JOURNAL OF THE
WORLD AQUACULTURE SOCIETY

Vol. 34, No. 3
September, 2003

Hematological Changes in Nile Tilapia (*Oreochromis niloticus*) Infected with *Streptococcus iniae* by Nare Inoculation

SHAWN T. MCNULTY, PHILLIP H. KLESIUS, AND CRAIG A. SHOEMAKER

USDA, ARS Aquatic Animal Health Laboratory, P.O. Box 952, Auburn, Alabama 36830 USA

JOYCE J. EVANS

USDA, ARS 151 Dixon Drive, Suite 4, Chestertown, Maryland 21620 USA

Tilapia has become an increasingly important food source, and production is predicted to double in the next decade (Stickney 2001). Hematology is a useful tool in monitoring the level of stress in fish (Blaxhall and Daisley 1973; Leamaster et al. 1990). This study was designed to examine the relationship between hematological variables as related to streptococcal disease of tilapia following infection with *S. iniae* by nare inoculation.

Streptococcal disease causes major economic losses to the global aquaculture industry (Boomker et al. 1979; Perera et al. 1994, 1997; Stoffregen et al. 1996; Shoemaker and Klesius 1997). In the U.S. alone the number exceeds $10 million annually (Shoemaker and Klesius 1997). *Streptococcus* spp. have been shown to gain entry into fish by ingestion (Minami 1979), through injured skin (Rasheed and Plumb 1984), by cohabitation of infected fish and immersion (Robinson and Meyer 1966). *Streptococcus iniae* has been identified as one of the main etiologic agents of streptococcal disease in cultured tilapia *Oreochromis* sp. (Perera et al. 1994, 1997; Eldar et al. 1995; Shoemaker and Klesius 1997). *Streptococcus iniae* is a coccus-shaped, aerobic, Gram-positive bacterium (Pier and Madin 1976).

Successful experimental *S. iniae* infection of tilapia following nare inoculation suggests the nares are a route of *S. iniae* infection in cultured tilapia (Evans et al. 2000, 2001). Based on this information, it is believed that nare inoculation provides for a more natural route of infection and possibly a more natural response. The objective of this study was to characterize hematological variables of juvenile and adult tilapia as they are affected by *S. iniae* infection by nare inoculation.

Materials and Methods

Fish

Two groups of Nile tilapia *Oreochromis niloticus* were used. The juvenile tilapia had a mean weight of 54.11 ± 4.7 g and length of 137.96 ± 11.4 mm. Juvenile tilapia were confirmed as juveniles by the absence of reproductive organs at the time of microbiological culture or at the termination of the experiment. The adult tilapia had a mean weight of 125.08 ± 9.3 g and length of 175.96 ± 15.5 mm. All fish were acclimated in flow-through 57-L glass aquaria supplied with 0.5 L/h dechlorinated water for 5 d prior to experimentation. A light and dark period of 12:12 h was maintained, and aeration was supplied through air stones. The fish were fed once daily to satiation with Aquamax Grower 400 (Brentwood, Missouri, USA).

Water Quality Evaluation

During the experiment the mean ± standard deviation of dissolved oxygen (mg/L) was 5.8 ± 0.512, temperature (C) was 26.2 ± 0.799, pH was 7.1 ± 0.253, salinity (%) was 0.1 ± 0.0, and hardness was 110 ± 10.0. Ammonia and nitrite concentrations were below detectable limits.

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Experimental Design

Tilapia maintained at the Aquatic Animal Health and Research Unit in Auburn, Alabama, USA, were used for this experiment. A subsample of the fish was microbiologically cultured to verify that the fish were disease free. Fifteen juvenile tilapia (mean weight 54.11 g) were placed into each of five 57-L aquaria. Aquaria 1 and 2 were designated as the control fish. Aquaria 3, 4, and 5 were designated as the 2, 5, and 7-d post-inoculated juvenile fish, respectively. Fifteen adult tilapia (mean weight 125.08 g) were placed into five 57-L aquaria. Aquaria 6 and 7 were designated as the control fish. Aquaria 8, 9, and 10 were designated as the 2, 5, and 7-d post-inoculated adult fish. The control fish received tryptic soy broth (TSB, Difco Laboratories, Sparks, Maryland, USA) only and all other fish received 10 μL/nare of a 1×10^8 CFU/mL inoculum of *S. iniae*. To collect blood for the control fish, fish were bled ($N = 25$) from each of the two control aquaria and the samples combined for analysis. When possible, 12 blood samples from challenged fish were collected on the designated day of the experiment. All control fish were bled on day 0. Aquaria 3 and 8 were bled on day 2, aquaria 4 and 9 were bled on day 5, and aquaria 5 and 10 were bled on day 7 of the experiment.

