1 **Case study development of a challenge test against *Edwardsiella ictaluri* in Mekong**
2 **striped catfish (*Pangasianodon hypophthalmus*), for use in breeding: Estimates of the**
3 **genetic correlation between susceptibility in replicated tanks**

4 **Running title: Challenge testing striped catfish**

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21 **Abstract**

22 Bacillary necrosis is a problematic disease in farming of Mekong striped catfish
23 (*Pangasianodon hypophthalmus*). The pathogenic bacterium is *Edwardsiella ictaluri*, causing

numerous white spots in swelled liver, kidney and spleen. An alternative to antibiotic treatment and vaccine is to select for improved genetic resistance to the disease that require to establish a proper challenge test. Here, four challenge tests of Mekong striped catfish against *E. ictaluri* are reported proposing three days acclimatization of test fish prior to the challenge, with restricted water level in the test, keeping a temperature of 26^0 C. In the challenge, cohabitant shedders should be released directly into the test tank and make up around ⅓ of the fish, and bacteria should be added directly to water. The last two experiments, with the highest mortality, suggest that any factor involving the dead cohabitants should be removed, and that additional experimentation should focus on bacteria (density) and timing for addition of bacteria to water. Genetic analyses revealed that resistance to bacillary necrosis tested in replicated tanks in the same experiment can be considered the same genetic trait.

Keywords: Bacillary necrosis; test environment.

1. Introduction

The Mekong striped catfish (*Pangasianodon hypophthalmus*) farming in Vietnam has faced increasing disease problems, particularly bacillary necrosis (BN). Clinical signs are internal numerous white spots of different sizes in swelled liver, kidney and spleen (Crumlish, Dung, Turnbull, Ngoc & Ferguson 2002). These authors described the histopathological lesions as acute to sub-acute multifocal areas of necrosis and pyogranulomatous inflammation, with several species of parasites associating with these lesions. Specifically, a variable number of large bacilli are usually seen at the margins of lesions. BN belongs to Edwardsiellosis and is caused by the *Edwardsiella ictaluri* (*E. ictaluri*) bacteria, first observed in 1998, with its cause identified in 2002 (Crumlish, Dung, Turnbull, Ngoc & Ferguson 2002). The disease has been found in the whole Mekong Delta, in all life stages of catfish, but is especially frequent in the

47 fingerling period. Unless antibiotic treatment is timely applied, fish mortality may rise as high
48 as 90% (Dong & Hoa 2008). In 2010, the first BN vaccine trial was conducted by Pharmaq Ltd.
49 Vietnam (Thanh & Berntsen 2012). The trial was successful with significant lower mortality of
50 vaccinated than non-vaccinated groups. The Alpha Ject® Panga 1 vaccine was licensed in 2013
51 (https://www.pharmaq.no/sfiles/8/66/4/file/2013_08-cty-pharmaq-vn_thuy-san-nam-14-so-
52 164.pdf). However, improved resistance to BN using vaccination is often costly and impractical
53 since it must be repeated for each fish, and in each generation. At current, to treat BN, antibiotics
54 is used which may lead to resistance and potential contamination of the final product (Chuah,
55 Effarizah, Goni & Rusul 2016). Moreover, reduction of mortality through drug application is
56 costly and does not solve the problem permanently (van Muiswinkel, Wiegertjes & Stet 1999).

57 Besides development of vaccines, improvement of resistance to BN through selective breeding
58 can be used as a tool for sustainable disease control. This is aimed at in the Mekong striped
59 catfish breeding program initiated by the Research Institute for Aquaculture No. 2 (RIA2),
60 Vietnam. Initially in this program, three base sub-populations were created from fish being
61 domesticated in three hatcheries, making up year-classes 2001, 2002 and 2003, respectively
62 (Sang, Klemetsdal, Ødegård & Gjøen 2012). So far, selection has been carried out for final
63 body weight and fillet yield. Heritability estimates have been found moderate for body weight
64 (0.21 - 0.34), while low estimates have been obtained for fillet yield (0.03 - 0.05) (Sang,
65 Klemetsdal, Ødegård & Gjøen 2012). Recently, Vu, Sang, Phuc, Vuong & Nguyen (2019)
66 reported on the genetic response achieved in the program.

67 Development of a challenge-test for BN was initiated in 2009. The objective was to rank
68 individuals with respect to disease resistance towards *E. ictaluri*. The developments were based
69 on the cohabitation method, known *a priori* to be difficult to standardize (Nordmo, Sevatdal &
70 Ramstad 1997) and even not always successful (Mahapatra, Gjerde, Sahoo, Saha, Barat, Sahoo,

71 Mohanty, Ødegård, Rye & Salte 2008). However, Gjøen, Refstie, Ulla & Gjerde (1997) have
72 shown that the cohabitation method mimics a natural infection well.

73 Here, findings in four experiments with family materials of Mekong striped catfish challenged
74 with *E. ictaluri* are summarized. The aim was to establish a challenge-test applicable in the
75 breeding program including to examine the genetic correlation of resistance to BN between two
76 replicated test-tanks within the same experiment.

77 **2. Materials and methods**

78 By granting the research, the Vietnamese Ministry of Agriculture and Rural Development pre-
79 approved the experiments carried out.

