

Effects of temperature and salt concentration on *Francisella noatunensis* subsp. *orientalis* infections in Nile tilapia *Oreochromis niloticus*

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ABSTRACT: Little is known about the environmental conditions that allow *Francisella noatunensis* subsp. *orientalis*, a worldwide emergent bacterial fish pathogen, to colonize and infect wild and cultured fish. We evaluated the effect of temperature and salinity on the infectivity of *F. noatunensis* subsp. *orientalis* in Nile tilapia *Oreochromis niloticus* (L). Immersion challenges of tilapia with *F. noatunensis* subsp. *orientalis* at water temperatures of 25 and 30°C in both sea and fresh water were conducted for 14 d. Morbidity and mortality were recorded daily, and at the completion of the study, a quantitative assessment of the splenic bacterial burden was performed in surviving fish. Fish maintained at 25°C developed francisellosis and had considerably higher mortality and splenic bacterial concentrations compared to control fish and fish maintained at 30°C. Moreover, increasing the water temperature from 25 to 30°C prevented the development of clinical signs and mortality in *Francisella*-challenged fish. In conclusion, temperature significantly influenced the development of francisellosis in tilapia, whereas salinity had no effect. Our findings may be useful in the establishment of improved prophylactic practices and in the management of outbreaks of francisellosis in the aquaculture industry.

KEY WORDS: Francisellosis · Aquaculture · Environment · Pathogenesis

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INTRODUCTION

Francisella noatunensis subsp. *orientalis* (syn. *F. asiatica*), a recently described member of the genus *Francisella*, is the causative agent of piscine francisellosis in warmwater fish (Birkbeck et al. 2011, Colquhoun & Duodu 2011). Francisellosis has been diagnosed in several important aquaculture species such as tilapia (*Oreochromis* spp.), hybrid striped bass *Morone chrysops* × *M. saxatilis*, threeline grunt *Parapristipoma trilineatum*, and ornamental fishes in the USA (Hawaii, California, Florida, Texas, and midwestern states), Taiwan, Costa Rica, Haiti, Jamaica, the UK, and Japan (Birkbeck et al. 2011, Colquhoun & Duodu 2011, Soto et al. 2011). Due to increased incidence, high infectivity rates, and a

wide range of fish hosts, francisellosis is becoming one of the most important emergent diseases affecting the aquaculture industry (Birkbeck et al. 2011, Colquhoun & Duodu 2011). In tilapia, francisellosis can present as an acute syndrome with high mortality or as a subacute to chronic syndrome with non-specific clinical signs (Soto et al. 2011). The bacterium can be highly infectious in tilapia fingerlings. Intracoelomical injection of low numbers (<10 colony-forming units, CFUs) is enough to cause colonization and significant lesions in the anterior kidney and spleen, while injection of slightly higher numbers (23 CFUs bacteria) results in mortality (Soto et al. 2009a). In the USA, the lack of commercially available and Food and Drug Administration approved vaccines, antimicrobials, and chemicals for the pre-

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vention and treatment of piscine francisellosis makes it an important economic and health concern amongst aquaculturists.

The combination of a pathogen in the water, a susceptible host, and an unfavorable environment defines the pathogenesis of many fish infectious diseases (Snieszko 1973, Plumb & Hanson 2011). Fish are completely dependent upon water to breathe, feed, excrete wastes, maintain osmolality, and reproduce. In this sense, the physical and chemical qualities of the aquatic environment in general, and the water in particular, are critical to understanding the pathogenesis of fish diseases and to developing efficacious preventative practices and successful therapies. Due to the emergent nature of francisellosis in warm- and coldwater fish, little is known of the ecology of this bacterium in water. Moreover, to develop an in-depth understanding of the pathogenesis of *Francisella noatunensis* subsp. *orientalis* infection, one must understand the environmental conditions that optimize the survival, infectivity, and pathogenicity of this bacterium in the fish host. Our objectives were to evaluate the capability of *F. noatunensis* subsp. *orientalis* to infect tilapia at different temperatures and salinities, and to evaluate the survivability of latently infected tilapia under different environmental conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Francisella noatunensis subsp. *orientalis* LADL 07-285A was isolated from cultured tilapia (*Oreochromis* sp.) as described previously (Soto et al. 2009b). The isolate was recovered from infected spleens presenting splenomegaly and multifocal white nodulations. The identity of the bacterium was confirmed following amplification and sequencing of the 16S rRNA and intracellular growth loci C gene (Soto et al. 2009a,b).

