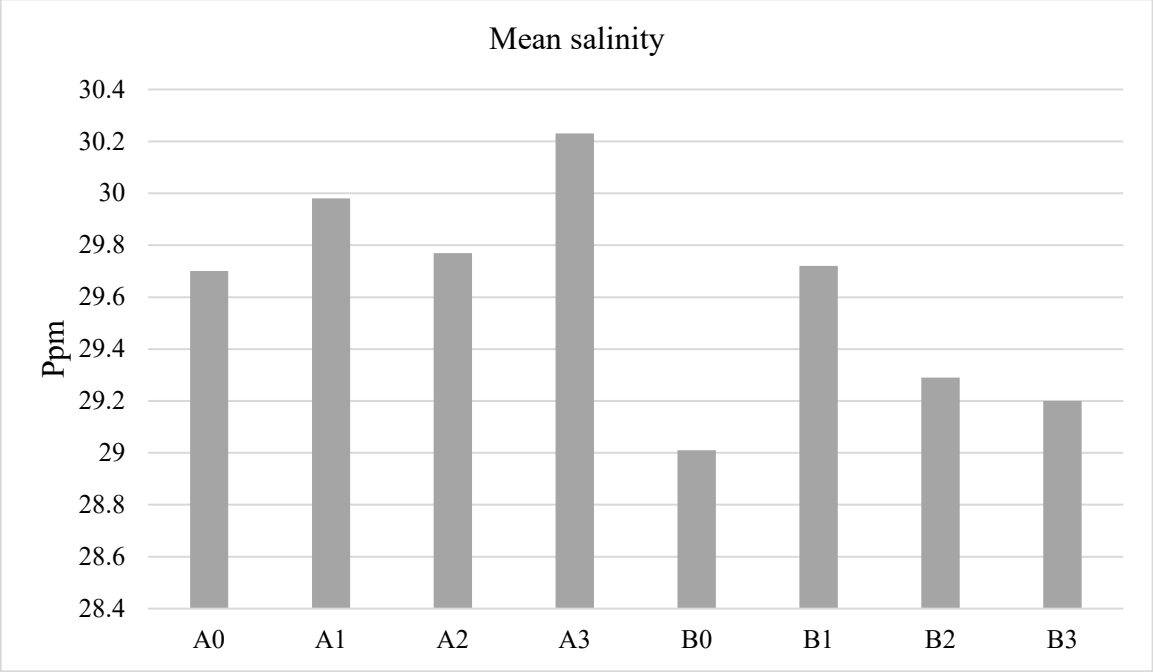

Figure 3: Mean value of water salinity

For the salinity of the water of all treatment groups, the mean was at 29.61 with range between 29.01 and 30.23. No significant different can be seen between the group that were assigned at temperature at 29°C±1.0 and the group at temperature 25°C±1.0.

