Effects of Ammonia Concentration on Histological and Physiological Status in Black Seabream (*Acanthopagrus schlegeli*)

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The histological changes of gill, liver, spleen and muscle, and respiration and blood variables and liver glycogen content were examined in black seabream, *Acanthopagrus schlegeli*. Fish were exposed to a high level of total ammonia nitrogen (10.4 mg/l) and recovered from exposure (0.4 ± 0.2 mg/l) in a closed recirculating seawater system. In the process of exposure, mortality was 9%, and hyperplasia, necrosis or inflammation appeared in all tissues except for muscle. Oxygen consumption was decreased by 49%, and red blood cell (RBC) number, hematocrit and hemoglobin concentration were significantly decreased, while plasma glucose contents, activities of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) increased. Liver glycogen content significantly increased from 6.6% to 10.4%. A large amount of hemosiderin was observed in the splenic tissue. During the recovery period, RBC number, hematocrit, hemoglobin concentration, GOT and GPT activities were returned to the normal status. Histological status of liver tissue was returned to the normal, but liver glycogen content was not recovered. During the recovery period, spleen melanin-macrophages temporarily increased, but subsequently decreased to the normal status.

Key words : black seabream, *Acanthopagrus schlegeli*, ammonia concentration, histology, physiological change

Introduction

It is well known that in teleosts, ammonia-N is the main end product of nitrogen metabolism (Foster and Goldstein 1969), and it can lead to death when its concentration is high in the environment (Alabaster et al. 1979; Tomasso et al. 1980; Wickins and Helm 1981; Goldstein et al. 1982; U.S. E.P.A. 1983). Ammonia levels in intensive fish ponds may seldom reach 6 mg/l of total ammonia nitrogen (TAN) (Wajsbrot et al. 1991). Kubota (1977) reported that, at an aquaculture farm of yellowtail in a bay, there was a case where levels increased within 2 hours from 2 mg/l to 20 mg/l of TAN after feed was supplied. Thus fish in aquaculture farms are sometimes in danger of being exposed to the high ammonia concentration.

Especially, when the water exchange was stopped by an unexpected event such as a typhoon or an interruption of electrical power, the ammonia concentration of the rearing water could be increased to a lethal level for fish within only several hours. Therefore, it is very critical that information on the physiological status of fish after exposure to the abruptly elevated levels of ammonia and during the process of recovery. Such information is one of the important concerns for the aquaculturists.

In the case of fresh-water fish, the damage of the gills (Thurston et al. 1978; Mallatt 1985), the suppression of the Krebs cycle (Sousa and Meade 1977) and the reduction of blood Na^+ concentration (Tomasso et al. 1980) resulted in lethal effects on fish by the high level of ammonia. Physiological effects of ammonia on marine fish, however, have been seldom reported (Wajsbrot et al. 1993). Moreover, little is known about the effects of abruptly elevated TAN in an actual aquaculture system.

In the presence of low dissolved oxygen, ammonia is more toxic to fish (Merkens and Downing 1957; Alabaster et al. 1979). Gilthead seabream, *Sparus aurata* also revealed that the toxicity increased at a low oxygen concentration (Wajsbrot et al. 1991). This relationship between ammonia toxicity and dissolved oxygen concentration may suggest that the process of death by ammonia toxicity is related to the disorder of the respiratory system. Furthermore, Heath (1987) indicated that frequently the first system to be affected by pollutants is the respiratory system, because it provides the most extensive interface of a fish with the aquatic environment. He also suggested that the disorder is often due to the failure of respiratory homeostasis. But there is not sufficient experimental evidence on various factors (Tomasso et al. 1980) such as gill damage, osmoregulation defect, blood pH reduction, hemoglobin deficiency, change of oxygen consumption and blood sodium reduction etc.

We induced an abrupt elevation of TAN concentration in a closed recirculating seawater system, and then investigated the changes of the respiratory and blood variables and histological status of fish. Fish were tested after exposure to an elevated level of ammonia concentration and during the recovery period. We used the black seabream, *Acanthopagrus schlegeli*, as it is one of the important species to commercial fish farming in Korea and Japan.