Bacteria were grown on Thayer Martin II plates (Becton Dickinson [BD] BBL) for 72 h at 27°C. The broth medium consisted of a modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL) and 0.1% glucose (MMH; Baker et al. 1985). Broth cultures were grown overnight at 25 or 30°C in a shaker at 175 rpm.

Growth curves of *Francisella noatunensis* subsp. *orientalis* were determined by inoculating triplicate culture flasks containing 50 ml MMH broth with

500 µl of a 0.5 McFarland suspension. The cultures were incubated at 25 or 30°C for 72 h on an orbital shaker (150 rpm), and growth was monitored at 0, 12, 24, 48, and 72 h post inoculation by measuring the optical density at 600 nm and dilution plating. Briefly, a sample from each flask was taken for bacterial plate counts. Serial dilutions of this bacterial preparation were used to assess plate counts. Each dilution was spread over agar plates and incubated at 27°C for 96 h. The mean number of the colonies counted was used to calculate CFU ml⁻¹. CFUs were expressed as the mean ± standard error of mean (SEM).

Fish

Nile tilapia *Oreochromis niloticus* fingerlings (mean weight 15.4 g for infectivity challenge or 28.7 g for latent infection challenge) were obtained from the St Kitts & Nevis Aquaculture Pilot Project and had no previous history of *Francisella noatunensis* subsp. *orientalis* infection. A sub-sample of the population (n = 20) was confirmed as negative for *F. noatunensis* subsp. *orientalis* infection by clinical, bacteriological, and molecular analysis as previously described (Soto et al. 2010). Briefly, fish were euthanized in tricaine methanesulfonate (FINQUEL MS-222, Argent Chemical Laboratories), at a dose of 100 mg l⁻¹, and a complete necropsy was performed in each individual fish to look for any morphological evidence of disease. The spleen was harvested, homogenized in 1× phosphate-buffered saline (PBS), and plated in Thayer Martin agar and tryptic soy agar supplemented with 5% sheep blood (blood agar) plates to ensure the fish were free of bacterial pathogens. Moreover, the total genomic DNA was extracted from ~20 mg of each recovered spleen, using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's directions, and DNA served as the template for a TaqMan real-time PCR assay of the intracellular growth loci C gene of *F. noatunensis* subsp. *orientalis* (Soto et al. 2010). Fish were maintained at 6 or 10 fish tank⁻¹ in 40 l aquarium tanks containing ~36 l unchlorinated fresh or seawater and fed commercial tilapia feed 3 times per week (Burriss Aquaculture Feeds) at 2% fish body weight per day. Each tank contained individual air stones and carbon filters (Marina-Hagen). The filters in each tank were replaced weekly, and water quality parameters (pH, parts per million [ppm] total hardness, ppm total alkalinity/buffering capacity, ppm nitrite, ppm nitrate, and ppm total ammonia) were measured 3 times per week using commercial

strips (Mardel-Sergeant's Pet Care Products). Fish were acclimatized for at least 1 wk prior to challenge.

Infectivity challenge

Eight different challenge treatments were investigated. Briefly, fish were maintained in fresh or marine water at either 25 or 30°C at a stocking density of 10 fish tank⁻¹, and were challenged with bacteria grown in MMH at either 25 or 30°C. Fish were randomly assigned to aquaria; each treatment group contained 3 replicate tanks. The day of the challenge, $\sim 10^8$ *Francisella noatunensis* subsp. *orientalis* CFU were added to each aquarium to achieve a final concentration of $\sim 10^4$ CFU ml⁻¹ of tank water. Triplicate tanks of non-infected treatments served as negative controls; they were maintained in fresh or marine water at 25 or 30°C and were treated the same as challenged fish without the addition of *F. noatunensis* subsp. *orientalis*.

Fish in each tank were observed twice daily during the 1 wk acclimation period and during the 2 wk post challenge period. Mortality in each tank was recorded twice daily, and feed was adjusted daily to avoid water quality deterioration. At the end of the challenge, 5 surviving fish from each treatment were euthanized and the spleen was harvested for CFU determination. Organs were weighed, homogenized in 0.3 ml sterile PBS, plated in triplicates on agar media, and incubated at 25°C for 4 d prior to CFU determination. For comparison, spleen weights from healthy non-infected animals were also evaluated. CFU were expressed as the mean \pm SEM.

