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Density and dose: factors affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*)

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Abstract

Fish density and infectious dose have been suspected to affect the mortality rate of cultured fish exposed to *Streptococcus iniae*. We determined the effects of *S. iniae* dose and tilapia (*Oreochromis niloticus*) density on streptococcal disease mortality. Tilapia with a mean weight of 12.7 g were used and maintained at 25 ± 1°C in aquaria supplied with flow-through water at 0.5 l/min with a 12 h light:12 h dark cycle. Density and dose were evaluated by stocking tilapia at low (5.6 g/l), medium (11.2 g/l) and high (22.4 g/l) density and administering 2.5×10^7, 5×10^7 and 1×10^8 colony-forming units (CFU)/ml of *S. iniae* by immersion (5 tanks per density and dose, 45 total tanks). Mortality was monitored for 28 days post challenge. A significant difference ($P < 0.05$) was seen in mortality when comparing low (4.8%) and medium (28.4%) and low and high (25.6%) density treatments. No significant difference was observed when comparing medium- and high-density treatments. Two-way analysis of variance demonstrated density had a significant effect on *S. iniae* mortality ($P = 0.0001$). Doses had little effect on mortality, except at high density by dose which did show a significant interaction ($P = 0.001$). We have demonstrated density has a significant effect on streptococcal disease mortality in tilapia exposed to *S. iniae* by immersion. We also evaluated infection of susceptible tilapia using

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dead/moribund *S. iniae* infected fish (i.e., cohabitation by placing five dead/moribund fish into tanks for 48 h). No significant difference in mortality pattern was observed between immersion in 8.6×10^7 CFU/ml *S. iniae* (37.6% and 34.6%) and cohabitation with *S. iniae* infected tilapia (24.0%). Although, densities used were less than in most water-reuse production systems (30–290 g/l), tilapia density of 11.2 g/l and above was an important factor in mortality of tilapia infected with *S. iniae*. A health-management strategy would be to reduce fish density thus lowering streptococcal disease mortality. Published by Elsevier Science B.V.

Keywords: Streptococcus iniae; Tilapia; Density; Dose; Mortality; Immersion; Cohabitation

1. Introduction

Streptococcal disease, caused by *Streptococcus* and *Lactococcus* spp., has increased with the intensification of cultured fish species susceptible to these Gram-positive bacteria (Eldar and Ghittino, 1999). The problem of streptococcal disease is worldwide (Muzquiz et al., 1999) and results in an estimated $150 million in losses annually (Shoemaker and Klesius, 1997). Few studies have examined the epidemiology of streptococcal infections in tilapia (*Oreochromis niloticus*). However, many inferences have been made to various factors (stressors) which influence mortality due to streptococcal disease caused by *Streptococcus iniae* (Plumb, 1997; Shoemaker and Klesius, 1997). Certain environmental factors have been researched and suggested to influence streptococcal disease mortality in tilapia. Perera et al. (1997) suggested that temperature (20°C) influenced streptococcal disease mortality in experimentally infected tilapia hybrids (*O. niloticus* × *O. aureus*). Bunch and Bejerano (1997) evaluated dissolved oxygen level and nitrite level on streptococcal mortality in hybrid tilapia. Their results suggested that low oxygen and high nitrite increased mortality due to *Streptococcus* sp. infection, but no additive effect (i.e., low dissolved oxygen and high nitrite in combination) was observed. A recent study by Bowser et al. (1998) suggested that low oxygen level was responsible for streptococcal mortalities observed in a water-recirculating production facility. However, upon trying to initiate the same results with experimental animals exposed to low oxygen levels, no death occurred suggesting that exposure to low oxygen for 8 h did not influence *S. iniae* induced mortality of tilapia. One factor that has not been quantified is the effect of high stocking densities on infectious disease problems in finfish (Bebak et al., 1999).