Materials and Methods

The black seabream obtained from the Yochon Hatchery, National Fisheries Research and Development Agency, Korea were maintained in a closed recirculating seawater system in laboratory for at least 1 month prior to use. Average length and weight of black seabream were 12.6 ± 1.8 cm and 32.4 ± 14.0 g, respectively. Fish were held in 280 L tanks at densities that never exceeded 5.4 kg/m^3. Experiments were carried out in triplicates. Fish were fed moist pellets that were made with mixed marine fish meat and commercial feed powder, and they were supplied by 2% of fish weight per day. Fish were not fed for 24 hours prior to the beginning of each test.

NH_4Cl as a chemical reagent was not used to adjust the ammonia concentration, because the organisms exhibited more sensitive at pure seawater than under actual operating conditions (Spotte 1979). Instead the test concentrations were achieved by adjusting the function of the biological filtration in the recirculating seawater system. The recirculating seawater system was stopped for 2 days to induce the abrupt elevating of ammonia concentration, and then the cycling resumed when the fish began to die at 10.4 mg/l. After 3 days of the resumption of water cycling, the TAN concentration in the experimental tanks was reduced to 0.4 ± 0.2 mg/l. Under these conditions, the survivors' recovery process was observed for 20 days. The test conditions were defined as follows: the beginning condition at 1.0 mg/l of TAN (0 day), after exposure for 5 days at 4.0~10.4 mg/l of TAN, the recovery period for 20 days at 0.4 ± 0.2 mg/l of TAN (Fig. 1). TAN

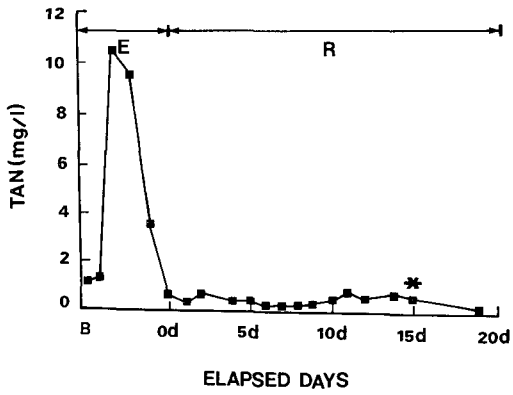

Fig. 1. Changes of total ammonia nitrogen (TAN) concentration through the whole experimental period. *: Complete change of the rearing seawater. B: the beginning of the experiment (1 mg/l TAN). E: exposed period for 5 days to ammonia of high concentration (4.0~10.4 mg/l TAN). R: recovery period. 0d-20d: after recovery for 0, 5, 10, 15, 20 days, respectively (0.4 ± 0.2 mg/l TAN).

concentrations were monitored by the phenol hypochlorite method (APHA 1989) in all tanks, and average concentrations were used. Physiological and histological status were investigated at 5-day intervals (i.e., at the beginning of the experiment, after exposure for 5 days, after recovery for 5 days, 10 days, 15 days, and 20 days, respectively). During the whole experimental period, the rearing water was exchanged by about 5% every 3~7 days for the removal of food remnants and feces, and then the rearing water was fully exchanged on the 15th day of the recovery period. Environmental conditions were sustained as follows: water temperature, 23.9~27.0℃; specific gravity, 1.0251~1.0259; pH, 6.32~7.72. Dissolved oxygen was maintained to 4.35~5.34 mg/l by continuous aeration.

Oxygen consumption was determined by the difference of dissolved oxygen concentration for 20 minutes without aeration within each experimental tank. Dissolved oxygen concentration was measured by Winkler's azide modification method (APHA 1989). Oxygen consumption rate was calculated using Wi and Chang (1976)'s formula. The ventilation rate was determined twice a day by visual measurement. The data of the ventilation rate used an average of 10 fish observed for a minute in each tank.

