Additive genetic variation in resistance of Nile tilapia (*Oreochromis niloticus*) to *Streptococcus iniae* and *S. agalactiae* capsular type Ib: Is genetic resistance correlated?

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ABSTRACT

Streptococcus iniae and *S. agalactiae* are both economically important Gram positive bacterial pathogens affecting the globally farmed tilapia (*Oreochromis* spp.). Historically, control of these bacteria in tilapia culture has included biosecurity, therapeutants and vaccination strategies.

Genetic gains in performance traits have been realized for Nile tilapia (*Oreochromis niloticus*) and interest in breeding for disease resistance has recently received attention. The goal of this study was three fold: 1) to verify previous results demonstrating heritability of *S. iniae* resistance in Nile tilapia families using increased numbers of fish per family; 2) to determine if realized genetic gain in resistance and/or susceptibility to *S. iniae* can be obtained following positive assortative mating between parents with high or low estimated breeding values (EBV); and 3) to determine if resistance to *S. iniae* and *S. agalactiae* capsular type Ib is genetically correlated. A total of 144 and 130 full sib families were challenged intraperitoneally with *S. iniae* and intramuscularly with *S. agalactiae* Ib, respectively. Cumulative mortality at test end was 46% for *S. iniae* and 68% for *S. agalactiae*. There was a high additive genetic component found for survival in fish injected with *S. iniae* (estimated heritability 0.52 ± 0.12) and with *S. agalactiae* (estimated heritability 0.38 ± 0.11). The *S. iniae* challenge results confirmed additive genetic variation in resistance of Nile tilapia to *S. iniae*. We also demonstrated via assortative mating that genetic gain for survival to *S. iniae* is possible. The genetic correlation between resistance to *S. iniae* and *S. agalactiae* Ib was not significantly different from zero ($r_g = -0.30 \pm 0.19$). The lack of correlation suggests if resistance to both *Streptococcus* sp. is desired, selection for both traits must be simultaneous. Selection of fish to improve survival to *Streptococcus* sp. may require a thorough understanding of the type of pathogen prevalent in the region so that custom genetic material may be tailored to meet the needs of the individual farm and/or region.

Keywords: *Streptococcus iniae*; *Streptococcus agalactiae* Ib; selective breeding; Nile tilapia

1. Introduction

Worldwide tilapia (*Oreochromis* spp.) sales were estimated at US \$7.6 billion in 2012 (FAO, 2014). Production is set to expand with estimates of production nearing 5,000,000 t (<http://ag.arizona.edu/azaqua/ista/markets.htm>, accessed March 4, 2016). Tilapia mortality due to climatic events and disease were recently suggested to be limiting growth of tilapia aquaculture (Thodesen et al. 2013). *Streptococcus iniae* (a non-groupable *Streptococcus* sp.; Pier and Madin, 1976) and *S. agalactiae* (group B *Streptococcus*; Vandamme et al. 1997) are economically significant Gram positive bacterial pathogens of tilapia (Eldar et al. 1994; Shoemaker et al. 2001; Agnew and Barnes 2007; Mian et al. 2009; Evans et al. 2015; Kayansamruaj et al. 2015; Soto et al. 2015; Delannoy et al. 2016) and have also been associated with potential for zoonotic infections (Weinstein et al. 1997; Liu et al. 2013; Delannoy et al. 2013). These reports highlight the negative impact of these emerging and/or reemerging diseases in farm raised tilapia. Tilapia are highly susceptible to *S. agalactiae* and capsular type Ib is virulent with lethal dose 50 values via intraperitoneal (ip) injection as few as 40 to 1000 colony forming units (cfu) fish⁻¹ (Evans et al. 2015; Soto et al. 2015; Delannoy et al. 2016). Capsular type Ib isolates appear to be associated with clonal complex 552 that has not been reported from human cases of *S. agalactiae* (Delannoy et al. 2013; Rosinski-Chupin et al. 2013; Delannoy et al. 2016). Vaccination has been utilized to prevent *S. iniae* and *S. agalactiae* infection; however, the most effective method is via injection delivery (Eldar et al. 1997; Evans et al. 2004; Sheehan et al. 2009; Locke et al. 2010; Shoemaker et al. 2010; LaFrentz et al. 2011; Chen et al. 2012) that may not be cost effective. Other fish health management strategies such as a reduction of fish density (Shoemaker et al., 2000) and use of antibiotics (Gaunt et al., 2010) to control the

infection have been practiced. Prevention and control of streptococcal disease in tilapia will require an integrated fish-health management strategy.

