RESEARCH ARTICLE

Elevated Salinity Tolerance of Reciprocal Hybrids of Improved Brackishwater Enhanced Selected Tilapia (*i*BEST) *Oreochromis* spp.

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ABSTRACT

This study evaluated the salinity tolerance of juvenile reciprocal hybrids of saline-tolerant tilapia strain in terms of salinity indices: Median Lethal Salinity (MLS), Mean Salinity Tolerance (MST), and Optimum Salinity Tolerance (OST). After the 25-day challenge of progressive salinity increase by 3 ppt, it was observed that the progenies of the two reciprocal crosses of *i*BEST showed no significant differences in terms of MLS (*i*BEST Hybrid 1=62.67±3.21 ppt; iBEST Hybrid 2=54.00±5.29 ppt), MST (*i*BEST Hybrid 1=54.33±3.06 ppt; *i*BEST Hybrid 2=56.33±4.73 ppt), and OST (*i*BEST Hybrid 1=36.00±2.65 ppt; *i*BEST Hybrid 2=37.67±6.03 ppt) values, indicating that the offspring of the reciprocal crosses of parents is lacking of maternal/paternal influence with regards to the salinity tolerance of its progenies. Therefore, reciprocal hybrids of the *i*BEST Parent Lines would have a comparable salinity tolerance during the grow-out culture of tilapia in a brackish and saline environment. Salinity tolerance values of *i*BEST is significantly higher compared to the MLS (31.33 ± 6.43 ppt), MST (35.67 ± 2.52 ppt), and OST (14.33 ± 2.52 ppt) value of *i*EXCEL (*Oreochromis niloticus*) juveniles. This study demonstrated the capacity of *i*BEST hybrids in terms of salinity tolerance and its potential in brackish and saline water aquaculture.

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1. INTRODUCTION

The aquaculture of tilapia in the Philippines started in the 1950s following the introduction of *Oreochromis mossambicus* (Guerrero 2019). However, due to its unwanted characteristics (*i.e.*, relatively slower growth rate, early maturation resulting in overcrowding in a pond and stunted fish at harvest, becoming pests in brackishwater ponds, and unappealing dark body coloration), the commercial culture of this tilapia species did not prosper (Bolivar 1993; Guerrero 1994; Ordoñez et al. 2014). Thus, several fast-growing tilapia species were introduced, including *O. niloticus*. Since then, various research and development projects have been launched to improve the performance, focusing on improving its growth performance (Ordoñez et al. 2014). Subsequently, the focus of the breeding program shifted to the development of tilapia strains that would perform well in different culture environments (*e.g.*, low water temperature and high-saline water) (Romana-Eguia and Eguia 1999; Tayamen et al. 2002; Rosario et al. 2004; Ordoñez et al. 2014). This led to the development of saline-tolerant tilapia strain by the Bureau of Fisheries and Aquatic Resources-National Freshwater Technology Center (formerly National Freshwater Fisheries Technology Center) (BFAR-NFTC) and BFAR-National Fisheries Development Center (formerly National Integrated Fisheries Technology Development Center) (BFAR-NFDC) in 1998 (Ordoñez et al. 2014).

The Brackishwater Enhanced Selected Tilapia (BEST) strain was produced through the breeding program initiated by the BFAR - NFTC in collaboration with Freshwater Aquaculture Center-Central Luzon State University (FAC - CLSU) and the University of the Philippines - Visayas (UPV) with the funding from the Bureau of Agricultural Research