80 ***2.1 Inoculums***

81 A strain of *E. ictaluri* Gly09M (Southern Monitoring Centre for Aquaculture Environment &
82 Epidemic, RIA2, Ho Chi Minh City, Vietnam) was used in all experiments. The strain was
83 isolated from diseased fish sampled in commercial striped catfish farms in the Mekong Delta
84 in 2009. Each year, reference stocks were sub-cultured and stored in a solution of Brain Heart
85 Infusion Broth (BHIB) and 20% glycerine supplementation, at -20^0 C. Working seed was
86 cultured in Sheep Blood Agar (SBA) and incubated for 24 hours at 30^0 C. Bacterial biomass
87 was suspended into a flask containing 500 ml BHIB, incubated with shaking for 18 hours. The
88 liquid culture was transferred into a 5 litter BHIB medium contained in a 10 liter fermenter,
89 cultured for 18 - 20 hours, stirred at 150 rounds/minute, and air supplied 5 vvm (in 1 minute, 5
90 liters of air are passing through 1 liter of medium). The density of bacteria was measured at 550
91 nm (OD_{550}) to calibrate the bacterial doses.

92 ***2.2 Cohabitant shedders***

93 Cohabitant shedders were randomly sampled from the hapas with the highest survival among
94 fish to be challenged, just prior to tagging of the latter fish. Cohabitants were not tagged while
95 test fish were, for discrimination. Shedders were acclimated in a separate tank and were
96 intraperitoneally injected at experimental day 0.

97 ***2.3 General test-environment: Tanks, water quality and feeding***

98 Experiment 1 was conducted in one outdoor fiber-glass tank at the National Breeding Centre
99 for Southern Freshwater Aquaculture (NABRECSOFA, in the Tien Giang province) in 2009
100 (Table 1). The tank was partly covered by net to prevent eutrophication. Experiments 2, 3 and
101 4 were carried out indoor in the Govap Experimental Centre (GEC), RIA2, in 2010, 2011 and
102 2012, respectively (Table 1). GEC is located 125 km away from NABRECSOFA, and the fish
103 were transported there by a lorry. In these three latter experiments, two replicated tanks were
104 used (Table 1). In all experiments, tagging was done when the fish were netted out of the
105 original hapas. In the last three experiments an equal number of randomly tagged fish from each
106 hapa were transferred to either of two tanks at NABRECSOFA, before transfer to GEC. In the
107 first experiment, tagged fish were kept in separate hapas for seven days prior to transfer to the
108 test tank.
109 The test tanks were of circular shape; diameter 4.0 m, depth 1.8 m, and water volume of 20 m^3.
110 Water was not exchanged unless considered necessary. Daily, the water quality was monitored
111 by the use of Sera Test Kits (Germany): Water oxygen levels were kept higher than 1.0 ppm,
112 using aeration to avoid losing energy on air-breathing behaviour (Lefevre, Jensen, Huong,
113 Wang, Phuong & Bayley 2011). Across experiments, water-pH levels varied from 6.5 to 7.5.
114 Fish were daily fed a standard commercial fingerling feed, 1% of total biomass.

115 ***2.4 Specific test-environment: Acclimatization, water temperature and level***

116 In experiment 1, fish to be challenged were transferred from the hapas to the test tank 22 days
117 prior to the experiment (Table 1). In experiment 2, tagged fish in the two tanks at
118 NABRECSOFA were transported separately to GEC in oxygenated containers and transferred
119 to the two replicated test tanks 14 days prior to the experiment. In experiments 3 and 4,
120 transportation was as in experiment 2, but just 3 days prior to challenge. Thus, in experiments
121 1 and 2 fish were more adapted to the new environment than in experiments 3 and 4 where, in
122 fact, a shortened acclimation period was used as a stressor.

123 Since experiment 1 was conducted outdoor, water temperature was 29.5° C (standard deviation
124 = 1.0° C), i.e., it followed the surrounding temperature (Table 1). In experiment 2, water
125 temperature was partly controlled using air conditioner; 29.0° C during the first 10 days post-
126 cohabitation and thereafter 26° C, until termination. In experiments 3 and 4, temperature was
127 set at 26° C because this temperature has been widely used in Enteric Septicaemia of Catfish
128 (ESC) studies with channel catfish (Camp, Wolters & Rice 2000; Lim & Klesius 1997; Patrie-
129 Hanson & Jerald Ainsworth 1999).

130 In experiment 1, the water level was kept constant during acclimatization and the entire testing
131 period (Table 1). In experiments 2, 3 and 4, water levels were halved 1 day prior to challenge
132 and kept at this level throughout the entire experimental period. This resulted in increased
133 density and posed additional stress to the fish.

134 ***2.5 Specific test-environment: Cohabitant shedder density, dose and placement, and addition***
135 ***of bacteria to water***

136 In experiment 1, the ratio of cohabitant shedders to test fish (for number of cohabitants and test
137 fish, see Tables 1 and 2, respectively) was 1:7, whereas in the other three experiments this ratio
138 was approximately 1:3 (Table 1).