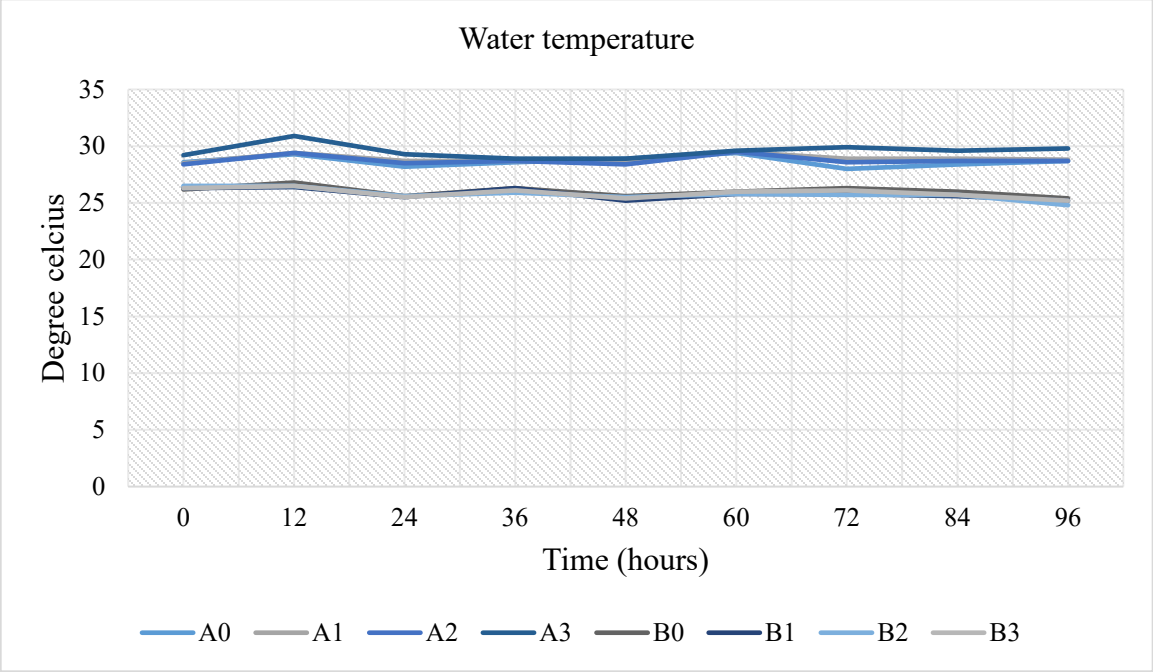

Figure 4: Value of water temperature

The water temperature for group A0, A1, A2, and A3 were able to maintain at the desire temperature which is at 29°C±1.0. This was shown by the average mean temperature for this 4 treatment groups were at 29.0°C with mean range at 28.7°C to 29.6°C. For the group B0, B1, B2, and B3, the mean temperature was at 25.9°C with the mean range for all four groups at 25.7°C to 26.0°C. The desire temperature assigned for group B0, B1, B2, and B3 was 25°C±1.0. The result above shows that the temperature was able to maintain at the desire temperature along the experiment for all groups.

Aquarium/Time (hours)	0	24	48	72	96
A0	0.03	0.03	0.87	1.34	1.86
B0	0.04	0.37	0.75	1.10	1.45
A1	1.09	1.17	1.23	3.5	4.4
A2	1.26	1.59	3.3	4.0	4.4
A3	3.0	3.5	4.5	4.8	5.3
B1	1.13	1.32	2.73	3.5	3.7
B2	1.35	1.51	3.5	3.8	4.5
B3	3.0	3.5	3.6	4.0	4.5

Figure 5: Total ammonia level in water

The total ammonia level for negative control group A0 and B0 was 0.03mg/L NH_3-N and 0.04mg/L NH_3-N. The total ammonia levels for all groups were lower than target total ammonia level for all groups. For group A1, A2, and A3 was at 1.09mg/L NH_3-N, 1.26mg/L NH_3-N and 3.0mg/L NH_3-N where the target concentration was at 1.5mg/L NH_3-N, 2.5mg/L NH_3-N and 3.5mg/L NH_3-N. For group B1, B2, and B3 was at 1.13mg/L NH_3-N, 1.35mg/L NH_3-N and 3.0mg/L NH_3-N where the target concentration was at 1.5mg/L NH_3-N, 2.5mg/L NH_3-N and 3.5mg/L NH_3-N. Yet, the level of the total ammonia increased drastically above the target concentration at the end of the experiment for all treatment groups including the negative control groups. The total ammonia level for group A0 and B0 at the end of the experiment was at 1.86mg/L NH_3-N and 1.45mg/L NH_3-N with the mean ammonia level at 0.83mg/L NH_3-N and 0.74mg/L NH_3-N. The ammonia level for group A1, A2, and A3 was 4.4mg/L NH_3-N, 4.4mg/L NH_3-N and 5.3mg/L NH_3-N at the end of the experiment with the mean ammonia level at 2.28mg/L NH_3-N, 2.91mg/L NH_3-N and 4.22mg/L NH_3-N. The ammonia level for group B1, B2,

and B3 was 3.7mg/L NH_3-N, 4.5mg/L NH_3-N and 4.5mg/L NH_3-N at the end of the experiment with the mean ammonia level at 2.48mg/L NH_3-N, 2.93mg/L NH_3-N and 3.72mg/L NH_3-N. This result show there was increased of ammonia level in the water along the experiment and the finding achieved far higher above the target concentration for all groups.