Bacteria

A beta hemolytic *Streptococcus iniae* isolate, originally isolated from a hybrid striped bass with natural streptococcal disease, was used to infect the fish. The isolate was identified as *S. iniae* according to tests described by Shoemaker and Klesius (1997) and the type isolate description of *S. iniae* by Pier and Madin (1976). The isolate was grown in TSB for 24 h at 27 C and then the culture was frozen in 0.2-mL aliquots at −70 C. The infectious isolate used in this study was prepared by inoculating 250 mL of TSB in a 500-mL culture flask with a thawed aliquot of the frozen isolate. Following incubation for 24 h at 27 C, the culture was adjusted spectrophotometrically to an optical density equal to 1.0 at 540 nm to give a concentration of 1×10^8 CFU/mL. The doses of *S. iniae* used in this study were then prepared by dilution in TSB, prior to the experiment, and the CFU was later confirmed by plate counts (Evans et al. 2000).

Nare Inoculation

Tilapia were netted out of the aquaria and placed in a holding tank and anesthetized with MS-222. Each individual fish was then picked up and inoculated via the nare with 10 μL/nare using a micropipet (Evans et al. 2001). Each nare was slowly instilled with the 10-μL aliquot of the culture suspension or control TSB. Special care was taken not to injure the olfactory mucosa during the procedure. The fish were then placed back into their respective aquaria and monitored for 5–10 min.

Blood Collection

The same experimental procedures were followed for all fish in aquaria. Fish were netted out of the aquaria and placed in a holding tank. The fish were then bled without the use of an anesthetic. Blood was drawn from the caudal vein into a 1-mL heparinized (Sodium heparin from porcine intestinal mucosa, Sigma, St. Louis, Missouri, USA) syringe. Twenty μL were used to run the total hemoglobin test (Sigma Diagnostics Total Hemoglobin kit 525-A). Hematocrit, total red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were tested and calculated manually by standard methods according to Harmening (1992). All control fish were bled on day one of the experiment.

Statistical Analysis

Data for this experiment were analyzed by one-way analysis of variance (SAS Institute 1997). Duncan's multiple range tests

TABLE 1. *Means and standard deviations of hematological variables for tilapia infected by* Streptococcus iniae *by nare inoculation.*

	N	Hematocrit (%)	RBC ($10^6/\mu L$)	HgB (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
				Juvenile Tilapia			
Control	25	24.12 ± 4.1[b,s]	2.88 ± 0.7[b]	7.63 ± 1.3[bc]	87.48 ± 21.4[c]	27.95 ± 8.2[bc]	32.26 ± 7.1[a]
2 DPI[t]	14	27.93 ± 4.9[ab]	2.23 ± 0.6[c]	7.85 ± 1.0[b]	128.68 ± 57.3[b]	38.45 ± 13.4[b]	28.9 ± 6.6[ab]
5 DPI	12	29.3 ± 3.5[a]	3.9 ± 1.6[a]	9.1 ± 2.1[a]	83.42 ± 27.6[c]	26.03 ± 11.1[c]	31.13 ± 6.3[a]
7 DPI	15	26.67 ± 7.6[ab]	1.4 ± 0.6[d]	6.45 ± 2.1[c]	209.42 ± 66.1[a]	53.25 ± 26.12[a]	24.55 ± 5.8[b]
				Adult Tilapia			
Control	25	29.14 ± 3.9[w]	4.64 ± 2.7[w]	7.75 ± 1.3[w]	78.93 ± 35[x]	20.93 ± 9.2[x]	26.94 ± 5.2[w]
2 DPI	12	25.88 ± 5[wx]	2.84 ± 0.7[x]	3.41 ± 1.5[x]	90.76 ± 23.9[x]	11.84 ± 5.2[y]	12.86 ± 3.5[x]
5 DPI	12	23.75 ± 4.8[xy]	3.03 ± 1.6[x]	9.1 ± 10.2[w]	94.57 ± 39.4[x]	23.94 ± 11.12[x]	28.14 ± 13.3[w]
7 DPI	10	21.9 ± 2.8[y]	1.19 ± 0.3[y]	6.6 ± 0.7[wx]	191.83 ± 44.5[w]	57.77 ± 12.6[w]	30.37 ± 3.1[w]

[s] Small letters denote significant difference in Duncan grouping ($P \leq 0.05$).
[t] Denotes day post inoculation of *S. iniae* by nares.

were used to determine significant differences between duration of *S. iniae* infection. All data were considered significant at $P < 0.05$.