(BAR) of the Department of Agriculture (DA) in which the program aimed to develop a saline-tolerant breed of tilapia for aquaculture which could withstand and thrive well in a brackish water environment. The founderstocks of BEST are composed of three euryhaline species of tilapia, namely: *O. aureus, O. spilurus, and O. mossambicus*, and three genetically improved strains of *O. niloticus*, namely 8th generation Genetically Improved Farmed Tilapia (GIFT), 13th generation Freshwater Aquaculture Center Selected Tilapia (FaST), and the YY males of the Philfishgen project were also integrated to the gene pool of the BEST (Tayamen et al. 2002). Products of the selection resulted in growth gains of about 86% compared to the F1 pure cross of *O. mossambicus* and 24% compared to F1 of *O. mossambicus* x *O. niloticus* Egypt strain. Survival also increased by 24% compared to the pure *O. mossambicus* cross and 35 % compared to the cross of *O. mossambicus* x *O. niloticus* Egypt strain (Tayamen et al. 2002). The BFAR-NFTC, being the National Broodstock Center for tilapia, is mandated to continuously improve different tilapia strains, which led to the amendment of the name BEST into *i*BEST (improved Brackishwater Enhanced Selected Tilapia). As of this writing, the fifth-generation *i*BEST is being propagated by the BFAR-NFTC and other BFAR production facilities that caters to the need for saline-tolerant tilapia strain nationwide, yet no available publish information on the salinity tolerance of its reciprocal progenies. Thus, this study was conducted to evaluate the salinity tolerance of the fifth-generation *i*BEST reciprocal hybrids.

2. MATERIALS AND METHODS

2.1 Production of reciprocal hybrids

Reciprocal crossing of *i*BEST parent line (PL) (ABW, >200g) (*i*BEST Hybrid 1= *i*BEST PL–A♂ x *i*BEST PL–B♀; *i*BEST Hybrid 2= *i*BEST PL–B♂ x *i*BEST PL–A♀) was done in 1x1x1 m fine mesh hapas installed in an earthen pond following a 1:1 sex ratio. The breeders were fed with commercial aquafeeds (brood stocks pellet, 36% CP) at 3% of their body weight during the conditioning and breeding period. Tilapia fry were obtained approximately 21 days after pairing of brood stock. Collected fry were reared in 1 x1 x 1 m fine-mesh hapas installed in an earthen pond at a rate of 1,000 fry per hapa. The stocks were fed with commercial aquafeeds (fry mash, 40% CP) at 20% of the fish's body weight and subsequently reared in b-net hapas with the same dimension until it reached a body weight of 0.61 - 1.0 gram or size 17, approximately after 45 days of rearing.

2.2 Experimental design and procedures

The study was conducted in triplicates and was laid out in a Completely Randomized Design (CRD) at the Indoor Laboratory of BFAR-NFTC. The fifth-generation *i*BEST, a saline-tolerant strain, was used in this study with the *i*EXCEL strain (*Oreochromis niloticus*) as a negative control since this species is less saline-tolerant than other tilapia species. Four (4) treatment groups (Table 1) were subjected to daily salinity increments following the method of Lemarié et al. (2004) with some minor modifications as per the recommendation of Rosario et al. (2004). It was suggested that a progressive salinity increase by 3 parts per thousand (ppt) is more conclusive in terms of salinity tolerance determination of stocks than in 6 ppt and above. Salinity was increased daily by 3 ppt until all the fish stocks in the experimental units had died. The progressive salinity increase was done by replacing 50% of the water in the container by siphoning out the fish's excrement and other organic wastes and then replaced with an appropriate salt solution via the water-to-fish method to lessen stress to the stocks. Fish were considered dead if there were no spontaneous movement and a lack of response to the mechanical stimuli (poking the stocks with a rod). The experiment lasted 25 days and reached a salinity of 75 ppt in a test aquarium.

Table 1. Treatment groups utilized in this study.

Treatment	Description
1	Negative control; 6th generation iEXCEL strain
2	Positive control; 5th generation iBEST strain
3	5th Generation iBEST Hybrid 1 (iBEST PL-A♂ x iBEST PL-B♀)
4	5th Generation iBEST Hybrid 2 (iBEST PL-B♂ x iBEST PL-A♀)

A total of 360 tilapia fingerlings (*i*EXCEL strain=0.60±0.12g; *i*BEST strain=0.64±0.08g; *i*BEST Hybrid 1=0.63±0.03 g; *i*BEST Hybrid 2=0.61±0.02 g) were stocked and conditioned for 7 days in a 12 rectangular plastic container (75 x 40 x 30 cm). The water volume was maintained at 15 liters with a stocking density of 2 fingerlings/L. Stocks were fed with commercial aquafeed (Fry mash, 40% CP) at 3% of their body weight throughout the conditioning and study period. Meanwhile, dissolved oxygen was

maintained at optimum throughout the study by providing an aeration system to the experimental units. Water parameters such as dissolved oxygen and temperature were monitored once a day using a digital water quality meter (AZ Instrument Corp, Taiwan R.O.C.).