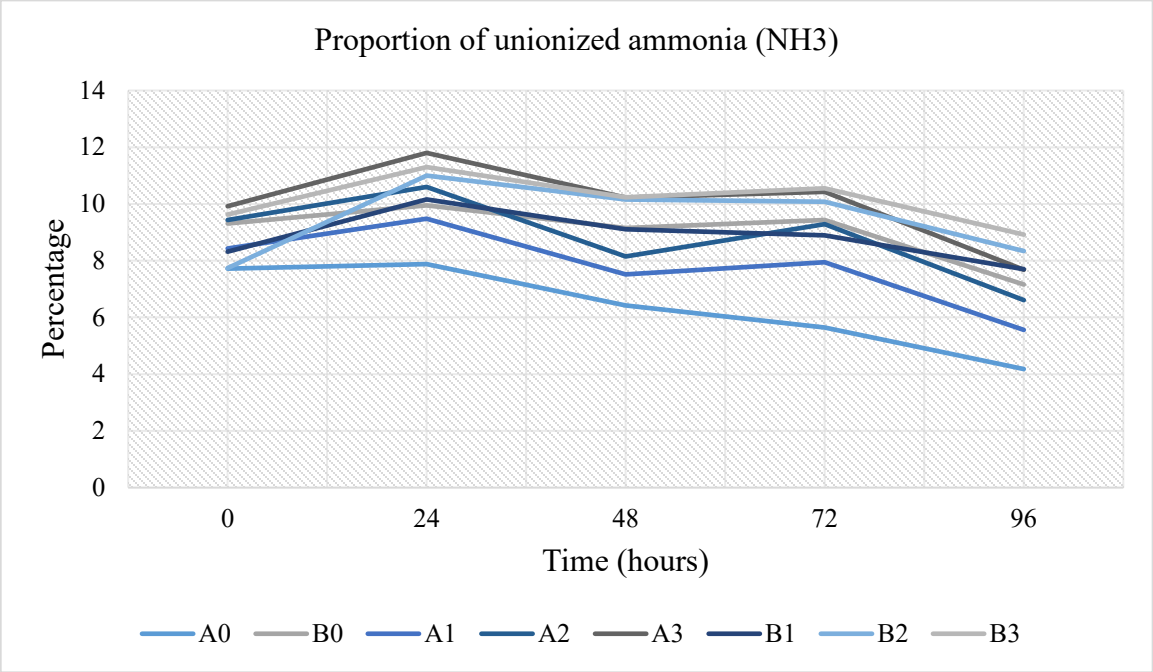

Figure 6: Percentage of unionized ammonia in water

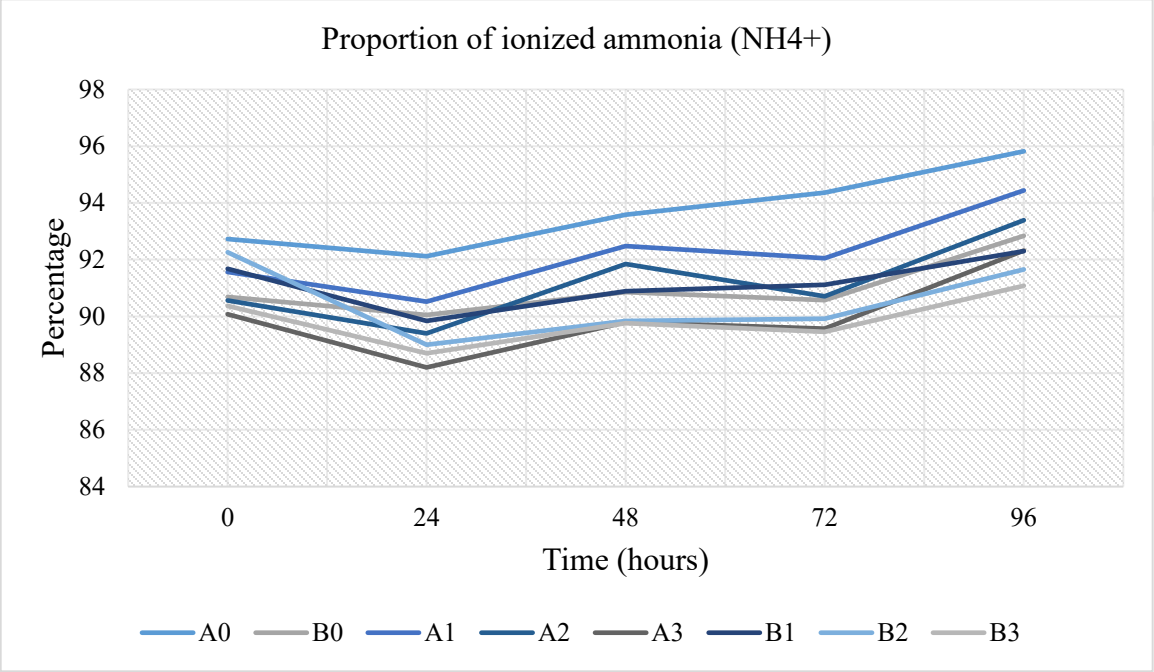

Figure 7: Percentage of ionized ammonia in water

As proportion of unionized and ionized ammonia in the water depends on the pH and temperature of the water (Randall & Tsui, 2002), the unionized and ionized ammonia level in water was calculated according to the pH and the temperature of the water following the table from Emerson *et al.,* (1979). The result showed that the amount of the unionized ammonia in the water reducing following to vice versa to the amount of the ionized ammonia level in the water. The mean proportion of the unionized ammonia for group A1, A2 and A3 was 7.79%, 8.82% and 10.01%. The mean proportion of the unionized ammonia for group B1, B2 and B3 was 8.84%, 9.46% and 10.13%. For the negative group A0 and B0, the mean proportion of the unionized ammonia was 6.37% and 9.0%. For the mean proportion of the ionized ammonia level for group A1 was 92.21%, group A2 was 91.18% and group A3 was 89.99%. For the group B1, B2 and B3,

the mean proportion of ionized ammonia level were 91.16%, 90.54% and 89.87%. The negative group A0 had the mean of 93.63% of the proportion of ionized ammonia where negative group B0 had the mean of 91% of ionized ammonia level.

There was no mortality observed along the experiment for all groups at temperature around 29°C±1.0 and 25°C±1.0 with target ammonia concentration of 1.5, 2.5 and 3.5 mg/L NH_3-N respectively and the negative control groups.

5.0 Discussion

The current study shows that the pattern of the water pH and dissolved oxygen of the group A0 to A3 that being assigned their temperature at around 29°C were lower to be compared to the group B0 to B3 that were assigned their temperature at around 25°C. This was due to the metabolism