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Impact of Elevated Aqueous Ammonia on Liver of Rabbitfish, *Siganus rivulatus.* Histopathological and Ultrastructure Studies

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Abstract: *The world's population is increasing while levels of capture fisheries remain roughly stable with limited potential of increase. The only choice to meet the world's increasing demand of fish is by farming them. Aquaculture is a developing industry and a large proportion of the industry is using aquaculture technology. A consequence of high-density aquaculture is the increased probability to exposure to elevated concentrations of nitrogenous wastes; the principal end product of amino acids metabolism that tends to accumulate and negatively affect the fish. Yet, the effects of ammonia on economically important rabbitfish (Siganus rivulatus) are not well documented. The present study aims to investigated the hypothesis that chronic exposure of fish to increased aqueous ammonia would affect certain parameters including, the structural liver integrity. Fish were treated with total ammonia nitrogen (TA-N) using 0- 12 mg/L. Results obtained indicated that fish reared in TAN- 0 mg/ L had 100% survival and 50% weight increase in 50 days. All fish at TAN- 2 and 4 mg/ L (low dose) died, whilst fish in 6 - 12 mg/ L TA-N survived and grew albeit less than in treatment 0 mg/ L. After 20 days of the chronic exposure, fish was randomly taken from each tank in treatments 0, 2 and 10 mg/ L TA-N and their liver were fixed and stained by routine methods and examined using light and electron microscopy. Results indicated that high dose ammonia (TAN -10) is deleterious to marbled rabbitfish since histologically liver exhibited a disrupted architecture, necrosis, blood congestion and sever ultrastructural alterations in comparison to low dose exposed to TA-N -2 and those exposed to TA-N -0. Lipid droplets and glycogen content were highly accumulated in hepatocytes as a result of exposure to TA-N, this could be due to induced imbalance between fat production and utilization and disturbance in lipid metabolism. Results obtained suggests chronic structural and functional damage, as a result of exposure to ammonia. It is recommended that ammonia levels be measured regularly during production of rabbitfish and be maintained below the threshold concentration used in this study to maximize production.*

Keywords: *Aqueous ammonia, Histology, Liver, Siganus rivulatus, Ultrastructure*

I. Introduction

I.1.The marbled spinefoot rabbitfish:

The marbled spinefoot rabbitfish belong to the family Siganidae that include a group of potentially economically important marine fish in tropical and subtropical areas of Indo-Pacific Ocean and Mediterranean Sea [1]. *Siganus rivulatus* rabbitfish, is of commercial importance in Cyprus [2], Turkey [3], Saudi Arabia [4] and Lebanon [5]. Due to the fact that rabbitfish, can tolerate a large range of salinities from 10 to 50 ppt [6], grow well in temperature between 23 and 36°C [7] predominantly herbivorous feeding habits they possess [8], and fast growth rates, they have recently attracted the attention of aquaculturists [1, 8], as a promising candidate for aquaculture.

I.2. Ammonia toxicity in aquaculture system:

In aquaculture systems the major harmful nitrogen metabolite is ammonia, representing about 70 % of nitrogenous waste excretion in fish [9; 10]. It exists in both ionized ammonium ($NH4^+$) and unionized ammonia (NH_3) forms [11;12]. Important to pointed that, NH_3 is 300–400 times more toxic than $NH4^+$ [13] probably because it is non-polar and readily soluble in lipids [14]. The chemical equilibrium and hence toxicity of ammonia is affected by environmental parameters, for instance temperature, pH, alkalinity, salinity and oxygen concentration [14]. Excessive ambient ammonia impairs NH_3 excretion which causes reduction and cessation of feeding and consequently leads to a net reduction in growth [15]. Ammonia also affects osmoregulation and ionic balance in fish, because NH^{4+} can substitute for K^+ in Na^+-K^+ - ATPase (NKA) and in Na^+/K+ /2Cl^- co-transport [16]. Also, ammonia disturbs the electrochemical gradient of nerve cells causing hyperexcitability and hyperventilation resulting in convulsions and death [17]. It is well known that the primary role of excretion in teleosts is to remove ammonia from the body [18], mainly by diffusion of NH_3 through the gills, exchange transport of NH^{4+} with Na^+ from water and/or conversion to a nontoxic compound as urea [11; 12]. An increase in water ammonia would decrease diffusion of this toxic metabolite out of the fish with deleterious effects culminating in mortality. Consequently, information on fish tolerance to ammonia is essential for accurate

design of aquaculture facilities[13]. Ammonia toxicity data vary from one species to another [14] and are not reported for *S. rivulatus*.

I.3. Liver histopathology

Due to its central role in metabolism, detoxification and its sensibility to pollutants in environment, the liver has been given a particular attention in toxicological investigations in different fish species [19; 20]. Many contaminants, therefore, tend to accumulate in fish liver, exposing the tissues of this organ to relatively higher levels than those experienced by other organs [21]. Authors suggested that histopathology can be used to monitor the effect of aquatic pollutants [22]. Moreover, ultrastructural changes in the liver have been used as biomarkers of toxic chemical in environmental risk assessment [23] since it has long proved to be valuable as a sensitive indicator of toxicants induced injury [24]. Alterations in liver of many species attributed to ammonia water exposure have been reported by Flis, [25](who studied the histopathological change induced in *Cyprinus carpio*), Thurston et al., 1978 (who studied acute toxicity of ammonia and nitrite to cutthroat trout fry) and Soderberg, [26] (who investigated the histopathological effect of unionized ammonia on rainbow trout).

Keeping the aforementioned informations in mind the present study, was initiated to evaluate the effects of hazardous exposure of chronic ammonia water on survival and growth of *S. rivulatus*. Also, to study detailed histological structure of normal liver of *S. rivulatus* because of lack of information about this species. In addition, the experiment aims to investigated the assumption that increased aqueous ammonia concentration probably affect liver integrity by studying cellular and subcelular changes using both light and electron microscopy. Lastly, to evaluate this fish as a useful indicator of ammonia toxicity or not.

II. Material And Methods

II.1. Fish used and Husbandry Conditions

"S. rivulatus" juveniles fish were trapped off in close meshed nets from the beach north of Beirut and transported live in well aerated tanks filled with sea water to the American University of Beirut aquaculture research facility. Acclimatizing of fish to culture circumstances were performed in $1m^3$ tank for 15 days before transfer to the laboratory research system. During acclimatization fish were offered a commercial trout diet twice daily ad libitum. The research system consisted of insulated glass aquaria 50L (60 x 30 x 30.5 cm; L x W x H) filled with filtered, chlorinated followed by dechlorinated sea water. Boyd and Tucker, (27) DPD method was used to measure total chlorine with residuals and found to be below detectable limits. A regenerative air blower and submerged diffusers were used for aeration. The system was housed in an environmentally controlled facility (Table 1). Lighting was set as 12 light:12 dark, dissolved oxygen (DO) concentration was maintained above 5 mg/ L while, salinity was maintained at 35± 1 ppt and water temperature at 25 ±1°C throughout the experiment. It must be pointed out that DO, salinity and temperature were measured daily using a YSI-85 salinometer (YSI, Yellow Springs, OH, USA). In addition, sodium bicarbonate was used to maintained pH between 7.9 & 8.1. Ammonia levels were adjusted using NH_4Cl of a known volume from 10 g /L stock solution NH_4 –N. This solution prepared by dissolving 38.2g of NH_4Cl in 1 L of DI water. Daily, ammonia concentrations in water were assessed [28], phenate and nitrite-N was measured biweekly [29].

