DEVELOPMENT OF THE IMMUNE SYSTEM OF PREVIOUSLY STARVED NILE TILAPIA (*Oreochromis niloticus* L.)

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Abstract

When young fishes are exposed to stressful conditions, such as starvation, the development of their immune system can be greatly affected. In this study, the effects of delayed initial feeding on the development of the immune system of *Oreochromis niloticus* were examined using histological techniques. Results showed a general decrease in the overall size of the fishes as the number of days of delayed initial feeding increased. The thymus and spleen also showed a decrease in size when the initial feeding of the fish was delayed for 4 days. The gut-associated lymphatic tissue (GALT) also showed a delay in development. Thus, it can be said that delaying the initial feeding of the fish caused a subsequent delay in the development of the organs of the immune system, specifically, the thymus and spleen, making the fish more susceptible to diseases.

Introduction

O. niloticus matures as early as two months of age (Herrera 1996). Being a mouth brooding fish, the eggs are incubated and hatched inside the mouth of the mother. The hatched fry would only be released when they have absorbed the reserved yolk and are able to swim. Nevertheless, in the presence of a predator, the fry would return to the mouth of their mother, seeking for cover and protection. In the wild environment, where the fry is exposed at once to danger upon its release from the mother's mouth, the mother would sometimes instinctively delay the release of her broods preventing the predators from eating or attacking her broods. This practice would ensure that the fry would survive; however, it also poses a problem for the fry since this would result in the delay of first feeding or inability to feed efficiently.

The delay of first feeding could also occur in hatchery conditions when the nursery containers are insufficient or unavailable. In these situations, the first feeding fry are held in hatching jars until the containers become available or until the containers are prepared. First feeding may also be delayed when the farmer unconsciously fail to check that the fry have already absorbed their reserved yolk and are ready to eat exogenous food.

This study aimed to determine if delayed first feeding has effects on the development of the immune system of *O. niloticus*.

Materials and methods

Collection and rearing of fish

Brood eggs of different paternal sources were collected from females crossed to YY males and incubated separately until hatched. Careful monitoring of the hatched fry was done to determine when they have already absorbed their reserved yolk and were ready to eat exogenous food. The broods that were selected to be used for the experiment were those that had at least a thousand fry.

The different broods were divided into 6 groups consisting of 200 fry. The different groups within the brood were given their first food at different times. Group 1 was the control group and was fed immediately after yolk absorption. Group 2 was fed 2 days after yolk absorption; group 3 was fed 4 days after yolk absorption. Group 4 was fed 6 days after yolk absorption, and finally group 5 was fed 8 days after yolk absorption.

The average weight of the fry was determined after the absorption of the yolk sac and each group was placed separately in plastic containers with water and was provided with aeration. Whenever a group was scheduled to be fed, the fry was stocked in a $1m^3$ meshed "hapa" installed in an earthen pond that was previously fertilized to enhance the growth of natural food. Supplemental food using commercial fry mass was given to stimulate actual hatchery practice in addition to the natural food.

The grow-out stage was carried out for four months. Prior to stocking, the fry was marked by clipping one of the fins to prevent contamination. Also, the initial size and number were determined prior to stocking. Monthly sampling was done to determine the growth curb and adjustment of feeding rate. After four months, the final individual weight and length were determined. Representative samples from each group were examined and physiological differences among the groups were also determined.

Histology

Dehydration of previously fixed samples was done in a series of ethyl alcohol concentrations, followed by clearing in xylene and infiltration and embedding in paraffin wax. Blocks were sectioned at 6 micrometers and stained with hematoxylin and eosin. Samples were mounted in entellan.

Observation

The thymus and spleen of the fishes were observed under 10x and 40x magnification, while the GALT was observed under 1000x magnification. Pictures of the organs were taken using Axio-Vision Software and a Carl-Zeiss digital photomicroscope.

Results

Generally, the overall fish length decreased as the number of days in which their initial feeding was delayed (data not shown).