of the fish in warm temperature is higher to be compared to the lower temperature. In addition, the fish metabolism is slightly higher and associated with higher of fish respiration rate. Thus, we can see the different of dissolved oxygen among the group of different temperature as the fish used more oxygen for respiration via gill. Furthermore, when the fish respiration rate getting higher, the more carbon dioxide being excreted out from the body to the water (Hargreaves & Bruson, 1996). This will then lead to the carbon dioxide being hydrolyze in the water and forming carbonic acid, which will release hydrogen ion that will cause reduction in the water pH.

Eventhough the pH and the dissolved oxygen of the group at temperature around 29°C and group at temperature around 25°C were different, both values are still in

acceptable range for the survival of fish and safe from any disease to be occur. This is according to the water quality set up by the Fish Research Institute following the data compiled from Environmental Protection Agency (1973), Wedemeyer *et al* (1976) and Albaster & Llyod (1982) stated that the acceptable range that suitable for the fish for water pH was at 6.5 to 8.5 and dissolved oxygen was at 5.0 to 8.0 mg/L. Besides that, our water salinity for all the groups was also at the suitable range which was at 15-37 g/L. Thus, we concluded that the constant variables were not affecting our experiment results.

For the water temperature, we assigned the temperature to be at around 29°C or 25°C. According to Moumita De *et al.,* (2016), the optimum temperature for the hybrid grouper TGGG is between 26°C and 30°C. The temperature of the groups that we assigned at 29°C was at the optimum temperature range but the groups at 25°C is slightly lower than the optimum temperature for the hybrid grouper TGGG.