To determine the effects of environmental ammonia on blood variables, fish were stunned by a blow to the head without the use of anesthetic, and blood was drawn from the caudal vein with a sodium-heparinized 1 ml syringe within 3 minutes. Blood samples were pooled from 4 fish with each replicated respectively at 5-day intervals. Immediately after blood sampling, a blood smear was made to observe the shape and size of the red blood cell (RBC), and RBC number was counted using a hemacytometer, and hematocrit (%) and hemoglobin (g/dl) were also measured. Plasma was separated by centrifugation at 2,000 g for 15 minutes, divided into aliquots, and frozen at −20℃ until further analysis. The plasma aliquots were used for glucose content, the activities of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) as measured by a kit for enzymatic methods (Asan Pharm. Co.).

The effects on the gills, liver, spleen and muscle were studied histologically at 5-day intervals. Tissues were fixed in Bouin's fixative. They were then dehydrated with a graded series of ethanol, embedded in paraffin, and sectioned at 5~6 μm thickness. Sections were stained with hematoxylin and eosin.

Water contents of the liver and muscle were determined by drying to a constant weight at 105℃. Hepatosomatic index (HSI) was calculated as the ratio of liver weight to fish weight. Liver glycogen content was estimated by using an anthrone reagent (Carrol et al. 1956).

The statistical differences of all variables according to the change of the TAN concentration were determined by analysis of variance, followed by Duncan's multiple range test ($P<0.05$) (Zar 1984).

Results

1. Fish behavior and mortality

By increasing the ammonia concentration, the fish showed abnormal behaviors such as rapid swimming and lying at the bottom of the tank. Especially when the ammonia concentration was sustained at a range of 9.5~10.4 mg/l of TAN, surface gulping and death of fish occurred. The total number of died fish was 21 individuals corresponding to 9% of the total fish. However, after the ammonia concentration was lowered to less than 3.5 mg/l, the death did not continue. During the recovery period, fish resumed its feeding schedule; nevertheless, there was no change in body weight.

2. Respiration

Changes of respiration during the exposure and recovery periods are shown in Table 1. The ventilation rate was not significantly different after exposure. For the recovery period, the occurrence of ventilation

Table 1. Respiratory frequency and oxygen consumption rate after exposure to an abruptly elevated level of TAN and during the recovery period in black seabream (replicates=3)

Test conditions	Respiratory frequency (times/min)	Oxygen consumption (mg/kg/hr)
Beginning	169.8 ± 19.07[a]	555.0 ± 275.9[b]
Exposed	166.5 ± 25.88[a]	271.2 ± 103.7[a]
Recovery 5d*	140.4 ± 6.97[b]	293.3 ± 36.0[a]
Recovery 10d*	145.4 ± 9.74[b]	666.8 ± 275.5[b]
Recovery 15d*	143.7 ± 14.26[b]	674.4 ± 399.8[b]
Recovery 20d*	143.7 ± 14.26[b]	304.0 ± 111.1[a]

Beginning: the beginning condition (1 mg/l TAN). Exposed: exposed condition for 5 days to a high level of ammonia (4.0~10.4 mg/l TAN). *5d-20d: recovered condition for 5, 10, 15, 20 days, respectively (0.4 ± 0.2 mg/l TAN). Values in the same column having different alphabetic superscripts are significantly different ($P<0.05$, Duncan's multiple range test).

Table 2. Parameters of red blood cells after exposure to an abruptly elevated level of TAN and during the recovery period in black seabream (replicates=3)

Test conditions	LD (μm)	SD (μm)	Counts ($\times 10^4$ cell/mm^3)	Hematocrit (%)	Hemoglobin (g/dl)
Beginning	10.1 ± 0.3[a]	6.8 ± 0.3[a]	115 ± 16[a]	26.9 ± 1.0[a]	14.7 ± 4.5
Exposed	10.1 ± 0.3[a]	6.8 ± 0.3[a]	46 ± 36[b]	8.0 ± 4.9	9.8 ± 1.8[bc]
Recovery 5d*	9.4 ± 0.3[b]	6.6 ± 0.3[a]	70 ± 12[ab]	31.0 ± 3.1[b]	5.6 ± 1.0[a]
Recovery 10d*	9.6 ± 0.3[b]	7.0 ± 0.2[a]	222 ± 50[ab]	29.9 ± 2.7[ab]	7.2 ± 1.1[ab]
Recovery 15d*	9.5 ± 0.3[b]	6.9 ± 0.2[a]	282 ± 48[c]	35.4 ± 0.8[c]	6.5 ± 0.8[a]
Recovery 20d*	9.9 ± 0.2[ab]	7.0 ± 0.3[a]	114 ± 14[a]	35.2 ± 1.4[c]	10.5 ± 1.1[c]