139 Injected doses are given in Table 1. Throughout the experiments, the doses for shedders were
140 reduced from 2.5 x 10^6 to 1.0 x 10^5 (in experiment 2, two doses were used), with the intention
141 to prolong the survival time and thus the time for pathogen dispersion.

142 In experiment 1, cohabitants were released into a 1 x 1 x 1 m^3 hapa located to the centre of the
143 tank, whereas they were released directly into the tanks in the other experiments (Table 1).
144 Release was done directly after injection.

145 In experiment 1, dead cohabitants were removed when they were observed lifeless on the
146 bottom of the hapa (Table 1), while in experiment 2 only floating dead fish (note that all dead
147 fish float for some time, before sinking and then floating again) were removed to mimic the
148 practice used by the industry. Additionally, in experiments 3 and 4 dead and floating cohabitants
149 were collected into plastic baskets, which were hung down into the water for another 2 days.

150 No pathogen was added directly to water in experiments 1 and 2. However, in experiment 3
151 external pathogen was added to the tanks after complete mortality of cohabitants, which
152 occurred at day 6 post-challenge. The density aimed at was 2.5 x 10^6 bacteria/ml water (Table
153 1), which was kept for another 8 days. In experiment 4, pathogen addition to water was started
154 earlier, at day 3 post-challenge and stopped at day 6. At day 3, cohabitant mortality was 30%,
155 while it peaked at day 6.

156 ***2.6 Test fish***

157 In all four experiments, spawning, incubation and nursing until tagging were done as described
158 by Pham, Ødegård, Sang, Gjøen & Klemetsdal (2020).

159 ***Experiment 1: Challenge started April 30, 2009, with year-class 2008***

Year-class 2008 was the third generation of the first sub-population in the breeding program. The fish were produced in June and July 2008 by use of a nested mating design, i.e. one male mated to two females. A total of 156 full-sib families were made. These were from 93 sires and 156 dams (Sang, Klemetsdal, Ødegård & Gjøen 2012). Due to high mortality in some families during nursing, only a total of 2,155 fish from 81 families could be included in the challenge test (Table 2). Mean weight of test-fish at the start of the experiment was 47.6 g.

Experiment 2: Challenge started February 23, 2010, with year-class 2009

Year-class 2009 was the third generation of the second sub-population. Using a nested mating design, as before, a total of 196 full-sib families (103 sires and 196 dams) were produced in July and August 2009. As in experiment 1, one hapa per family was used for nursing. High mortality after nursing resulted in only 64 full-sib families available for challenge (Table 2). All families were represented in tank 1 (1,019 fish), whereas only 60 families were represented in tank 2 (969 fish). Mean fish weight at the start of the experiment was 22.6 g in tank 1 and 22.4 g in tank 2.

Experiment 3: Challenge started January 14, 2011, with year-class 2010

Year-class 2010 was again the third generation, with parents mainly from the third sub-population. Families were produced from July to September 2010, by use of a nested mating design. A total of 233 families were made from 137 sires and 230 dams. Parents were mainly from year-class 2007 (88 sires and 151 dams), but parents from the two preceding year-classes were also used; 2005 (second generation of first sub-population: 21 sires and 32 dams) and 2006 (second generation of second sub-population: 12 sires and 18 dams) as well as some parents of wild type (16 sires and 29 dams). Each family was nursed in a separate hapa until tagging. A total of 187 full-sib families took part in the challenge (Table 2). The numbers of

183 fish were 2,944 and 2,745 in the two replicated tanks, respectively, and mean fish weight at the
184 start of test was 20.4 and 19.5 g.

185 ***Experiment 4: Challenge started January 3, 2012, with year-class 2011***

186 Year-class 2011 was the fourth generation of the first sub-population. Fish were produced in
187 June and July 2011 with a nested mating design. Most parents came from year-class 2008 (93
188 sires and 154 dams), but parents from the other three preceding year-classes were also included:
189 2005 (3 sires), 2006 (16 sires and 37 dams), 2007 (20 sires and 43 dams) as well as parents of
190 wild type (8 sires, 13 dams). The 250 families (from the 140 sires and 247 dams) produced were
191 raised in a total of 269 hapas, meaning that some families were represented in more than one.
192 Of these, 233 families were utilized in the challenge test (Table 2). A total of 3,246 and 2,931
193 fish were tested in the two replicated tanks, respectively. Mean fish weight at the start of test
194 was 20.0 and 20.2 g, for tanks 1 and 2, respectively.

195 ***2.7 Recoding of data***

196 Collection of dead fish was carried out twice daily, either at 8:00 or 14:00, throughout the test
197 period. PIT-tag (Passive Integrated Transponder) identity was recorded for tested fish as well
198 as weight, day and time of death. Moreover, time of death of cohabitants was recorded.
199 Mortality was observed for 22 - 23 days in experiments 1 and 2 and for 18 - 19 days in
200 experiments 3 and 4, respectively. In the period with high daily mortality, random samples of
201 dead fish were examined for presence of typical colonies of *E. ictaluri*, by having kidney
202 samples grown on sheep blood agar plates, incubated at 30^0 C for 24 hours (Crumlish, Dung,
203 Turnbull, Ngoc & Ferguson 2002). In experiment 2, with low daily mortality, one awaited it to
204 increase implying that sampling was postponed, and in the end, it was not carried out. The
205 sample sizes were 40, 100 and 100 in experiments 1, 3 and 4, respectively. After the challenge,

206 survivors were biosecure-buried, following the national veterinary regulations (Department of

207 Animal Health, Vietnam).

208 **2.8 Statistical analysis**

209 In experiments 3 and 4, dead or alive (= 0/1) at the end of the test in the two replicated tanks

210 defined traits 1 and 2, respectively. Bivariate analyses across tanks were performed by a cross-

211 sectional linear sire-dam model.