Selective breeding for growth and resistance to disease is a complimentary strategy for improving fish performance and health. LaFrentz et al. (2016) recently demonstrated that resistance to *S. iniae* is heritable (heritability estimate = 0.42 ± 0.07 , observed binary scale) in Nile tilapia (*O. niloticus*). This is similar to reports in other fish species and pathogens illustrating that resistance to infectious disease can be moderately heritable (Silverstein et al., 2009; Leeds et al. 2010; Ødegård et al., 2011; Evenhuis et al., 2015). Research has illustrated the potential for genetic improvement of disease resistance in fish without adversely affecting performance (Bangera et al. 2011; Ødegård et al., 2011; Wiens et al., 2013). The goal of the study reported here was three fold: 1) to verify previous results demonstrating heritability of resistance to *S. iniae* in Nile tilapia using increased numbers of fish per family; 2) to determine if realized genetic gain in resistance and/or susceptibility to *S. iniae* can be obtained following positive assortative mating between parents with high or low estimated breeding values (EBV); and 3) to determine if resistance to *S. iniae* and *S. agalactiae* capsular type Ib is genetically correlated.

2. Materials and methods

2.1. Fish, rearing conditions and transport

Fish from the fourth generation (G4B1) of the Spring Genetics Nile tilapia breeding program were used for this study. Further details of the genetic material are provided in LaFrentz et al. (2016). A total of 144 full sib families were produced in June 2015 using 72 sires and 144 dams from the previous generation. Families were produced by natural mating in single

pair breeding units over a period of 18 d. A hierarchical nested design was used where each male was mated with two different females. Most of the families were produced using parents with the highest EBVs for growth (LaFrentz et al. 2016). However, 12 of the families were produced by positive assortative mating between parents within the 6 best and 6 worst families ranked according to their EBV for survival to *S. iniae* using the linear model (LaFrentz et al. 2016).

On average 61 d after egg collection fish from all families were pit-tagged and representatives from all families were communally stocked in two separate test units prior to transport from Spring Genetics' nucleus breeding center in Homestead, FL to the Aquatic Animal Health Research Unit (AAHRU) in Auburn, AL. Two groups of fish were transported. Sex was not recorded for the fish in the first group destined for *S. iniae* challenge (avg. weight, 29.5 g [sd = 14.9]), since they were too young to record sex visually in a reliable way. Sex was recorded for all fish in the second group destined for *S. agalactiae* challenge (avg. weight, 179 g [sd = 74.6]).

2.2. Fish rearing conditions at AAHRU

2.2.1. Group 1 fish for *S. iniae* challenge

Upon arrival at the AAHRU, the tilapia for the *S. iniae* challenge were stocked into eight 340 L troughs for acclimation (~ 356 fish trough⁻¹) by placing transport water into the receiving tanks via pump. Afterwards, fish were netted into each tank and the local water supply was added to each tank ($\sim 0.5 - 1$ L min⁻¹) to allow for a slow increase in water temperature (26 ± 2 °C) and adjustment to local water quality conditions. The fish were acclimated for a period of 20 d prior to bacterial challenge and fed daily (2-3% body weight) with Aquamax Grower feed

(PMI Nutrition International, Inc., Brentwood, MO, USA). The PIT tag number and date of death was recorded using an ISO handheld reader (ARE H5; AEG ID, Ulm, Germany) for mortalities that occurred during the acclimation period. The tilapia family information and PIT tag numbers were not provided to AAHRU in order for the experiment to be blinded. Water supply to the tanks was de-chlorinated city water at 26 ± 2 °C with flow rates that allowed for the tank water to be exchanged ~ 3 times every 24 h for acclimation. Upon challenge tilapia were moved to a 5500 L tank receiving ~ 13 L min⁻¹ with water quality similar to LaFrentz et al. (2016). All procedures utilizing fish were approved by the USDA-ARS, AAHRU Institutional Animal Care and Use Committee.