II.2. Experimental design

To evaluated the aqueous ammonia concentrations that did not cause any mortality of fish for 96h; Rabbitfish juveniles were size sorted (n =252, 6.1 ± 1.1 g; mean ± SE) and stocked at 12 fish/ tank into 21 tanks. Tanks were randomly assigned into one of 7 concentration: 0, 2, 4, 6, 8, 10 & 12 mg/ L TA-N, with three replicate tanks/ concentration. One extra fish /tank was added in concentration 0, 2 and 10 mg/ L (Table 2). Chronic ammonia exposure was started by adding sufficient amounts of stock solution (10g/L NH_4Cl) to each aquarium to obtain preferred TA-N concentrations in each tank. TA-N in each tank was measured and enough water was replaced daily to maintain the favorite concentrations. About 80% of water in each tank was replaced once weekly with water containing desired concentrations of TA-N for that tank and then the final concentration was adjusted by adding seawater. To reduce microbial conversion of ammonia-N to nitrite-N two drops of Florfenicol were added to each tank. TA-N concentration never varied more than 0.5 mg/ L from the concentration chosen for the particular tank. Fish were offered a 50% crude protein, 20% lipid trout commercial diet (Golden Extruded, Chile) at 4% body weight of control fish daily divided into two feedings at 8:00 and 17:00 hours. Uneaten feed particles were removed during the adjustment of TA-N concentration water. Behavioral and morphological changes were monitored and mortalities recorded. Worthy to mention, that all fish exposed to 2 and 4 mg/ L died by the end of 3rd week of the experiment, but fish in all other concentrations survived. Therefore, the experiment was terminated and restarted with new fish and new seawater. The same event was observed again so the experiment was terminated a 2nd time at the end of the 3rd week. The work was then reinitiated for the 3rd time and when the event was observed yet again, the work was not discontinued. The experiment was terminated after 50 days. All surviving fish were harvested, individual weight, survival and growth recorded.

II.3. Histological and ultrastructure studies:

After 20 days of chronic exposure TA-N, fish was randomly taken from each tank in concentration 0, 2 and 10 mg/ L TA-N and assigned the names; TAN-0, (control), TAN-2 (low dose-group) and TAN-10 (high dose group). Liver of fish from different groups were removed and quickly fixed in both Bouin's solutions for light microscopy examination. Small pieces of fixed specimens were dehydrated in increasing concentrations of ethanol, cleared in xylene, embedded in paraffin wax and finally 5µm thick section were stained with haematoxylin and eosin [30]. For electron microscopy, small blocks of liver ($1mm^3$) from different groups were quickly removed and $_4F_1G$ fixatives (Formalin-Glutaraldehyde buffered mixture) immediately poured on them for 24 h. After two rinses in phosphate buffer, fixed samples were postfixed in 1% buffered osmium tetraoxide for 2 h at 4°C. Samples were washed in phosphate buffer for 30 min and dehydrated in ascending concentrations of ethanol. Liver samples were infiltrated with propylene oxide and embedded in 1:1 Epon-Araldite mixture at 58°C in a gelatin capsule. Polymerization was performed at 65°C for 24 h. Ultrathin sections were sliced using LKB ultratome and mounted on copper grids. Sections were then double stained with uranyl acetate for 2 h and lead citrate for 30 min, and inspected using JEOL 100CX transmission electron microscope. In addition to usual observation and qualitative analysis of the light and electron micrographs, quantitative analysis was established to highlight the histological alterations in liver of experimental groups. Hepatocytes were measured in light micrographs to determine any variations in size among concentration. Dimensions of nuclei, mitochondria, lipid droplets and lysosomes measured using electron micrographs of hepatocytes to determine significant differences among concentration. One way analysis of variance (ANOVA) was performed on data assuming a completely randomized experimental design. Student-Newman-Keuls multiple comparison test was used to determine significant differences among means.

III. Results

III.1. Mortality, and body weight changes as a results of chronic ammonia toxicity

In the present study by the end of the 3rd week of the experiment 90% of fish exposed to 2 & 4 mg/L aqueous ammonia died while, all fish survived in TAN-0 mg/L. Some mortality was observed in treatments 6, 8 &10 mg/L, although no significant difference than control (reared 0 g/L TA-N) detected. Results of the present experiment showed also, that mortality was 50% among fish exposed to 12 mg/L, i.e. significantly greater than all other treatments ($P<0.05$). Also, the final weight of fish in concentration 0 mg/L (used as control) was greater than the final weight of fish in all other treatments. There was no significant differences in final weight among treatments 6, 8, 10 and 12 mg/L, although a descending trend in growth was observed as TA-N concentration increased (Table 2). There was no significant difference in initial condition index (CI_i) and in final condition index (CI_f) of fish in different groups detected.

III.2. Behavioral changes as a results of chronic ammonia toxicity

During the 1st week of ammonia exposure important to point that, fish exposed to 0 - 8 mg/ L TA-N exhibited normal behaviour and appetite. However, fish exposed to 10 and 12 mg/ L TA-N (high dose) had erratic swimming and reacted slowly to feed being dropped into the aquarium and consumed very little. This mode of swimming was all the time followed by some mortality. In contrast, fish at 0 mg/ L TA-N consumed feed particles as soon as they were dropped in water. Furthermore, fish exposed to high TA-N concentrations appeared disturbed. Some fish were found stayed in the back corner of the aquarium, others were lying on their side and morphologically, there was an obvious darkening in skin color of a lot of these fish. In the 2nd and 3rd weeks, it was clearly noticed that, although a few fish periodically exhibited erratic swimming at 6 and 8 mg/ L, fish in the 10 and 12 mg/ L TA-N exhibited signs of disoriented swimming, but started to feed somewhat better than they did during the 1st week of exposure. Some fish exposed to high dose TAN hovered and gulped air at the surface and others were lethargic and lay at the bottom with their mouths wide open. Worthy note that by the end of 2nd week, fish in treatments 2 & 4 mg/ L started dying without exhibiting any abnormal behaviour preceding to death. By the middle of 3rd week more than 80% had deceased. On the 4th and 5th week there was an obvious shift in fish behaviour and appetite in all remaining groups. All fish appeared in good health with an excellent appetite compared with previous weeks. However, during the 6th week and until the end of experiment, most fish in concentration 8, 10 and 12 mg/ L TA-N were lethargic and lost their appetite for food. At harvest, fish in treatments 10 and 12 mg/ L TA-N had slimy skins with high mucous production.