A salt stock solution was prepared by mixing an appropriate amount of commercial rock sea salt and unchlorinated tap water in a 100-liter tub until the desired salinity was achieved. The salt stock solution was prepared 24 hours prior to use to allow the complete dissolution of the salt and achieve a relative water temperature with those in experimental units. Salinity reading was determined using a handheld refractometer (Atago Co. Ltd., Japan) and adjusted to the desired salinity level if necessary.

2.3 Determination of median lethal salinity, mean salinity tolerance, and optimum salinity tolerance

2.3.1 Median lethal salinity (MLS)

Cumulative mortality was recorded as the occurrence of mortality at the progressive change in salinity level. The curves of the cumulative mortalities from 0% to 100% were drawn; the equations of the linear regressions and their correlation coefficients (r) were determined using Microsoft Excel 2016. The equation for the calculation of Median Lethal Salinity (MLS=salinity value for which 50% survival rate is recorded) for each replicate is as follows (Lemarié et al. 2004; Mateo et al. 2004):

$$y=a+bx$$

Where: y = Median Lethal Salinity
x = median survival (50%)
a = slope
b = intercept

2.3.2 Mean salinity tolerance (MST)

The Mean Salinity Tolerance (MST) is the average salinity where the fish dies at a certain salinity level. The MST serves as an index for determining the average salinity tolerance of a certain group of hybrids or pure species. This was calculated by multiplying the number of dead fish in each replicate with the respective salinity it died (Mateo et al. 2004). The products were summed and divided by the total number of fish per replicate. The MST was computed as follows:

$$MST=(f1 * s1 + f2 * s2 + \cdots + fN * sN)/N$$

Where: f = fish
s = salinity
N = number of fish observed

2.3.3 Optimum salinity tolerance (OST)

The Optimum Salinity Tolerance (OST) was determined using the break-line analysis (Mateo et al. 2004). It is used to determine the maximum salinity at which there was no mortality or low mortality, even at longer exposure to a saline environment. Briefly, the values were plotted into a graph to determine the formed plateau. The value was from the start or at the salinity where the mortality starts to occur. The slope of the regression line ($Y=a_1+b_{1x}$) was tested for significance. Once significance was observed, stepwise calculations of linear regression were done until the results became insignificant. The remaining plotted values were subjected to linear regression 2 ($Y=a_2+b_{2x}$), connecting to the first value of the regression plateau. The OST was determined using the two linear regression functions ($a_1+b_{1x}= a_2+b_{2x}$). The value where the two regression lines intersect was the breakpoint value (Mateo et al. 2004).

2.4 Data analysis

The determinations of Median Lethal Salinity (MLS), Median Salinity Tolerance (MST), and Optimum Salinity Tolerance (OST) were computed in Microsoft Excel 2016. Meanwhile, Levene's and Shapiro-Wilk's tests were used to evaluate the homogeneity of variance and normality, respectively. Afterwards, comparisons of the salinity indices (MLT, MST, and OST) between the treatment groups were subjected to the CRD One-way Analysis of Variance (ANOVA) and Tukey's HSD as a post-hoc comparison with a 95% confidence level using IBM SPSS Statistical Package 23.

3. RESULTS

3.1 Median lethal salinity (MLS)

The mean MLS value for *i*BEST Hybrid 1 was 62.67 ± 3.21 ppt, followed by *i*BEST Hybrid 2 (54.00 ± 5.29 ppt), *i*BEST strain (52.00 ± 4.85 ppt), and *i*EXCEL strain (31.00 ±6.47 ppt). No significant difference

was observed between the reciprocal progenies of *i*BEST Parent Lines (Hybrid 1 and 2). Interestingly, a significantly ($p<0.01$) higher mean MLS value of *i*BEST groups than in *i*EXCEL strain (31.33 ± 6.43 ppt) was observed (Figure 1).