Beginning: the beginning condition (1 mg/l TAN). Exposed: exposed condition for 5 days to a high level of ammonia (4.0~10.4 mg/l TAN). *5d-20d: recovered condition for 5, 10, 15, 20 days, respectively (0.4 ± 0.2 mg/l TAN). Values in the same column having different alphabetic superscripts are significantly different ($P<0.05$, Duncan's multiple range test). LD: long diameter, SD: short diameter, Hb: Hemoglobin, Ht: Hematocrit.

was reduced as compared to the levels prior to exposure ($P<0.05$). Oxygen consumption significantly decreased from 555 ml/kg/hr to 271 ml/kg/hr immediately after exposure ($P<0.05$), and then greatly increased by 667 ml/kg/hr during the recovery period ($P<0.05$), and was reduced again by 304 ml/kg/hr at the end of recovery ($P<0.05$).

3. Blood variables

Changes of the RBC parameters during exposure and recovery are shown in Table 2. Long diameters of RBC did not show a significant difference immediately after exposure; however, during the recovery period they became significantly smaller than that of the normal ($P<0.05$). There were no significant changes in the short diameters of the RBC through the whole period of the experiment. Just after exposure, RBC number was greatly reduced from 115×10^4 cells/mm^3 to 46×10^4 cells/mm^3 ($P<0.05$). Henceforth it increased largely by 282×10^4 cells/mm^3 ($P<0.05$) and subsequently was restored to the initial level. The hematocrit varied similarly to the RBC counts. Hemoglobin concentration was significantly reduced from 14.7 ± 4.5 g/dl to 9.8 ± 1.8 g/dl immediately after exposure, then sustained this level, but finally increased by 10.5 ± 1.1 g/dl ($P<0.05$).

Regardless of the recovery period, the plasma glucose concentration continuously increased ($P<0.05$)

Table 3. Plasma glucose contents and the activities of GOT and GPT after exposure to an abruptly elevated level of TAN and during the recovery period in black seabream (replicates=3)

Test conditions	Glucose (mg/dl)	GOT (Karmen unit)	GPT (Karmen unit)
Beginning	72.17 ± 0.38^{ab}	27.90 ± 5.40^{a}	20.08 ± 7.14^{ab}
Exposed	43.99 ± 8.23^{c}	72.58 ± 24.15^{b}	32.27 ± 14.94^{bc}
Recovery 5d*	60.40 ± 14.32^{abc}	79.00 ± 30.23^{b}	35.47 ± 13.89^{c}
Recovery 10d*	61.42 ± 2.48^{abc}	74.27 ± 27.32^{b}	27.33 ± 1.25^{bc}
Recovery 15d*	82.41 ± 6.59^{a}	36.53 ± 6.04^{a}	20.50 ± 2.04^{ab}
Recovery 20d*	57.61 ± 6.67^{bc}	16.00 ± 4.00^{a}	7.00 ± 4.08^{a}

Beginning: the beginning condition (1 mg/l TAN). Exposed: exposed condition for 5 days to a high level of ammonia (4.0~10.4 mg/l TAN). *5d-20d: recovered condition for 5, 10, 15, 20 days, respectively (0.4 ± 0.2 mg/l TAN). Values in the same column having different alphabetic superscripts are significantly different ($P<0.05$, Duncan's multiple range test). GOT: glutamate-oxaloacetate transaminase, GPT: glutamate-pyruvate transaminase.