The total ammonia level for all treatment groups were far lower that the target test concentration. This due to the fact that the natural volatilization of the NH_3 gas to the atmosphere which similar from previous finding (Økelsrud & Pearson, 2007). However, after 96 hours, the ammonia levels of the water were significantly higher from the target test concentration. Eventhough, before the experiment started the fishes were fasted and no feed given along the experiment to prevent the buildup of ammonia in the water. In fact, some starved fish also produced some ammonia (Francis-Floyd *et al.*, 2015). In addition, stress fish was resulted in increased in cortisol level, which will then stimulate the glycogenesis and gluconeogenesis as well as increased protein catabolism and ammonia production (Wendelaar Bonga, 1997; Mommsen *et al,* 1999). This can be seen

as the ammonia level slowly increased at day one for all the groups except group A0 which is the negative group for water temperature at around 29°C. This is due to fish is being stress from the added ammonia or the water temperature plus starvation. The ammonia level in group A0 start increased on day two may due to the fish starting felt stress of starvation.

The ammonia in the water was produced by the amino acid catabolism by transdeamination inside the fish. It was explained by Randall & Wright, (1987) that amino acid converted to ammonia in the liver. Transaminase in the liver convert the amino acid and the alpha-ketoglutarate to form glutamate and accompanying by alpha-keto acid. Glutamate will then oxidized to yield ammonia and alpha-ketoglutarate. The cycle then continued again.

Ammonia that being produced then will be excreted into the water via gill as explain by Chasiotis (n.d). The ammonia can be excreted by passive diffusion of ammonia into the water transcellularly or paracellularly or active transport into the gill by replacing K^+ in NA^+-K^+-ATPases and into water by replacing H^+ in H^+-Na^+-Exchanger (HNEs).

From Randall & Tsui (2002), acute toxicity of ammonia is mainly due to its effect on the central nervous system of vertebrates. It will cause acute ammonia intoxication following by convulsions and death. The elevated ammonia in the water will cause toxicity by depolarization effect of NH_4^+ on neurons leading to excessive activation of NMDA receptors. This will not only cause ATP depletion but also lead to influx of Ca^{2+} and Na^+ into the cell. This will cause increasing glutamate, a neurotransmitter, release and

decreasing glutamate synaptic reuptake. In fact, high level of glutamate in the brain will cause excitotoxicity (Schubert & Piasecki, 2001). This will then eventually lead to subsequently neuronal cell death.

In this study, the concentration of ammonia level in the water is as high as 1.45mg/L NH_3-N to 5.30mg/L NH_3-N which was far higher than the acute ammonia toxicity level for 17 seawater species according to USEPA which at 1.86mg/L NH_3-N, there were no mortality was observed in all groups. As stated by Randall & Tsui (2002), acute ammonia toxicity in fish effect on the central nervous system of vertebrae and most of the fish cannot tolerate by the high environmental ammonia level but there are some species of fishes are ammonia tolerant. This is due to the fishes convert the ammonia to less toxic substance to avoid ammonia toxicity.

Many fish detoxify ammonia to glutamine when exposed to elevated environmental ammonia which is less toxic substance. This was started by alpha-ketoglutarate formed glutamate by detoxify ammonia ions with glutamate dehydrogenase. From the glutamate and ammonia ion, glutamine is formed by the enzyme glutamine synthetase (Levi *et al.,* 1974; Ip *et al.,* 2001). An advantage of this strategy is that glutamine can be stored in the tissues and is readily utilized as an oxidative substrate upon return to normal conditions (Randall & Tsui, 2002).

Besides that, fish able to convert ammonia to less toxic urea via ornithine urea cycle. The ammonia will bind with bicarbonate to form carbomyl-phosphate by carbomyl-phosphate synthase. The carbomyl-phosphate will be converting the ornithine into

citruline which will then converting into arginisosuccinate and arginine before becoming urea. Furthermore, the urea that had been excreted to the water by gill or kidney will then be hydrolyze become ammonium carbamate (Taranaki, 1998).

In conjunction, the proportion of unionized and ionized ammonia in the water depends on the water pH and temperature (Randall & Tsui, 2002). We can see from the result that the level of unionized ammonia in the water is decreasing and the level of the ionized ammonia in increasing over the period. A unionized ammonia is more toxic to be compare to ionized ammonia due to the fact that it diffused through the epithelial membrane of aquatic animals more readily than the charged ammonium ions (USEPA, 1999).