2.2.2. Group 2 fish for *Streptococcus agalactiae* challenge

Following arrival at the AAHRU, the tilapia for the *S. agalactiae* challenge were acclimated for 13 d by stocking into sixteen 340 L troughs due to their larger average size (~170 fish trough⁻¹). All procedures were as described in the previous section.

2.3. *Streptococcus iniae* growth conditions

An archived stock of *S. iniae* generated following passage through tilapia was used for the *S. iniae* challenge (LaFrentz et al. 2016). Briefly, 50 µL of thawed stock was inoculated into each of seven - 50 mL tubes containing tryptic soy broth (TSB; BD, Sparks, MD, USA) and then incubated at 28 °C with shaking at 140 rotations per minute (rpm). After 24 h of growth, the tubes were aseptically combined into a single flask, the optical density (OD) measured at 540 nm was 1.3, and this culture was then diluted 1:5 for use in the bacterial challenge. The number of cfu mL⁻¹ was determined by spread plating 100 µL volumes of 10-fold serial dilutions (in duplicate) onto sheep blood agar (SBA; Remel, Lenexa, KS, USA). Plates were incubated for 48

h at 28 °C, and colonies were counted and averaged to enumerate the cfu mL⁻¹ using standard procedures.

2.4. *Streptococcus iniae* challenge

The average weight of fish was 29.5 g (sd = 14.9 g) determined ~ 25 d prior to *S. iniae* challenge (i.e., at transport). All tilapia (groups of 20-50 fish) were anesthetized in 18 L buckets with 120 mg L⁻¹ buffered tricaine methane sulfonate (MS-222; Argent Chemicals, Redmond, WA, USA) and injected ip with 100 µL of 1:5 diluted *S. iniae* culture using a 26 gauge 3/8 inch needle. Each fish received 5.31×10^7 cfu, a dose that resulted in ~ 50-70% mortality in preliminary experiments (data not shown). Prior to injection, the PIT tag number of each fish was recorded and following injection, fish were placed in clean water and transported to a 5,550 L tank to monitor recovery after handling and pathogen injection. Following challenge, fish were fed to satiation daily and dead fish were removed once or twice daily for 21 d and the PIT tag numbers and date of death were recorded. Re-isolation of *S. iniae* was attempted daily from a minimum of 20% of the dead fish by inoculating brain tissue onto SBA. Plates were incubated at 28 °C for 48 h and examined for the presence of beta-hemolytic colonies indicative of *S. iniae*. Upon termination of the challenge, all surviving fish were euthanized and PIT tag numbers were recorded.

2.5. *Streptococcus agalactiae* Ib identification, growth conditions and passage through fish

A virulent isolate of *S. agalactiae* LADL-05-108A was used for the bacterial challenge. This isolate was recovered from a diseased tilapia in Honduras that had been submitted to the Louisiana Animal Diagnostic Laboratory, Baton Rouge, LA (Soto et al. 2015). The capsular type of the isolate was confirmed based on the method of Imperi et al. (2010) with some

modifications. Briefly, the method consisted of a multiplex PCR using the following primers cpsL F/R; cpsG F/R and cpsG-2-3-6 R; cpsJ 2-4 F, cps J-2 R; and cps J Ib F/R that allow identification of the most common capsular types associated with aquaculture. The capsular type was also confirmed serologically using antisera directed against capsular types Ia, Ib, II and III (Denka Seiken Co., Ltd., Japan; data not shown). Both methods confirmed the LADL-05-108A isolate as capsular type Ib. The *S. agalactiae* isolate was grown for 24 h at 28 °C with shaking at 150 rpm in TSB and used to pass the isolate through fish. Briefly, 100 µL of the 24 h culture was injected into the peritoneal cavity (ip injection) of three to five tilapia (avg. weight, 48 g). At 24 h post-injection, brain tissue from moribund fish were aseptically plated onto SBA and incubated at 28 °C for 48 h. A colony of *S. agalactiae* recovered from fish was passed from the SBA plate into TSB, incubated, and used to inject another group of tilapia as described above. This process was repeated one additional time for a total of three passages through tilapia. After the final passage, a colony was passed into TSB, grown, and then 20% glycerol stocks were prepared and frozen at -80 °C. The isolate was confirmed as *S. agalactiae* capsular type Ib by PCR as described above.