Latent infection challenge

To evaluate the effect of water temperature on the transition of *Francisella noatunensis* subsp. *orientalis* from a latent stage to clinical infection, 14 recirculating freshwater tanks, with 6 fish tank⁻¹, were maintained at 25°C for 2 wk. Eight tanks were challenged with $\sim 10^7$ *F. noatunensis* subsp. *orientalis* CFU to achieve a final concentration of $\sim 10^3$ CFU ml⁻¹ of tank water. The other 6 tanks served as non-infected controls. Four days post challenge, fish from 2 of the challenged tanks were euthanized with an overdose of MS-222, and the spleen from individual fish was collected, homogenized in 1 \times PBS, and plated in agar media. The splenic bacterial burden was estimated by counting CFUs 4 d post inoculation. Four days post challenge, the water temperature in 3 of the chal-

lenged tanks and 3 of the non-infected control tanks was increased to 30°C. The different treatment groups (triplicate tanks per group) consisted of: (1) challenged at 25°C and maintained at a constant temperature of 25°C, (2) challenged at 25°C and subjected to increase water temperature to 30°C 4 d post challenge, (3) negative control at 25°C and maintained at a constant temperature of 25°C, (4) negative control at 25°C and subjected to increased water temperature to 30°C 4 d post challenge. Triplicate tanks of non-infected treatments served as negative controls and were treated similarly as challenged fish without the addition of *F. noatunensis* subsp. *orientalis*.

Fish in each tank were observed twice daily during the 2 wk acclimation period and during the 3 wk post challenge period. Mortality events and bacterial loads in surviving fish were monitored as in the previously described challenge.

Francisella noatunensis subsp. *orientalis* genome equivalents (GE) were determined 0, 7, 14, and 21 d post challenge by collecting water from challenged tanks, and a previously described real-time PCR was used to quantify bacterial DNA loads in water (Soto et al. 2010).

Statistical analysis

The Statistical Analysis System (SAS Institute) was used with the general linear model procedure (PROC GLM) to conduct analysis of variance (ANOVA) of factorial arrangements of treatments. When the overall test indicated significance, pairwise comparisons of main effects were calculated with Tukey's test. Interaction effects were examined with a pairwise *t*-test comparison of mean square means. For the mortality studies, the percent mortalities were arcsine-transformed to normalize the data. CFUs recovered were log₁₀-transformed for statistical analysis. All comparisons were considered significant at $p < 0.05$.

RESULTS

Bacterial growth at different temperatures

Francisella noatunensis subsp. *orientalis* was able to grow at both 25 and 30°C in the broth media. Exponential growth was observed during the first 48 h post inoculation at both 25 and 30°C. Bacteria grown at both temperatures achieved stationary phase 72 h post inoculation.

Infectivity challenge

Water quality parameters were maintained within the appropriate ranges described for tilapia (Popma & Masser 1999). After statistical analysis of the mortality events and bacterial loads in surviving fish, the only variable with a significant effect was the water temperature ($p < 0.0001$, $F = 40.89$, $df = 1$). The temperature at which the bacteria were grown in the broth media and water salinity did not show any significant effect ($p = 0.19$, $F = 1.86$, $df = 1$; and $p = 0.47$, $F = 0.54$, $df = 1$, respectively).

Fish maintained at 25°C succumbed to disease and had a significantly higher mortality and bacterial concentration in the spleen than fish maintained at 30°C ($p < 0.0001$; Fig. 1). Fish maintained in fresh-water at 25°C and challenged with bacteria grown at 25 or 30°C had mean mortality of $66.6 \pm 11.8\%$ and $46.6 \pm 7.20\%$, respectively. Similarly, fish maintained at 25°C in seawater and challenged with bacteria grown at 25 or 30°C had mean mortality of $56.6 \pm 18.8\%$ and $33.3 \pm 10.8\%$, respectively. Moreover, splenic bacterial concentrations in surviving fish were similar for all challenged fish maintained at 25°C. Fish maintained at 25°C in fresh or seawater and challenged with bacteria grown at 25 or 30°C had 3.79 ± 0.3 , 5.13 ± 1.4 , 3.15 ± 0.9 , and 5.26 ± 0.6 log(CFU) mg^{-1} of spleen, respectively. Typical clinical signs (anorexia, skin lesions, abnormal swimming) and gross pathological findings (splenomegaly and renomegaly with multifocal white nodulations throughout the spleen, anterior and posterior kidney) were observed in fish maintained at 25°C.