212 In matrix notation, the bivariate model can be written:

213
$$\begin{bmatrix} \boldsymbol{y_1} \\ \boldsymbol{y_2} \end{bmatrix} = \begin{bmatrix} \boldsymbol{X_1} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{X_2} \end{bmatrix} \begin{bmatrix} \boldsymbol{b_1} \\ \boldsymbol{b_2} \end{bmatrix} + \begin{bmatrix} \boldsymbol{Z_1} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{Z_2} \end{bmatrix} \begin{bmatrix} \boldsymbol{a_1} \\ \boldsymbol{a_2} \end{bmatrix} + \begin{bmatrix} \boldsymbol{W_1} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{W_2} \end{bmatrix} \begin{bmatrix} \boldsymbol{c_1} \\ \boldsymbol{c_2} \end{bmatrix} + \begin{bmatrix} \boldsymbol{e_1} \\ \boldsymbol{e_2} \end{bmatrix}$$

214 where $\boldsymbol{y_1}$ and $\boldsymbol{y_2}$ are vectors of the observed survival to BN in tanks 1 and 2, respectively; the

215 vectors $\boldsymbol{b_i}$ both contain the intercept (b_0), fixed regression coefficient on number of days from

216 spawning until tagging (b_1) and the fixed regression coefficient on number of days from tagging

217 to start of experiment (b_2); $\boldsymbol{a_i}$ is a vector that for each trait contains the random additive genetic

218 effect of sire l (s_l), and the random additive genetic effect of dam m (d_m); $\boldsymbol{c_i}$ is a vector that for

219 a trait contains the random common environmental effect of full-sib family lm (c_{lm}), and $\boldsymbol{e_i}$ is a

220 residual vector for a trait with the random error term for fish n (e_{lmn}) ~ N (0, $\mathbf{I}\sigma^2_e$), with σ^2_e

221 being the error variance. This assumes that the residual covariances were zero which is

222 appropriate since a fish could only be represented in one tank. The design matrices for $\boldsymbol{b_i}$, $\boldsymbol{a_i}$ and

223 $\boldsymbol{c_i}$ are denoted $\boldsymbol{X_i}$, $\boldsymbol{Z_i}$ and $\boldsymbol{W_i}$, respectively. The $\boldsymbol{a_i}$, $\boldsymbol{c_i}$ and $\boldsymbol{e_i}$ effects were distributed as follows:

224 $\begin{bmatrix} \boldsymbol{a_1} \\ \boldsymbol{a_2} \end{bmatrix} \sim \boldsymbol{N}\left(\boldsymbol{0}, \boldsymbol{A} \otimes \begin{bmatrix} \sigma^2_{sd_1} & \sigma_{sd_1, sd_2} \\ \sigma_{sd_1, sd_2} & \sigma^2_{sd_2} \end{bmatrix}\right)$,

225 $\begin{bmatrix} \boldsymbol{c_1} \\ \boldsymbol{c_2} \end{bmatrix} \sim \boldsymbol{N}\left(\boldsymbol{0}, \boldsymbol{I} \otimes \begin{bmatrix} \sigma^2_{c_1} & \sigma_{c_1, c_2} \\ \sigma_{c_1, c_2} & \sigma^2_{c_2} \end{bmatrix}\right)$, and

226 $$\begin{bmatrix} \boldsymbol{e_1} \\ \boldsymbol{e_2} \end{bmatrix} \sim \boldsymbol{N}\left(\boldsymbol{0}, \begin{bmatrix} \boldsymbol{I}\sigma^2{}_{e_1} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{I}\sigma^2{}_{e_2} \end{bmatrix}\right),$$

227 where $\boldsymbol{A}$ is the additive genetic relationship matrix, and $\boldsymbol{I}$ are an identity matrices of appropriate

228 sizes, while $\begin{bmatrix} \sigma^2{}_{sd_1} & \sigma_{sd_1,sd_2} \\ \sigma_{sd_1,sd_2} & \sigma^2{}_{sd_2} \end{bmatrix}$ and $\begin{bmatrix} \sigma^2{}_{c_1} & \sigma_{c_1,c_2} \\ \sigma_{c_1,c_2} & \sigma^2{}_{c_2} \end{bmatrix}$ are the sire-dam and common

229 environmental (co)variance matrices, respectively, and $\sigma^2{}_{e_1}$ and $\sigma^2{}_{e_2}$ are the residual variances

230 of the two traits. The relationships were traced back to the base. In experiments 3 and 4, $\boldsymbol{A}$

231 contained 6239 and 6896 animals, respectively.