Thymus

The thymus appeared as a mass of purple and reddish tissue embedded in a loose investment of connective tissue. The organ was divided into a cortex and medulla that are composed of epithelial cells and thymocytes. The thymocytes were generally immature lymphocytes of T cell lineage. The cortex was denser with a color of a deeper purple than the medulla. The medulla appeared pink when stained with hematoxylin and eosin. The medulla consists of several epithelial cells that are intimately in contact with lymphocytes. Lymphocytes are more numerous in the medulla than in the cortex.

The thymocytes that were observed in all groups appeared to be immature T cells. They were seen dispersed all over the thymus. However, for the starved fishes, the thymocytes were less densely packed than those in the control.

Generally, the thymus of the fishes in the same group increased in size as the fish grew older. However, the size of the thymus of the control (unstarved fish) differed with those that were previously starved, such as T1D8 (8 day old) and T2D8 (10 day old).

The thymus of the T2D8 (10-day old fish that was starved for 2 days before initial feeding) group was smaller than the control group. The biggest decrease in thymus size was seen in the group 3 fish. These were the fish whose initial feeding was delayed for 4 days (Figs. 3 and 4). The thymus of these fishes were almost half the size of those in the group 2 fishes and were less than half the size of those observed in the control. In the next groups, the thymus showed an increase in size but were still smaller than the control. The thymus of T5D30 (38 day old) was the biggest thymus observed in all starved fishes. But considering that this fish was the oldest among all observed fishes (38 days), the size of the thymus was still considered small since it was just a few micrometers larger than the thymus of the unstarved 8-day old control.

Hassall's bodies were seen only in the 32-day old group 2 fish (Fig. 4) and in group 4 fishes. The Hassall's bodies of the thymus, which are located in the medulla, have a hyaline center that stains red with eosin.

Spleen

The spleen was reddish in color when stained with hematoxylin and eosin, with the red pulp stained as red while scattered through this area were tissues stained in deep purple, which corresponded to the white pulp (Figs.5 and 6). The spleen had no cortex or medulla. The red pulp was made up of reticular tissue and it contained all types of blood cells. The erythrocytes gave this region the distinct red color. Macrophages were also distributed in the red pulp and their vesicular nuclei stained readily with eosin. The white pulp was also made up of reticular tissue but contained large numbers of lymphocytes, monocytes and plasma cells. The white pulp also contained T and B cells. The red and white pulps were seen to be scattered in the organ. The red and white pulps were first seen in the group 2 fishes that were fed for 4 days already.

In the control group, the spleen was recognizable at 4 days (Figure 5). There was a general increase in size of the spleen in the fishes in the same group. However, the spleen size of the control differed with those of the starved fishes. The spleen of the group 2 fishes was still larger than the control. These were the fishes that were previously starved for 2 days. However, the spleen of the group 3 fishes was smaller than the control. From then on, the spleens of the group 4 and group 5 fishes decreased in size when compared to the control, except for T4D4 (10-day old fish that was starved for 6 days before initial feeding), which showed a slight increase in spleen size.

GALT

The gut-associated lymphatic tissue or GALT was also observed in the fishes and was first seen in 8-day old unstarved control (Figs.7-8). GALT was also observed for the starved fishes. It first appeared in the group 2 fishes and was similar in appearance with the control group (Fig. 8). Some fishes of group 3, 4 and 5 also showed the presence of GALT. However, for some fishes in group 4 and 5, the GALT became unrecognizable due to the regression of the intestinal lining. The GALT consists of lymphoid aggregates and follicles. The lymphoid follicles contain both B and T lymphocytes. The lymphocytes were embedded in the intestinal submucosa and were observed as darker cells. However, in the starved fishes, some as old as 36 days, the intestinal lining was not intact or it was too thin to see the GALT even under 1000x magnification.

Discussion

The results show that starvation delays the development of the immune system of the fish. This indicates that the delay in initial feeding stressed the fish and affected the development of the immune system.