Fish maintained in marine or fresh water at 25°C were similarly affected by *Francisella noatunensis* subsp. *orientalis* and presented equivalent mortality rates and persistent infection of the spleen. On the other hand, fish maintained at 30°C had no mortality during the experimental challenge, presented no pathological findings upon necropsy, and no *F. noatunensis* subsp. *orientalis* was recovered from the spleens of survivors (Fig. 1).

Latent infection challenge

Four days post challenge, 7 out of 12 fish contained viable *Francisella noatunensis* subsp. *orientalis* in the spleen. The mean log(CFU) mg^{-1} of spleen in these fish was 1.5 ± 0.14 . However, there were no apparent gross changes in the spleen. Three weeks post infection, fish maintained at a constant temperature of 25°C developed typical clinical signs (anorexia, skin lesions, abnormal swimming) and gross pathological findings (splenomegaly and renomegaly with multifocal white nodulations throughout the spleen, anterior and posterior kidney) of francisellosis. Moreover, significantly greater ($p < 0.0001$) cumulative mortality and higher splenic bacterial concentration of surviving fish occurred in these tanks than in fish subjected to an incremental increase in water temperature to 30°C 4 d post challenge (Fig. 2). Fish subjected to an incremental increase of water temperature to 30°C 4 d post challenge had no mortality, no lesions were found at necropsy, and no *F. noatunensis* subsp. *orientalis* were recovered from the

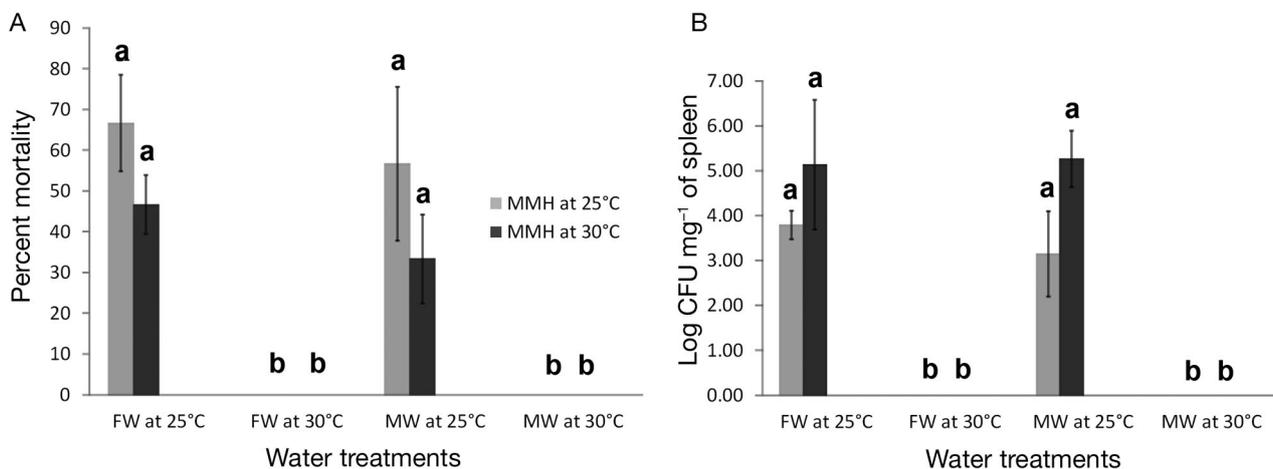


Fig. 1. *Oreochromis niloticus*. (A) Mean percent cumulative mortality in triplicate tanks containing 10 fish tank^{-1} challenged with *Francisella noatunensis* subsp. *orientalis* $\sim 10^4$ CFU ml^{-1} in either fresh (FW) or marine water (MW) at 25 or 30°C. Error bars represent SEM. Letters denote statistical differences among treatments ($*p < 0.05$). (B) *F. noatunensis* burden in spleens of surviving tilapia. Error bars represent SE of CFU mg^{-1} tissue weight