Management strategies and environmental conditions vary among the various types of fish culture systems. A common practice that is employed in both closed culture and pond aquaculture is the use of high stocking densities per unit area (Teichert-Coddington and Green, 1997). Intensive culture of susceptible fish species has resulted in severe losses due to streptococcal disease with reports of up to 75% mortality in closed culture systems (Perera et al., 1994; Stoffregen et al., 1996) and 50% in ponds (Eldar et al., 1997). High fish density and infectious dose have been suspected to affect the mortality rate of cultured fish infected with *S. iniae*. The objective of this study was to determine the effects of *S. iniae* dose and tilapia density and mode of infection by cohabitation on streptococcal disease mortality.

2. Materials and methods

2.1. Experimental animals

Tilapia with an average weight of 11.9 g for cohabitation experiments and 12.7 g for density by dose immersion experiments were used as experimental animals. A sample of fish was culture negative for *S. iniae* by standard methods (Shoemaker and Klesius, 1997) prior to experimentation. Fish were stocked into 57-l glass aquaria supplied with $25 \pm 1^{\circ}C$ flow-through water at 0.5 l/min with daily dissolved oxygen level of $5.5 + 0.7$ mg/l (Klesius et al., 1999). Fish were fed Purina catfish chow (Purina Mills, St. Louis, MO) at 2–3% body weight daily and kept on a 12 h light:12 h dark cycle. In the experiment to evaluate density and dose, fish were stocked at low density (25 fish or 5.6 g/l), medium density (50 fish or 11.2 g/l) and high density (100 fish or 22.4 g/l) (five replicate aquaria for each density and dose, 45 total tanks). In a separate experiment to evaluate infection of tilapia by *S. iniae* from exposure to fresh dead and/or moribund *S. iniae* infected fish (cohabitation) as compared to immersion exposure and noninfected control treatments; fish were stocked in three replicates per treatment at a density of 100 fish per tank (21 g/l). Fish in both experiments, density and cohabitation, were stocked into the respective tanks and acclimated for 2 weeks prior to experimentation.

2.2. Infection

An isolate of *S. iniae* (ARS-98-60) obtained from diseased tilapia in the laboratory and identified biochemically (Pier and Madin, 1976; Shoemaker and Klesius, 1997) was used to challenge the tilapia. The isolate used was not passed in/on media more than two times prior to infecting the fish. Briefly, the isolate of *S. iniae* taken from a blood agar plate was grown in tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) for 24 h prior to use. Tilapia in the density by dose experiment were infected by immersion exposure to *S. iniae*. Tilapia were added at a density of 25 fish per liter of water with aeration (e.g., 50 fish into 2 l water; 100 fish into 4 l water) with the appropriate amount of broth culture of *S. iniae* added followed by a 15-min timed exposure before release to aquarium. Plate counts from immersion water (triplicate) showed that the dose used was about 2.5×10^7, 5×10^7 and 1×10^8 colony-forming units (CFU)/ml (Table 1). The infectious dose used to immerse the fish to compare to infection by cohabitation was 8.6×10^7 CFU/ml. Cohabitation was accomplished by placing five moribund or fresh dead *S. iniae* infected tilapia into each tank for 48 h prior to removal. The dead or moribund tilapia used to infect by cohabitation were previously infected by immersion exposure to 2×10^7 CFU/ml and showing signs of streptococcal disease prior to addition to the tanks. No attempt was made to quantify the number of bacteria in the cohabitation experiment using dead or moribund fish. After fish were infected, mortalities were monitored twice daily for 28 days. Fresh dead fish were cultured (brain and anterior kidney) on blood agar (Remel, Lenexa, KS) to confirm *S. iniae*.