III.3. Histological and histopathological observation of liver of "S. rivulatus" in different groups

Serious examination of light micrographs of livers of *S. rivulatus* fish in TAN-0 (control group) revealed normal architecture (Fig. 1). Liver sections showed masses of hepatocytes not organized into distinct lobules and interrupted by sinusoids. The hepatocytes were polyhedral in shape with a mean diameter of 33.9 ± 1.6 µm (Table 3). Hypatocytes housed spherical central nuclei (with 4.2 ± 0.2 µm mean diameter) surrounded with eosinophilic granular cytoplasm. Blood vessels and bile ducts were randomly found throughout the hepatic

parenchyma. Few melanomacrophage centers were found among hepatic parenchyma, and were usually located in the vicinity of blood vessels and bile ducts. Examination of light micrographs of livers of fish exposed to low dose (Fig.2a), revealed that chronic exposure to TAN-2 resulted in discernible liver damage. The major histological alterations in TAN-2 include, loss of cord-like arrangement; dilation of intercellular space and greater incidence of melanomacrophage centers (Fig. 2b) than those observed in control fish (TAN-0). Moreover, tissue necrosis and blood infiltration into the parenchyma was also detected. Morphmetric measurements based on light micrographs showed that there was a significant increase in size of hepatocytes since, their mean diameter equal 44.5 ± 2 µm, compared to the mean diameter of liver cells of fish in control group (Table 3). On the other hand, light micrographs of fish livers exposed to high dose (TAN- 10) displayed similar but more pronounced histological alterations than those observed in low dose group ((TAN-2). These alterations included loss of cord-like arrangement (Fig. 3a); dilation of intercellular space (Figs.3a, b) and blood congestion (Figs. 3b, c, d); and extensive blood infiltration into the parenchyma (Fig. 3c). One of the most significant observations in liver of fish exposed to high dose was severe hyperplasia of hepatocytes since, the size of hepatocytes was significantly greater (hepatocytes possess mean diameter =50.3 ± 1.7 µm), if compared to hepatocytes of fish in control and low dose ammonia groups (Table. 3).

III.4. Ultrastructural observation of liver of S .rivulatus exposed to aqueous ammonia:

Electron microscopic preparations of liver of fish of control group exposed to 0 mg/L TA-N reveal similar observations to those observed in light preparations as well as numerous fine details. In hepatocytes of control liver nuclei generally had regular envelopes with distinguishable pores (Figs. 4a, b), prominent nucleoli in most of them and heterochromatin often attached to the nucleoli and the inner nuclear membrane. Extended layers of organelles were arranged around the nuclei and consisted of parallel stacks of endoplasmic reticuli (Fig. 4a), numerous spherical mitochondria (with 0.8 ± 0.1 µm mean diameter) and ovoid mitochondria (with 1.6 ± 0.1 µm L x 0.6 ± 0.1 µm W). These mitochondria possess short tubular cristae. Few lysosomes (with 0.8 ± 0.1 µm mean diameter) also observed. Numerous glycogen granules were found accumulated between organelles in the cytoplasm. Lipid droplets (with 0.8 ± 0.1 µm mean diameter) were also found in hepatocytes cytoplasm. TEM micrographs of fish exposed to low dose ammonia (TAN- 2) (Figs. 5a-d) confirmed results obtained by light microscopy and showed significant ultrastrucural variations caused as a result of chronic ammonia exposure. Low dose - treated fish revealed pleomorphic hepatocytes surrounding blood sinusoids congested with blood. Hepatocytes showed altered eccentric nuclei and altered cytoplasmic organelles. Nuclear changes included slightly altered nuclei (with 4.3 ± 0.3 µm L x 3.8 ± 0.3 µm W). Some nuclei showed centric nucleoli (Figs. 5a,b) and displayed heterochromatin dissolution and irregular nuclear envelopes (Fig. 5d). Cytoplasmic alterations included accumulation of huge amounts of various size lipid droplets (Figs. 5a-d). Large lipid droplets (with 2.4 ± 0.1 µm L X 2.0 ± 0.1 µm W) and small size lipid droplets (with 1.6 ± 0.1 µm L X 1.4 ± 0.1 µm W) detected. It is suggested that accumulation of lipid responsible for displacement of nuclei and increase the size of hepatocytes as previously mentioned. Considerable amounts of primary and secondary lysosomes as well as autophagosomes with mean diameter equal 1 ± 0.1 µm were observed. The presence of different stages of lysosomes associated with many altered membranous structures is expected. Also, altered mitochondria (with 1.3 ± 0.1 µm L x 0.6 ± 0.1 µm W) were observed. Some of them depicted partial loss of cristae and others exhibited swelling and vesiculation of cristae (Figs. 5b & d). On the other hand, examination of liver of fish exposed to high dose ammonia (10mg/ L TA-N) showed pleomorphic hepatocytes with altered oval nuclei and altered cytoplasmic organelles (Figs. 6a-d). Nuclear alterations included abnormally shrunk nuclei (Fig.6a) (with 2.9 ± 0.2 µm L x 2.3 ± 0.3 µm W) with centric nucleoli (Fig.6 b). Increased incidence of pyknotic nuclei with decrease in heterochromatin content of other were observed (Figs.6 a-d). Cytoplasmic alterations included accumulation of enormous amounts of glycogen granules and disorganized cisternae of ER (Figs.6c,d). A moderate amount of lipid droplets of various sizes were observed. Large lipid droplets had a mean diameter of 1.7 µm and small lipid droplets had a mean diameter of 0.6 µm detected. Additionally, an increase in numbers of primary and secondary lysosomes (with mean diameter of 0.9 µm ± 0.1 was obvious (Fig. c). Anomalous mitochondria with indistinct membranes and with 0.9 ± 0.2 µm L X 0.5 ± 0.1 µm W were observed. Some of these mitochondria exhibited partial loss and others exhibited complete loss of cristae and others exhibited swelling and vesiculation of cristae observed (Fig. 6c). Several of the above described parameters varied significantly among groups. Nuclear size in hepatocytes from fish exposed to high dose ammonia was significantly less than in thier corresponding control and low dose. Mitochonrial length in hepatocytes from high dose was smaller than in control and low dose but mitochondrial width was similar among treatments. Large lipid droplets in hepatocytes from low dose were significantly larger than lipid droplets in high dose who in turn were significantly larger than lipid droplets in control. Small size and numerous lipid droplets were found in hepatocytes of liver of fish exposed to both dose. lipid were significantly larger in size in low dose than in high dose. Furthermore, lysosome size in low dose hepatocytes was significantly larger than in control.

IV. Discussion

As the human population continues to grow, finding way to feed those people is one of the most significant challenges faced around the sphere. Fish farming is an important way to meet the heavy demands of food and nutrition values needed per human being, which seafood best provides. The convention laboratory toxicity test, in which condition may be controlled within desired limits, as in the case of aquaculture is valuable. The present study aims to investigated the suggestion that increased ammonia in aquaculture system would affect behavior, body weight and liver integrity. It should be pointed out that rabbitfish belonging to Siganids which are algaevorous and should require less protein in their diets and thus should be relatively safer to aquaculture from a nitrogenous waste standpoint were used.