2.6. *Streptococcus agalactiae* challenge

The frozen stock of *S. agalactiae* following passage through tilapia was used for the challenge. Briefly, 100 µL of thawed stock was inoculated into each of three 50 mL TSB broth tubes and then incubated at 28 °C with shaking at 150 rpm. After 24 h of growth, each of the tubes were aseptically combined into a single flask and the OD measured at 540 nm was 0.88. Seven replicate serial dilution series of the culture were prepared from 1:10 to 1:100000 in TSB and then the 1:100000 tubes were combined and placed on ice for use in injection of fish. The

number of cfu mL⁻¹ was determined by spread plating 100 µL volumes of 10-fold serial dilutions (in duplicate) onto SBA. Plates were incubated for 48 h at 28 °C, and colonies were counted and averaged to enumerate the cfu mL⁻¹ using standard procedures.

Intramuscular (im) challenge was used based on pilot studies that indicated delivery of 1,998 cfu fish⁻¹ resulted in 76% mortality at 21 d post challenge. Tilapia with an average weight at transport of 179.2 g (sd = 74.6) were anesthetized with 120 mg L⁻¹ buffered tricaine methanesulfonate and im injected with 1,850 cfu fish⁻¹ using a 26 gauge 3/8 inch needle. Briefly, groups of 20-50 fish were added to 18 L buckets containing anesthetic solution, anesthetized, removed with a net, pit tag recorded electronically, injected with *S. agalactiae* and then placed into a bucket containing clean water for transport to a single 5,500 L tank for monitoring of mortality post challenge. Fish were fed to satiation daily during challenge and dead fish were removed once or twice daily for 33 d and the PIT tag numbers and date of death were recorded. Re-isolation of *S. agalactiae* was completed on at least 20% of the fresh dead fish each day by culture of the brain on sheep blood agar. Plates were incubated at 28 °C for 48 h and then examined for bacterial colonies phenotypic of non-hemolytic *S. agalactiae*. A subsample of isolated bacteria was confirmed using capsular PCR typing as described above (data not shown). Upon termination of the challenge, all surviving fish were euthanized, examined for clinical signs of disease, and PIT tag numbers were recorded.

2.7. Data analysis

Survival at end of test (scored as 1 for fish alive and 0 for dead fish) was analyzed for both *S. iniae* and *S. agalactiae*. Fish from families with less than five fish were discarded from analysis. A total of 2681 records (representing 144 families) were used for *S. iniae* and 1900

records for *S. agalactiae* (representing 130 families).

Univariate animal linear (1) and sire-dam threshold (2) models were used to obtain estimates of variance components of random effects for survival by the Restricted Maximum Likelihood (REML) using the ASReml software (Gilmour et al., 2009).

2.7.1. Univariate linear animal model (Model 1)

Model 1 (Univariate linear animal model): $\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{Wc} + \mathbf{e}$; where $\mathbf{y}$ is the vector of the phenotypic observations for survival; $\mathbf{X}$ is a design matrix for the fixed effects that links individual observations to the fixed effect class; $\mathbf{b}$ is the vector with the estimates of the fixed effects (sex only for *S. agalactiae*, production week, weight at start of challenge test as a covariate); $\mathbf{Z}$ is an incidence matrix that links the observations to the animal additive genetic effects, $\mathbf{a} \sim N(\mathbf{0}, \mathbf{A}\sigma_a^2)$ is a vector of random animal additive genetic effect, $\mathbf{W}$ is an incidence matrix that links the observations to the random environmental effects common to full-sibs, $\mathbf{c} \sim N(\mathbf{0}, \mathbf{I}\sigma_c^2)$ is a vector of random environmental effects common to full-sibs other than additive genetics; and $\mathbf{e}$ is a vector of random residuals. Finally, $\mathbf{A}$ is the numerator relationship matrix that describes the additive genetic relationship among all individuals included in $\mathbf{a}$, and $\mathbf{I}$ is an identity matrix of appropriate size.