232 The estimated heritability for each trait was calculated as:

233 $$h^2 = \frac{4\sigma^2{}_{sd}}{2\sigma^2_{sd} + \sigma^2_c + \sigma^2_e}$$

234 Additive genetic ($r_{a1,a2}$) and common environmental correlations ($r_{c1,c2}$) between traits were

235 estimated as:

236 $$r_{a1,a2} = \frac{\sigma_{sd1,sd2}}{\sigma_{sd1}\sigma_{sd2}}$$

237 and

238 $$r_{c1,c2} = \frac{\sigma_{c1,c2}}{\sigma_{c1}\sigma_{c2}}$$

239 The ASREML software (Gilmour, Gogel, Cullis, Welham & Thompson 2015) was used to

240 estimate (co)variance components and genetic parameters.

241 **3. Results**

242 In experiment 1, the overall cumulative mortality of test fish approached 25% (Figure 1). First

243 mortality of cohabitants was recorded 2 days post-challenge and reached 100% on day 3 (Table

3). First mortality of test-fish was recorded on day 8, while daily mortality peaked at day 11, at 7.6% dead/day, and remained low until the end of challenge (Figure 2).

In experiment 2, cumulative mortality was only 3.0% and 5.7% for tanks 1 and 2, respectively (Figure 1). In both tanks, mortality among cohabitants started 2 days post-challenge and reached 100% on day 8 (Table 3). In tanks 1 and 2, first mortality of fish was at days 11 and 9, respectively, while daily peak mortality was reached on day 12, in both tanks (0.7% and 1.6%, respectively) (Figure 2).

In experiment 3, cumulative mortality was considerable in both tanks: 84.0% and 83.1%, respectively (Figure 1). Cohabitants in both tanks started to die 2 days post-challenge, and all were dead (both tanks) on day 6 (Table 3). In both tanks, first mortality of fish was at day 3 but with a much-delayed development of daily mortality in tank 2 compared to tank 1 (Figure 2).

In the fourth experiment, cumulative mortality was 87.1% and 87.7% in tanks 1 and 2, respectively (Figure 1). In both tanks, first mortality of cohabitants occurred at day 2 and reached 100% on day 8 (Table 3). Moreover, in both tanks, fish started to die at day 6, with similar daily mortality patterns, peaking at days 12 and 11, respectively (Figure 2).

Test-fish that died during the challenge showed typical signs of BN; bugged-eyes, less reactive to sound, and with jumping and spinning before death. Internal clinical signs were numerous white spots of different sizes (0.3 - 3 mm) in swelled liver, kidney and spleen. The white spots appeared primarily on the kidney, making them swollen, but could also in critical cases be found in liver and spleen. In all samples, *E. ictaluri* was identified.

Estimated variance components and heritabilities of the traits (tank) as well as additive genetic and common environmental correlations between the traits (tank), using the cross-sectional linear sire-dam model, are presented in Table 4. In experiment 3, heritabilities were similar in

267 the two tanks (0.09 ± 0.05 and 0.08 ± 0.04, respectively), and the genetic correlation between
268 tanks was close to unity (fixed at boundary). Moreover, the estimated correlation between
269 common environmental effects in the two tanks was negative (-0.29), with a large standard error
270 (0.78). In experiment 4, the estimated heritabilities were also low (0.08 ± 0.04 for tank 1 and
271 0.04 ± 0.03 for tank 2, Table 4). Again, the estimated additive genetic correlation was high
272 (0.95 ± 0.19), while the common environmental correlation was fixed at boundary (0.99).

273 **4. Discussion**

274 In challenge testing of fish, a natural reference is a mortality of 50% because it maximizes the
275 phenotypic variance for a binary trait (Gjøen, Refstie, Ulla & Gjerde 1997). This reasoning is
276 as follows: If the infection load becomes too low/massive, all the fish will eventually live/die,
277 and any phenotypic variance will not exist when analysing endpoint data (at least as dead/alive).
278 More recently, Ødegård, Baranski, Gjerde & Gjedrem (2011) have argued that mortality should
279 naturally cease in the endpoint because surviving fish then are more likely to be non-susceptible
280 to the disease. Establishing such a testing practice will have implications not only for genetic
281 analysis, but also when examining effects of treatments, for example effects of vaccines
282 (Drangsholt, Gjerde, Ødegård, Finne-Fridell, Evensen & Bentsen 2012; Fredriksen, Olsen,
283 Furevik, Souhoka, Gauthier & Brudeseth 2013) or effects of feed ingredients/feed additives
284 (Ward, Bengtson, Lee & Gomez-Chiarri 2016).

285 In the first experiment, mortality was only 25%. To increase mortality in the second experiment,
286 several of the environmental factors were made more extreme: Acclimatization shortened,
287 temperature more stressful, water level reduced, direct contact between the test fish and the
288 cohabitants, a larger fraction of cohabitants per test fish, and cohabitant shedders expected to
289 contaminate over a longer time-period (half the cohabitants given a reduced dose) (Table 1).

