

Enhanced mortality in Nile tilapia *Oreochromis niloticus* following coinfections with ichthyophthiriasis and streptococcosis

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ABSTRACT: *Ichthyophthirius multifiliis* Fouquet (Ich) and *Streptococcus iniae* are 2 major pathogens of cultured Nile tilapia *Oreochromis niloticus* (L). Currently there is no information available for the effect of coinfection by Ich and *S. iniae* on fish. The objective of this study was to determine the effects of parasite load and Ich development size on fish mortality following *S. iniae* infection. Low mortality ($\leq 20\%$) was observed in tilapia exposed to Ich or *S. iniae* alone. Mortalities increased from 38% in tilapia exposed to Ich at 10 000 theronts fish⁻¹ to 88% in fish at 20 000 theronts fish⁻¹ following *S. iniae* exposure. The median days to death were significantly fewer (7 d) in fish exposed to Ich at 20 000 theronts fish⁻¹ than fish exposed to 10 000 theronts fish⁻¹ (10 d). A positive correlation (correlation coefficient = 0.83) was noted between tilapia mortality and size of Ich trophonts at the time of *S. iniae* challenge. Fish parasitized with well-developed trophonts (Day 4, $2 \times 10^7 \mu\text{m}^3$ in volume) suffered higher mortality (47.5%) than fish (10.0%) infested by young trophonts (Hour 4, $1.3 \times 10^4 \mu\text{m}^3$ in volume) after *S. iniae* challenge. The results of this study demonstrated that both parasite load and trophont size increased susceptibility and mortality of tilapia to *S. iniae* infection.

KEY WORDS: Coinfections · Mortality · Nile tilapia · *Ichthyophthirius* · *Streptococcus* · Immersion

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INTRODUCTION

Tilapia are among the most important farm-raised fish, and worldwide farmed-tilapia production is estimated to exceed 3 000 000 metric tons by 2010 (Fitzsimmons 2006). Production of tilapia is 50% in semi-intensive or intensive ponds, 25% in cages, and 10% in intensive recirculating systems (Fitzsimmons 2006). Tilapia production is ~75% Nile tilapia *Oreochromis niloticus* (L.) and continues moving towards intensive culture systems (Fitzsimmons 2006).

The ciliated protozoan *Ichthyophthirius multifiliis* Fouquet (Ich) is a virulent parasite of freshwater fish. Almost all freshwater fish are susceptible to infestation (Paperna 1972, Jessop 1995, Dickerson & Dawe 1995, Traxler et al. 1998). The life stages of the parasite include an infective theront, a parasitic trophont, and a reproductive tomont (MacLennan 1935, Hines & Spira

1974, Matthews 2005). Severe Ich infestations occur most commonly in dense populations of fishes (Dickerson & Dawe 1995). Tilapia are most likely to suffer losses due to Ich infestation when water temperatures are optimum for Ich development from 20 to 25°C (Shoemaker et al. 2006).

Streptococcus iniae is a bacterial pathogen responsible for severe streptococcal disease outbreaks in various species of commercially raised or wild fish, and losses occur in most regions of the world where tilapia are cultured (Plumb 1997, Agnew & Barnes 2007, Klesius et al. 2008). 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, Agnew & Barnes 2007). In laboratory studies, however, mortalities are usually low following immersion exposure to *S. iniae* (Chang & Plumb 1996, Shoemaker et al. 2000).

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Most studies of tilapia diseases in the laboratory have been conducted for a single pathogen (a parasite, a bacterium, or a virus). Such a study design does not fully reflect conditions in aquaculture systems, where tilapia may be concurrently infected with 2 or more pathogens. Some studies have demonstrated enhanced bacterial invasion where parasitic injuries serve as portals of entry (Cusack & Cone 1986, Busch et al. 2003, Evans et al. 2007, Xu et al. 2007). Parasites may also act as mechanical vectors to transfer bacterial pathogens. Currently no information is available for the effect of coinfections by Ich and *Streptococcus iniae* on fish. The objective of this study was to determine the effects of parasite load and Ich trophont size on fish mortality following *S. iniae* infection.

MATERIALS AND METHODS

Water quality. During trials, dissolved oxygen (DO) and temperature in tanks were measured daily using a YSI 85 oxygen meter. The pH, hardness, ammonia, and nitrite were determined using a Hach CEL/890 Advanced Portable Laboratory. The mean \pm SD of DO was 6.5 ± 0.7 mg l⁻¹, temperature was 24.6 ± 0.6 °C, pH was 7.4 ± 0.2 , ammonia was 0.2 ± 0.1 mg l⁻¹, and hardness was 91.9 ± 12.3 mg l⁻¹. Nitrite concentrations were below the detection limit.