For Model 1 the significance of both the additive genetic effect and the effect common to full-sibs was assessed with the likelihood-ratio test (Lynch and Walsh, 1998):

$LR = -2(\text{Log}L_R - \text{Log}L_F)$, where $\text{Log}L_R$ is the log of the restricted likelihood of the reduced model (excluding each random effect at a time) and $\text{Log}L_F$ is the log of the restricted likelihood of the full model. The significance of LR was tested with a χ^2 test with $df = 1$ (number of omitted variance components) and $\alpha = 0.05$ for both random effects. For model 1, estimated

heritability for survival was calculated as $h^2 = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_a^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2}$, and the relative proportion of the

environmental effects common to full-sibs as $c^2 = \frac{\hat{\sigma}_c^2}{\hat{\sigma}_a^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2}$.

2.7.2. Univariate threshold sire-dam model (Model 2)

Model 2 (Univariate threshold sire-dam model): $\lambda = \mathbf{Xb} + \mathbf{Z}_S \mathbf{a}_S + \mathbf{Z}_D \mathbf{a}_D + \mathbf{Wc} + \mathbf{e}$; where λ_i is the underlying liability which is assumed to be associated to the binary observation of animal i (y_i) such that $\lambda_i \leq 0$ gives $y_i = 0$, and $\lambda_i > 0$ gives $y_i = 1$. The vector λ is a vector of all underlying liabilities. A Probit link function was used to relate the linear predictor scale to the observation scale for *S. iniae*. Due to problems with convergence, a Logit link function was used for *S. agalactiae*. The variance components were estimated on the underlying scale, where residual variance (σ_E^2) was restricted to 1.0, implying that $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I})$; $\mathbf{X}$, $\mathbf{b}$, $\mathbf{W}$, $\mathbf{c}$ and $\mathbf{I}$ are the same as described in Model 1; $\mathbf{Z}_S$ and $\mathbf{Z}_D$ are incidence matrices linking observations to the sire and dam additive genetic effects; $\mathbf{a}_S \sim N(\mathbf{0}, \mathbf{A}\sigma_S^2)$ and $\mathbf{a}_D \sim N(\mathbf{0}, \mathbf{A}\sigma_D^2)$ are vectors of the random sire and dam additive genetic effects, respectively; sire and dam variance are assumed to be equal $\sigma_S^2 = \sigma_D^2 = \sigma_u^2$. The effect common to full-sibs (c) included the environmental tank effects caused by separate rearing of the families until tagging and potential dominance and maternal effects. For each family, these effects are confounded, and thus fitted as a single random effect. Log-likelihood ratio tests cannot be used to test significance of random effects in the threshold models. For model 2, estimated heritability for survival was calculated as $h^2 = \frac{4\hat{\sigma}_u^2}{2\hat{\sigma}_u^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2}$,

and the relative proportion of the environmental effects common to full-sibs as

$$c^2 = \frac{\hat{\sigma}_c^2}{2\hat{\sigma}_u^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2}.$$

2.7.3. Genetic correlation between survival to *S. iniae* and *S. agalactiae*

The genetic correlation between survival to *S. iniae* and *S. agalactiae* was estimated using a bivariate model 1 (including sex effect only for *S. agalactiae*). The residual correlation was set to zero for the bivariate model 1 since only one trait was measured per fish. A reduced model where the additive genetic covariance between the two traits is assumed to be equal to zero was used to perform the likelihood-ratio test previously described and determine if the genetic correlation between both traits was significantly different from zero.

3. Results

3.1. Fish acclimation and *Streptococcus iniae* challenge

During transport and acclimation a total of 9 fish died. Accumulated mortality at the end of the challenge test was 46%. Most of the fish died on day 1 and 2 after injection and by day 5 mortalities were less than 1% daily (Figure 1). The challenge was terminated at day 21. Brain tissue samples were collected from 265 fish that died during the trial and 99% of these samples yielded pure cultures of *S. iniae*. There was high variation among mean family survival for fish injected ip with *S. iniae*, with survival ranging from 0 to 100%, and a coefficient of variation of 55% (Figure 2). The Pearson correlation between the mean family weight at shipment and *S. iniae* survival was negative but very low in magnitude and not statistically significant ($r = -0.10$, $P > 0.05$), suggesting that within the weight range for the families tested the initial weight of fish had little impact on survival.