Table 1
Density and dose effect on mortality following *S. iniae* immersion challenge in tilapia held for 28 days

Dose CFU/ml (plate count)	No. fish per tank (five tanks for each dose)	No. dead/ no. total*	% Mortality (SEM)**
2.5×10^7	25	6/125	4.8 ($\pm$1.5)^{a}
5.4×10^7	25	4/125	3.2 ($\pm$0.8)^{a}
1.7×10^8	25	8/125	6.4 ($\pm$1.6)^{a}
	Low density***	Mean % mortality (SEM)†	4.8 ($\pm$0.8)^{A}
2.6×10^7	50	87/250	34.8 ($\pm$2.1)^{a}
4.2×10^7	50	59/250	23.6 ($\pm$2.6)^{b}
7.3×10^7	50	67/250	26.8 ($\pm$3.7)^{a,b}
	Medium density‡	Mean % mortality (SEM)	28.4($\pm$2.0)^{B}
2.9×10^7	100	78/500	15.6 ($\pm$1.9)^{a}
5.4×10^7	100	133/500	26.6 ($\pm$5.1)^{a,b}
1.2×10^8	100	171/500	34.2 ($\pm$4.6)^{b}
	High density$^{\#}$	Mean % mortality (SEM)	25.6 ($\pm$3.0)^{B}

* Fresh dead fish were cultured on blood agar and *S. iniae* was isolated from the brain and kidney.

** Lower case superscripts denote differences in the means on each group of mortality data in low, medium and high treatments (i.e., dose) by Duncan's multiple range test. Significance is indicated by different superscripts at $P < 0.05$.

*** Low density = 5.6 g/l.

†Upper case superscripts denote differences in the mean percent mortality as compared by two-way analysis of variance using Duncan's multiple range test to compare means. Significance level was equal to $P < 0.05$.

‡Medium density = 11.2 g/l.

$^{\#}$High density = 22.4 g/l.

2.3. Statistical analysis

Data for the density by dose experiment were analyzed by two-way analysis of variance (SAS Institute, 1997). Duncan's multiple range test was used to determine significant differences due to dose, density and dose by density interactions. Data in the cohabitation experiment were analyzed by analysis of variance with Duncan's multiple range test to compare means. All data were considered significant at $P < 0.05$.

3. Results

Mortalities in the low-, medium- and high-density treatments began occurring 2–4 days post infection. Typical signs of streptococcal disease [i.e., erratic swimming, darkening of the fish, exophthalmia and cloudy eyes, (Plumb, 1997, 1999)] were observed in the dead and dying tilapia following infection. *S. iniae* was isolated from both brain and anterior kidney of fresh dead tilapia on blood agar. Density was shown to significantly affect mortality of tilapia infected by immersion exposure to *S. iniae*

Table 2
Density effect on route of infection with *S. iniae*

Treatment	No. of fish per tank (three tanks/treatment)	No. dead/no. total*	% Mortality (SEM)**
Noninfected***	100	0/300	0.0 ($\pm$0.0)^{A}
8.6×10^{7} (rep 1)†	100	113/300	37.6 ($\pm$7.3)^{B}
8.6×10^{7} (rep 2)	100	104/300	34.6 ($\pm$2.8)^{B}
Cohabitation‡	100	72/300	24.0 ($\pm$2.5)^{B}

* Fresh dead fish were cultured on blood agar and *S. iniae* was isolated from the brain and kidney.

** Significant differences in the means are noted by different letters ($P = 0.0008$). The standard error of the mean is presented in parentheses.

*** Noninfected tilapia (controls) which were immersed in tryptic soy broth only.

† Tilapia infected with *S. iniae* (CFU/ml) as determined from triplicate plate counts of immersion water on blood agar plates.

‡ Cohabitation infection was accomplished by adding five moribund or fresh dead tilapia to each tank for 48 h before removal.

(Table 1). A significant difference ($P < 0.05$) was seen in the mortality when comparing low (4.8%) and medium (28.4%) and low and high (25.6%) density treatments. No significant difference was observed when comparing medium- and high-density treatments. Two-way analysis of variance demonstrated density had a significant effect on *S. iniae* mortality ($P = 0.0001$). Dose utilized had little effect on mortality. High density by dose however, did show a significant interaction ($P = 0.001$), which is evident when looking at the mortality of each dose in the high-density trial (Table 1).