Fish and parasite. Nile tilapia fry hatched and collected from a pond were reared to experimental size in tanks using filter-recirculated water at the US Department of Agriculture (USDA) Agricultural Research Service, Aquatic Animal Health Research Laboratory, Auburn, Alabama. Ich was originally isolated from an infected channel catfish *Ictalurus punctatus* from a fish pond at New Hope, Alabama, and the parasite isolate was maintained by serial transmission on catfish held in 50 l glass aquaria as previously described (Xu et al. 2000). Fish infected with maturing trophonts were anesthetized with 150 mg l⁻¹ tricaine methanesulfonate (Argent Chemical Laboratories) and rinsed in tank water, and the skin was gently scraped to dislodge the parasites. Isolated trophonts were placed in a tank with 20 l of water and incubated at 22 to 24°C. Theronts for infection trials were enumerated with the aid of a Sedgewick-Rafter cell.

Bacterial isolation. An isolate of *Streptococcus iniae* (ARS-98-60) was obtained from diseased tilapia in the laboratory and identified biochemically as described previously (Shoemaker et al. 2000). The isolate of *S. iniae* from a sheep blood agar plate was grown in tryptic soy broth (Difco, Becton Dickinson) for 24 h and used to challenge the tilapia.

Dead or moribund fish were removed twice daily during the infection trial, and bacterial samples asepti-

cally obtained from brain tissue were streaked onto 5% sheep blood agar plates (Remel). Bacterial colonies with beta-hemolysis, testing negative for catalase production, positive by Gram-stain, and having a coccoid morphology were considered *Streptococcus iniae* positive.

Effect of parasite load on tilapia mortality after exposure to *Streptococcus iniae*. In total, 310 Nile tilapia, which ranged from 8.5 ± 0.9 (mean \pm SD) cm in total length and 9.6 ± 2.8 g in body weight (N = 12) were used in this trial. Fish were distributed among 12 tanks (57 l), with 25 fish tank⁻¹ and 2 tanks group⁻¹. Ten tilapia were examined and cultured to verify their pathogen-free status of Ich and *S. iniae* prior to the trial as described previously (Xu et al. 2007), and all fish were negative for *S. iniae* and Ich. Fish in 4 replicate tanks were exposed to Ich theronts at one of 3 Ich theront levels (0, 10 000, or 20 000 theronts fish⁻¹). The water volume was adjusted to 10 l for each tank, and the required number of live theronts was added to each tank. Fish were exposed to theronts for 2 h, and then water flow (0.5 l min⁻¹) was resumed. The trophonts create tissue spaces in the epithelial layers, and trophonts within these vesicles can be seen by the naked eye as 'white spots' 5 d post theront infection. Parasite loads of 'white spots' on fish skin/fins was determined while fish were kept in each aquarium and assessed as none, light (<50 trophonts fish⁻¹), medium (50 to 100 trophonts fish⁻¹), and heavy infection (>100 trophonts fish⁻¹; Xu & Klesius 2004).

Fish in 6 tanks (half of the fish in the trial) were challenged with *Streptococcus iniae* and the others served as non-*S. iniae* challenge controls (i.e. only exposed to parasites). Twelve buckets were filled with 2 l of tank water, and 25 fish were added per bucket with aeration. For fish challenged with *S. iniae*, the bacterial suspension was added to the bucket at the rate of 10⁷ colony forming units (CFU) ml⁻¹. After immersion for 1 h, the fish from each bucket were moved to a 57 l aquarium with flowing water at 0.4 to 0.5 l min⁻¹ with aeration. Mortality of fish was recorded, and dead or moribund fish were examined for *S. iniae* infection twice daily for 2 wk.

Effect of Ich development size on tilapia mortality after exposure to *Streptococcus iniae*. Ich theronts penetrate into fish skin and gills and become parasitic trophonts. As trophonts grow in size, the parasites cause more damage to fish skin and gills. This trial examined the effect of Ich trophont size on tilapia mortality after exposure to *S. iniae*. In total, 270 tilapia (8.7 ± 0.6 cm and 10.2 ± 2.1 g) were used in this trial, with 20 fish tank⁻¹ and 2 tanks per treatment. Ten fish were sampled prior to the trial, and all fish were negative for Ich and *S. iniae*. The following treatments were con-