290 However, the temperature was first changed from 29° C to 26° C, at day 10 of the experiment
291 (Table 1), when realizing that mortality would be low, but this change probably occurred too
292 late to have any effect since the cohabitants had already died out at day 8 (Table 3). The
293 experience in the Mekong Delta is that outbreak of BN is highly affected by the temperature,
294 since it is mostly found in the winter when water temperature is low and fluctuating. Moreover,
295 in channel catfish, a temperature range of 22 - 28° C has been found to stimulate outbreaks of
296 ESC, a disease also caused by *E. ictaluri* (Hawke, Durborow, Thune & Camus 1998; Patrie-
297 Hanson & Jerald Ainsworth 1999; Thune, Fernandez, Benoit, Kelly-Smith, Rogge, Booth,
298 Landry & Bologna 2007). Mortality in cohabitation challenge with *Aeromonas salmonicida* in
299 Atlantic salmon can also to a large degree be adjusted by varying the water temperature
300 (Nordmo & Ramstad 1999) and led us to choose a temperature of 26° C in the challenge test
301 with Mekong striped catfish. Changes of the remaining environmental factors in experiment 2
302 were, however, decided *a priori* to carrying out the experiment. It was assumed that a shortened
303 acclimatisation time, i.e., the time from arriving GEC (test station) to the release of the
304 cohabitants (day 0), would pose stress to the fish. The same was assumed for halving the water
305 level, despite Mekong striped catfish being an air breather. Regarding cohabitants, they were
306 released directly into the test tanks with direct exposure of cohabitants to test fish, expected to
307 increase the infectious pressure. So was the increased density of cohabitants (from 1:7 to 1:3)
308 and the reduced dose applied for half the number of cohabitants, expected to increase the time
309 for pathogen dispersion because those given the lowest dose should live longer. Despite all
310 these environmental factors being more extreme in experiment 2 than in experiment 1, still one
311 factor was less extreme, the earlier removal of the dead fish, and mortality became very low (<
312 5.7%).

313 The results of experiments 1 and 2 proposed that the practise around removal of the dead
314 cohabitants affected mortality, meaning that the infectious load of the water could be
315 instrumental to regulation of the disease frequency. Thus, in experiment 3 the load was
316 increased by adding bacteria directly to water (from day 6 to day 13) but also from collecting
317 dead cohabitants into plastic baskets that were hung down into the water for another 2 days.
318 Additionally, smaller modifications were done to the experimental factors applied in
319 experiment 2; the acclimatisation time was shortened from 14 to 3 days, and the lowest dose
320 for the cohabitants was kept (10^5 bacteria). Now, the mortality increased considerably relative
321 to the first two experiments, reaching 83 - 84% in the two test tanks at termination.

322 In order to reduce the high disease frequency obtained in experiment 3, addition of bacteria to
323 water was done earlier in experiment 4 and for a shorter time (days 3 to 6), otherwise for the
324 same environmental factors as in experiment 3. Despite the change, mortality was still high and
325 approached 87% in the two test tanks at termination. However, mortality curves were more
326 similar in experiment 4 than in experiment 3 suggesting that the bacteria need to be added to
327 the water already in the initial phase of the challenge test.

328 An experiment 5 was reported by Vu, Sang, Trong, Duy, Dang & Nguyen (2019), also in
329 replicated tanks with fish of size ~ 20 g. To reduce the pathogen load of experiments 3 and 4,
330 the factor involving the dead cohabitants was removed from the experiment. Relative to the
331 first four experiments, one awaited 2 days from cohabitant injection to release to the test tanks
332 (released at day 0 in experiments 1 - 4). The bacterium used was now the most virulent
333 (determined by lethal dose 50, LD_{50}, i.e. the dose needed for 50% of fish to die) of four
334 serotypes (found by use of biochemical tests and polymerase chain reaction, PCR), as tested
335 prior to the experiment. Cohabitant dose was 10^6 bacteria, and bacteria were added to water
336 only once, 4 days after cohabitant injection (10^5 bacteria/ml). Otherwise the same

337 environmental factors were as established; acclimatisation time of 4 days, water temperature 338 26° C, restricted water level (2/3) and cohabitant density 1:3, with direct release into the test 339 tanks. The reported average mortality across the two test tanks in experiment 5 became 39%, 340 close to the desired frequency of 50%. This disease frequency is considered highly acceptable 341 because the phenotypic variance is not much affected by frequencies deviating slightly from 342 50% (= $2pq$, where p and q are the frequencies of dead and alive, respectively). In a longer time 343 perspective, however, one should take into account that resistance due to selection will increase 344 over time, meaning that a higher BN dose will likely be required to obtain the desired frequency.

345 A linear cross-sectional sire-dam model was applied to the data in experiment 3 and 4, and the 346 estimates of the heritability were limited since they are frequency dependent (Gianola & 347 Foulley 1983). Moreover, the estimated genetic correlations were consistently high, while the 348 common environmental correlations were imprecisely estimated. The precision of the estimate 349 is highly affected by the many parameters determined and from the nested mating design, 350 known to weakly separate the common environmental and additive genetic effects (Berg & 351 Henryon 1998). Genetic analyses revealed that resistance to bacillary necrosis tested in 352 replicated tanks (in the same experiment) can be considered the same genetic trait.

353 In these experiments, no outbreak of disease was observed during the nursing period. However, 354 note that the water supply during the hapa period was from the Mekong river, such that the fish 355 might have been exposed to the pathogen *a priori* to the challenge.