Stress is defined as physical or chemical factors that cause bodily reactions that may contribute to disease and death (Rottmann *et al*. 1992). Fishes used in agriculture are weakened by stressful conditions such as increased fish density, poor water quality, injury during handling, inadequate nutrition and poor sanitation (Rottmann *et al*. 1992). Delaying the initial feeding of the fish may also be considered as a stressful condition as well as exposure to extreme temperatures. In fact, the two factors, which strongly influence the immune response and antibody production in fishes, are seasonal changes and temperature (Olabuenaga 2004). These conditions may decrease the fish resistance to disease, which may eventually lead to early death.

Aside from the observed delay in the development of the immune system, stress could also affect the osmoregulation and breeding of the fish, thus, shortening its natural life span. Fishes, however, did not die at once upon exposure to a stressful condition. They were able to adapt to stress, but only for a certain period of time. As the stress levels increase, the fish's ability to cope with it decreases. However, once their energy reserves become depleted

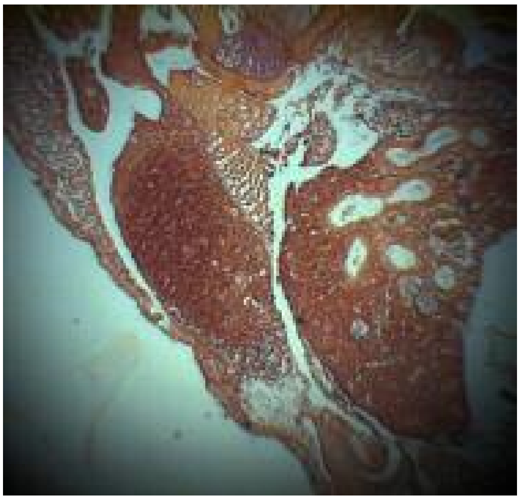

Figure 1. Cross-section of the thymus (a) of the unstarved fish after 2 days of feeding. The thymus is seen as an elongated organ beside the anterior kidney (k). (H.E. x 100)

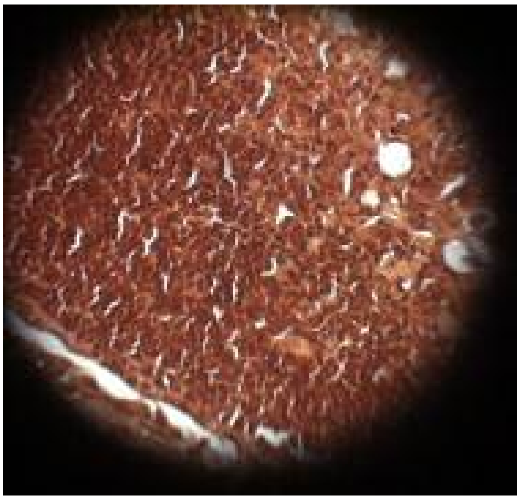

Figure 2. Cross-section of the thymus, with distinct thymocytes (arrow), of the unstarved fish after 2 days of feeding. Most of the thymocytes are immature T cells. (H.E. x 400)

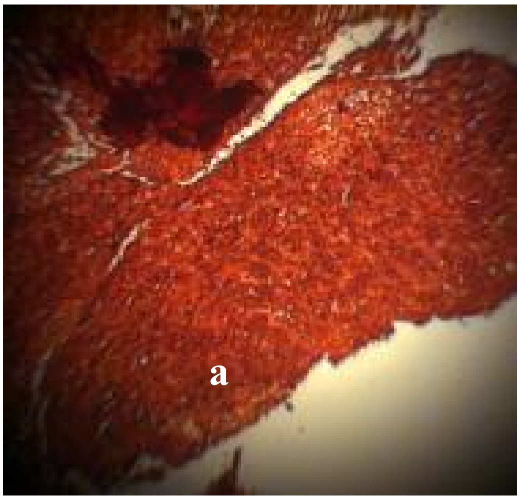

Figure 3. Cross-section of the thymus (a) of the 2-day starved fish after 30 days of feeding. A slight decrease in size can be observed when compared to the control in Figure 10. (H.E. x 100)