Much more interesting that, according to the Director of IPMB of UMS in (2006), hybrid grouper was reported to be higher resilience to environmental variation with better disease resistance and have a survival rate as high as 40%. The hybrid grouper was also being reported for having high tolerance to low water salinity (Liang *et al.,* 2013), tolerance to low water pH (Mustafa *et al.,* 2013) and had wide rang range of salinity tolerance (Amni *et al.,* 2015). Eventhough there were no studies done on the tolerance of the hybrid grouper on acute ammonia toxicity, we had the reason to believe that from the few other researches done on the hybrid grouper showed that this fish had ability to resist with the elevated ammonia level in the water.

6.0 Conclusion

From this study, we can conclude that there is no significant difference on acute ammonia toxicity in juvenile hybrid grouper at different temperature. This is due to the metabolism of the juvenile hybrid grouper had the ability to tolerate with elevated ammonia level in the water. However, more study should be done as there were no mortality observed in this experiment and we did not know the sub-lethal effect of the elevated ammonia level on the fish, and also the effect of the elevated ammonia on the histology of the fish.

7.0 Recommendation Study

Future experiment can be done by making replication for each group in the experiment to achieve the precision result. Then, follow up the experiment on the effect of the histology of the fish brain, gill, kidney and liver. It is also good to be able to calculate the glutamine level of the fish. Finally, make a same experiment but choose the fry hybrid grouper as the sample experiment. This is to detect the different of the acute ammonia toxicity on the fry to be compare to the juvenile fish as the fry is much more sensitive to the ammonia than juvenile fish.

8.0 References

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Appendices

Appendix A: Result of the water temperature

Aquarium/Time (hours)	0	12	24	36	48	60	72	84	96
A0	28.6	29.3	28.2	28.6	28.9	29.4	28.0	28.4	28.7
B0	26.2	26.8	25.6	26.3	25.6	26.0	26.3	26.0	25.4
A1	28.5	29.4	28.7	28.7	28.7	29.5	28.9	28.9	28.8
A2	28.4	29.4	28.5	28.7	28.4	29.5	28.6	28.7	28.7
A3	29.2	30.9	29.3	28.9	28.9	29.6	29.9	29.6	29.8
B1	26.3	26.4	25.5	26.3	25.2	25.8	25.8	25.6	25.2
B2	26.5	26.5	25.6	25.9	25.5	25.8	25.7	25.7	24.8
B3	26.3	26.5	25.5	26.1	25.4	26.0	26.1	25.7	25.2

Appendix B: Result of water pH

Aquarium/Time (hours)	0	12	24	36	48	60	72	84	96
A0	8.03	8.12	8.08	7.70	7.96	7.83	7.93	7.87	7.76
B0	8.22	8.26	8.27	8.15	8.23	8.09	8.22	8.21	8.12
A1	8.10	8.14	8.15	7.88	8.04	7.89	8.06	8.04	7.90
A2	8.16	8.18	8.21	8.04	8.09	7.97	8.14	8.10	7.98
A3	8.16	8.23	8.24	8.09	8.18	8.04	8.16	8.13	8.02
B1	8.16	8.26	8.28	8.17	8.24	8.13	8.21	8.24	8.16
B2	8.12	8.29	8.32	8.22	8.28	8.17	8.27	8.27	8.21
B3	8.23	8.28	8.33	8.23	8.29	8.19	8.28	8.29	8.23

Appendix C: Result of water dissolved oxygen

Aquarium/Time (hours)	0	12	24	36	48	60	72	84	96
A0	4.43	4.95	5.51	5.31	4.92	5.16	5.21	5.31	5.00
B0	5.04	6.08	5.84	6.38	5.47	5.48	5.54	5.31	5.53
A1	5.41	5.31	5.09	5.01	5.29	5.15	5.04	5.31	5.28
A2	4.76	5.24	5.14	5.75	5.31	5.07	5.16	5.02	5.02
A3	4.94	5.20	4.96	5.71	5.01	5.18	5.08	5.34	5.33
B1	5.30	5.95	5.90	6.21	6.01	5.58	6.12	5.57	6.24
B2	5.21	6.00	5.93	6.01	5.98	5.85	6.03	6.02	6.10
B3	5.68	5.64	5.68	5.98	5.88	5.67	5.85	5.71	5.32