356 **5. Conclusions**

357 The four challenge tests of Mekong striped catfish against *E. ictaluri* propose three days 358 acclimatization of test fish prior to the challenge, with restricted water level in the test, keeping 359 a temperature of 26^0 C. In the challenge, cohabitant shedders should be released directly into

360 the test tank and make up around ⅓ of the fish, and bacteria should be added directly to water.
361 The last two experiments, with the highest mortality, suggest that any factor involving the dead
362 cohabitants should be removed, and that additional experimentation should focus on bacteria
363 (density) and timing for addition of bacteria to water. Genetic analyses revealed that resistance
364 to bacillary necrosis tested in replicated tanks (in the same experiment) can be considered the
365 same genetic trait.

366 **Conflict of interest statement**

367 There is no conflict of interest.

368 **References**

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444

Table 1. Environmental factors varied across the four challenge-test experiments (Exp) with Mekong striped catfish.

Factor	Exp1	Exp2	Exp3	Exp4
Year	2009	2010	2011	2012
Test-tank location	Outdoor at NABRECSOFA	Indoor at GEC	Indoor at GEC	Indoor at GEC
No. of replicated tanks	1	2	2	2
No. of days of acclimatization prior to experiment	22	14	3	3
Water temperature, ^{o}C	29.5	29 (the first 10 days post-cohabitation) and 26 (until termination)	26	26
Reduction of water level	No	Halved 1 day prior to test	Halved 1 day prior to test	Halved 1 day prior to test
No. of cohabitants	323	340 in tank 1 320 in tank 2	1,000 in tank 1 1,000 in tank 2	1,280 in tank 1 1,240 in tank 2
Ratio of cohabitant: test fish	~1:7	~1:3	~1:3	~1:3
No. of bacteria in cohabitant-dose	2.5×10^6	2.5×10^6 for half the cohabitants, and 2.5×10^5 for the rest	1×10^5	1×10^5
Placement of cohabitants	In hapa, located central in tank	Direct into tank	Direct into tank	Direct into tank
Dead cohabitant removal	When dead and sunk down to bottom of hapa	When dead and floating	Dead and floating fish collected into plastic baskets hung down into water for another 2 days	Dead and floating fish collected into plastic baskets hung down into water for another 2 days
Addition of pathogen to water	No	No	Post-exposure to cohabitants: From day 6 to day 13, to reach a density of 2.5×10^6 bacteria/ml	Post exposure to cohabitants: From day 3 to day 6, to reach a density of 2.5×10^6 bacteria/ml

Table 2. Numbers of families and fish per tank in the four challenge-test experiments (Exp) with *E. ictaluri* in Mekong striped catfish: Experiment 1 had one tank (t1), while the other experiments had two replicated tanks.

No. of families and fish	Tank	Exp1	Exp2	Exp3	Exp4
No. of families	t1	81	64	187	233
	t2	-	60	187	233
No. of fish	t1	2,155	1,019	2,944	3,246
	t2	-	969	2,745	2,931

Table 3. Number of days from injection (experimental day 0) to first and complete death of cohabitants in four challenge-test experiments with *E. ictaluri* in Mekong striped catfish: Experiment (Exp) 1 had one tank (t1), while the other experiments had two replicated tanks.

	No. of days to	
Experiment	First mortality of cohabitants	Complete mortality of cohabitants
Exp1-t1	2	3
Exp2-t1	2	8
Exp2-t2	2	8
Exp3-t1	2	6
Exp3-t2	2	6
Exp4-t1	2	8
Exp4-t2	2	8

Table 4. Estimates (linear model) of genetic sire-dam variance (σ^2_{sd}), common environmental variance (σ^2_c), heritability (h^2), and additive ($r_{a1,a2}$) and common environmental correlations ($r_{c1,c2}$) between survival (dead = 0/alive = 1) in the endpoint of two replicated tanks, defining traits 1 and 2, in two experiments (Exp) of Mekong striped catfish with *E. ictaluri*.

	Trait	Exp3	Exp4
σ^2_{sd}	Trait 1	0.0030	0.0020
	Trait 2	0.0029	0.0010
σ^2_c	Trait 1	0.0048	0.0037
	Trait 2	0.0020	0.0020
h^2 †) ± SE	Trait 1	0.09 ± 0.05	0.08 ± 0.04
	Trait 2	0.08 ± 0.04	0.04 ± 0.03
$r_{a1,a2} \pm$ SE		0.99‡)	0.95 ± 0.19
$r_{c1,c2} \pm$ SE		-0.29 ± 0.78	0.99‡)

†) $h^2 = \frac{4\sigma^2_{sd}}{2\sigma^2_{sd}+\sigma^2_c+\sigma^2_e}$.

‡) Fixed at boundary.