3.2. Fish acclimation and *Streptococcus agalactiae* challenge

During transport and acclimation a total of 68 fish died, and most acclimation mortalities occurred shortly after shipment (data not shown). Accumulated mortality at the end of the challenge test with *S. agalactiae* was 68%. Mortalities began at 3 d post im injection, with the majority of the mortality occurring between days 5-11 after injection (Figure 1). After day 20 daily mortalities were less than 1%. Mortality for *S. agalactiae* was higher than for *S. iniae*, but *S. agalactiae* mortality curve was less acute (Figure 1). Brain tissue samples were collected from 297 fish that died during the trial and 99% of the fish samples collected yielded cultures of *S. agalactiae*. High variation among mean family survival for the fish injected with *S. agalactiae* (range from 0 to 100%, CV 72%) was observed (Figure 3). The Pearson correlation between the mean fish weight at shipment and *S. agalactiae* survival was close to zero ($r = -0.04$, $P > 0.05$), suggesting that weight of fish at shipment did not influence survival.

3.3 Additive genetic and environmental effect common to full-sibs

There was a high additive genetic component found for survival in fish injected with either *S. iniae* or *S. agalactiae* (Table 2). The heritability estimate in the observed scale was 0.52 ± 0.12 for *S. iniae* and 0.38 ± 0.11 for *S. agalactiae*. As expected, heritabilities were higher in the underlying liability scale, 0.74 ± 0.16 and 1.04 ± 0.10 for *S. iniae* and *S. agalactiae*, respectively. The additive genetic effect was significantly different from zero for both traits ($P < 0.001$; Log-likelihood ratio test Model 1). The environmental effect common to full-sibs was not significant for either trait ($P > 0.05$; Log-likelihood ratio test Model 1), suggesting that separate rearing, maternal or epistatic effects did not affect the survival of the fish to the *Streptococcus* sp. tested.

3.4 Phenotypic and genetic correlation between survival to *S. iniae* and *S. agalactiae*

The Pearson correlation between the phenotypic survival to *S. iniae* and *S. agalactiae* was negative but very low in magnitude and not statistically significant ($r = -0.07$, $P > 0.05$), suggesting there is no relationship between survival to *S. iniae* and *S. agalactiae*. The genetic correlation between *S. iniae* and *S. agalactiae* obtained with bivariate model 1 was not significantly different from zero ($r_g = -0.30 \pm 0.19$, $P > 0.05$ in Log-likelihood ratio test). When the number of full-sibs per family is high the correlation between the mid-parent EBV (using model 1) for *S. iniae* and *S. agalactiae* is close to the true genetic correlation between the traits. In this case this correlation was negative but not significant ($r = -0.08$, $P > 0.05$), confirming the lack of genetic relationship between survival to *S. iniae* and *S. agalactiae*.

3.5 *S. iniae* parental groups

Streptococcus iniae survival for the G4B1 families produced by assortative mating (see 2.1) is shown in Table 1. As expected based on the estimated heritability, offspring from families with the best *S. iniae* performance in 2014 had high survival in 2015 challenge test (average 88%). Similarly, families with the worst parental performance had low survival in 2015 (10%). The correlation between the mid-parent phenotypic *S. iniae* survival (based on data from 2014) and the offspring *S. iniae* survival (data from 2015) was positive and significant ($r = 0.63$, $P < 0.001$). The regression of progeny survival on mid-parent offspring phenotypic survival was 0.77, which is a rough approximation of the realized heritability. In contrast, results suggest no relationship between the survival to the *S. agalactiae* in 2015 and the G4B1 families produced by assortative mating based on *S. iniae* index (Table 1).