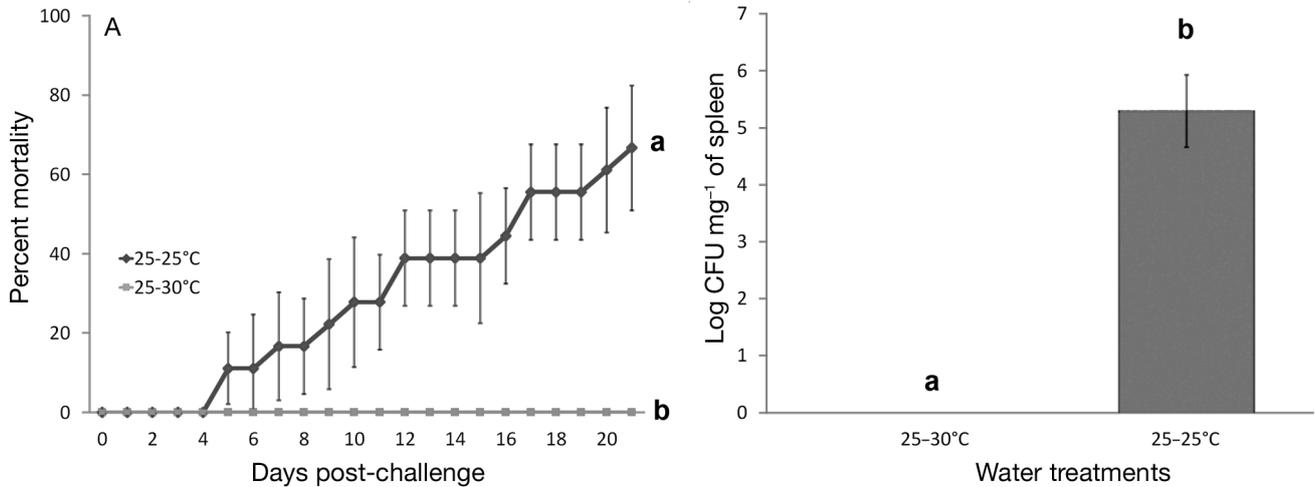


Fig. 2. *Oreochromis niloticus* challenged with *Francisella noatunensis* subsp. *orientalis*. (A) Mean percent cumulative mortality in triplicate tanks containing 6 latently infected fish tank⁻¹ in fresh water. Different challenged groups (triplicate tanks per group) consisted of fish challenged at 25°C and maintained at a constant temperature of 25°C (25–25°C) and fish challenged at 25°C and subjected to increased water temperature to 30°C 4 d post challenge (25–30°C). Error bars represent SEM. Letters denote statistical differences among the different treatments (**p* < 0.05). (B) *F. noatunensis* burden in spleens of surviving tilapia. Error bars represent SE of CFU mg⁻¹ tissue weight

spleens of survivors. Furthermore, significantly greater *F. noatunensis* subsp. *orientalis* GE were detected in the water of challenged fish maintained at a constant temperature of 25°C than those subjected to an incremental increase of water temperature to 30°C 4 d post challenge (Fig. 3).

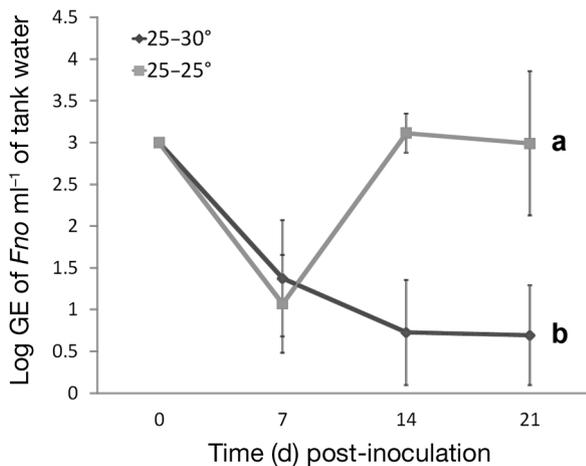


Fig. 3. *Francisella noatunensis* subsp. *orientalis*. Persistence of *F. noatunensis* (*Fno*) genome equivalents (GE) in tank water of tilapia challenged by immersion exposure. Different challenged groups (triplicate tanks per group) consisted of fish challenged at 25°C and maintained at a constant temperature of 25°C (25–25°C) and fish challenged at 25°C and subjected to increase water temperature to 30°C 4 d post challenge (25–30°C). Error bars represent SE of triplicate samples. Letters denote statistical differences among treatments (**p* < 0.05)