Table 4. Liver glycogen, liver water, HSI and muscle water content after exposure to an abruptly elevated level of TAN and during the recovery period in black seabream (replicates=3)

Test conditions	Glycogen (% of LWT)	Liver water (%)	HSI	Muscle water (%)
Beginning	6.66 ± 2.65^{ab}	67.63 ± 6.46	1.61 ± 0.50	76.11 ± 3.06
Exposed	10.39 ± 4.09^{cd}	66.47 ± 5.01	1.51 ± 0.67	74.51 ± 2.90
Recovery 5d*	9.16 ± 4.66^{bc}	68.77 ± 3.63	1.19 ± 0.28	76.97 ± 2.26
Recovery 10d*	9.15 ± 3.10^{bc}	66.04 ± 8.18	1.42 ± 0.18	76.44 ± 2.15
Recovery 15d*	12.41 ± 8.62^{d}	65.13 ± 2.98	1.32 ± 0.24	76.29 ± 1.45
Recovery 20d*	5.52 ± 0.16^{a}	65.42 ± 6.02	1.49 ± 0.33	77.72 ± 0.49

Beginning: the beginning condition (1 mg/l TAN). Exposed: exposed condition for 5 days to a high level of ammonia (4.0~10.4 mg/l TAN). *5d-20d: recovered condition for 5, 10, 15, 20 days, respectively (0.4 ± 0.2 mg/l TAN). Values in the same column having different alphabetic superscripts are significantly different ($P<0.05$, Duncan's multiple range test). HSI: hepatosomatic index, LWT: liver weight.

and when the rearing water was completely exchanged, it returned to the initial level (Table 3). The activities of GOT and GPT exhibited a similar tendency, and those values increased also during the recovery period, but from 10th day of the recovery sharply decreased ($P<0.05$) (Table 3).

4. Liver glycogen content, liver and muscle water content and HSI

As shown in Table 4, liver glycogen (% of liver wet weight) increased significantly from 6.6% to 10.4% ($P<0.05$). It thereafter sustained this level, and finally returned to the initial level. The water content of liver and muscle and HSI did not change significantly by exposure to ammonia at high level.

5. Histology

After being exposed to ammonia at a high level, the gill tissues revealed edema, hyperplasia, fusion, and necrosis of the gill lamella as compared with the normal status (Fig. 2a & 2b). The damaged gills were not restored during the recovery period, 0.4 ± 0.2 mg/l of TAN, until 15th day when the hyperplasia and fusion were reduced. The liver tissue showed necrosis, inflammation and atrophy as compared with the normal status (Fig. 2c & 2d). The damaged liver tissue was not restored during the recovery period, 0.4 ± 0.2 mg /l of TAN, until 10th day of the recovery. The sple-