4. Discussion

The importance of tilapia to global aquaculture production has been increasing and led us to examine if selective breeding for resistance to *Streptococcus* spp. was possible. LaFrentz et al. (2016) pioneered the first disease resistance phenotyping of individual families in Nile tilapia against *S. iniae* and this report serves to validate and expand on the initial study. We have verified the first study results and confirmed the additive genetic variation in resistance of Nile tilapia to *S. iniae* using a larger number of fish per family (at least 14 fish represented for each family). The study reported here utilized smaller fish (29 g) as compared to larger sized fish (180 g) reported previously. The heritability estimate based on the observed binary scale was 0.52 ± 0.12 , similar to 0.42 ± 0.07 reported by LaFrentz et al. (2016). Although injection of fish bypasses the innate immune response to some degree (i.e., skin and mucosal defenses), injection challenge has been utilized for assessing disease resistance to many bacterial fish pathogens (e.g., Leeds et al. 2010; Gjedrem and Gjøen 1995; Yáñez et al. 2013). In some cases, the genetic resistance observed following injection challenge has mirrored resistance in field trials (Gjøen et al. 1997; Wiens et al. 2013). Immersion or cohabitation challenges with *S. iniae* or *S. agalactiae* often result in low grade mortality and inconsistent results (Shoemaker et al. 2000; Soto et al. 2015). Our studies utilized injection challenge to ensure that each fish received the same pathogen dose and also used a large tank where all fish were maintained in the same environmental conditions throughout the challenge test.

Twelve tilapia families were produced via assortative mating in this study. Six families were produced based on high parental breeding values for survival to *S. iniae* and six families were produced based on low parental breeding values for survival to *S. iniae* (Table 1). Results demonstrated that mean survival of the offspring was 88% (range 60 – 100%) for tilapia families

produced using the high parental breeding values and only 10% (range 0 – 42%) for families produced using poorer performing parents. These results clearly demonstrate that resistance to *S. iniae* is heritable and can be improved upon through selective breeding.

Streptococcus agalactiae has emerged as the predominant disease of tilapia (Mian et al. 2009; Chen et al. 2012; Delannoy et al. 2013; Evans et al. 2015). Due to the emergence of *S. agalactiae*, we hypothesized that disease resistance to *S. iniae* may be correlated with disease resistance to *S. agalactiae* due to the gross disease signs being similar (i.e., meningitis, cloudy eyes, pustule formation, exophthalmia, fecal casts, spiral swimming, etc.). In this regard, the same tilapia families were challenged with a *S. agalactiae* Ib isolate to examine for additive genetic variation in resistance to *S. agalactiae*. Results demonstrated substantial additive genetic variation for survival to *S. agalactiae* following im injection challenge with a heritability estimate of 0.38 ± 0.11 based on the observed binary scale derived from a linear animal model. Since cumulative end of test mortality was close to 50% in both traits, a simple cross sectional model such as Model 1 allows for accurate ranking of the families with respect to genetic merit (Ødegård et al. 2011).

For the *S. agalactiae* Ib challenge, we used im injection because pilot studies using ip injection were extremely lethal (< 100 cfu fish⁻¹ resulted in rapid and near 100% mortality, data not shown). Soto et al. (2015) and Delannoy et al. (2016) found similar results using ip injection challenge in gulf killifish (*Fundulus grandis*) and Nile tilapia, respectively. Our preliminary experiments showed a higher number of bacteria could be injected im that resulted in delayed mortality and reproducible results. Although, two different routes of challenge were used, results revealed no genetic correlation between resistance to *S. iniae* and *S. agalactiae* Ib.

No association was suggested when looking at the best and worst performing families for

S. iniae survival (Table 1). Evenhuis et al. (2015) reported a small, favorable genetic correlation in resistance between two *Flavobacterium* spp. in rainbow trout (*Oncorhynchus mykiss*); however, our results suggest no association for *S. iniae* and *S. agalactiae* Ib in tilapia. Therefore, selection for disease resistance to *S. iniae* is not expected to indirectly affect resistance to *S. agalactiae* Ib. Bangera et al. (2011) reported high heritabilities and low or no genetic correlation among growth rate, resistance to *Vibrio anguillarum* and resistance to viral nervous necrosis in Atlantic cod (*Gadus morhua* L.) and suggested that genetic improvement for all traits was feasible. Based on our results in Nile tilapia, if resistance for both *Streptococcus* spp. is desired, selection must be for both traits simultaneously.