ducted: (1) no Ich and no *S. iniae* infection, (2) no Ich infestation and challenged with *S. iniae*, (3) Ich infestation and no *S. iniae*, (4) challenged with *S. iniae* at Hour 4 post Ich infestation, (5) challenged with *S. iniae* on Day 2 post Ich infestation, and (6) challenged with *S. iniae* on Day 4 post Ich infestation. An additional tank (Tank 13) with 20 fish was exposed to Ich theronts, but no *S. iniae*, to evaluate trophont development size. To induce fish infestation by Ich, fish were exposed to live theronts at 20 000 theronts fish⁻¹ for 2 h. At Hour 4, Day 2, and Day 4 post Ich exposure, fish in corresponding tanks were challenged with *S. iniae* at the rate of 10⁷ CFU ml⁻¹ for 1 h as described above. Four tilapia from Tank 13 were sampled at the time of *S. iniae* challenge to evaluate trophont development size. Caudal fin and gill filaments were clipped from each tilapia and compressed in wet mounts by applying pressure using a cover slip. Fifteen trophonts were randomly measured for each tissue per fish using a calibrated ocular micrometer with a compound microscope (Olympus). Most trophonts were assumed to be spherical in wet mount samples. If trophonts were not uniformly spherical, long and wide diameters (perpendicular to each other) were measured and averaged as diameter (*D*). Trophont volumes were calculated using the formula volume = $\pi \times D^3/6$ as described previously (Xu et al. 2000).

Statistical analysis. All data analysis was performed with SAS software (SAS Institute 1989). Median days to death (MDD) were calculated by the Lifetest procedure (Kaplan-Meier method). Mortalities and MDD of fish from different treatment groups were compared with Duncan multiple range tests. The relationships between Ich trophont doses and parasite infestation level, fish survival, and MDD were evaluated with Pearson correlation. Probabilities of 0.05 or less were considered significant.

RESULTS

Effect of parasite load on tilapia mortality after exposure to *Streptococcus iniae*

Tilapia showed a light parasite load (<50 trophonts fish⁻¹) when exposed to 10 000 theronts fish⁻¹ and medium infection (50–100 trophonts fish⁻¹) when exposed to 20 000 theronts fish⁻¹ (Table 1). The light to medium parasite loads caused no or low mortality in tilapia if the fish were not subsequently infected with *S. iniae*. Mortalities were less than 5% for fish

exposed to 0, 10 000, or 20 000 theronts fish⁻¹ without challenge with *S. iniae* (Table 1). Tilapia infected only with *S. iniae* showed a 20% cumulative mortality. The increase of parasite dose significantly increased fish mortality when fish were also exposed to the bacterial infection (correlation coefficient = 0.95, *p* < 0.01). Mortalities increased from 38% in tilapia exposed to Ich at 10 000 theronts fish⁻¹ to 88% in fish at 20 000 theronts fish⁻¹ following *S. iniae* exposure. Fish had the highest mortality when exposed to both parasite at 20 000 theronts fish⁻¹ and *S. iniae* infection.

A low mortality rate (<5%) commenced on Day 5 post Ich infestation in the group using a dose of 20 000 theronts fish⁻¹. The mortality reached a peak 7 to 8 d post Ich infestation or 3 to 4 d post challenge with *Streptococcus iniae*. The MDD was significantly shorter (*p* < 0.05) in fish exposed to Ich theronts at 20 000 theronts fish⁻¹ than fish exposed to 10 000 theronts fish⁻¹ (Table 1). The MDDs were 7.1 and 10.1 d, respectively, for fish exposed to 20 000, and 10 000 theronts fish⁻¹.

Fish died mainly in groups exposed to both Ich and *Streptococcus iniae*. *S. iniae* was isolated from 94.1% and 90.7% of moribund or dead tilapia exposed to Ich theronts at 10 000 and 20 000 theronts fish⁻¹, respectively (Table 2). Ich trophonts were found in the skin from 50% and 95% of fish exposed to Ich theronts at 10 000 and 20 000 theronts fish⁻¹, respectively.

Effect of development size of trophonts on tilapia mortality after exposure to *Streptococcus iniae*

Trophonts expanded in size significantly in both skin and gills (Table 3). The volumes of trophonts were 1.3 × 10⁴ μm³, 3.1 × 10⁵ μm³, and 2 × 10⁷ μm³ in skin at Hour 4, Day 2, and Day 4 post Ich infestation, respectively.