Results

The mean baseline hematocrit level for control juvenile and control adult tilapia before *S. iniae* infection was 24.1% and 29.1%, respectively. Hematocrit levels in the experimentally infected juveniles increased to 26.7% after 7 d of infection, but adult levels decreased to 21.9% after 7 d of infection. The mean RBC count for both fish sizes declined throughout the experiment from 2.9 ($10^6/\mu L$) to 1.4 ($10^6/\mu L$) for the juveniles, and 4.6 ($10^6/\mu L$) to 1.2 ($10^6/\mu L$) for the adults. Pre-inoculation and 7 d post-inoculation (DPI) hemoglobin levels for the juveniles (7.6 g/dL, 6.5 g/dL) and adults (7.8 g/dL, 6.6 g/dL) were not significantly different from each other. The juvenile levels, however, were much more constant than the adult levels, which dropped to 3.41 g/dL on day 2, then increased to 9.1 g/dL on day 5, and then returned to 6.6 on day 7. *S. iniae* infection caused the MCV in both groups to increase by more than 200%. The MCV for both groups was not significantly different before infection. The MCV for the juveniles increased from 87.5 fL at day 0 to 209.4 fL at day 7, and the MCV levels for adults increased from 78.9 fL to 191.8 fL. MCH levels followed a similar pattern in which the juvenile values increased from 27.9 pg pre-inoculation to 53.3 pg at 7 DPI. The adult values increased from 20.9 pg pre-inoculation to 57.8 pg at 7 DPI. The MCHC levels for the juveniles had a close range from 32.3 g/dL on day 0 to 24.6 g/dL on day 7 and were significantly different only on 2 DPI. Adult MCHC varied little throughout the experiment and was only significantly different on day 2 (Table 1).

The first signs of streptococcal disease occurred on day 2 of the experiment with at least one fish in all aquaria, except the control aquaria, exhibiting increased pigmentation. By the end of the experiment, all typical signs of *S. iniae* infection were observed, including increased pigmentation, eye opacity, erratic swimming, and lethargy. According to our observations and microbiology, we believe that all treated aquaria were successfully infected with *S. iniae*.

Discussion

As the aquaculture industry expands, the need for early disease detection will increase. This work indicates that changes in hematology, before clinical signs appear, are an early sign of disease. However, the role that hematology will play in the monitoring of fish health has yet to be deter-

mined mainly because of a lack of species normal values and variance due to sampling techniques (Houston 1997).

From this study we believe that the hemolytic action of *S. iniae* alters all of the hematological properties examined, thus decreasing the overall health of the fish. Two possible stressors that could occur as a result of the infection are the loss of oxygen carrying capacity and increases in the iron levels. This work indicates that as RBC levels decrease the overall ability of the blood to transport essential oxygen could be decreased because of the loss of erythrocytes and hemoglobin. MacMillan and Mulcahy (1980) observed that chum salmon *Oncorhynchus keta* that had lost RBCs due to viral erythrocytic necrosis died at significantly higher oxygen concentrations than did non-infected chum salmon. It is likely that the tilapia with reduced RBCs would have a similar response to low oxygen concentrations.

The release of excess iron from the breakdown of hemoglobin is another possible stressor caused by *S. iniae* infection. Weinberg (1992) states that all vertebrate hosts possess a system termed "iron withholding," which is an anti-infective, antineoplastic defense system. This system attempts to deny available iron to invading microbial and neoplastic cells. However, as hemoglobin is released when the RBC is lysed by *S. iniae,* iron is released and "iron withholding" is circumvented. Weinberg (1992) claims that as the host becomes iron overloaded, the risk of developing neoplastic growths and intracellular microbial infections is increased. It is probable that enough iron is released during the breakdown of hemoglobin molecules from *S. iniae* infections that the immune system is stressed. However, further research must be done to verify this effect of *S. iniae.*

A distinct hematological trend was observed in the adult fish. First, throughout the experiment, the RBC counts decreased (the difference between 2-d and 5-d post-infection values is not significant). As the mature RBCs died, due to the hemolytic nature of *S. iniae,* hemoglobin was lost during the first 2 d post *S. iniae* inoculation. This is reflected in the substantial drop in total hemoglobin from the controls to the 2-d treatment. Because the number of RBCs was reduced, immature RBCs were released into the blood stream. It has been documented that immature RBCs are larger than mature ones (Hardig and Höglund 1983; Harmening 1992). The increase in number of immature RBCs is reflected in the increased MCV throughout the experiment. The initial increase in immature RBCs is also reflected in the fact that MCH and MCHC dropped during the first 2 d of the experiment but returned to normal levels afterward. Hardig and Höglund (1983) state that it appears that hemoglobin synthesis occurs after the immature RBCs are released into the blood stream.

The effects of *S. iniae* infection on the hematology of tilapia have not been defined. Our work shows that *S. iniae,* administered through the nares, causes infections and significantly alters the hematological characteristics of both adult and juvenile tilapia, even if the infection does not cause mortality. Literature that describes other species with similar stresses and infections (Farghaly et al. 1973; Reno et al. 1985; Haney et al. 1992), states that possible negative effects that could be caused by *S. iniae* infections include a reduced tolerance to low oxygen concentrations and lowered immune system efficiency.

Acknowledgments

We would like to thank Dr. John Grizzle and Dr. Yolanda Brady for reviewing this manuscript.

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