These results in part confirm the difficulty to control streptococcosis in commercial aquaculture. Research suggests that *S. iniae* and *S. agalactiae* are antigenically distinct as vaccination with a *S. iniae* bacterin did not provide protection against *S. agalactiae* Ia challenge (Evans et al. 2004). Furthermore, two main biotypes of *S. agalactiae* have been identified and it has been demonstrated that immunity to *S. agalactiae* is biotype specific (Sheehan et al. 2009). In this study, the challenge isolate used was a non-hemolytic *S. agalactiae* Ib (similar to biotype 2 described by Sheehan et al. 2009) that appears to be the predominant type in Asia (China, Indonesia, Vietnam and Philippines) and Central, North and South America (Soto et al. 2015), and has recently been suggested to be fish associated and/or fish specific (Rosinski-Chupin et al. 2013; Delannoy et al. 2016). Future testing may include the other predominant capsular types (Ia and III; biotype 1) that are more prevalent in *S. agalactiae* infected tilapia in the Middle East, Thailand, Malaysia and Singapore (Evans et al. 2002; Sheehan et al. 2009; Liu et al. 2013; Kayansamruaj et al. 2015; Delannoy et al. 2016). Selection of fish to improve survival to *Streptococcus* sp. may require a thorough understanding of the type of pathogen prevalent in the

region so that custom genetic material may be tailored to meet the needs of the individual farm and/or region.

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Table 1

Survival results for the families produced with assortative mating for high and low resistance to *Streptococcus iniae*.

Assortative mating groups	Number of families	Avg_ <i>S. iniae</i> index parents*	Percentage survival to <i>S. iniae</i> in 2015			Percentage survival to <i>S. agalactiae</i> in 2015		
			Average**	Min	Max	Average***	Min	Max
none	132	101.6	54	0	100	31	0	89
yes_high	6	121.9	88	60	100	19	7	50
yes_low	6	80.2	10	0	42	40	18	59
Total	144							

*Index (mean = 100, sd=10). Avg = (sire index + dam index)/2

**Mean family survival to *S. iniae* during challenge test.

***Mean family survival to *S. agalactiae* during challenge test.

Table 2

Survival variance components, estimated heritability ($h^2 \pm se$) and effect common to full-sibs ($c^2 \pm se$) for the univariate linear animal model (Model 1) and the univariate threshold sire-dam model (Model 2) for traits (*Streptococcus iniae* and *S. agalactiae*).

<i>Parameter</i>	Model 1 (observed scale)		Model 2 (underlying scale)	
	<i>S. iniae</i>	<i>S. agalactiae</i>	<i>S. iniae</i> *	<i>S. agalactiae</i> **
σ^2 sire	-	-	0.326	0.544
σ^2 dam	-	-	0.326	0.544
σ^2 additive	0.126	0.083	1.305	2.175
σ^2 common env.	0.009	0.001	0.000	0.257
σ^2 e	0.109	0.136	1.000	1.000
$h^2 \pm se$	0.52 ± 0.12	0.38 ± 0.11	0.74 ± 0.16	1.04 ± 0.10
$c^2 \pm se$	0.04 ± 0.04	0.00 ± 0.04	0.03 ± 0.05	0.00 ± 0.00

* PROBIT link function

** LOGIT link function

Fig. 1. Cumulative percent mortality in Nile tilapia following challenge with *Streptococcus iniae* by intraperitoneal injection (●) and *S. agalactiae* Ib challenge by intramuscular injection (■).

Fig. 2. The number of fish per family (line; right y-axis) and mean survival (bars; left y-axis) of 144 Nile tilapia families following challenge with *Streptococcus iniae* by intraperitoneal injection. Families with less than five fish at the onset of the bacterial challenge were excluded from the data analysis (see section 2.7. *Data Analysis*).

Fig. 3. The number of fish per family (line; right y-axis) and mean survival (bars; left y-axis) of 130 Nile tilapia families following challenge with *Streptococcus agalactiae* by intramuscular injection. Families with less than five fish at the onset of the bacterial challenge were excluded from the data analysis (see section 2.7. *Data Analysis*).

Figure(s)

Figure(s)

Figure(s)