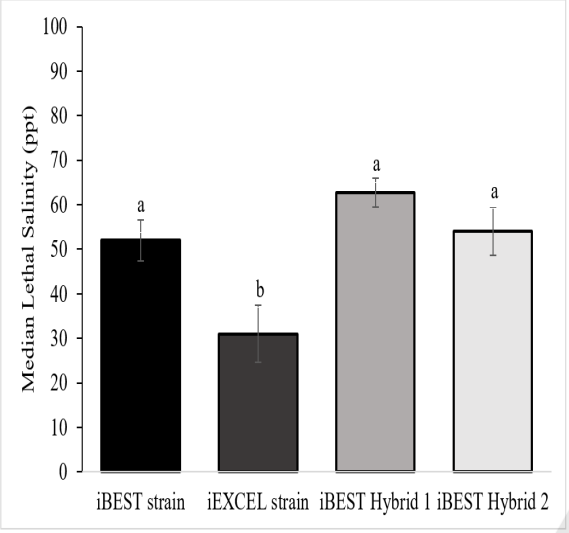

Figure 1. Mean lethal salinity of *i*BEST hybrid.

3.2 Mean salinity tolerance (MST)

The mean MST value for *i*BEST Hybrid 1 was 56.33 ± 4.73 ppt, followed by *i*BEST Hybrid 2 (54.33 ± 3.06 ppt), *i*BEST strain (53.33 ± 3.51 ppt), and *i*EXCEL strain (35.67 ± 2.52 ppt). Post-hoc analyses revealed no significant differences among the MST values of *i*BEST groups. On the other hand, a significantly ($p<0.01$) higher mean MST value was observed in *i*BEST groups as compared to *i*EXCEL strain (Figure 2).

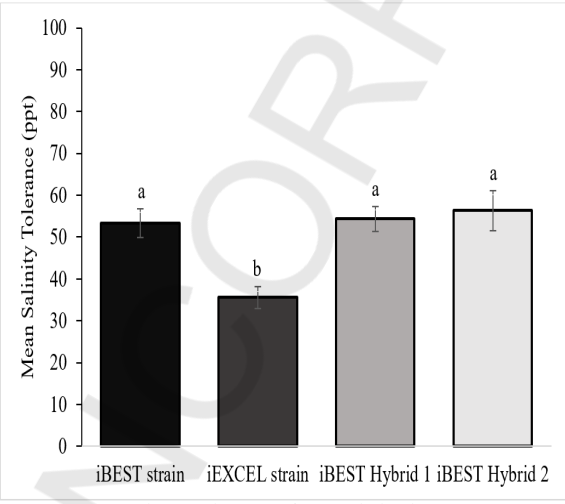

Figure 2. Mean salinity tolerance of *i*BEST hybrid.

3.3 Optimum salinity tolerance (OST)

*i*BEST Hybrid 2 had the highest mean OST value of 37.67 ± 6.03 ppt, followed by *i*BEST Hybrid 1 (36.00 ± 2.65 ppt), *i*BEST strain (33.67 ± 1.15 ppt), and *i*EXCEL strain (14.33 ± 2.52 ppt). Statistical analyses showed no significant differences among the *i*BEST groups. Remarkably, the mean OST value of *i*BEST groups was significantly higher ($p<0.01$) than in *i*EXCEL strain (14.33 ± 2.52 ppt) (Figure 3).

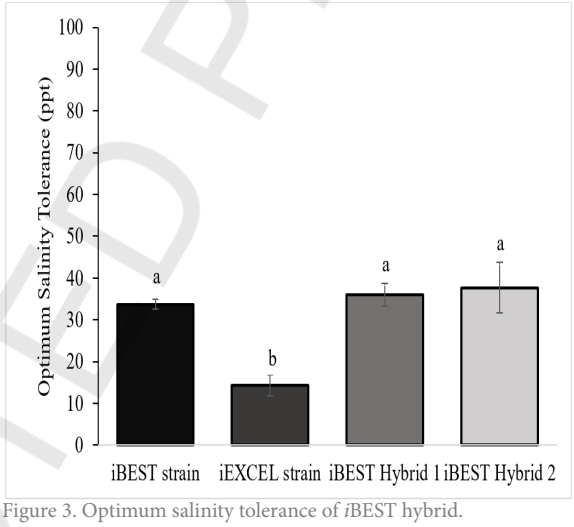

Figure 3. Optimum salinity tolerance of *i*BEST hybrid.