All fish survived in 0 mg/ L ammonia-N treatment and although some mortality occurred in treatments 6, 8 and 10mg/ L TA-N, survival difference was insignificant among treatments. Survival decreased significantly at 12 mg/ L TA-N. Lemarié et al. (31) reported 100% survival of European seabass (*Dicentrarchus labrax*) in controls and up to 0.71 NH_3 mg/L but at greater ammonia concentrations, survival decreased. All fish in treatments 2 and 4 mg/ L TA-N died within 3 weeks of experiment initiation. It was not possible to explain this event, but are quite certain that it is not a random event. The challenge was repeated three times using new fish, yet results did not change. Fish in 0 and 6 mg/ L TA-N survived whilst fish in 2 and 4 mg/ L TA-N died. Worthy to mention that it is not believe that mortality was caused by disruptions to the nervous system since fish did not show any signs of hyperexcitability or lethargy before death. Ammonia might have caused a disruption in osmoregulation, but that would have also affected the fish maintained in greater ammonia concentrations. Possibly, the fish have a defense mechanism to tolerate chronic ammonia exposure, but the threshold for activation of those defenses is >4 mg/ L. Moreover, it was noticed that fish exposed to 0 mg/ L TA-N grew faster than fish in all other treatments. Investigators described the effect of graded ammonia concentration on fish growth [31; 32;33] and found a correlation between ammonia concentration and growth. Most studies report that exposure to ammonia reduce growth of juvenile marine fish, as gilthead sea bream "*S. auratus*" [34], turbot "*Scophthalmus maximus*" [35], and Atlantic cod "*Gadus morhua*" [33]. On the other hand, Lemarie et al., [31] reported that ammonia is stressful for fish even at low concentrations and the effect of ammonia is dose dependant. No significant differences in final weight among treatments 6, 8, 10 and 12 mg/ L, although a diminishing trend in growth was observed as TA-N concentration increased. It must be pointed out that, it is expected that if the experimental period had been allowed to persist longer, growth differences among treatments might have widened and become statistically significant. It is suggested that ammonia induced reduction in growth which is largely attributed to a decrease in food intake with increasing ammonia concentration [33]. In addition, increased energy expenditure by disrupted Na-K-ATPase [15] might also have contributed to reduction in growth.

Erratic behavior and loss of equilibrium in fish exposed to high levels of ammonia are well documented in the literature for tilapia [9; 36], and European seabass juveniles [31]. It was noticed in the present study that *S. rivulatus* exposed to high levels of ammonia (TAN-10) exhibited erratic, unbalanced swimming within 24 h of exposure. Thus, results of the present study are in agreement with previously mentioned results. Disorientation and erratic behavior assumed to be due to the fact that ammonium competes with extracellular potassium on the serosal side of nerve cells, increasing plasma potassium levels and disrupting cellular electrochemical gradients[17]. Furthermore, Daoust and Ferguson [37] reported that fish exposed to excessive ammonia soon developed clinical symptoms suggestive of neurological dysfunction, but subsequently recovered. Recovery notwithstanding, a reduction in erratic behaviour is not enough to deduce a decrease in stress. It is of interest to mention that, *S. rivulatus* can easily withstand the short postprandial spikes in ammonia that occur in recirculating systems, and if biological filters are working well or enough water is being exchanged, TA-N should not affect production. On the other hand, anatomical examination of fish exposed to 10 and 12 mg/ L TA-N (high dose) showed that they had a slimy skin with increased mucus production, a typical stress response in fish [9; 38], yet swam normally. It is believe that hovering, and increased ventilation observed due to elevated ammonia were probably due to gill tissue deterioration resulting in a reduction in oxygen transfer to the blood. Fish would react similarly if exposed to low ambient oxygen. Benli and Kŏksal [9] reported similar results when studying the effects of ammonia toxicity on fish. However, some fish at increased ammonia concentrations appeared to have acclimatized and both feeding behavior and normal swimming resumed. Possibly, as fish got habituated to their environment, stress and thus respiratory needs decreased. In aquaculture systems, ammonia will rarely remain at or above 2 ppm for 3 weeks or more without water being changed or treated. Accordingly, the marbled spinefoot rabbitfish is a good aquaculture candidate from an ammonia tolerance standpoint. From a biological standpoint, further investigations are necessary to determine the reason fish died at 2 and 4 mg /L TA-N, but survived at greater concentrations.

Liver, is an organ of vital importance and it is considered to be the first organ in detoxification of any toxicant. In the present study it was observed that normal liver of *S. rivulatus* comprises of a continuous mass of hepatic cells arranged in cords. There is no clear division of hepatic cells into lobules. The hepatocytes

possess large size and the nucleus is centrally situated. Similar description was reported by Ravindrababu and Neeraja [39], who studied histologically the liver of *Oreochromis mossambicus*. In the present study micrographs depicted noticeable histopathological alterations of liver histology in low dose ammonia challenged *S. rivulatus*, after 21 days, as compared to non-challenged fish i.e. those exposed to 0 mg/ L TA-N. The pathological changes include, both nuclear and cytoplasmic degeneration of hepatocytes. Formation of clear vacuoles in hepatocytes cytoplasm, rupture in blood vessels and appearance of blood cells amongst hepatocytes were clearly noticed in light micrographs. But the severity of pathological changes in liver is evident in high dose ammonia exposed fish. The changes include pushing of nucleus to peripheral region, pyknotic nuclei and fragmentation of nuclear material, disarray of hepatocytes. Thus, fish respond in a quantitative manner to the stress of ammonia in water. Several authors have reported similar alterations in liver tissue resulting from ammonia exposure [40; 41; 42]. Preparations of the present study showed also, that ammonia exposure resulted in tissue necrosis, loss of cord-like arrangement, blood congestion in sinusoids and blood infiltration between parenchyma cells. Similar results were reported by, Flis [25] in carp (*Cyprinus carpio*) and by Smith and Piper [42] in fish after chronic exposure to ammonia. It is of interest to mention that effects of various pollutants on liver histology resemble effects of ammonia observed in the present work. Wahaby and El-Greisy, [43] reported disruption of cordial arrangement in hepatocytes of *S. rivulatus* exposed to pollutants from different effluent sources. Li et al., [44] recorded disruption of hepatic cords companied with intrahepatic hemorrhage. Sinusoid dilation and blood congestion was reported by Thophon et al., (2003) in white seabass as a result of acute and subchronic cadmium exposure. Many studies described alterations in fish livers because of exposure to chemical contaminants [22; 45; 46]. Also, nuclear alterations including abnormally shrunk nuclei, pyknotic nuclei, centric nucleoli, irregular heterochromatin distribution and irregular nuclear envelopes was observed easily in electron microscopy preparations. It was also noticed that there was a significant decrease in nuclear dimensions after high dose (TAN -10) exposure, as compared to control (TAN-0) and low dose (TAN-2). Worthy to mention that these changes probably leads to apoptosis. Several studies on effects of water contaminants on fishes report similar hepatic cell alterations [45; 47; 48]. Accumulation of large lysosomes in hepatocytes of ammonia challenged fish was observed in the present study. Similar effects were reported following exposure of fish to xenobiotics and is a typical response to heavy metal exposure [48]. Lysosomal increase in number constitutes one of the primary phases of intoxication processes and manifestation of cell injury, and reflects the attempt made by the lysosomes to digest toxicants and remove any damaged organelles [48]. Worth note that, data indicated that liver respond to ammonia in a dose dependent manner. Melanomacrophage centers also known as macrophage aggregates, are distinctive groupings of pigment-containing cells within the tissues of heterothermic vertebrates. Agius and Roberts, [49] reported that they are normally situated in the stroma of the haemopoietic tissue of the kidney and spleen in fish, although in amphibians and reptiles, and some fish, they are also found in the liver. They added that an increase in size or frequency in conditions of environmental stress and have been suggested as reliable biomarkers for water quality in terms of both deoxygenation and chemical pollution. In agreement with these results Wahaby and El-Greisy,[43] reported an increase in number of melanomacrophage centers in hepatocytes of *S. rivulatus* exposed to waste pollutants. Similar observation were observed in the present work. In addition, various cellular organelles, especially the mitochondria and ER, were particularly affected by ammonia exposure. Giari et al., [48] reported mitochondrial swelling and cristae regression in hepatic cells, as a result of mercury exposure in sea bass (*Dicentrarchus labrax*). Such observations are similar to observations of anomalous mitochondria in hepatic cells of *S. rivulatus* exposed to ammonia in the present work. Hypertrophy of endoplasmic reticula in hepatocytes was also reported by researchers and is believed to be a hepatic detoxification mechanism [48]. A comparison of symptoms of liver ultrastructure modifications resulting from various pollutant exposures suggests that *S. rivulatus* livers react similarly to exogenous pollutants as they do to the endogenous toxicant ammonia. Although fish have developed mechanisms to avoid toxicity when exposed to elevated levels of environmental ammonia [12], it seems that long term exposure is ultimately deleterious to their livers. One explanation could be that in nature fishes are exposed to pulses of ammonia rather than continuously elevated ammonia concentrations [25,50] and thus have evolved mechanisms that help them survive periodic ammonia increases rather than chronic exposure. Severity of effects of ammonia exposure seems to be correlated with ammonia concentration. Damage to hepatocyte structure was much more severe in high dose TA-N than in low dose TA-N. However, when fish were challenged with pulses of ammonia, damage appeared to be related more to the frequency of exposure rather than ammonia concentration [50].