Appendix D: Result of water salinity

Aquarium/Time (hours)	0	12	24	36	48	60	72	84	96
A0	29.26	29.73	29.55	29.61	29.77	29.64	29.89	29.87	29.94
B0	29.02	29.82	28.74	29.13	29.01	28.84	28.84	28.87	28.84
A1	29.31	29.66	29.81	29.81	30.02	30.13	30.28	30.36	30.47
A2	29.44	29.55	29.69	29.75	29.87	29.26	30.05	30.12	30.17
A3	29.27	29.88	30.05	30.29	30.37	29.96	30.55	30.72	30.96
B1	29.22	29.62	29.76	29.78	29.82	29.67	29.85	29.90	29.90
B2	29.27	29.15	29.25	29.28	29.30	29.11	29.36	29.37	29.49
B3	29.31	29.08	29.16	29.18	29.21	29.04	29.26	29.27	29.29

Appendix E: Tabulation of percent unionized ammonia

Temperature (°C)/pH	7.70	7.73	7.76	7.80	7.83	7.85	7.87	7.89	7.90	7.93
28.2	3.44	3.68	3.93	4.30	4.59	4.80	5.01	5.23	5.35	5.71
28.4	3.49	3.73	3.99	4.35	4.65	4.86	5.08	5.30	5.42	5.78
28.6	3.54	3.78	4.04	4.41	4.71	4.92	5.14	5.37	5.49	5.86
28.8	3.58	3.83	4.09	4.47	4.77	4.99	5.21	5.44	5.56	5.94
29.0	3.63	3.88	4.15	4.53	4.84	5.05	5.28	5.51	5.64	6.01
29.2	3.68	3.93	4.20	4.59	4.90	5.12	5.35	5.59	5.71	6.09
29.4	3.73	3.98	4.26	4.65	4.97	5.19	5.42	5.66	5.78	6.17
29.6	3.78	4.04	4.31	4.71	5.03	5.25	5.49	5.73	5.86	6.25
29.8	3.83	4.09	4.37	4.77	5.10	5.32	5.56	5.81	5.93	6.33
30.0	3.88	4.14	4.43	4.83	5.16	5.39	5.63	5.89	6.01	6.41

Temperature (°C)/pH	7.96	7.97	7.98	8.00	8.01	8.02	8.03	8.04	8.05	8.06
28.2	6.09	6.23	6.36	6.64	6.79	6.93	7.08	7.24	7.39	7.55
28.4	6.17	6.31	6.44	6.73	6.87	7.02	7.17	7.33	7.49	7.65
28.6	6.25	6.39	6.53	6.82	6.96	7.11	7.27	7.42	7.58	7.75
28.8	6.33	6.47	6.61	6.90	7.05	7.21	7.36	7.52	7.68	7.85
29.0	6.42	6.56	6.70	6.99	7.14	7.30	7.46	7.62	7.78	7.95
29.2	6.50	6.64	6.79	7.08	7.24	7.39	7.55	7.71	7.88	8.05
29.4	6.58	6.73	6.87	7.17	7.33	7.49	7.65	7.81	7.98	8.15
29.6	6.67	6.81	6.96	7.27	7.42	7.58	7.74	7.91	8.08	8.25
29.8	6.75	6.90	7.05	7.36	7.52	7.68	7.84	8.01	8.18	8.36
30.0	6.84	6.99	7.14	7.45	7.61	7.78	7.94	8.11	8.29	8.46

Temperature(°C)/pH	8.07	8.08	8.09	8.10	8.11	8.12	8.13	8.14	8.15
28.2	7.71	7.88	8.05	8.22	8.40	8.57	8.76	8.94	9.13
28.4	7.81	7.98	8.15	8.33	8.50	8.68	8.87	9.06	9.25
28.6	7.91	8.08	8.25	8.43	8.61	8.79	8.98	9.17	9.36
28.8	8.01	8.19	8.36	8.54	8.72	8.90	9.09	9.29	9.48
29.0	8.12	8.29	8.47	8.65	8.83	9.02	9.21	9.40	9.60
29.2	8.22	8.39	8.57	.876	8.94	9.13	9.32	9.32	9.72
29.4	8.32	8.50	8.68	8.87	9.05	9.25	9.44	9.44	9.84
29.6	8.34	8.61	8.79	8.98	9.17	9.36	9.56	9.56	9.96
29.8	8.54	8.72	8.90	9.09	9.28	9.48	9.68	9.68	10.09
30.0	8.64	8.83	9.01	9.20	9.40	9.60	9.80	9.80	10.21