Table 1. *Oreochromis niloticus*. Cumulative mortality and median days to death (MDD) of Nile tilapia infested by different numbers of *Ichthyophthirius multifiliis* (Ich) theronts and subsequently exposed to *Streptococcus iniae* in Trial 1. Fish mortality was observed for 15 d post challenge with *S. iniae*. Within a given column, means (± SD) followed by different superscripts are significantly different (*p* < 0.05). Parasite loads of Ich trophonts in tilapia were evaluated as none, light (<50 trophonts fish⁻¹), medium (50–100 trophonts fish⁻¹), and heavy infection (>100 trophonts fish⁻¹). NA: not applicable

Theronts fish ⁻¹	Bacterial challenge	Parasite load	No. of dead fish	Mortality (%)	MDD
0	None	None	0	0 ± 0 ^a	NA
10000	None	Light	0	0 ± 0 ^a	NA
20000	None	Medium	2	4 ± 5.6 ^a	10 ± 1 ^a
0	<i>S. iniae</i>	None	10	20.0 ± 5.7 ^b	15 ± 3 ^b
10000	<i>S. iniae</i>	Light	19	38.0 ± 8.5 ^c	10 ± 2 ^a
20000	<i>S. iniae</i>	Medium	44	88.0 ± 5.6 ^d	7 ± 0 ^c

Table 2. *Oreochromis niloticus*. Number and percentage of moribund or dead tilapia positive for *Streptococcus iniae* or *Ichthyophthirius multifiliis* (Ich). Number of fish sampled, number positive, and percentage for Ich are shown in parentheses. Only moribund or dead fish were sampled during 10 d post challenge with *S. iniae* in Trial I

Theronts fish ⁻¹	Challenge	No. of fish sampled	Positive	% positive
20000	None	2 (2)	0 (2)	0 (100)
0	<i>S. iniae</i>	5 (4)	5 (0)	100 (0)
10000	<i>S. iniae</i>	17 (16)	16 (8)	94.1 (50)
20000	<i>S. iniae</i>	43 (40)	39 (38)	90.7 (95.0)

Table 3. Trophont development size in skin and gills of tilapia at Hour 4, Day 2, and Day 4 post theront exposure. Values are means \pm SD (N = 60). Within a given column, means followed by different superscripts are significantly different ($p < 0.05$)

Time	Trophonts in skin		Trophonts in gill	
	Diameter (μm)	Volume $\times 1000$ (μm^3)	Diameter (μm)	Volume $\times 1000$ (μm^3)
Hour 4	29 \pm 5 ^a	13 \pm 6 ^a	27 \pm 3 ^a	11 \pm 3 ^a
Day 2	81 \pm 17 ^b	312 \pm 202 ^b	58 \pm 10 ^b	108 \pm 55 ^b
Day 4	325 \pm 71 ^c	20490 \pm 13062 ^c	238 \pm 44 ^c	7799 \pm 4731 ^c

Table 4. *Oreochromis niloticus*. Mortality of Nile tilapia infested with *Ichthyophthirius multifiliis* (Ich) theronts and challenged with *Streptococcus iniae* at Hour 4, Day 2, and Day 4 post Ich infestation. Cumulative mortality of fish was observed for 15 d post challenge with *S. iniae*. Within a given column, means (\pm SD) followed by different superscripts are significantly different ($p < 0.05$). MDD: median days to death; NA: not applicable

Theronts fish ⁻¹	Challenge	Time	No. of dead fish	Mortality (%)	MDD
0	None	Hour 4	0	0 \pm 0 ^a	NA
0	<i>S. iniae</i>	Hour 4	2	5.0 \pm 0 ^b	10 \pm 8
20000	None	Hour 4	0	0 \pm 0 ^a	NA
20000	<i>S. iniae</i>	Hour 4	4	10.0 \pm 7.1 ^b	7 \pm 1
20000	<i>S. iniae</i>	Day 2	12	30.0 \pm 15.0 ^c	7 \pm 1
20000	<i>S. iniae</i>	Day 4	19	47.5 \pm 7.5 ^d	9 \pm 1

The size of trophonts in the skin increased 24-fold in volume from Hour 4 to Day 2 and 66 times from Day 2 to Day 4. Similar size increments of trophonts were also noted in gills at Hour 4, Day 2, and Day 4 post exposure.

When fish were infested with Ich theronts and then challenged with *Streptococcus iniae* on the same day, fish showed low mortality (Table 4). Fish mortalities significantly increased when subsequently exposed to *S. iniae* on Day 2 or Day 4 post Ich infestation. A positive correlation ($r = 0.83$, $p < 0.05$) was noted between tilapia mortality and size of Ich at time of *S. iniae* chal-

lenge. Fish mortalities increased from 10.0% to 47.5% when fish were challenged with *S. iniae* from Hour 4 to Day 4 post Ich infestation. Fish parasitized with well-developed trophonts (Day 4, $2 \times 10^7 \mu\text{m}^3$ in volume) suffered higher mortality than fish infested by young trophonts (Hour 4, $1.3 \times 10^4 \mu\text{m}^3$) after *S. iniae* challenge.