V. Conclusion

It is concluded that under normal culture conditions (TA- 0) marbled spinefoot rabbitfish (*S. rivulatus*) will survive and grow in contrast, at TAN-2 and TAN-10 concentrations, behavior, growth and survival of *S. rivulatus* are affected. In addition, histopathological alterations in the liver of these fish were observed after 21 days from the start of experiment in response to the deleterious level of ammonia and the extent of damage

varies depending upon the dose of ammonia. It is worth mentioning that the notable recovery in fish behavior which was observed starting from the 4th week of the chronic experiment with normal swimming performance and good appetite suggest a possible acclimation to ammonia. Further studies have to be performed to determine the reason why fish died after chronic exposure to 2 and 4 mg/L TA-N but survived at greater concentrations.

Table 1. Water quality characteristics during chronic ammonia exposure of *S. rivulatus* for 50 days.

Parameters	**Range**	**Mean ± SD**
Dissolved oxygen (mg/l)	6.5-7.2	6.9 ± 0.5
Temperature (ºC)	23.5-27.5	25.5 ± 2.8
Salinity (ppt)	34.5-35.5	35.0 ± 0.5
PH	8.1 -8.3	8.2 ± 0.1

Table 2. Initial weight, final weight, survival, initial condition index and final condition index of *S. rivulatus* exposed 50 days to various TA-N concentrations. Values in the same column sharing the same letter are not significantly different from each other (α = 0.05).

TN-T concentration	**Weight (g)**		**Survival**	**Condition index**	
	Wt_i (g)	Wt_f (g)	S%	CI_i	CI_f
0	6.1	9.4a	100a	1.2	1.23
2	6.1	-2	0.0 c	1.20	-2
4	6.1	-2	0.0 c	1.20	-2
6	6.1	8.7 a,b	97.2a	1.20	1.21
8	6.1	7.5 a,b	792a	1.20	1.13
10	6.1	6.8 b	87.5a	1.20	1.20
12	6.1	6.4 b	50.0b	1.20	1.11
PSE[1]		**0.53**	**7.11**		**0.05**

[1]PSE = Pooled standard error, [2] Fish at 2 and 4 mg/l didn't survive the 50-days test and, thus, values couldn't be estimated.

Table 3. Illustrates morphometric measurements in μm of hepatocyte, nucleus, mitochondria, lipid and lysosomes of hepatocytes of rabbitfish in control and ammonia treated fish (values expressed as means ± standard error). Values in the same column sharing the same letter are not significantly different from each other (α = 0.05).

Groups	**Size**	**Dimensions**			**Size**		**Diameter**
Ammonia - treated	**Hepatocyte**	**Nucleus**	**Mitochondria**		**Lipid droplets**		**Lysosome**
			Spherical	**Oval**	**Large**	**Small**	
TAN- 0mg/L	33.9 ± 1.6 [c]	4.2 ± 0.2 [a] L 4.2 ± 0.2 [a] W	0.8 ± 0.1	1.6 ± 0.1 [a] L 0.6 ± 0.1[a] W	0.8 ± 0.1 [c] L 0.8 ± 0.1 [c] W	–	0.8 ± 0.1 [b]
TAN- 2mg/L "Low dose"	44.5 ± 2.0 [b]	4.3 ± 0.3 [a] L 3.8 ± 0.3 [a] W	–	1.3 ± 0.1 [a] L 0.6 ± 0.1 [a] W	2.4 ± 0.1 [a] L 2.0 ± 0.1 [a] W	1.6 ± 0.1 [a] L 1.4 ±0.1 [a] W	1 ±0.1 [a]
TAN- 10 mg/L "High dose"	50.3 ± 1.7 [a]	2.9 ± 0.2 [b] L 2.3 ± 0.3 [b] W	–	0.9 ± 0.2 [b] L 0.5 ± 0.1 [a] W	1.7 ± 0.2 [b] L 1.7 ± 0.2 [b] W	0.6 ± 0.04 [b] L 0.6 ± 0.04 [b] W	0.9 ± 0.1 [a&b]
Pooled standard error= PSE1	6.1	0.4 L 0.5W	–	0.2 L 0.1W	0.2 L 0.2W	0.1 L 0.1W	0.1

(PSE[1]= Pooled standard error).