Temperature (°C)/pH	8.16	8.17	8.18	8.19	8.20	8.21	8.22	8.23	8.24
28.2	9.32	9.52	9.72	9.93	10.13	10.3	10.6	10.8	11.0
28.4	9.44	9.64	9.84	10.05	10.26	10.5	10.7	10.9	11.1
28.6	9.56	9.76	9.97	10.17	10.39	10.6	10.8	11.0	11.3
28.8	9.68	9.88	10.09	10.30	10.52	10.7	11.0	11.2	11.4
29.0	9.60	10.01	10.22	10.43	10.65	10.9	11.1	11.3	11.6
29.2	9.92	10.13	10.34	10.56	10.78	11.0	11.2	11.5	11.7
29.4	10.05	10.26	10.46	10.69	10.91	11.1	11.4	11.6	11.8
29.6	10.17	10.38	10.60	10.82	11.05	11.3	11.5	11.7	12.0
29.8	10.30	10.51	10.73	10.95	11.18	11.4	11.6	11.9	12.1
30.0	10.43	10.64	10.86	11.09	11.32	11.6	11.8	12.0	12.3

Temperature(°C)/pH	8.09	8.12	8.13	8.15	8.16	8.17	8.19	8.21	8.22
25.2	6.63	7.07	7.22	7.53	7.70	7.86	8.20	8.56	8.74
25.4	6.71	7.16	7.32	7.63	7.80	7.96	8.31	8.67	8.85
25.6	6.80	7.25	7.41	7.73	7.90	8.07	8.42	8.78	8.96
25.8	6.89	7.35	7.51	7.83	8.00	8.17	8.53	8.89	9.08
26.0	6.98	7.45	7.61	7.94	8.11	8.28	8.64	9.01	9.20
26.2	7.08	7.54	7.71	8.04	8.21	8.39	8.75	9.12	9.31
26.4	7.17	7.64	7.81	8.14	8.32	8.50	8.86	9.24	9.43
26.6	7.26	7.74	7.91	8.25	8.43	8.60	8.97	9.36	9.55
26.8	7.36	7.84	8.01	8.36	8.53	8.72	9.09	9.48	9.68

Temperature (°C)/pH	8.23	8.24	8.26	8.27	8.28	8.29	8.31	8.32	8.33
25.2	8.92	9.11	9.50	9.70	9.90	10.11	10.5	10.8	11.0
25.4	9.04	9.23	9.62	9.82	10.03	10.24	10.7	10.9	11.3
25.6	9.15	9.35	9.75	9.95	10.16	10.37	10.8	11.0	11.4
25.8	9.27	9.47	9.87	10.08	10.29	10.50	10.9	11.2	11.5
26.0	9.39	9.59	10.00	10.20	10.42	10.63	11.1	11.3	11.7
26.2	9.51	9.71	10.12	10.33	10.55	10.77	11.2	11.5	11.8
26.4	9.63	9.83	1025	10.46	10.68	10.90	11.4	11.6	12.0
26.6	9.75	9.96	10.38	10.60	10.82	11.04	11.5	11.7	12.1
26.8	9.88	10.09	10.51	10.73	10.95	11.18	11.6	11.9	12.3

Appendix F: Result of percent unionized ammonia

Aquarium/Time (hours)	0	24	48	72	96
A0	7.27	7.88	6.42	5.64	4.18
B0	9.31	9.95	9.15	9.43	7.16
A1	8.43	9.48	7.52	7.95	5.56
A2	9.44	10.6	8.15	9.29	6.61
A3	9.92	11.8	10.22	10.43	7.68
B1	8.32	10.16	9.11	8.89	7.70
B2	7.74	11.0	10.16	10.08	8.34
B3	9.63	11.3	10.24	10.55	8.92

Appendix G: Result of percent ionized ammonia

Aquarium/Time (hours)	0	24	48	72	96
A0	92.73	92.12	93.58	94.36	95.82
B0	90.69	90.05	90.85	90.57	92.84
A1	91.57	90.52	92.48	92.05	94.44
A2	90.56	89.4	91.85	90.71	93.39
A3	90.08	88.2	89.78	89.57	92.32
B1	91.68	89.84	90.89	91.11	92.3
B2	92.26	89.0	89.84	89.92	91.66
B3	90.37	88.7	89.76	89.45	91.08