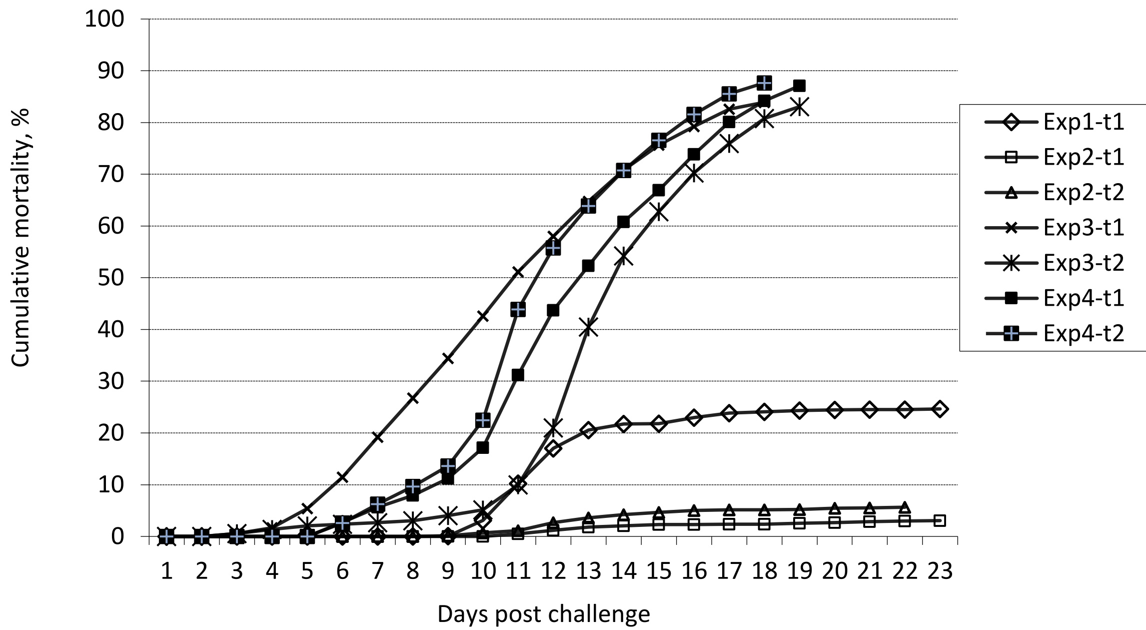

Figure 1. Cumulative mortality observed in four challenge-test experiments with *E. ictaluri* in Mekong striped catfish: Experiment (Exp) 1 had one tank (t1), while the other experiments had two replicated tanks.

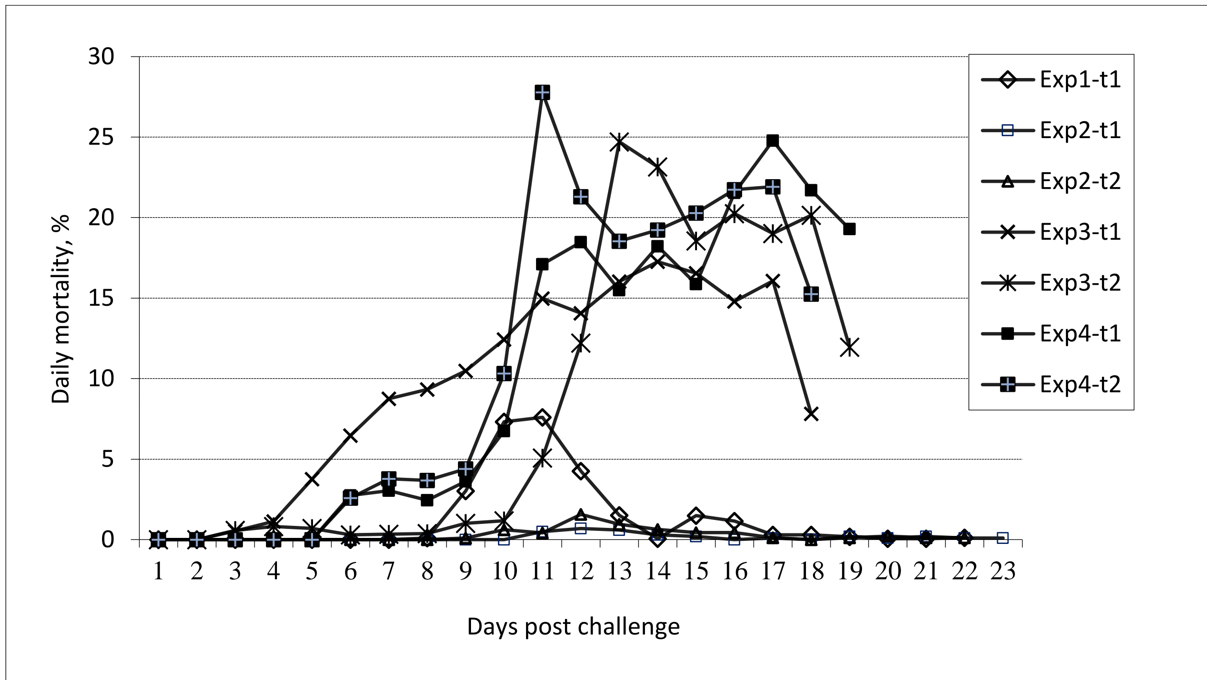

Figure 2. Daily mortality in four challenge-test experiments with *E. ictaluri* in Mekong striped catfish: Experiment (Exp) 1 had one tank (t1), while the other experiments had two replicated tanks.