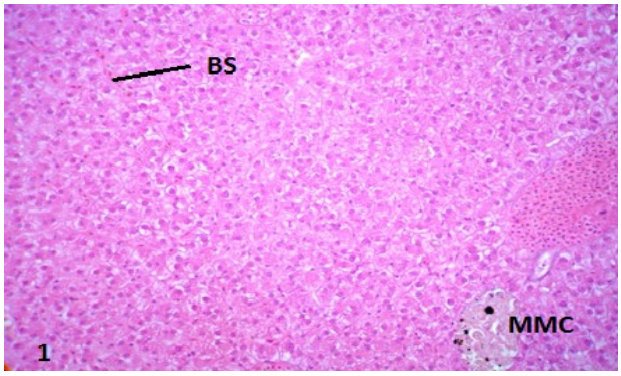
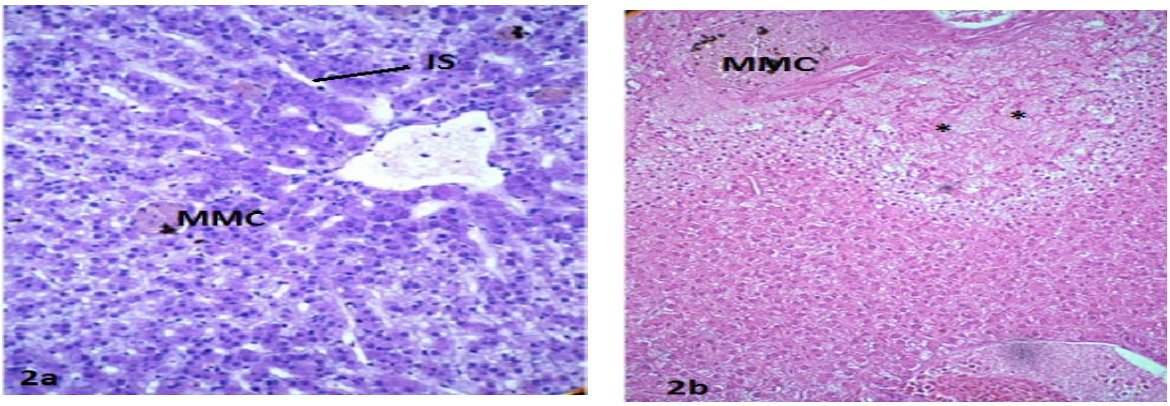
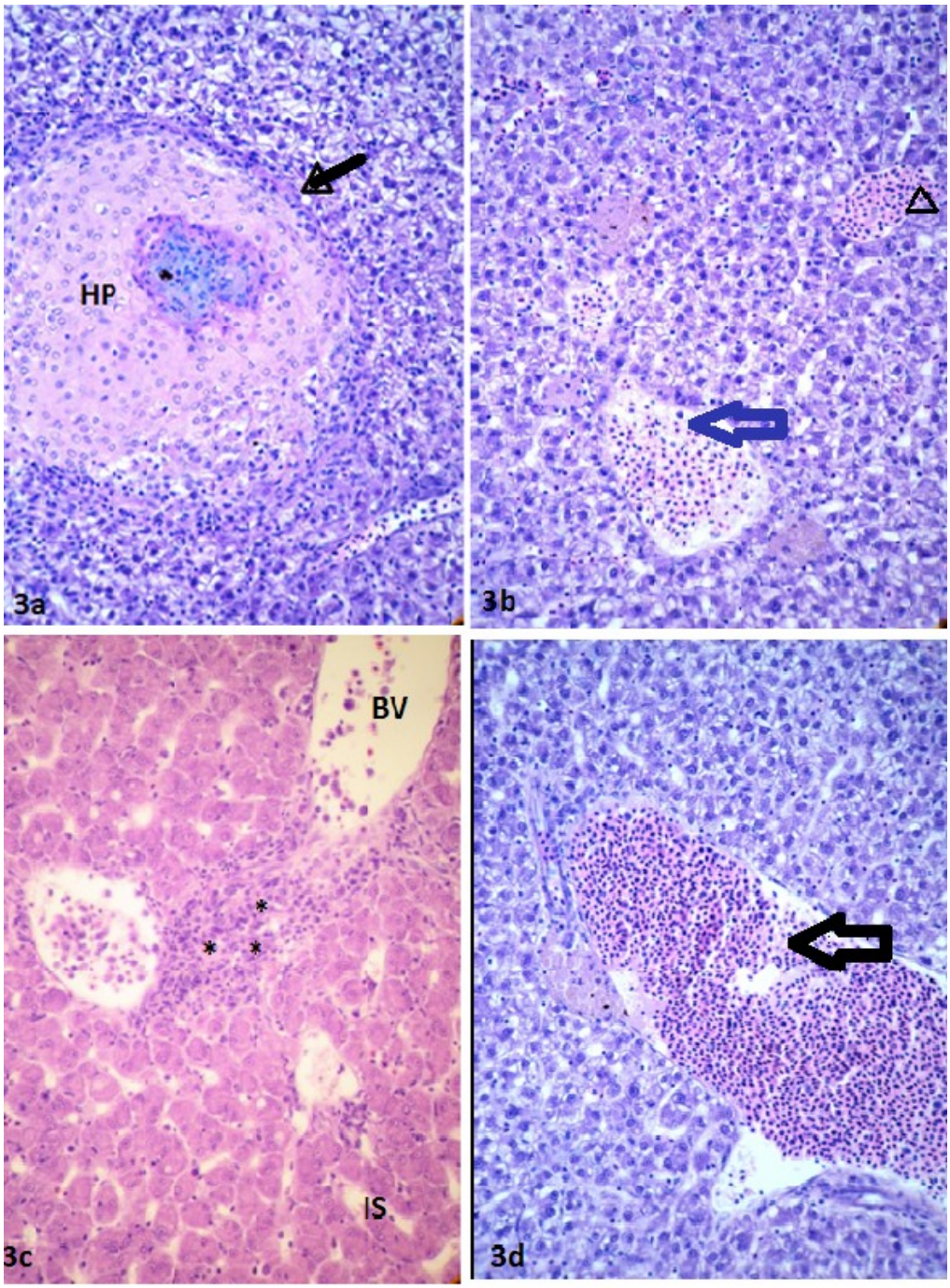