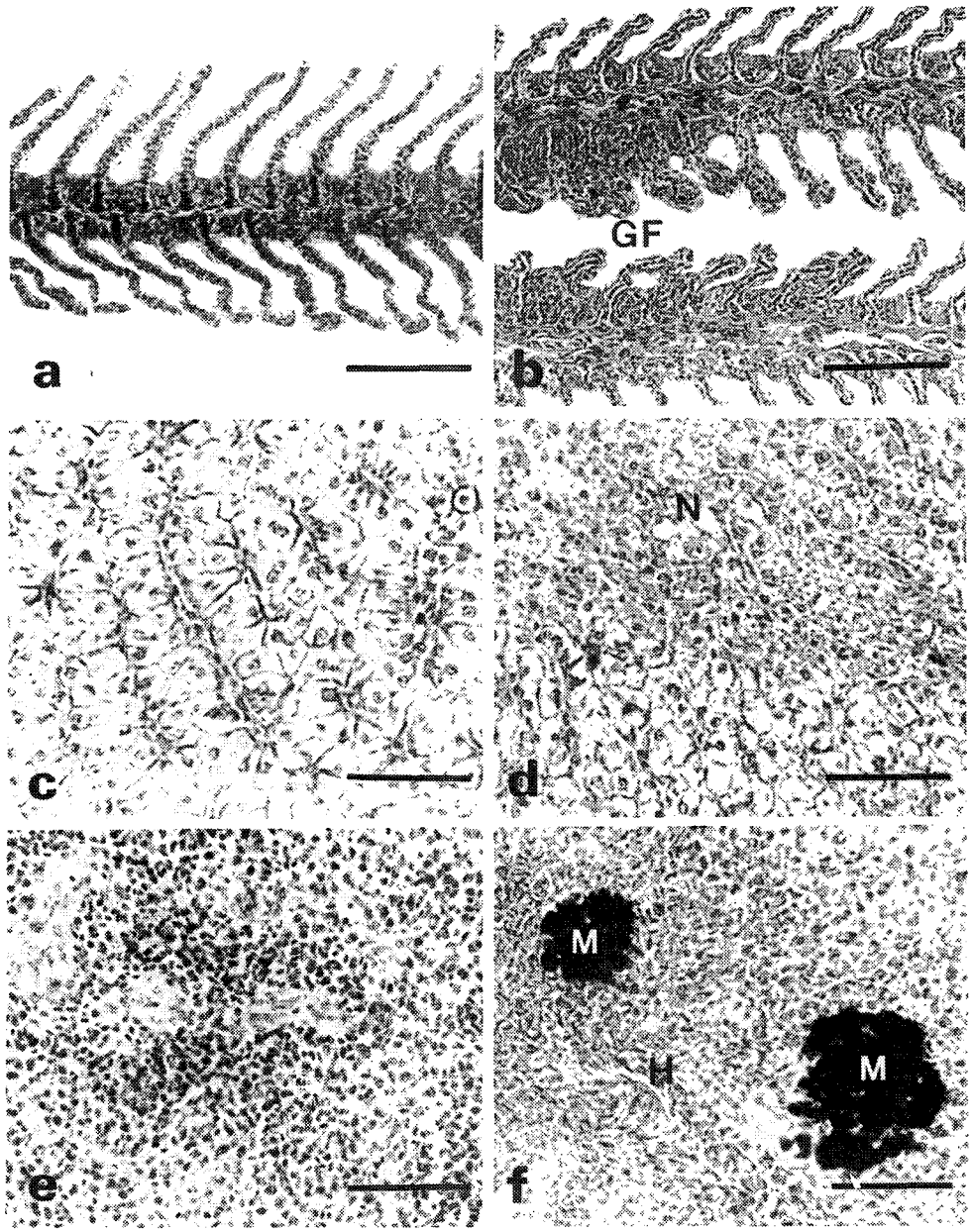

Fig. 2. Histological changes of the gills (a and b, scale bar=100 μm), liver (c and d, scale bar=50 μm) and spleen (e and f, scale bar=50 μm). a, c and e: the beginning conditions; b, d and f: exposed conditions for 5 days to a high level of ammonia. GF: gill fusion. N: necrosis. H: hemosiderin. M: melanin-macrophage.

nic tissue showed a severe deposition of hemosiderin as well as an increase in number and size of the melanin-macrophage by exposure to ammonia (Fig. 2e & 2f). During the time of tissue recovery, the deposition of hemosiderin gradually disappeared, but number and size of the melanin-macrophage temporally increased. However, at the end of the recovery, most of the tissues exhibited a normal state. The muscle

tissue did not show apparent changes after exposure to the high level of ammonia.

Discussion

When the ammonia concentration was raised to 10.4 mg/l of TAN, fish moved to the water surface and death appeared. Thus high ammonia concentration revealed a fatal effect on black seabream. Lethal concentration of ammonia in fish has been suggested at 3.0~4.9 mg/l of TAN for juvenile red seabream, *Pagrus major* (Kido et al. 1991), 4.2~12.0 mg/l of TAN for rainbow trout, *Oncorhynchus mykiss* (Thurston et al. 1986) and 8.2~13.0 mg/l of TAN for the gilthead seabream, *S. aurata* (Wajsbrot et al. 1993), respectively. On the other hand, Wickins and Helm (1981) demonstrated that ammonia concentration for culture of marine animals has to sustain less than 17.5 mg/l of TAN. Black seabream, however, began to die at 10.4 mg/l of TAN in the present study. These discrepancies probably may be due to the differences of fish size, species, or water quality.