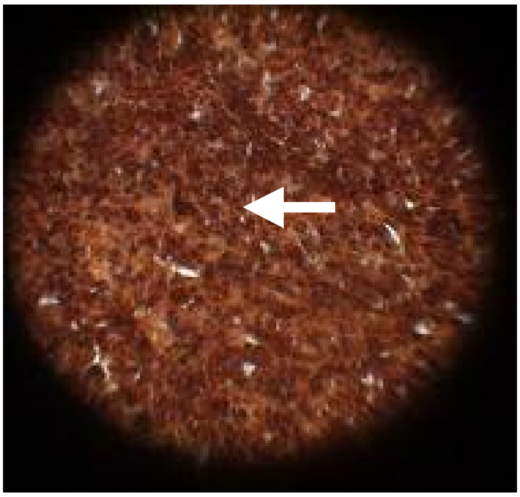

Figure 4. Cross-section of the thymus of the 2-day starved fish after 30 days of feeding. Hassall's bodies are seen embedded in the medulla of the thymus (pointed). (H.E. x 400)

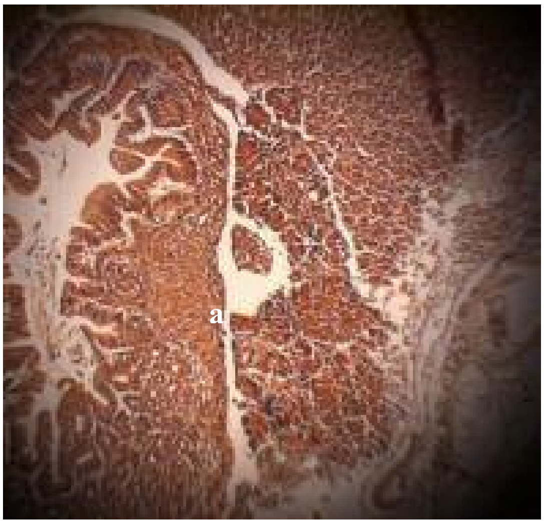

Figure 5. Cross-section of the spleen (a) of the unstarved control after 4 days of feeding. The spleen is reddish in color and is seen close to the pancreas. (H.E. x 100)

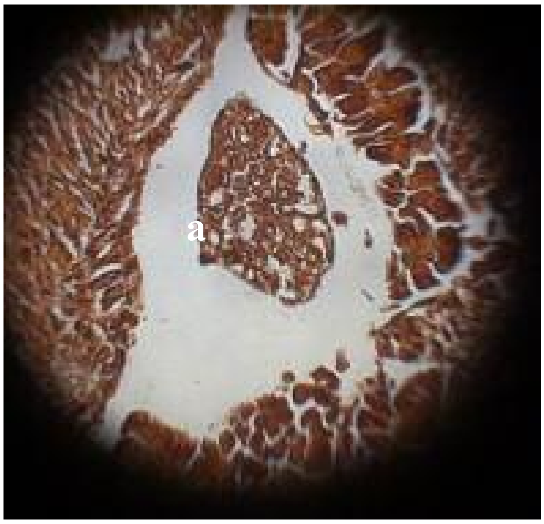

Figure 6. Cross-section of the spleen (a) of the unstarved control after 4 days of feeding. However, in this figure, the red and white pulp cannot be seen distinct from each other. (H.E. x 400)

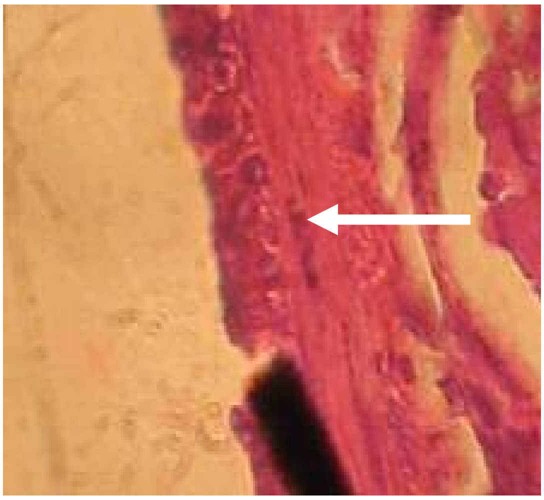

Figure 7. Cross-section of the GALT of the unstarved control after 8 days of feeding. The GALT is seen embedded in the intestinal submucosa. (H.E. x 1000)