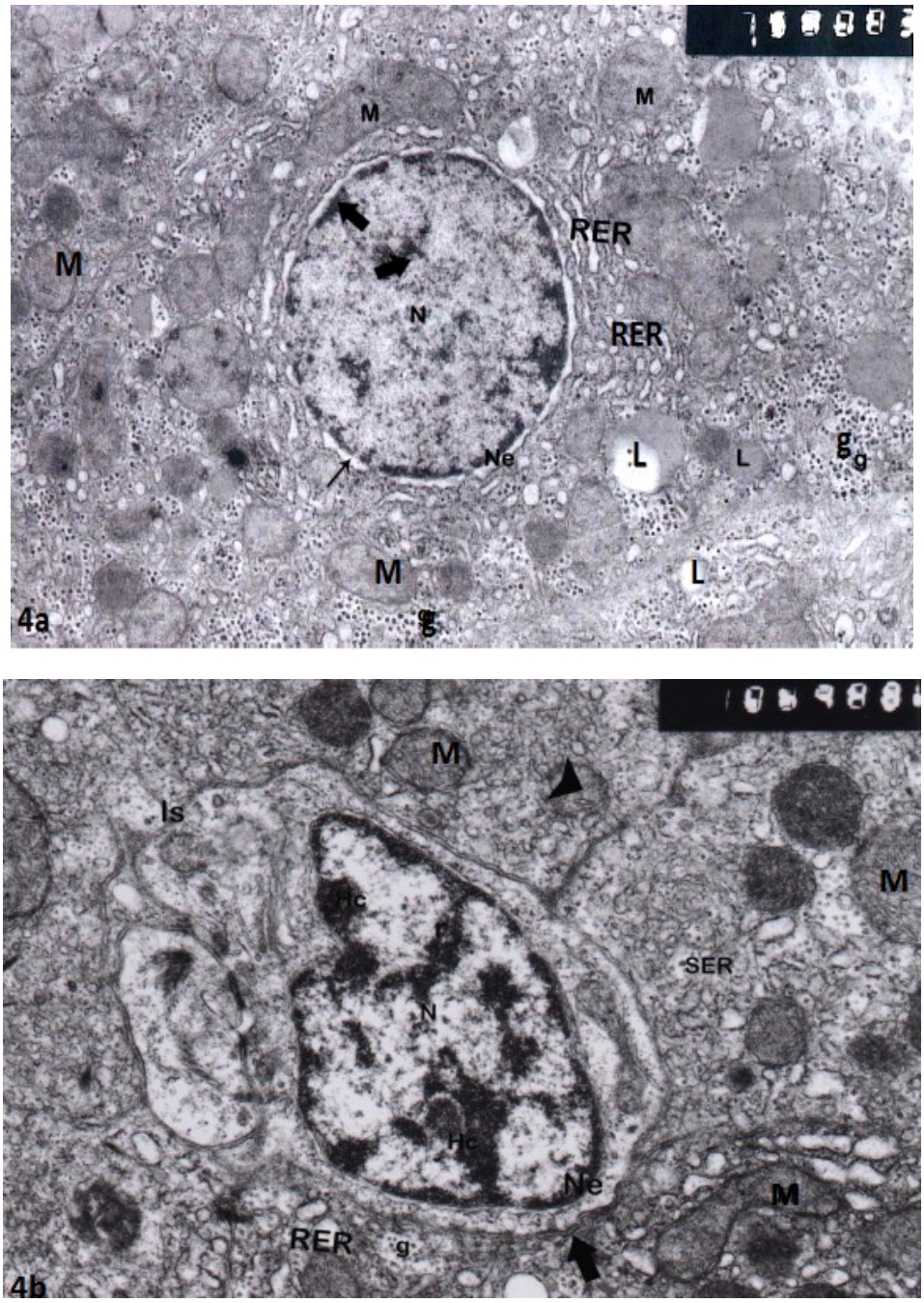
Fig.1. Light micrograph (LM). Formalin fixed- H&E stained preparation. Liver of *S. rivulatus* (0 mg/L TA-N). Showing, normal liver archticture with regular cord-like arrangement of hepatocytes, interrupted by blood sinusoids (BS), normal appearing hepatocytes with central nuclei in most of the parynchmal cells [X400].



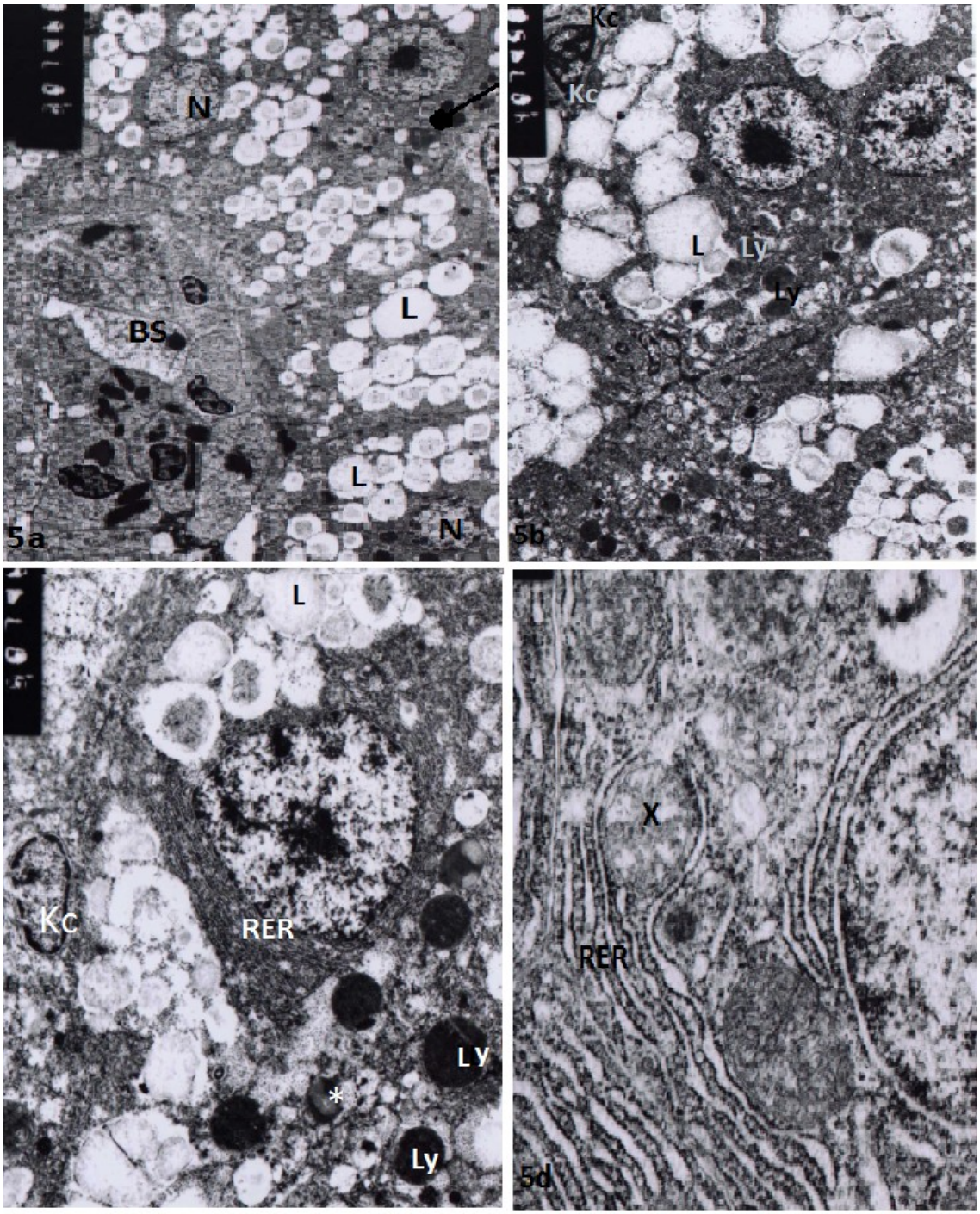
Figs. 2 a&b. LM. Formalin fixed- H&E stained preparations. Liver of *S. rivulatus* corresponding to low ammonia concentration (2mg/L TA-N). a) Showing, apparent alterations as dilation of intercellular space (IS) and higher frequency of melanomacrophage centers (MMC) [X400]. b) Showing loss of cord-like arrangement and normal hepatic organization with evident dilation of intercellular spaces; obvious occurrence of melanomacrophage centers (MMC), marked necrosis:(*) [X 100].