3.4 Mortality trend

Figure 4 shows the cumulative mortality of test groups during the progressive increase of salinity by 3 ppt. The onset of mortalities in *i*EXCEL was observed at 12 ppt, and subsequent mortalities were observed until all stocks died at 57 ppt. Meanwhile, the onset of mortalities in *i*BEST groups was observed at higher salinities (*i*BEST strain=27 ppt; *i*BEST Hybrid 1=33 ppt; *i*BEST Hybrid 2=21 ppt) and total mortalities of stocks were observed at higher salinities in *i*BEST groups (*i*BEST strain=75 ppt; *i*BEST Hybrid 1=72 ppt; *i*BEST Hybrid 2=75 ppt) than in *i*EXCEL.

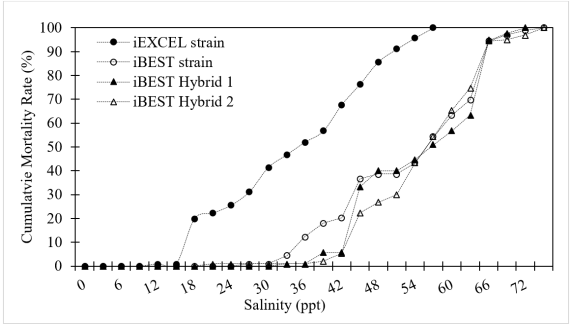

Figure 4. The cumulative percentage mortality of experimental tilapia throughout the 25-day salinity tolerance test.

4. DISCUSSION

The earliest and majority of the breeding program for tilapia is geared towards the improvement of traits with economic importance, primarily growth and survival. However, considering the potential culture of tilapia in brackish and saline water environments, attention has been drawn in developing a fast-growing tilapia that can withstand high saline environments. Selective breeding (Jaspe and Caipang 2011) and hybridization (Gurrero et al. 1996) are among the approaches that are widely used to improve the ability of tilapia to withstand high salinity levels. Some of the earliest reports on the utilization of tilapia species and hybrids in brackishwater environments were demonstrated in the Philippines since the 1980s. Several tilapia species, including *O. niloticus, O. mossambicus, O. niloticus* x *O. mossambicus* hybrids, and red tilapia hybrids, were commercially cultured in ponds, tanks, and cages (Gurrero et al. 1996; Romana–Eguia et al. 2020). However, the poor growth performance of *O. mossambicus* and *O. niloticus* in brackishwater environments has led to the demand for the availability of fast-growing and saline-tolerant tilapia. The *i*BEST is systematically bred to perform and thrive well in a brackish and saline-water environment (Tayamen et al. 2002).

In this study, the salinity tolerance of fifth-generation *i*BEST Hybrid 1 and Hybrid 2 was evaluated following the method of Lemarié et al. (2004). Upon the 25-day challenge to progressive salinity increase, the study revealed no difference in the salinity tolerance indices (MLS, MST, and OST) value of *i*BEST Hybrid 1 and Hybrid 2. These results were similar to the data obtained by Apaga (2014) where the salinity tolerance levels of the fourth generation *i*BEST reciprocal offspring in terms of MLS (*i*BEST Hybrid 1=29.75 ± 0.26 ppt; *i*BEST Hybrid 2=29.75 ± 2.23 ppt), MST (*i*BEST Hybrid 1=32.47 ±0.40 ppt; *i*BEST Hybrid 2=32.20 ± 2.01 ppt), and OST (*i*BEST Hybrid 1=15.99 ± 0.95 ppt; *i*BEST Hybrid 2=13.23 ± 2.18 ppt) does not differ significantly. This may be explained by the maternal/paternal influence on the salinity tolerance of the fish. Parents of the *i*BEST Hybrid 1 and Hybrid 2 may lack strong maternal influence pertaining to salinity tolerance. This would result in similar salinity tolerance for their reciprocal progenies. The higher maternal influence compared with the paternal influence may be due to the presence of extrachromosomal genes in the cytoplasm of the eggs (Shikano et al. 1997; Mateo et al. 2004). Meanwhile, the paternal effects represent contributions to fitness outside the effects of nuclear genes and may include cytoplasmic effects (Roach and Wulff 1987; Wade 1998; Levin 2003; Burgess and Husband 2004). Since no maternal effect was observed in the *i*BEST Hybrid 1 and Hybrid 2 in terms of salinity tolerance, it is indicative that the progenies of the *i*BEST reciprocal pairs would have a similar salinity tolerance in a brackish and saline water environment during the grow-out period. In terms of growth and survival performance of *i*BEST in the actual brackish water conditions, Labastida et al. (2014) evaluated its performance in organically fertilized brackishwater ponds (6.3–6.9 ppt) for 120 days with semi- and intensive management. At harvest, fish reported average body weight (ABW) was 223.1g and 214.5g, with a survival rate of 80 and 84%, respectively.