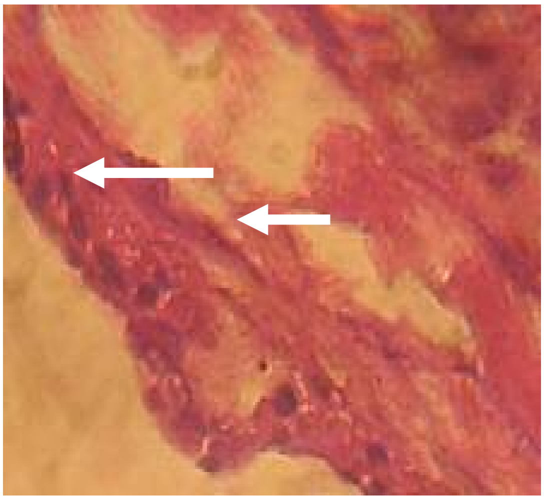

Figure 8. Cross-section of the GALT of the 2-day starved fish after 8 days of feeding. The GALT is seen embedded in the intestinal submucosa. (H.E. x 1000)

and hormone imbalance occurs, their immune system becomes suppressed, thus increasing their susceptibility to infectious diseases (Rottmann *et al*. 1992). Thus, in this study, the fishes under the group 2 (initial feeding delayed for 2 days) probably were still able to adapt to the stressful condition brought about by delayed feeding as shown by the size of the thymus that was almost of the same size as the control. However, delaying initial feeding for 4 days (group 3) may have resulted to depletion of energy reserves and hormonal imbalance, thus a large decrease in the size of the thymus and spleen was observed. The small increase in size of the thymus and the spleen seen in the fishes starved for 6 and 8 days before initial feeding were just probably due to the fishes getting older.

The thymus and its microenvironment change throughout life under different intrinsic and extrinsic factors including disease, stress, environmental conditions, and many more. In response to these factors, the thymus' response is usually transient involution. The degree of involution is highly dependent on the type and duration of the stimulus (Dominguez-Gerpe *et al*. 2003). Thus, in this study, the observed decrease in the size of the thymus in groups 2-5 may be attributed to involution of the organ. In some cases, the thymus is also able to recover after the cessation of stress (Dominguez-Gerpe *et al*. 2003). This may be the reason why there was an observed increase in size of the thymus of the fishes of the same group, as the fish grew older, since the feeding of the fishes became regular after the initial feeding.

The importance of the thymus in young animals have been demonstrated by several experiments in which removal of the organ in newborn animals resulted to the animal failing to develop immunological competence, thus resulting to death (Ham 1974). Also, they failed to reject transplants of foreign tissue and their capacity to produce antibodies was also diminished (Ham 1974). The thymus is an important organ for an animal to become immonologically competent during its first week of life since the epithelial cells of the thymus secretes some humoral factor, such as a hormone, into the blood. Thus, in this study, despite the stress caused by the delayed initial feeding, the thymus of the starved fishes (groups 2-5) was still able to continue its development. However, for these groups, the starvation caused a delay in the development causing the thymus to be smaller and the thymocytes to be less densely packed. The abnormal growth of the thymus observed in the oldest fish in group 5 may also indicate that there is already tumor growth and hyperplasia in the organ.

The spleen is also important to the immune system of the body of an animal. This organ serves as a site where antigens in the blood can activate suitably programmed lymphocytes to develop into immunologically functioning cells (Ham 1974). In this study, delayed development was also observed in the spleen of the fishes that were starved (groups 2-5). This again proves that starvation stresses the fish and that the inadequate nutrition that the starved fish received affected the development of the spleen. However, despite the delayed development of the spleen that was caused by starvation, it still developed in the starved fishes and confirmed that the organ is really important for the survival of the fish.

Thus, it is important that fishes are not exposed to stressing conditions for a long time. Exposure to prolonged stress reduces the effectiveness of the immune system, and therefore, increasing the opportunities for disease-causing organisms and could also induce tumor growth. (Rottmann *et al.* 1992).

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