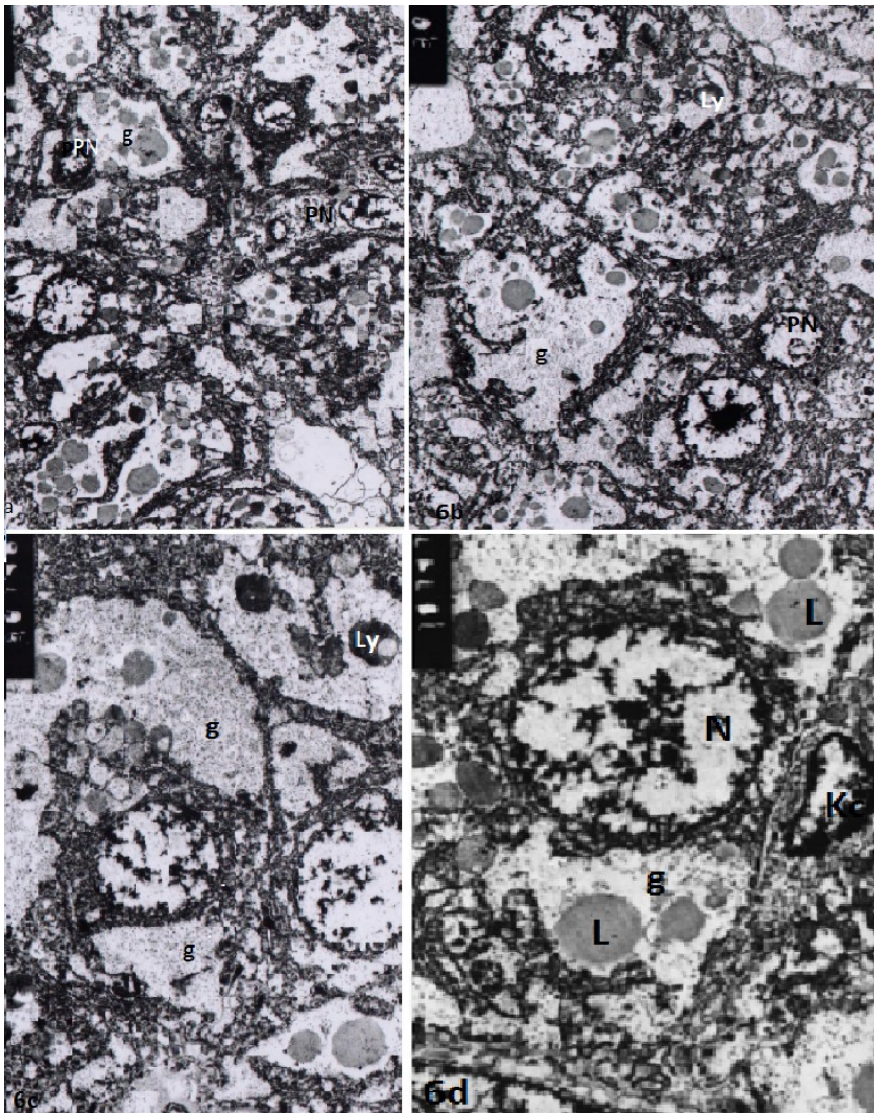
Figs.3. (a-d). LM. Formalin fixed- H&E stained preparations. Liver of S. rivulatus exposed to high dose ammonia(10mg/L TA-N). a) showing, loss of cord-like arrangement, and loss of normal hepatic organization, severe hyperplasia (HP) forming an uncapsulated nodule like structure (arrow) [X400]. b) Showing, aggravated congestion (arrow)[X 400]. c) Revealing, severe blood infiltration into hepatic parenchyma (arrow) and extensive dilation of intercellular spaces (IS). BV: indicates blood vessel [X,400]. d) Revealing, severe blood congestion in blood vessels (arrow) [X400].



Figs. 4a & b. Transmission electron micrographs (TEM). Liver of S. rivulatus exposed to TAN-0. Specimen fixed in 4F1G - OsO4 and stained with uranyl acetate-lead citrate. a) Revealing, polyhedral hepatocyte with distinct boundaries; spherical central nucleus (N), normal heterochromatin distribution); regular nuclear envelope, distinguishable pores (thin arrow), RER completely surrounding the nucleus; mitochondria (M) with different shape, with distinct membranes; lipid droplet (L); high content of glycogen (G) granules accumulated between organelles of the cytoplasm [X 14,000]. b) Showing, nucleus (N) surrounded with parts of hepatocytes that differ in their cytoplasmic densities; dense heterochromatin; more or less regular nuclear envelope, the cytoplasm contains few organelles including mitochondria (M) with dense matrix; cristae in theform of incomplete transverse septa of variable length; distinct outer and inner mitochondrial membrane; RER; SER; glycogen (g) distributed randomly [X 20,000].



Figs. 5 a-d. TEM. Liver of *S. rivulatus* exposed to low ammonia concentration. Specimen fixed in $_4F_1G$ - OsO_4 and stained with uranyl acetate-lead citrate. **a)**Showing, abnormally elongated hepatocytes surrounding blood sinusoids congested with erythrocytes, euchromatic nuclei (N), and dissolution of heterochromatin; the cytoplasm possesses different sizes lipid droplets (L) accumulated near blood sinusoids, lysosomes and autophagosomes (arrow) [X 6˛000]. b) Showing, hepatocyte with central nucleus (N), decreased heterochromatin, with indistinct membranes; RER surrounding mainly the nucleus; large accumulation of different-sized lipid droplets in cytoplasm (L), the presence of dense lysosomes (Ly), he presence of kuppfer cell (Kc)[X 8˛000]. **c)** Showing, hepatocyte with central nucleus (N), RER surrounding the nucleus, large accumulation of different-sized lipid droplets in cytoplasm (L); primary and secondary lysosomes (Ly); oval mitochondria with partial loss of cristae (M). (*): indicates secondary lysosome [X 10˛000]. **d)** Showing, mitochondria with distinct membranes; some exhibit partial or complete loss of cristae (X); others exhibit swelling and vesiculation (M), dilation of RER, numerous ribosomes [X 20˛000].



Figs.6 a- d. TEM. Liver of *S. rivulatus* exposed to low ammonia concentration. Specimen fixed in $_4F_1G$ - OsO_4 and stained with uranyl acetate-lead citrate. **a)** Showing, overall tissue damage and severe hepatic alteration; increased incidence of pyknotic nuclei (PN) altered shrinked nuclei, aggravated glycogen (g) accumulation in cytoplasm, (Kc): indicates kupffer cell [X 6,000**].b)** Showing, severe hepatic alteration including both nucleus and cytoplasm. Some nuclei decrease greatly in size, pyknotic nuclei (Pc) also observed, heterochromatin decrease in most nuclei, the cytoplasm houses numerous glycogen (g) granules, primary and secondary lysosomes (Ly) [X 6,000]. **c)** Demonstrating, severe hepatic alteration; altered nuclei, decrease heterochromatin, disorganized cisternae of RER surrounding the nuclei, aggravated glycogen (g) granules in cytoplasm and secondary lysosomes (Ly) [X 10,000]. **d)** Showing, altered hepatocyte with loss of normal cellular arrangement and extensive glycogen accumulation (g),lipid (L) droplets, anomalous mitochondria (M) with complete loss of cristae. Kc: indicates Kupffer cell with its characteristic triangular nucleus, Is: intercellular space [X 14,000].

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