Higher MLS (66-100%), MST (50-58%), and OST (135-163%) values of *i*BEST Hybrid 1 and Hybrid 2 were observed in this study as compared to *Oreochromis niloticus*. This is attributed to the superior tolerance to salinity of *i*BEST's foundation stocks, *O. spilurus, O. aureaus, O. mossambicus* (Tayamen et al. 2004). In the study of Mateo et al. (2004), they observed that the salinity tolerance values of *O. mossambicus* expressed in MLS, MST, and OST were 108.1, 113.5, and 163.7%, respectively, which is significantly higher than in *O. niloticus*. Moreover, it is known that *O. mossambicus* is among the most saline-tolerant of the tilapias and reported to grow well in the pond at a salinity from 32 to 40 ppt (Popper and Lichatowich 1975; Villegas 1990), spawning at 34 ppt (Liao and Chang 1983; Villegas 1990) and 49 ppt (Popper Lichatowich 1975; Villegas 1990).

Literature suggests that the tolerance of tilapia to salinity is attributed to endogenous (*i.e.*, age, body size, species, strain) and exogenous factors (*i.e.*, diet, temperature, dissolved oxygen, and ammonia) (Prunet & Bornacin, 1989). Commonly, the salinity tolerance of the fish is attributed to its body size. This is explained by the body surface-to-volume relationship. Larger fish experience less osmotic stress than smaller fish due to the ratio of gill area to the body of the fish, wherein the ratio of gill area to body weight decreases as the body weight of the fish increases (Parry, 1960; Watanabe et al. 1985; Villegas, 1990). However, according to Watanabe et al. (1985) and Villegas (1990), though the body size and development come along during the ageing and development of the fish, the ontogenical development of the fish has more influence on the salinity tolerance rather than the body size alone. This could be attributed to the functional development of the osmoregulatory system, particularly the gills and

kidneys in the developing fish (Ewing et al., 1980; Mateo et al., 2004). Furthermore, various literatures (Nordlie et al., 1982; Watanabe et al., 1985) suggests that once the fish has reached its developmental stage, its salinity tolerance may not be influenced by the body size. In *O. mossambicus*, a second hemoglobin appears at 47 days post-hatching, in which it increases its tolerance to salinity by increasing its capacity to transport oxygen within its body (Perez and Maclean, 1976). Since the size and age of the fish has a positive relationship with its salinity tolerance (Watanabe et al. 1985), we decided to use the bigger size (size 17 or bigger) of tilapia, which are also preferred for cage culture (Romana-Eguia et al. 2020), to assess the salinity tolerance of *i*BEST hybrid.

Even though tilapia is known due to its euryhalinity, one way to ensure higher survival rate of tilapia in brackish- and saline-water is through the acclimatization. According to literature (Lemarié et al. 2004), tilapia could be effectively acclimated to the desired salinity by progressively increasing the water salinity by 2 to 8 ppt daily until it reaches the salinity of cultured water.