DISCUSSION

Both temperature and salinity play a role in the development of several diseases in cultured fish, such as edwardsiellosis caused by *Edwardsiella tarda* in Japanese flounder *Paralichthys olivaceus* and Japanese eel *Anguilla japonica* (Zheng et al. 2004, Hossain et al. 2011). Water temperature also plays an important role in the development of enteric septicemia of catfish (ESC) caused by *E. ictaluri* (Francis-Floyd et al. 1987, Plumb & Shoemaker 1995). For example, channel catfish latently infected with *E. ictaluri* and held at 15°C had significantly greater mortality when the water temperature was raised to 25°C than when it remained constant at ~15°C, or even when it was increased to 30°C (Plumb & Shoemaker 1995). Based on our results, an incremental increase in water temperature of only 5°C, from 25 to 30°C, prevented the development of clinical signs, gross changes, and mortality events in fish infected with *Francisella noatunensis* subsp. *orientalis*. On the other hand, fish that were challenged with the same number of bacteria but in which water temperature remained constant developed significantly greater mortality events due to francisellosis. In our study, cumulative mortality and splenic bacterial concentrations in surviving fish 14 d post challenge indicated that water temperature is a significant factor in the development of francisellosis in tilapia fingerlings. Fish maintained at 25°C had significantly (*p* < 0.001) higher mortality than those

maintained at 30°C, regardless of salinity or the temperature at which the bacteria were incubated (25 or 30°C). This is in accordance with the prior observation that the incidence of francisellosis in cultured tilapia is higher when the water temperature is <26°C (Mauel et al. 2003). Interestingly, salinity does not appear to play a significant role in the development of francisellosis in tilapia since fish maintained in fresh water were as susceptible as those maintained in seawater.

It appears that environmental conditions (temperature, pH, salinity) in the water may favor the expression of virulent factors in certain bacterial pathogens (Ebanks et al. 2006, Fernández et al. 2007, Rogge & Thune 2011). For example, it has been reported that temperature induces the expression of virulence factors in the fish pathogen *Yersinia ruckeri*, the causative agent of enteric redmouth disease or yersiniosis in salmonid fish. Studying outbreaks of this disease, Fernández et al. (2007) found higher levels of expression of the gene encoding a putative protein involved in the secretion/activation of a hemolysin, when outbreaks occurred at 18°C rather than at 28°C, the optimal growth temperature of this bacterium *in vitro*.

Environmental conditions, including water temperature, can play a critical role in the development of both specific and nonspecific immunity in cold-blooded animals, such as fish (Collins et al. 1976, Hrubec et al. 1996, Watts et al. 2001). Following vaccination of rainbow trout *Oncorhynchus mykiss* with a bacterin of *Yersinia ruckeri*, the upregulation of cytokine genes was generally faster and higher at high water temperature, with major expression at 25°C (Raida & Buchmann 2007). Temperatures of 10 and 18°C decreased the magnitude and delayed the time of the antibody response to *Aeromonas salmonicida* in sunshine bass *Morone chrysops* × *M. saxatilis* when compared to fish reared at 24 or 29°C (Hrubec et al. 1996). Ndong et al. (2007) investigated the susceptibility of tilapia *Oreochromis mossambicus* to *Streptococcus iniae* infections when the fish were subjected to temperature fluctuation stress. Total leucocyte count, respiratory burst phagocytic activity, phagocytic index, and alternative complement pathways significantly decreased when fish were transferred from water at 27°C to water at 19 or 23°C (Ndong et al. 2007). Moreover, tilapia became more susceptible to stress-induced streptococcosis when fish were subjected to a rapid temperature change (Ndong et al. 2007). In this experiment, the fish were subjected to a 1 wk acclimation period, and although cortisol as a stress indicator was not measured, the

normal swimming pattern and normal appetite of the fish together with the absence of dead animals in any of the control treatments indicated that fish were not stressed.

In our study, the specific mechanisms that underlie the increased pathogenicity of *Francisella noatunensis* subsp. *orientalis* at lower water temperatures in challenged tilapia remain unclear. Future proteomic analysis of *F. noatunensis* subsp. *orientalis*, upon culture and incubation under different conditions, should elucidate the expression of different virulence factors and immunogens that may play a pathogenic role. Proteomic analysis of the mammalian pathogen *F. tularensis* grown at different temperatures confirmed the significantly decreased expression of virulent factors, like the pathogenicity determinant protein C (PdpC) and the intracellular growth proteins C and D (IglC and IglD), at 25°C as compared with 37°C (Lenco et al. 2009), indicating that in this particular case, the expression of virulence factors by the pathogen is favored at normal mammal body temperature.

A better understanding of the tilapia innate and adaptive immune responses could benefit the development of environmentally friendly prophylactic and therapeutic practices, thereby reducing the chemical and antimicrobial residues generated by the aquaculture industry. Progress in this area will benefit not only wild fish populations but other aquatic animals, and possibly terrestrial animals, including humans.

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