Recently, Wajsbrot et al. (1991) pointed out that the less dissolved oxygen present, the more toxic ammonia is to gilthead seabream. In fresh-water fish, these results were reported (Alabaster et al. 1979; Meade 1985). It suggests that there is a close relationship between ammonia concentration and respiration. In the present study, results showed that decreased oxygen consumption and severely damaged gills just after exposure to high ammonia concentration indirectly supported such a relationship.

During the recovery period sustaining 0.4 ± 0.2 mg /l of TAN, fish showed low oxygen consumption at an early period, perhaps resulting from remaining severe gill damage. In the process of the restoration of the damaged gill tissue, oxygen consumption was greatly increased. It could be explained by that the requirement of metabolic energy would be increased for the recovery of fish.

In the present study, the reduction of RBC counts and hematocrit caused hypo-chromic anemia by decreasing hemoglobin concentration. The results suggest that respiratory efficiency had decreased.

In general, it has been well known that plasma glucose content could be increased by the environmental stress (Brown et al. 1984). This coincided with the results of the present study, although the increasing rate of plasma glucose content was lower than expected. It was difficult to compare the results of previous studies with those of the present study, because those were mainly on fresh-water fish or did not investigate effects caused by ammonia. However, as the general mechanism of the plasma glucose elevation related to stress in fresh-water fish considerably coincided with the results of the present study, it could be similarly applied to marine fish.

The increase of GOT and GPT activities corresponded to damage of the liver, spleen and muscle etc. Wajsbrot et al. (1993) studied chronic ammonia toxicity with exposure of a concentration of 8~15 mg/l of TAN for 20 days in marine fish, gilthead seabream, and the results showed the occurrence of necrosis and atrophy in the liver tissue, as well as reduced growth and survival rate. These results coincide with our study.

The elevations of plasma glucose and liver glycogen were not likely to be metabolized body fat. The high levels of plasma glucose and liver glycogen were probably derived from the absorbed food materials which should have been used for somatic growth (Tam et al. 1987). In the present study, during the recovery period, there was practically no change of body weight despite no reluctance of the fish to feed.

It has been well known that black seabream is a species in which the hematopoesis function of the spleen is more active than the kidney, as compared with others (Kim 1989). Hibiya (1982) demonstrated that the apparent deposition of hemosiderin may appear as hemolytic anemia. The observed apparent deposition of hemosiderin in splenic tissue implied that serious

hemolysis occurred. If it is true, it can support the claim that RBC counts, hematocrit and hemoglobin concentration are greatly reduced in black seabream exposed to ammonia.

Splenic tissue especially reflected the recovery status. During the early recovery period, increased size or number of melanin-macrophages may contribute to phagocytosis-destroyed destructed RBC. Thereafter, the decreased size or number of melanin-macrophages may suggest that fish recovered to normal status. Changes of muscle tissue were not apparent, but if the exposure period was longer, some damage would appear.

Judging from the above discussion, the process of physiological changes leading to the death of black seabream under the condition of high ammonia concentration in this study was suspected as follows: gill necrosis→hemolysis→deficiency of hemoglobin→reduced oxygen consumption→disturbance of respiration→suffocation→death. Thus we considered that death by ammonia toxicity was primarily caused by a functional disorder of the circulatory and respiratory systems.

In conclusion, when water exchange was stopped by an unexpected event such as a typhoon or an interruption of electric power in the fish farm, the ammonia concentration of rearing water was rapidly elevated, and mass mortality of the fish occurred, or survivors would be impaired by severe physiological stress. Therefore, in the land-based culture farms for marine fish, which rear fish with high density, scientific provisions, such as a closed recirculating water system (Saeki 1958; Sano 1987; Chang and Yoo 1988) should be prepared to prevent the elevation of ammonia concentration. During the recovery period, it is likely better to enhance aeration, because the oxygen requirement increases for physiological recovery of fish.

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