Several physiological mechanisms are attributed to the salinity tolerance of tilapia. This includes the role of osmoregulation in fishes primarily through the chloride cells in the branchial epithelium as a medium of salt uptake in freshwater and salt excretion in a saltwater environment (Fosket and Scheffey 1982; Uchida et al. 2000; Lemarié et al. 2004). According to the investigations of Potts et al. (1967), about 75% of the total influx of seawater during the acclimatization of *O. mossambicus* in seawater occurs in branchial and skin organs, whereas the remaining 25% is due to the drinking of seawater by the fish. Moreover, various hormones, including rapid-acting hormones (catecholamines, somatostatin, glucagon, vasoactive intestinal peptide, urotensins) and slow-acting hormones (prolactin, cortisols, and thyroid hormones), are also being attributed to the osmoregulation mechanisms in tilapia (Prunet and Bornacin, 1989). In *O. mossambicus*, gene transcripts for ion transporters, enzymes, hormones, and components of cellular stress signaling are found in the brain, gill, gut, and kidney upon exposure to salinity (Fiol et al. 2006; Cnaani et al. 2011).

In the 1990s, an outbreak of bacterial diseases due to Luminous Vibrio Bacteria (LVB) created havoc in the Philippine shrimp industry that resulted in massive economic losses. In response, several studies were conducted to address this problem of LVB outbreaks, including the use of chlorination in culture waters, vaccinations, therapeutics in the form of antibiotics, and management measures. One of the interesting developments is the polyculture of saline-tolerant tilapia (*e.g.*, red tilapia, *O. mossambicus* x *O. niloticus hybrids,* and *O. mossambicus* x *O. hornorum* hybrids) with tiger shrimp (*Penaeus monodon*) in brackish water fishponds. This polyculture of two commodities resulted in a beneficial effect, thereby producing "green water effect", an effective and low-cost measure in inhibiting the growth of pathogenic LVB (Guerrero 1999). Furthermore, saline-tolerant tilapia species could serve as an alternative biocontrol of *Cryptocaryons irritans* tomonts in the heavily infested *Trachinotus ovatus* (Zhong et al. 2021).

5. CONCLUSION

Fifth-generation *i*BEST hybrids displayed no significant differences in terms of MST, MLS, and OST values. Therefore, reciprocal pairs of both parent lines can be used in the mass hatchery production of *i*BEST fingerlings having a comparable salinity tolerance. Furthermore, fifth-generation *i*BEST fingerlings exhibited a higher salinity tolerance (MLS=66–100%; MST=50–58%; and OST=135–163%) as compared to the *O. niloticus*.

The recorded median lethal salinity or the salinity level where 50% of the population died ranged from 54.00 to 62.67 ppt. Meanwhile, the mean salinity tolerance or the average salinity level that the *i*BEST reciprocal hybrids can tolerate ranged from 54.33 to 56.33 ppt. Lastly, the optimum salinity tolerance for the *i*BEST ranged from 33.67 to 37.67 ppt upon proper acclimation to the actual culture condition.

Around 239,323 hectares of existing brackish water fishponds and roughly 20 million hectares of marine coastal waters for mariculture production are available for aquaculture production. Utilization of saline-tolerant tilapia in these culture areas was seen to be strategic to further the aquaculture production of tilapia. Saline-tolerant tilapia strains could also serve as an alternative brackish water culture species during the scarcity of seedstocks and polyculture with high-valued species such as shrimps (*Penaeus spp.*), pompano (*Trachinotus spp.*) and milkfish (*Chanos chanos*). Also, the polyculture of tilapia with Penaids in a brackish water environment could create a healthy environment that is effective in the control of pathogenic *Vibrio* bacteria.

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AUTHOR CONTRIBUTIONS

Francis Gerald N. Madrid: Investigation, Writing-Original Draft. **Lyda B. Balagtey:** Visualisation, Writing- Review & Editing. **Milagros M. Apaga:** Methodology, Conceptualization. **Jesusa Q. Undan:** Conceptualization. **Jhonny L. Munar:** Investigation. **Eric J. Morales:** Writing- Review & Editing. **Roniño C. Del Pilar:** Formal Analysis. **Archebald N. Valiente:** Conceptualization. **Ma. Jodecel C. Danting:** Supervision, Conceptualization. **Casiano H. Choresca Jr.:** Writing- Review & Editing, Supervision

CONFLICTS OF INTEREST

The authors declared no conflict of interest.

ETHICS STATEMENT

The researchers followed all institutional and national guidelines for the care and use of laboratory animals.

DATA AND REPRODUCIBILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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