DISCUSSION

Ich infestation causes direct damage to fish skin and gills and leads to fish mortality. However, it is not clear whether fish with non-lethal Ich infestation (low parasite number) suffer mortality following bacterial infection. Here we investigated the effect of parasite load and trophont size on tilapia mortality following *Streptococcus iniae* exposure.

Our results demonstrated that the mortalities increased in fish with a high parasite load when compared to fish having a low parasite load after tilapia were exposed to an equal number of *Streptococcus iniae* and subjected to the same exposure conditions. Tilapia showed light (<50 trophonts fish⁻¹) and medium (50–100 trophonts fish⁻¹) infestation of Ich when exposed to Ich at doses of 10000 and 20000 theronts fish⁻¹, respectively. Infection by the parasite alone caused no or low mortality at these infestation levels. Tilapia are more resistant to ichthyophthiriasis than other warm-water-cultured fish (Subasinghe & Sommerville 1986, Xu et al. 2008). In a previous study, tilapia were exposed to 40000 to 80000 theronts fish⁻¹ in 2 challenge trials (Xu et al. 2008). In the current study, mortalities increased from 38% in tilapia exposed to Ich at 10000 theronts fish⁻¹ to 88% in fish exposed to 20000 theronts fish⁻¹ when fish were further exposed to *S. iniae*. It seems reasonable that more parasites create more lesions in the skin and gills which in turn help *S. iniae* to enter fish tissues more easily.

This study also demonstrated that trophont size enhanced bacterial invasion. Fish mortalities increased from 10.0% to 47.5% when fish were challenged with *Streptococcus iniae* from Hour 4 to Day 4 post Ich infestation. Fish parasitized with well-developed trophonts (Day 4, $2 \times 10^7 \mu\text{m}^3$ in volume) suffered higher mortality than fish infested by young trophonts (Hour 4, $1.3 \times 10^4 \mu\text{m}^3$) after *S. iniae* challenge. The newly established trophonts had a small volume and caused less damage to fish tissues. Ich trophonts were observed to rotate within tissues after penetration into the skin or gills. Continuous rotation of trophonts created tissue vesicles and damaged fish skin and gills (Xu et al. 2000). The increase in trophont volume caused more tissue damage in fish. The relocation of

trophonts within skin or gill and exiting of trophonts from the host resulted in empty intercellular spaces at the original settlement sites. Surface mucus and skin in fish are the first line of physical defense against bacteria, as they contain antibacterial factors. Earlier studies suggested that damaged skin, lesions, and ulcers were putative routes for bacterial invasion and subsequent manifestation of disease (Kanno et al. 1990, Pylkkö et al. 2006, Evans et al. 2007, Xu et al. 2007). Parasite infestation has been shown to result in increased stress and depressed immune responses, which are believed to be linked to decreased disease resistance (Bowers et al. 2000, Tully & Nolan 2002). The exact pathogenesis mechanisms of coinfection by Ich and *S. iniae* need further study.

In naturally occurring infections, *Streptococcus iniae* transmission is thought to be by contact (Plumb 1999) and/or oral and olfactory routes (Shoemaker et al. 2000, Evans et al. 2001). Infections by *S. iniae* were difficult to establish by immersion transmission under laboratory conditions in tilapia unless the skin was scarified before bacterial exposure (Chang & Plumb 1996). In our study, captive tilapia infected with Ich theronts or tilapia infected with *S. iniae* had low mortality ($\leq 20\%$). However, fish mortality increased significantly with coinfection by Ich and *S. iniae*. The results are in agreement with several previous studies that demonstrated enhanced bacterial invasion by parasite infestation (Cusack & Cone 1986, Busch et al. 2003, Evans et al. 2007, Xu et al. 2007).

Our results could be considered in fish health management to aid in disease prevention. Necessary steps (such as parasite quarantine, good water quality, reduction of fish density, and early diagnosis) can be used to prevent the parasite from entering culture facilities or building up in fish. The combined efforts would not only reduce the direct damage by parasites but also minimize fish mortality due to secondary bacterial infection.

In summary, our results demonstrated that coinfections by Ich and *Streptococcus iniae* greatly increased tilapia mortality. Both parasite load and trophont size directly affected the mortality of tilapia following subsequent infection of *S. iniae*.

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