Mortalities in the cohabitation treatment began to occur between day 5 and day 7 after addition of the dead/moribund fish. Dead fish were seen earlier (day 2) in the immersion treatments. Signs of streptococcal disease were observed in both cohabitation and immersion infected treatments and *S. iniae* was isolated from fresh dead fish on blood agar. No mortality occurred in the noninfected tilapia for the 28 day trial. No significant difference ($P > 0.05$) in mortality was observed between replicates 1 and 2 of the immersion exposed treatments (37.6% and 34.6%, respectively) and of the cohabitation treatment (24.0%, Table 2). The infected treatments all exhibited significantly higher ($P = 0.0008$) mortality when compared to the control treatment in which no fish died.

4. Discussion

Significant increases in mortality due to *S. iniae* were shown to occur in tilapia held at densities ≥ 11.2 g/l. We could not find any other study from literature searches that demonstrated the importance of density on mortality of fish infected with *S. iniae* by immersion. Water-reuse fish-production facilities often use densities greater than those used in this study with little or no water exchange. In our study, the fish were housed in aquaria that allowed for one-half water exchanges per hour. In intensive closed culture systems, density is probably even more important because little or no water is ex-

changed, which leads to the high levels of mortality (70% or more) that have been observed in these systems (Perera et al., 1994). Increased density of fish probably also favors transmission of *S. iniae*. We suspect increased contact with a greater concentration of *S. iniae* in water from dead diseased fish may explain the resulting increase in mortality. Direct contact and subsequent inoculation via abrasion may also be responsible. Rasheed and Plumb (1984) and Chang and Plumb (1996) were only successful in infecting fish with *Streptococcus* sp. after abrasion of the skin prior to immersion. This may explain why only a few fish died in the low-density treatments where less fish to fish contact occurred.

Dose of *S. iniae* used to infect the tilapia by immersion exposure did not appear to influence the mortality in the different density treatments of tilapia, with the exception of the highest density treatment (22.4 g/l). In this treatment, significantly higher mortality occurred in tilapia with the greater number of *S. iniae* used to infect the fish. The number of *S. iniae* used to inoculate the low-density treatments resulted in few mortalities. Dose of *S. iniae* used to infect tilapia by different modes of infection in previously published works have varied greatly; however, the doses would have been in the range of those used in this study.

We were successful in inducing mortality in tilapia following cohabitation by placing previously infected tilapia in the same tank for 48 h exposure before removal. Cohabitation was demonstrated by Robinson and Meyer (1966) using golden shiners (*Notemigonus crysoleucas*) and group B nonhemolytic streptococci. Cumulative mortality in our study, however, was not significantly different between the immersion exposure treatments (37.6% and 34.6%) and the cohabitation treatment (24.0%). Perera et al. (1997) reported that cohabitation with infected dead tilapia failed to infect susceptible tilapia with *S. iniae*, although they only used five to seven fish for their experiment. While the dead and/or moribund fish were in the tanks, we observed cannibalism of eyes and viscera. This suggests an oral and/or olfactory mode of infection may be responsible for infection by waterborne *S. iniae*. Perera et al. (1997) were able to infect tilapia using direct delivery of *S. iniae* to the gut. Ingestion of fish feed containing *Streptococcus* sp. was also shown to cause streptococcal disease in yellowtail *Seriola quinqueradiata* (Minami, 1979). However, with the high density of fish used in our study, transmission from fish to fish via contact and abrasion could also have occurred. The number of *S. iniae* or dose used in the cohabitation experiment was not quantified. Because of the observed cannibalism and suggested oral and/or olfactory routes of infection, a management strategy should be employed to quickly remove dead and moribund fish from intensive culture operations.

Densities in intensive commercial water-reuse systems are greater than used in these experiments. It has been reported that densities used range from 30 to 290 g/l (the authors' personal communication with commercial tilapia producers in the United States). We used densities ranging from 5.6 to 22.4 g/l, which were below those used by commercial producers. Significantly less mortality (4.8%) was found in the lowest density (5.6g/l) treatment upon infection by immersion with *S. iniae*. Densities of 11.2 and 22.4 g/l had significantly higher mortalities of 28.4% and 25.6%, respectively. Reducing fish density may be another management strategy to limit transmission of *S. iniae* in intensively farmed tilapia.

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